Development of new vaccine strategies against *Streptococcus pneumoniae*

by

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For my family

"Found this spoon, sir!"

Statement of Originality

This accompanying thesis submitted for the degree of PhD entitled "Development of a new vaccine strategy against *Streptococcus pneumoniae*" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation of the University of Leicester during the period between August 2001 and September 2004.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of this work has been submitted for another degree in this or any other University.

Signed: _____ Date: _____

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Abstract

The polysaccharide capsule is the most important virulence factor of *S. pneumoniae*, which remains a major cause of morbidity and mortality in both the developing and developed worlds. Unencapsulated, or rough pneumococcal strains have been shown to be completely avirulent and the most effective host defence against *S. pneumoniae* is opsonisation of the polysaccharide capsule with type-specific antibody. For this reason, the polysaccharide capsule has been a major target for vaccine research.

There are over 90 chemically distinct capsular types and protection is specific for each serotype. In order to thoroughly protect against pneumococcal disease, a vaccine must include as many serotypes as possible. Furthermore, capsular polysaccharide antigens are T-independent type 2 (TI-2) antigens, which means that T cell help is not required to induce an immune response. Because of this TI-2 antigens characteristically fail to stimulate a protective immune response in the most high-risk groups of pneumococcal disease, the elderly and in infants. To circumvent this problem, a vaccine was produced in which the polysaccharide is chemically conjugated to a protein. However, this is an expensive approach, which limits the possibility of developing countries undertake a pneumococcal vaccination programme.

In this thesis I have shown that peptide mimotopes of both serotype 6B and 9V pneumococcal polysaccharide, following vaccination, can induce specific anti-CPS antibodies and can protect mice against developing pneumococcal disease. This was achieved using previously described monoclonal antibodies (mAb) directed against the capsular polysaccharide of *S. pneumoniae* serotype 6B and 9V. These mAbs were shown to protect mice in passive immunisation experiments, and were then used to screen phage-displayed peptide libraries. In total four peptide mimics of serotype 9V pneumococcal polysaccharide and seven mimics of serotype 6B pneumococcal polysaccharide were identified. The peptides were conjugated to keyhole limpet haemocyanin (KLH) and were used to immunise mice. Two peptides, MP7 and MP13, were found significantly protect mice against a lethal challenge with *S. pneumoniae* and induce anti- capsule specific antibodies. This project showed that the poor immunogenicity of capsular polysaccharide could be improved using peptide mimics.

Secondly, this thesis confronts the problem of high vaccine production costs by developing a transgenic plant capable of manufacturing pneumococcal polysaccharide. The four genes involved in type 3 pneumococcal capsular polysaccharide synthesis are closely linked on the bacterial chromosome, arranged within a single locus (cassette). Previous work had shown that the recombinant expression of two of these proteins, $Cps3D^{+}S^{+}$, resulted in the synthesis of extra-cellular type 3 polysaccharide in *Lactococcus lactis* and expression of Cps3S alone led to the synthesis of type 3 polysaccharide in both unencapsulated pneumococci and *E. coli*, provided that the nucleotide precursors of the capsular polysaccharide (UDP-Glc and UDP-GlcA) were also present. Therefore, I cloned a copy of the serotype 3 capsule synthase gene, *cps3S*, into a plant expression vector, which was transformed into tobacco plants by *Agrobacterium*-mediated gene transfer. After cultivating a second generation of transgenic plants, we have shown the stable integration of the *cps3S* gene and production of type 3 pneumococcal polysaccharide. This discovery aims to confront the high vaccine production costs associated with pneumococcal vaccines.

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Abbreviations

Alum	Aluminium hydroxide gel
AP	Alkaline phosphatase
BAB	Blood agar base
6-BAP	6-benzylaminopurine
BCIP	5-Bromo-4-Chloro-3'-Indolyphosphate p-Toluidine Salt
BHI	Brain heart infusion broth
bp	base pairs
BSA	Bovine serum albumin
CD	cluster of differentiation
cfu	colony forming units
CPS	capsular polysaccharide
CRM_{197}	cross reactive material 197
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FCS	Foetal calf serum
FITC	fluorescein isothiocyanate
HRP	Horse Radish Peroxidase
Ig	Immunoglobulin
IPD	invasive pneumococcal disease
IPTG	Isopropyl-ß-D-Thiogalactopyranoside
KLH	Keyhole limpet haemocyanin
LPS	lipopolysaccharide
LT-B	B subunits of the heat labile toxin (LTB) of enterotoxigenic *E. coli*
M13	filamentous phage M13
M13 K07	wild-type filamentous phage M13
mAb	monoclonal antibody
MHC	major histocompatibility complex
MP	Messina/Palermo collaboration
MS	Murashige and Skoog media
NAA	α-napthalene acetic acid
NBT	Nitro-Blue Tetrazolium Chloride
NCTC	National Collection of Type Cultures, Central Public Health Laboratory, Colindale Avenue, London NW 9 5HT, UK
OMPC	outer membrane protein complex from *N. meningitidis*
PCR	polymerase chain reaction
PEG	Polyethylene glycol
PnCPS	pneumococcal capsular polysaccharide
R_i	Root inducing virulence plasmid
SDS	Sodium dodecyl sulphate
TBS	Tris Buffered Saline
TCA	Trichloroacetic acid
TCR	T cell receptor
TD	T-cell dependant antigen
T_i	Tumour inducing virulence plasmid
TI	T-cell independent antigen
TP	Time Point (days)
TU	Transducing units
Wt	Wild type
X-Gal	5-bromo-4-chloro-3-indolyl-ß-D-galactoside
X-Gluc	5-bromo-4-chloro-3-indolyl-ß-D-glucuronic acid

Symbols

ϕ	Phage
§	Section

CHAPTER ONE – INTRODUCTION

1.1 *Streptococcus pneumoniae*: The pneumococcus

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive, encapsulated, haemolytic, coccus of approximately 1 μm in length (Bannister *et al.* 1996; Kaltoft *et al.* 2000; Kamerling 2000). The *Streptococcus* genus comprises over 250 species (Bischoff *et al.* 2004), which include several known human pathogens. *S. pneumoniae* was first isolated more than 120 years ago (Sternberg 1881), yet today it remains a major cause of morbidity and mortality in both the developing and developed worlds (Lesinski *et al.* 2001). It is the causative agent of many types of infectious disease, including pneumonia, septicaemia, meningitis and otitis media (Magee and Yother 2001). This important pathogen has a significant impact in public health, as cases of invasive pneumococcal diseases (IPD) are substantially high, especially in developing countries and in individuals predisposed to infection, e.g. elderly, young, diabetic, asplenic, immunocompromised persons (i.e. those with impaired IgG synthesis, impaired phagocytosis, or defective clearance of pneumococci) (Mitchell and Andrew 1995).

The pneumococcus is reportedly the most widely studied microorganism in medicine, biology and chemistry (Kamerling 2000). One reason for this is because an investigation performed in the late 1920s showed that an unencapsulated strain could be transformed to an encapsulated strain (Griffith 1928). This transformation later proved that DNA was the genetic material (Avery *et al.* 1944).

Streptococcus pneumoniae possesses a capsule of high molecular weight polysaccharides which is situated external to the cell wall (see Figure 1.1) (Kamerling 2000). These polysaccharides form a highly hydrated shell around the bacterium (Kamerling 2000), which provides protection against phagocytosis by host cells and is important in determining virulence (see §1.2).

1.1.1 Serotypes

There are currently over 90 chemically distinct capsular types (Henrichsen 1995), although not all of them cause fatal infections. Differences in the composition of the capsular types is thought to be responsible for the alteration in virulence, although the reason for this remains unknown (Kamerling 2000).

Each serotype is defined by unique antigenic determinants on the capsular material, which can be used to confirm the serotype of a strain. Studies conducted at the beginning of the twentieth century showed that humoral immunity against pneumococcal capsular polysaccharide (PnCPS) protected against the experimental infection, and that protection was specific for each serotype (Griffith 1928; MacLeod *et al.* 1945). The serotyping technique, known as the Quellung (Neufeld) reaction, causes pneumococci to appear to swell in the presence of specific antiserum (Heineman 1973). The Quellung reaction can be used to conduct epidemiological studies, to associate certain serotypes to their diseases for example, or for determining which serotypes are the most prevalent in certain areas (see §1.3), thus aiding the decision of which serotypes to include in the preparation of a vaccine.

There are two different systems of nomenclature for the pneumococcal serotypes, namely the Danish system and the American system (Kamerling 2000). In the American system serotypes are numbered sequentially, based on the order of discovery. The alternative Danish system classifies serotypes based on cross-reaction between the different serotypes, so that serologically cross-reactive types are assigned to a common serogroup (Kamerling 2000). This is the system that will be used throughout this thesis.

As suggested above, some serotypes are antigenically related to each other and share some degree of cross-reactivity. These are classified in serogroups, for example, 9A, 9L, 9N and 9V all belong to serogroup 9. Serotypes that do not share a close antigenic relationship to other serotypes are given numbers only, for example, types 1, 2, 3, 4, etc. The extent of cross-reactivity between serotypes within the respective groups may differ. For example, types 6A and 6B are extensively cross-reactive. The two capsules have identical chemical compositions except for one of the bonds between two sugars (Robbins *et al.* 1983a; Henrichsen 1995). In contrast, types 19F and 19A are less cross-reactive (Penn *et al.* 1982). This has been illustrated in a chinchilla model of otitis media in which a tetravalent polysaccharide vaccine composed of types 6B and 19F (and types 14 and 23F) conjugated to OMPC (see Abbreviations) conferred protection against types 6A, 6B and 19F but not 19A (Giebink *et al.* 1996).

In addition to the capsular polysaccharide, the pneumococcus produces two common antigenic polysaccharides: a teichoic acid (C-polysaccharide) and a lipoteichoic acid

(Forssman antigen; F-antigen) (Kamerling 2000; Tuomanen and Masure 2000). These, along with several other pneumococcal surface components, for which roles in virulence and/or elicitation of protection have been established, are shown in Figure 1.1.

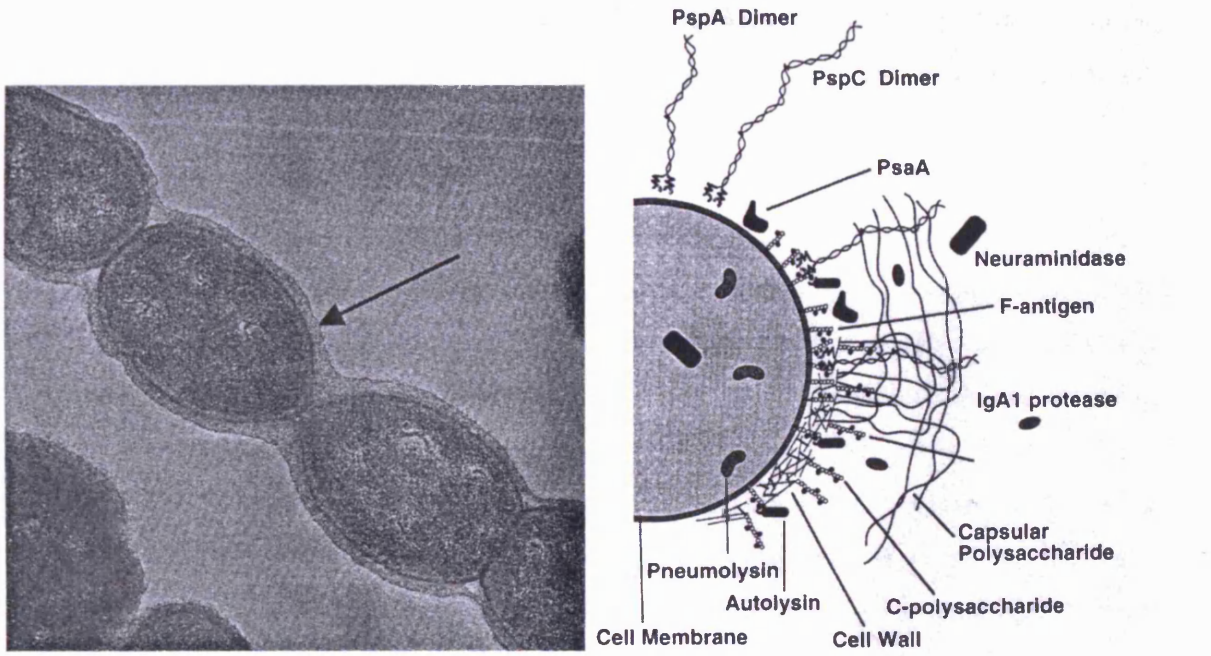

Figure 1.1. (A) The structure of *Streptococcus pneumoniae*. A chain of an encapsulated strain of *S. pneumoniae*. The arrow identifies the capsular polysaccharide (the low-density layer enveloping the bacterium). This was taken from (Tuomanen and Masure 2000). **(B) The surface components and virulence factors of *S. pneumoniae*.** A diagrammatic representation of *S. pneumoniae* depicting several surface components for which roles in virulence and/or elicitation of protection have been established. This was modified from that of Briles *et al.* (2000).

1.2 The role of the polysaccharide capsule in the virulence of *S. pneumoniae*

Numerous proteins have been shown to play an important role in the virulence of *Streptococcus pneumoniae*. These include pneumococcal surface protein A (PspA) neuraminidase, pneumolysin, and autolysin (Paton *et al.* 1993) (see Figure 1.1b). Mutations in the genes encoding any one of at least three of these proteins (pneumolysin, autolysin, and PspA) have been shown to significantly reduce virulence of the bacterium (Paton *et al.* 1993). However, some researchers believe that the polysaccharide capsule is the single most important virulence factor of *S. pneumoniae* (Hardy *et al.* 2001). This is because unencapsulated, or rough pneumococcal strains have been shown to be completely avirulent (Griffith 1928).

The polysaccharide capsule has many functions that benefit the pneumococcus. It is sufficiently permeable to modulate the passage of molecules, ions or even large proteins to the bacterial cell envelope, but prevents dehydration, protects against bacteriophage infection (Kamerling 2000) and is thought to aid in the adherence of pneumococci to biological (Magee and Yother 2001) and inanimate surfaces (Jones 1998; Tuomanen and Masure 2000). Its most important function is to inhibit phagocytosis by particular host cells (Kamerling 2000). Phagocytosis is the ingestion of particulate foreign material by a polymorphonuclear leukocyte (such as a neutrophil or macrophage). An invading pathogen is identified by a receptor on the surface of the phagocyte (see §1.2.1), which proceeds to engulf the pathogen by endocytosis. Once internalised it is contained in a vesicle (phagosome), which fuses with one or more lysosomes to form a phagolysosome. The lysosomal enzymes then break down and destroy the microbe (Janeway *et al.* 1999).

1.2.1 Mechanisms of evasion of host cell phagocytosis

Phagocytes can identify foreign cells in several ways. Neutrophils, for example, possess several receptors (the mannose receptor, the glycan receptor and CD14) that bind bacterial cell wall components directly (Wood 2001). Encapsulated pneumococci are thought to avoid non-specific phagocytosis because their surface has a negative charge (Van Oss and Gillman 1973). When confronted with an equally negatively charged phagocytic receptor (Van Oss and Gillman 1973) this is thought to result in mutual repulsion (Moxon and Kroll 1990). However, this has not been thoroughly investigated, since, in practice, the

measurements needed to test the physical chemistry involved have been difficult to make (Moxon and Kroll 1990).

The host attempts to phagocytose the encapsulated pathogen do not end there. Bacterial surface polysaccharides are thought to activate the complement system by binding to mannan-binding lectin (MBL) (Ezekowitz 2003). MBL is constitutively present in serum, even before the development of a specific antibody response. It binds a broad spectrum of polycarbohydrate hexoses, such as mannose, glucose, fucose and N-acetylglucosamine groups present in glucans, lipophosphoglycans and glycoinositol-phospholipids on the surface of pathogens (Epstein *et al.* 1996) and once bound, it is thought to activate the complement cascade via the most recently characterised system of complement activation, the lectin pathway (Ezekowitz 2003). The complement cascade results in the production of the complement protein C3b, which can penetrate the polysaccharide capsule without difficulty, reaching and binding to the bacterial cell wall beneath (Roberts *et al.* 1989). Complement receptors (CRs), such as CR1 and CR3, on the surface of phagocytes then bind the bacterial-bound C3b to initiate phagocytosis of the pathogen (Roberts *et al.* 1989). However, the pneumococcal capsular polysaccharide layer is very thick and cell wall bound C3b molecules can be masked or hidden by the capsular polysaccharide. This prevents engagement of the complement receptors on the phagocyte, which are unable to initiate phagocytosis (Roberts *et al.* 1989).

1.2.2 Control of pneumococci requires anti-capsular IgG

The most effective host defence against *S. pneumoniae* is opsonisation (coating) of the polysaccharide capsule with type-specific antibody (Lee *et al.* 1996). Although, some pneumococcal protein antigens have been shown to be protective without stimulating opsonisation, pneumolysin, for example (Ogunniyi *et al.* 2001). Opsonisation is dependant on the existence of anti-capsular IgG antibodies, which bind to and coat the pathogen at various sites. These could originate from prior exposure to the pathogen, from immunisation, or from the transmission of maternal antibodies (Janeway *et al.* 1999). Once bound, a conformational change occurs at the Fc portion of the antibody that allows it to be recognised by specific FcγRIIA (also known as CD32) receptors on the surface of the phagocyte (Stein *et al.* 2000). The interaction of these antibodies (now known as opsonins) also results in the activation of complement via the classical pathway, thus maximising

phagocytosis of the pneumococcus via both FcγRIIA and C3b receptors on the phagocyte (Roberts *et al.* 1989).

The presence of type-specific antibody is a therefore a major defence against pneumococcal infection. The passive administration of anti-capsular antibody has been shown to be effective at protecting both humans and animals against developing pneumococcal disease. This observation led to the development of pneumococcal vaccines targeting the capsular polysaccharide. This was because active immunisation is an effective means of producing anti-capsular IgG antibody. However, this was not an absolute solution. The first immunoglobulin class to be produced in response to an antigen and the first to be synthesised by a neonate is IgM, therefore a successful pneumococcal vaccine must induce class switching to IgG in all individuals. This has proved to be a difficult task, since the best vaccine candidate, the capsular polysaccharide, is a T-independent antigen, which produces a predominantly IgM response and also stimulates poor immunological memory. This is explained further in §1.4.3.

1.3 Epidemiology

The epidemiology of pneumococcal infection and disease is an important consideration for vaccine development, since, as will be discussed, it influences vaccine composition.

1.3.1 Carriage

Like many microorganisms, carriage plays an important role in the aetiology of pneumococcal disease. The nasopharynx is an ideal location for the pneumococcus to spread to new hosts (by droplet secretions) and it is presumed that all invasive serotypes are carried, at least transiently, prior to invasion (Barthelson *et al.* 1998; Dagan *et al.* 2004). The pneumococcus is commonly isolated from the human nasopharynx where asymptomatic colonisation can occur shortly after birth (Austrian 1986). The scale of colonisation can differ between individuals, although up to 40 % of the general population are thought to carry it in small numbers (Austrian 2000; Tuomanen and Masure 2000). Studies have shown that as many as four different capsular serotypes maybe carried simultaneously during childhood (Austrian 2000; Tuomanen and Masure 2000). In certain environments, particularly closed environments such as military barracks, individuals have been shown to be colonised with as many as eight different capsular types over a period of

10 weeks (Hodges *et al.* 1946). Colonisation or carriage is therefore influenced by the individual's exposure to one or more of the 90 pneumococcal types. Therefore, it comes as no surprise that the serotypes that account for the majority of nasopharyngeal isolates in children are included in the pneumococcal vaccines (see §1.4.2) (Hausdorff *et al.* 2005). However, if an individual develops antibodies to a particular serotype after colonisation this does not eliminate the carrier state (Austrian 2000). In contrast, individuals that develop antibody to a given pneumococcal capsular type prior to colonisation are half as likely to be colonised with that serotype (MacLeod *et al.* 1945). Therefore, early immunisation is the best defence. This is not a solution for infants, the group most at risk from pneumococcal disease, as they do not readily raise an immune response to capsular polysaccharides (see §1.4.2.3).

1.3.2 Disease

As stated earlier, the diseases caused by *S. pneumoniae* are a major cause of morbidity and mortality throughout the world (Gilbert *et al.* 2000). Table 1.1 shows a summary of the incidence of IPD worldwide and in the United States. Actual accounts of the incidence of IPD and the distribution of particular serotypes within a population can change due to age, time and geographically. This is discussed in the following sections.

Table 1.1 The Annual Incidence of Pneumococcal Disease

I. Worldwide
- A. Pneumonia
  1. Estimated number of cases: 20, 000, 000
  2. Estimated number of deaths: 1, 050, 000
- B. Meningitis
  1. Estimated number of deaths: 75, 000
- C. Total pneumococcal deaths: 1, 125, 000 (9 % of all childhood deaths)

II. United States
- A. Pneumonia - estimated number of cases 500, 000
- B. Meningitis - estimated number of cases 3, 000
- C. Bacteraemia - estimated number of cases 50, 000
- D. Otitis Media - estimated number of cases 7, 000, 000

Taken from Klein (2000)

1.3.3 Age

The World Health Organization estimates that at least 1.2 million children die of pneumococcal disease each year (World Health Organisation 1996). In developing

countries Pneumonia is the main cause of death in young children and overall diseases caused by *S. pneumoniae* account for 30 % of deaths in children less than 5 years old (Lesinski *et al.* 2001). In Europe, it is estimated that the pneumococcus is responsible for 25 % to 50 % of bacterial meningitis in children (Connolly and Noah 1996) and in the USA cases of invasive pneumococcal disease, including meningitis and bacteraemia, are estimated to be as high as 160 cases per 100,000 in children less than two years of age (Advisory Committee on Immunization Practices 1997).

As shown in Figure 1.2, the number of IPD cases in infants can vary by age. A study performed on 806 invasive pneumococcal isolates from Danish children aged between 0 - 23 months during 1981 - 1999 showed that children were most at risk of developing pneumococcal meningitis at 10.2 months old, and bacteraemia at 15.9 months old (Kaltoft *et al.* 2000). In the UK, surveillance data during 1996 - 1998 showed that the incidence of invasive and other pneumococcal disease was highest among children during the first year of life (Miller *et al.* 2000). The reason for the higher susceptibility of IPD in infants is discussed in §1.4.2.3

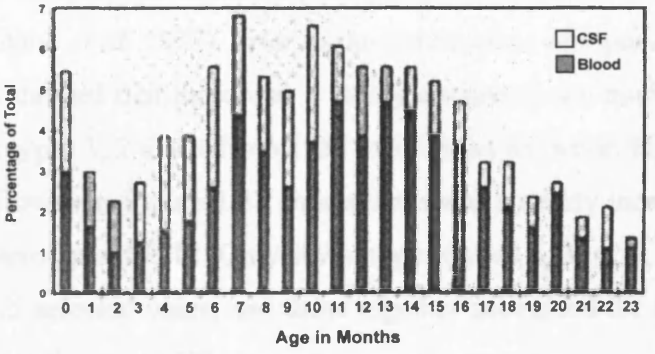

Figure 1.2. Age distribution of 806 invasive pneumococcal isolates from Danish children ages 0-23 months during the period 1981-1999. The percentage of total by site of isolation is also displayed. Modified from Kaltoft *et al.* (2000)

Old age is also a risk factor for infection, although the reason for this is not as well understood as for infants (Artz *et al.* 2003). Reported annual incidences of IPD among the elderly in North America and Europe range from 25 to 90 cases/100 000 persons (Butler and Schuchat 1999). Mortality caused by pneumococcal infections is highest among those aged >65, with nearly 20 % cases resulting in death (Butler and Schuchat 1999). This has

been shown to rise to as much as 40 % for those aged 85 years or older (Artz *et al.* 2003). This threatens to become a critical public health issue, since the elderly population is expected to triple by 2050 (Artz *et al.* 2003).

1.3.4 Time

The distribution of specific serotypes implicated in disease can also vary with time. During the winter months the number of cases of pneumococcal disease has been shown to increase. This is thought to be because pneumococci are usually transmitted by droplet secretions, from person to person (O'Brien and Nohynek 2003) and during these months the course of other respiratory infections and secretions, coughing and sneezing are increased (Kaltoft *et al.* 2000). Kaltoft and colleagues investigated the seasonal distribution of 777 episodes of pneumococcal bacteraemia and 346 episodes of pneumococcal meningitis in Danish children aged 0 - 6 years old during 1981 – 1999. They found that there were significantly fewer episodes of invasive pneumococcal infections during the summer months (June, July and August) when respiratory infections overall are low compared with the rest of the year (Kaltoft *et al.* 2000).

In long-term studies the serotypes most frequently implicated in disease has been shown to vary over years (Finland *et al.* 1977). Prior to the introduction of a pneumococcal vaccine, Finland and Barnes showed that there was a steady decline in the number of bacteraemic cases caused by serotypes 5, 2 and 1 from 1935 to 1974 (as shown in Figure 1.3a) (Finland and Barnes 1977). However, Figure 1.3b shows there was a steady increase in the number of cases caused by serotypes 14, 18. Only seven types (types 1, 3, 4, 7, 8, 14 and 18) were found in all of the 15 selected years, and these together accounted for 60 % of the strains identified (Finland and Barnes 1977).

1.3.5 Geography

The incidence of IPD can also vary geographically. There is an unequal distribution of pneumococcal serotypes causing disease throughout the world. For example, in Europe, the literature cited suggests that Italy has the lowest incidence with 1.1 reported cases per 100 000 population (Principi and Marchisio 2000) and Greece has the highest incidence with 100/100 000 (Syriopoulou *et al.* 2000). Denmark and the UK have 16.8/100 000 (Kaltoft *et al.* 2000) and 6.6/100 000 (Miller *et al.* 2000) reported cases of IPD respectively. The

incidence and differences in the prominent serogroups isolated from children under 5 years old are illustrated in Table 1.2.

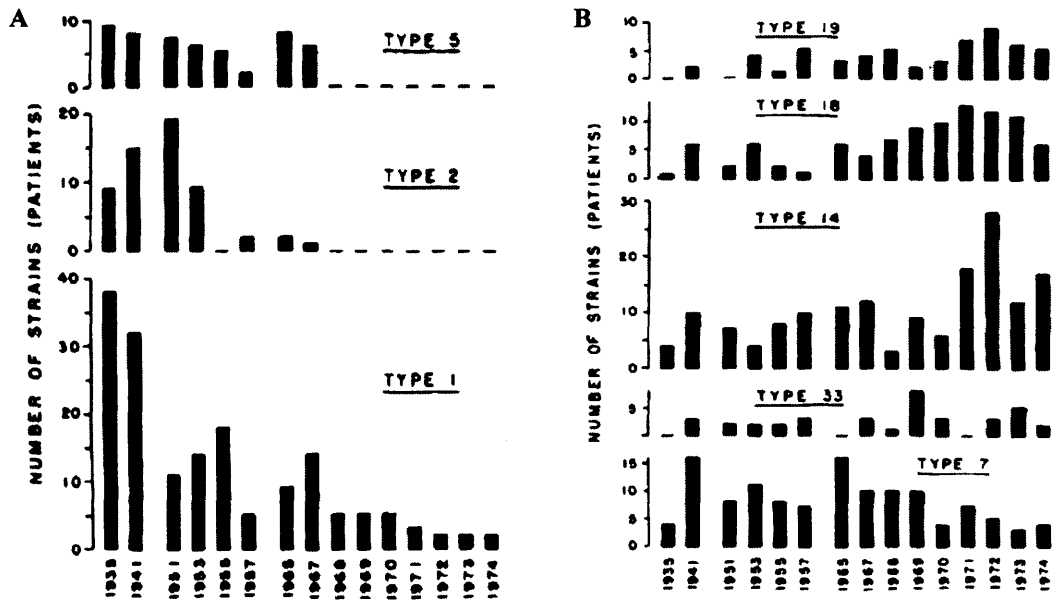

Figure 1.3. Occurrence of *S. pneumoniae* types 1, 2, 5, 14, 18, 19, and 33 in bacteraemic patients at Boston City Hospital in 15 selected years between 1935 and 1974. Taken from Finland *et al.* (1977)

Table 1.2. Geographic distribution of the most prevalent pneumococcal serotypes in Europe. The five most prominent serogroups isolated from children under 5 years old are described (incidence is described in parentheses).

Country	*Rate/1000 for IPD*	*Predominant serotypes*	*Reference*
Greece	(100/100 000)	**14, 19, 6, 18, 23**	(Syriopoulou *et al.* 2000)
Denmark	16.8/100 000	**1, 4, 6, 7, 9, *14***	(Henrichsen and Nielsen 2000; Kaltoft *et al.* 2000)
The Netherlands	8.2/100 000	**3, 6, 7, 9, 14**	(Spanjaard *et al.* 2000)
UK	6.6/100 000	**14, 19, 6, 18, 1**	(Miller *et al.* 2000)
Sweden	5.8/100 000	**9, 19, 51, 6, 23**	(Eriksson *et al.* 2000)
Italy	1.1/100 000	**14, 6, 23, 1, 4**	(Principi and Marchisio 2000)
Spain	N/a	**6, 19, 23, 1, 14**	(Fenoll *et al.* 2000)

n/a, not available

These differences are thought to be due to the location of particular ethnic backgrounds. For example, persons in certain racial groups, including African-Americans, American Indians, Native Alaskans and Australian Aborigines, have all been shown to have an increased risk of disease (Butler and Schuchat 1999). A person's socio-economic status is also said to be a risk factor (Butler and Schuchat 1999). Alternatively, these differences could be due to the differences in carriage (see §1.3.1), and the virulence of the predominant serotypes, since different capsular serotypes are known to differ in virulence. Serotype 3, for example, requires 1 cfu to kill 50 % of mice and rats, whereas serotype 37, which possesses a capsule composed of similar sugars and is the same size as serotype 3, requires 10^7 cfu to cause the same effect (Knecht *et al.* 1970). A 10 -year (1952 - 1962) study at a New York medical centre showed that 56 % of all deaths due to pneumococcal pneumonia were only caused by six serotypes (Austrian and Gold 1964). The pneumococcal vaccine, Pneumovax, is composed of 23 capsular serotypes and is designed to provide coverage of approximately 90 % of the most frequently reported serotypes from the US, Europe and East Africa (see §1.4.2.2 for more details).

These observations have led to the suggestion that geographic- and age-specific vaccine formulations should be manufactured (Austrian 1985; Lee 1987) but unless relatively low cost solutions are found, this aim will not become a reality.

1.4 Treatment

1.4.1 Antibiotics

In the 1960s, penicillin was fully effective against nearly all strains of *S. pneumoniae*, to such an extent that the surgeon-general at the time, William Stewart, felt confident enough to declare: 'The time has come to close the book on infectious diseases. We have basically wiped out infection in the United States' (Anonymous 2001). Even as he spoke, and increasingly, strains were being isolated that were resistant to not only penicillin, but also to erythromycin, macrolides, clindaycin and the quinolones (Baquero *et al.* 1991). A 3-year multi-centre surveillance study in the United States showed that nearly 20 % of strains (ranging from 13 % to 24 % between centres) isolated from IPD cases in children were resistant to penicillin and 7 % (ranging 0 % - 13 %) were resistant to ceftriaxone (Arditi *et al.* 1998). These authors also noted that the number of penicillin resistant strains and ceftriaxone resistant strains had increased over the same period (1993-1996) (Arditi *et al.*

1998). Vancomycin used to be the last resort for the treatment of pneumococcal infections (because of dosing and tissue penetration issues) but, worryingly, strains resistant to vancomycin have also started to emerge (Novak *et al.* 1999). This can lead to dramatic treatment failure in some patients with pneumococcal infections. But what is the alternative to treatment? Traditionally, preventive measures, such as the use of vaccines are considered to be a relatively inexpensive and effective alternative approach to reduce the incidence of pneumococcal disease (Andrew *et al.* 1994).

1.4.2 Pneumococcal vaccines

As discussed in §1.2.2, anti-capsular IgG controls pneumococcal disease by opsonising the bacterium in preparation of host cell phagocytosis. Currently, immunisation is the only available practical tool to develop anti-capsular IgG, since antibodies are too expensive and difficult to administer (World Health Organisation 1999). Immunisation therefore aims to elicit antibodies against the polysaccharide capsule of the bacterium (Janeway *et al.* 1999).

1.4.2.1 "a specific soluble substance"

Carbohydrate vaccine research began in 1917 when Dochez and Avery stumbled across what they referred to as a 'specific soluble substance' secreted by pneumococci during growth (Dochez and Avery 1917). They had discovered pneumococcal capsular polysaccharide and soon after it was found that immunisation with purified capsular polysaccharide provided serotype-specific protection, in mice and humans (MacLeod *et al.* 1945; Jennings 1990).

The impact of this discovery was not limited to *S. pneumoniae* and, since then, capsular polysaccharides have formed the target antigen for vaccines against organisms such as Group B Streptococcus, *Haemophilus influenzae* (Anderson *et al.* 1972), and *Neisseria menigiditis* (Frasch 1995). Yet it is not a vaccine strategy without its limitations.

1.4.2.2 Pneumococcal polysaccharide vaccines

Prior to the introduction of a pneumococcal vaccine, the protective efficacy of six and twelve valent vaccines (composed of purified capsular polysaccharide) was investigated in controlled studies of gold miners in South Africa, in whom there was a high attack rate for pneumococcal pneumonia (Smit *et al.* 1977). The number of cases caused by those

serotypes included in the vaccine was observed for the period from 2 weeks to about 1 year after immunisation. The rates for pneumonia caused by the capsular types included in the vaccine decreased from 38.3/1000 to 9.2/1000 for the 6-valent vaccine and from 22/1000 to 1.8/1000 for the 12-valent vaccine (as shown in Table 1.3) (Smit *et al.* 1977). This shows that increasing the number of capsular types included does not decrease the overall protective efficacy of the vaccine.

Table 1.3 The rate of pneumonia cases in South African gold miners following immunisation

Number of Capsular serotypes in Vaccine	*Rate/1000 for Pneumonia caused by Homologous Capsular serotypes*		*Protective Efficacy*
	Immunised Groups	*Control Groups*	
6	9.2	38.3	76%
12	1.8	22.0	92%

Taken from Smit *et al.* (1977)

In 1989 a commercial vaccine (Pneumovax, Pasteur Merieux, France) containing capsular polysaccharide from 23 of the most prevalent or invasive pneumococcal serotypes in the US and Europe (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F, 33F), was licensed for use in the United Kingdom (Kamerling 2000). It is given mainly to people over the age of 50 and to those with chronic heart, lung, or liver disease. It costs the National Health Service (NHS) £9.49 per dose (HTML1 2002).

Pneumovax consists of 25 μg of highly purified capsular polysaccharide from each serotype (Baxendale *et al.* 2000). Despite the vaccine representing only 23 of the 90 different pneumococcal serotypes, and that protection is serotype-specific, this vaccine covers almost 90 % of the infections caused by *S. pneumoniae* in the United States (Kamerling 2000). However, as discussed earlier, large variations in the incidence of infections caused by particular pneumococcal serotypes can exist due to age and in different locations vaccine coverage can be considerably less (see §1.3) (Jennings 1990). Some studies have shown coverage rates among adults predisposed to infection (see §1.4.2.3) ranged from 11.8 % (aged 18 - 49 years) to 20.1 % (aged 50 - 64 years) in 1995

(Singleton *et al.* 2000), and remained low (19.1 %) among persons aged 18 - 64 years in 2002 (Willis *et al.* 2005).

Unfortunately, the Pneumovax vaccine does not provide adequate protection to children <2 years of age (who are at greatest risk of disease), in addition to immunocompromised patients and the extreme elderly (Klein 2000). Even in healthy adults, this vaccine only offers a protective efficacy of 60 - 70 % and, as explained in §1.4.2.3, this is even less in young children (World Health Organisation 1999). Furthermore, a gradual decline in antibody titre can be detected one year after a single immunisation of healthy young adults (Mufson *et al.* 1987). After five years antibody titres are about 75 % of their peak value and ten years after, the anti-capsular antibody remained high for only two out of six serotypes tested (Mufson *et al.* 1987). As a result, the Pneumovax vaccine has proved to be ineffective for the most at risk groups, and infections with *S. pneumoniae* continue to cause significant morbidity and mortality worldwide.

This lack of responsiveness among various high-risk groups is due to their inability to mount a T-cell dependent immune response to free polysaccharide antigens. This is discussed in §1.4.2.3.

1.4.2.3 Polysaccharides are T-independent antigens

Antigens can be classified as T cell dependant (TD) or T cell independent (TI) depending on the host's response (Lesinski and Westerink 2001). TD antigens (proteins or peptides) produce an immune response that involves T cells and results in immunological memory. The protein antigen enters the cell (B cells, macrophages, and other antigen presenting cells) by phagocytosis, where it is degraded into peptides. Some of these peptides will associate with class II MHC molecules (see §1.5.1.4), which transports the bound peptide to the cell surface where the antigen is presented to helper T cells (Wood 2001). In response, cytokines released by the T cell help B cells proliferate and differentiate into antibody producing cells and memory B-cells (Wood 2001). Consequently, a strong antibody response against the protein antigen/infection is provoked. Helper T cells also possess a CD154 receptor, which binds CD40 on the B cell to switch to the production of an alternative class of antibody (Wood 2001), such as IgG.

There are two classes of T-cell independent (TI) antigen, TI-1 and TI-2, both of which induce a humoral response without the involvement of T cells (Lesinski and Westerink 2001). TI-1 antigens, such as bacterial lipopolysaccharides, are potent B-cell mitogens, which directly induce non-specific polyclonal activation of B cells (Lesinski and Westerink 2001). TI-2 antigens, such as pneumococcal capsular polysaccharides, do not function as B cell mitogens but instead activate mature B cells by cross-linking surface exposed immunoglobulins (as shown in Figure 1.5) (Lesinski and Westerink 2001). The mechanism for immune responses to TI-2 antigens is not completely understood, but it is thought they provide a strong stimulus to activate the B cell to become an antibody secreting plasma cell in the absence of T cell help (Eales 1997; Lesinski and Westerink 2001). However, as helper T cells are not involved, there is no antibody class switch or affinity maturation and only low affinity IgM is produced (Klein 2000). IgM is very efficient at fixing complement and this can provide protection against infection, but it does not provoke immunological memory or opsonise bacteria.

As stated previously in §1.4.2.2, infants cannot raise an antibody response to polysaccharide antigens efficiently. This is thought to be because TI-2 antigens only activate mature B cells and infants only have a repertoire of immature B cells (Eales 1997). Another reason for the high incidence of IPD in children could be because they have yet to develop a particular subset of B cells, known as B-1 cells (or CD5 B cells). These cells respond to several TI antigens and antibodies that are of B-1 cell origin and are reported to provide optimal protection from virulent pneumococcal infection (Shaw *et al.* 2000). B-1 cells secrete high levels of anti-capsular polysaccharide IgM antibody upon primary response to the antigen, which are thought to act as innate opsonins (see §1.2.1). However, their precise role in host defence against *S. pneumoniae* remains uncertain and, although B-1 cells arise early in development, they do not make a fully effective response to carbohydrate antigens until about 5 years of age (Eales 1997).

To circumvent the problems associated with TI antigens, major research efforts have focussed on producing a vaccine in which the polysaccharide is chemically conjugated to a protein (Klein 2000).

Table 1.4 A summary of the characteristics of a T-independent immune response

Produces a disproportionate amount of IgM antibody
Is unable to provoke immunological memory and therefore cannot induce a booster response upon repeated immunisations
Produces an immune response which is short lived
Fails to produce high affinity antibody

Taken from Klein (2000)

1.4.3 Polysaccharide-protein conjugate vaccines

Once protein and polysaccharide are conjugated, the polysaccharide acquires the immunogenic character of the protein carrier and is converted to a T-dependant (TD) antigen (Lesinski and Westerink 2001). This concept dates back to 1929 when Avery and Goebel conjugated *S. pneumoniae* serotype 3 polysaccharide to horse serum globulin (Goebel and Avery 1931). They noticed that immunisation with this conjugate was able to induce polysaccharide-specific antibody in rabbits previously unresponsive to the pure polysaccharide (Goebel and Avery 1931). It is thought that polysaccharide-specific B cells internalise the conjugate. Proteolysis of the carrier protein produces peptides that bind to class II MHC molecules and activate helper T cells (see Figure 1.4) (McCool *et al.* 1999). As a result, the B cells can mature to antibody producing plasma cells or into memory cells (Lesinski and Westerink 2001).

Polysaccharide conjugate vaccines have been introduced for *Haemophilus influenzae* type b, *Neisseria meningitidis* serogroup C and *S. pneumoniae.* A brief review of the successes following their introduction is discussed in the following sections. It should be noted that conjugate vaccine developments against Group B Streptococci, *Salmonella typhi*, *E. coli*, *Shigella sonnei* and *Shi. flexneri* are also underway.

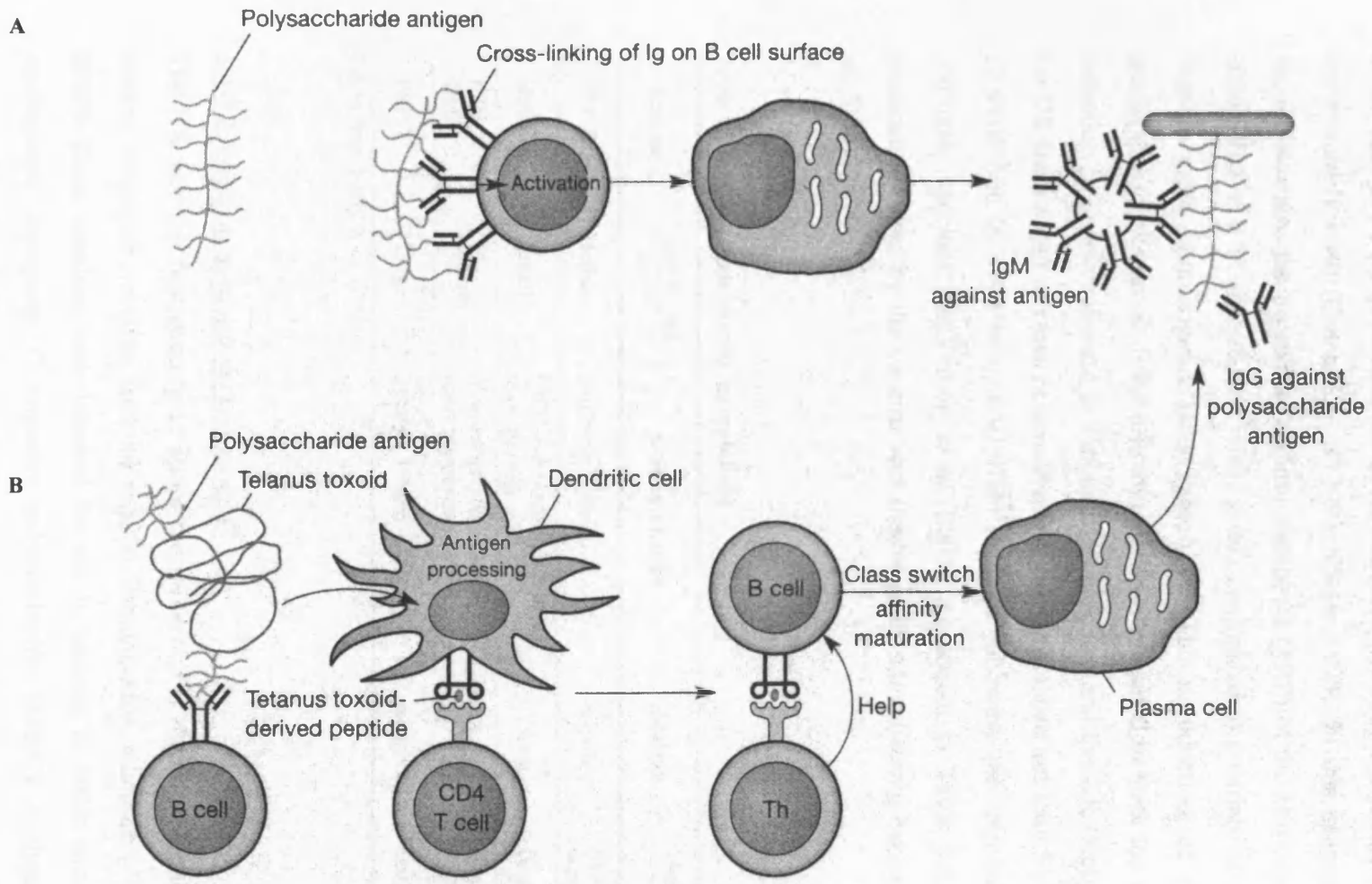

Figure 1.4. The immune responses to polysaccharide antigens (A) The typical TI-2 immune response to a pneumococcal capsular polysaccharide antigen. **(B)** If it is conjugated to a protein such as diptheria or tetanus toxoid the polysaccharide acquires the immunogenic character of its carrier and is converted to a T-dependant (TD) antigen. Taken from Wood (2001).

1.4.3.1 *Haemophilus influenzae* type b

The first ever polysaccharide-protein conjugate vaccine used in humans was against *H. influenzae* type b (Hib) (infections with other serotypes of *H. influenzae* (types a, c, d, e, f) are extremely rare) (Granoff *et al.* 1993; Cadoz 1998). In this example, the conjugate vaccine contains the polyribosyl ribitol phosphate (PRP) of the Hib capsule (Kelly *et al.* 2004). PRP is a TI antigen, but when it was conjugated to a variety of carrier proteins it became more immunogenic (see Table 1.5). The introduction of the PRP vaccine conjugated to tetanus or diphtheria toxoids were estimated to have led to a 90 to 100 % reduction in invasive disease in Finland, USA, Africa and the UK (Kelly *et al.* 2004). In the UK the number of cases of invasive Hib disease in those less than 5 years old fell from 22.9/100 000 in 1990 to 0.65/100 000 in 1998 following the introduction of the Hib conjugate vaccines (McVernon *et al.* 2003). As shown in Table 1.5, the duration of protection offered by the vaccine was improved by administering booster doses (Kelly *et al.* 2004).

Table 1.5 Hib conjugate vaccine formulations

Vaccine	*Polysaccharide size*	*Carrier protein*	*Linkage*	*Antibody response in infancy*
PRP-D	Medium	Diptheria Toxoid	6-carbon	Moderate, after 2nd dose
HbOC	Small	CRM_{197} mutant *C. diptheria* toxin protein	None	Good, after 2nd dose
PRP-OMP	Medium	*N. meningitidis* protein outer membrane complex	Thioether	Moderate, after 2nd dose
PRP-T	Large	Tetanus Toxoid	6-carbon	Good, after 2nd dose

Taken from Kelly *et al.* (2004)

1.4.3.2 *Neisseria meningitidis* serogroup C

The UK was the first country to adopt the serogroup C meningococcal polysaccharide–protein conjugate vaccines into its routine immunisation schedule (Snape and Pollard 2005). Three vaccines were licensed for use in humans in 2000: two consisting of *N. meningitidis* serogroup C capsular polysaccharide (MenC) conjugated to CRM_{197} (produced by Wyeth Vaccines and Chiron Vaccines) and one conjugated to tetanus toxoid conjugate (produced by Baxter) (Jennings 1990; Snape and Pollard 2005). Together these resulted in a 81 % reduction in the number of confirmed cases of invasive meningococcal

C disease, from 537 cases in July 1998 to April 1999 to 103 in the equivalent period from 2001 to 2002 (Miller *et al.* 2002).

However, concerns that immunisation against MenC alone may result in an increase in meningococcal disease due to non-C serogroups have arisen (Snape and Pollard 2005). Of the 9 known serogroups of *N. meningitidis* capsular polysaccharide, the majority of meningococcal strains associated with epidemics or outbreaks are in groups A and C (Lesinski and Westerink 2001). Therefore, combination conjugate vaccines (containing A, C, Y, and W polysaccharides) are thought to be the way forward. Clinical trials are currently underway, and it is likely these broadly protective vaccines will become available in the near future (Snape and Pollard 2005). A bivalent menAC polysaccharide-protein conjugate has already been developed (Anderson *et al.* 1994; Twumasi *et al.* 1995). However, no vaccine has yet to be effective against group B polysaccharide, even after conjugation to a carrier (Lesinski and Westerink 2001).

1.4.3.3 *Streptococcus pneumoniae*

As stated previously, Avery and Goebel pioneered the concept of polysaccharide conjugate vaccines back in 1929, by conjugating serotype 3 PnCPS to horse serum globulin (Goebel and Avery 1931). However, the coupling procedures they used (diazotization of *p*-aminobenzyl ether substituents on the polysaccharide) were too drastic to be used on some of the highly sensitive polysaccharides currently used in human vaccines (Jennings 1990) and so it wasn't until very recently that a licensed pneumococcal conjugate vaccine (PCV7) became available. This vaccine, known as Prevenar (PCV7) (Wyeth-Lederle Vaccines and Paediatrics), consists of 7 pneumococcal capsular polysaccharides (serotypes 4, 6B, 9V, 14, 18C, 19F, 23F) conjugated to a non-toxic variant of diptheria toxoid (CRM_{197}) (Kamerling 2000).

PCV7 is proving to be success (Brown 2001; Shields 2001; Kaplan *et al.* 2004). A 9-year surveillance study of all invasive infections caused by *S. pneumoniae* among children in the United States showed that the annual number of invasive pneumococcal infections for children <2 years of age declined by 66 % (from 1994 to 2002) following the introduction of PCV7 (Kaplan *et al.* 2004). The number of cases caused by those serogroups included in the vaccine declined further, by 77 % in 2002 (Kaplan *et al.* 2004).

The PCV7 vaccine has been shown to cover 80 – 90 % of childhood IPD in USA, Canada, and Australia (Hausdorff *et al.* 2005). In Europe and Africa the highest serogroup coverage reported was 75 %, and Latin America and Asia have reported coverages of approximately 65 % and 50 % respectively (Hausdorff *et al.* 2005). Other pneumococcal conjugate vaccines are being evaluated (Lesinski and Westerink 2001). Kaltoft and colleagues showed that including more serotypes (PCV7 plus serotypes 1 and 5) in the vaccine formula (PCV9) increased the coverage in Denmark from 61 % to 70 % in 0 - 6 year olds (Kaltoft *et al.* 2000). An 11-valent vaccine (PCV11, PCV7 plus serotypes 1, 3, 5, and 7F) further increased the coverage to nearly 80 % in USA, Canada, Australia, Europe, Africa, Latin America and Asia (Kaltoft *et al.* 2000; Hausdorff *et al.* 2005). Despite the success of increasing the valency it is though a pneumococcal conjugate vaccine including >11 individual serotypes is unlikely, due to antigenic competition (Lesinski and Westerink 2001).

1.4.4 Problems associated with pneumococcal conjugate vaccines

1.4.4.1 High cost

The introduction of the Hib conjugate vaccine to non-industrialised countries (Qatar, Kuwait, Chile, Uruguay) showed vaccine cost is a prominent issue in its uptake and the largest single obstacle to its wider use (Wenger *et al.* 1999). Three of the four countries investigated noted cost as the major factor in the decision making process (Wenger *et al.* 1999). The new 7-valent pneumococcal conjugate vaccine (PCV7) is the most expensive vaccine introduced to date. It costs £49 per dose (McIntosh *et al.* 2003) and 3 doses are required to obtain complete protection. Yet, it is those countries with the highest incidence of disease that are least able to afford vaccines and, importantly, the current vaccine may have limited potential in these countries, since the vaccine has been designed for the western market and the serotypes it covers may not be the most prevalent in developing countries.

The price of this vaccine is high due to the complicated and expensive production methods. These include the purification of polysaccharide, the need for multiple protein conjugations (see §1.4.4.2 for more details) and the quality control issues (including the removal of any toxins) (Klein 2000).

1.4.4.2 Serotype replacement

One problem associated with conjugate vaccines is the need for multiple conjugations. Increasing the coverage (including as many of the 90 different pneumococcal serotypes as possible) would further increase the end cost. But low serotype coverage increases the opportunity for serotype replacement (Mbelle *et al.* 1999). Kaplan and colleagues noted that after the introduction of PCV7, the number of isolates caused by the serogroups not included in the vaccine increased by 28 % in 2001 and by 66 % in 2002, in particular serogroups 15 and 33 (Kaplan *et al.* 2004). This means that eventually there would be a reduction in vaccine coverage in this area. An alternative solution would be to develop specific vaccine formulations and doses to accommodate different populations, age groups, and geographical areas (Klein 2000). However, this is also an expensive approach and there is no guarantee this would be as successful as PCV7, since conjugate vaccine antigens are not created equally; each conjugated antigen is a unique, separate vaccine with different immunological properties and the capsular polysaccharides may behave differently when conjugated to a larger protein and in the presence of many different antigens (Klein 2000).

Despite these difficulties, polysaccharide-protein conjugates remained the preferred approach for human immunisation against bacterial polysaccharide antigens. This thesis describes two alternative approaches: one to improve pneumococcal vaccine manufacture and another method to tackle the poor immunogenicity of pneumococcal polysaccharides. These approaches will be discussed in the next two parts of this chapter.

Part A: Peptide Mimicry

Mimic or mimotope?

The terms 'peptide mimic' and 'peptide mimotope' are used throughout this thesis. The classification depends on the immunological properties of the antigen. Peptides that mimic and compete with the native antigen for antibody binding will be referred to as mimics; if they are able to induce antibodies with the same biological activity (for example bacterial opsonisation) as the antibody used as template, they will be called mimotopes.

1.5 Novel vaccine strategies to polysaccharide antigens

As discussed in §1.4.2.3, pneumococcal capsular polysaccharide is a T-independent type 2 (TI-2) antigen, and is not an ideal vaccine candidate since it does not produce memory or require T cell help to elicit an immune response (Lesinski and Westerink 2001).

Antibodies directed against capsular polysaccharides protect against pneumococcal infection (Griffith 1928; MacLeod *et al.* 1945) and therefore the antigens they recognise are attractive vaccine candidates. So, to overcome the problems associated with its TI nature, vaccine strategies against capsular polysaccharides have focused on attempting to convert the TI-2 immune response to that of a TD immune response (Lesinski and Westerink 2001). These strategies include conjugating the protein to a carrier protein, which was discussed in §1.4.3, anti-idiotypic antibodies and peptide mimics of polysaccharide antigens (Lesinski and Westerink 2001). The latter two strategies use the concept of molecular mimicry, which will be discussed in the following sections.

1.5.1 Molecular mimicry

Nearly 30 years ago molecular recognition, the specific recognition of one molecule by another, was recognised as a major driving force of life (Manning 1976). Figure 1.5 illustrates the principle of molecular mimicry, which is thought to occur in three different ways: conformationally (the two antigens have the same shape), immunochemically (both antigens share identical patterns of antibody binding) and immunogenically (both antigens elicit a similar immune response) (Baxendale *et al.* 2000). Not surprisingly, it directs the discovery of prime targets for the pharmaceutical industry and biomedical research (Meloen *et al.* 2000). In particular, antibodies that recognise protective B- and T cell epitopes have been utilised to identify mimics of the native antigen.

As shown in Figure 1.5, an antibody-binding site only binds a small proportion of the total antigen molecule. In terms of proteins it recognises amino acid sequences of about 6 - 15 residues in length (Harlow and Lane 1988). Therefore it is not necessary to mimic the whole antigen, but instead a molecule representing the ligand involved in the recognition event could be used (Meloen *et al.* 2000). This means that discontinuous epitopes can also be mimicked (Meloen *et al.* 2000). The majority of interaction sites are discontinuous (i.e. epitopes located on different parts of the primary structure that are brought near each other

due to folds in the molecule). Anti-idiotypic antibodies, and peptide mimics of polysaccharide antigens have been produced by using molecular mimicry to linearise sequences of discontinuous epitopes recognised by mAbs specific for polysaccharide antigens (Lesinski and Westerink 2001). These types of vaccines (which will be discussed further in the following sections) have an advantage over polysaccharide-based vaccines since antibody molecules and peptides are TD antigens, and able to stimulate a memory immune response. This means that it could be possible to find a pneumococcal vaccine that can stimulate an anti-polysaccharide antibody production but is immunogenic like a protein.

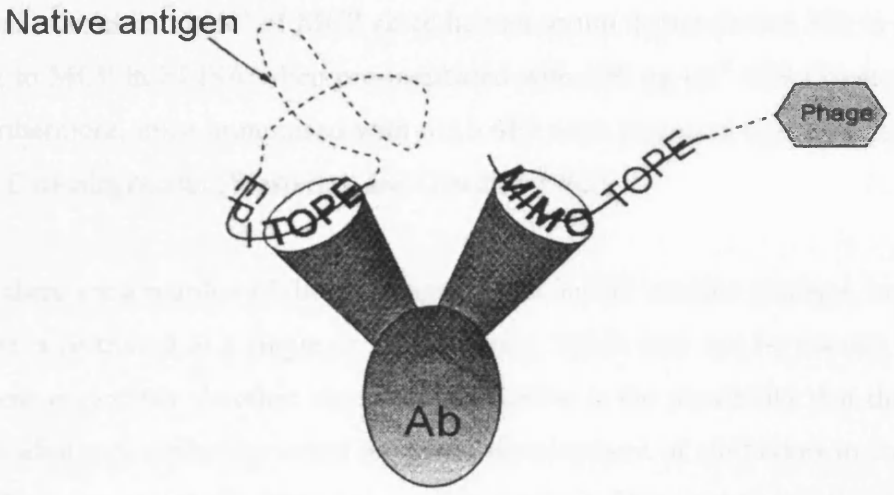

Figure 1.5. The principle of molecular mimicry. The native antigen (epitope) and the peptide (mimotope), here displayed on the surface of a filamentous phage, both occupy the same antigen-binding site of the antibody (Ab). MIMO therefore, mimics the structural characteristics of TOPE. This diagram was modified from that of Partidos (2000)

1.5.1.1 Anti-idiotype vaccines

The specific antigen-binding site of an antibody molecule, or its variable region, is often referred to as the idiotypic determinant (Id). Antibodies raised against a specific antibody idiotype (anti-idiotypes) have been shown to mimic the conformation and shape of a protective epitope of an infecting agent even if this is lipid, carbohydrate or glycolipid in nature (Brown *et al.* 1993). Therefore, if the native antigen protects against disease caused by the pathogen, it could be assumed that an anti-Id would display identical binding characteristics as the epitope and could elicit the same response upon immunisation. The first example of this biological phenomenon was reported in 1978 when Sege and Peterson

developed an anti-Id against insulin (Sege and Peterson 1978). They demonstrated that the anti-Id mimicked the biological actions of insulin, by regulating blood glucose level. They also showed that anti-Id mimics of insulin bound insulin receptors on rat intestinal epithelial cells and blocked the uptake of insulin by these cells (Sege and Peterson 1978). Since this discovery, anti-idiotypic antibodies have been, and are being used to identify immunogenic mimics of viral, bacterial and parasitic pathogens (Lesinski and Westerink 2001) including hepatitis B virus (Rajadhyaksha and Thanavala 1995), *Neisseria gonorrhoeae* (Gulati *et al.* 1996) and *Schistsoma mansoni* (Grzych *et al.* 1985). Westerink and colleagues developed an anti-idiotype monoclonal, known as 6F9, that mimicked meningococcal group C capsular polysaccharide (MCP) (Westerink *et al.* 1988). This mAb was defined as a 'true mimic' of MCP since human serum demonstrated 100 % inhibition of binding to MCP in ELISA when pre-incubated with 200 $\mu g\ ml^{-1}$ 6F9 (Westerink *et al.* 1988). Furthermore, mice immunised with mAb 6F9 were protected against infection with serogroup C meningococci (Westerink and Giardina 1992).

However, there are a number of disadvantages to the anti-Id vaccine strategy, in particular the vaccine is restricted to a single or few epitopes, which may not be enough to protect against some organisms. Another significant limitation is the possibility that the multiple use of anti-idiotypes antibodies could led to the development of antibodies to the constant region of the immunoglobulin (the hinge and Fc portion). This might prejudice subsequent immunisations, and antibody-antigen complexes of this region could lead to immunopathological damage (Mackett and Williamson 1995).

1.5.1.2 Peptide mimics

Peptide mimicry takes the principle of anti-Id vaccines (that antibodies recognise a molecular shape rather than a particular chemical formula (Kieber-Emmons 1998)) a step further. Like anti-Ids, peptide mimicry entirely substitutes the TI epitope with a TD one, and mimic the binding properties of native antigens (see Figure 1.5). However, unlike anti-Ids, peptide mimotope vaccines are sequences selected from a pool of short random amino acid sequences (peptides). Antibodies raised against a specific epitope can be used to select mimics of that epitope from a library of different molecular shapes. Peptide mimotopes of some bacterial polysaccharides have already been shown to (1) structurally mimic carbohydrate antigens; (2) induce carbohydrate-reactive B- and T-cell responses after

immunisation; and (3) after priming, enhance responses upon boosting with carbohydrate (Kieber-Emmons 1998). This is discussed further in §1.5.2.2.

1.5.1.3 Advantages to peptide mimicry

Peptide antigens offer a number of advantages over carbohydrate and anti-Id vaccines.

- Due to their TD nature, peptide mimics are highly immunogenic, if conjugated to a larger protein, and can induce immunological memory (Lesinski and Westerink 2001).
- They are cheap and easy to produce and require little downstream processing (Lesinski and Westerink 2001).
- The peptide product is stable at ambient temperatures, and can be stored as a freeze-dried powder over prolonged periods (Partidos 2000) eliminating the need for cold chain storage and handling from manufacturing to administration (Lesinski and Westerink 2001).
- The vaccine is defined in chemical terms and is free of nucleic acid contamination (Lesinski and Westerink 2001).
- They can be designed to stimulate broad immunity to cover different strains or serotypes (Partidos 2000).
- They can be formulated to include several T-cell epitopes covering the MHC haplotypes for immunising an outbred population such as humans (see §1.5.1.4) (Partidos 2000).
- Peptide mimics elicit a predetermined type of immune response. Therefore undesired epitopes and side effects can be avoided. (Meloen *et al.* 2000; Partidos 2000).

These advantages have led to the suggestion that peptide mimotope vaccines may lead to a breakthrough in the area of vaccines required for inducing anti-polysaccharide antibodies (Meloen *et al.* 2000).

1.5.1.4 Limitations of peptide vaccines

There are some important limitations to peptide mimic vaccines that need to be considered. As discussed in §1.4.2.3, the antibody response to a protein antigen requires the involvement of helper T cells. Briefly, to summarise, after internalisation protein antigens

are broken down into peptides, some of which associate with class II MHC molecules (Wood 2001). The MHC-antigen complex is then transported to the cell surface where the antigen is presented to helper T cells (Wood 2001). Once bound, the T cell releases cytokines which help B cells proliferate and differentiate into antibody producing cells and memory B-cells (Wood 2001). Importantly, a T cell cannot recognize a foreign antigen unless it is presented in the context of an MHC protein.

The human and mouse MHC molecules are known as HLA (human leukocyte antigens) and H-2, respectively. For the purposes of this study, the organisation of the MHC class II proteins in humans will be discussed. The MHC class II molecules consist of two non-covalently linked glycosylated polypeptides, called α and β. These two chains form a large groove, within which the MHC molecule binds foreign antigens of around 12-16 amino acids long. The genes that encode the α- and β-chains are arranged into three pairs of loci, called HLA-DQ, -DP and -DR on chromosome 6. The organisation of these genes is shown in Figure 1.6.

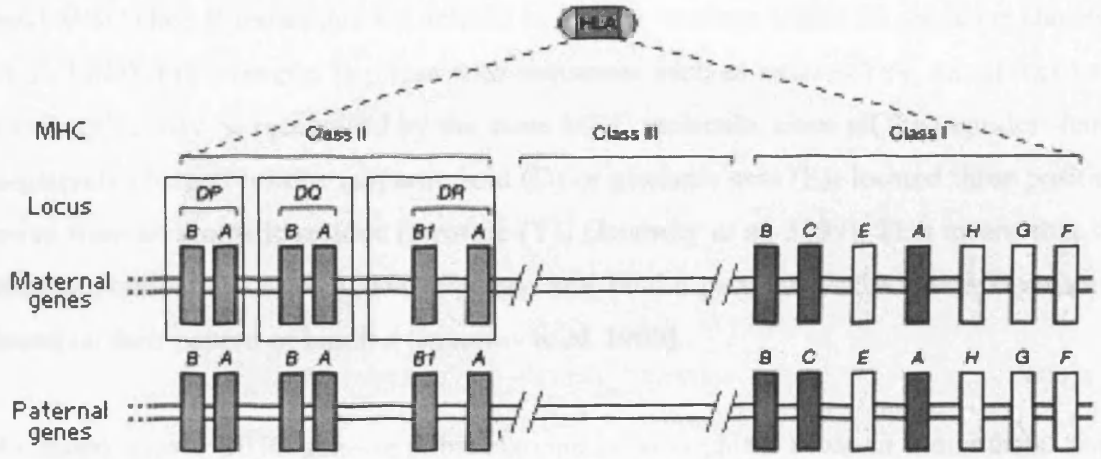

Figure 1.6. The organisation of human MHC (HLA) genes. The genes coding the class II MHC molecules are arranged into three pairs of loci, called DQ, DP and DR and each contain a α-gene, which codes for the α-chain, and a β-gene, which codes for the β-chain. This is highlighted by grey boxes. Furthermore, the DR locus may contain one or two DRB genes, each coding for a separate β-chain . Therefore an individual possesses a DRB1 allele alone or a DRB1 allele plus one of DRB3, DRB4, or DRB6. Diagram modified from that in Wood (2001).

The DP and DQ loci each contain a functional α-gene, which codes for the α-chain, and a functional β-gene, which codes for the β-chain (this is shown in Figure 1.7 with each molecule highlighted by grey squares). The DR locus is more complicated than DP and DQ; it contains one functional α-gene, but different versions of the locus contains one or two functional DRB genes, each coding for a separate β-chain (not shown on Figure 1.7). Therefore an individual can express at least 6 different MHC proteins. The number of proteins is higher in an outbred population, since MHC genes are expressed co-dominantly. Like any other gene, individuals inherit one copy of an MHC gene from their mother and one from their father. Therefore, if two different alleles of a gene are inherited (as is likely the case given the extreme polymorphism demonstrated in these genes (see below)), an individual will make both forms of the protein. This is not the case for inbred populations, since both alleles are the same (Wood 2001).

Because of the limited individual variation, the MHC proteins do not recognise every antigenic peptide with an individual MHC molecule as TCRs and antibodies do. Instead the peptide-binding groove recognises particular patterns of binding. The peptides that can bind MHC class II molecules are defined by anchor residues within its sequence (Janeway *et al.* 1999). For example, peptides with sequences such as xxDxxxYxx, xxxxDxxxYx or xxxExxxYx may be recognised by the same MHC molecule, since all the peptides share a negatively charged residue (aspartic acid (D) or glutamic acid (E)) located three positions away from an aromatic residue (tyrosine (Y)) (Janeway *et al.* 1999). This means that it is usually possible to detect which sequence will bind a particular MHC class II molecule based on their pattern of binding (Janeway *et al.* 1999).

As stated above, MHC genes exhibit extreme polymorphism between individuals. Some MHC loci in humans have over one hundred alleles and the amino acid sequence between alleles can vary by more than 20 amino acids. This is a big problem for peptide vaccine research as, consequently, some peptide antigens will not be recognised by some MHC alleles and will therefore appear invisible to the immune system of some individuals. On the other hand, some peptide sequences can bind to many MHC class II alleles albeit with different avidity, implying that the same peptide can immunise individuals of diverse MHC haplotypes. These types of peptides are infrequent and generally this is a problem in vaccine development (Schuler-Thurner *et al.* 2002). Therefore, in an outbred population, a

single peptide mimotope is unlikely to work as a vaccine; instead it may be necessary to use a vaccine composed of multiple peptide sequences.

1.5.2 Identifying peptide mimics

As previously discussed in §1.5.1.2, the detection of peptide mimics begins with the identification of an antibody that binds to the epitope of interest. The antibody is then used to select peptides that mimic that epitope. For developing mimics to polysaccharide antigens, this generally occurs by one of two methods: one based on the structure and sequence of the anti-idiotype antibody, the other based on the use of random peptide libraries. These methods and examples of their success will be discussed in the following sections.

1.5.2.1 Design of mimics based on the sequence of the anti-idiotype antibody

One way to identify mimics is to construct peptides using the protein sequence of the variable region of the anti-idiotypic antibody (see §1.5.1.1). As described previously, Westerink and colleagues developed an anti-idiotype mAb (6F9) that mimicked meningococcal group C capsular polysaccharide (MCP) (Westerink *et al.* 1988). They went on to analyse the sequence of the variable regions, V_L and V_H, of the mAb 6F9 and delineated potential immunogenic regions using computer-assisted molecular modelling (Westerink *et al.* 1995). The analysis, known as surface simulation (Meloen *et al.* 2000), has been used to develop peptide mimics based on the three dimensional structure of anti-Id. Westerink and colleagues demonstrated the presence of several potentially immunogenic epitopes that included a unique sequence region of the complementary determining region 3 (CDR3) of the V_H of 6F9 (Westerink *et al.* 1995). Immunisation with the peptide spanning this region of the anti-idiotypic antibody produced an anti-MCP response that was significantly greater than that achieved from immunisation with the native antigen, MCP (Westerink *et al.* 1995; Prinz *et al.* 2003). The immunogenicity of the peptide was further enhanced following conjugation to a carrier protein and 100 % of the BALB/c mice that received four doses of this conjugate survived challenge (Westerink *et al.* 1995). They also showed that immunisation led to a faster clearance of bacteria, as no bacteria were recovered from the blood of infected mice 24 hours after challenge. This indicated that the peptide induced a protective, TD antibody response (Westerink *et al.* 1995).

1.5.2.2 Selection of peptide mimics using random peptide libraries

It is impossible to know how many epitopes there are in nature. It is, however, possible to calculate an estimate of the number of potential epitopes. There are 20 different amino acids. This means that the structure/sequence of a completely undescribed antibody-binding site, which can bind sequences up to 15 amino acids in length, has the capacity to recognise in excess of 3×10^{19} peptide sequences (the number of permutations of 20 amino acids in 15 spaces), although some residues share similar binding properties. Therefore for the antibody to select a binder (mimic), a peptide library should represent a large number of epitopes.

1.5.2.3 Synthetic peptide libraries

Synthetic libraries were the first method used to identify peptide mimics or mimotopes (Geysen *et al.* 1984; Geysen *et al.* 1985; Geysen *et al.* 1986). These libraries have a major disadvantage in that they represent only a small array of peptides, since large synthetic libraries could not be produced at that time (Meloen *et al.* 2000). Therefore, their use was mainly out of necessity, although some researchers have found advantages to using small libraries. This is because, unlike recombinant libraries, which suffer from a much lower signal to background noise ratio (see §1.5.2.4) many weakly binding peptides can be obtained from synthetic peptide libraries (Meloen *et al.* 2000). This is beneficial, since the characteristics of these peptides can be used to define a 'quality' array of mimics. Repeated screening of this array with the antibody produces peptides with improved binding properties until peptides with appropriate binding properties can be obtained (Meloen *et al.* 2000). In this way the need for large libraries is circumvented (Meloen *et al.* 2000). Nevertheless I found no published examples of this type of library being used to screen for peptide mimics of polysaccharide antigens, and the majority of successful examples have been obtained using recombinant libraries.

1.5.2.4 Recombinant peptide libraries.

Recombinant peptide libraries have the capacity to represent a much larger number of peptides than synthetic libraries. Recombinant libraries have been constructed in bacteria (e.g. *Streptococcus gordonii*, (Maggi *et al.* 2002)), but the majority of successful examples in the literature have been obtained by screening peptide libraries displayed on the surface of filamentous phage (e.g. fd (Smith 1985; Parmley and Smith 1988) and M13 (Felici *et al.* 1991; Meloen *et al.* 2000). This is because using phage libraries increases the number of

clones that can be handled in a single experiment 1000-fold compared to synthetic libraries. Phage libraries are also relatively easy to produce and use (see §1.5.3 for construction of phage libraries) (Meloen *et al.* 2000).

Bacteriophage are viruses capable of infecting prokaryotic cells (Madigan *et al.* 1997). The phage genome of the filamentous phage M13 and, similarly, the fd phage (which have both been used to construct peptide libraries), consists of single stranded circular DNA (Gao *et al.* 1999), which encodes all ten phage proteins that are involved in replication, morphogenesis, and the formation of the virus coat (Viti *et al.* 2000). In 1977 Messing discovered that the length of the coat proteins of M13 reflected the size of the ssDNA it carried (Messing *et al.* 1977). In 1985, Smith demonstrated that when DNA coding for a peptide sequence was inserted into the pIII coat protein gene it caused the filamentous phage fd to display the peptide as a fusion protein on its exterior surface (Smith 1985). Proteins as large as 50kDa have been displayed (Smith and Scott 1993).

Most phage display libraries are constructed by insertion of a random peptide sequence into either the pIII (Smith 1985; Scott and Smith 1990) or the pVIII (Il'ichev *et al.* 1989; Iannolo *et al.* 1995; Malik *et al.* 1996) coat protein of the fd or M13 phage (Lesinski and Westerink 2001). Libraries have also been constructed using the pVI (Jespers *et al.* 1995), pVII and pIX (Gao *et al.* 1999) coat proteins of M13. These libraries contain $>10^7$ clones, each of which displays a unique peptide sequence (Lesinski and Westerink 2001). Using monoclonal antibodies or polyclonal antisera, the phage library can be screened by successive cycles of selection and amplification. A single round of this (affinity purification) can enrich antibody-binding phage by a factor of 10^5 (Lesinski and Westerink 2001). As a result, antibody-binding phage can be selected from a vast background of non-binding phage (see §1.5.4 for more details) (Smith and Scott 1993). Figure 1.5 illustrated how an anti-polysaccharide mAb could select a peptide that functionally mimicked a carbohydrate antigen.

Phalipon and colleagues described two immunogenic nonapeptide mimics (known as p100c and p115) that were selected from a phage-display library using IgA monoclonal antibodies specific for the O-antigen of *Shigella flexneri* serotype 5a lipopolysaccharide (Phalipon *et al.* 1997). Immunisation with the phage-bound peptide induced an O-antigen specific immune response (Phalipon *et al.* 1997). Mice immunised with wild-type phage

did not elicit an anti-LPS response. To examine whether these antibodies could play a role in protection against the development of disease, they were tested for their ability to bind the bacterium *in vitro*. As shown in Figure 1.7, peptide-induced sera from mice immunised with p100c or p115 bound serotype 5a *S. flexneri*. The same sera did not bind to serotype 2a bacteria, which was used as a control (Phalipon *et al.* 1997).

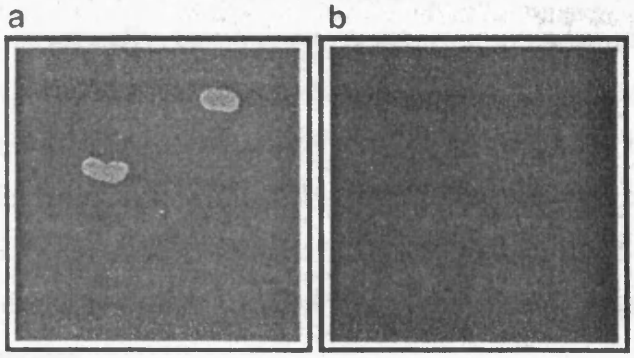

Figure 1.7. Labelling of *Shigella flexneri* bacteria with peptide mimic-induced antibodies. Labelling of *S. flexneri* serotype 5a (A) or serotype 2a (B) bacteria previously centrifuged and fixed onto coverslips was performed with sera of mice immunised with wild-type phage, p100c, or p115. Rhodamine-conjugated goat anti-mouse IgG was used as a secondary antibody. Results shown in this figure were obtained with p115-induced antibodies (results from p100c serum were the same as p115). Taken from Phalipon *et al.* (1997).

These results imply that immunisation with p100c or p115 induced antibodies capable of specifically neutralising and disrupting the pathogenic process of serotype 5a *S. flexneri*. Similar results have been found using peptide mimics of Group B streptococcal type III capsular polysaccharide (Pincus *et al.* 1998) and *Brucella* LPS (De Bolle *et al.* 1999), both of which were selected from a phage-display library. In the case of the latter, four mAbs, named A15-6B3, A76-12G12, B66-4F9 and B66-2C8, directed against the O-chain of *Brucella* LPS were used to select peptide mimics from five different phage display libraries (De Bolle *et al.* 1999; Mertens *et al.* 2001). Interestingly, they found that those mAbs that bound the native antigen with high affinity (12G12 and 4F9) were poor targets for selecting peptide mimics, as biopanning with 12G12 resulted in the isolation of no binders. In contrast 6B3 and 2C8, that presented low affinity for LPS, selected plenty of positive clones (Mertens *et al.* 2001).

All the mimics selected by mAbs 6B3 and 2C8 were shown to compete with LPS for the binding to the mAb (De Bolle *et al.* 1999). However, 95 % of mice immunised with the phage-bound peptide failed to produce any antibody that reacted with LPS (De Bolle *et al.* 1999). These data suggested that the peptides may present different conformations, and only a few of which actually mimicked the O-chain of *Brucella* LPS (Mertens *et al.* 2001). This was thought to be due to instability of the peptide structure. For this reason some peptide libraries are structurally constrained, using two cysteine residues to form a disulphide bond, which circularises and stabilises the shape of the peptide. Investigators also often screen multiple libraries with a panel of mAbs and align the resulting sequences (Lesinski and Westerink 2001). Using this strategy, a consensus sequence can be obtained, or even a matched sequence from separate libraries (Lesinski and Westerink 2001). Additional peptides can then be constructed based on the sequence and success of previously selected peptides.

1.5.3 Construction of a phage display peptide library

As stated in §1.5.2.4, many phage display libraries have been constructed using the major coat protein pVIII of the M13 phage (Felici *et al.* 1991). M13 is a lysogenic bacteriophage, which infects F' episome-bearing bacterial cells through binding of the adsorption end of the phage to the tip of the F pilus. The pilus retracts and the phage genome penetrates the bacterial membrane. As the bacterial cell replicates, it translates the phage genome, which is now double stranded, to produces all the phage proteins needed for complete construction of new phage particles (see Figure 1.8) (Felici *et al.* 1991).

The pVIII coat protein is preferred since it is present in more copies than any other coat protein. For example, pVIII is present in about 2700 copies per phage particle whereas the pIII coat protein (as used by Smith (1985) and Scott *et al.* (1990)) is displayed in only 3 - 5 copies. The N-terminal region of pVIII was used for recombinant protein expression for two reasons. Firstly, mutations in the C-terminus have been shown to interfere with phage infectivity (the C-terminus is involved in membrane anchorage) (Smith and Scott 1993). This means that such phage could not be selected from panning and be amplified via bacterial infection (see §2.5.3). Secondly, as the N-terminus is on the exterior and is next to a hydrophobic region, the inserted peptide will be displayed on the outside of the phage and can therefore be tested for antibody binding (Smith and Scott 1993).

Table 1.6 An overview of attempts to develop mimotopes against bacterial polysaccharide antigens

Antigen	*Method*	*Mimic*	*Mimotope*	*Protection*	*Reference*
Polysaccharide conA ligand	Phage display library	+	-	ND	
Group C meningococcal polysaccharide	CDR3- anti idiotype mimic	+	+	+	(Westerink *et al.* 1995)
C. neoformans polysaccharide	Phage display library	+	+	ND	(Valadon *et al.* 1996)
Shi. flexneri 5a LPS	Phage display library	+	+	ND	(Phalipon *et al.* 1997)
Streptococcal polysaccharide	Phage display library	+	+	ND	(Pincus *et al.* 1998)
Group B meningococcal polysaccharide	Phage display library	+	-	ND	(Moe *et al.* 1999)
Brucella LPS	Phage display library	+	+	ND	(De Bolle *et al.* 1999)
Serotype 4 pneumococcal CPS	Phage display library	+	-	ND	(Lesinski *et al.* 2001)
Serotype 6B pneumococcal CPS	Phage display library	+		ND	(Shin *et al.* 2002)

(-) Indicates a negative result (ND) indicates that the property was not investigated

Adapted from Meloen *et al.* (2000).

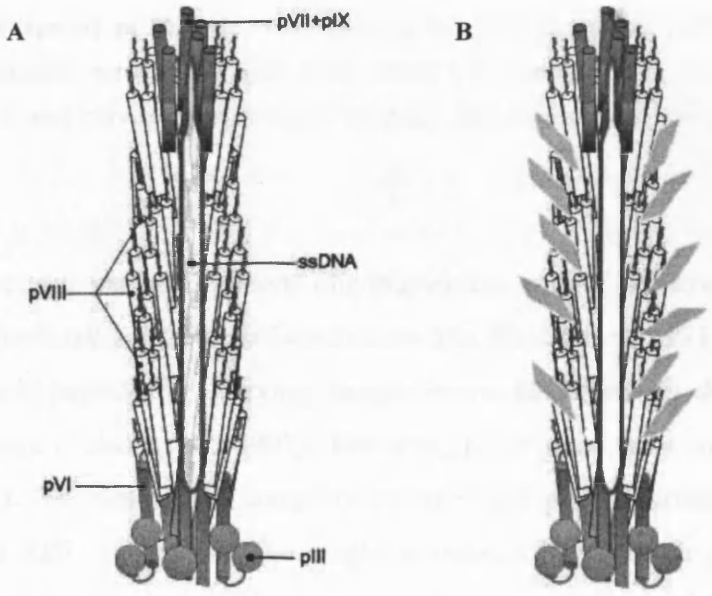

Figure 1.8. The morphology of wild-type and recombinant M13 filamentous phage. This diagram identifies the coat proteins pIII, pVI, pVII, pVIII, and pIX, which have been previously modified to display random peptide and create libraries of peptide antigens. (A) shows the wild-type phage. (B) shows the recombinant phage with the additional oligopeptide displayed on the pVIII coat protein. This diagram was provided by F. Felici.

1.5.3.1 Phagemid vectors

Felici and colleagues developed a phage display peptide library by constructing a phagemid vector that encodes the M13 phage coat protein gene, *pVIII*, under the control of the pLac promoter (Felici *et al.* 1991). The vector, known as pC89, contains both phage and plasmid (hence the name phagemid) origins of replication (fl and ColEl, respectively), which allow expression of antibiotic resistance markers in transformed bacteria and expression of the recombinant phage coat protein. Figure 1.9 shows the pC89 vector map.

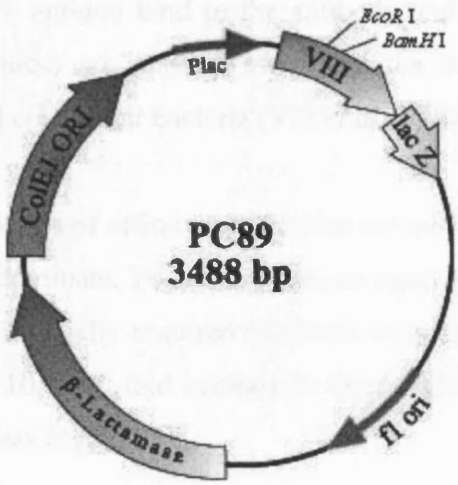

Figure 1.9. The phagemid pC89. This vector contains the *pVIII* phage coat protein gene. Insertion of random coding sequences between the *EcoR* I and *BamH* I restriction sites of this gene allows random peptide sequences to be displayed on the surface of the phage. This diagram was provided by F. Felici

Felici and colleagues inserted random oligonucleotide coding sequences into the *pVIII* gene, between the *EcoR* I and *BamH* I restriction sites (Felici *et al.* 1991). This meant that, once transformed, peptides of varying lengths were displayed on the surface of the recombinant phage (Felici *et al.* 1991). However, pC89 does not contain the complete genome of M13. To replicate a complete recombinant phage particle a helper phage (known as M13 K07) is needed. The single stranded DNA of both phage are quickly converted to double stranded DNA upon entry to the cell (by cellular enzymes) and replication begins. However, M13 K07 has a mutated pII (coat protein II) origin of replication (Barbas *et al.* 2001). This means that preferential replication of the recombinant phage pII, which does not display this mutation, and therefore the complete construction of

new recombinant phage particles will occur. In doing this libraries containing up to 9.4 x 10^7 independent clones have been produced (Felici *et al.* 1991).

1.5.4 The Recovery of Specific Peptide Mimics

Phage-displayed peptide mimics are often isolated by affinity selection (Harrison *et al.* 1996). The principle of phage selection is outlined in Figure 1.10. Basically, an antibody raised to the native antigen is fixed to a solid support or column. The phage library is then passed through the column and the peptides that recognise the antibody paratope, and therefore mimic the native antigen bind to the antibody and remain in the column. The unbound phage (non-mimics) are washed away and the bound phage are eluted and propagated by infection of competent bacteria (Viti *et al.* 2000).

Typically, three or four rounds of affinity purification are needed to obtain a suspension in which reactive clones predominate. To achieve this, an input of 10^{12} TU (transducing units of phage) for each round is usually required to obtain an output of 10^5 - 10^8 TU. This has been shown to result in a 10 - 100 fold increase in the number of reactive clones after the third or fourth round (Barbas *et al.* 2001).

1.5.5 The aims

At the start of this study there were no published examples of peptide mimic vaccine candidates for *S. pneumoniae*. Since then, two studies have developed mimics to serotype 4 (Lesinski *et al.* 2001a) and 6B (Shin *et al.* 2002) pneumococcal capsular polysaccharide (Table 1.6). Both studies reported the production of cross-reactive anti-polysaccharide antibodies upon immunisation (Lesinski *et al.* 2001a; Shin *et al.* 2002). This was one aim of our investigation. Also, Westerink and colleagues indentified a mimotope that induced a protective immune response against capsular polysaccharide of *N. meningitidis* (Westerink *et al.* 1995). There are no reports of mimotopes that can induce protection against pneumococcal disease. One of the aims of this project was to identify mimics of pneumococcal capsular polysaccharide that could induce protection against disease upon immunisation.

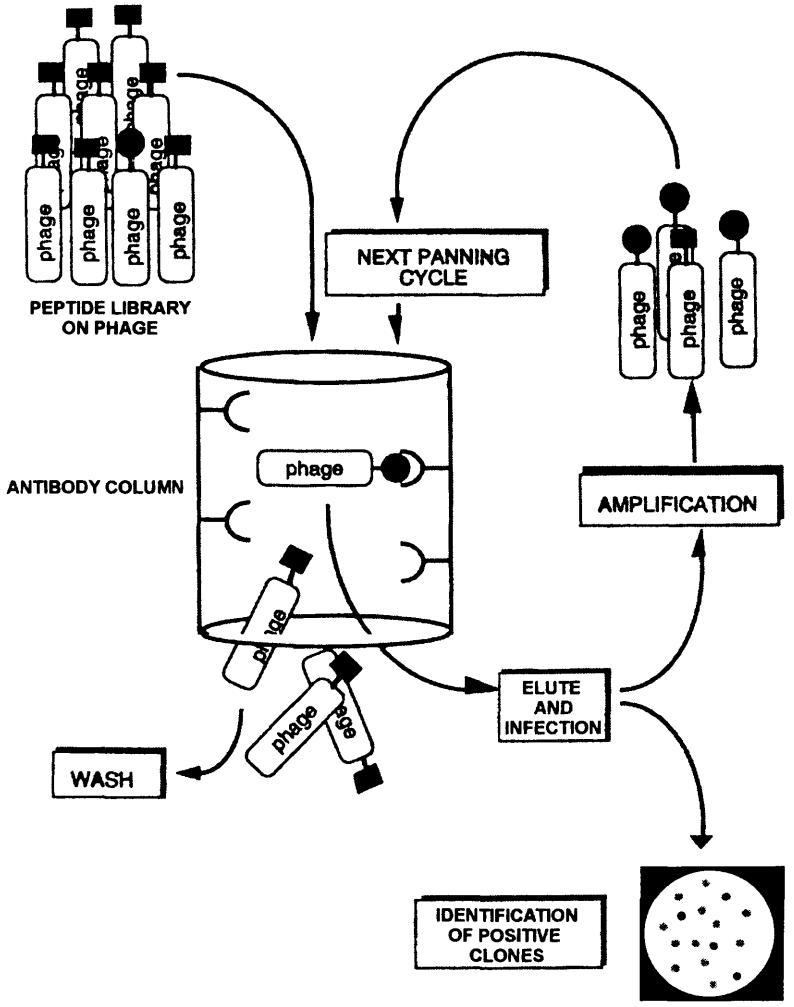

Figure 1.10. A schematic representation of the selection of peptide mimics from a phage-display library. Phage are passed through an antibody column. The phage with the desired binding specificity remain bound to the antibody immobilised on the support. Those that do not bind are removed by washing. After several rounds of panning, peptide mimics can be identified by immunoscreening, see §1.5.4. This diagram was modified from Viti *et al* (2000).

Part B: Heterologous expression of pneumococcal polysaccharide *in planta*

1.6 An alternative vaccine production system

In the past, most medicinal compounds were derived from plants. Then about 60 years ago, the pharmaceutical industry began generating new drugs synthetically in the lab and this has remained the case since (Moffat 1995). Today, with the development of biotechnology, plants are reclaiming their therapeutic potential, in part by becoming a useful system for vaccine production. Plant systems were preferred because of the advantages they offer over cell-culture based expression systems (Mason and Arntzen 1995). Currently, cell-culture based expression systems such as genetically engineered bacteria, yeast, and insect cells are the most common large-scale production systems for proteins, since these organisms are relatively easy to manipulate and have rapid predictable growth (Richter and Kipp 1999). However, the recombinant proteins they express need to be extensively purified to remove toxins or pyrogens and these processes add to the overall production cost (Richter and Kipp 1999).

The main advantage of producing vaccines in transgenic plants is the ability to directly use edible plant tissues for oral immunisation without purification, removing the need for expensive down-stream processing (Richter and Kipp 1999). Furthermore, the ability to grow plants on a large scale introduces extra economies of scale that will drive down costs, this being the most important factor to consider when developing a vaccine for third world countries.

Transgenic plants are already recognised as a valuable expression system for the production of proteins. The idea was first discussed in 1990 in a patent application which reported the expression of a surface protein of *Streptococcus mutans* surface protein antigen A (SpaA) in tobacco plants (Curtiss and Cardineau 1990). Although no further details of this study have been reported, it paved the way for others, including the expression of Hepatitis B virus surface antigen (HbsAg) (Mason and Arntzen 1995; Gao *et al.* 2003), enterotoxins such as *E. coli* heat-labile enterotoxin B subunit (LTB) (Haq *et al.* 1995; Mason *et al.* 1998; Lamphear *et al.* 2002), and a *M. tuberculosis* antigen (ESAT-6) (Rigano *et al.* 2004) in plants. A summary of recent developments is shown in Table 1.7.

Table 1.7 Chronological Overview of Some Recent Plant Systems Developed for Antigen Expression

Antigen	*Plant Expression System*	*Expression*	*Antibody response*	*Reference*
LT-B of enterotoxigenic *E. coli*	Tobacco and Potato	+	+	(Haq *et al.* 1995)
LT-B of enterotoxigenic *E. coli*	Potato	+	+	(Mason *et al.* 1998)
Hepatitis B surface antigen (HbsAg)	Potato	+	+	(Richter *et al.* 2000)
N-terminal domain of the spike protein (S) from Coronavirus	Tobacco	+	+	(Tuboly *et al.* 2000)
N-terminal domain of the spike protein (S) from Coronavirus	Potato	+	+	(Gomez *et al.* 2000)
VP1 epitope of foot and mouth disease virus	Potato	+	-	(Carrillo *et al.* 2001)
B subunits of the cholera toxin of *Vibrio cholerae*	Tomato	+	ND	(Jani *et al.* 2002)
LT-B of enterotoxigenic *E. coli*	Maize	+	+	(Lamphear *et al.* 2002)
Hepatitis B surface antigen (HbsAg)	Tomatillo	+	-/+	(Gao *et al.* 2003)

(-) Indicates a negative result or that the property was not investigated, (-/+) indicates only a booster response was noted (ND) not done.

1.6.1 Plants that have been used for recombinant vaccine production

Typically, readily transformed plant species such as tobacco, potato, tomato and maize (Haq *et al.* 1995; Mason *et al.* 1998; Jani *et al.* 2002; Lamphear *et al.* 2002) have been used for *in planta* recombinant protein expression (Richter and Kipp 1999). Historically, transgenic work was carried out in tobacco due to its ease of transformation, rapid growth, and robust regeneration (Richter and Kipp 1999). However, recently novel transformed species, such as banana (May *et al.* 1995), corn, leek (Eady *et al.* 2005), garlic (Eady *et al.* 2005), lettuce, walnut and others have been used for the same purpose (Sala *et al.* 2003). This is because transgenic tobacco tissue is not an ideal material for oral vaccine delivery since it contains high levels of toxins, such as alkaloids and nicotine, although low alkaloid varieties do exist (Richter and Kipp 1999) and immunisations with recombinant plant tissues have not been shown to cause adverse effects (Modelska *et al.* 1998). The novel plant species benefit from having edible, palatable tissue, which can be fed to animal subjects (such as mice) uncooked. This is discussed further in §5.9.3.

1.6.2 Plant transformation and expression vectors

Transgenic plants are commonly produced by two methods, *Agrobacterium*-mediated gene transfer (Valentine 2003) or particle bombardment (Takeuchi *et al.* 1992). The method of particle bombardment is illustrated in Figure 1.12. Tungsten or gold particles coated in plasmid DNA are pipetted onto the centre of a micro-carrier (Fig. 1.11). This is placed upside down, in front of a stopping screen (wire mesh) and the plant cells (or tissue) are positioned under the stopping screen at a distance of approximately 8 cm. A helium pressure of 1100psi to applied to accelerate the micro-carrier towards the stopping screen (Kost *et al.* 1998). Once the micro-carrier hits the stopping screen the DNA coated particles are fired into the plant cells (Kost *et al.* 1998).

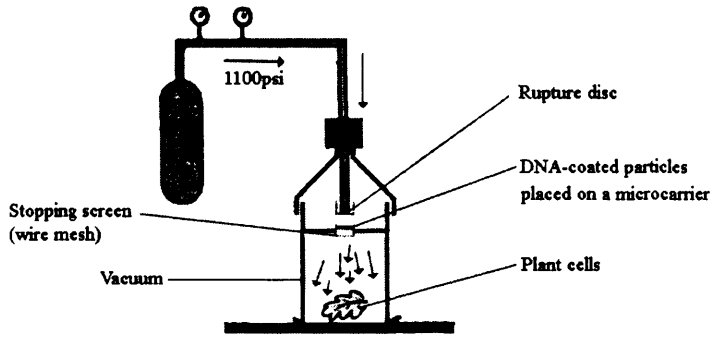

Figure 1.11. Graphic illustration of the particle gun used to transform plant cells. This diagram was modified from that of (Sagi *et al.* 1995), using the methods described by Kost *et al.* (1998).

However, *Agrobacterium*-mediated transformation is thought to offer significant advantages over particle bombardment (Travella *et al.* 2005). In a recent study, it was found that *Agrobacterium*-mediated transformation of barley was twice as efficient than that obtained by particle bombardment (Travella *et al.* 2005). This is because, as described in §1.6.2.1, more cells are exposed to recombinant *Agrobacterium* than the individual cells that are exposed to particles. Travella and colleagues also noted that in most *Agrobacterium*-derived lines the integrated DNA was stable and inherited as a simple Mendelian trait, whereas transgene silencing was frequently observed in the first filial (F1) generation of the particle bombardment-derived lines (Travella *et al.* 2005).

1.6.2.1 *Agrobacterium*-mediated transformation of plants

Agrobacterium is a soil-borne bacterium that opportunistically infects wounded plant cells to cause tumours such as crown gall (*A. tumefaciens*) and hairy root (*A. rhizogenes*). The compounds released by the wounded plant attract *Agrobacterium*, which attaches to the cell (Valentine 2003). The bacterium then transforms the cell by mobilising a region of

DNA located in the virulence (*vir*) region of the bacterial plasmid (tumour inducing (T_i) in *A. tumefaciens* and root inducing (R_i) in *A. rhizogenes*), known as T-DNA (transfer DNA), which becomes integrated almost anywhere in the plant's genome (Valentine 2003). The T-DNA carries the virulence genes needed for tumour formation and also the production of metabolic enzymes (opines), which provide an extra source of carbon and nitrogen for *Agrobacterium* (Armitage *et al.* 1988; Valentine 2003).

For the purposes of cloning, a binary vector that can replicate in both *Escherichia coli* and *Agrobacterium* (hence the term "binary vector"), and also possesses a T-DNA region is cloned into *Agrobacterium* (Lessard *et al.* 2002). Again, when exposed to compounds released from a wounded plant, the T-DNA region from both the binary vector and the T_i (or R_i) plasmid are mobilised by transcription of the *vir* region of the T_i plasmid (Armitage *et al.* 1988) and integrated into the plant genome. To prevent disease, the oncogenes, which are not required for the transfer of the T-DNA, have been removed from attenuated bacteria and replaced with multiple cloning sites where genes of interest can be inserted (Armitage *et al.* 1988; Lessard *et al.* 2002).

1.6.2.2 A plant-derived antigen can also be immunogenic

As Table 1.7 shows, some plant-derived protein antigens have also been shown to elicit an immune response in mice, with varying success. In 1995, Haq and colleagues published the first 'proof of concept' for edible vaccines. They demonstrated that mice that had been fed doses of transgenic potatoes expressing the B subunit of the heat labile enterotoxin (LT-B), which is known to protect against enterotoxigenic *E. coli* (ETEC), produced both serum IgG and mucosal IgA antibodies against LT-B (Haq *et al.* 1995). They also showed that these antibodies were capable of neutralising the enterotoxin *in vitro* cell protection assays (Haq *et al.* 1995).

In another study, Mason and colleagues fed mice three doses (one a week for three weeks) of raw transgenic potatoes expressing LT-B (Mason *et al.* 1998). After three weeks, the immunised mice developed anti-LT-B serum IgG and anti-LT-B faecal IgA responses equivalent to or greater than responses developed by mice gavaged orally with purified LT-B (Mason *et al.* 1998). However, this was not enough to protect mice *in vivo* against the effects of LT upon challenge (Mason *et al.* 1998).

Gao and colleagues found that feeding mice daily with recombinant HbsAg from a transgenic cherry tomatillo plant was not enough to elicit an immune response in naïve mice (Gao *et al.* 2003). However, once primed with a dose of the commercial HbsAg vaccine, mice fed recombinant HbsAg had a significantly boosted antibody response compared to mice fed wild-type cherry tomatillos (see Figure 1.12) (Gao *et al.* 2003). Alternatively, Richter and colleagues fed mice daily with recombinant HbsAg from a transgenic potato plant, but this response declined within weeks. Upon boost with a low level of the commercial vaccine, the antibody level was significantly boosted (Richter *et al.* 2000). This indicates that the potato vaccine had led to a memory immune response.

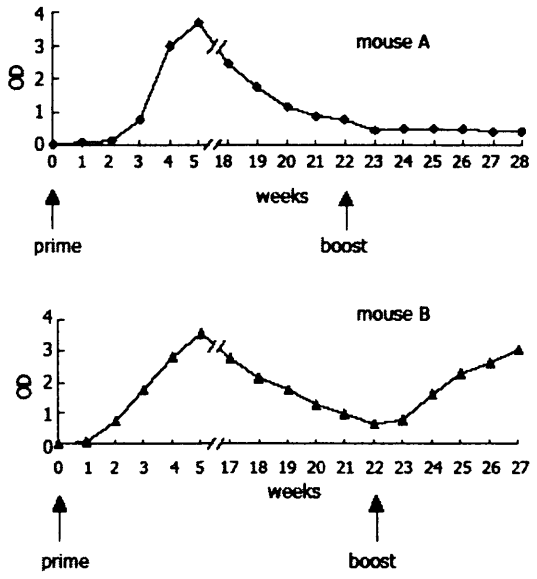

Figure 1.12. Serum antibody response elicited by oral boost with transgenic cherry tomatillo. Mouse A was primed parenterally with 2 μg commercial HBsAg, and when its serum antibody level descends to OD<1.0, mouse was fed with untransformed tissues; Mouse B was primed parenterally with 2 μg yeast-derived rHBsAg, and when the serum antibody levels descended to OD<1.0, mice were fed with transformed tissues as boost. Taken from Gao *et al.*, (2003)

1.6.3 Can transgenic plants offer a pneumococcal vaccine solution?

Despite the success of expressing protein antigens in plants (§1.6.1), the concept of engineering the plant's own metabolism to produce a heterologous polysaccharide as a vaccine antigen had not yet been reported. Therefore, the aim of this project was to develop a transgenic plant capable of synthesising pneumococcal polysaccharide. It was thought this would contribute to solving three problem areas of pneumococcal vaccine development. Firstly it would result in large-scale manufacture and reduce the cost of pneumococcal polysaccharide production and, ultimately of the conjugate vaccine. Additionally, an edible plant vaccine would reduce costs further by removing expensive down-stream processing, i.e. the need for antigen purification. Second, it would aid better adoption of the vaccine into national programmes by enabling local production (a scheme

of 'grow you own vaccines') and finally it would make the vaccine more palatable to recipients by dispensing with the need for needles.

In order to determine whether heterologous expression of pneumococcal polysaccharide is possible, it was necessary to understand how pneumococcal polysaccharide is synthesised and then, how this compares to polysaccharide synthesis in plants.

1.7 Polysaccharide synthesis

1.7.1 Capsular polysaccharide synthesis in pneumococci

As stated in §1.1.1, there are currently over 90 chemically distinct types of pneumococcal capsular polysaccharide (Henrichsen 1995). Each is defined by unique antigenic determinants (Griffith 1928; MacLeod et al. 1945). Capsular polysaccharides are usually composed of repeating oligosaccharide subunits containing several different monosaccharides and, in many cases, are branched structures (Kamerling 2000). The structures of some of these polysaccharides are shown in Table 1.8.

The biosynthesis of pneumococcal capsular polysaccharide has been described for serotypes 3 (Dillard *et al.* 1995; Cartee *et al.* 2000; Forsee *et al.* 2000), 14 (Kolkman *et al.* 1997) and 19F (Morona *et al.* 1997; Kamerling 2000). The genes involved in pneumococcal capsular polysaccharide synthesis are closely linked on the bacterial chromosome, arranged within a single locus (cassette) (Caimano *et al.* 2000). These cassettes (*cps*) are termed "type-specific" because, although the genes involved in capsule synthesis of different serotypes share little homology they may occupy identical sites in the chromosome (Caimano *et al.* 2000). Furthermore, in most cases, genetic exchange of the type-specific cassettes results in transformation to the new type and the loss of the ability to express the original polysaccharide (Dillard *et al.* 1995).

The Sanger Institute has recently finished sequencing all 90 capsular polysaccharidebiosynthetic cassettes (*cps*) of *S. pneumoniae* by amplifying each *cps* cluster by PCR using primers in the flanking regions of the cassette (http://www.sanger.ac.uk/Projects/S_pneumoniae/CPS/). This is because flanking either side of the type-specific genes are sequences common to all capsule types

Table 1.8 The Primary Structure of Some Pneumococcal Polysaccharides

Serotype	*Structure*
3	1→4)-β-D-Glc*p*-(1→3)-β-D-GlcA*p*-(1→4
4	1→3)-β-D-Man*p*NAc-(1→3)-α-L-Fuc*p*NAc- (1→3)-α-D-Gal*p*NAc-(1→4)-α-D-Gal*p*2,3(S)Pyr-(1→3
6B	P→2)- α-D-Gal*p*-(1→3)-α-D-Glc*p*-(1→3)-α-L-Rha*p*-(1→4)-D-Rib-ol-(5→P→2
9V	1→4)-α-D-Glc*p*A-(1→3)-α-D-Gal*p*- (1→3)- β-D-Man*p*NAc-(1→4)- β-D-Glc*p*-(1→4)-α-D-Glc*p*-1→4) : : : 2Ac (17%) 4Ac (6%) 2Ac (17%) 3Ac (25%) 6Ac (55%) 3Ac (4%)
18C	Gro(1→P ↓ 3 1→4)-β-D-Glc*p*-(1→4)-β-D-Gal*p*-(1→4)-α-D-Glc*p*-(1→3)- β-L-Rhap-(1→4 2 ↑ 1 α-D-Glc*p*6Ac$_{0.3}$

Taken from Kamerling (2000)

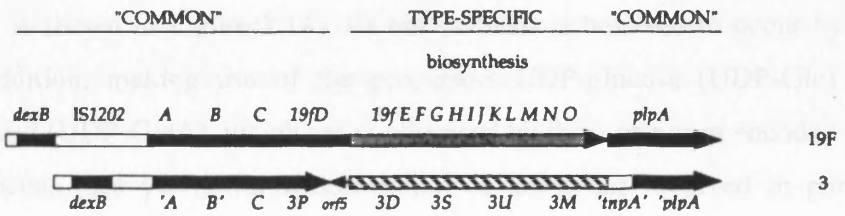

Figure 1.13. Conserved structure of the capsule loci. The maps are derived from the sequence data of the type 19F (Guidolin *et al.* 1994) and type 3 loci (Arrecubieta *et al.* 1995; Dillard *et al.* 1995). The common downstream sequence containing *plpA* has also been identified adjacent to the type 2, 5, 6B, and 14 loci (refs). ■, homologous sequences; ■ and ◪, type specific sequences; □, non-homologous sequences; ', harbouring a deletion. Taken from Caimano *et al.* (2000)

(Cartee *et al.* 2000). Figure 1.13 shows the alignment of the conserved and unique regions of the capsular biosynthesis cassettes of type 3 and type 19F *S. pneumoniae*. These common genes have been proposed to play a role in polysaccharide transport and, in some serotypes, in determining polysaccharide chain length (Cartee *et al.* 2000)

The possibility of synthesising serotype 3 capsular polysaccharide in plants was chosen for this investigation since, chemically, it is the simplest of the capsular polysaccharides. It is composed of repeating D-glucose (Glc) and D-glucuronic acid (GlcA) units, known collectively as cellobiuronic acid [1→4)-β-D-Glc*p*-(1→3)-β-D-GlcA*p*-(1→4] (Reeves and Goebel 1941; Arrecubieta *et al.* 1996). It also has the simplest genetic organisation (see §1.7.2), and the biosynthesis and heterologous expression of type 3 capsular polysaccharide has already been described in *E. coli* and *Lactococcus lactis* (Dillard *et al.* 1995; Arrecubieta *et al.* 1996a; Cartee *et al.* 2000; Forsee *et al.* 2000; Gilbert *et al.* 2000) (see §1.7.2). Furthermore, type 3 pneumococci are among the most abundant and frequently isolated strains from disease cases in developed and developing communities (Robbins *et al.* 1983a; Kalin 1998), the latter countries being those that would benefit the most from a reduced cost in vaccine production. Diseases caused by type 3 strains are often reported to have a higher mortality rate than disease due to other serotypes and therefore, present significant clinical problems (Munoz *et al.* 1997).

As stated above, type 3 polysaccharide has a simple structure. It is composed of repeating units of cellobiuronic acid; a disaccharide composed of glucose (Glc) and glucuronic acid (GlcA) (this is shown in Figure 1.14). Its biosynthesis is believed to occur by successive monomer addition, making use of the precursors UDP-glucose (UDP-Glc) and UDP-glucuronic acid (UDP-GlcA), which are synthesised by three enzymes encoded by the type 3 pneumococcus (see §1.7.2 for a description of the genes involved in pneumococcal capsule synthesis). The enzymes are a glucose-1-phosphate uridyltransferase (Cps3U) and a putative phosphoglucomutase (Cps3M), which synthesise one of the precursors, UDP-Glc, from the central metabolic pathway of Glc-6-P, and a glucose dehydrogenase (Cps3D), which converts UDP-Glc to the other precursor, UDP-GlcA (Dillard *et al.* 1995). A fourth enzyme, known as the type 3 synthase (Cps3S), catalyses the formation of bonds between the UDP precursors to form the polymer (Forsee *et al.* 2000). The proposed biosynthetic pathway is presented in Figure 1.15 (Dillard *et al.* 1995).Whilst the two UDP sugar precursors (UDP-Glc and UDP-GlcA) of type 3 polysaccharide are present in the

cytoplasm, the type 3 synthase is thought to be bound to the plasma-membrane (Cartee *et al.* 2000). Cartee and colleagues showed that, in the presence of the two precursors and Mn^{2+}, membrane preparations of type 3 *S. pneumoniae* displayed Cps3S activity by producing a polymer composed of Glc and GlcA that bound to a type 3 polysaccharide-specific monoclonal antibody (Cartee *et al.* 2000).

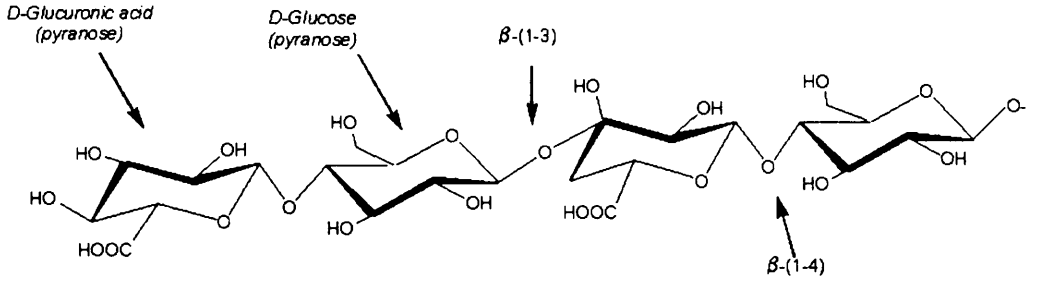

Figure 1.14. The structure of serotype 3 pneumococcal polysaccharide. As described by Kamerling (2000).

Cps3S is thought to possess two separate binding sites for each of the UDP sugar precursors, with each site catalysing the formation of a distinct glycosidic bond (Forsee *et al.* 2000). This assumption is based on hydrophobic cluster analysis, which compared the sequences of several processive β-glycosyltransferases from prokaryotes and eukaryotes, including hyaluronic acid synthase (HasA) of *Streptococcus pyogenes*, with which Cps3S shares significant homology (Keenleyside and Whitfield 1996; Saxena and Brown 1997). The study, performed by Keenleyside and Whitfield, revealed two conserved domains that are believed to be responsible for binding the nucleotide sugars and catalysing the formation of the glycosidic linkages between them (Keenleyside and Whitfield 1996). Loss of the enzymatic activities of either domain resulted in an inability to synthesise type 3 polysaccharide (Dillard *et al.* 1995).

During synthesis, the polymer is believed to grow from the non-reducing (C=O) end of the molecule (the right side of the molecule shown in Figure 1.14) (Cartee *et al.* 2000), which

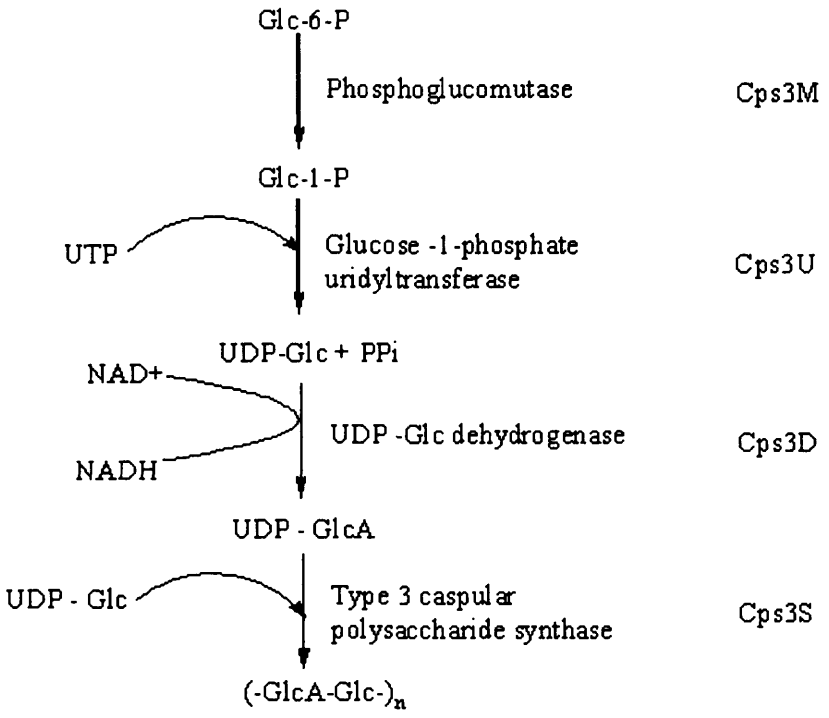

Figure 1.15. Biosynthetic pathway for type 3 capsular polysaccharide (Dillard *et al.* 1995).

continues until one of the binding sites is filled, but the concentration of the other precursor is insufficient to fill the other binding site (Forsee *et al.* 2000). At this point the protein releases the polysaccharide chain. Heterologous expression of Cps3S in *E. coli* showed that the recombinant membrane-bound synthase released all of the polysaccharide when incubated with either UDP-Glc or UDP-GlcA alone (Cartee *et al.* 2001). Although, in contrast to *S. pneumoniae*, release of the polysaccharide from type 3 synthase in *E. coli* was not accompanied by release from the membranes (Cartee *et al.* 2001). The recombinant enzyme was also able to reinitiate polysaccharide synthesis following polymer release. This was thought to be due to the retention of a phospholipid anchor at the reducing end of the polymer, which also acted as a primer to reactivate growth of the polymer chain (Cartee *et al.* 2000). No other proteins have yet been identified that are likely to be involved in transport of the type 3 polysaccharide, and this function may be performed by the type 3 synthase (Cartee *et al.* 2000).

1.7.2 The molecular biology of type 3 pneumococcal capsular polysaccharide biosynthesis

The genes required for the synthesis of the enzymes involved in the biosynthesis of type 3 pneumococcal capsular polysaccharide (see §1.7.1) have been cloned, sequenced, and expressed in both *E. coli* and *L. lactis* (Dillard *et al.* 1995; Arrecubieta *et al.* 1996a; Gilbert *et al.* 2000; Cartee *et al.* 2001). Like other serotypes, the type 3-specific genes are closely linked, arranged within a single locus (cassette) on the bacterial chromosome (these were designated 3D, 3S, 3U and 3M in Figure 1.13) (Caimano *et al.* 2000). Flanking either side of the type 3-specific genes are sequences common to all capsule types. However, in type 3 strains most of these sequences are mutated and are not required for polysaccharide synthesis or transport (Cartee *et al.* 2000).

The type 3-specific genes encode a UDP-glucose dehydrogenase (known as *cps3D*), the type 3 synthase (*cps3S*), glucose-1-phosphate uridyltransferase (*cps3U*) and a putative phosphoglucomutase (*cps3M*) (Dillard *et al.* 1995). An alternative nomenclature for the type 3 specific locus and genes, *cap*, has been used previously. This is based on the findings of Arrecubieta *et al.* (1995). However, this thesis will follow the molecular characterisation of Dillard and colleagues, since these are named based on the expected function of the protein. Therefore, the locus genes are prefixed *cps* (Dillard *et al.* 1995).

As discussed in §1.7.1, Cps3M and Cps3U synthesise the precursor UDP-Glc from the central metabolic pathway of Glc-6-P, while Cps3D catalyses the conversion of UDP-Glc to the other precursor of type 3 capsular polysaccharide, UDP-GlcA (Garcia *et al.* 1997). Cps3S is the capsule synthase that polymerises the polysaccharide chain. The proposed biosynthetic pathway was described and presented in §1.7.1.

The functions encoded by these genes were originally demonstrated by mutational analyses, which showed that only two genes were essential for type 3 capsule production in pneumococci: *cps3D* and *cps3S* (Dillard *et al.* 1995). The mutations and their effects are shown in Figure 1.16 and Table 1.9. *S. pneumoniae* strains JD900 and MC1092, which contained an insertional mutation in *cps3U* and *cps3M*, respectively, showed no reduction in capsule production, as judged by colony morphology (see Figure 1.17) (Dillard *et al.* 1995; Caimano *et al.* 2000). On the other hand, *S. pneumoniae* strains JD611 and JD619, which contained stop mutations in *cps3D*, failed to synthesise a 'detectable' level (< 0.9 μg

PnCPS mg^{-1} total protein) of capsular polysaccharide when specific Ag-Ab complexes were measured at 650 nm and quantitated by comparison with a standard curve of purified type 3 polysaccharide (Dillard *et al.* 1995). However, when supplied with UDP-GlcA (the product of the reaction involving *cps3D*) both JD611 and JD619 mutants synthesised type 3 polysaccharide (9.8 μg PnCPS mg^{-1} protein and 5.7 μg PnCPS mg^{-1} protein, respectively) (Dillard *et al.* 1995). This showed that mutants that lacked the ability to convert UDP-Glc to UDP-GlcA (lacked UDP-Glc dehydrogenase activity) could synthesise type 3 polysaccharide if supplied with the product if this reaction (UDP-GlcA) (Dillard *et al.* 1995). These findings were confirmed by heterologous expression of type 3-specific genes in other pneumococcal serotypes (*cps3S*) (Arrecubieta *et al.* 1996a), in *E. coli* (*cps3D* (Arrecubieta *et al.* 1996) and *cps3S* (Arrecubieta *et al.* 1996a)), and *Lactococcus lactis* (*cps3S*) (Gilbert *et al.* 2000). Recombinant expression of $Cps3D^{+}S^{+}U^{+}M^{-}$ in *L. lactis* resulted in the synthesis of copious amounts (120 mg $litre^{-1}$) of extracellular type 3 polysaccharide (Gilbert *et al.* 2000). This is probably because *L. lactis* contains two native phosphoglucomutase that may replace Cps3M. Furthermore, when expression was reduced to just two enzymes, Cps3D and Cps3S, the production of type 3 polysaccharide was still detected, but at a substantially lower concentration than in the recombinant with Cps3DSU (Gilbert *et al.* 2000). This implied that a glucose-1-phosphate uridylyltransferase (GalU) was also present in *L. lactis* that may fulfil the role of Cps3U. However this enzyme appeared to be rate limiting as when the pneumococcal gene, *cps3U*, was expressed, type 3 capsular polysaccharide production was far higher (Gilbert *et al.* 2000). Arrecubieta and colleagues (1996) reduced expression further to just Cps3S and found that unencapsulated pneumococci and *E. coli* were still capable of synthesising type 3 polysaccharide, provided that the nucleotide precursors (UDP-Glc and UDP-GlcA) were also present (Arrecubieta *et al.* 1996a).

(Following page) Figure 1.16. DNA sequence of the region containing *cps3D*, *cps3S*, and *cps3U*, and upstream flanking DNA. The sequence of *cps3M* is not presented. Endpoints of insertion mutations shown in **A** are indicated by triangles and are labelled with the name of the strain containing the mutation. Point mutations in *cps3D* are labelled with the sequence of the mutation and the name of the strain containing the mutation. Sequencing of the *Pvu* II-*Ssp* I fragment of A66R2 began at bp 1921, thus additional mutations between the Pvu II site and this point are possible. Selected restriction sites are shown. These sequence data are available from GenBank under accession number U15171. (Dillard *et al.* 1995)

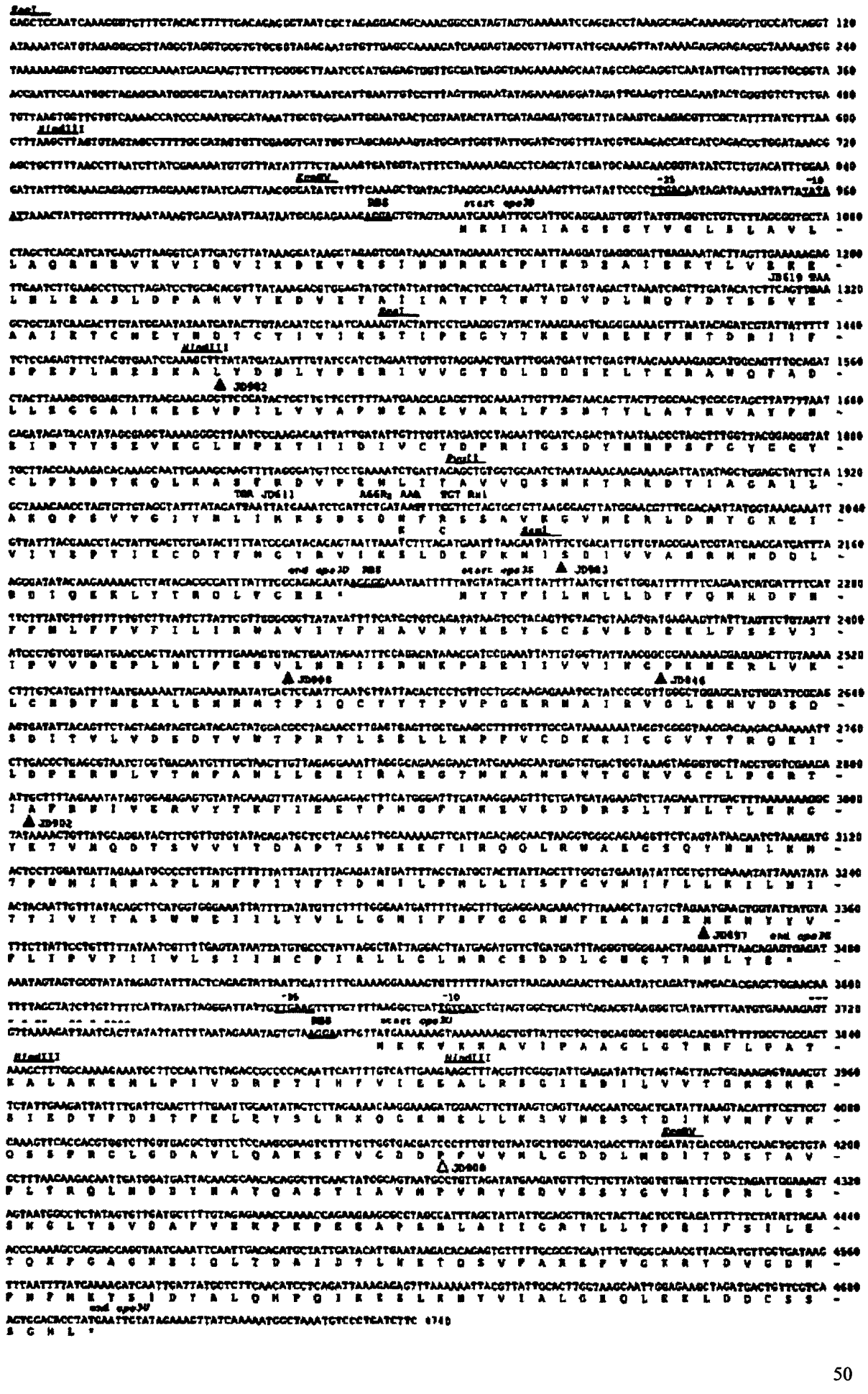

Table 1.9 *In vitro* Capsule Synthesis

Bacteria (strain)	*CPS Phenotype*	*Presence of UDP-GlcA*	*Type 3 capsule production*	*Reference*
S. pneumoniae (WU2)	$Cps3D^+S^+U^+M^+$	+	+	(Dillard *et al.* 1995)
		-	+	(Dillard *et al.* 1995)
S. pneumoniae (JD611)	$Cps3D^-S^+U^+M^+$	+	+	(Dillard *et al.* 1995)
		-	-	(Dillard *et al.* 1995)
S. pneumoniae (JD619)	$Cps3D^-S^+U^+M^+$	+	+	(Dillard *et al.* 1995)
		-	-	(Dillard *et al.* 1995)
S. pneumoniae (S3-[a], 15783/94[b], 8595/95[c], 6028[d])	$Cps3D^-S^+U^-M^-$	n/a	+	(Arrecubieta *et al.* 1996a)
E. coli (pTBP3)	$Cps3D^-S^+U^-M^-$	n/a	+	(Arrecubieta *et al.* 1996a)
S. pneumoniae (JD902)	$Cps3D^+S^-U^+M^+$	+	-	(Dillard *et al.* 1995)
S. pneumoniae (JD908)	$Cps3D^+S^-U^+M^+$	+	-	(Dillard *et al.* 1995)
S. pneumoniae (JD900)	$Cps3D^+S^+U^-M^+$	n/a	+	(Dillard *et al.* 1995)
L. lactis (UCP1618)	$Cps3D^+S^+U^-M^-$	n/a	+	(Gilbert *et al.* 2000)
S. pneumoniae (MC1092)	$Cps3D^+S^+U^+M^-$	n/a	+	(Caimano *et al.* 2000)
L. lactis (UCP1619)	$Cps3D^+S^+U^+M^-$	n/a	+	(Gilbert *et al.* 2000)
S. pneumoniae (D39[e])	$Cps2^+$	+	-	(Dillard *et al.* 1995)

[a], unencapsulated; [b], serotype 2; [c], serotype 5; [d], serotype 8; [e], serotype 2, n/a, not applicable

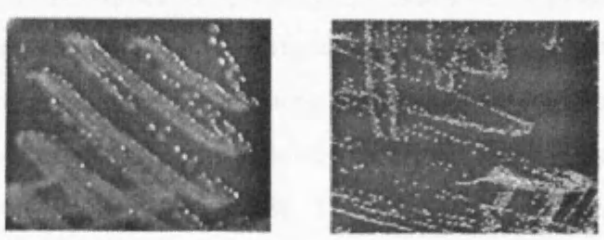

Figure 1.17. The differences in colony morphology between an encapsulated and an unencapsulated serotype 3 strain of pneumococcus when grown on blood agar. Taken from Hardy *et al* (2000).

The two type 3-specific genes, *cps3U* and *cps3M*, are therefore not essential for type 3 capsule production (Dillard *et al.* 1995; Caimano *et al.* 2000), suggesting that they are compensated by other genes in the host chromosome, possibly by essential cellular enzymes such as those involved with cell wall and teichoic acid synthesis (Caimano *et al.* 2000; Hardy *et al.* 2001). They are also not required for pneumococcal virulence in mice (Hardy *et al.* 2001). Mice infected with type 3 deletion mutants ΔCps3U (CV1047 and CV1048) and ΔCps3M (CV1037 and CV1044) were just as virulent as the parent strain (Hardy *et al.* 2001). This has prompted some researchers to question their presence in the type-specific cassette (Dillard *et al.* 1995). Although possibly, they function to regulate capsule production (Dillard *et al.* 1995) and to avoid depletion of cellular pools of UDP-Glc under conditions of enhanced capsule synthesis, such as during infection (Hardy *et al.* 2000; Hardy *et al.* 2001). For example, a type 3 mutant (JY1060) with reduced cellular phosphoglucomutase activity, displayed reduced virulence and, *in vitro* assays showed JY1060 bound seven times more complement (C3) (Hardy *et al.* 2001). An alternative explanation is that these genes were obtained along with the type-specific genes in a horizontal transfer from another organism and have not been lost (Dillard *et al.* 1995). Whatever the explanation, it can be assumed that for cloning and expressing type 3 polysaccharide, it is necessary that the capsule synthase and high concentrations of the precursors UDP-glucose and UDP-glucuronic acid are present.

1.7.3 Could heterologous expression of Cps3S lead to the synthesis of type 3 polysaccharide in plants?

As discussed in §1.7.2, at least two host functions are necessary for the production of heterologous type 3 polysaccharide: the synthesis of the precursors UDP-glucose (UDP-Glc) and UDP-glucuronic acid (UDP-GlcA), and their polymerisation into the polysaccharide (Dillard *et al.* 1995). A hypothesis to how this could be possible in plants will now be discussed.

1.7.3.1 Synthesis requires the presence of the precursors

Growing plant cells, particularly roots cells, continuously synthesise new wall polymers in order to maintain thickness and integrity (Northcote 1974). A typical dicotyledonous plant species has three classes of primary cell wall polysaccharide, which differ greatly in sugar composition (Fry 1988). They are classified as cellulosic, pectic, and hemicellulosic. Cellulose microfibrils are cross-linked into a network by H-bonded hemicellulose, to

provide tensile strength. The cross-linking of cellulose to pectin allows the cell to resist compression (Alberts *et al.* 1994). The major sugars are: cellulose (glucose (Glc)), pectins (galacturonic acid (GalA), arabinose (Ara), galactose (Gal), rhabinose (Rha)), hemicellulose (Glc, xylose (Xyl), Ara, GlcA) (Fry 1988)

The hemicelluloses are a heterogenous group of branched polysaccharides, which include the same monosaccharides that form type 3 polysaccharide, Glc and GlcA. Like type 3 polysaccharide, the hemicelluloses are derived from UDP-sugars such as glucuronic acid (UDP-GlcA) (Fry 1988). In plants UDP-GlcA is synthesised from UDP-glucose (UDP-Glc) by an endogenous UDP-Glc dehydrogenase, although there is evidence for an alternative pathway which involves the oxidative cleavage of inositol derivatives (see Figure 1.19) (Robbins *et al.* 1967; Haug and Larsen 1974). In some plant species as much as a 50 % of the cell wall mass is derived from UDP-GlcA derived precursors and most of the UDP sugars present in plant cells are used exclusively for the synthesis of cell wall polysaccharides (Fry 1988).

Therefore, given the relative abundance of UDP-glucose and UDP-glucuronic acid in plants, it was predicted that cloning Cps3U, Cps3M and Cps3D into a plant system was not necessary to achieve synthesis of type 3 polysaccharide. Instead, the introduction of a transgene encoding the type 3 capsular synthase was expected to lead to a diversion of a pool of UDP-glucuronic acid and UDP-glucose from normal matrix polysaccharide (hemicellulose) biosynthesis and into the synthesis of type 3 polysaccharide. Similar 'metabolic engineering' has been described before with success (see §1.7.3.2)

1.7.3.2 Metabolic engineering in plants

Metabolic engineering is defined as the redirection of one or more enzymatic reaction to increase the production of a specific compound, to down regulate a specific unwanted compound, or to produce a novel compound (i.e. a molecule that is produced in nature, but not usually in the host plant, or a completely novel compound) (DellaPenna 2001; Capell and Christou 2004). Over the past 25 years there have been many success stories of using of cloned genes to engineer plant metabolism. A review of some recent findings can be found in §5.11.3.1. However there are no published examples that describe the expression

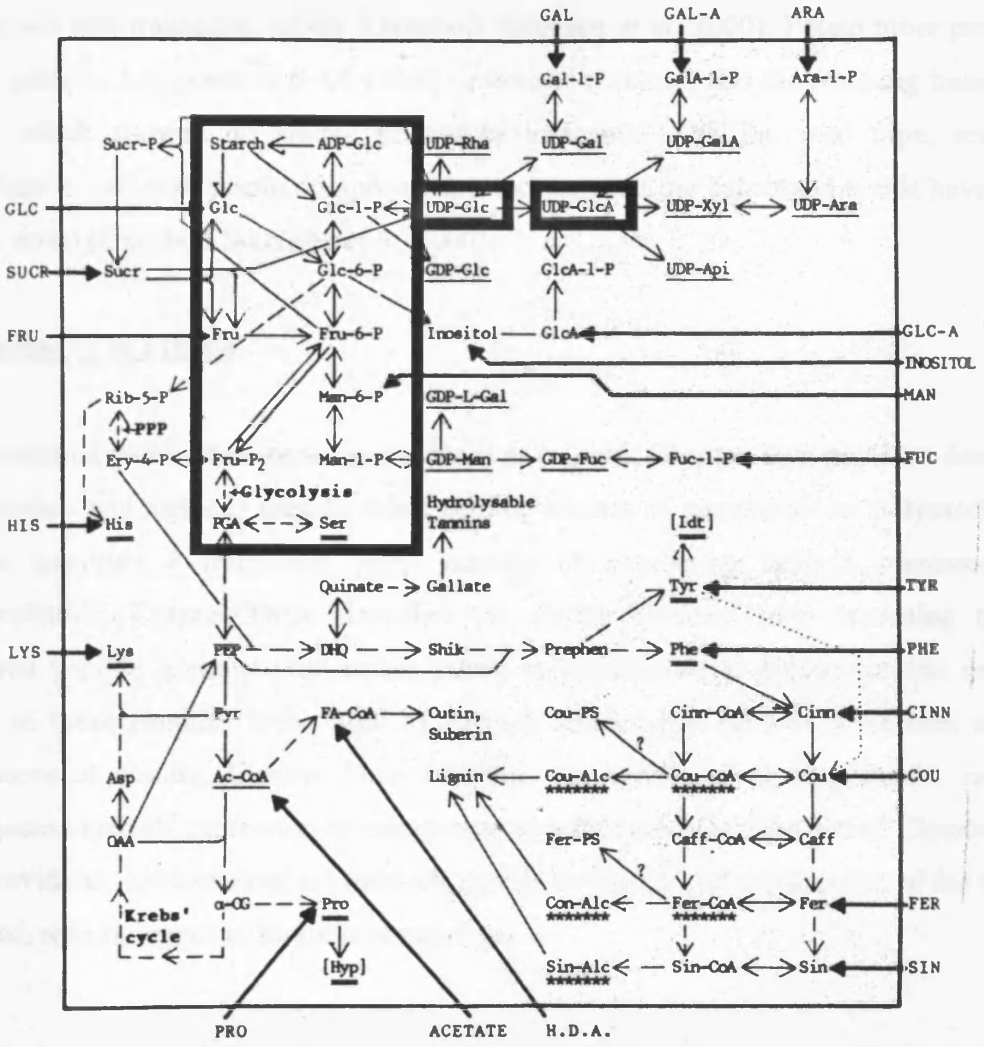

Figure 1.19: Metabolic map showing the activation and inter-conversion of some precursors involved in cell wall synthesis. The metabolic pathways that could lead to the expression of type 3 pneumococcal polysaccharide precursors are highlighted by grey boxes. The diagram was taken from Fry (1988)

Abbreviations: Caff, Caffeate; Con-Alc, Coniferyl alcohol; Cinn, Cinnamate; Cou, Coumarate; Cou-Alc, *p*-coumaryl alcohol; DHQ, Dehydroquinate; Ery, Erythrose; FA, Fatty acid; Fer, Ferulate; Fru-P_2, Fructose 1,6-biphosphate; HAD, Hexadecanoic acid (palmitic acid); OAA, Oxaloacetate; PEP, Phosphoenolpyruvate; PGA, 3-Phosphoglyceric acid; PPP, Pentose-phosphate pathway; Prephen, Prephenate; PS, Polysaccharide; Pyr, Pyruvate; Shik, Shikimate; Sin, Sinapate; Sin-Alc, Sinapyl alcohol; Sucr, Sucrose; α-OG, α-Oxoglutarate.

of microbial polysaccharide antigens in plants. At the start of this project, one paper discussed the introduction of a fungal gene (from *Aspergillus aculeatus*) encoding an endo-galactanase into transgenic tubers (Oxenboll Sorensen *et al.* 2000). Potato tuber pectin is rich in galactan (oligomer of ß-1,4-linked galactosyl residues) and the resulting transgenic plants, which showed no altered phenotype compared with the wild type, revealed alterations in cell wall pectin composition, indicating that the galactosyl bonds have been broken down (Oxenboll Sorensen *et al.* 2000).

1.8 Outline of this thesis

The remaining part of this thesis is structured as follows. Chapter Two provides details of the materials and methods used to select peptide mimics of pneumococcal polysaccharide and to construct a transgenic plant capable of expressing type 3 pneumococcal polysaccharide. Chapter Three describes the results obtained from screening phage-displayed peptide libraries with mAbs raised to pneumococcal polysaccharide and the ability of these peptides to be used as vaccine mimics and provide protection against pneumococcal disease. Chapter Four describes the results of cloning Cps3S and the consequent *in planta* expression of pneumococcal polysaccharide. The aim of Chapter Five is to provide an assessment of the methods used in this study and a discussion of the results obtained, with reference to future investigations.

CHAPTER TWO - MATERIALS AND METHODS

2.0 Chapter overview

This chapter of the thesis is divided into two main parts. The first part (Part A) describes the methods used to select peptide mimics of serotype 6B and 9V pneumococcal polysaccharides using a phage-display library. The second part (Part B) describes the methods used to develop a transgenic plant capable of synthesising pneumococcal polysaccharide. First the general methods used by both projects are described.

2.1 Bacterial strains and growth conditions

Escherichia coli strains were cultured aerobically in either Luria Broth (LB (1 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 1 % (w/v) NaCl) or Luria Agar (LB, as above, containing 1.5 % (w/v) agar) and incubated at 37°C.

Streptococcus pneumoniae serotypes 3 (WU2) 4, 6B, 9V and 18C strains were obtained from the frozen stocks of Lab 227 Department of Infection, Immunity and Inflammation, University of Leicester, UK. Cultures of *S. pneumoniae* were grown at 37°C in a candle jar (raised CO_2 conditions) in either Brain Heart Infusion (BHI) (Oxoid, Basingstoke, Hants, UK; product BO0366) broth, BHI containing 10 % (v/v) Foetal calf serum (FCS) or on Blood Agar Base (BAB) (Oxoid, Basingstoke, Hants, UK; product CM0055) containing 5 % (v/v) defibrinated horse blood for culture on solid medium.

Strains of *Agrobacterium tumefaciens* were cultured aerobically in YEP broth (10 g l^{-1} Yeast Extract, 10 g l^{-1} Peptone, 5 g l^{-1} NaCl, pH 7.0). For culture on solid medium the YEP broth was supplemented with 1.5 % (w/v) agar. Growth occurred following incubation at 28°C for 2-3 days. Strains of *A. rhizogenes* were grown aerobically in YMB broth (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 10 g l^{-1} Mannitol, 0.4 g l^{-1} Yeast Extract, pH 7.0). For culture on solid medium the YMB broth was supplemented with 1.5 % (w/v) agar (YMB). Growth occurred following incubation at 28°C for 2-3 days.

All media were autoclaved at 121°C for 20 minutes prior to use. Agar media were allowed to cool to 50°C before the addition of media supplements.

Table 2.1 Bacterial strains used to identify peptide mimics of pneumococcal polysaccharides from a phage-display library. The Lab reference refers to the location of the bacterial bead stocks, which are stored in 10 % (v/v) glycerol at -70°C in Lab 227, Dept of I.I.I, University of Leicester, UK.

Bacterial Strain	*Lab Reference*	*Reference or Source*
E. coli TG1	Lab Stock, University of Messina, Italy	(Harrison *et al.* 1996)
S. pneumoniae serotype 4 *(TIGR4)*	P202	Tim Mitchell, University of Glasgow
S. pneumoniae serotype 6B *(23477)*	P164	Marie Trombe, Universitaire de Rangueil, Toulouse, France
S. pneumoniae serotype 9V *(907/88)*	P187	RIVM (Kolberg and Jones 1998)
S. pneumoniae serotype 18C *(NCTC11905)*	P233	NCTC
Agrobacterium tumefaciens (GV3101)	S39	Biology Dept. University of Leicester
A. rhizogenes (LBA9402)	S38	Biology Dept. University of Leicester

2.1.1 Antibiotics and media supplements

All media supplements are shown in Table 2.2 below. Those dissolved in water were filter-sterilised through a 0.2 μm acrodisc filter (Acrodisc, Gelman Laboratories), aliquoted and stored at -20°C

Table 2.2 Antibiotics and media supplements

Media supplements	*Stock*	*Working Concentration*
Ampicillin	50 mg ml^{-1} in water	100 μg ml^{-1}
Chloramphenicol	100 mg ml^{-1} water	34 μg ml^{-1}
Cefotaxime	200 mg ml^{-1} in water	250 – 500 μg ml^{-1}
Gentamicin	100 mg ml^{-1} in water	150 μg ml^{-1}
Kanamycin	100 mg ml^{-1} in water	100 μg ml^{-1}
Rifampicin	10 mg ml^{-1} in methanol	100 μg ml^{-1}
Spectinomycin	1mg ml^{-1} in water	100 μg ml^{-1}
6-benzylaminopurine (6-BAP)	2 mg ml^{-1} in 0.1 M KOH	1 μg ml^{-1}
Isopropyl-ß-D-thiogalactopyranoside (IPTG)	200 mM	0.5 mM
Napthaleneacetic acid (NAA)	1 mg ml^{-1} in water	0.1 μg ml^{-1}
5-bromo-4-chloro-3-indolyl-ß-D-galactoside (X-Gal)	32 mg ml^{-1} in dimethylformide	80 μg ml^{-1}

2.1.2 Serotyping pneumococci

Serotyping the pneumococcal strains was done by the capsular reaction test (Quellung reaction) using diagnostic pneumococcal antiserum. This test detects the presence of type specific capsular polysaccharide (Heineman 1973).

A loop of overnight culture of *S. pneumoniae* grown in BHI containing 20 % (v/v) Foetal Calf serum was smeared onto a microscope slide and allowed to air-dry. 10 μl of serotype specific anti-pneumococcal capsular polysaccharide antiserum (Statens Serum Institute, Copenhagen, Denmark) was placed onto a coverslip and mixed with 10 μl 1 % (w/v) methylene blue in water, before placing the coverslip, fluid side down, onto the slide. Bacteria were examined under x1000 magnification and were compared to a control slide

prepared with non-immune serum (heat inactivated foetal calf serum). Bacteria were counted as reactive if the capsule was distinctly outlined around the blue stained cells.

2.2 Mice

Female, specific pathogen free outbred mouse strain MF1 (from Harlan Olac Ltd., UK) were used throughout this study. They were at least 9 weeks old at the beginning of each experiment. The mice were housed in groups of five under standard conditions with regulated day length, temperature and humidity, and were given tap water and pelleted food *ad libitum*.

2.2.1 Infection of mice

2.2.1.1 Dose preparation

To challenge mice, *S. pneumoniae* strains were first passaged through female MF1 mice (Harlan Olac Ltd., Bicester, UK). A 10 ml culture of *S. pneumoniae* in BHI was grown overnight at 37°C from a single colony. The following day the bacteria were harvested by centrifugation at 600x *g* for 15 minutes and the pellet was re-suspended in 5 ml sterile PBS. 200 µl of this suspension was injected intraperitoneally into female MF1 mice. 24 hours following infection the bacteria were recovered via cardiac puncture, as described previously (Canvin *et al.* 1995). 100 µl of blood was used to inoculate a 10 ml BHI broth and this was incubated overnight at 37°C. The following day the bacteria were harvested by centrifugation, as before, and the pellet re-suspended in 1 ml BHI containing 20 % (v/v) Foetal Calf serum. This was used to inoculate a fresh 10 ml BHI broth containing 20 % (v/v) FCS with a starting OD_{500} of 0.7 and samples were grown at 37°C until OD_{500} was 1.6. At this time samples were aliquoted and stored at -70°C. When required, the suspension was thawed at room temperature, pelleted by centrifugation at 600x *g* for 10 minutes at room temperature and re-suspended in the same volume of sterile phosphate-buffered saline (PBS).

2.2.1.2 Dose confirmation and viable counts

Doses and viable counts were calculated as an average colony forming units (cfu) formed from triplicate 10 µl spots of Miles-Misra plating onto Blood Agar Base (BAB) with 5 % (v/v) defibrinated horse blood following ten-fold serial dilutions in sterile PBS (see Figure 2.1).

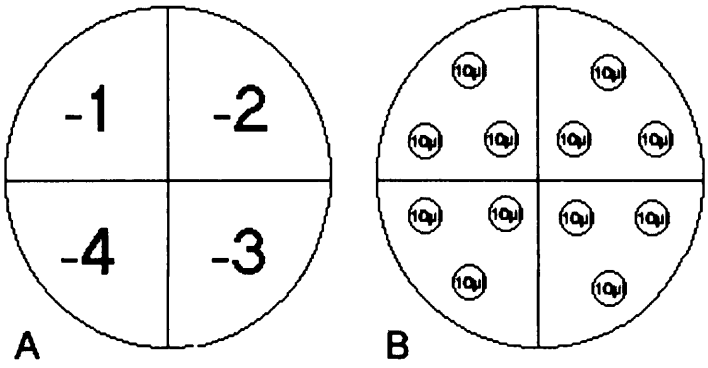

Figure 2.1. Miles-Misra style plating of diluted bacterial suspensions to achieve a viable count. A represents the dilution factor performed, whereas B represents the placement of the triplicate 10µl spots for each dilution. Calculation of cfu/ml was attained by the following equation; cfu/ml = y x 10^d x 100, where y is the average colony count in 10µl per dilution and d is the dilution factor.

2.2.1.3 Virulence testing

To determine the appropriate challenge dose, serial 10-fold dilutions of pneumococci were given intraperitoneally (i.p.) to 5 mice. The technique used to perform i.p. injections with mice was described by Harlow *et al.* (1988). Each group received an estimated dose of between 1 x 10^6 – 1 x 10^7 cfu passaged bacteria diluted in 100 µl sterile PBS. For more virulent strains, experiments were repeated with smaller dilution steps of the inoculum in order to determine the useful dose more precisely. The number of bacteria present in the suspensions was confirmed by the method described in §2.2.1.2.

Following infection, mice were observed for 10 days, except for serotype 6B, which showed a delayed onset of symptoms and therefore required a longer period of observation (15 days). The signs of disease were monitored and signs were scored based on the scheme of Morton *et al.* (1985). This is shown in Table 2.3. If significant morbidity (Lethargic ++) was observed (≥80 % mice affected) this dose was used in subsequent experiments.

Table 2.3 The scores given to mice suffering from signs of pneumococcal disease, based on the scheme of Morton *et al.* (1985).

Sign	*Score*
No sign	0
Hunched +	1
Hunched++	2
Starey coat +	3
Starey coat ++	4
Lethargic +	5
Lethargic ++	6
Moribund	7
Found Dead	8

2.3 ELISA

Three different enzyme-linked immunosorbent assays (ELISA) techniques were used in this study. The methods used were adapted from those described by Harlow *et al.* (1988). These are illustrated in Figure 2.2.

The phage ELISA technique was used to give a quantitative determination of the number of recombinant phage present in the suspension. As shown in Figure 2.2, an anti-pIII coat protein was used to coat the microtitre plate. This orientated the recombinant phage so that more pVIII coat proteins were available for binding the primary mAb. Since the phage was coating the plate and the monoclonal was in abundance, the amount of monoclonal that binds represented the number of positive clones. This was detected using a labelled secondary antibody. This is described further in §2.5.7.2.

The direct ELISA technique was used to assess the immunological quality of the serum produced from mice immunised with peptide-conjugates. 96-well microtitre plates (Maxisorp, Nunc) were coated with either 2 $\mu g\ ml^{-1}$ pneumococcal polysaccharide, or 5 $\mu g\ ml^{-1}$ of the peptide conjugated to BSA (or unconjugated peptide). The serum was then added at different dilutions and the IgG concentration (or titre) was detected by an anti-mouse secondary antibody conjugated to alkaline phosphatase. This is described further in §2.8.1.

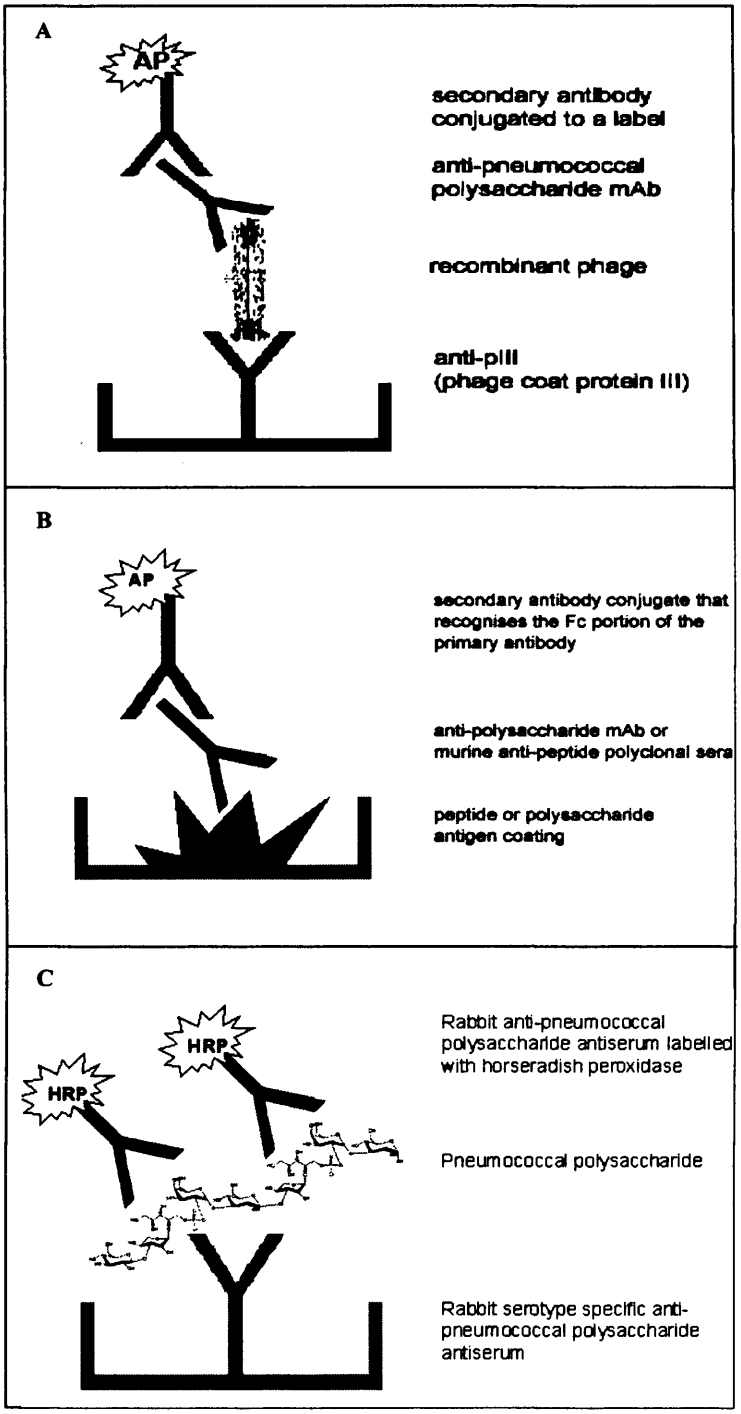

Figure 2.2 (A) Phage ELISA: This technique gives a quantitative account of the number of recombinant phage present in the suspension. **(B) Direct ELISA.** This is the ELISA technique used to assess the immunological quality of the serum produced from mice immunised with peptide-conjugates. **(C) Capture ELISA.** This technique was used to detect the presence of type 3 pneumococcal polysaccharide in plant tissue extract (see §2.15.4.2).

The capture ELISA technique was used to detect the presence of type 3 pneumococcal polysaccharide in plant tissue extracts (see §2.15.4). Rabbit anti-type 3 polysaccharide specific antiserum was used to coat the plate, the plant cell extract or purified pneumococcal polysaccharide (control) was then added and this was detected with a horseradish peroxidase conjugate of the coating antibody. This is described further in §2.15.4.

2.3.1 Reagents

- Coating Buffer: 50nM $NaHCO_3$, pH9.6, 0.02 % (w/v) NaN_3
- Washing Buffer: PBS containing 0.5 % (v/v) Tween20
- Milk-Blocking buffer: PBS containing 0.5 % (v/v) Tween20, 1 mg ml^{-1} non-fat dry milk (Difco, Product Number 232100) and 0.1 % (w/v) NaN_3).
- Developing solution: 1 mg ml^{-1} p-nitrophenyl phosphate (Sigma N9389) dissolved in 1 M diethanolamine pH9.8, 0.5 M $MgCl_2$ with 0.05 % (w/v) NaN_3.

Part A: Peptide Mimicry

2.4 Monoclonal antibodies

Monoclonal antibodies (mAb) raised to serotype 9V (one IgA and one IgG1 (6bC3, 206,F-5)), 4 (two IgG2 (CbB2 and Db7D4)), 6B (two IgG2 (DM5 and Db3G9)) and 18C (one IgG3 (6b53)) have been previously described (Kolberg and Jones 1998; Baxendale *et al.* 2000).

The murine mAb 206,F-5 was produced from six-week old female BALB/c mice immunised i.p. with a heat treated (30 min at 56°C), sonicated serotype 9V pneumococcal suspension. Booster injections with the same mixture were administered 2 weeks later and 4 days prior to fusion (5 months after the primary immunisation). Spleen cells were fused with NSO myeloma cells and hybridoma supernatants were screened by ELISA (see §2.8.1). This work was performed by Jan Kolberg, (Norwegian Institute of Public Health, Division of Infectious Disease Control, P.O. Box 4404 Torshov, N-0403 Oslo, Norway)

The human mAbs were produced from healthy volunteers given a single dose of the 23-valent polysaccharide vaccine (Pneumovax II, Pasteur Mérieux, France) (6b53) containing

25 μg of each serotype, or the 7-valent vaccine (7V-CRM_{197}) containing 2 μg each of 4, 9V, 14, 18C, 19F, 23F and 4 μg of 6B (CbB2, Db4D7, DM5 and Db3G9) conjugated to a mutant diptheria toxoid (Wyeth Lederle Vaccines and Paediatrics, NY, USA). Lymphocytes recovered from venepuncture on Day 7 post immunisation were fused to the non-secreting mouse myeloma line OURI using PEG (Thompson *et al.* 1991). Fused lymphocytes were plated onto a mouse fibroblast feeder layer in 96-well tissue culture plates and were incubated at 37°C in 5 % CO_2 for 2-3 weeks. The cells were screened for pneumococcus-specific antibody production by ELISA (see §2.4.2). Positive wells were subcloned by limiting dilution and the cell lines then expanded in standard tissue culture flasks. This work was performed by Helen Baxendale from the Institute for Child Health, London, UK.

2.4.1 Determination of antibody isotype

To determine the antibody isotype, monoclonals were re-screened on single-serotype polysaccharide coated ELISA plates (as §2.4.2). The isotype of the antibody was determined using a panel of isotype-specific HRP-conjugated goat anti-human Ig antibodies (Sigma, Dorset, GB) for the human mAbs (Baxendale *et al.* 2000)., or alkaline phosphatase-conjugated goat anti-mouse IgG antibodies (Sigma, Dorset, GB) for the murine mAb (Kolberg and Jones 1998)

2.4.2 Determination of mAb concentrations

The mAb 206,F-5 was obtained as an ascites. The reactivity of ascites to capsular polysaccharide was analysed by ELISA (as described by Kolberg *et al.*, 1998). Flat-bottomed microtitre plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated overnight at 4°C with serotype 9V PnCPS (2 μg ml^{-1}) in PBS (pH 7.4) with 0.02 % (w/v) sodium azide, 50 μl per well. The plate was blocked using 200 μl PBS containing 3 % (w/v) BSA for 1 hour at 37°C. Hybridoma culture supernatant dilutions were prepared in PBS containing 3 % (w/v) BSA and 50 μl was added to the wells. The plate was then incubated for 2 hours at 37°C. Alkaline phosphatase-conjugated goat anti-mouse-Ig (Sigma, St. Louis, MO, USA) was used at a dilution of 1:2000. p-Nitrophenyl-phosphate was used as the substrate with 1 mg ml^{-1} in 10 % (w/v) diethanolamine buffer (pH 9.8) containing 5 mM $MgCl_2$. The plate was washed between each step using PBS containing 0.05 % (v/v) Tween 20 (Kolberg and Jones 1998)

Serotype specific pneumococcal antibody titres of human mAbs were determined by ELISA as previously described (Baxendale *et al.* 2000). 96-well plates were coated with 7-valent antigen mix (containing purified pneumococcal polysaccharide (ATCC) of serotypes 4, 6B, 19F (all at a concentration of 20 μg ml^{-1}), 9V, 14 and 23F (10 μg ml^{-1}) and 18C (2 μg ml^{-1})), 100μl per well. ELISA was performed as described above, except that the secondary antibody used was goat anti-human polyvalent IgG conjugated to Horse Radish Peroxidase (HRP). Those that gave a positive result (absorbance at A_{450} greater than 1.0) were re-screened on plates coated with polysaccharide of a single serotype.

Serotype-specific IgG, IgM and IgA titres in pre- and postimmunisation sera (day 0 and day 28, respectively) were determined for each of the five serotypes for which hybridomas were made, using ELISA (as above). A standard serum, 89SF (supplied by Carl Frasch, Center for Biologics Evaluation and Research, Rockville, MD, USA) was used as the reference serum. All samples were assayed in duplicate. The second antibody, HRP-conjugated goat anti-human IgG, IgM or IgA (Sigma, Dorset, GB), was used at a dilution of 1:1000, 1:500 and 1:1000, respectively. Ig concentrations were calculated using ELISA software (Revelations 2.0; Dynatech, GB) from the standard curve produced by 89SF. Antibody concentration is expressed in μg ml^{-1}. See Table 3.2.

2.4.3 Identifying protective antibodies

Mice were primed by passive intraperitoneal immunisation with 100μl ascites fluid containing 206,F-5 or tissue culture medium containing human mAb diluted 1:2 in PBS (as advised by Kolberg). Mice received doses 24 hours prior to infection and were boosted with the same dose 2 hours prior to infection. Test mice and Control mice, which received PBS alone, were infected with a dose of bacteria previously shown to cause disease (see Table 3.1). Following infection, the signs of disease were monitored for 10 or 15 days as described in §2.2.1

2.5 Selection of peptide mimics of pneumococcal polysaccharide using phage display libraries

Peptide mimics of pneumococcal polysaccharide were selected by panning four different phage libraries: pVIII-9aa, pVIII-12aa and pVIII-12aa.Cys, and pVIII-15aa. These libraries display peptides composed of random 9 amino acid, 12 amino acid, and 15 amino acid

residues, respectively. The pVIII-12aa.Cys library was cyclically restrained by the presence of two cysteine residues at positions 5 and 9, the remaining positions were occupied by random amino acids. The construction of these libraries has been previously described (Felici *et al.* 1991; Luzzago *et al.* 1993). The methods for screening phage display libraries (Harrison *et al.* 1996) were described in §1.5.2.

Escherichia coli TG1 cells were used to propagate phage in this study. This strain was chosen for two reasons. 1) It displayed the sex pilus encoded by the F episome (F') that allowed the filamentous phage to attach to the bacterium and infect. 2) TG1 was a suppressor strain, which maintained that transcription of the pVIII fusion proteins in pC89 was under the control of the Lac promoter. This meant that fusion gene transcription levels are low in the absence of IPTG so that only one or two copies of the oligopeptides were displayed on each phage particle surface. The presence of multiple copies of wild-type pVIII coat proteins ensured efficient phage packaging and infectivity.

Two methods were used in this study to select peptide mimics of pneumococcal polysaccharide from the phage-displayed peptide libraries (these are outlined in §2.5.1 and 2.5.2). This was to eliminate any bias that may occur from using the same selection technique for all rounds of selection. The methods used for each round of panning for each monoclonal antibody are shown in Table 2.4. Subsequent rounds of selection will increase the prevalence of the reactive clones and the sequence of the selected peptide can be deduced from the sequence of the phagemid DNA (Felici *et al.* 1991).

Dr Carla LoPasso performed the selections with mAb 206,F-5, whereas I performed those with mAbs Db3G9, DM5 and 6b53. All were achieved in the facilities provided by the University of Messina under the direction of Prof Franco Felici.

2.5.1 Phage selection by Biopanning

The library was screened by the biopanning technique according to (Parmley and Smith 1988). The mAb was incubated overnight at 4°C with 10^{10} transducing units (see §2.5.6.1 for quantification of transducing units) of library in a total volume of 20 µl PBS. Under the same incubation conditions, a 6 cm Falcon Plate was coated with 10 µg streptavidin (stored at -20°C as 1 mg ml^{-1} lyophilised streptavidin (Sigma) in 10 mM PBS, 15 mM NaCl and 0.05 % (w/v) NaN_3) in a volume of 4 ml 50 mM sodium carbonate pH 9.6.

Table 2.4 The concentration of mAb and methods used for different rounds of phage panning to select peptide mimics of pneumococcal polysaccharides.

Antibody reference	*Libraries screened*	*First round of selection*	*Second Round of selection*	*Third round of selection*	*Fourth round of selection*
206,F-5	12aa, 12aa.Cys 15aa	Dynabeads mAb conc^{n}: 1μg	Dynabeads mAb conc^{n}: 1μg	Biopanning mAb conc^{n}: 10nM	
			Biopanning mAb conc^{n}: 1μg	Dynabeads mAb conc^{n}: 1μg	
DM5	9aa 12aa 12aa.Cys	Biopanning mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM	Biopanning mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM
Db3G9	9aa 12aa 12aa.Cys	Biopanning mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM	Biopanning mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM
6b53	9aa 12aa 12aa.Cys	Dynabeads mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 5μM	
			Biopanning mAb conc^{n}: 10μM	Biopanning mAb conc^{n}: 10μM	Dynabeads mAb conc^{n}: 10μM

The mAb-library mixture was then incubated with 0.25x concentration (in PBS) biotin conjugated goat anti-mouse IgG secondary antibody (Sigma) for 4 hours at room temperature or goat anti-human IgG-conjugated to biotin (Sigma B1140) for the human mAbs. Before use, the secondary antibodies were pre-adsorbed overnight at 4°C with 3.2 x 10^{10} UV killed M13 K07 phage particles to prevent non-specific binding. The streptavidin-coated Falcon plates were blocked with 10 ml BSA-Blocking Buffer (Stored at -20°C as 5 mg ml^{-1} dialyzed BSA (Sigma A3912), 0.1 M $NaHCO_3$ (pH 8.6), 0.1 μg ml^{-1} streptavidin) for 1 hour at room temperature and then the phage-mAb-secondary antibody complex was added to the plate. The plate was incubated for 10 minutes on a rocking platform at room temperature before an extensive washing step; a fresh 5 ml of Washing Buffer (PBS

containing 0.5 % v/v Tween20, pH7.5) was added at 3 minute intervals 10 times. Bound complexes were eluted by the addition of 800 µl Elution buffer (0.1 M HCl-glycine pH2.2 containing 0.2 M 10 mg ml^{-1} BSA (added prior to use)) and incubated on a rocking platform for 10 minutes at room temperature.

2.5.2 Phage panning using Protein-G coated Dynabeads

Phage capture using Dynabeads was performed as described by the manufacturer (Instruction manual for Dynabeads coated in protein G (Dynal Biotech, Oslo, Norway, Product #100.04)). 10 µl of Dynabeads (as above) were used for each selection. At each step the reaction was incubated on a rotating stage. The beads were first washed twice by the addition of 500 µl of Washing Buffer (PBS containing 0.5 % (v/v) Tween20) and a magnetic particle separator (Dynal) was used to aspirate the supernatant.

The mAb was added at the required concentration (see Table 2.3). This mixture was incubated for 1 hour at room temperature. Then, using the magnetic particle separator the mAb-beads were washed 3 times for 10 minutes each with 500µl Washing Buffer. Unbound beads were blocked with 1 ml Milk-Blocking Buffer (PBS containing 0.5 % (v/v) Tween20, 1 mg ml^{-1} Non fat dry milk (Difco, Product Number 232100) and 0.1 % (w/v) NaN_3) for 30 minutes at room temperature. Beads were separated from the Blocking Buffer with a magnetic particle separator and the solution was replaced with fresh Blocking Buffer for 30 minutes.

The beads were then incubated with 10 µl of phage (approx. 10^{11} TU) in 990 µl Blocking Buffer on a rotating rack for 4 hours at room temperature. Again the beads were washed (as before). Bound complexes were eluted by the addition of 450 µl Elution Buffer (0.1 M HCl-Glycine pH2.2 containing 0.2 M 10 mg ml^{-1} BSA (added fresh prior to use)) for 10 minutes at room temperature. The mixture was neutralised by the addition of 32 µl 2 M Tris pH9.6 and eluted phage were stored at 4°C.

2.5.3 Phage Amplification

Phage concentrations following elution from panning were usually in the range of 1×10^5 – 1×10^8 TU ml^{-1} (Barbas *et al.* 2001). Increasing the size of the repertoire led to improved binding affinities (Harrison *et al.* 1996). Therefore the number of phage were amplified by

bacterial infection to around 10^{12} TU (see §2.5.6 for determination of phage concentration) before another round of selection was performed.

To amplify the numbers of phage, 200 µl of the eluted phage from each round of selection were added to 800 µl *E. coli* (TG1) (OD_{600} 1.5 - 2.0) and incubated in a Thermomixer set to 37°C (Eppendorf Inc.) for 15 minutes and a further 30 minutes shaking. This method was modified from that described in Barbas *et al.*, (2001). The 1 ml of infected bacteria was spread onto a large Petri dish containing Luria Agar, 100 mg l^{-1} ampicillin and 1 % (w/v) glucose and incubated overnight at 37°C. The following day the confluent bacterial lawn was scraped from the plate uaing 15 ml Luria broth (LB) containing 100 mg l^{-1} ampicillin and 1 % (w/v) glycerol. 50 µl of the bacteria-phage suspension was grown at 37°C until the OD_{600} was 0.2, at which time 10^9 TU ml^{-1} of helper wild-type phage (M13 K07) was added. This was because pC89 did not contain the complete genome of M13. Therefore, a helper phage (known as M13 K07) was needed to begin complete phage replication. M13 K07 had a mutated gene II product that interfered less efficiently with its own origin of replication. Therefore, it preferentially bound to the recombinant origin of replication and completed the construction of the recombinant phage particles. 20 µg l^{-1} IPTG was also added to promote growth of recombinant phage. Incubation continued for a further 4 hours and phage were recovered in the supernatant by centrifugation at 600x *g* for 40 minutes at 4°C. The phage supernatant was stored at 4°C until use.

2.5.4 Small-scale preparation of phage

If, after quantification (see §2.5.6), the phage concentration following amplification was not high enough, the phage were precipitated by the addition of polyethylene glycol (PEG). This method was adapted from Barbas (Barbas *et al.* 2001). PEG is a water-soluble waxy solid that binds to the phage to increase its molecular weight (MW). As the temperature decreased its solubility was greatly reduced, and it (and the phage) precipitated out of solution easily.

1.6 ml phage suspension was precipitated by the addition of 4 % (w/v) PEG8000 and 0.5 M NaCl and incubated at 4°C overnight. The following day, the phage were pelleted by centrifugation at 10 000x *g* for 30 minutes and re-suspended in 160 µl TBS (1.21 g l^{-1} Tris, 8.76 g l^{-1} NaCl, pH to 7.9). The precipitation step was repeated by the addition of 10 % (w/v) PEG8000 and 1.25 M NaCl and incubation for 1 hour on ice. The precipitate was

recovered by micro-centrifugation at 10 000x *g* for 30 minutes. The supernatant was discarded and the walls of the eppendorf were dried with tissue and then placed in an incubator at 37°C for 5 minutes. Finally, the pellet was re-suspended in 32 µl TBS and stored at 4°C.

2.5.5 Large-scale preparation of phage

This method, adapted from Smith and Scott (Smith and Scott 1993), was particularly effective as a means of removing residual PEG, nucleases, proteases, and other contaminants from PEG-purified phage. Caesium chloride (CsCl) creates a density gradient when spun in an ultracentrifuge. Highly purified phage were then extracted from the supernatant. This method was recommended for large-scale purification and long-term storage of phage libraries (Barbas *et al.* 2001) and was performed by Dr Carla LoPasso (University of Messina, Sicily, Italy).

The phage were recovered from a 1 Litre overnight culture of phage-infected *E. coli* TG1 cells by two successive 10 minute centrifugations (5000 and 8000 rpm in three 500 ml bottles in a Sorvall (Norwalk, CT) GS3 rotor at 4°C); the final supernatant was distributed equally in three 500 ml centrifuge bottles. After adding 0.15 vol PEG/NaCl solution (100 g PEG8000, 116.9 g NaCl and 475 ml water), the supernatant was thoroughly mixed and incubated overnight at 4°C. Precipitated phage were collected by a 30 minute centrifugation (8000 rpm in a Sorvall GS3 rotor at 4°C), removing all supernatant. The precipitate from all bottles were resuspended in a total of 30 ml TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.02 % (w/v) NaN_3) and transferred to a single Oak Ridge tube (Nalge, Rochester, NY). Any contaminants were cleared by a 10 minute, 15000 rpm centrifugation in a Sorvall SS34 rotor, and the phage were re-precipitated from the supernatant by adding 4.4 ml PEG/NaCl solution and incubating for 1 hr at 4°C. Phage were then collected by centrifugation at 4°C in a Sorvall SS34 rotor, again taking care to remove all supernatant, and finally resuspended in 10 ml TBS.

The PEG precipitated phage supernatant was transferred to a tared vessel and TBS added to bring the total net weight to 10.75g; 4.83g CsCl was added to bring the density to 1.30 g ml^{-1}. The CsCl solution was transferred into a polyallomer tube (topped with mineral oil if necessary - tubes had to be filled to the top to prevent collapse during centrifugation) and centrifuged at 37 000 rpm for at least 40 hr in a SW41 rotor (Beckman Instruments) at 4°C.

After centrifugation the tubes were carefully removed from the rotor and placed in a rack in a clamp stand. The clamped tube was then illuminated from the top with a strong visible light source (halogen lamp). By shining a bright light downward through the tube and looking through the wall of the tube at right angles to the light beam a translucent, non-flocculent phage band was visualised near the middle of the tube just above a sharp, white, flocculent band (probably PEG); 10^{15} particles can give a band ~1 cm wide (Smith and Scott 1993). The fluid overlying the phage was carefully aspirated and discarded to within 2 mm of the band and the phage collected in a sterile glass pipetter attached to a peristaltic pump. If a quickseal polyallomer tube was used the phages were collected by puncturing the side of the tube with a 16G needle attached to a 3 ml syringe.

Phage were pooled (~10ml) and transferred to a 26 ml polycarbonate bottle for a Beckman 60Ti rotor; the bottles were filled to the shoulder with TBS, inverted several times to mix and centrifuged at 50 000 rpm for 4 hr at 4°C to pellet the phage. The supernatant was poured off and discarded and the phage pellet was dissolved in 12 ml TBS per litre equivalent of starting culture; this gave an anticipated concentration of 3 x 10^{13} phage/ml. This was transferred to a sterile Oak Ridge tube and centrifuged at 6500x *g* for 10 minutes. Sodium azide was then added (to final concentration of 0.02 % (w/v)) to prevent the growth of contaminants and the phage-containing supernatant was stored at 4°C away from the light or in 50 % (v/v) glycerol at -18°C for long-term storage. Under these conditions, titres were stable for several years.

2.5.6 Phage quantification

2.5.6.1 Transducing Units

100 µl of phage was ten-fold serially diluted in 900 µl TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.02 % (w/v) NaN_3) to a concentration of 10^{-5}. 10 µl of each dilution was then used to infect 100 µl of *E. coli* TG1 (that had previously grown to an OD_{600} 0.2 in LB) and incubated in a Thermomixer set to 37°C (Eppendorf Inc.) for 15 minutes and a further 30 minutes shaking. The infected bactreria were then spread onto LA containing 100 mg l^{-1} ampicillin and viable counts were performed as described in §2.2.1.2. This method was described by Smith *et al.* (1993).

2.5.6.2 Phage particles

Phage particles were quantified spectrophotometrically by measuring the optical density of a purified suspension at 269 nm and 320 nm (Day 1969). The phage preparation was first diluted 1:50 in TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.02 % (w/v) NaN_3). The equation shown in Figure 2.3, was used to calculate the number of phage particles (physical particles per litre), as mg l^{-1}. This method was described by Barbas *et al.* (2001).

$$= \frac{(OD_{269} - OD_{320}) \times \text{dilution factor} \times 6e16}{3488}$$

Figure 2.3. Equation for the calculation of phage particles (μg ml^{-1}). As described by Barbas *et al.*, (2001). An explaination to the constant values was not given.

2.5.7 Identifying individual reactive phage clones

The following sections describe the method of immunoscreening, which was used to identify individual clones that were selected for further investigation. Also described is a method of ELISA that was used to assess the mAb binding capabilities of individual recombinant phage (see also §2.3). The method used was described by Luzzago *et al.* (1993)

2.5.7 Reagents

- Washing Buffer: PBS containing 0.5 % (v/v) Tween20
- Milk-Blocking buffer: PBS containing 0.5 % (v/v) Tween20, 1 mg ml^{-1} non-fat dry milk (Difco, Product Number 232100) and 0.1 % (w/v) NaN_3).
- Substrate Buffer: 100 mM Tris-HCl pH9.6, 5 mM $MgCl_2$, 100 mM NaCl
- Developing Solution: 100 mM Tris-HCl pH9.6, 5 mM $MgCl_2$, 100 mM NaCl containing 3.3 mg ml^{-1} nitro-blue tetrazolium chloride (NBT), and 1.65 mg ml^{-1} 5-bromo-4-chloro-3'-indolylphosphate p-toluidine salt (BCIP)

2.5.7.1 Immunoscreening

Infected bacteria display phage coat proteins on the cell surface. Therefore the mAb was used to identify reactive colonies, following adsorption to a nitrocellulose filter.

In preparation, phage infected *E. coli* (TG1) cells (that were scraped from the plate §2.5.3) were grown to mid-log phase (OD_{600} 0.4) by incubation in LB containing 100 mg l^{-1} ampicillin, and 100 mg l^{-1} kanamycin. A ten-fold serial dilution in TBS (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.02 % (w/v) NaN_3) to 10^{-4} was performed and 100 µl of the 10^{-3} and 10^{-4} dilutions were spread onto Luria agar containing 100 mg l^{-1} ampicillin, 100 mg l^{-1} kanamycin and 20 µg l^{-1} IPTG and incubated overnight at 37°C.

A plate containing between 10-100 colonies was covered with a nitrocellulose filter (BIORAD Laboratories, Richmond, CA, USA; Catalogue number 170-3202) for 1 hour at room temperature. Absorbed colonies and the remaining filter were blocked with 8 ml of Milk-Blocking Buffer for 30 minutes at room temperature. Any remaining colony debris was removed by gentle rubbing with gloved hands under a steady stream of cold tap water. The blocking step was then repeated for a further 30 minutes.

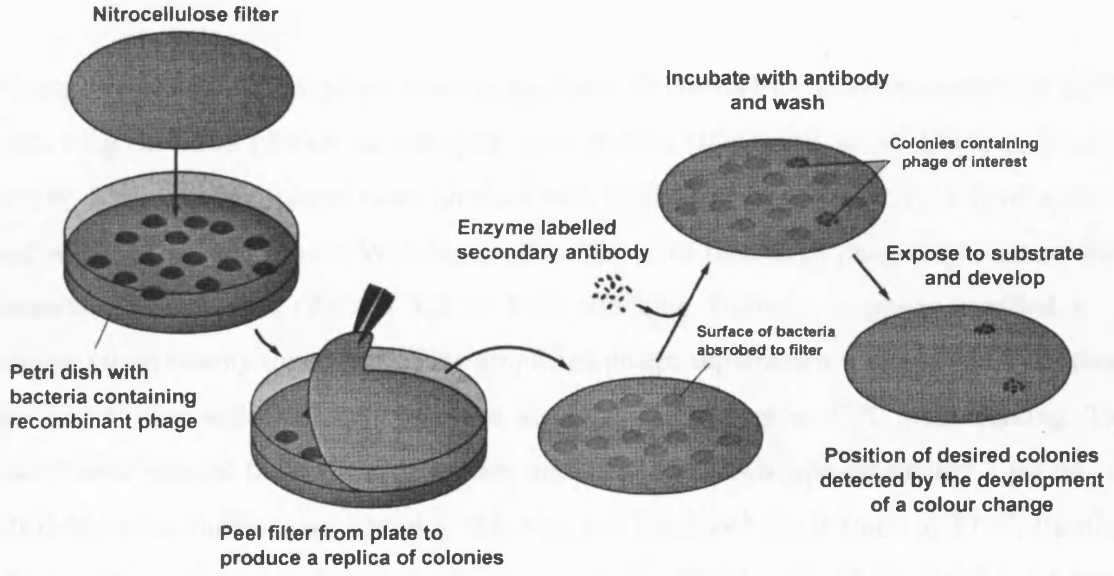

Figure 2.4. The protocol of immunoscreening as described by Luzzago *et al.* (1993). This diagram was modified from Alberts *et al.* (1994).

5 ml of mAb (diluted 1:100 in Milk-Blocking Buffer) was then added to the filter and incubated for 2 hours at room temperature. The filter was washed three times for 5 minutes in Washing Buffer, and then 5 ml of secondary antibody (as before §2.3) diluted 1:5000 in Milk-Blocking buffer was incubated on the filter for 1 hour shaking at room temperature.

This procedure was followed by a thorough washing step of 10 applications of 5 ml Washing Buffer for 3 - minute intervals. 5 ml Substrate Buffer was then added to the filter for 3 minutes at room temperature. The Substrate Buffer was removed and replaced with 5 ml Developing Solution. The reaction was stopped after 10 minutes by saturating the filter in tap water. Positive/reactive colonies were noted by a purple colour change on the filter and were traced to the corresponding colony on the agar plate. Colonies were removed from the plate using a toothpick into 50 µl PBS. Bacteria were heat inactivated at 70°C for 15 minutes and phage were amplified as described in §2.5.3.

2.5.7.2 Determining the reactivity of positive clones by phage ELISA

A phage ELISA was used to test the interaction of the individual phage with the mAb. Here a fixed concentration of each clone was bound to the plate, and therefore the results were used to compare the reactivity of each clone to identify those that demonstrated superior binding capabilities. The method used was previously described by Dente *et al.* (1994).

Ninety-six well microtitre plates (Maxisorp, Nunc, Denmark) were coated overnight at 4°C with 1 µg ml^{-1} 100 µl/well rat anti-pIII (coat protein III) (Dente *et al.* 1994) in Coating Buffer. The next day, plates were blocked with Milk-Blocking Buffer for 1 hour at 37°C and washed three times with Washing Buffer. 100 µl of recovered phage supernatant from bacterial amplification (diluted 1:2 in Milk-Blocking Buffer), or phage purified to a concentration twenty times that of the amplified phage supernatant (using a $CsCl_2$ gradient see §2.5.4) was added to each well and incubated for 1 hour at 37°C with shaking. The plates were washed three times as before, and 100 µl of mAb (approximately 1 µg ml^{-1} in Milk-Blocking Buffer) was added to the well and incubated for 2 hours at 37°C. Binding of the mAb to phage was detected using a secondary antibody (see §2.3.1.1) diluted 1:5000 in Milk-Blocking Buffer and incubated for 1 hour at 37°C. Plates were developed using Developing solution and the absorbance was read at 405 nm after 1 hour at 37°C.

To determine the specificity of binding, approximately 1 µg ml^{-1} mAb solution was pre-incubated for 1 hour with either 1 µg or 10 µg of purified serotype specific pneumococcal capsular polysaccharide (ATCC). The DNA from the clones that showed a reduction in

binding following pre-incubation with PnCPS was recovered and the amino terminus of the pVIII gene was sequenced (see §2.5.8). This provided the sequence of the inserted peptide.

2.5.8 Determination of the peptide sequence of the clones

DNA from the recombinant phage was obtained from recombinant phage by the method described by Felici *et al.* (1991). Phage infected bacterial colonies that were scraped from the agar plate in §2.5.3, were used to prepare plasmid DNA. 20 ml of this suspension was precipitated with PEG8000 (see §2.5.4) and finally resuspended in 200 µl TBS. This should contain at least 10^{11} phage particles. Phage were extracted once with an equal volume of phenol and once with an equal volume of chloroform in a 500 µl eppendorf tube. The final aqueous phase (100 - 150 µl) was transferred to a 1.5 ml eppendorf tube containing 250 µl TE and 40 µl 3M sodium acetate buffer (pH 6.0 with acetic acid), and the viral ssDNA was preciptated by adding 1 ml ethanol. This was incubated at 4°C for 1 hour and the ssDNA was collected by centrifugation at 10 000x *g* for 30 minutes. The DNA was briefly dried by aspirating the supernatant and gently washing in 70 % (v/v) ethanol. The pellet was finally dissolved in 7.5 µl water and stored at -20°C.

DNA of the recombinant *pVIII* gene was amplied by polymerase chain reaction (PCR) as described by Felici *et al.* (1991) and sequenced by the dideoxy-mediated chain termination method as described by Sanger *et al.* (1977). Sequences were analysed with the DNA Strider 1.2 software.

2.5.9 Analysis of the peptide sequences

2.5.9.1 Determining whether the occurrence of residues is significant or random

To do this we determined a ratio (frequency) of occurrence of each amino acid. First the expected frequencies of each residue were calculated based on the number of oligonucleotides that encoded each amino acid that were used to construct the libraries. If the observed frequency was the same as the expected frequency (a ratio of 1.0) then the presence of these residues in the sequence was a random event, since the library was constructed randomly. Any ratio >1.0 indicated an over-representation of that residue (Mertens *et al.* 2001)

The theoretical (expected) frequency of each residue was calculated based on the proportion of each codon present in the randomly generated oligonucleotides used to

construct the libraries, with each one coding a particular amino acid. This is shown in Table 2.5. Cysteine was excluded from the calculations since it was not randomly generated; it was fixed in the sequences of the pVIII-12aa.Cys peptides and did not appear in the 6B mimics. The 'Z' residue corresponds to the stop codon, since all peptides were of an expected length, it was logical to observe no stop codons (Mertens *et al.* 2001). The expected frequencies are shown below:

Table 2.5. The expected frequency of amino acid residues in a random 12aa peptide sequence based on the proportion of trinucleotides coding each residue

A	D	E	F	G	H	I	K	L	M	N	P	Q	R	S	T	V	W	Y	Z
6.45	3.13	3.13	3.13	6.25	3.13	4.69	3.13	9.38	1.56	3.13	6.25	3.13	9.38	9.38	6.25	6.25	1.56	3.13	4.69

2.5.9.2 Sequence alignment

ClustalW was used to align the peptide mimics selected for each pneumococcal serotype. This programme was described by Thompson *et al.* (1994) and was freely available on the EMBL-EBI website (http://www.ebi.ac.uk/clustalw).

In the aligment sequence '*' signified that the residues or nucleotides in that column were identical in all sequences in the alignment. ':' signified that conserved substitutions had been observed, according to the COLOUR in Table 2.6. '.' signified that semi-conserved substitutions were observed.

Table 2.6 Colour designations for the alignment of peptide sequences as depicted by the ClustalW program

Amino acid	*Designated colour*[a]	*Group*
AVFPMILW	RED	Small and hydrophobic (including aromatic -Y)
DE	BLUE	Acidic
RHK	MAGENTA	Basic
STYHCNGQ	GREEN	Hydroxyl, Amine and Basic - Q

[a] from http://ebi.ac.uk and based on the algorithms of Thomson *et al.* (1994)

The ClustalW program presented a score (percentage identity). A score above 30 - 40 % has been designated significant by previous researchers (A. Stark, personal communication). Therefore, since the peptides in this study were short and only exact matches were scored, the lower range of 30 % was used.

2.6 Immunisation with peptide mimics

2.6.1 Immunisation phage-displayed mimotopes

2.6.1.1 Preparation of phage

Filamentous phage M13 displaying the peptide MP58 on its pVIII coat protein, that had been purified through a caesium chloride gradient, were used to immunise mice (see §2.5.4). Immunisation following purification of phage by this method has been shown to elicit a good quality antibody response towards the displayed protein and to reduce, but not completely eliminate, the induction of antibodies to contaminant bacterial antigens (Galfre *et al.* 1996).

2.6.1.2 Immunisation procedure

Previous studies where the phage-bound peptide was used as an immunogen to mice (Westerink *et al.* 1995; Galfre *et al.* 1996; Phalipon *et al.* 1997; Mertens *et al.* 2001) found that a dose of approximately 10^{11} phage particles (see §2.5.6.2) was sufficient to produce a specific response to the mimotope. This corresponds to about 1.3 μg of protein in pC89 phagemid-derived libraries (Felici *et al.* 1991). The stock phage suspension was stored at 4°C, at a concentration of 1 x 10^{14} phage particles ml^{-1} in PBS. Therefore, the stock phage suspension was diluted 1:10 in PBS and 10 μl of this was mixed with 57 μl PBS and 33 μl of ImjectAlum adjuvant (Pierce, Rockford, IL, USA). The phage and adjuvant were mixed for 30 minutes at room temperature prior to injection. 100 μl (10^{11} phage particles) of the immunisation solution was administered intraperitoneally per mouse. This route was shown to elicit a good immune response to peptide mimotopes in mice (Galfre *et al.* 1996).

Doses were administered according to the schedule described in Table 2.7 (groups MP58PHV1-4). Control mice received 33 μl ImjectAlum adjuvant in a total volume of 100 μl PBS. This was also according to the same schedule. Following immunisation, two groups of mice immunised with the peptide mimics were challenged intraperitoneally with a dose of serotype 9V pneumococci previously shown to cause disease (see Table 3.1). The

health status of animals was monitored for 10 days, as described in §2.2.1.3. The remaining group of mice was used to obtain a high quantity of serum by exsanguination.

2.6.2 Immunisation with KLH-displayed mimotopes

2.6.2.1 Peptide synthesis

All 9V peptide mimics were synthesised to immunograde purity (~70%) and conjugated to Keyhole Limpet Hemocyanin (KLH) or Bovine Serum Albumin (BSA) by Thermohybaid (Thermo Biosciences GmbH, Ulm, Germany). 6B mimics were conjugated to KLH or BSA by Cambridge Research Biochemicals (CRB, Billingham, Cleveland, UK). Cambridge Research Biochemicals used *m*-Maleimidobenzoyl-*N*-hydroxysuccinimide ester (MBS) to link the peptide to the carrier protein through free amino groups. Thermohybaid coupled the peptides to a carrier protein using Carbodimide. Both methods are described in Harlow *et al.* (1988).

Peptides MP10 and MP2 were purified by HPLC prior to conjugation to KLH and 5 mg was provided as unconjugated peptide. This additional purification step inflated the cost of peptide synthesis dramatically and not all the peptides could be prepared this way. All peptides were supplied lyophilised.

For long-term storage, peptides were diluted to a concentration of 0.5 mg ml^{-1} in sterile PBS, aliquoted into 1 ml volumes, frozen at -70°C and lyophilised overnight. Samples could then be stored at -20°C.

2.6.2.2 Immunisation procedure

Three groups of 10 female MF1 mice (Harlan Olac, Bicester, UK) were given doses of 30 µg peptide conjugated to KLH in 67 µl PBS and 33 µl ImjectAlum adjuvant (Pierce, Rockford, IL, USA). The peptide and adjuvant were mixed for 30 minutes at room temperature prior to injection. Mice were immunised intraperitoneally with 100 µl of the immunisation solution. This corresponded to a dose of 5 - 7 µg of the peptide mimic per mouse (A. Phalipon, personal communication). Doses were administered according to the schedule described in Table 2.7.

To determine whether pneumococcal polysaccharide could boost the immune response to the peptide mimic, some groups of immunised mice received two doses of peptide

followed by an intraperitoneal dose of 30 μg pneumococcal polysaccharide (serotype 9V or 6B) in 67 μl PBS and 33 μl ImjectAlum adjuvant. The groups of mice that received polysaccharide are shown in Table 2.7.

Control mice received 30 μg KLH and 33 μl ImjectAlum adjuvant in a total volume of 100 μl PBS. This was also according to the same schedule. Blood samples were extracted from the tail vein on the days described in Table 2.7. The blood was left to clot at room temperature for 1 hour and the serum was recovered by centrifugation at 5000x *g* for 10 minutes. The serum was stored at -70°C until needed.

Following immunisation, mice were challenged intraperitoneally with a dose of pneumococci previously shown to cause disease (see Table 3.1). The health status of animals was monitored for 10 day or 15 days (for 6B infections), as described in §2.2.1.3.

2.7 Immunisation schedules

Immunisation protocols with phage displayed mimotopes have been widely publicised (Westerink *et al.* 1995; Galfre *et al.* 1996; Phalipon *et al.* 1997; Mertens *et al.* 2001). On the basis of the findings of these studies, the following immunisation protocol was designed. The schedule we used was modified from that described by Galfre *et al* (1996) on the advice of A. Weintraub and K. Daalsgard (personal communications). Galfre *et al* (1996) recommends the following protocol: Day 0, obtain a sample of pre-immune serum and priming i.p injection of antigen, Day 21, boost with antigen, Day 42 second boost. As short peptides take longer to activate B-cells than proteins (because more epitopes are present) it was recommended that the first booster dose occurred 5 - 6 weeks following the primary dose (A. Weintraub and K. Daalsgard, personal communications). This would allow for the delayed primary response and enhance the booster response. Test bleeds were performed 10 days after immunisation

Table 2.7 The immunisation schedules of peptide mimotopes selected from the phage-display library.
Day of action is represented by TP#. (–) represented no action taken

DAY OF ACTION						
	GROUP					
ACTION	MP2V1-3	MP2V4	MP7V1	MP7V2	MP7V3	MP10V1
Tail Bleed + Immunisation I	TP0 MP2-KLH	TP0 MP2-KLH	TP0 MP7-KLH	TP0 MP7-KLH	TP0 MP7-KLH	TP0 MP10-KLH
Tail Bleed	TP14 TP36	-	TP42	TP42	TP42	TP14 TP36
Immunisation II	TP42 MP2-KLH	-	TP42 MP7-KLH	TP42 MP7-KLH	TP42 MP7-KLH	TP42 MP10-KLH
Tail Bleed	-	-	TP56	TP56	TP56	TP56
Immunisation III	-	-	TP70 MP7-KLH	TP70 MP7-KLH	TP70 MP7-KLH	TP63 MP10-KLH
Tail Bleed	-	-	TP84	TP84	TP84	TP80
Tail Bleed + Challenge	-	TP98	TP112	TP112	TP112	TP105

	GROUP					
ACTION	MP10V2	MP10V3	MP12V1	MP12V2	MP12V3	MP13V1-2
Tail Bleed + Immunisation I	TP0 MP10-KLH	TP0 MP10-KLH	TP0 MP12-KLH	TP0 MP12-KLH	TP0 MP12-KLH	TP0 MP13-KLH
Tail Bleed	TP14 TP36	TP14 TP36	TP42	TP42	TP42	TP42
Immunisation II	TP42 MP10-KLH	TP42 MP10-KLH	TP42 MP12-KLH	TP42 MP12-KLH	TP42 MP12-KLH	TP42 MP13-KLH
Tail Bleed	TP56	TP56	TP56	TP56	TP56	TP56
Immunisation III	TP63 MP10-KLH	TP63 6B PnCPS	TP70 MP12KLH	TP70 MP12KLH	TP70 MP12KLH	TP63 MP13-KLH
Tail Bleed	TP80	TP80	TP84	TP84	TP84	TP77
Tail Bleed + Challenge	TP105	TP105	TP112	TP112	TP112	TP105

	GROUP					
ACTION	MP13V3	MP14V1	MP14V2	MP15V1	MP15V2	MP15V3
Tail Bleed + Immunisation I	TP0 MP13-KLH	TP0 MP14-KLH	TP0 MP14-KLH	TP0 MP15-KLH	TP0 MP15-KLH	TP0 MP15-KLH
Tail Bleed	TP42	TP14 TP42	TP14 TP42	TP21	TP21	TP21
Immunisation II	TP42 MP13-KLH	TP42	-	TP42 MP15-KLH	TP42 MP15-KLH	TP42 MP15-KLH
Tail Bleed	TP56	-	-	TP56	TP56	TP56
Immunisation III	TP63 6B PnCPS	-	-	TP63 MP15-KLH	TP63 MP15-KLH	TP63 6B PnCPS
Tail Bleed	TP77	-	-	TP77	TP77	TP77
Tail Bleed + Challenge	TP105	-	TP140	TP98	TP98	TP98

DAY OF ACTION						
	GROUP					
ACTION	MP17V1-3	MP18V1-3	MP55V1	MP55V2	MP55V3	MP58V1
Tail Bleed + Immunisation I	TP0 MP17-KLH	TP0 MP18-KLH	TP0 MP55-KLH	TP0 MP55-KLH	TP0 MP55-KLH	TP0 MP58-KLH
Tail Bleed	TP21	TP21	-	-	-	-
Immunisation II	TP42 MP17-KLH	TP42 MP18-KLH	TP42 MP55-KLH	TP42 MP55-KLH	TP42 MP55-KLH	TP42 MP58-KLH
Tail Bleed	-	-	TP56	TP56	TP56	TP56
Immunisation III	-	-	TP63 MP55-KLH	TP63 MP55-KLH	TP63 MP55-KLH	TP63 MP58-KLH
Tail Bleed	-	-	TP77	TP77	TP77	TP77
Tail Bleed + Challenge	TP98	TP98	TP103	TP103	TP103	TP103

	GROUP					
ACTION	MP58V2	MP58V3	MP58PHV1	MP58PHV2	MP58PHV3	MP58PHV4
Tail Bleed + Immunisation I	TP0 MP58-KLH	TP0 MP58-KLH	TP0 PHAGE 58	TP0 PHAGE 58	TP0 PHAGE 58	TP0 PHAGE 58
Tail Bleed	-	-	-	-	TP37	TP37
Immunisation II	TP42 MP58-KLH	TP42 MP58-KLH	TP42 PHAGE 58	TP42 PHAGE 58	TP42 PHAGE 58	TP42 PHAGE 58
Tail Bleed	TP56	TP56	TP56	TP56	TP56	TP56
Immunisation III	TP63 MP58-KLH	TP63 MP58-KLH	TP63 PHAGE 58	TP63 PHAGE 58	TP70 PHAGE 58	TP70 PHAGE 58
Tail Bleed	TP77	TP77	TP77	TP77	TP84	TP84
Tail Bleed + Challenge	TP103	TP103	TP113	TP113	TP112	TP112

2.8 Analysis of the immune response in immunised mice

A convenient assessment of the response to immunisation is quantifying the production of antibodies to the peptide mimic. The most practical test to assess antibody titres and/or concentrations is by an enzyme-linked immunosorbent assay (ELISA). This assay was widely described in literature (Harlow and Lane 1988)

2.8.1 Direct ELISA

This method will also be referred to as ELISA. An ELISA was performed as illustrated in Figure 2.2. See §2.3.1 for a description of the reagents used. 96-well microtitre plates (Maxisorp, Nunc) were coated with either 2 μg ml^{-1} of the appropriate pneumococcal polysaccharide, or 5 μg ml^{-1} of the peptide conjugated to BSA (or unconjugated peptide). These were diluted in Coating buffer and 100 μl was added to each well. The next day, plates were blocked with Milk-Blocking Buffer for 1 hour at 37°C and washed three times with Washing Buffer. The plates were washed three times as before, between each subsequent step prior to developing.

2.8.1.1 Standard curve

To produce a standard curve, from which the antibody concentration in the mouse serum could be determined, the monoclonal antibody used to screen the phage libraries (206,F-5 or Db3G9) was added to the peptide and polysaccharide coated plates. These mAbs are both of the isotype IgG and their concentrations are known (4 μg ml^{-1} and 3 μg ml^{-1}, respectively). The reactivity of these mAbs, at different concentrations, to the antigen provided a range of absorbencies from which unknown IgG concentrations could be determined.

The mAbs were diluted in Milk-Blocking buffer as two-fold dilutions, starting at a concentration of 4000 ng ml^{-1} for 206,F-5 and 3000 ng ml^{-1} for Db3G9 and subject to ELISA as §2.8.1. A standard curve (for each peptide and polysaccharide) of A_{405} measurements versus antibody concentration was constructed by linear regression (PRISM 4).

2.8.1.2 Determining the anti-peptide and anti-polysaccharide IgG concentration in mouse sera

The serum from each mouse was assayed individually. Primarily, sera from time point TP77 or TP84 (see Table 2.7) were diluted two-fold in Milk-Blocking Buffer starting at 1:100 or 1:250. This time point was used as it was thought the highest antibody concentration would be observed following the second boost immunisation. An ELISA was performed as described in §2.8.1, although the secondary antibody was goat anti-mouse IgG conjugated to alkaline phosphatase (Sigma A2064). The dilution of serum, which gave an absorbance within the linear range of the standard curve, was chosen for

future work. Serum antibody concentrations were calculated using the linear regression equation from the respective standard curve.

To assess anti-peptide and anti-polysaccharide antibody production throughout the immunisation schedule, the dilution determined to be within the linear range for TP77/TP84 was performed for all time points and subjected to ELISA as above. Assays for all time points were assessed once the immunisation schedule was completed. This dilution was also used for competition ELISA and affinity binding assays.

2.8.2 Competition of the immune response

This inhibition assay was modified from that described by Mertens *et al.* (2001). It was performed primarily as described in §2.8.1, except that the mouse serum taken at time point T77 or T84 (depending on the schedule) was pre-incubated with either 0 μg, 20 μg or 100 μg purified serotype specific pneumococcal capsular polysaccharide (253-X (9V), or 225-X (6B) all from the ATCC), or 100 μg *E. coli* strain 0111:B4 lipopolysaccharide (Sigma) as a negative control. These concentrations of polysaccharide were added to 200 μl diluted serum in Milk-Blocking buffer and the suspension was mixed on rotating rack at room temperature for 45 minutes. The suspension was then divided equally between peptide-coated wells and the ELISA done as described as §2.8.1.1. The absorbance was determined at 405 nm after 1 hour.

The inhibition of antibody binding was calculated (as a percentage) from the difference in antibody concentration obtained from all immunised mouse serum incubated with 0 μg to 100 μg PnCPS. Eight randomly selected sera from each peptide immunisation were used to obtain an average (mean) inhibition of binding using *E. coli* LPS.

2.8.3 Antibody avidity for peptide and polysaccharide antigens

This assay was done as described in §2.8.1, except that following incubation with the mouse serum taken at time point T77 or T84 (depending on the schedule) the peptide or polysaccharide coated plate was treated with a concentration gradient of ammonium thiocyanate (4 M, 2 M, 1 M, 500 mM, 250 mM, 125 mM, 62.5 mM NH_4SCN in distilled water) for exactly 15 minutes at room temperature. This method had been described previously (Goldblatt *et al.* 1998; Guttormsen *et al.* 2002). Different concentrations of ammonium thiocyanate (as before) were added to a single dilution of serum in order to

dissociate low avidity antibody from the antigen-coated plates. The molar concentration of ammonium thiocyanate resulting in 50 % reduction in the amount of antibody that remained bound was termed the avidity.

2.8.4 Fluorescent labelling of bacteria

Antibodies generated from immunisation with peptide mimics were incubated with pneumococci in suspension. This determined whether these antibodies could bind to pneumococci and could potentially act to opsonise the bacteria. The presence of bound antibody was detected by using a fluorescent-labelled secondary antibody. This method was modified from that of Mounier *et al.* (1997) (Mounier *et al.* 1997).

A 10 ml culture of pneumococci was grown statically overnight at 37°C in BHI. This was spun at 600x *g* for 10 minutes and re-suspended in 10 ml 3.7 % (w/v) paraformaldehyde in PBS for 20 minutes at room temperature. The suspension was centrifuged as before and the pellet washed four times by re-suspension in an equal volume of sterile water. Approximately 1 x 10^6 cfu pneumococci (determined by viable count prior to formaldehyde treatment (see §2.2.1.2)) were incubated overnight with approximately 100 µg anti-peptide serum (determined as in §2.8.1) from a mouse that was identified to produce polysaccharide-reactive antibodies following immunisation with a peptide mimic (see §2.8.1). The antibody-bacteria complex was centrifuged (600x *g* for 5 minutes) and washed in PBS three times. This was then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti mouse IgG (Sigma F5387) diluted 1:2000 for 1 hour at 37°C. Identification of fluorescent bacteria was performed at a magnification of x1000 under oil immersion using a conventional fluorescent microscope (Nikon Diaphot) with a FITC/Ex507nm filter and emission of 529nm.

2.8.5 Toxicity studies

To determine the cause of the toxicity observed during immunisation with peptides MP2, MP14, MP17 and MP18, a further peptide was synthesised. This was referred to MP2m (a modified sequence of the peptide MP2) and was conjugated to KLH prior to immunisation. The sequence of MP2m was NH_2 –AEGEFWGPRVIF**QN**VTVGDPAK – COOH (the modified residues are highlighted in boldface). The glutamic acid (E) and aspartic acid (D) residues present in MP2 were replaced by glutamine (Q) and asparagine (N) respectively,

as it was thought that the presence of acidic amino acids in the sequence maybe the cause of the toxic effects, since these were absent in the peptides that were safe.

Groups of five female MF1 mice were given a dose of 30 μg antigen (peptide MP2, MP2m or serotype 6B capsular polysaccharide) in PBS with Alum Imject adjuvant, see schedule in Table 2.8. Mice were immunised intraperitoneally with 100 μl.

As is seen in Table 2.8, mice belonging to group V6 received an oral dose of the antihistamine (H1 blocker), cetirizine hydrochloride (Molekula, Dorset, UK) prior to challenge. Mice received a single dose of 0.6 mg diluted in 100 μl sterile water by oral gavage (using a 18G x 30mm canula) each day for 3 days, the last dose was administered 1 hour prior to challenge. This was based on the published examples of cetirizine use in mice (Musoh *et al.* 2000; Kayasuga *et al.* 2002; Shimizu *et al.* 2004). Control mice were, in this case, group V1 which received no antihistamine.

Mice belonging to group V5 were primed with 100μl of the mAb Db3G9 (tissue culture medium diluted 1:4) by passive intraperitoneal immunisation 24 hours prior to challenge and boosted with the same dose 1 hour prior to challenge. Control mice, in this case group V1, received PBS prior to challenge.

Blood samples were extracted from the tail vein and then mice were challenged intraperitoneally with a dose of 30 μg antigen (peptide MP2, MP2m or serotype 6B polysaccharide) in PBS with Alum Imject adjuvant. The health status of animals was monitored critically for a maximum of 2 hours. Mice were culled if significant morbidity (Lethargic ++) was observed. Cardiac puncture under terminal anaesthesia was performed to recover blood from mice that have reached the end stage of the experiment or at the same time point from mice from the control group that did not develop disease signs.

To elucidate the mechanisms of peptide toxicity, the body temperature of all mice was recorded before and immediately following challenge using a 1319 K-type rectal thermometer (TES, Taipei, Taiwan). The temperature was recorded once the digital display rested at either its maximum value or displayed a value for more than 10 seconds.

Table 2.8 The immunisation schedules of peptide MP2 and MP2m.

DAY OF ACTION						
	GROUP (numbers)					
ACTION	MP2V5	MP2V6	V1	V2	V3	V4
Immunisation I	MP2	PBS	MP2	MP2	MP2m	MP2
Action	-	-	-	-	-	-
Tail Bleed	TP21	TP21	TP21	TP21	TP21	TP21
Immunisation II	TP21 PNEUMO	TP21 PNEUMO	TP21 MP2	TP21 MP2m	TP21 MP2m	TP21 6b PnCPS
Tail Bleed	-	-	CARDIAC PUNCTURE	CARDIAC PUNCTURE	CARDIAC PUNCTURE	CARDIAC PUNCTURE

	GROUP (numbers)					
ACTION	V5	V6	V7	V8		
Immunisation I	MP2	MP2	MP2m	6B PnCPS		
Action	Db3G9	CETIRIZINE	-	-		
Tail Bleed	TP21	TP21	TP21	TP21		
Immunisation II	TP21 MP2	TP21 MP2	TP21 MP2	TP21 MP2		
Tail Bleed	CARDIAC PUNCTURE	CARDIAC PUNCTURE	CARDIAC PUNCTURE	CARDIAC PUNCTURE		

Day of action is represented by TP#. (–) represents no action taken. Each group contained five mice.

Serum was collected by cardiac puncture at the end of the experiment and was analysed for histamine contents by a method previously described (Sanmugalingam *et al.* 2000). This was performed by Dr Peter Bradding (Department of Infection, Immunity and Inflammation, University of Leicester, UK). Briefly, this method incorporated a radioactive label (^{3}H) into any histamine present by the addition of rat histamine methyl transferase. A solvent was then used to separate the organic layer containing the ^{3H} histamine, and the level of radioactivity was used to quantitate the histamine concentration based on a standard.

2.9 Statistical analyses

Survival data were analysed by the Kaplan-Meier survival curve analysis using the PRISM 4.0 (GraphPad) statistical programme. The median survival time was calculated by sorting all the survival times for each group into ascending order. The median was the middle value. If this could not be calculated the mean of all the survival times was calculated. The TD50 (Time to death 50 %) was also calculated. This is a measurement of time taken for 50 % of the mice killed by challenge to die.

Signs of disease in mice were scored as described in §2.2.1.3 and analysed using a non-parametric Kruskal-Wallis test and/or a one tailed non-parametric T-test (PRISM 4.0 (GraphPad)).

To determine the serum antibody concentrations collected from immunised mice, linear regression (using MS-Excel (Microsoft)) was performed on a standard curve of absorbances determined by a dilution series of a known concentration of monoclonal antibody (see §2.8.1.1).

The differences in antibody titres and/or concentrations obtained by experimental and control groups of mice from ELISA data were analysed by a T-test (PRISM 4.0 (GraphPad)). To determine whether the antibody response changed following subsequent boost immunisations, a repeated measure ANOVA was performed, since this method analyses the same subject at different time points.

Avice on the use of statistical analyses was given by Miss Zanaib Ansari (Medical Statistician, GSK, UK).

Part B: Heterologous Expression of Pneumococcal Polysaccharide

2.10 DNA techniques

2.10.1 Isolation of pneumococcal chromosomal DNA

This method was adapted from Davies (1990). *S. pneumoniae* (strain WU2) serotype 3 was grown overnight at 37°C in BHI containing 10 % (v/v) FCS. 1 ml of the starter culture was used to inoculate 10 ml BHI with 10 % (v/v) FCS and after 6 hours incubation at 37°C was centrifuged at 600x *g* for 10 minutes. The pellet was resuspended in 200 µl TE with 20 % (w/v) sucrose. To this 50 µl of 75 mM EDTA containing 1 % (w/v) SDS and 2.5 mg proteinase K (Sigma) was added and left overnight at 37°C. The following morning the clear solution was made up to a total volume of 1 ml with nanopure water and phenol extracted and ethanol precipitated (see §2.10.2.1.2). The resulting DNA pellets were resuspended in 50 - 100 µl nanopure water and stored at 4°C.

2.10.2 Preparation of plasmid DNA

The reagents and methods used for the extraction of plasmid DNA were taken from Maniatis *et al.* (1982). The solutions listed below were autoclaved after preparation, except Solution II, which was prepared fresh prior to use.

2.10.2 Reagents

Solution I: 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0

Solution II: 0.2 M NaOH, 1% (w/v) SDS

Solution III: 3 M potassium acetate pH 4.8 containing 11.5% (v/v) glacial acetic acid

A single colony was removed from an overnight culture of *E. coli* on LA containing the appropriate antibiotic and used to inoculate a 10 ml Luria Broth (LB) starter culture (including appropriate selective antibiotic). This was incubated overnight at 37°C in a shaking incubator at 220rpm.

2.10.2.1 Small-scale plasmid preparations ("miniprep")

1.5 ml of the overnight culture was poured into a microcentrifuge tube and centrifuged at 3500x *g* for 1 minute. This was repeated and the bacterial pellet was finally resuspended in 100 µl of Solution I and incubated at 15 - 25°C for 5 minutes. Then 200 µl of freshly

prepared Solution II was added to lyse the cells and the contents mixed by gentle inversion and incubated on ice for 5 minutes. 150 µl of Solution III was then added to neutralise the pH and complex denatured chromosomal DNA to protein and SDS. This was mixed gently until a white precipitate formed. The insoluble protein-DNA-SDS complex was then removed by centrifugation at 12, 000x *g* for 5 minutes at 2 - 8°C. The plasmid-containing supernatant was transferred to a sterile microcentrifuge tube, to which 2.5 volumes (approx. 450 µl of cold absolute ethanol was added and incubated at room temperature for 30 minutes to precipitate the DNA. The precipitate was recovered by centrifugation at 12, 000x *g* for 10 minutes at 2 - 8°C and the pellet was washed cold 70 % (v/v) ethanol. The pellet was then dried at 37°C for 20 minutes and re-suspend in 50 µl sterile redistilled water. 0.5 µl (~20 mg ml^{-1}) RNase, DNase-free (Roche) was added to degrade RNA at 37°C for 30 minutes. The sample was then extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol as described in §2.10.2.1.1.

2.10.2.1.1 Phenol extraction of proteins

To remove contaminating proteins from a DNA solution, an equal volume of phenol:chloroform (48 % (v/v) liquid phenol, 48 % (v/v) chloroform and 2 % (v/v) isoamyl alcohol) was added to the sample and gently mixed. The top aqueous phase containing the DNA was separated from the lower organic phase by centrifugation at 12, 000x *g* for 5 minutes. The top aqueous layer was transferred to a fresh microcentrifuge tube and another equal volume of phenol:chloroform was added and the step above repeated. A further step to remove any remaining phenol was to add an equal volume of chloroform-isoamyl alcohol (96 % (v/v) chloroform, 4 % (v/v) isoamyl alcohol). Again, the sample was spun and the top aqueous phase was retained. The DNA in solution was then ethanol precipitated as described in §2.10.2.1.2.

2.10.2.1.2 Ethanol precipitation

DNA was precipitated from the sample by the addition of 1/10 volume 3M sodium acetate (pH5.2) and 2 – 3 volumes of 100 % ethanol. This was placed on ice for 30 minutes (or at -20°C overnight) and then spun at 12, 000x *g* for 20 minutes. The pellet was washed in fresh 70 % (v/v) ethanol and recentrifuged as above. The pellet was then air dried for 10 minutes and resuspended in 20 µl sterile redistilled water.

2.10.2.2 Large scale plasmid preparations ("maxiprep")

When a higher concentration of plasmid DNA was needed, the following modifications to the protocol outlined in §2.10.2.1 were performed.

5 ml of the overnight culture was used to inoculate 500 ml of LB (including appropriate selective antibiotic) and grown overnight at 37°C in a shaking incubator. The following day the culture was dispensed into 400 ml centrifuge tubes (brand) and centrifuged (using the GS-3 rotor of the Sorval centrifuge) at 9000x *g* for 10 minutes at 4°C. The pellet was resuspended in 25 ml Solution I containing 4 mg ml^{-1} lysozyme powder. This was mixed gently by inversion and incubated at room temperature for 10 minutes.

50 ml Solution II was rapidly mixed with the viscous lysate by inversion 4 or 5 times (until a clear lysate appeared). 37.5 ml cold Solution III was mixed thoroughly into the lysate by inversion and incubated on ice for 10 minutes. The precipitate was removed by centrifugation at 15, 000x *g* for 30 minutes at 4°C and the supernatant was promptly filtered through a 5 ml gilson tip plugged with polymer wool taped to top of a clean 250 ml centrifuge pot containing 2/3 volume (~70 ml) isopropranol (propan-2-ol). This was incubated at room temperature for 10 minutes. The precipitate was recovered by centrifugation (as above), now using the small rotor at 10, 000x *g* for 10 minutes at room temperature (to prevent salt co-precipitating) and the pellet was carefully washed (so as to not disturb the pellet) in 5 ml 70 % (v/v) ethanol. This was allowed to drain and then dried for 10 minutes at 37°C. The pellet was finally resuspended in 3 ml sterile redistilled water. 10 µl (~20 mg ml^{-1}) RNase, DNase-free (Roche) was added to degrade RNA at 37°C for 30 minutes. The sample was then extracted twice with phenol/chloroform and once with chloroform/isoamyl alcohol as described in §2.10.2.1.1.

2.10.3 DNA isolation from plant tissue

DNA was isolated from plant tissue using the DNeasy Plant Mini Kit (Qiagen Cat #69104) and by following the protocol outlined in the manufacturer's handbook 100 mg of leaf tissue was crushed with a pellet pestle under liquid nitrogen in an eppendorf tube. 400 µl of buffer AP1 (provided in the kit) containing 10 mg ml^{-1} of RNase A was added to the 100 mg of plant tissue and vortexed vigorously. The mixture was incubated at 65°C for 10 minutes to lyse the cells and mixed a couple of times during incubation. 130 µl of buffer AP2 (provided in the kit) was then added to the lysate to precipitate the detergent, protein

and polysaccharides. This mixture was incubated on ice for 5 minutes. The lysate was then applied to the QIAshredder Mini Spin Column and centrifuged at 20, 000x *g* for 5 minutes (as recommended in the manufacturer's handbook). The lysate was recovered from the collection tube and transferred to a fresh tube containing 1.5 volumes buffer AP3/E (provided in the kit) and mixed by pipetting. 650 µl of this mixture was then added to a DNeasy Mini Spin Column and centrifuged for 1 minute at 6000x g. The flow-through was discarded and the spin was repeated with the remaining sample. 500 µl of buffer AW (provided in the kit) was then added to wash the column by centrifugation at 6000x g for 1 minute. This washing step was repeated, but centrifugation occurred for 2 minutes at 20, 000x *g*. The column was left to dry at room temperature for 5 minute, to rid the column of any residual ethanol. DNA was then eluted by the addition of 50 µl of buffer AE (provided in the kit), which was incubated at room temperature for 5 minutes and then spun at 6000x g for 1 minute. This was repeated with a further 50 µl of buffer AE. Eluted DNA was stored at 4°C.

2.10.4 Polymerase chain reaction (PCR)

2.10.4 Reagents

- Nanopure water, autoclaved and exposed to UV light for 20 minutes
- 125 mM of each deoxynucleotide; dATP, dTTP, dGTP, dCTP (Advanced Biotechnologies Ltd). This concentration was achieved by mixing 12.5µl of each dNTP solution supplied with 950µl of nanopure water. These were aliquoted and stored at -20°C.
- 5 pM of forward and reverse primer
- 1x ThermoPol Reaction Buffer: 20 mM Tris-HCl (pH8.8), 10 mM $(NH_4)_2SO_4$, 10 mM KCl, 2 mM $MgSO_4$, 0.1 % Triton X-100 (New England Biolabs, Beverly, MA, USA).
- 1 unit / 50 µl of Deep VentR DNA Polymerase (New England BioLabs, Beverly, MA, USA).

2.10.4.1 Colony PCR

Colonies were picked from the agar with a sterile toothpick and vigorously mixed in 20 µl nanopure water in a small eppendorf tube. A hole was made in the cap with a needle and

the eppendorf tube was placed in a polystyrene rack in a boiling water bath for 5 minutes. 5 µl of this suspension was used as a template for PCR.

2.10.4.2 PCR of *cps3S*

PCR reactions (Saiki *et al.* 1988) were done to amplify the *cps3S* gene from the serotype 3 *S. pneumoniae* capsular polysaccharide biosynthesis cassette, the sequence of which was obtained from GenBank (www.ncbi.nlm.nih.gov) accession number U15171 (Dillard *et al.* 1995). The following oligonucleotide primers were used: sense (CPSFOR) 5'-CT**G GTA CCC** ATG TAT ACA TTT ATT TTA ATG TTG TTG G-3' corresponding to 2227 bp – 2254 bp with a *Kpn* I restriction site inserted at the 5' end (indicated in bold); anti-sense (CPSREV) 5'- TCA TCA CTC TGT TAA ATT CCT AGT TCC -3', corresponding to 3454 bp – 3479 bp of the type 3 capsular polysaccharide gene cassette. A primer was also constructed that would bind 2863 bp – 2891 bp of the *cps3S* gene 5'- TGC TTA CCT GGT CGA ACA ATT GCT TTT AG -3'. This was named CPSMID as it was used to sequence the middle region of the *cps3S* gene. All primers were constructed by MWG-Biotech (MWG-Biotech, Ebersberg, Germany). To incorporate a histidine-tag to C-terminus of the protein, a further anti-sense primer was constructed (CPSHIS) 5'- TCA TCA ATG ATG ATG ATG ATG ATG TCA TCA CTC TGT TAA ATT CCT AGT TCC -3'.

The PCR amplification was performed with the reagents described above, in a total volume of 50 µl. For the initial cloning of the *cps3S* gene from type 3 pneumococcal chromosomal DNA, 2 µg genomic template (a 1:10 dilution of extracted DNA) was used. For identification of recombinant plasmids, 2 µg of purified plasmid DNA was used. An initial denaturation was performed at 94°C for 4 minutes and then a cycling procedure comprising denaturation at 94°C for 45 seconds, annealing at 58°C for 1 minute, and extension at 72°C for 1 minute 30 seconds, which was repeated either 10 or 30 times, and a final extension at 72°C for 10 minutes. The amplification was carried out on a Thermocycler PCR machine (PTC-200, MJ Research) and the amplified products of all PCRs were analysed by agarose gel electrophoresis, see §2.10.5.

2.10.4.3 PCR of the Multiple Cloning Site of pRSET C

PCR reactions (Saiki *et al.* 1988) were done to amplify the *cps3S* gene from pRSET in order to identify clones containing the pCMS5 vector. The following oligonucleotide primers were used: sense (T7) 5'- TAA TAC GAC TCA CTA TAG GG - 3'; anti-sense

(pRSET-REV) 5'- TAG TTA TTG CTC AGC GGT GG -3'. These were constructed by MWG-Biotech (MWG-Biotech, Ebersberg, Germany). These primers flank the multiple cloning site in pRSET C and were used to determine whether the cloned gene had been correctly introduced to the pRSET vector. If *cps3S* was present in pRSET C, PCR using these primers should amplify a band of ~1.3 Kbp, whereas if transformation of a vector containing an empty multiple cloning site had occurred, a band of only 42bp would be amplified.

The PCR amplification was performed with the reagents described above, in a total volume of 50 µl. 2 µg of small-scale purified plasmid DNA was used as a template. An initial denaturation was performed at 95°C for 5 minutes and then a cycling procedure comprising denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 1 minute 30 seconds, which was repeated times, and a final extension at 72°C for 10 minutes. The amplification was carried out on a Thermocycler PCR machine (PTC-200, MJ Research) and the amplified products of all PCRs were analysed by agarose gel electrophoresis see §2.10.5.

2.10.4.4 PCR of the Multiple Cloning Site of pCambia 2301

The sequence of the vector pCambia 2301 was obtained from GenBank (www.ncbi.nlm.nih.gov) accession number AF234316. Oligonucleotide primers sense 5'- CGG CTC GTA TGT TGT GTG GAA TTG TGA -3' (CAMFOR); anti-sense 5'- GGG GAT GTG CTG CAA GGC GAT TAA -3' (CAMREV), were constructed by MWG-Biotech (MWG-Biotech, Ebersberg, Germany). These primers bind to regions flanking the multiple cloning site of pCambia 2301 and were used to determine whether the cloned gene had been correctly introduced into the pCambia vector. If *cps3S* was present in pCambia 2301, PCR using these primers should amplify a band of ~2.5 Kbp, whereas if transformation of a vector containing an empty multiple cloning site had occurred, a band of only 106bp would be amplified. These primers were used to identify clones containing the pCMS4 vector.

2 µg of small-scale purified plasmid DNA was used as a template and an increased Mg^{2+} concentration, 187.5 µM, was needed to assist primer annealing. An initial denaturation was performed at 94°C for 4 minutes and then a cycling procedure comprising denaturation at 94°C for 45 seconds, annealing at 63°C for 45 seconds, and extension at 72°C for 2

minute 30 seconds, which was cycled 35 times, and a final extension at 72°C for 10 minutes. The amplification was carried out on a Thermocycler PCR machine (PTC-200, MJ Research) and the amplified products were analysed by agarose gel electrophoresis see §2.10.5.

2.10.4.5 PCR for the detection of *Agrobacterium sp.* contamination

Oligonucleotide primers (VCF/VCR) were designed based on the sequences of the *virC* operon located on T_i and R_i plasmids of phytopathogenic *Agrobacterium* strains to detect these plasmids by PCR (Sawada *et al.* 1995). The *virC* operon is absent in the binary vector (pCMS3 or pCMS4) therefore this method could be used to detect the presence of *Agrobacterium sp.* as a contaminant of transgenic plants. This would support conclusions that the amplified *cps3S*, from the PCR described in §2.10.4.2, was due to the presence of the transgene in plant cells rather than the presence of the binary vector in residual bacteria.

A universal primer set (VCF/VCR) for PCR analysis based on the sequence of the virulence region C (*virC*) operon located on T_i and R_i plasmids have been designed to detect these plasmids. The *virC* operon is a conserved region of both Ti and Ri plasmids required for T-DNA processing and transfer and therefore, can be used to detect the presence of most phytopathogenic *Agrobacterium* strains (Sawada *et al.* 1995). The oligonucleotide primers used were sense (VCF) 5'- ATC ATT TGT AGC GAC T -3' corresponding to 1289 bp – 1375 bp of the *virC* operon and anti-sense (VCR) 5'- AGC TCA AAC CTG CTT C -3' corresponding to 604 bp – 589 bp (Sawada *et al.* 1995). These primers define a 730 bp region on the *virC* operon, and the size of the amplified DNAs will coincide with that of the target region flanked by the primer set. Both primers were constructed by MWG-Biotech (MWG-Biotech, Ebersberg, Germany).

The PCR amplification was performed with the reagents described before in a total volume of 50 µl. 2 µg of small-scale purified plasmid DNA was used as a template. An initial denaturation was performed at 95°C for 2.5 minutes and then a cycling procedure comprising denaturation at 95°C for 1 minute, annealing at 55°C for 1 minute, and extension at 72°C for 2 minutes, which was cycled 40 times, and a final extension at 72°C for 7 minutes (Sawada *et al.* 1995). The amplification was carried out on a Thermocycler

PCR machine (PTC-200, MJ Research) and the amplified products were analysed by agarose gel electrophoresis, see §2.10.5.

2.10.4.6 Purification of PCR products

Amplified DNA resulting from PCR were purified from the PCR reagents using the QIAquick PCR purification kit (Qiagen). This was done following the manufacturer's instructions. 5 volumes of buffer PB was mixed with 1 volume of PCR sample. The mixture was placed in a QIAquick spin column rested in a 2 ml collection tube. The apparatus was spun at 12, 000x *g* for 1 minute, to bind the DNA. The flow-through was discarded and the bound DNA was washed by the addition of 750 µl buffer PE. The tube was then recentrifuged for 1 minute. DNA was eluted from the column into a fresh eppendorf tube by the addition of 50 µl buffer EB. After a minute or two, the column was spun at 12, 000x *g* for 1 minute and the eluted DNA was collected in a fresh collection tube.

2.10.5 TAE-agarose gel electrophoresis

This method was described by Maniatis *et al* (1982). Agarose gel electrophoresis was used to separate different MW DNA fragments. The agarose concentration of the gel was dependant on the size of DNA to be observed, usually 0.7 % (w/v) for fragments larger than 3 Kb and 1.5 % (w/v) for fragments larger than 10 Kb. Therefore 0.7g Agarose (Seakem) was dissolved in 100 ml 1x TAE buffer (50x TAE buffer: 57.1 ml glacial acetic acid, 0.5 M EDTA (pH8), 242 g Tris-base, in a total volume of 1000 ml water, pH 8.0) by heating for a few minutes in a microwave oven. The solution was allowed to cool before the addition of 0.5 µg ml^{-1} ethidium bromide. The gel was poured into a suitable mould, Once set, the gel was transferred to a gel tank containing 1x TAE buffer (as above). Each DNA sample was mixed with 1x loading buffer (6x loading buffer: 15 % (w/v) Ficoll 400 in water, 0.25 % (w/v) xylene cyanol FF, 0.25 % (w/v) bromophenol blue) and loaded into the wells produced by the removal of the gel comb. The first well was filled with either a 1 Kb ladder (Gibco/BRL, or New England Biolabs) or λ/*HinD* III ladder (Gibco/BRL) (for fragments >9Kb) to determine the size of the DNA samples.

A constant voltage of 70 mV for an 8cm x 10 cm gel was applied to separate the DNA fragments. DNA was visualised under UV light on a long-wave UV transilluminator.

2.10.5.1 Gel extraction of DNA

DNA fragments were excised and purified directly from the agarose gel. This was done using the QIAEX II gel extraction kit (Qiagen) and following the manufacturer's instruction. The DNA was visualised under the UV light of a long-wave UV transilluminator. With a clean scalpel, the portion of gel containing the DNA band of 1200 bp was excised. The gel slice was placed into a previously weighed eppendorf tube and the weight of the gel slice alone was determined. 3 volumes of buffer QX1 to 1 volume of gel was added to the gel. Then 10 µl of QIAEX II was then added to the sample and the mixture incubated in a water bath set to 55°C for 10 minutes with regular inversion to aid dissolution of the gel. The colour of the mixture remained yellow, indicating a neutral pH. The suspension was centrifuged to remove bound DNA and the pellet was washed with 500 µl QX1 to remove residual agarose contaminants. The pellet was further washed twice with buffer PE to remove residual salt contaminants and then was left to air dry for 10 - 15 minutes. DNA was eluted in 20 µl 10 mM Tris-HCl, pH8.5 and spun at 12, 000x *g* for 1 minute to remove any insoluble contaminants.

2.10.6 Spectrophotometric quantification of DNA

DNA concentrations were quantified from A_{260nm} using an UV/visible spectrophotometer (Ultrospec 3000, Pharmacia Biotech). DNA was prepared as a 1:70 dilution in 1 ml sterile redistilled water, and 100µl was placed inside a quartz cuvette. Sterile redistilled water was used as the blank. The absorbance of the DNA was measured at 260 nm and 280 nm. DNA was quantified based on 50 µg ml^{-1} double stranded DNA having an optical density of 1.0 at 260 nm (using a 10 mm pathlength cell) (Brown 1991). Therefore the concentration of DNA in the sample was calculated from the equation:

DNA (µg ml^{-1}) = A_{260} x 50 x dilution factor.

The ratio of absorbance at 260 nm and at 280 nm was recorded to assess the purity of the DNA solution. A pure DNA solution has an A_{260}:A_{280} absorbance ratio of 1.8; a ratio below this may indicate protein contamination.

2.10.7 DNA sequencing

All DNA sequencing was performed by MWG-Biotech (MWG-Biotech, Ebersberg, Germany). For each sequencing reaction, DNA was provided at a concentration of 0.01 -

0.06 μg μl^{-1}. Also 10 μl of each primer was provided at a concentration of 10 pM μl^{-1}, as recommended by the MWG-Biotech. Analysis of sequencing data was performed using Genetool (Version 2.0, BioTools Inc.).

2.10.8 Restriction Digestion

DNA restriction digests were performed by restriction endonucleases that cut double-stranded DNA at specific recognition sites, generating sticky (staggered) or blunt ended fragments. The restriction digests were performed as described before (Maniatis *et al.* 1982). All restriction endonucleases were purchased from Gibco/BRL and were generally used at a concentration of 5 units (0.5 μl) in a total volume of 20 μl (2 μl 10x reaction buffer (Gibco/BRL) made up with nanopure water). Generally, the reaction was incubated with 500 ng ml^{-1} of target DNA for 30 minutes or 2 hours at 37°C, but if a different concentration of DNA was used, it is stated in the cloning method. When completed, the digestion reaction was either analysed by agarose gel electrophoresis or, if to be used for ligation the enzymes were first heat inactivated for 10 minutes at 65°C.

2.10.9 Ligation

Ligations were performed by the methods described by Maniatis *et al.* (1982). The efficiency of ligation was directly correlated to the ratio of vector to insert; a molar ratio of 1:3, vector:insert was generally used. To calculate the appropriate quantity of insert to include in the ligation reaction, the following equation was used:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector ratio} = \text{ng of insert}$$

To maximise the probability of intermolecular interactions, the ligation reaction was performed in as small a volume as possible. Typically this volume was set to 10 μl. If the concentration of DNA was too low for this volume, the sample was ethanol precipitated (see §2.10.2.1.2) and resuspended in a smaller volume. To perform the ligation reaction, the DNA of the vector and insert were added to 1 μl of 10x T4 DNA ligase buffer (Promega) and 0.5 μl (3 units) T4 DNA ligase (Promega).

If the ligation reaction was to be used for transformation by electroporation, the DNA was first ethanol precipitated (§2.10.2.1.2) to reduce the concentration of salt and the pellet resuspended in an equal volume of nanopure water.

2.11 Cloning

2.11 Overview

cps3S was cloned into five different vectors, which are listed in Table 2.6. These vectors allowed stable conservation of the *cps3S* gene following PCR from the genomic DNA of the type 3 pneumococcus (pCR-4-TOPO and pGEM-T Easy), analysis of protein expression *in planta* (pCHF2 and pCambia 2301) and analysis of protein expression in recombinant bacteria (pRSET C).

The scheme for cloning of Cps3S is shown in Figure 2.5. This figure shows that the *cps3S* gene cloned into all expression vectors was the product of the same PCR reaction. This maintained the same sequence and allowed comparisons to be made between the expression of the protein in each plasmid. As each plasmid contained the same sequence of *cps3S*, no differences in the transcription of the gene would be expected.

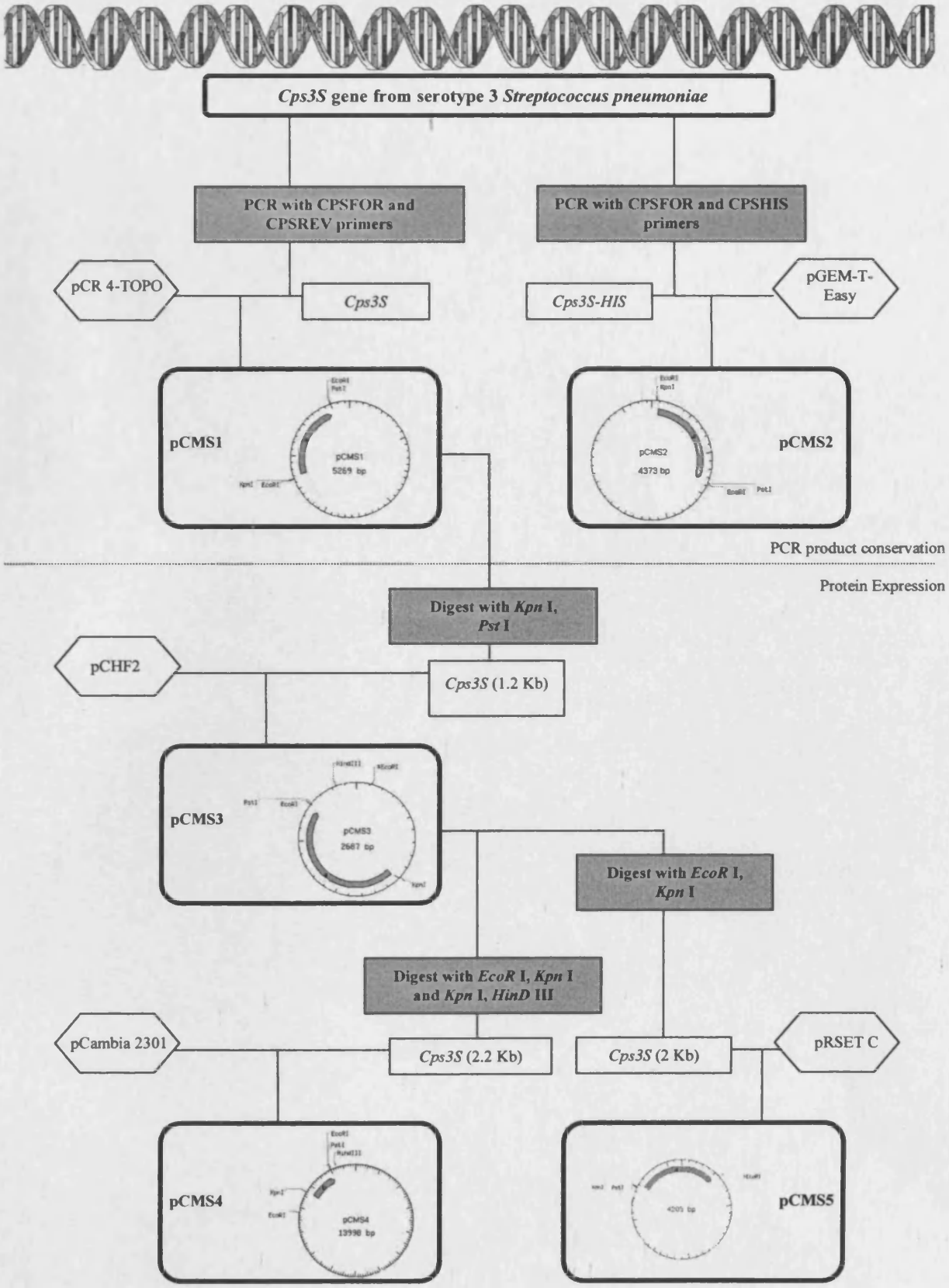

Figure 2.5. The cloning of *cps3S* from the genomic DNA of the type 3 pneumococcus. Plasmid restriction maps were obtained using NEBcutter from www.neb.com. See Figure 2.6 for detailed view of plasmids.

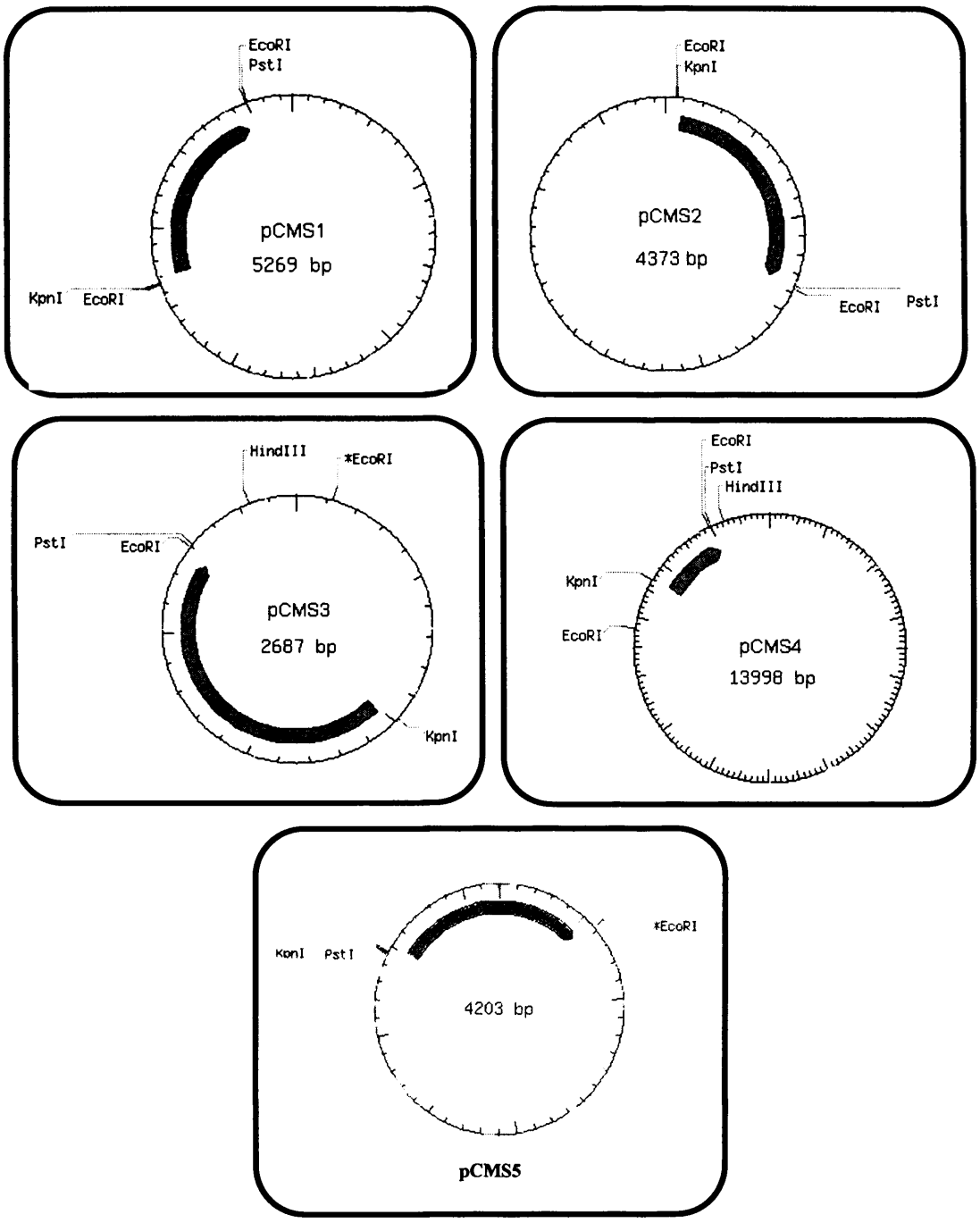

Figure 2.6. Plasmid restriction maps constructed during the cloning of *cps3S* from the genomic DNA of the type 3 pneumococcus. The ORF shown represents *cps3S*. Restriction maps were obtained using NEBcutter from www.neb.com

Table 2.9 Vectors used for the maintenance and expression of *cps3S* in different systems.

Vectors	*Description*	*Reporter genes*	*Reference*
pCR-4-TOPO	This vector was used for cloning blunt ended PCR products. The *EcoR* I restriction sites flanking the MCS and the 3' *Pst* I site was also convenient for future cloning work in this study. Transformed bacteria expressed ampicillin resistance and were identified by blue/white screening. Bacteria containing re-circularised pCR-4-TOPO were eliminated by the expression of the lethal *E.. coli* gene *ccdB*.	*bla, LacZ, ccdB*	Manufacturer's handbook (Invitrogen)
pGEM-T Easy	This vector was used for cloning PCR products. The presence of a 3' T overhang improves efficiency of ligation by preventing re-circularisation of the plasmid. A 3' *Pst* I site was also convenient for future cloning work in this study. Transformed bacteria expressed ampicillin resistance and blue/white screening identified those containing the recombinant plasmid.	*bla, LacZ*	Manufacturer's handbook (Promega)
pCHF2	This vector is a derivative of pPZP222, which is a plant expression vector used for *Agrobacterium* mediated gene transfer. Transformed bacteria expressed spectinomycin resistance, whilst transformed plants expressed gentamycin resistance.	*aadA, aacC1*	(Hajdukiewicz *et al.* 1994)4) GenBank U10463
pCambia 2301	This vector also derives from the pPZP family of plant expression vectors used for *Agrobacterium* mediated gene transfer. Transformed bacteria and plants expressed kanamycin resistance and blue/white screening of the roots using the Gus assay identified those containing the recombinant plasmid.	*nptII, gusA*	CAMBIA, Canberra, Australia
pRSET C	This vector was used to determine whether recombinant bacteria expressed Cps3S. This was because bacterial culture is a faster system than plant. pRSET C provided in frame transcription and an upstream Shine-Delgano sequence for ribosome binding. The presence of the pLysS plasmid in transformed bacteria allowed stricter control of the expression levels of the cloned protein.	*bla*	Manufacturer's handbook (Invitrogen)

2.11 Recombinant Plasmids

Table 2.10 Plasmids constructed for the maintenance and expression of *cps3S* in different organisms. The table shows the source of the *cps3S* gene and the restriction enzymes used for construction of the recombinant plasmids. Also shown are the organisms transformed with the plasmid.

Plasmid	*Technical name*	*Source of transgene*	*Enzymes used for construction*	*Organisms Transformed*
pCMS1	pTOPO-*cps3S*	*cps3S* PCR product	-	*Escherichia coli* Top10F'
pCMS2	pGEM-*cps3S HIS*	His-tagged c*ps3S* PCR product	-	*E. coli* DH5α
pCMS3	pGEM-*cps3S*	pCMS1	*Kpn* I, *Pst* I	*E. coli* Top10F' *Agrobacterium tumefaciens* *Arabidopsis thaliana*
pCMS4	pCambia-*cps3S*	pCMS3	*EcoR* I, *Kpn* I and *Kpn* I, *HinD* III	*E. coli* Top10F' *A. tumefaciens* *A. rhizogenes* *Nicotinia tobacum* cv Samsun *Arabidopsis thaliana* *Lotus corniculatus*
pCMS5	pRSET-*cps3S*	pCMS3	*EcoR* I, *Kpn* I	*E. coli* BL21(DE3)pLysS

2.11.1 Recombinant plasmids for PCR product conservation

2.11.1.1 Cloning of *cps3S* into pCR4-TOPO

pCR4-TOPO was used as a PCR conservation vector because amplified PCR products made using Thermostable DNA polymerases with proof-reading activity, such as Deep Vent polymerase, are blunt ended fragments. This vector allowed the cloning of blunt-ended fragments. See Table 2.9 for more details.

DNA from 10 cycles of two PCR reactions with the CPSFOR and CPSREV primers were combined and purified using the QIAquick PCR purification kit (Qiagen) see §2.10.4.6. The PCR fragment was ligated into the blunt ended insertion site of the vector pCR4Blunt-TOPO (Invitrogen, Carlsbad, CA, USA). Approximately 120 ng of the purified PCR product was ligated to 40 ng of pCR4-TOPO, using 0.5 µl (3 units) of T4 DNA ligase (Promega) 0.5 µl T4 DNA ligase buffer (Promega) in a total volume of 5 µl. The reaction was incubated at room temperature for 10 minutes as described in the manufacturer's handbook for pCR4Blunt-TOPO (Invitrogen). All of the 5 µl was then transformed into 45 µl of $CaCl_2$ competent *E. coli* Top10F' by heat shock (see §2.12.1.1.1). Cells were cultured on LA containing 0.5 mM IPTG, 80 µg ml^{-1} X-Gal and 100 µg ml^{-1} ampicillin. White colonies harbouring the recombinant plasmid were picked and screened by colony PCR with the CPSFOR and CPSREV primers (see §2.10.4.2). Purification of plasmids containing an insert were performed as described in §2.10.2.2. The pCR4-TOPO vector carrying the *cps3S* gene was named pCMS1.

pCMS1 was used to sequence the amplified *cps3S* PCR product. This was achieved using T7 and SP6 commercially available primers of the pCR4-TOPO vector. These primers sequenced through the MCS of the pCR4-TOPO vector, which contained the amplified *cps3S* PCR product. The primer CPSMID (see §2.10.4.2) was also used to sequence the middle region of the *cps3S* gene. This was performed by MWG-Biotech (Ebersberg, Germany) see §2.10.7.

2.11.1.2 Cloning of *cps3S* into pGEM-T Easy

pGEM-T Easy has a 3'-T overhang at the insertion site and therefore, did not allow the cloning of blunt ended PCR products. Therefore, the *cps3S-HIS* PCR fragment was modified using an A-tailing procedure. This method ensures that only one insert will be

ligated into the vector as opposed to multiple insertions that can occur with blunt-ended cloning.

DNA from 10 cycles of two PCR reactions using the CPSHIS and CPSREV primers were combined and purified using the QIAquick PCR purification kit (Qiagen), see §2.10.4.6. To add an A-tail, 7 µl of purified PCR product was mixed with 1 µl (5 units) *Taq* DNA polymerase (Bioline), 1 µl 10x *Taq* DNA polymerase buffer with $MgCl_2$, 0.2 mM dATP to a final reaction volume of 10 µl in distilled water.

This mixture was incubated at 70°C for 20 minutes. 150ng (3 µl) of the purified PCR product was ligated into 50 ng (1 µl) of the pGEM-T Easy vector using the pGEM-T Easy Vector system following the method described in the manufacturer's handbook (Promega, Madison, WI, USA). 0.5 µl (3 units) of T4 DNA ligase (Promega) and 5 µl Rapid ligation buffer (Promega) was used to perform the ligation in a total volume of 10 µl. The reaction was incubated at room temperature for 1 hour as recommended in the pGEM-T Easy cloning kit. 2 µl of this reaction was transformed into 40 µl electrocompetent *E. coli* DH5α (see §2.12.1.1.1). Transformed cells were selectively grown on LA containing 0.5 mM IPTG, 80 µg ml^{-1} X-Gal and 100 µg ml^{-1} ampicillin. White colonies harbouring the recombinant plasmid were picked and screened by colony PCR with the CPSFOR and CPSREV primers (see §2.10.4.2). The purification of plasmids containing an insert was performed as described in §2.10.2.2. The pGEM-T Easy vector carrying the *cps3S* gene with the 3' Histidine tag was named pCMS2.

2.11.2 Expression Vectors

2.11.2.1 Cloning of *cps3S* into pCHF2

The *cps3S* gene was ligated into the multiple cloning site of a derivative of the binary vector pPZP222 (Hajdukiewicz *et al.* 1994). The derivative used was designated pCHF2 and contained, within the T-DNA, a duplicated CaMV35S promoter upstream of a PR1B signal sequence coding region, the multiple cloning site and the rbcS (ribulose-1, 5-bisphosphate carboxylase small subunit) terminator sequence from pea. The CaMV35S promoter was thought to drive strong constitutive expression of the transgene and it has been shown to function efficiently in all plants, as well as green algae, yeast and *E. coli* (Cummins *et al.* 2000). A low level of expression has also been detected in *Agrobacterium* (McIntosh *et al.* 2004). The PR1B signal sequence was thought to direct secretion of the

transgene product via the endoplasmic reticulum to the apoplast (Denecke *et al.* 1990). The apoplast is the site of plant cell wall polysaccharide synthesis and so the type 3 synthase (Cps3S) was expected to have access to the required precursor pools in that compartment. The pPZP222 vector also contains, within the T-DNA region, a gentamicin acetyltransferase (*aacC1*) gene that will confer gentamicin resistance on transformed cells. Outside the T-DNA region the pPZP222 vector has a bacterial spectinomycin resistance gene *aadA*, encoding aminoglycoside-3-adenyltransferase that will confer spectinomycin resistance to transformed bacterial cells. See Figure 2.7.

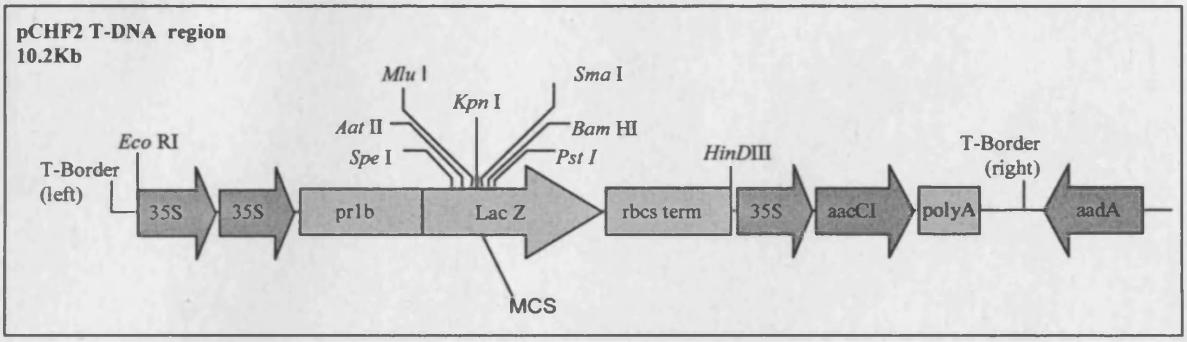

Figure 2.7. The vector pCHF2. A CaMV35S promoter, rbcS terminator region, and a PR1B signal sequence were cloned into the T-DNA region of the pPZP222 vector (Hajdukiewicz *et al.* 1994). The unique restriction endonuclease sites are also shown.

pCMS1 and pCHF2 were first prepared by large-scale DNA extraction (see §2.10.2.2). 17 µg of a large-scale preparation of pCMS1 was digested with restriction endonucleases *Kpn* I and *Pst* I in a total volume of 25 µl (see §2.10.8). This was because a *Pst* I restriction site was needed downstream of the 3' end of *cps3S* for sub-cloning into pCHF2. 5 µg of pCHF2 was similarly digested with *Kpn* I and *Pst* I in a total volume of 20 µl. This was so the *cps3S* fragment would be ligated in the correct orientation. Digested DNA fragments were separated on a 0.7 % (w/v) TAE-agarose gel electrophoresis (see §2.10.5) and the bands of 1331 bp and 10.2 Kbp, for the *cps3S* gene and the pCHF2 vector respectively, were extracted from the agarose gel using the QIAEX II gel extraction kit (Qiagen), see §2.10.4.6.

Gel-extracted DNA from *cps3S* and pCHF2 were ligated using T4 DNA ligase (Promega), see §2.10.5.1. 1 µg of the pCHF2 fragment was ligated with 1 µg of the *cps3S* fragment (approximately a 1:3 molar ratio) in a total volume of 10 µl. The reaction was incubated at room temperature for 1 hour and 2 µl was transformed into 50 µl of $CaCl_2$ competent *E. coli* Top10F' by heat shock (see §2.12.1.1.1). Cells were cultured on LA containing 100µg ml^{-1} spectinomycin. Spectinomycin resistant colonies harbouring the recombinant plasmid were screened by small scale DNA extraction. DNA was digested with *Kpn* I and *Pst* I. Digested DNA fragments were analysed on a 0.7 % (w/v) TAE-agarose gel. pCHF2 carrying the *cps3S* gene was named pCMS3.

2.11.2.2 Cloning of *cps3S* into pCambia 2301

The plant expression vector pCambia 2301 was used for a number of reasons. 1) It contained a *gusA* gene, which allowed analysis of gene function or presence in plants by the β-glucuronidase (GUS) assay (see §5.11.1). To eliminate the possibility of background expression by residual bacteria the *gusA* gene coding sequence was interrupted by a castor bean catalase intron, since *Agrobacterium* was incapable of processing introns. 2) It conferred kanamycin resistance to transformed plants (by expression of the *nptII* gene), which was technically easier to select for than gentamicin resistance (which was conferred by transformation with pCHF2). 3) The pUC18 polylinker within the DNA for the LacZα peptide fragment allowed blue/white screening of clones in *E. coli*, which allowed enhanced selection of transformants.

pCMS3 was digested with either *EcoR* I and *Kpn* I or *Kpn* I and *HinD* III into two fragments of 850 bp and 1460 bp respectively. Together these fragments form an important region of the T-DNA region of pCMS3. As with pCMS3, once these fragments are ligated into pCambia 2301 the duplicate CaMV35S promoters and PR1B signal sequence will drive strong constitutive expression of the *cps3S* gene and direct secretion of the transgene product to the apoplast, respectively.

pCMS3 was prepared by large-scale DNA extraction (see §2.10.2.2) and 2 µg was digested in two separate reaction tubes with either *EcoR* I and *Kpn* I, or *Kpn* I and *HinD* III using 10 units of each enzyme (Gibco/BRL), in a total volume of 20 µl nanopure water containing 2 µl 10x React buffer 4 (Gibco/BRL). This was incubated for 30 minutes at 37°C. The enzymes were then heat inactivated for 10 minutes at 65°C

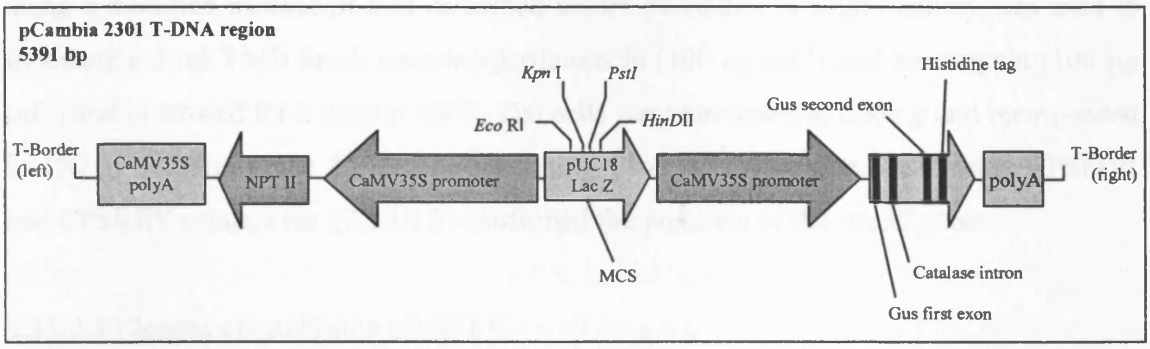

Figure 2.8. The T-DNA region of the pCambia 2301 vector. The unique restriction endonuclease cutting sites are also shown.

2 µg of pCambia 2301 (CAMBIA, Canberra, Australia) was digested with 10 units of each enzyme, *EcoR* I and *HinD* III (Gibco/BRL), in a total volume of 20 µl nanopure water containing 2 µl 10x React buffer 4 (Gibco/BRL). This was incubated for 30 minutes at 37°C. The enzymes were then heat inactivated 10 minutes at 65°C.

The two pCMS3 fragments were simultaneously re-ligated with the pCambia 2301 vector in a molar ratio of 3:3:1 respectively (using approximately 8 ng of the 850 bp pCMS3 fragment: 13 ng of the 1900 bp pCMS3 fragment: 100 ng of the 11.6 Kbp pCambia vector). 2 µl of this reaction was transformed into 50 µl of electrocompetent *E. coli* (DH5α) (see §2.12.2.2). Cells were cultured on LA containing 100 µg ml^{-1} kanamycin. Colonies harbouring the recombinant plasmid were km^r and colony PCR using the CPSFOR and CPSREV primers (see §2.10.4.2) further revealed the presence of the *cps3S* gene by PCR. The colonies that produced a PCR product of ~1.3Kb were prepared by small-scale purification (§2.10.2.1) and digested with *EcoR* I (see §2.10.8). Digested DNA fragments were analysed on a 0.7 % (w/v) TAE-agarose gel. pCambia 2301 carrying the CaMV35S promoters, PR1B signal sequence, *cps3S* gene and terminator sequence was named pCMS4.

The pCMS4 plasmid was then prepared by large-scale DNA extraction (see §2.10.2.2) and transformed into *Agrobacterium tumefaciens* strain GV3101 by heat shock (see §2.12.1.2.1). pCMS4 was also cloned into *A. rhizogenes* strain LBA9402 by triparental mating (see §2.12.3). Transformed *A. tumefaciens* and *A. rhizogenes* were screened on YEP or YMB agar respectively (see §2.1), containing 100 µg ml^{-1} rifampicin (to maintain *Agrobacterium* chromosomal DNA) and 100 µg ml^{-1} kanamycin. Plasmids were purified

using a modified method of that described before §2.10.2.1. A single colony was used to inoculate a 5 ml YMB broth containing rifampicin (100 μg ml^{-1}) and kanamycin (100 μg ml^{-1}) and incubated for 2 days at 28°C. The cells were harvested at 600x *g* and resuspended in 150 μl Solution I (see §2.10.2.1) containing 4 mg ml^{-1} lysozyme. PCR using CPSFOR and CPSREV primers (as §2.10.4.2) confirmed the presence of the *cps3S* gene.

2.11.2.3 Cloning of *cps3S* into pRSET C

The cultivation of a bacterial culture has many advantages over whole plant culture, especially for protein expression work. Bacterial cell suspension cultures grow more rapidly and have greater homogeneity and, consequently a high proportion of the cells are in direct contact with the antibiotic selection in the culture medium. This is unlike whole plant cell culture as antibiotic selection is ceased after rooting and transfer to soil. Therefore, we cloned *cps3S* into the bacterial expression plasmid pRSET C. This vector is a pUC-derived expression vector used for high-level expression and purification of recombinant proteins in *E. coli* (pRSET technical manual, Invitrogen). The strong T7 promoter controlled expression of the transgene.

Two studies have previously reported the expression of Cps3S in *E. coli* (Arrecubieta *et al.* 1996a; Cartee *et al.* 2001). This was not without difficultly, as Cps3S was thought to be toxic to *E. coli* when over expressed (Cartee *et al.* 2001). The level of expression therefore needed to be closely regulated. Arrecubieta *et al* (1996) found success cloning into *E. coli* containing the DE3 bacteriophage (Arrecubieta *et al.* 1996). The DE3 bacteriophage contains a *lac* repressor, which represses expression of T7 RNA polymerase. In this study, we used *E. coli* BL21(DE3)pLysS (Invitrogen, Carlsbad, CA, USA) cells, which were designed for expression of genes regulated by the T7 promoter. T7 RNA polymerase specifically recognised this promoter and the delivery of T7 RNA polymerase to the cells was necessary for expression of the transgene. The BL21(DE3)pLysS *E. coli* strain carried the DE3 bacteriophage and the pLysS plasmid. The pLysS plasmid produced T7 lysozyme that bound to T7 polymerase to inhibit transcription. Both DE3 and pLysS acted to reduce basal expression of T7 RNA polymerase and therefore, completely blocked expression of the transgene. Expression of the transgene could be induced by the addition of IPTG (pRSET technical manual, Invitrogen).

The CaMV35S promoter present in pCMS3 also functioned as a promoter in *E. coli.* It was important that this was not cloned into the pRSET C, as it would drive expression of *cps3S* independently of the T7 regulators.

pCMS3 was prepared by large-scale DNA extraction (see §2.10.2.2). 9 μg of pCMS3 was digested with 10 units of *EcoR* I and *Kpn* I in a total volume of 50 μl and buffer 4 (Gibco). This reaction was incubated for 2 hours at 37°C and then heat inactivated for 10 minutes at 65°C. pCMS3 was digested at two restriction sites to ensure the gene ligated into pRSET C in the correct orientation. (see Figure 2.7).

7.6 μg of pRSET C was also prepared by large-scale DNA extraction and digested with 10 units of *EcoR* I and *Kpn* I in a total volume of 50 μl nanopure water containing 5 μl of buffer 4 (Gibco). This reaction was incubated for 2 hours at 37°C and then heat inactivated for 10 minutes at 65°C. The digested products were analysed on a 0.7 % (w/v) TAE-agarose gel and the vector fragment was gel extracted (see §2.10.5.1).

Digested DNA of *cps3S* and pRSET C were ligated using T4 DNA ligase (Promega) see §2.10.9. 2 μg of the pRSET C fragment was ligated with 2 μg of the *cps3S* fragment (approximately a 1:3 molar ratio) in a total volume of 20 μl. The reaction was incubated at room temperature for 1 hour and 20 ng of the ligation mixture was transformed into *E. coli* BL21(DE3)pLysS by heat shock see §2.12.1.1.1. Cells were cultured on LA containing 34 μg ml^{-1} chloramphenicol (to maintain the pLysS plasmid) and 100 μg ml^{-1} ampicillin (for selection of clones containing pCMS5). pRSET carrying the *cps3S* gene was named pCMS5. Chloramphenicol and ampicillin resistant colonies harbouring the pLysS and pCMS5 vectors, respectively, were screened by small-scale purification of plasmids as described in §2.10.2.1. The identification of clones containing the pCMS5 plasmid and not the reformed pRSET C vector were analysed by PCR, using the PRSET-REV and T7 primers (see §2.10.4.3).

2.12 Bacterial Transformation

Table 2.11 Plasmids and the method used to transform bacteria

Plasmid	*[DNA] used for transformation*	*Species (strain)*	*Method*	*Selection*
pCMS1	20 ng/ 50 µl	*E. coli* (Top10F')	Heat shock	Ampicillin, IPTG, X-Gal
pCMS2	10 ng / 40µl	*E. coli* (DH5α)	Electroporation (pGEM manual (Promega))	Ampicillin, IPTG, X-Gal
pCMS3		*E. coli* (Top10F')	Heat shock	Spectinomycin
pCMS4	33 ng / 40 µl	*E. coli* (DH5α)	Electroporation (T4 DNA ligase manual (Invitrogen))	Kanamycin
	-	*A. rhizogenes* (LBA9402)	Triparental mating	Kanamycin Rifampicin[a]
	1 µg / 50 µl	*A. tumefaciens* (GV3101)	Heat Shock	Kanamycin Rifampicin[a]
pCMS5	20 ng / 40 µl	*E. coli* (BL21(DE3)pLysS)	Heat shock (One Shot cells manual (Invitrogen))	Ampicillin, Chloramphenicol[b]

[a] Rifampicin is needed to maintain the Ti or Ri plasmids in *Agrobacterium* species. These plasmids essentially transfer the T-DNA region of the binary vector into the infected plant cell genome.

[b] Chloramphenicol is needed to maintain the pLysS plasmid in the BL21(DE3) cells. This plasmid allowed stricter regulation of transgene expression (see §2.11.2.3).

2.12.1 Bacterial Transformation by heat shock

Cells washed in $CaCl_2$ (see §2.12.1.1) achieve a state of competence during which DNA molecules may be admitted to the cell (Maniatis *et al.* 1982). When these cells were

subjected to a sudden increase in temperature (shock) the pores enlarged allowing foreign DNA to enter.

2.12.1.1 *Escherichia coli*

2.12.1.1 Preparation of $CaCl_2$ competent *E. coli* cells

The preparation of *E. coli* cells for heat shock transformation was done as described by Maniatis *et al.* (1988) (Maniatis *et al.* 1988). A 20 ml overnight culture of *E. coli* DH5α or Top10F' was used to inoculate a fresh 200 ml LB broth (a 1:10 dilution) and the cells were grown at 37°C to an OD_{600} between 0.4 - 0.6 (2 - 4 hours) to ensure the cells were in mid-log phase. The cells were harvested by centrifugation at 600x g for 10 minutes at 4°C in 50 ml Falcon tubes and were drained until all traces of LB broth were removed. At all times the cells were kept on ice. The pellet was then resuspended in 50 ml cold 100 mM $CaCl_2$ (in 10 mM Tris pH 8) and incubated on ice for 30 minutes. This was repeated and cells were harvested as before. Finally the cells were gently resuspended in 4.5 ml 100 mM $CaCl_2$ (in 10 mM Tris pH 8) containing 10 % (v/v) glycerol, aliquoted into 40 µl volumes, snap-frozen on dry ice and stored at -70°C.

2.12.1.1.1 Transformation

$CaCl_2$ competent *E. coli* Top10F' or *E. coli* DH5α cells (see §2.12.1.1) were transformed as described by Maniatis *et al.* (1988). 2 µl of plasmid DNA was added to the cells and left on ice for 20 minutes. The cells were then subjected to 42°C for 45 seconds and then resuspended in 1 ml SOC broth (2 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM, $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose). Cells were incubated for 1 hour shaking at 37°C before plating onto LA agar containing a selective antibiotic (see Table 2.11).

2.12.1.2 *Agrobacterium tumefaciens*

2.12.1.2 Preparation of $CaCl_2$ competent *A. tumefaciens* cells

The preparation of *A. tumefaciens* cells for heat shock transformation was modified from that described by Draper *et al.* (1988). A culture of *A. tumefaciens* was grown overnight at 28°C in 5 ml YEP broth. The following day, 2 ml of the overnight culture was added to 50 ml YEP and grown at 28°C to an OD_{500} between 0.5 – 1.0 (2 - 4 hours) to ensure the cells were in mid-log phase. The cells were harvested by centrifugation at 3000x *g* for 5 minutes at 4°C in 50 ml Falcon tubes and were drained until all traces of YEP broth were removed.

At all times the cells were kept on ice. The pellet was then resuspended in 1 ml cold 20 mM $CaCl_2$ (in 10 mM Tris pH 8) and aliquoted into 100 µl volumes into pre-chilled eppendorf tubes. Cells were stored at -70°C.

2.12.1.2.1 Transformation

Agrobacterium tumefaciens strain GV3101 was transformed by heat shock following a modification of the method described by Draper *et al.* (1988), using 0.5 µl (1 µg) pCMS4 and 0.1 ml of frozen $CaCl_2$ competent *A. tumefaciens* cells (see §2.12.1.2). Cells were thawed at 37°C for 5 minutes, resuspended in 1 ml of YEP broth (10 g l^{-1} Yeast Extract, 10 g l^{-1} Peptone, 5 g l^{-1} NaCl, pH 7.0) and incubated at 28°C for 2 hours with gentle shaking. Cells were harvested by centrifugation at 600x *g* for 10 minutes, the pellet resuspended in 0.1 ml YEP broth and spread onto YEP agar containing 100 µg ml^{-1} kanamycin and 100 µg ml^{-1} rifampicin. Colonies appeared after incubation at 28°C for 48 hours.

2.12.2 Bacterial Transformation by Electroporation

2.12.2.1 Preparation of Electrocompetent *E. coli*

The preparation of *E. coli* cells for electroporation was done as described by Maniatis *et al.* (1982). A streaked culture of *E. coli* DH5α was grown overnight at 37°C overnight on LA. A single colony was used to inoculate a 10 ml LB broth and re-incubated shaking at 37°C overnight. All 10 ml was used to inoculate a 400 ml LB broth and grown to an OD_{600} between 0.4 and 0.6. The culture was then transferred to 4 x chilled 50 ml falcon tubes and incubated on ice for 15 minutes. At all times the cells were kept on ice. Cells were harvested by centrifugation at 600x *g* for 15 minutes and gently resuspended in 50 ml cold sterile nanopure water and recentrifuged. This washing step is repeated before the addition of 5 ml cold 10 % (v/v) glycerol. Again the cells were pelleted by centrifugation and finally the cells were resuspended in 500 µl cold 10 % (v/v) glycerol. Cells were aliquoted on dry ice into 40 µl or 80 µl volumes into pre-chilled microfuge tubes and stored at -70°C for up to 6 months.

2.12.2.2 Transformation

The process of freezing bacteria can weaken cell walls and extensive washing with nanopure water to ensure the removal of salts causes the cells to become electrocompetent. Exposure to an electrical charge destabilizes the bacterial membranes and induces the formation of transient membrane pores through which DNA molecules can enter (Maniatis

et al. 1982). As with heat shock, the cells are allowed to divide before selection is analysed. However, this process is at least 10 times as effective at producing transformed cells as chemical (heat shock) transformation (Maniatis *et al.* 1982)

This method was performed as described by Maniatis *et al.* (1982). 2 µl of a ligation mixture was mixed with 40 µl of cells. This was transferred to an ice-cold 0.2 cm electroporation cuvette (Bio-Rad). The cuvette was placed in the cuvette holder of the Bio-Rad Gene Pulser apparatus and the electroporation was performed under an electrical discharge of 2.5 kV (Kilovolts), a capacitance of 25 µF (micro Faraday) and a resistance load of 200Ω (Ohms). The cuvette was removed from the apparatus and 1 ml of ice-cold SOC broth (2 % (w/v) Tryptone, 0.5 % (w/v) Yeast Extract, 10 mM NaCl, 2.5 mM KCl, 10 mM, $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose) was added directly to the cell suspension. This was mixed gently and transferred to a 1.5 ml eppendorf tube. The eppendorf tube was then strapped firmly into a shaking incubator (200rpm) and incubated for 1 hour at 37°C. This gives the cells time to recover from the stress of electroporation and begin to express the antibiotic resistance genes. The cells were then plated out on to LA agar containing the appropriate selective antibiotics. The plates were dried and placed inverted in a 37°C incubator overnight.

2.12.3 Bacterial Transformation by Triparental Mating

This method was performed as described by Draper *et al.* (1988). The aim of the triparental mating was to transfer pCMS4 into *Agrobacterium rhizogenes* strain LBA9402, which contained the virulence loci (present in the R_i plasmid) necessary for T-DNA transfer into plants. This was achieved by using the mobilizing (mob) functions of the broad host range plasmid pRK2013 present in *E. coli* HB101. pRK2013 (Ditta *et al.* 1980) is a transfer helper plasmid that is used for mobilization of non-self-transmissible plasmids. During triparental mating, pRK2013 mobilized itself into both *A. rhizogenes* and *E. coli* containing pCMS4. The presence of pRK2013 in the latter, mobilized both plasmids (pCMS4 and pRK2013) back into *E. coli* HB101 and *A. rhizogenes.*

100 µl of a 5 ml culture of *A. rhizogenes* (strain LBA9402) that had been grown for 48 hours in YMB broth (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 10 g l^{-1} Mannitol, 0.4 g l^{-1} Yeast Extract, pH 7.0) containing 100 µg ml^{-1} rifampicin, was mixed with an equal volume (50 % of each) of fresh overnight cultures of *E. coli* pCMS4 (see

Table 2.11) and *E. coli* strain HB101 containing pRK2013 (Clontech). *Escherichia coli* HB101::pRK2013 was maintained by growth in the presence of 100 μg ml^{-1} kanamycin in LB broth. Cells were incubated at 37°C overnight.

The tri-species mixture was placed on YMB agar containing no antibiotics and incubated overnight at 28°C. The following day all the colonies were scraped from the agar and resuspended in 500 μl YMB broth. A loop full of this suspension was streaked onto YMB agar containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin, and incubated for 2-4 days until colonies formed. As a control, wild-type *A. rhizogenes* was grown on YMB agar containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin for 4 days.

Colonies were sub-cultured onto fresh YMB agar containing rifampicin and kanamycin (as above) and the same colony was used to inoculate a Simmons citrate agar slope see §2.12.3.1 below. This was to confirm that the transformed colony was *Agrobacterium rhizogenes* and not an *E. coli* contaminant.

Glycerol stocks were prepared by adding 10 % (v/v) glycerol to a 5 ml 48-hour old culture grown at 28°C in YMB broth containing the appropriate antibiotics. Cells were aliquoted into 0.1 ml volumes and stored at -70°C

2.12.3.1 Citrate utilisation

To confirm that the transformed colonies from tri-parental mating were *Agrobacterium* and not *Escherichia coli* contaminants, the colony was tested for citrate utilisation (Simmons 1926). A loop of each clone was streaked onto Simmons Citrate agar slopes (0.2 g l^{-1} $MgSO_4$, 0.2 g l^{-1} ammonium dihydrogen phosphate, 0.8 g l^{-1} sodium ammonium phosphate, 2 g l^{-1} sodium citrate (tribasic), 5.0 g l^{-1} NaCl, 0.08 g l^{-1} Bromothymol blue, 13.0 g l^{-1} Agar, pH 7.0) (Oxoid) and incubated overnight at 37°C. *Agrobacterium rhizogenes* ferment citrate (i.e. utilise citrate) and therefore produce an acid, usually the product of fermentation. This lowers the pH and changes the colour of the medium from green to bright blue. *E. coli* do not ferment citrate and therefore the colour of the medium remains unchanged (Madigan *et al.* 1997).

2.13 Plant transformation

2.13.1 Transformation of *Arabidopsis thaliana* cv. Columbia

2.13.1.1 Floral dip method

The floral dip method used here has been described by Clough *et al.* (1998), and it is a variation on vacuum infiltration as described by Bechtold *et al.* (1998) (Bechtold and Pelletier 1998). A 5 ml starter culture of either *Agrobacterium rhizogenes*::pCMS4 or *A. rhizogenes*:: pCMS3 was grown in YMB broth (0.5 g l^{-1} K_2HPO_4, 0.2 g l^{-1} $MgSO_4.7H_2O$, 0.1 g l^{-1} NaCl, 10 g l^{-1} Mannitol, 0.4 g l^{-1} Yeast Extract, pH 7.0) containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin (or 150 μg ml^{-1} gentamicin to select for pCMS3) at 28°C until an OD_{600} of 1.8-2.0 (2 days incubation). Cells were harvested by centrifugation for 20 minutes at room temperature at 5500x *g* and resuspended in 500 ml dipping medium (50g l^{-1} sucrose and 2.03 g l^{-1} $MgCl_2.6H_2O$), containing 0.05 % (v/v) (i.e. 500μl l^{-1}) Silwet L-77, a detergent (Cat#VIS-02, LEHLE SEEDS, Tuscon, AZ, USA).

The inoculated dipping medium was poured into a large beaker. 6 - 8 week old *Arabidopsis thaliana* cv. Columbia plants were stripped of siliques (seedpods) and floral buds (if present) and inverted into the beaker, such that all exposed plant tissue was submerged in the *Agrobacterium* suspension. After 15 minutes the plants were lifted out of the suspension, gently shaken to remove excess inoculum, and placed horizontally onto a clean tray. These were covered in cling film to prevent moisture loss (and also to improve the transformation rate) and left overnight at room temperature (Clough and Bent 1998). Plants were then grown as normal in an isolated room away from other plants and watered once a week, for 3 - 5 weeks until siliques had developed. At this time no additional water was supplied, to allow the seeds to dry out and prevent contamination. The stalks were then cut 1 cm above the topsoil and the bundle of bolts from each pot was placed upside down in a thin paper bag. The seeds were harvested from their seedpods by gentle shaking (gripping the stalks at the top of the bag). The majority of stem and pod material was removed by sifting. Desiccated seeds were stored in microcentrifuge tubes and kept at 4°C.

Seeds were sterilised as described in §2.13.1.2 and then resuspended in a final volume of 6 ml sterile water and stored at 4°C for 3 days. This was to ensure all seeds were simultaneously exposed to an increase in temperature and will therefore germinate at an equal rate.

Approximately 50 seeds (1.5 ml) were sprinkled onto a 9 cm diameter Petri dish containing Murashige and Skoog basal medium (MS) (Sigma M5524) agar supplemented with 3 % (w/v) sucrose, 100 μg ml^{-1} kanamycin (or 150 μg ml^{-1} gentamicin for pCMS3), 250 μg ml^{-1} cefotaxime, 100 μg ml^{-1} ampicillin. Transgenic shoots harbouring the pCMS4 plasmid were kan^r, those harbouring the pCMS3 plasmid were $gent^r$. The addition of a further carbon source was to ensure that the shoots did not rely on photosynthetic fixation for growth. However, full greening of the leaves does require a light source and so seeds were incubated in a growth room with controlled 16 hour light cycles for 10 days, until shoots were >1 cm.

The shoots that displayed no signs of chlorosis (yellowing or whitening of plant tissue) were aseptically transferred with a sterile scalpel into individual universals containing 10 ml of the selective medium (as for germination) and incubated in the same growth room for a further 1 - 2 weeks. This limited the possibility of contamination spreading to all seedlings and confirmed antibiotic resistance. Those shoots showing signs of chlorosis were discarded, as this was an indication of antibiotic sensitivity. After this time the plants were transferred to a 6 cm Falcon plate containing MS agar containing 3 % (w/v) sucrose, 250 μg ml^{-1} cefotaxime, 100 μg ml^{-1} napthaleneacetic acid (NAA) and 1 mg ml^{-1} 6-benzylaminopurine (6-BAP). The plasmid selection was no longer needed as the plants where, by now, substantially developed through selection. NAA is a plant auxin and 6-BAP is a cytokinin. NAA is a synthetic indole-like compound that stimulates cell expansion, particularly cell elongation. Auxins also promote adventitious root development (Mineo 1990). Synthetic auxins are more potent than naturally occurring auxins and only small amounts (<1 μM) are required for a tissue response. This is because they are not readily recognised by degrading enzymes and they are therefore, more effective hormones and last for an extended length of time (Lindsey 1991). Cytokinins (6-BAP) are derived from adenine and produce two immediate effects on undifferentiated cells: the stimulation of DNA synthesis and increased cell division (Ting and Wang 1982). Cytokinins also produce a delayed effect, which is the formation of shoot primordia (Lindsey 1991). Together these compounds stimulate the transgenic plants to produce stronger shoots and, more importantly, the growth of roots, which is necessary for transfer of the plantlet to soil. Plates were incubated in the growth room for a further 1 - 2 weeks.

Once roots were formed and the shoots had reached 2 - 3 cm in height, the agar was removed and the plantlets were transferred to a plant pot containing soil that was covered with a transparent plastic lid. These plants were grown in an incubator at 23°C with 8-hour light cycles. To maintain high humidity and allow the development of a harder waxier cuticle (leaf coat), plants remained covered for a further 2 weeks.

After one week a single leaf was removed and DNA was isolated from the plant tissue using the DNeasy Plant Mini Kit (Qiagen) see §2.10.3. PCR was performed using the extracted DNA from all 14 plants to detect the presence of the *cps3S* gene and residual *Agrobacterium* contamination.

2.13.1.2 Plant tissue sterilisation

Seeds or leaves were sterilised in a volume of 10 % (v/v) commercial bleach necessary to cover the surface. This was usually 10 ml (in a universal) for seeds and 500 ml (in a glass bowl containing a magnetic stirrer) for leaves. If some leaf surface was not exposed to bleach a piece of blue roll was placed on top of the bowl to submerge all the tissue. This was mixed (for 10 minutes and washed four times with an equal volume of sterile tap water.

2.13.2 Transformation of *Lotus corniculatus*

Seeds of *Lotus corniculatus* were sterilised in 10 % (v/v) commercial bleach for 10 minutes and thoroughly washed 4 times in sterile water (see §2.13.1.2). The seeds produced by *L. corniculatus* are larger than those of *Arabidopsis thaliana*, therefore only 3 seeds were placed onto each 6 cm diameter Petri dish. Seeds germinated on MS agar containing 3 % (w/v) sucrose in a growth room with controlled 16 hour light cycles for 10 days, until shoots were >3 cm in length.

Agrobacterium rhizogenes::pCMS4 was grown at 28°C on YMB agar containing 100 μg ml^{-1} rifampicin and 100 μg ml^{-1} kanamycin. A fresh colony was picked with a small syringe needle (0.33 mm or 29G) and was used to infect *L. corniculatus* seedlings about 5 mm above the media/roots (Webb *et al.* 1990).

Seedlings were incubated in the growth room for 10 - 14 days, until hairy roots developed from the infection site. Hairy roots were excised, decontaminated for 5 hours in MS

medium with 500 μg ml^{-1} carbenicillin and transferred to solid or liquid MS medium containing 3 % (w/v) sucrose, 500 μg ml^{-1} ampicillin, 250 μg ml^{-1} cefotaxime, 500 μg ml^{-1} carbenicillin and 100 μg ml^{-1} kanamycin, to select for transgenic roots. Cefotaxime and carbenicillin were used to eliminate any remaining *Agrobacterium*.

3 - 4cm long apical pieces from 4-week-old secondary root cultures were harvested and sub-cultured for growth MS broth pH 6.8 on a rotary shaker (110rpm) at 27°C. DNA was isolated from plant tissue using the DNeasy Plant Mini Kit (Qiagen) see §2.10.3.

2.13.3 Transformation of *Nicotinia tobacum* cv. Samsun

The *Agrobacterium*-mediated transformation of *N. tobacum* was performed as described by Draper *et al.* (1988). An *A. rhizogenes*::pCMS4 starter culture was grown as described in §2.13.1. The leaves from *N. tobacum* cv. Samsun were sterilised in 10 % (v/v) commercial bleach for 10 - 15 minutes in a large beaker with a magnetic stirrer, ensuring all leaves were submerged, and then thoroughly washed 4 times in an equal volume of sterile tap water. Each leaf was cut into 1 cm^2 discs (or squares) avoiding leaf veins and incubated in the *Agrobacterium* culture for 5 minutes at room temperature. Leaf discs were then transferred to a MS agar plate containing 3 % (w/v) sucrose, which was sealed with parafilm and incubated in the dark at room temperature for 2 days. Discs were then transferred to selective medium (MS agar containing 3 % (w/v) sucrose, 100 μg ml^{-1} kanamycin, 250 μg ml^{-1} cefotaxime, 100 μg ml^{-1} ampicillin, 100 μg ml^{-1} napthaleneacetic acid (NAA) and 1 mg ml^{-1} 6-benzylaminopurine (6-BAP), (to promote shoot and root growth, see §2.13.1)) and incubated in a growth room with controlled 16-hour light cycles for 1 - 2 weeks. Those discs showing shoot or callus formation were transferred to fresh selective medium (as before) and re-incubated for approximately 1 - 2 weeks. Larger shoots were transferred to powder rounds (jam jars) containing the same medium and incubated for a further 3 weeks, or until roots started to form.

Once roots were formed, the agar was removed and the plantlets were transferred to a plant pot containing soil and covered with a transparent plastic lid and placed in greenhouse conditions. To maintain high humidity and allow the development of a harder waxier cuticle (leaf coat), plants remained covered for a further 2 weeks. Once the plants had grown to exceed the size of the pot they were in they were transferred into a larger pot. DNA was isolated from plant tissue using the DNeasy Plant Mini Kit (Qiagen) see §2.10.3.

For continued cultivation, the plants were allowed to flower, the buds were covered in a paper bag to ensure self-pollination and the seeds collected. These were sterilised in 10 % (v/v) bleach as §2.13.1.2 and were stored desiccated at 4°C.

2.14 Detecting the Expression of the Type 3 Capsule Synthase *in planta*

This section of the thesis describes the methods used to detect the expression of Cps3S and type 3 polysaccharide in plants.

2.14.1 Plant culture and growth conditions

2.14.1.1 Whole plant culture

Plants were generally cultured on Murashige - Skoog (MS) medium (Sigma M5524) containing 3 % (w/v) sucrose and 0.8 % (w/v) agar in the presence of a suitable selection marker and incubated in a growth room with controlled 16 - hour light cycles. Larger plants (> 5 cm in height) were rooted in soil and kept in greenhouse conditions (see §2.13.1)

2.14.1.2 Plant Tissue culture

Plant cell suspension cultures were used in preference to callus cultures, due to their more rapid growth, greater homogeneity and ease of manipulation (Lindsey 1991). This is because continued agitation used in culture aerates the medium and helps break up the cell aggregates. Consequently, a high proportion of the cells were in direct contact with the culture medium and antibiotic selection added to it. This is unlike callus culture where only the cells at the base of the callus were in contact with the solid medium. Callus' were grown as described by Draper *et al.* (1988)

2.14.2 Analysis of gene expression

2.14.2.1 Isolation of total RNA from plant cells

100 mg of leaf tissue was harvested and ground under liquid nitrogen with a pellet pestle. Frozen tissue was not allowed to thaw and the RNA was immediately extracted using the RNeasy Plant Mini Kit (Qiagen Cat #74903) and following the protocol outlined in the manufacturer's handbook: 450 µl buffer RLT (provided in the kit) was added and the sample was vigorously vortexed. The lysate was transferred to a QIAshredder spin column (provided in the kit) rested in a 2 ml collection tube and centrifuged at 12, 000x *g* for 2

minutes to remove cell debris and homogenise the lysate. The supernatant was retained in a fresh microcentrifuge tube and ½ volume of 100 % ethanol was added to precipitate the RNA and DNA. This was immediately transferred to an RNeasy mini column rested in a 2 ml collection tube and spun at 12, 000x *g* for 15 seconds. DNA was removed by on-column digestion using the RNAse-Free DNase set (Qiagen Cat #79254).

500 µl of buffer RPE (provided in the kit) was added to wash the RNeasy column by centrifugation at 12, 000x *g* for 15 seconds into a fresh collection tube. This step was repeated except centrifugation occurred at 12, 000x *g* for 2 minutes. To elute the RNA, 50 µl of RNase free water was added directly to the RNeasy silica-gel membrane and the column was spun at 12, 000x g for 1 minute. Eluted RNA was collected in a fresh microcentrifuge tube. This step was repeated to increase total RNA yield and samples were stored at -20°C.

2.14.2.2 Reverse Transcriptase-PCR

5 µl of each RNA extraction was run on a 1 % (w/v) TAE-agarose gel (as §2.10.5) to confirm that the sample contained RNA and was free of noticeable contaminants.

RNA samples were then treated with reverse transcriptase to produce cDNA. The method used here was taken from the manufacturer's manual for SuperScript II RNase H$^-$ reverse transcriptase (Invitrogen). 5 µl of the RNA extract was mixed with 250 ng random hexamer primers (Invitrogen, Cat #48190-011) and 10mM of each deoxynucleotide; dATP, dTTP, dGTP, dCTP (Advanced Biotechnologies Ltd) in a total volume of 12 µl RNase-free water. This was heated to 65°C for 5 minutes, quickly chilled on ice and the contents of the tube were collected by centrifugation at 12, 000x *g* for 30 seconds. Then 4 µl of 5x First Strand Buffer (from the RNase H$^-$ reverse transcriptase kit (Invitrogen)), 1 mM DTT and RNase-free water was added to a final volume of 20 µl. This suspension was mixed and incubated at 42°C for 2 minutes. 1 µl (200 units) of SuperScript™ II reverse transcriptase was added and mixed by gentle pipetting up and down. This was incubated at 25°C for 10 minutes followed by 42°C for 50 minutes. The enzyme was inactivated, and the reaction stopped by heating at 70°C for 15 minutes.

PCR was then performed using the CPSFOR and CPSREV primers as described in §2.10.4.2, RNA extracts without prior reverse transcriptase treatment were used as a control as this would indicate the presence of *cps3S* specific DNA.

2.15 Analysis of polysaccharide production *in planta*

2.15.1 Polysaccharide isolation

2.15.1.1 Crude extraction

Leaf tissue was harvested and the mid-rib removed. The tissue was cut into pieces small enough to fit in a 1.5 ml eppendorf tube (maximum weight of 1 g) and then ground into a fine powder under liquid nitrogen using a pellet pestle. 1 ml nanopure water (or to a concentration no less than 0.5 g ml^{-1}) was added and the sample put on ice. The cells were then lysed by sonication with the small sonicator (Sonitech) probe, to break open the cells and release the polysaccharide. This was performed on ice at an amplitude of 50 microns in 1 minute intervals with 30 second sonication and 1 minute cooling, repeated for 6 - 8 bursts, or until the colour of the plant sonicate turned more green compared to the original cell suspension before sonication. The cell lysate was then centrifuged for 5 minutes at 10 000x *g* and supernatant transferred to a fresh 1.5 ml eppendorf tube. This was stored at 4°C.

Alternatively the above method was repeated, except 3 - 6M NaOH was used as the diluent. Hemicelluloses were precipitated by neutralisation using glacial acetic acid. This was based on the method to extract plant hemicelluloses taken from Fry (1988).

2.15.1.2 Extraction of apoplastic fluid

This method was modified of that described by Fry (1988). Leaf tissue was harvested and the mid-rib removed. Sections 1 cm square were cut (total weight of 1 g) and placed into a small beaker containing 50 ml 50 mM $CaCl_2$ (BDH). A conical flask that fitted flush to the inside of the beaker was then placed on top of the liquid to ensure full immersion of the tissue pieces. The leaf pieces were then vacuum-infiltrated for a period of 30 minutes by which time the tissue had taken on a transparent appearance. They were then removed and dried gently on a paper towel before being transferred to the barrel of a 25 ml syringe with the plunger removed. This was placed in a 50 ml Falcon tube and the assembly spun at 800x *g* at 10°C for 10 minutes. The extra-cellular wash accumulated at the bottom of the

tube and was retrieved after the spin. A subsequent identical spin ensured full recovery of the wash and the tissue was then processed again to produce a second wash extraction. The sample was stored at 4°C.

2.15.2 Precipitation of polysaccharides

2.15.2.1 Acetone

The precipitation of polysaccharides was done by the method described by Gilbert *et al* (2000). 25 % (v/v) TCA (made to a stock concentration of 220 % w/v) was added to an overnight pneumococcal culture or plant tissue preparation (see §2.15.1) and proteins were precipitated after 15 minutes incubation on ice. The supernatant was recovered from centrifugation at 12, 000 x *g* at 4°C and the protein pellet was discarded. An equal volume of ice-cold 100 % acetone was added to precipitate polysaccharides, which were pelleted by centrifugation (as before), washed with acetone, and re-dissolved in 5 % (w/v) sodium acetate. The pellet was extracted twice with 1/5 volume chloroform:butanol (5:1), precipitated again in an equal volume of acetone and finally dissolved in nanopure water. If not completely dissolved, the solution was placed in a 70°C water bath for 5 - 10 minutes.

2.15.3 Quantification of polysaccharide: Phenol-Sulphuric acid method

Polysaccharides and other sugars contain methyl ethers with free or potentially free reducing groups that cause a colour change when combined with phenol and sulphuric acid (Dubois *et al.* 1956). This colour change (to orange-yellow) has been shown to be proportional to the sugar concentration (Dubois *et al.* 1956). Therefore the quantification of polysaccharides was done by a modified version of that described by Dubois *et al.* (1956).

25 µl of an 80 % (v/v) phenol solution in water was mixed thoroughly with 1 ml nanopure water containing approximately 10 - 100 mg ml^{-1} polysaccharide. To this, 2.5 ml concentrated sulphuric acid was added quickly (to avoid evaporation from the strong exothermic reaction). The mixture was incubated at room temperature for 10 minutes and at 30°C for 20 minutes. The total uronic acid content of the sample (type 3 pneumococcal polysaccharide is composed of cellobiuronic acid units) was measured at 480 nm using a spectrophotometer (the extinction coefficient for type 3 polysaccharide was 1.39 µg ml^{-1} at A_{480}) (Ultrospec 3000, Pharmacia Biotech). Nanopure water was used as a reference and

different concentrations of type 3 polysaccharide (ATCC) were used to form a standard curve from which relative sample concentrations were determined.

2.15.4 Analysis of the type 3 pneumococcal polysaccharide contents of plant extracts

2.15.4.1 Ouchterlony

The immuno double-diffusion technique used to detect the presence of type 3 pneumococcal polysaccharide in plant extracts was a modified version of that described by Ouchterlony *et al.* (1973) and Cherniak *et al.* (1972) (Cherniak and Henderson 1972; Ouchterlony and Nilsson 1973). The antigen and antibody molecules diffuse through the agar and precipitate as an arc upon binding at equivalence to form an antibody-antigen complex. 0.2 % (w/v) Ouchterlony agarose (Difco) in Barbitone buffer (1.84 g l^{-1} Diethylbarbituric acid, 10.3 g l^{-1} Sodium diethylbarbiturate, pH8.6 with NaOH) was used to coat microscope slides (76 mm x 26 mm x 1mm). These were left to dry overnight at room temperature and then overlaid with 4.5 ml 1 % (w/v) agarose in Barbitone buffer (as above). Once set, holes of 4 mm in diameter were cut using a cork borer in the pattern shown below (actual size).

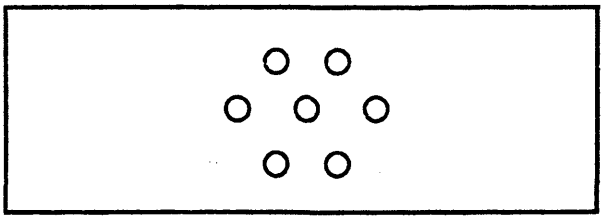

Figure 2.9. The pattern of holes made with a cork borer on a 76 x 26 x 11 mm microscope slide overlaid with 4.5 ml Ouchterlony agar. Diagram is actual size.

20 µl of each sample was placed in the outer holes. 10 µg type 3 polysaccharide (ATCC) was used as a positive control. The central hole contained 20 µl neat rabbit anti-type 3 polysaccharide antiserum (Statens Serum Institute, Copenhagen, Denmark). The slide was incubated at 4°C for at least one week (and no more than 2 weeks). To prevent the slide drying out it was placed in a humidity box and remained covered.

2.15.4.2 ELISA

The capture ELISA technmique described in §2.3 was unsuccessful within the time scale of this thesis. However, a secondary antibody was prepared and so for future reference the method is given below.

2.15.4.2.1 Preparation of the secondary horseradish peroxidase antibody conjugate.

Rabbit anti-type 3 polysaccharide antiserum (Statens Serum Institute) was conjugated to horseradish peroxidase for use as a secondary antibody in ELISA using EZ-link Activated Peroxidase (Pierce, Rockford, IL, USA #31496). The protocol for conjugation was described in the product information sheet for EZ-link Activated Peroxidase.

The antiserum to be conjugated had to be free of salts and most importantly free of sodium azide, as this is an inhibitor of horseradish peroxidase. These were removed by dialysis using a Slide-A-lyser (Perbio). 100 µl of antibody solution was added to the bottom of three Slide-A-lysers and placed in a floating rack in a large beaker containing 400 ml cold, sterile PBS. This was incubated on a magnetic stirrer for 1 hour at room temperature. The PBS was exchanged with 400 ml of fresh PBS every 20 minutes. The antibody solutions inside the Slide-A-lysers were combined and transferred to an eppendorf tube. Samples were then frozen at -70°C and lyophilised overnight.

0.3 mg lyophilised antibody was then dissolved in 50 µl Conjugation buffer (0.1 M $NaHCO_3$, pH9.5, 0.9 % (w/v) NaCl). 1 mg of EZ-link Activated Peroxidase was reconstituted in 50 µl distilled water and chilled on ice. Immediately the EZ-link Activated Peroxidase was mixed with the IgG and incubated overnight at 4°C. The following day 30 µl of Quench buffer (0.2M Lysine, pH8.0) was added and allowed to react for 2 hours at room temperature.

For storage, 350 µl Stabilising solution (1 % (w/v) BSA in distilled water) was added to the sample and dialysed extensively (as before) against dialysis buffer (50 mM sodium phosphate, pH 6.8, 0.9 % (w/v) NaCl, 0.02 % (w/v) thimerosal). Horseradish peroxidase conjugated antibodies were stored at -20°C.

CHAPTER THREE - THE SELECTION AND USE OF PEPTIDE MIMICS OF PNEUMOCOCCAL POLYSACCHARIDE AS VACCINES

3.1 Chapter overview

The aim of this investigation was to select peptides that mimicked a protective epitope on the capsular polysaccharide *S. pneumoniae* using monoclonal antibodies. It was thought that vaccines composed of these peptides could circumvent the problems of the TI characteristics of pneumococcal capsular polysaccharide vaccines, since proteins are TD antigens and therefore, superior immunogens (see §1.4.3).

This chapter of the thesis describes the results of the selection of peptide mimics and their use as vaccines against pneumococcal disease. First the results of virulence testing four different serotypes of *S. pneumoniae* (4, 6B, 9V and 18C) in mice are described. The results of these assays determined the appropriate challenge dose for immunisation investigations. If the dose was too high, the numbers of bacteria could overwhelm the animal and it may die before any differences between the experimental and control groups could be observed. If the dose was too low the bacteria maybe cleared by innate immune responses and the effects of immunisation and the presence of anti-capsular antibodies would be irrelevant.

Subsequently the survival of mice passively immunised with several monoclonal antibodies (mAbs) raised against one of the pneumococcal serotypes (4, 6B, 9V, 18C) prior to challenge is described. This was to determine the best antibody to use for identifying pneumococcal mimics. If the antibodies were shown to protect mice from developing pneumococcal disease, they were thought to bind to a protective epitope, of which a mimic could elicit a protective immune response as a vaccine. Three mAbs, 206,F-5, Db3G9 and 6b53 were used to screen phage-displayed peptide libraries. The peptide mimics of different pneumococcal serotypes, 9V, 6B and 18C, which were selected by these mAbs and their use as vaccines against pneumococcal disease are described in part A, B and C of this chapter, respectively.

3.2 Bacterial virulence tests

Mice were infected intraperitoneally (i.p.) with 100 µl or intranasally with 50 µl containing an estimated dose of 1 x 10^5 - 1 x 10^7 cfu passaged bacteria (see §2.2.1). The animals were frequently monitored following infection. The characteristic signs of disease were scored

according to the scheme outlined in §2.2.1. Infected animals moved progressively from exhibiting signs of piloerection, to a hunched appearance, lethargy and culminated in a moribund state prior to death. Any animal judged to have become severely lethargic (++) was culled.

3.2.1 Serotype 4

A dose of 1 x 10^6 cfu serotype 4 pneumococci was shown to cause significant mortality when administered through the intraperitoneal route. As shown in Figure 3.1a, no mice (0/5) survived an intraperitoneal challenge of 1 x 10^6 cfu serotype 4 pneumococci. All the mice (5/5) died when the dose was increased to 3 x 10^6 cfu. All the deaths occurred 24 - 72 hours after infection and as the doses of around 1 - 3 x 10^6 cfu appeared more reproducible it was concluded that the lower dose of 1 x 10^6 cfu should be used in future experiments.

The virulence of the type 4 pneumococci was significantly decreased by inoculation through the intranasal route. A dose of 1 x 10^7 cfu serotype 4 pneumococci was shown to cause significant mortality when administered intranasally. Forty percent of mice (2/5) survived an intranasal challenge of 1 x 10^7 cfu serotype 4 pneumococci. However, when the dose was increased to 1 x 10^8 cfu all the mice challenged were dead within 4 hours.

As shown on Figure 3.1b, increasing intranasal infective dose correlated with increased killing. Fifty percent of challenged mice (3/6) survived a dose of 6 x 10^6 cfu, 60 % (3/5) survived a challenge of 3 x 10^6 cfu, 80 % (4/5) survived a challenge of 6 x 10^5 cfu and 100 % (3/3) survived when a dose of 5 x 10^5 cfu was used. These data implied that as the intranasal dose was halved, the overall survival rate increased by 10 %.

3.2.2 Serotype 6B

A general observation that was made during infections with serotype 6B pneumococci was that infected mice took longer to establish diseases of disease compared to the other serotypes investigated (see Table 3.1). Aaberge *et al* also reported a protracted course of disease with some strains in their investigations of the virulence of different serotypes of pneumococci in mice (Aaberge *et al.* 1995). Therefore, the health status of mice challenged with serotype 6B pneumococci was monitored for an extended time of 15 - 20 days, compared to 10 days for the other serotypes.

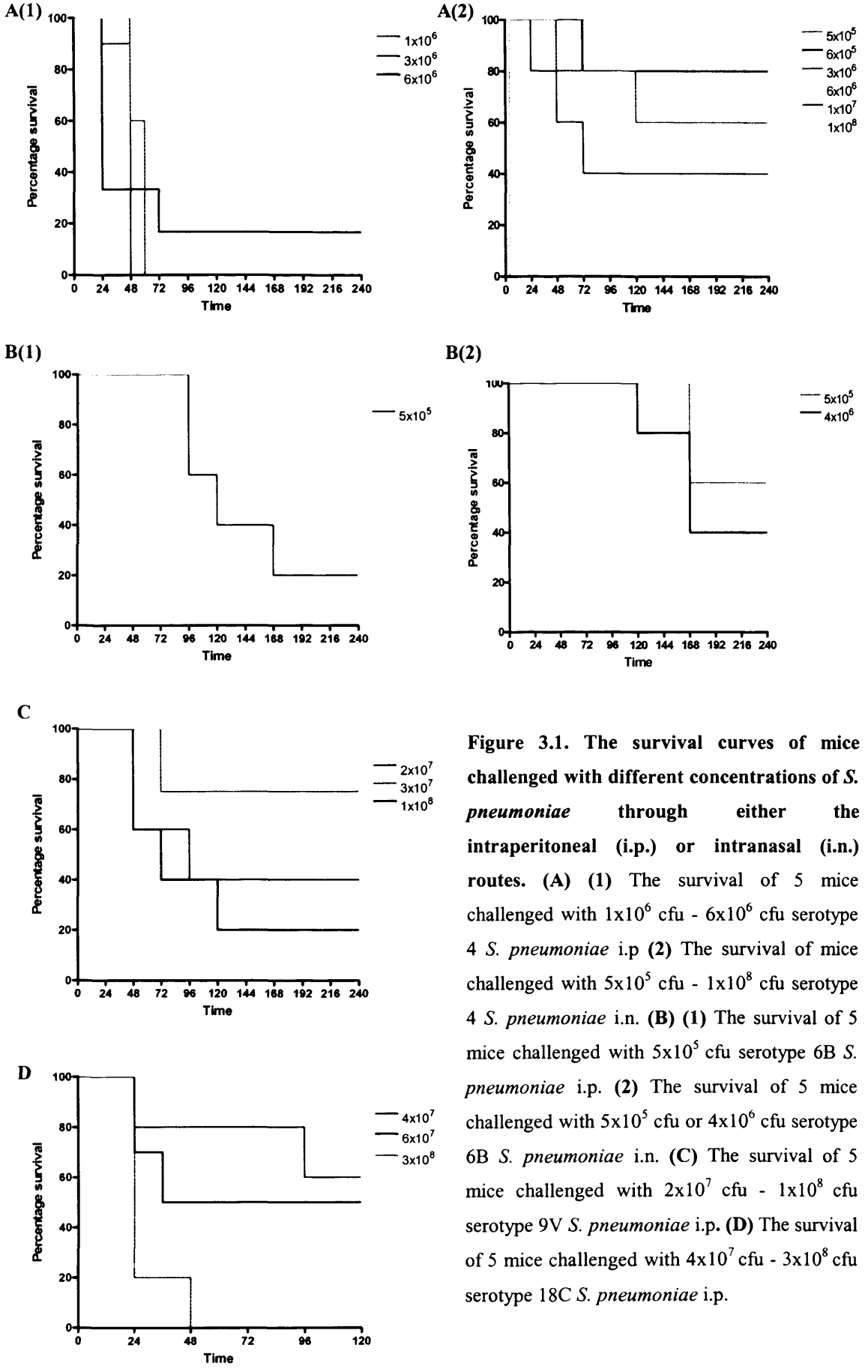

Figure 3.1. The survival curves of mice challenged with different concentrations of *S. pneumoniae* through either the intraperitoneal (i.p.) or intranasal (i.n.) routes. (A) (1) The survival of 5 mice challenged with $1x10^6$ cfu - $6x10^6$ cfu serotype 4 *S. pneumoniae* i.p **(2)** The survival of mice challenged with $5x10^5$ cfu - $1x10^8$ cfu serotype 4 *S. pneumoniae* i.n. **(B) (1)** The survival of 5 mice challenged with $5x10^5$ cfu serotype 6B *S. pneumoniae* i.p. **(2)** The survival of 5 mice challenged with $5x10^5$ cfu or $4x10^6$ cfu serotype 6B *S. pneumoniae* i.n. **(C)** The survival of 5 mice challenged with $2x10^7$ cfu - $1x10^8$ cfu serotype 9V *S. pneumoniae* i.p. **(D)** The survival of 5 mice challenged with $4x10^7$ cfu - $3x10^8$ cfu serotype 18C *S. pneumoniae* i.p.

A dose of 5 x 10^5 cfu serotype 6B pneumococci was shown to cause significant mortality when administered through the intraperitoneal route. As shown in Figure 3.1a, only 20 % of challenged mice (1/5) survived an intraperitoneal dose of 5 x 10^5 cfu serotype 6B pneumococci.

As with serotype 4, the virulence of serotype 6B pneumococci was decreased by intranasal inoculation. The same dose of 5 x 10^5 cfu led to a survival rate of 60 % (3/5). When the dose was increased 8 - fold, to 4 x 10^6 cfu, the percentage survival decreased to 40 % (2/5). However, to establish this level of disease took 48 hours longer than when infection occurred through the intraperitoneal route (see Figure 3.1). A dose of 5 x 10^5 cfu i.p. was considered appropriate for future investigations.

3.2.3 Serotype 9V

Forty percent of infected mice (2/5) survived an intraperitoneal (i.p.) challenge of 2 x 10^7 cfu serotype 9V pneumococci. This is shown in Figure 3.1. When the dose was increased to 1 x 10^8 cfu the survival decreased to 20 % (1/5).

In conclusion, a higher dose (1 x 10^8 cfu) of serotype 9V pneumococci was needed to produce the same level of disease in mice challenged intraperitoneally with serotypes 4 (1 x 10^6 cfu) and 6B pneumococci (5 x 10^5 cfu).

3.2.4 Serotype 18C

A dose of 6 x 10^7 cfu serotype 18C pneumococci was shown to cause significant mortality when administered i.p. Fifty percent of infected mice (5/10) survived an intraperitoneal challenge of 6 x 10^7 cfu. This is shown in Figure 3.1. When the dose was increased to 3 x 10^8 cfu no mice survived beyond 48 hours (0/5). This was a large number of bacteria and it was possible these effects were due to toxicity rather than the infective process. Therefore, doses this high were considered inappropriate for future investigations.

3.2.5 Conclusion

An intraperitoneal dose of 5 x 10^5 - 1 x 10^8 cfu pneumococci 100 μl^{-1} was found to be necessary to induce diseases of pneumococcal disease. Although the doses varied, it was aimed to determine an appropriate dose that took approximately 48 hours to establish

infection. Table 3.1 showed that the doses used for each serotype took approximately two days to kill at least 50 % of mice.

Table 3.1 The infective intraperitoneal dose of different pneumococcal serotypes needed to cause significant mortality in groups of 5 mice. The table also shows the median end point recorded for the mice that died (referred to here as TD50). The Lab reference number refers to the location of the bacterial stocks in Lab 227, Department of III, University of Leicester, UK, which are stored in 10 % (v/v) glycerol at -70°C.

Serotype (strain)[a]	*Lab reference Number*	*Infective dose (cfu 100 μl^{-1})*	*Percentage mice killed*	*TD_{50}*[c]
4 *(TIGR4)*	P202	1 x 10^6cfu	100 % (5/5)	2.50
6B *(23477)*	P164	5 x 10^5cfu	80 % (4/5)	4.08
9V *(907/88)*	P187	1 x 10^8cfu	80 % (4/5)	2.00
18C *(NCTC11905)*	P233	6 x 10^7cfu	50 % (5/10)	1.00

[a] see Table 2.1 for more details

[c] The time (days) where 50 % of the total mice killed were dead (time to death 50, TD50) as calculated from the survival curve.

3.3 Identifying protective antibodies

3.3.1 To recapitulate

The monoclonal antibodies (mAbs) used in this study had been previously described ((Kolberg and Jones 1998; Baxendale *et al.* 2000)). They included two antibodies raised to serotype 9V *S. pneumoniae* (one IgA and one IgG1 (6bC3 and 206,F-5[a])), two antibodies raised to serotype 4 (both IgG2 (CbB2 and Db4D7)), two raised to serotype 6B (both IgG2 (DM5 and Db3G9)) and one raised to serotype 18C (IgG3 (6b53)). The concentrations and isotypes were determined as described in §2.4.2. A summary of the mAb characteristics is shown in Table 3.2.

Mice were passively immunised with the monoclonal antibody 24 hours and 2 hours prior to challenge (as described in §2.7). This was to examine the ability of the mAbs directed against the capsular polysaccharide of serotypes 4, 6B, 9V and 18C (described above) to protect against pneumococcal infection of that serotype. Statistical differences between the

[a] Kolberg. All other mAbs were acquired from H. Baxendale.

survival curves obtained from vaccinated mice and control mice was determined by Kaplan-Meier survival analysis and is shown as a P value on Table 3.2. The mAbs shown to protect against the development of pneumococcal disease are shown in boldface.

The disease scores were determined as described previously (§2.2.1). Briefly, at the end of each 24 hours the disease of the mouse was assessed and a score was calculated based on a schedule described previously (Morton and Griffiths 1985). The sum of the scores from each day formed the cumulative disease score. These data are presented in the following pages.

3.3.2 mAbs CbB2 and Db4D7 (Serotype 4)

Mice were passively immunised with two human IgG2 antibodies (CbB2 or Db4D7) raised against serotype 4 pneumococcal polysaccharide. Mice immunised with CbB2 produced a significantly different ($P< 0.05$) survival curve to the sham vaccinated (control) mice. There was a 50 % (5/10) survival rate and a median survival time of 174 hours, compared to 0 % (10/10) and 24 hours respectively in the control mice. This is shown in Figure 3.2a. The results of the disease scores (shown in Figure 3.2b) also showed that CbB2 protected mice from developing type 4 pneumococcal disease by passive immunisation. The median cumulative disease score from mice that received CbB2 was 3.0, which was lower than the control mice (4.0). The range of diseases suffered also decreased, although these changes were not shown to be statistically different from the control when analysed by ANOVA and T-test.

The survival curve of mice vaccinated with Db4D7 was not significantly different ($P> 0.05$) from the control and produced a similar median survival time of 24 hours and average (mean) cumulative disease score of 4.0. These differences between the activities of these mAbs maybe accounted to the difference in antibody concentrations administered. Mice vaccinated with CbB2 received a total of 1000 ng of antibody whereas mice vaccinated with Db7D4 received 400 ng.

Table 3.2 A summary of the results from passive vaccination with monoclonal antibodies raised to different pneumococcal polysaccharide serotypes. The concentrations of the mAbs were determined from a standard curve produced by ELISA (see §2.4.2). The concentrations used to inoculate the mice varied, as the concentrations were unknown at the time of vaccination. The antibodies that significantly protected mice against pneumococcal disease (when the survival curve of challenged mice is compared to that of the sham-vaccinated mice ($P < 0.05$)) are shown in *boldface*.

Antibody reference	Serotype	Isotype	Concentration	*Total mAb administered*	*P value*
CbB2	**4**	**IgG2**	**10 μg ml^{-1}**	**1000 ng**	**(P<0.05)**
Db7D4	4	IgG2	4 μg ml^{-1}	400 ng	(P>0.05)
Db3G9	**6B**	**IgG2**	**3 μg ml^{-1}**	**300 ng**	**(P<0.05)**
DM5	**6B**	**IgG2**	**1 μg ml^{-1}**	**100 ng**	**(P<0.05)**
206,F-5	**9V**	**IgG1**	**4 μg ml^{-1}**	**400 ng**	**(P<0.05)**
6bC3	9V	IgA	10 ng ml^{-1}	1 ng	(P>0.05)
6b53	18C	IgG3	10 μg ml^{-1}	1000 ng	(P>0.05)

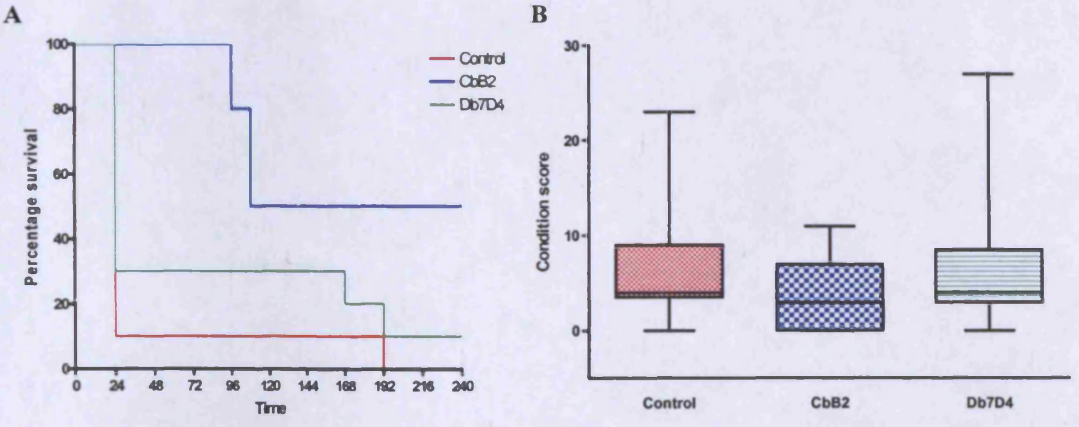

Figure 3.2 (A) shows the survival curve of 10 mice passively vaccinated against serotype 4 pneumococci with either mAb CbB2 or Db7D4 compared to 10 sham-vaccinated control mice when challenged with 1x10^{6}cfu. **(B)** shows the box-plot of disease scores accrued after challenge with serotype 4 *S. pneumoniae* in a group of 10 mice primed with an anti-type 4 human monoclonal antibody (6b53) compared to 10 sham-vaccinated mice. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

3.3.3 mAbs DM5 and Db3G9 (Serotype 6B)

Immunisation with both of the two human serotype 6B mAbs, DM5 and Db3G9 (IgG2), significantly ($P < 0.05$) extended the survival of mice compared to the sham-vaccinated mice. Figure 3.3 showed that 100 % (10/10) of the mice passively immunised with DM5 or Db3G9 survived till the end of the experiment, but only 10 % (1/10) of the sham-vaccinated mice survived the same period following infection.

Passive vaccination with DM5 or Db3G9 also protected mice from developing diseases of pneumococcal disease. These results (shown in Figure 3.3b) showed that mice vaccinated with DM5 or Db3G9 produced a median cumulative disease score of 0.0 (no diseases observed), which was significantly lower ($P < 0.05$) than the control mice (7.0) when analysed by ANOVA (a T-test could not be performed as the data for DM5 and Db3G9 is one repeated value).

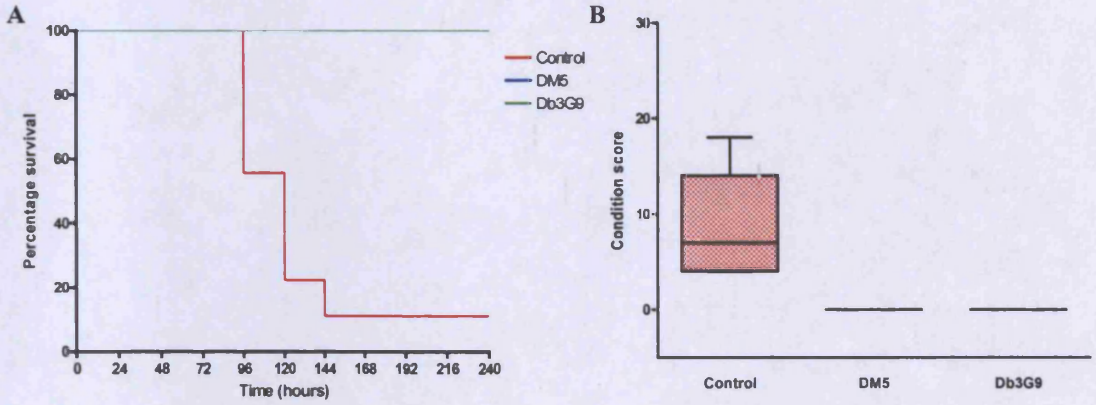

Figure 3.3 (A) shows the survival curve of 10 mice passively vaccinated against serotype 6B pneumococci with either mAb DM5 or Db3G9 compared to 10 sham-vaccinated control mice when challenged with $5x10^5$cfu. Since both DM5 and Db3G9 demonstrated the same survival rate the graph lines are superimposed **(B)** shows the box-plot of disease scores accrued after challenge with serotype 6B *S. pneumoniae* in a group of 10 mice primed with an anti-type 6B human monoclonal antibody (6b53) compared to 10 sham-vaccinated mice. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

The results obtained were the same for both anti-6B mAbs despite the mice receiving different concentrations of mAb. Mice immunised with DM5 received a total of 100 ng of antibody whereas mice vaccinated with Db3G9 received 300 ng.

3.3.4 mAbs 206,F-5 and 6bC3 (Serotype 9V)

Mice were passively immunised with two anti-type 9V antibodies (206,F-5 or 6bC3). Those mice immunised with the murine mAb 206,F-5 (IgG1) received a total of 400 ng of antibody. This was shown to significantly protect against the development of serotype 9V pneumococcal disease ($P< 0.05$, Kaplan Meier survival analysis). There was a 60 % (6/10) survival rate and mean survival time of 120 hours, compared to 20 % (2/10) and 74 hours in the control mice (the mean survival time is used here instead of the median as this could not be calculated a single value (all mice died at the same time)). The disease scores for mice passively vaccinated with 206, F5 were unavailable for recording in this thesis.

An IgA mAb (6bC3) raised against serotype 9V pneumococci was also tested for its ability to passively protect against the development of pneumococcal disease. Since mucosal immunity is important in the host defence against pneumococci, it was thought that this mAb could be a valuable tool for this project. However, the survival curves for the control mice and vaccinated mice were not shown to be significantly different ($P> 0.05$) and the median survival time and percentage survival for both groups were equal at 48 hours and 20 % (2/10) respectively. Mice received 1 ng of antibody.

However, the results of the disease scores (shown in Figure 3.4b) show that passive immunisation with 6bC3 produced a lower median cumulative disease score than the control mice (7.0 and 9.5 respectively). The number of mice that suffered diseases also decreased, although this was not a significant difference ($P> 0.05$, as established by a T-test). These results may have been expected, since primarily, the IgG class activates the classical complement pathway whereas IgA does not. This could mean that immunisation with 206,F-5 stimulated the involvement of complement proteins but those mice immunised with 6b53 did not. In effect there is increased killing with 206,F-5.

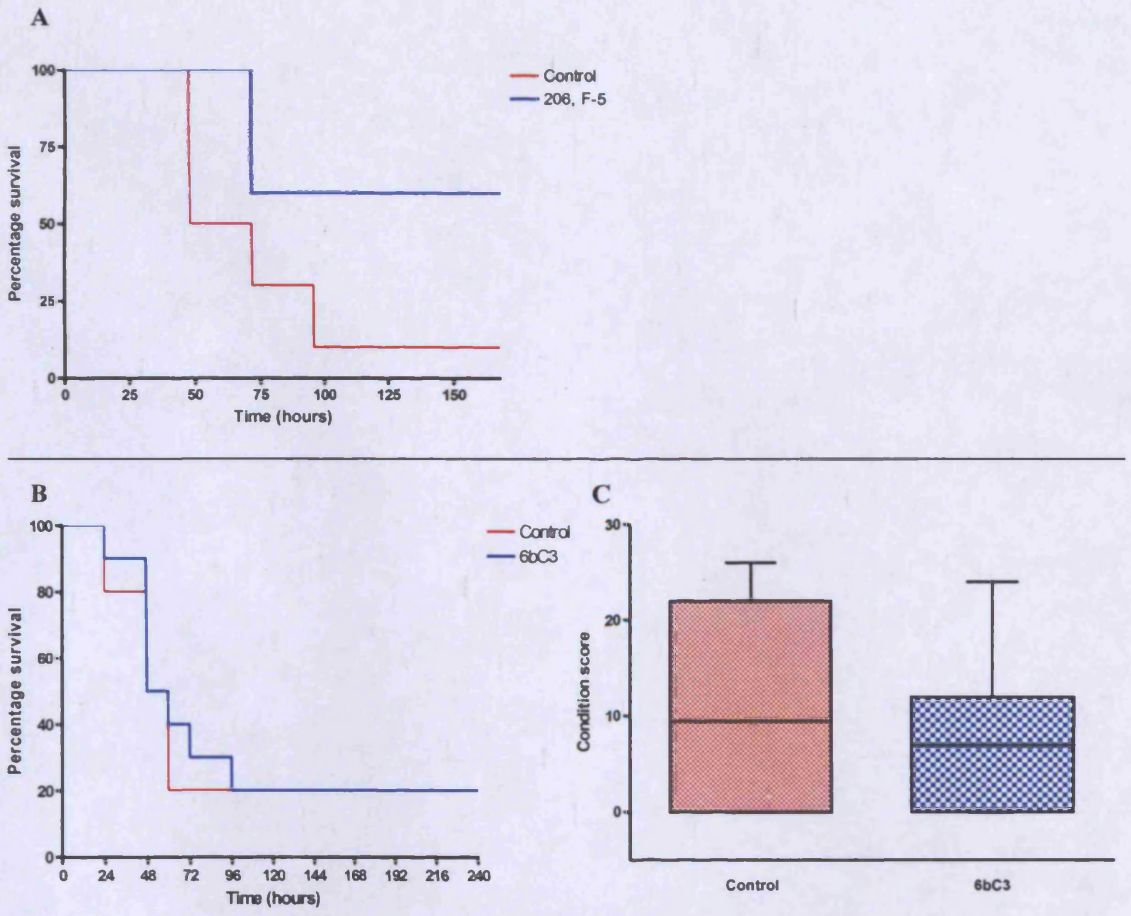

Figure 3.4 (A) shows the survival curve of 10 mice passively vaccinated against serotype 9V pneumococci with mAb 206,F-5 (IgG1) compared to 10 sham-vaccinated control mice when challenged with a dose of $1x10^7$cfu i.p. **(B)** shows the survival curve of 10 mice passively vaccinated against serotype 9V pneumococci with mAb 6bC3 (IgA) compared to 10 sham-vaccinated control mice when challenged with a dose of $3x10^5$cfu i.p. **(C)** the box-plot of disease scores accrued after challenge with serotype 9VC *S. pneumoniae* in mice passively vaccinated with 6bC3 compared to sham-vaccinated mice. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

3.3.5 mAb 6b53 (Serotype 18C)

The human mAb 6b53 (IgG3) was not shown to produce a significantly different survival curve from the control group ($P > 0.05$). Mice immunised with 6b53 received a total of 1000 ng of antibody. Immunisation with 6b53 produced an 80 % (8/10) survival rate and mean survival time of 204 hours, compared to 50 % (5/10) and 123 hours in the control mice, but this was not significantly different (the mean survival time is used here instead of the median since it could not be calculated from few time points) (see Figure 3.5a).

However, protection was implied when the cumulative disease scores were considered. Figure 3.5b showed that only one mouse vaccinated with 6b53 developed notable signs of pneumococcal disease. This resulted in a median cumulative score of 0. However, 9/10 of the sham immunised mice developed diseases, producing a median score of 19.5 (Figure 3.5b). These scores are statistically different ($P < 0.05$, T-test).

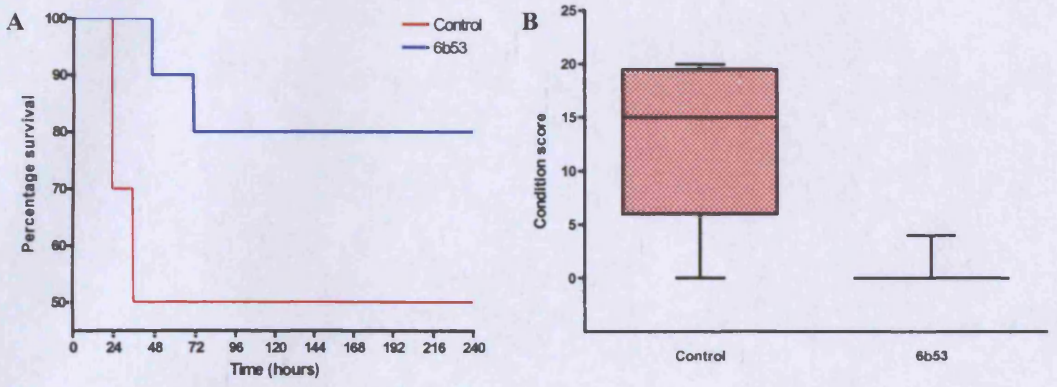

Figure 3.5 (A) shows the survival curve of 10 mice passively vaccinated against serotype 18C pneumococci with mAb 6b53 compared to 10 non-vaccinated mice when challenged with $6x10^7$cfu. **(B)** shows the box-plot of disease scores accrued after challenge with serotype 18C *S. pneumoniae* in a group of 10 mice primed with an anti-type 18C human monoclonal antibody (6b53) compared to 10 sham-vaccinated mice. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

3.4 Summary of results so far

The mAbs 206,F-5, Db3G9, DM5 were shown to protect mice against developing pneumococcal disease and were subsequently used to select peptides mimics of pneumococcal polysaccharide by screening phage libraries. Although CbB2 was shown to be protective against the development of serotype 4 pneumococcal disease, the quantity of mAb available was insufficient to begin selections. Therefore, because of time-restraints the mAb 6b53 (raised to serotype 18C) was used as an alternative.

3.4.1 Overview of the remaining chapter

Peptide mimics were identified for all three mAbs: the murine mAb 206,F-5 (these results are described in Part A), the human mAb Db3G9 (described in Part B) and the human mAb 6b53 (described in Part C). Colony immunoscreening, the primary method of identifying reactive phage clones, was performed on phage selected from three or four rounds of panning. A Phage ELISA confirmed these results and the sequences of the recombinant peptide was deduced and analysed following the methods described in Chapter 2. Mice were then immunised with three doses of the peptide. Serum samples were collected for analysis of the immune response at various time points throughout the schedule (see Table 2.7 for more details) and finally immunised mice were challenged with virulent pneumococci to determine whether immunisation with peptide mimics of pneumococcal polysaccharide protected mice from developing pneumococcal disease. Control mice were challenged at the same time as the experimental groups. The results of this challenge and the immune response to the peptide mimics are also described in the following sections.

When comparing the immunological properties of the peptide mimics it was important to note that the number of peptides conjugated to each carrier protein was unknown, and it was unlikely each peptide will be represented equally in their respective conjugate. A low number of peptides (displayed conjugated to the carrier protein) may have elicited a low-level antibody response, which was insufficient to confer protection upon challenge. A peptide displayed more abundantly may have generated a more antibodies, but it could be that few isotypes bound pneumococcal polysaccharide and conferred protection against pneumococcal disease. Consequently, peptides were assessed for their ability to protect against the development of pneumococcal disease (see §3.5 and §3.7), and also for their

ability to elicit an immune response that cross-reacted with *S. pneumoniae* capsular polysaccharide (§3.6 and §3.8).

Part A: The selection and use of peptide of type 9V pneumococcal polysaccharide selected using a murine mAb

3.5. Selection of peptide mimics to serotype 9V pneumococcal polysaccharide

3.5.1 Overview

The screening of the phage displayed peptide libraries with the murine mAb, 206,F-5, was performed by Carla LoPasso (University of Messina, Italy). However, some of her results were not made available and are therefore, not recorded in this thesis.

The monoclonal antibody 206,F-5 (of the isotype IgG1) had been used to screen three different peptide phage-displayed libraries displayed on the pVIII phage coat protein. The libraries displayed random sequences of either 12 amino acids (pVIII-12aa), 15 amino acids (pVIII-15aa) or 12 amino acids restrained by 2 cysteine residues at positions 5 and 9 (pVIII-12aa.Cys). Four positive phage clones were isolated from three rounds of panning, two from pVIII-12aa (designated MP7 and MP12) one from pVIII-12aa.Cys (MP55), and one from pVIII-15aa (MP58). Peptides were selected on the basis that they bound to 206,F-5 in colony immunoscreening (data not available) and ELISA, and binding was specifically competed by 9V polysaccharide.

3.5.2 Phage ELISA

Figure 3.6 shows the reactivity of the phage-displayed peptides to the mAb in phage ELISA (as described in §2.3). This technique gives a quantitative account of the number of recombinant phage present in the suspension. Since the phage coated the plate and the monoclonal was in abundance, the amount of bound monoclonal represented the concentration of reactive phage. As shown in Figure 3.6, the amount of antibody that bound to the recombinant phage was greater than to the wild-type phage (pC89). This showed that the monoclonal was binding to the recombinant peptide, as this was absent in the wild-type pC89 phage. However, in the case of the clones displaying the mimotopes MP55 and MP58, the concentration of phage had to be increased twenty-fold in order to achieve an absorbance comparable to that achieved by MP7 and MP12. In each case a

statistically greater ($P < 0.05$, T-Test) absorbance was recorded when the mAb was incubated on the plate compared to the control (when no mAb was used). This indicated that the secondary labelled antibody did not bind the phage.

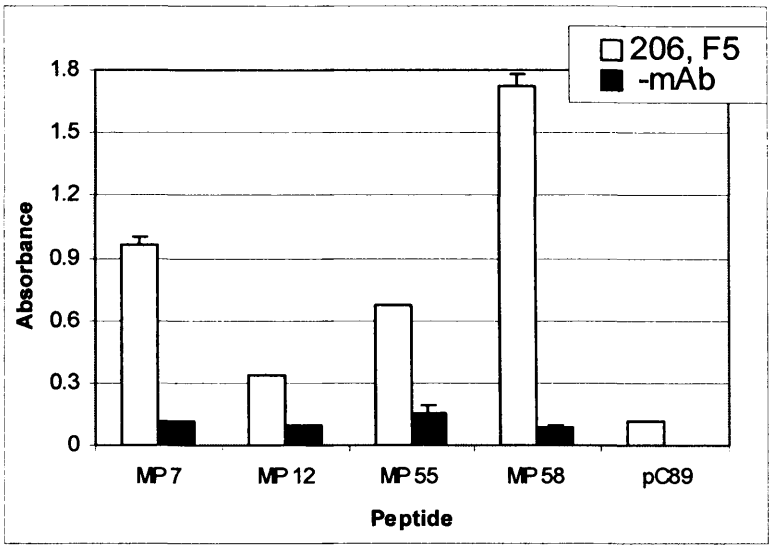

Figure 3.6. Reactivity of the selected phage clones with the monoclonal antibody 206,F-5 in PHAGE ELISA. MP55 and MP58 clones were twenty times concentrated. The absorbance in ELISA of the non-recombinant phage (pC89) to mAb 206,F-5 (□) and the absorbance recorded when no mAb was used in the ELISA to the recombinant phage (■) are also shown as negative controls. Absorbance was read at 405nm. N=2

3.5.3 Competition ELISA

The type 9V pneumococcal polysaccharide mimicry properties of the phage displayed peptides were confirmed by competition ELISA. The binding mAb 206,F-5 to three of the four phage clones that were selected could be inhibted by first incubating the mAb with 1 μg of serotype 9V pneumococcal polysaccharide. These data are shown in Figure 3.7. These results indicated that the peptide mimotope and native polysaccharide antigen occupied the same antibody binding site of 206,F-5, as preincubation with polysaccharide, inhibited the mAb binding to the peptide. The peptide was therefore a mimic of serotype 9V pneumococcal polysaccharide.

MP7 demonstrated the greatest inhibition to the binding of 206,F-5 with an average (mean) percentage inhibition of 79 %. The binding of 206,F-5 to MP55 was also inhibited (31 %). However, the binding to MP12 was only inhibited by 9 % and binding to MP58 was not

inhibited. This implied that the peptide mimotope MP7 may display a stronger likeness to serotype 9V polysaccharide than the other mimotopes. This was explored further by investigating the ability of the peptide mimotope MP7 to not only bind, but also compete for the binding of human polyclonal serum to serotype 9V pneumococcal polysaccharide. These results are discussed in §3.5.4.

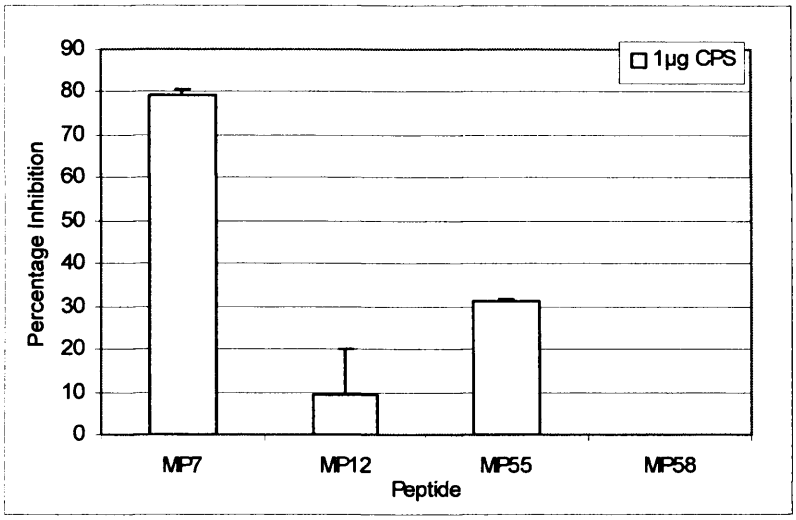

Figure 3.7. Competition ELISA. This assay shows whether pre-incubation with the mAb's native antigen (9V pneumococcal polysaccharide) can inhibit binding to the phage supernatant in PHAGE ELISA. This figure shows the inhibition of reactivity (□) recorded when 1 µg 9V pneumococcal polysaccharide was incubated with mAb 206,F-5 prior to ELISA with the plate coated with the peptide. N=2

3.5.4 The murine peptide mimic MP7 also binds human antibodies

MP7 was selected using a murine antibody (206,F-5), it was therefore, interesting to discover whether MP7 could function as a pneumococcal polysaccharide mimic vaccine for use in humans.

Serum was obtained from three volunteers vaccinated with either the Pneumovax pneumococcal polysaccharide vaccine (Cc and Dc) or Prevenar pneumococcal polysaccharide conjugate vaccine (6c). Each of the sera bound MP7. Binding was then inhibited by prior incubation with 9V polysaccharide; the morphologically different serotype 4 polysaccharide was used as a control. As can be seen in Figure 3.8, the binding

of MP7 by human serum could be inhibited as much as 27 % by serotype 9V polysaccharide. All inhibitions by serotype 9V polysaccharide were shown to be statistically different ($P < 0.05$) from the control (in a T-test). These results indicate that MP7 could possibly be used as a human vaccine against serotype 9V pneumococcal disease.

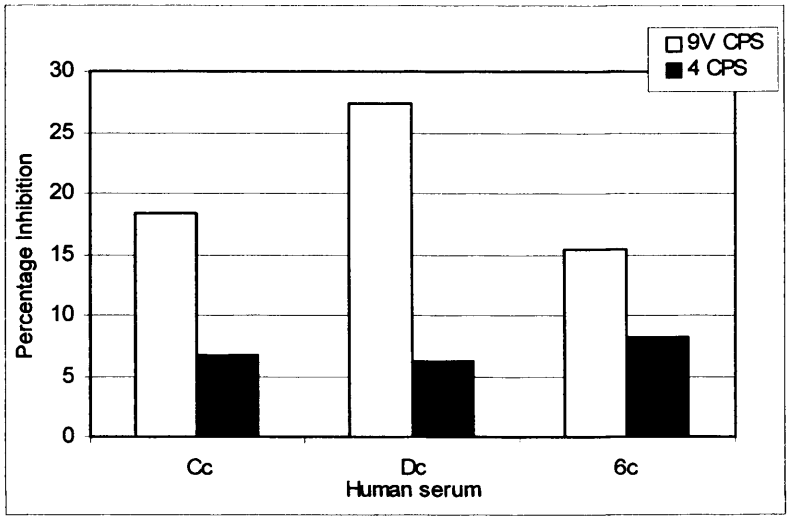

Figure 3.8. Inhibition of the binding of peptide MP7 and human serum by pre-absorption with 9V polysaccharide. Serum was obtained from human volunteers vaccinated with polysaccharide conjugate vaccine (Cc and Dc) or the polysaccharide vaccine Pneumovax (6C). The chart shows the absorbance readings determined by ELISA using human sera pre-adsorbed with 9V polysaccharide (□), or serotype 4 pneumococcal polysaccharide (■). N=1

3.5.5 The sequence of the serotype 9V peptide mimics

The four peptides described in §3.5.3 (MP7, MP12, MP55 and MP58) were sequenced. The sequences obtained are shown in Table 3.3.

N.B. As is standard for many researchers, peptide structures were written so that the amino group (as H_2N -) was at the left and the carboxyl group (as COOH) was at the right. Consequently, the left and right ends of the peptide are referred to as the N terminus (amino) and the C terminus (carboxyl), respectively (Atkins and Carey 2001).

Table 3.3 Sequences of peptides selected from phage-displayed peptide libraries using the murine mAb 206,F-5.

Clone	*Library*	*Sequence* [a]
MP7	pVIII-12aa	NH_2 AEGEF-KFHPKDIPYQVW-GDPAK COOH
MP12	pVIII-12aa	NH_2 AEGEF-HKKDFARGPGWS-GDPAK COOH
MP55	pVIII-12aa.Cys	NH_2 AEGEF-RQFECYTTTCVG-GDPAK COOH
MP58	pVIII-15aa	NH_2 AEGEF-RQGEEVYMWRDSMPA-GDPAK COOH

[a] The first five amino acids (AEGEF) and the last five (GDPAK) are constant in the library, as they belong to the pVIII coat protein sequence in the pC89 phagemid vector, and are therefore invariant in the random peptide libraries used for this study.

3.5.5.1 Analysis of the peptide sequences

3.5.5.1 The occurrence of certain residues is not random

A method used by Mertens *et al.* (2001) was applied to determine a ratio (frequency) of occurrence of each amino acid. First the expected frequencies of each residue were calculated based on the number of oligonucleotides that encoded each amino acid that were used to construct the libraries. If the observed frequency was the same as the expected frequency (a ratio of 1.0) then the presence of these residues in the sequence was a random event, since the library was constructed randomly. Any ratio >1.0 indicated an over-representation of that residue (Mertens *et al.* 2001). Figure 3.9 shows that the occurrence of each amino acid residue in the four 9V peptide mimics is not random. There appears to be a biased proportion of residues, as many residues had a ratio >1.0. Interestingly, all four aromatic amino acids [histidine (H), phenylalanine (F), tyrosine (Y) and tryptophan (W)] were over-represented, in particular phenylalanine and tryptophan. Some researchers do not regard histidine as an aromatic amino acid (Stryer 1995). However, previous work on peptide mimics (Hoess *et al.* 1993; Phalipon *et al.* 1997) and protein structure (Sternberg 1996) does includes histidine in this group and therefore this is the classification used in this thesis. This is discussed more in §5.4.1.1

MP7 contained 33 % aromatic amino acids, MP12 25 %, MP55 17 % and MP58 13 %. Additionally glycine (G), histidine (H), lysine (K), and glutamine (Q) are over represented in the 9V mimics, whereas A and S, and T and V were underrepresented

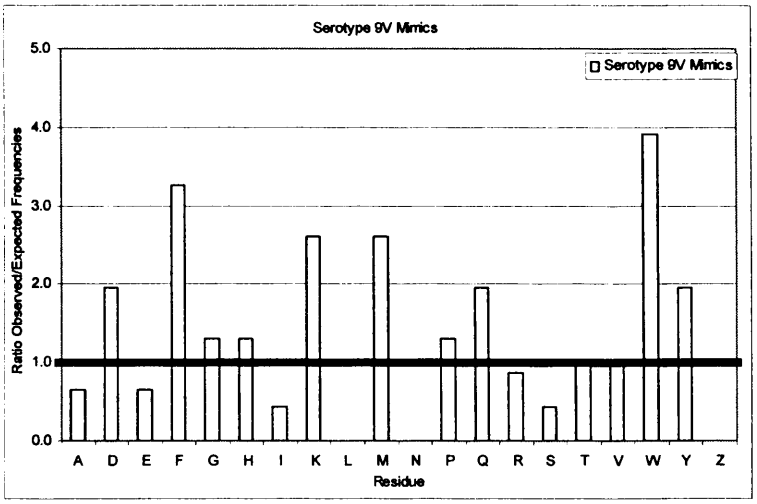

Figure 3.9. Occurrence frequencies of each residue in the peptides selected with mAb 206,F-5 from the pVIII-12aa, pVII-12aa.Cys and pVIII-15aa libraries (□). The vertical axis is the ratio between the observed frequency of each residue in the peptides and the theoretical frequency of each residue calculated based on the proportion of each codon present in the randomly generated oligonucleotides used to construct the libraries, each coding for a particular amino acid. A ratio above 1.0 indicates over-representation of a particular residue in the peptides. Cysteine has been excluded from this graph, as it is not a randomly generated residue. It is fixed in the sequences of the pVIII-12aa.Cys peptides and did not appear in the 6B mimics. The 'Z' residue corresponds to the stop codon, since all peptides are of the expected length, it is logical to observe no stop codons.

3.5.5.2 Sequence alignment

The sequences of the serotype 9V peptide mimics were aligned using the ClustalW program described by Thompson *et al.* (1994). This scored the identity of two peptides based on exact matches. Rarely do two aligned sequences share 100 % identity, even though the function of both proteins could be similar. Therefore, many researchers use a 30 - 40 % significant level (A. Stark, personal communication). Any two sequences that shared greater than this percentage identity were considered to be significantly similar. Therefore, since the serotype 9V pneumococcal polysaccharide mimics display a maximum of 25 % identity (Table 3.4) there is no consensus sequence.

Table 3.4 Alignment and consensus identity score of serotype 9V peptide mimics

Peptide1	*Peptide 2*	*Identity (%)*
MP7	MP12	25
MP55	MP58	16
MP7	MP58	8
MP12	MP55	8
MP12	MP58	8

```
MP7     KFHPKDIPYQV-W--  COOH
MP12    --HKKDFARGPGWS-  COOH
MP58    RQGEEVYMWRDSMPA  COOH
MP55    ---RQFECYTTTCVG  COOH
```

The four serotype 9V pneumococcal polysaccharide peptide mimics were arranged using the ClustalW version 1.82 program. Each peptide was placed based on its identity to the adjacent peptides. Colour codes: Small + hydrophobic (including aromatic -Y) residues (RED), Acidic residues (BLUE), Basic residues (MAGENTA), Hydroxyl + Amine + Basic - Q (GREEN).

3.6 Immunisation with peptide mimics of type 9V pneumococcal polysaccharide

3.6.1 To recapitulate

To determine if the peptide sequences described above could elicit potentially protective antibodies *in vivo*, corresponding peptides were synthesised, conjugated to KLH, and used to immunise mice. The immunisation schedules were shown in Table 2.7. The peptide MP58 bound to the filamentous phage (M13) was also used to immunise mice (see §2.6.1). Control mice received KLH alone. Serum samples were collected by tail bleeding at various time points throughout the schedule (see Table 2.7 for more details).

To determine whether immunisation with a peptide mimic of serotype 9V pneumococcal polysaccharide protected mice from developing pneumococcal disease, immunised mice were challenged with a dose of serotype 9V pneumococci previously shown to cause disease (see §3.2.3). Control mice were challenged at the same time as the experimental groups.

3.6.2 Immunisation with MP7 protected mice from serotype 9V pneumococcal disease

Following challenge with serotype 9V pneumococci, the survival curve of mice immunised with MP7 conjugated to KLH was significantly different ($P< 0.05$) from the control mice that received KLH alone. Kaplan-Meier survival analyses compared the survival curves from the 20 MP7-KLH immunised mice and the 19 control mice that were challenged. These are shown in Figure 3.13a. Sixty percent of mice (12/20) immunised with MP7-KLH survived an intraperitoneal challenge of 1×10^7 cfu serotype 9V pneumococci, compared to the control mice where 38 % (7/19) survived to 10 days after challenge with the same dose of bacteria. This implies that the sequence of MP7 possesses the ability to elicit a functional immune response against serotype 9V pneumococcal disease.

The challenged mice were also monitored for any signs of pneumococcal disease, which were scored by the scheme of Morton *et al.* (1985). The scoring system was shown in Table 2.3. These results (shown in Figure 3.13b) showed the median cumulative disease sign score from mice immunised with MP7-KLH was 2.0, which was lower than from mice that received MP12-KLH or KLH alone (9.0 and 6.0 respectively). However, this was not significantly different from the control mice (ANOVA, T-test).

These results indicate that immunisation with MP7 prevented more mice from developing signs of pneumococcal disease signs compared to those that received either the control or MP12 vaccines. However, if these mice began to develop signs of disease, the disease progressed at a similar rate for all the mice, irrespective of the vaccine they had received. This is indicated by the difference in the average disease sign scores between the groups, as some mice developed no signs of disease.

Mice receiving MP7-KLH, MP12-KLH or KLH alone (control) were immunised and challenged at the same time and received an identical challenge dose. The challenge results from of all these immunisations confirms the specificity of the anti-MP7 protective response, since the mice immunised with KLH alone or MP12-KLH were not protected against type 9V pneumococcal disease (see §3.6.3).

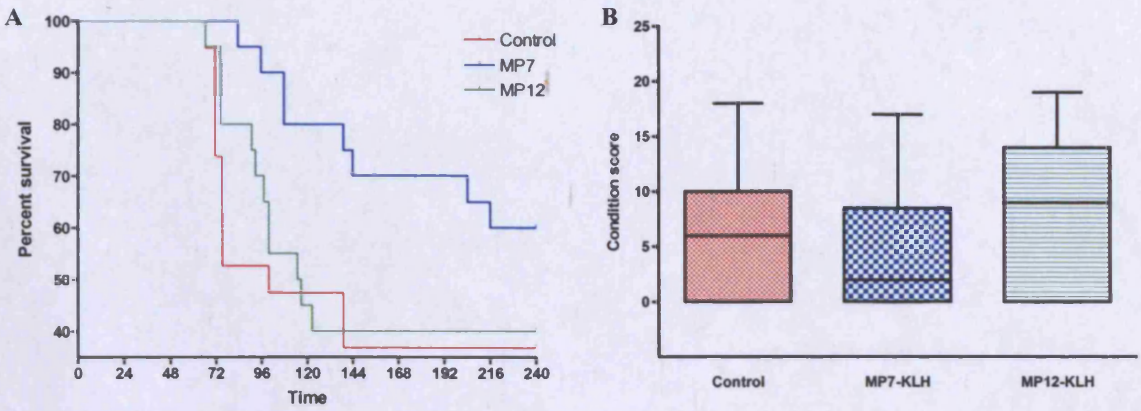

Figure 3.10. (A) Survival of mice challenged intraperitoneally with 1 x 10^7cfu 9V pneumococci. The graph shows the Kaplan-Meier survival curves of 20 mice immunised with peptide MP7-KLH (■) and 20 mice immunised with MP12-KLH (■) compared to 20 mice immunised with KLH alone (■) prior to challenge. **(B)** The Box-plot of disease sign scores of mice receiving 3 doses of with peptide MP7-KLH (■) and 20 mice immunised with MP12-KLH (■) compared to 20 mice immunised with KLH alone (■) followed by challenge with 1 x 10^7cfu type 9V pneumococcus i.p. The whisker bars represent the range of conditions scores, the borders of the box are the 25 % and 75 % percentiles and the bar dividing the box is the median.

3.6.3 Immunisation with MP12, MP55 or MP58 did not protect mice from serotype 9V pneumococcal disease

Immunisation with MP12-KLH, MP55-KLH, MP58-KLH or MP58-PH (phage bound peptide MP58) did not confer protection against the development of pneumococcal disease in mice. Figures 3.10, 3.11 and 3.13 showed that the survival following challenge with serotype 9V pneumococci of mice immunised with MP12-KLH, MP55-KLH, MP58-KLH and MP58-PH was not significantly different ($P > 0.05$) from the contol mice (immunised with KLH alone).

Of these three peptides, immunisation with peptide MP58 bound to phage (MP58-PH) produced the highest rate of survival in an immunised group of mice, although this was not statistically different from the control group (see Figure 3.11). Fifty percent (10/20) of mice immunised with MP58-PH survived an intraperitoneal challenge of 3 x 10^8 cfu serotype 9V pneumococci, compared to the control mice where 21 % (3/14) survived

challenge with the same dose of bacteria. The probability that these survival curves belonged to different distributions was close to the 95 % significance level (P=0.089).

The results of the disease sign scores (shown in Figure 3.12) showed that the median cumulative disease sign score from mice that received MP58-PH was 7.0, which was lower than from mice that received KLH alone (11.5). This is not a statistical different (ANOVA, T-test). However the mice immunised with MP58-PH received a high dose of pneumococci (3 x 10^8 cfu), therefore it could be possible the mice were overwhelmed by the infection and could not elicit a high enough response to reduce the signs of disease, irrespective of the effects of immunisation. To determine whether this was an important consideration in protection studies, the immunisation schedule for MP58-PH was repeated.

The second experimental group of MP58-PH immunised mice received a challenge dose of 3 x 10^7 cfu serotype 9V pneumococci. Forty seven percent (9/19) mice survived. However, 40 % (8/20) of the control mice also survived challenge (the survival curve is shown on Figure 3.14b). Kaplan-Meier survival curve analysis judged the difference to be non-significant (P> 0.05). There was also no significant difference (P> 0.05) between the disease sign scores acquired for the groups (see Figure 3.12b). The median cumulative disease sign score from mice that received MP58-PH was 4.0, which was similar to that from mice that received KLH alone (5.0).

Immunisation with three doses of MP58-KLH led to a survival rate of 47 % (9/19) (Figure 3.13). However, the significance of this survival rate was much less than for the MP58-PH immunised mice, as 50 % (10/20) of the control mice also survived. Kaplan-Meier survival analysis showed no significant difference (P> 0.05). The median cumulative disease sign score was lower in the peptide-immunised mice, at 4.0, compared to 8.0 for the control group; however, this was also not a significant difference (P> 0.05) (Figure 3.14).

Forty percent of mice (8/20) immunised with three doses of MP12-KLH survived an intraperitoneal challenge of 1 x 10^7 cfu serotype 9V pneumococci, compared to the control mice where 38 % (7/19) survived challenge with the same dose of bacteria. These data are shown on Figure 3.10 alongside the survival results for MP7-KLH. Not only were the survival curves for the MP12-KLH-immunised and control groups similar, but also the

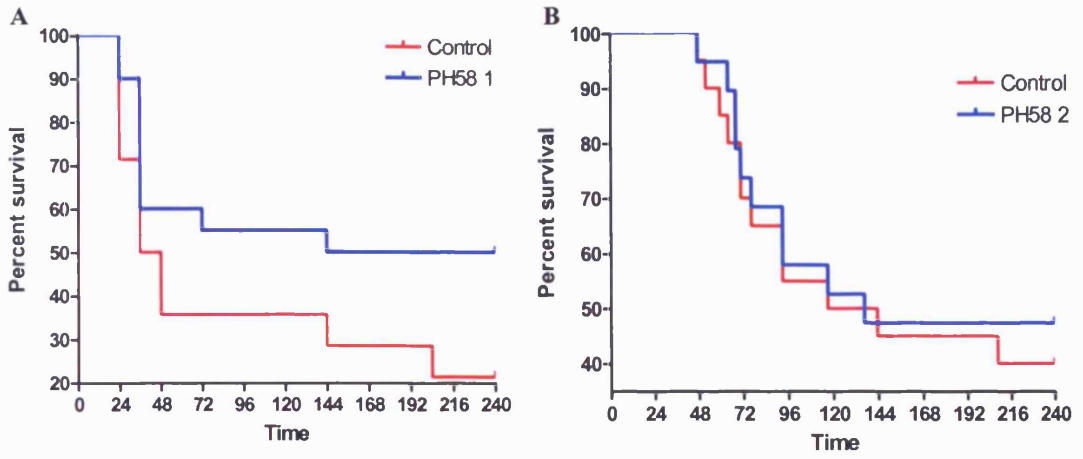

Figure 3.11. (A) Survival curve for mice immunised against type 9V pneumococcus with peptide 58 (■) bound to the phage compared to PBS immunised control mice (■) when challenged with $3x10^8$cfu type 9V pneumococcus i.p. **(B)** The survival of a second group of mice receiving 3 doses of phage borne-peptide 58 (■) followed by challenge with $3x10^7$cfu type 9V pneumococcus i.p. compared to PBS immunised control mice (■).

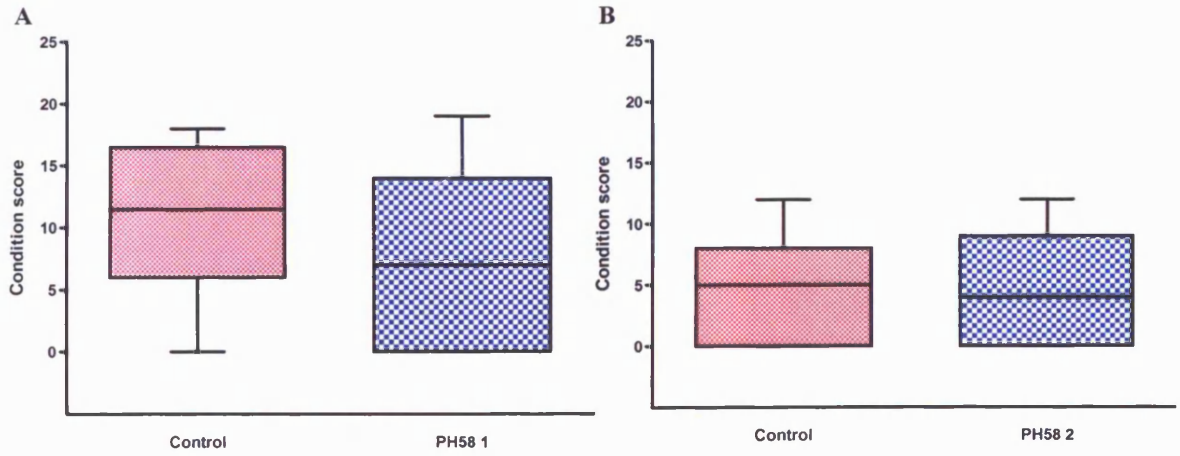

Figure 3.12. (A) Box-plot of disease sign scores for mice immunised against type 9V pneumococcus with peptide 58 (■) bound to the filamentous phage M13 compared to PBS immunised control mice (■) when challenged with $3x10^8$cfu type 9V pneumococcus i.p. **(B)** The Box-plot of disease sign scores of a second group of mice receiving 3 doses of phage borne-peptide 58 (■) followed by challenge with $3x10^7$cfu type 9V pneumococcus i.p. The whisker bars represent the range of conditions scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

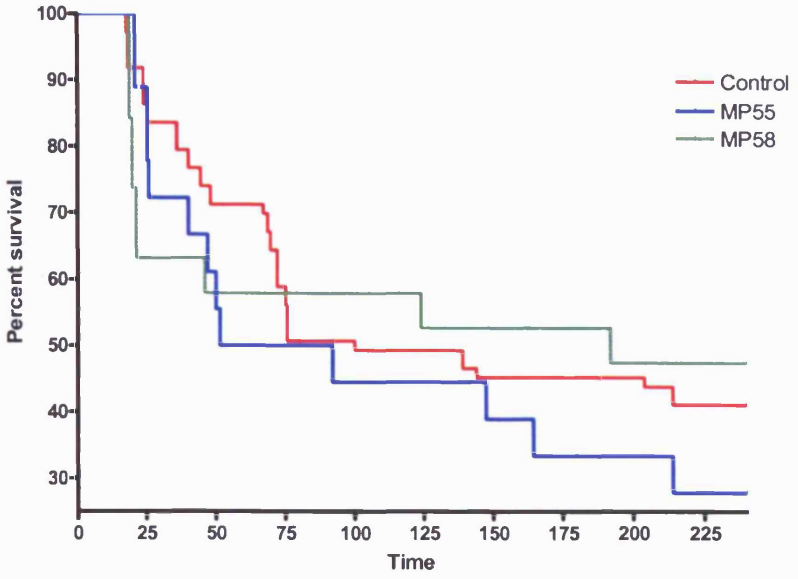

Figure 3.13. Survival of mice challenged intraperitoneally with $1x10^7$cfu 9V pneumococci. The graph shows the Kaplan-Meier survival curves of 20 mice immunised with peptide MP55-KLH (■) and 20 mice immunised with MP58-KLH (■) compared to 20 mice immunised with KLH alone (■) prior to challenge.

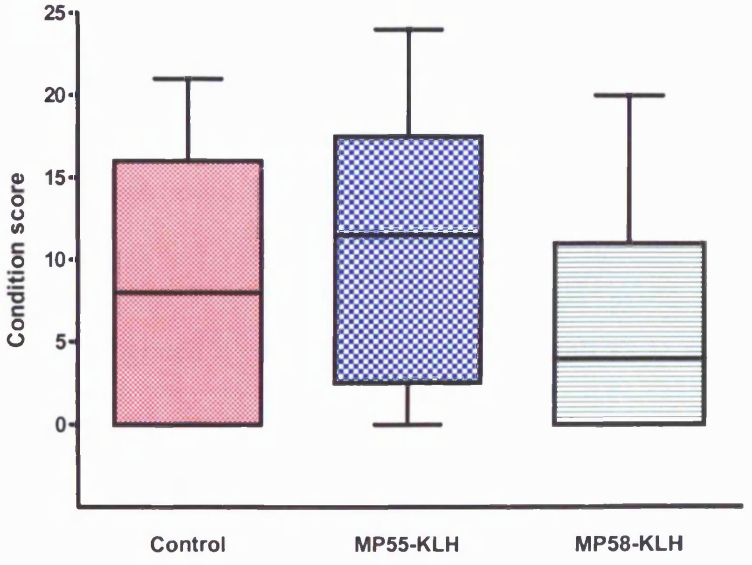

Figure 3.14. The Box-plot of disease sign scores of mice immunised with KLH alone (■), peptide MP55-KLH (■) or with MP58-KLH (■) when challenged with $1x10^7$cfu type 9V pneumococcus i.p. The whisker bars represent the range of conditions scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median.

median disease sign scores for mice that received MP12 were higher (9.0) than those of the control group (6.0) (Figure 3.10). Therefore, it was concluded that immunisation with MP12-KLH does not protect mice against developing serotype 9V pneumococcal disease.

Immunisation with three doses of MP55-KLH produced the lowest chance of survival after challenge (Figure 3.13). Upon challenge with 1 x 10^7 cfu 9V pneumococci only 28 % (5/18) of the mice that received MP55-KLH survived, compared to 50 % (10/20) in the control group. The median disease sign score for the group that were immunised with the peptide was also higher (11.5) than those of the mice that received KLH alone (8.0) (Figure 3.14). Therefore, it was concluded that immunisation with MP55-KLH does not protect mice against developing serotype 9V pneumococcal disease.

3.6.4 The antibody response in mice immunised with serotype 9V peptide mimics

3.6.4.1 Recapitulation

The development of specific anti-peptide and anti-polysaccharide antibodies in mouse serum following immunisation with peptide mimics was investigated by ELISA (see §2.8.1). Microtitre plates were coated with either the peptide conjugated to BSA (Bovine serum albumin) or serotype 9V capsular polysaccharide. The BSA conjugate was used to coat the plate as this eliminated the detection of any anti-KLH antibodies. A goat anti-IgG molecule conjugated to alkaline phosphatase was used to detect the bound antibody. The production of IgG indicated the stimulation of a secondary immune response. It also suggested the antibodies could mimic the mAb 206,F-5 (isotype IgG1) that selected the peptides.

3.6.4.2 Antibodies generated by control immunisation with KLH did not bind peptide or polysaccharide

Immunisation with KLH alone did not elicit a significant concentration of anti-peptide antibodies compared to the mice that were immunised with the peptide mimotopes conjugated to KLH. An ELISA (see §2.8.1) was performed to determine the IgG concentrations following the final boost immunisation of mice (TP77 or TP84 (see Table 2.7)). This time point was used as it was thought the highest antibody concentration would be found at this point of the schedule.

Figure 3.15 shows that the average (mean) serum anti-peptide IgG concentration from mice immunised with the peptide conjugates of MP7, MP12, MP55, and MP58, was >900 μg ml^{-1}. The average anti-MP7, MP12, MP55 or MP58 serum IgG concentration found in mice immunised with KLH alone was significantly lower ($P< 0.05$, T-test). Less than 2 μg ml^{-1} serum IgG from mice immunised with KLH alone bound to peptides MP12, MP55, or MP58. A greater quantity of control IgG serum bound to MP7 (13.9 μg ml^{-1}), but this is still significantly different ($P< 0.05$) from the sera of mice immunised with the MP7-KLH (916 μg ml^{-1}). This showed that the mice immunised with KLH alone did not develop antibodies that cross-reacted with any of the serotype 9V peptide mimotopes.

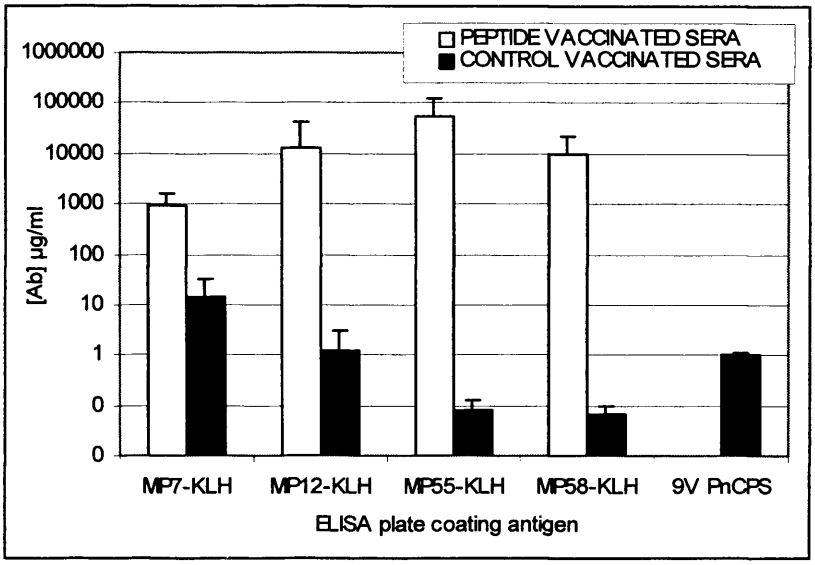

Figure 3.15. The average (mean) serum anti-peptide IgG concentration from mice immunised with three doses of the peptide mimics MP7 (n=4), MP12 (n=19), MP55 (n=25) and MP58 (n=29) conjugated to KLH compared to mice immunised with KLH alone (n=8). The chart shows the reactivity of serum antibodies to the coating antigen from mice immunised with peptide mimics of serotype 9V pneumococcal polysaccharide (□) (at time point TP77 or TP84 (depending on schedule, see §2.7)). Also shown is the reactivity of control serum to the coating antigen, taken from mice immunised with KLH alone (■). Data are from ELISA with microtitre plates coated with 5 μg ml^{-1} peptide-BSA or 5 μg ml^{-1} 9V capsular polysaccharide.

3.6.4.3 Antibodies generated from immunisation with MP58-PH cross-reacted with MP58-KLH, MP58-BSA, MP58-GST and serotype 9V pneumococcal polysaccharide

A phage ELISA plate, as shown in Figure 2.2, was coated with an anti-pIII antibody (anti-M13 phage coat protein III), which allowed the phage to be displayed in an orientation that increased the availability of the pVIII proteins to the mAb (see §1.5.3). However, we were not given permission to use of this antibody outside the lab in Sicily. Therefore, to detect the titre of anti-peptide IgG in immunised mouse serum, separate microtitre plates were coated with MP58 conjugated to KLH, BSA (Bovine serum albumin), or GST. Plates were also coated with serotype 9V capsular polysaccharide to detect whether anti-PnCPS antibodies had been generated in mice immunised with the peptide mimics. A goat anti-IgG molecule conjugated to alkaline phosphatase was used to detect the bound antibody (as before) and the titre was defined as the number of times higher than the absorbance when no serum was used.

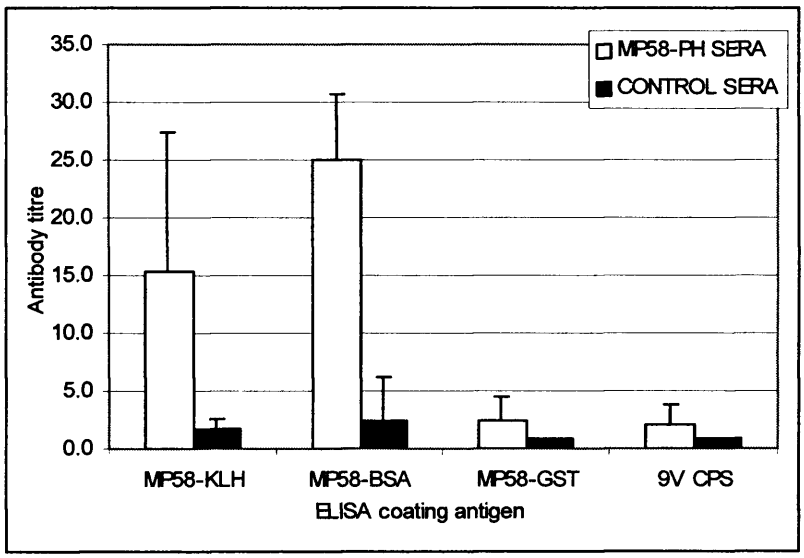

Figure 3.16. The anti-peptide IgG titre from mice immunised with the phage bound peptide mimic MP58 as determined by ELISA. Microtitre plates were coated with MP58 conjugated to KLH, BSA, or GST. Plates were also coated with serotype 9V capsular polysaccharide to detect the presence of anti-PnCPS antibodies. The titre was defined as the number of times higher than the negative control (no sera). The chart shows the ability of antibodies (pooled serum (n=20) generated from immunisation with MP58-PH to bind to MP58-KLH, MP58-BSA, MP58-GST and to serotype 9V pneumococcal polysaccharide (□). The titres recorded were significantly higher ($P<0.05$) than those achieved from control mice that received KLH alone (n=10) (■).

Figure 3.16 shows that antibodies generated against MP58 displayed on phage (MP58-PH) bound to MP58-KLH, MP58-BSA, MP58-GST and to serotype 9V pneumococcal polysaccharide in ELISA. The control serum was obtained from mice immunised with PBS alone (as no wild-type phage was available). A one-tailed T test showed that the average (mean) antibody titres to MP58-KLH, MP58-BSA, MP58-GST and to serotype 9V pneumococcal polysaccharide generated from MP58-PH immunised mice (n=20) were significantly higher ($P< 0.05$) than those achieved from the control serum (n=10).

The titre of the anti-PnCPS IgG antibody response is significantly less ($P< 0.05$) than the anti-peptide IgG titre. On average (mean n=20) the anti-PnCPS IgG titre represents only 8.4 % of the anti-MP58-BSA response. This will be discussed further in §5.2.6.

3.6.4.4 Antibodies generated from immunisation with MP7, MP12 or MP58 bound to serotype 9V pneumococcal polysaccharide

For each mouse, the concentration of IgG in the pre-immune serum was used to determine the background absorbance. This value was subtracted from the concentration obtained at the other time points to indicate any increase in antibody levels after immunisation. The serum collected from mice immunised with KLH alone was used as a negative control; these results were discussed in §3.6.4.2. Only those mice that demonstrated an antibody level greater than zero after subtraction of the background level were used to determine the average (mean) IgG concentration. The standard deviation for each time point (see Figure 3.17) was also determined.

The anti-MP7 and anti-serotype 9V capsular polysaccharide (anti-PnCPS) specific IgG concentrations in mice were significant boosted ($P< 0.05$, repeated measures ANOVA) following the administration of a second and third dose and MP7-KLH (Figure 3.17a). Thirteen mice from the 30 immunised with MP7-KLH were shown to elicit an anti-peptide immune response that was higher than in the pre-immune sera. The average IgG concentration was 2.1 $\mu g\ ml^{-1}$ 42 days after the first dose of peptide, which rose ten - fold 14 days after the second dose to 23.3 $\mu g\ ml^{-1}$. Fourteen days after the final boost the average IgG concentration was nearly 300 - fold higher than at day 42, at 603.3 $\mu g\ ml^{-1}$. These data are shown in Figure 3.17a

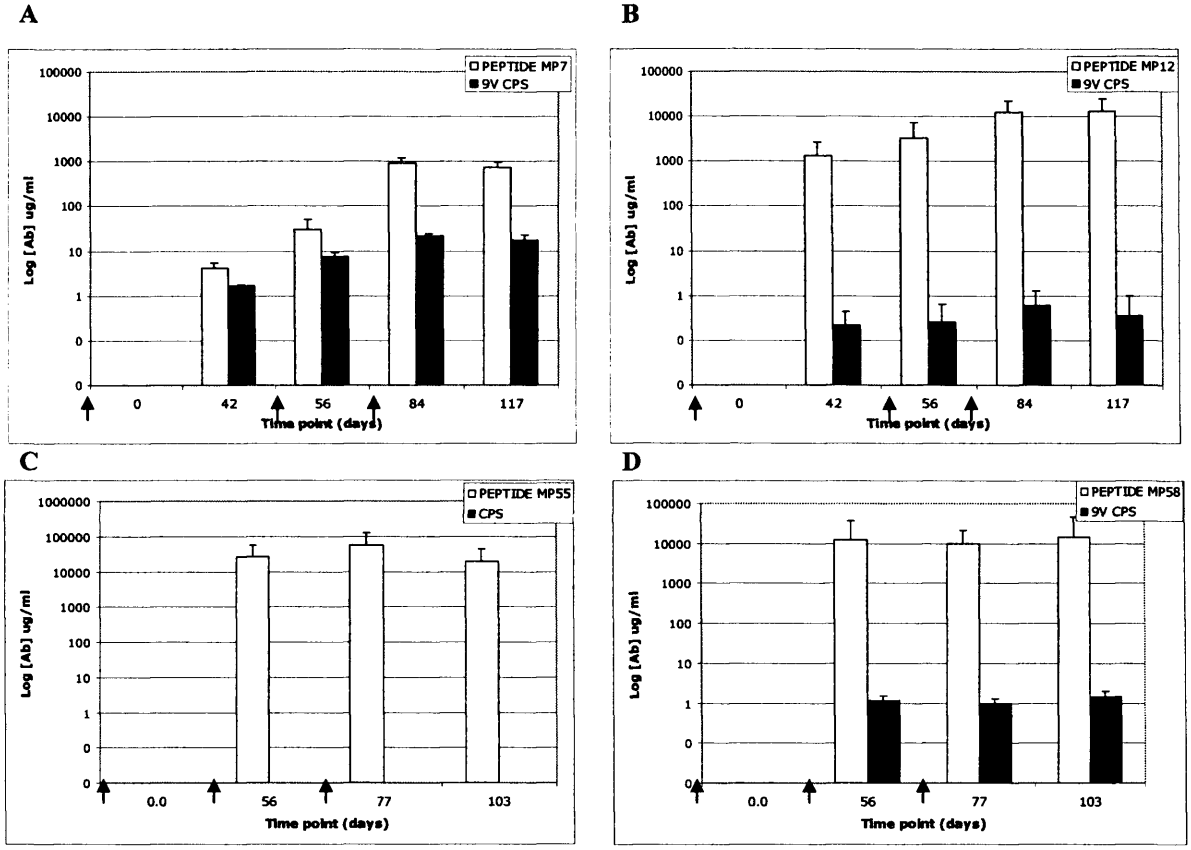

Figure 3.17. The humoral IgG response of mice immunised with peptide conjugates MP7-KLH, MP12-KLH, MP55-KLH, or MP58-KLH. **(A)** shows the mean (n=13) anti-MP7 response (□) and the mean (n=3) anti-9V capsular polysaccharide response (■) from mice immunised with MP7-KLH. **(B)** shows the mean (n=20) anti-MP12 response (□) and the mean (n=5) anti-9V capsular polysaccharide response (■) from mice immunised with MP12-KLH. **(C)** shows the mean (n=26) anti-MP55 response (□) from mice immunised with MP55-KLH. No anti-9V capsular polysaccharide IgG antibodies were detected by ELISA (■). **(D)** shows the mean (n=29) anti-MP58 response (□) and the mean (n=5) anti-9V capsular polysaccharide response (■) from mice immunised with MP58-KLH. Primary and booster doses are marked on the graphs by arrows (see §2.7 for more details).

The IgG concentrations were determined by ELISA. Microtitre plates were coated with 5 μg ml^{-1} the BSA conjugated peptide or 5 μg ml^{-1} 9V capsular polysaccharide, 100 μl per well. The concentration of antibody at time point 0 was used to determine the background level. This value was subtracted from the concentrations obtained from the other time points to indicate any increase in antibody levels after immunisation. Only those mice that demonstrated an increase in antibody levels were used to determine the mean IgG concentration and the error bars represent the standard deviation for each time point.

The highest concentration of anti-MP7 IgG achieved by a single mouse was 1502.3 μg ml^{-1}. The anti-PnCPS IgG concentration from the same mouse was 19.1 μg ml^{-1}; which is 1.3 % of the anti-peptide IgG. Another mouse that responded well to immunisation produced 614.5 μg ml^{-1} anti-MP7 IgG, the anti-PnCPS IgG concentration was 23.5 μg ml^{-1} (3.8%). Both these mice survived challenge.

Mice immunised with MP12-KLH, MP55-KLH or MP58-KLH produced a higher concentration of anti-peptide antibody after two doses of antigen (TP56) than mice immunised with three doses of MP7-KLH. At this time point, the highest average (mean) anti-peptide IgG concentration recorded was 12.45 mg ml^{-1} from mice immunised with MP58-KLH (n=29), mice immunised with MP55-KLH (n=26) elicited 25.87 mg ml^{-1} IgG and those that received MP12-KLH (n=20) produced 3 mg ml^{-1} anti-peptide IgG. The lowest of which, MP12-KLH, was still 1500-fold higher than in mice immunised with MP7-KLH. Blood samples prior to TP56 for mice immunised with MP55-KLH and MP58-KLH were not collected. However, at TP42 (prior to the first boost dose of MP12-KLH) the mean anti-peptide response was already 600 - fold higher (1338 μg ml^{-1}) than when mice were immunised with MP7-KLH.

Despite these high concentrations, no significant boost ($P > 0.05$, repeated measures ANOVA) of the anti-peptide specific IgG concentrations was recorded from mice immunised with MP12-KLH. The anti-PnCPS IgG concentration elicited by these mice was also lower compared to MP7-KLH (Figure 3.17b). Five mice immunised with MP12-KLH, from the 20 investigated, elicited an average (mean) anti-PnCPS IgG concentration of 0.6 μg ml^{-1} after three doses of antigen (TP84). This was also shown to not be a significant boost ($P > 0.05$, repeated measures ANOVA).

Immunisation with multiple doses of MP55-KLH (n=26) did lead to a boost in the anti-peptide response ($P < 0.05$, repeated measures ANOVA). The IgG concentrations recorded were 25.87 mg ml^{-1} (TP56), 54.6 mg ml^{-1} (TP77), which decreases to 18.9 mg ml^{-1} 33 days after the final dose (TP103), see Figure 3.17c. No increase in the anti-PnCPS IgG concentration was detected in these mice, as the concentrations were the same or less than the background level.

However, immunisation with MP58-KLH did result in a significant boost ($P< 0.05$, repeated measures ANOVA) of the anti-MP58 and anti-serotype 9V capsular polysaccharide (anti-PnCPS) specific IgG concentrations (Figure 3.17d). Five mice, immunised with MP58-KLH elicited a mean anti-PnCPS IgG concentration of 1.0 $\mu g\ ml^{-1}$ after three dose of antigen (TP77). This represents only 1/125th of the average anti-MP58 response (n=29). This is lower than the 8.4 % seen in mice immunised with MP58-PH (see §3.6.4.3).

The difference between the responses to immunisation with the peptide mimics could be due to genetic restrictions in the mice. The outbred strain of mouse used, MF1, possess genetically dissimilar individuals with different repertoires of MHC proteins. MHC molecules are present in phagocytes. Once the antigen enters the cell it binds to the MHC class II molecule, which presents the peptide on the cell surface to make it available to helper T cells. Once the T cell receptor binds to the peptide, cytokines are released which stimulate antibody production in B cells.

Therefore, antibody production is dependant on the MHC class II molecule recognising the peptide. From the results described here, it appears that the sequence of MP7 maybe less efficient at binding to the murine MHC II molecules than peptides MP12, MP55 and MP58. This is because immunisation with MP7 stimulated the lowest concentration of antibody of all the serotype 9V peptide mimics. Consequently, the MP7 sequence may not have stimulated B cell proliferation as efficiently, resulting in decreased anti-peptide antibody production. This could be examined by vaccinating other mouse species, and does not necessarily imply that this restriction would be present in humans.

An alternative approach is to substitute residues of the MP7 sequence with some from MP12, MP55 or MP58. All four peptides bound to the mAb 206,F-5 (see §3.5.2) and therefore, must have similar binding characteristics. Replacing amino acids could maintain the binding avidity and improve MHC recognition. A further method to improve immunogenicity is to test alternative immunisation protocols. Boosting at different intervals or using an alternative adjuvant may stimulate an increased antibody response. However, as discussed above, an IgG response of 23.5 $\mu g\ ml^{-1}$ (from immunisation with MP7) was sufficient to protect against developing serotype 9V pneumococcal disease.

3.6.4.5 Immunisation with MP55-KLH elicited antibodies with the highest avidity to the peptide vaccine and the strength of binding correlated to an extended survival time in challenged mice.

Antibody avidity is a measure of the functional strength of serum antibody to bind to antigen. In this study, we compared the avidity of antibodies elicited by immunisation with four peptide mimics of serotype 9V pneumococcal polysaccharide. The serum samples were obtained from mice immunised with three doses of MP7, MP12, MP55 or MP58 conjugated to KLH (from time point TP77 or TP84). Microtitre plates were coated with the respective peptide conjugated to BSA. Bound antibodies were then treated with a concentration gradient of ammonium thiocyanate (see §2.8.3) to dissociate low avidity antibody from the antigen-coated plates. The molar concentration of ammonium thiocyanate resulting in 50 % reduction in antigen binding (antibody concentration) was termed the avidity.

We showed that mice immunised with MP55 evoked antibody of highest avidity. This was significantly greater than recorded for MP12 ($P< 0.05$) but equal to that of MP7 or MP58 ($P> 0.05$). Figure 3.18a shows the mean avidity of antibody elicited by MP55 was 1.827 M. This was higher than MP7 (1.567 M) and MP58 (1.289 M), and MP12 (0.969 M).

All the mice, that were tested, that received MP7-KLH (2/2) developed antibody avidity greater than 1.0 M. Eighty percent (8/10) of those that received MP58-KLH, and 67 % (6/9) of the mice given MP55-KLH developed avidity values in excess of 1.0 M. However, only 56 % (5/9) of those that received MP12-KLH developed avidity values in excess of 1.0 M.

Previous studies have shown that the avidity of anti-pneumococcal capsular antibodies can be related to their ability to confer protection against disease (Usinger *et al* 1999). Mice immunised with MP55-KLH or MP12-KLH that generated antibodies with higher avidity showed an extended survival time compared to low-avidity antibody in survival assays. This is shown in Figure 3.18b. The correlation coefficients for mice immunised with MP12 (n=9), MP55 (n=9), and MP58 (n=10) are shown. The correlation coefficient for MP7 could not be calculated from two values. The positive correlation coefficients of 0.0379 and 0.058 were observed for MP12 and MP55, respectively. The antibodies with high avidity to MP58 did not correlate with an extended survival time.

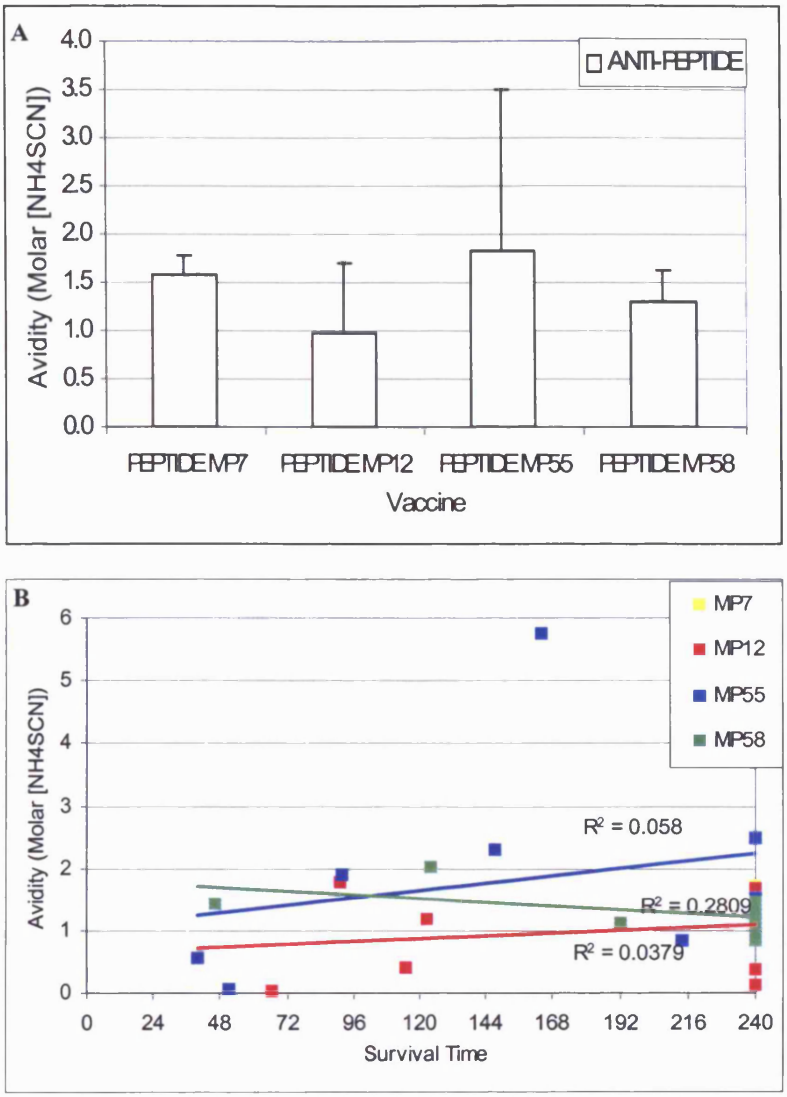

Figure 3.18 (A) The mean avidity of binding of anti-peptide mouse antibodies to MP7-KLH (n=2), MP12-KLH (n=9), MP55-KLH (n=9) or MP58-KLH (n=10). **(B)** the correlation of mice that produced high avidity antibody and their survival.

3.6.4.6 Serum antibodies developed from immunisation with peptides MP7, MP12 or MP55 compete for binding of serotype 9V pneumococcal polysaccharide and the peptide mimic equally.

To determine whether anti-peptide antibodies bound to serotype 9V polysaccharide, an ELISA was performed (as §3.6.4.5) except the serum was pre-incubated with either purified serotype 9V pneumococcal capsular polysaccharide, or *E. coli* lipopolysaccharide (LPS) as a negative control.

Figure 3.19 shows the average (mean) percentage inhibition and standard deviation of antibodies to bind to peptides MP7-BSA, MP12-BSA, MP55-BSA and MP58-BSA by pre-adsorption to capsular polysaccharide. Inhibition of antibody binding indicated a mutual recognition of the antibody-binding site by both antigens and, therefore, the act of mimicry.

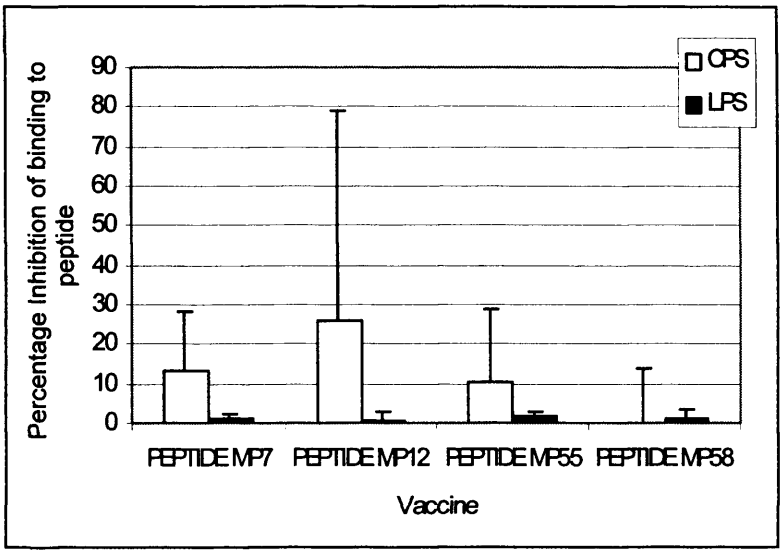

Figure 3.19. The average (mean) percentage inhibition of binding of anti-peptide specific mouse antibodies to MP7-KLH, MP12-KLH, MP55-KLH or MP58-KLH following pre-absorption with 9V polysaccharide. Serum was obtained from mice immunised with the peptide-conjugate and incubated with either 100 μg 9V polysaccharide (□) or *E. coli* lipopolysaccharide (■) prior to ELISA. Microtitre plates were coated with peptide-BSA to detect the presence of anti-peptide antibodies, prior to, and following the addition of polysaccharide. The percentage inhibition was determined from the difference in IgG concentrations recorded, divided by the absorbance recorded prior to addition of polysaccharide. The error bars represent the standard deviation of the mean.

The inhibition of binding to peptides MP7, MP12 and MP55 by serotype 9V pneumococcal capsular polysaccharide was significantly greater ($P < 0.05$, T-test) than when *E. coli* LPS was used (see Figure 3.19). Antibodies generated by immunisation with MP12 displayed the greatest average (mean) inhibition at 26 %. This was double that recorded for MP7 at 13 %, and higher still than for MP55 and MP58 (10 % and 0 %, respectively).

These results show that the inhibition by serotype 9V pneumococcal polysaccharide is specific and, therefore, peptides MP7-KLH, MP12-KLH and MP55-KLH mimic serotype 9V pneumococcal polysaccharide. To examine the cross-reactivity of these antibodies further, antibodies to MP7-KLH were tested on their ability to bind serotype 9V pneumococci *in vitro*. These results are described in §3.6.4.7.

3.6.4.7 Serum antibodies developed from immunisation with MP7 bound to serotype 9V bacterium *in vitro*

In addition to binding 9V polysaccharide (as described in §3.6.4.4), antibodies raised by immunisation with MP7-KLH also bound to serotype 9V pneumococci. A chain of fluorescent pneumococci was observed when bound antibodies were detected using a FITC-conjugated anti-mouse IgG antibody (see Figure 3.20a). Antibodies from mice immunised with KLH alone did not bind pneumococci and no fluorescence was observed when control serum was added as a primary antibody (Figure 3.20b). These results confirm the opsonic activity of the cross-reactive antibody that was observed with protection.

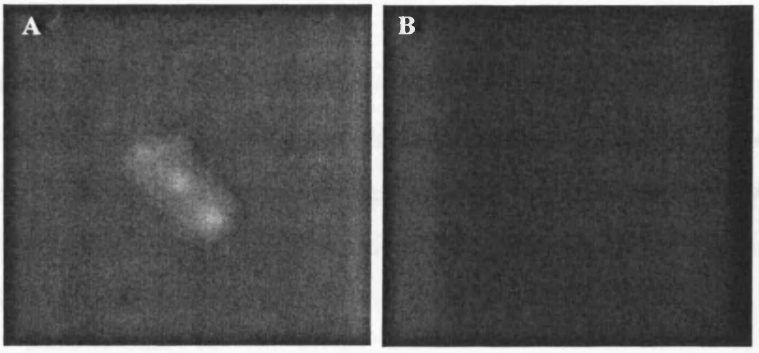

Figure 3.20. (A) Immunofluorescent labelling of pneumococci with murine polyclonal antibodies induced by immunisation with peptide mimic MP7-KLH conjugate. Serotype 9V pneumococci were incubated overnight with protein A purified mouse IgG from serum. This was followed by the addition of FITC-labelled goat anti-mouse IgG secondary antibody. A chain of fluorescent pneumococci is shown by the arrow. **(B)** pneumococci incubated with serum derived from mice immunised with KLH alone. No fluorescence was observed.

Part B: Mimics of type 6B pneumococcal polysaccharide selected using a human mAb

3.7 Selection of peptide mimics to serotype 6B pneumococcal polysaccharide

3.7.1 Section overview

Peptides were selected from four rounds of panning the pVIII-12aa library with Db3G9. Alternate panning procedures were used starting with Biopanning and then Dynabeads for the following round (see Table 2.4). This was to eliminate any bias that maybe introduced from using a single technique. For example, during biopanning, some peptides may bind to streptavidin or the milk used to coat the plate and be eluted along with the mAb selected phage.

No positive clones were identified from screening with mAb DM5, although it was noted that the clones selected by Db3G9 also bound DM5 in ELISA (see Figure 3.22 and §3.7.4).

Peptides were selected for further characterisation on the basis that they bound to DM5, Db3G9, or both in colony immunoscreening and ELISA, and if binding was specifically competed by 6B pneumococcal polysaccharide.

3.7.2 Immunoscreening

E. coli TG1 infected with the M13 phage, displayed phage coat proteins on the external surface of the cell membrane. Therefore, phage-infected bacterial colonies were bound to a nitrocellulose filter and the presence of reactive phage were identified by incubation with either mAb Db3G9 or DM5 (see §3.7.3).

Figure 3.21a shows the presence of reactive clones on a filter containing colonies infected with phage carrying the pVIII-12aa library, after screening with Db3G9. The purple pigment represents where the colony had adsorbed to the filter. The clones that were selected for further characterisation are shown in Figure 3.21b. These clones were selected based on the intensity of the pigment they produced and also the ease of picking a pure colony (i.e. clearly separate from other clones).

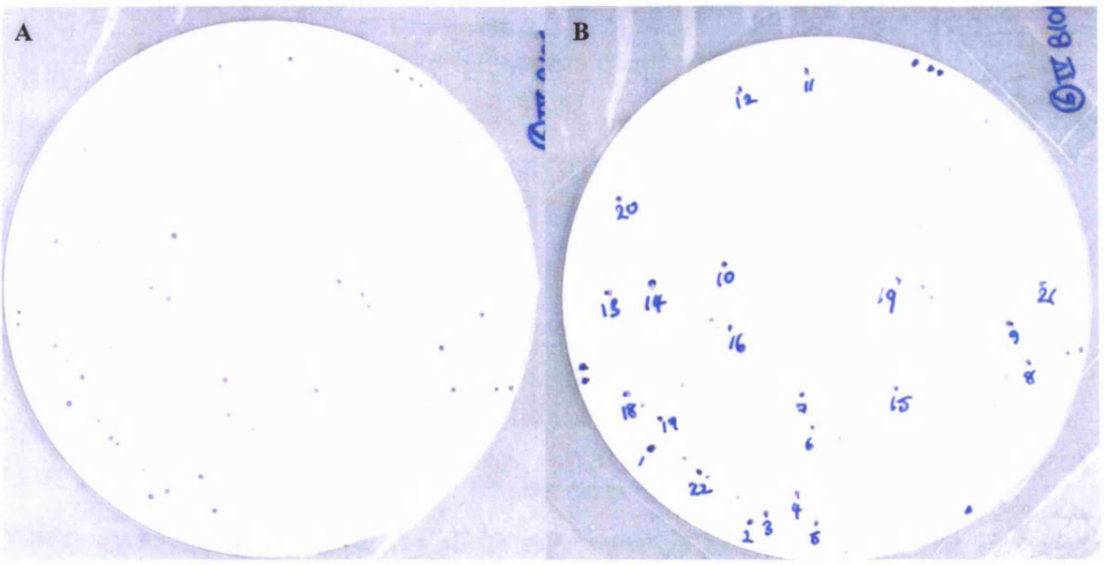

Figure 3.21. The positive clones identified by phage infected *E. coli* colony immunoscreening. **(A)** positive clones were identified by the development of a purple pigment where the colony had adsorbed to the nitrocellulose filter. **(B)** showed the positive clones that were picked from the agar and used in subsequent experiments

3.7.3 Phage ELISA

Individual phage clones were cultured as described in §2.5.7. The recovered phage supernatant was analysed for reactivity to DM5 or Db3G9 in phage ELISA (as described in §2.5.7.2).

As Figure 3.22 shows, the clones bound more of the mAb Db3G9 than DM5. This maybe due the mAb concentration used in ELISA, as both mAbs were diluted 1:50. All the phage clones (with the exception of 4, 17, 19 and 20) bound more of the mAb Db3G9 than the wild-type phage (pC89). This binding is also dependant on the presence of recombinant peptides, since a higher absorbance ($P< 0.05$) was observed compared to the wild-type pC89 phage. Only clones 1 and 9 bound a significantly higher ($P< 0.05$) proportion of mAb DM5 compared to the control phage.

Seven of these peptides were chosen for further investigation. These peptides were termed MP2, MP10, MP13 MP14, MP15, MP17, and MP18 (referring to clones 1, 2, 5, 9, 10, 11 and 15 respectively).

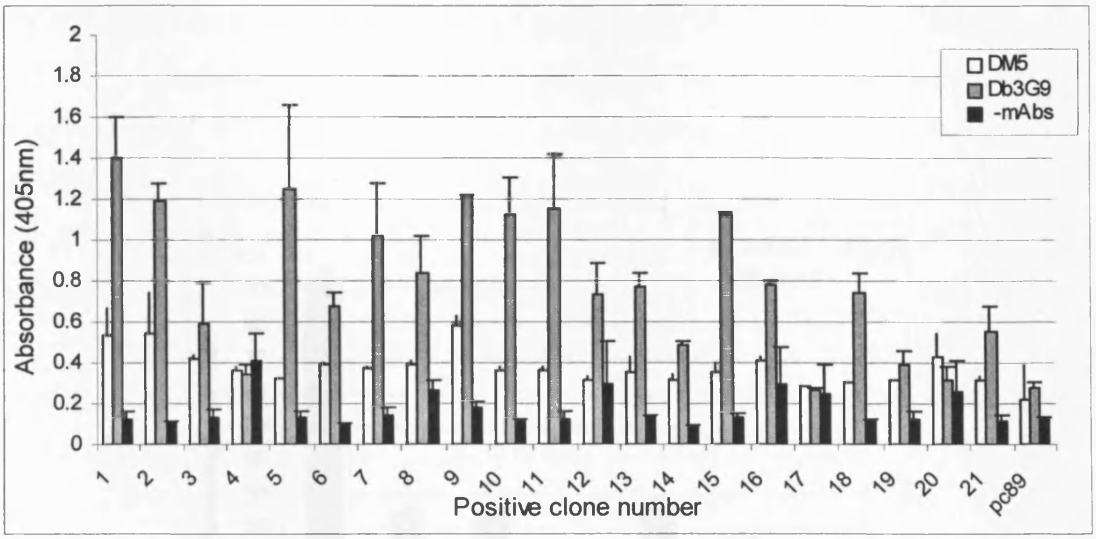

Figure 3.22 The reactivity of the phage supernatant in PHAGE ELISA from 21 colonies identified by immunoscreening. The phage supernatant was used to coat the plate, and the monoclonal antibody (either DM5 (□) or Db3G9 (■)) was added in abundance (or no monoclonal (■), the amount of antibody that binds represented the number of reactive phage in the supernatant. N=2

3.7.4 Competition ELISA

The binding of all seven peptide mimics to mAb Db3G9 or DM5 could be inhibited by first incubating the mAb with serotype 6B pneumococcal polysaccharide. Statistical analysis could not be performed on these data since there was no negative control. Figure 3.23a showed that the binding of Db3G9 to the peptide MP2 was inhibited by 94 % following pre-absorption with serotype 6B pneumococcal polysaccharide. Binding pf Db3G9 to the peptides MP10, MP13 and MP15 was inhibited ~25 %. These results showed that both the peptides and native polysaccharide antigen occupy the same antibody binding site of the mAb. This is because preincubation with polysaccharide blocks the ability of the mAb to bind to the peptide, as that region of the antigen binding site is already occupied. Therefore it was concluded that these peptides mimic an epitope of serotype 6B pneumococcal polysaccharide.

As a matter of interest, the binding of the mAb DM5 to the peptide mimics was also investigated. When these results (shown in Figure 3.23b) were compared to the competitive inhibition of Db3G9, an interesting phenomenon was observed. The results showed that binding to the peptide MP2, which was almost entirely inhibited by

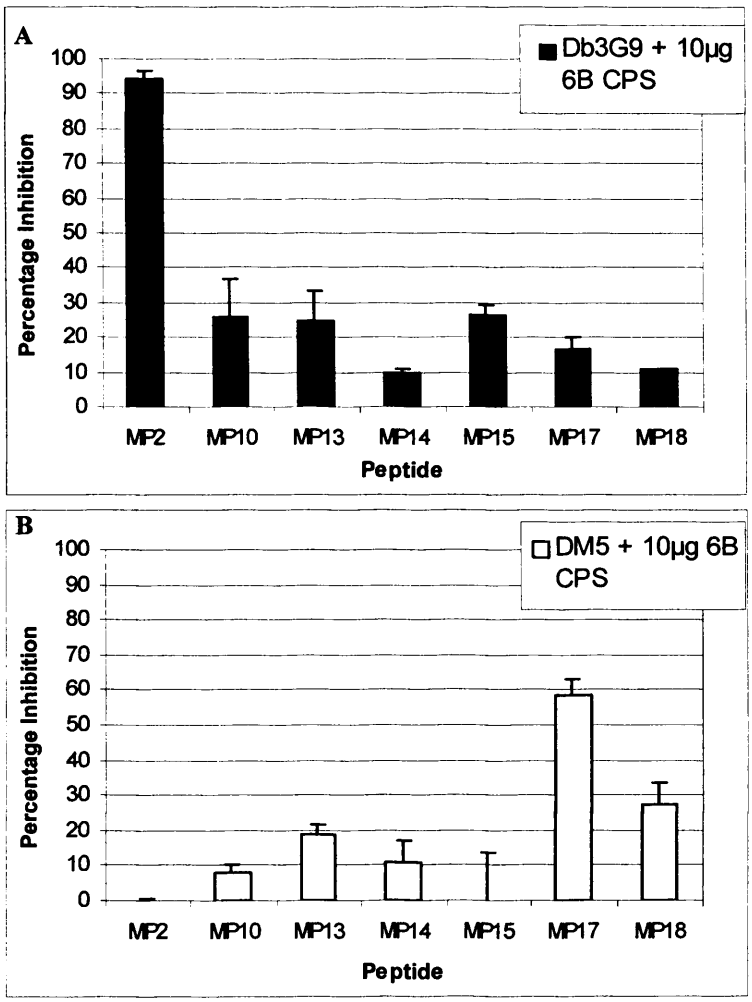

Figure 3.23. Competition ELISA. This technique shows whether pre-incubation with the mAb's native antigen (6B pneumococcal polysaccharide) can inhibit the mAb binding to the phage in ELISA. (A) showed the inhibition of binding each clone with mAb Db3G9 following pre-incubation of the mAb with 10 µg 6B pneumococcal polysaccharide (■). **(B)** showed the inhibition of binding each clone with mAb DM5 following pre-incubation of the mAb with 10 µg 6B pneumococcal polysaccharide (□). N=2

6B pneumococcal polysaccharide when Db3G9 was used, displayed no sign of inhibtion to DM5. This was also observed for peptides MP10 and MP15, both of which demonstrated a lower percentage inhibition to DM5 than to Db3G9 (7 % and 0 % respectively). However, the preincubation of DM5 with serotype 6B pneumococcal polysaccharide resulted in a higher inhibition of binding to peptides MP17 and MP18 compared to when Db3G9 was used. Binding of the mAb to MP17 was inhibited by 59 % and to MP18 by 27 %, compared to 17 % and 11 % with Db3G9. These results indicated that although both mAbs do recognise and bind to the peptide mimics, they are infact binding to different epitopes within the sequence. If they recognised the same epitopes then the degree of inhibition would be equal.

3.7.5 The sequence of the serotype 6B peptide mimics

The seven peptide mimics of serotype 6B pneumococcal polysaccharide described in the previous sections (MP2, MP10, MP13 MP14, MP15, MP17, and MP18) were sequenced as described in §2.5.8. Table 3.5 shows the sequences obtained.

Table 3.5 Sequences of peptides selected from phage-displayed peptide libraries using the human mAb Db3G9

Clone	*Library*	*Sequence* [a]
MP2	pVIII-12aa	NH_2 AEGEF- WGPRVIFEDVTV -GDPAK COOH
MP10	pVIII-12aa	NH_2 AEGEF- EIYPWYPMAERS -GDPAK COOH
MP13	pVIII-12aa	NH_2 AEGEF- DWPALIFDQDML -GDPAK COOH
MP14	pVIII-12aa	NH_2 AEGEF- WEWDWPRIELNI -GDPAK COOH
MP15	pVIII-12aa	NH_2 AEGEF- YSLLVEPYTFDP -GDPAK COOH
MP17	pVIII-12aa	NH_2 AEGEF- DWPAVIFEDTRA -GDPAK COOH
MP18	pVIII-12aa	NH_2 AEGEF- WPQIWFDDEEDV -GDPAK COOH

[a] The first five amino acids (AEGEF) and the last five (GDPAK) are constant in the library, as they belong to the pVIII coat protein sequence in the pC89 phagemid vector, and are therefore invariant in the random peptide libraries used for this study.

3.7.5.1 Analysis of the peptide sequences

3.7.5.1 The occurrence of certain residues is not random

A ratio (frequency) of occurrence was determined from the expected frequencies based on the codon frequencies in the oligonucleotides that were used to construct the libraries. Therefore, any ratio >1.0 indicated an over-representation of that residue (Mertens *et al.* 2001). Figure 3.24 showed the frequency of occurrence for each randomly generated amino acid residue present in the seven 6B peptides selected from the pVIII-12aa phage display library. As described in §3.5.5.1, these are compared to the expected frequencies based on the codon frequencies in the oligonucleotides that were used to construct the libraries. As with the 9V peptide mimics, there does appear to be a biased proportion of residues. However, unlike the 9V mimics, the peptides selected here also appear to have a consensus sequence (see §3.7.5.2).

Only three (F, W, Y) of the four aromatic amino acids are over-represented in these peptides, in particular tryptophan (W) is represented more than 6 times the expected frequency. MP2 contains 17 % aromatic amino acids, MP10 25 %, MP13 17 %, MP14 25 %, MP15 25 %, MP17 17 %, and MP18 25 %. There is also a high proportion of aspartic acid (D) and glutamic acid (E) residues, both (as their name suggests) are acidic, and negatively charged. Isoleucine (I), methionine (M) and valine (V) are also over represented in the 6B mimics.

3.7.5.2 Sequence alignment

The sequences of the serotype 6B peptide mimics were aligned using the ClustalW program described by Thompson *et al.* (1994). This scored the identity of two peptides based on exact matches. Sequences sharing greater than 30 % identity was considered to be significant (see §3.5.5.3). There does appear to be a significant amount of homology between the serotype 6B pneumococcal polysaccharide mimics. As shown in Table 3.6, peptides MP2 and MP17, and peptides MP13 and MP17 share 50 % identity. Additionally, peptides MP13 and MP17 share 33 % identity to MP18. This means that four of the seven peptide mimics share a significant degree of similarity. In fact, the similarity is so apparent that it can be seen without analysis and just from colour coding the amino acids (Table 3.6). The pairing of tryptophan and proline residues (either PW, WP or WGP) is found in all seven peptides (except MP15 where proline is adjacent to tyrosine, another aromatic

pairing). This feature has been described before (Phalipon *et al.* 1997; Mertens *et al.* 2001) and is discussed in §5.4.1.1.

All peptides contain at least one proline (P) residue and MP2 and MP15 both contain 2 proline residues, which sandwich 2 aromatic amino acids between them. This was seen more clearly when displayed in Table 3.6. The significance of these findings is discussed in §5.3.

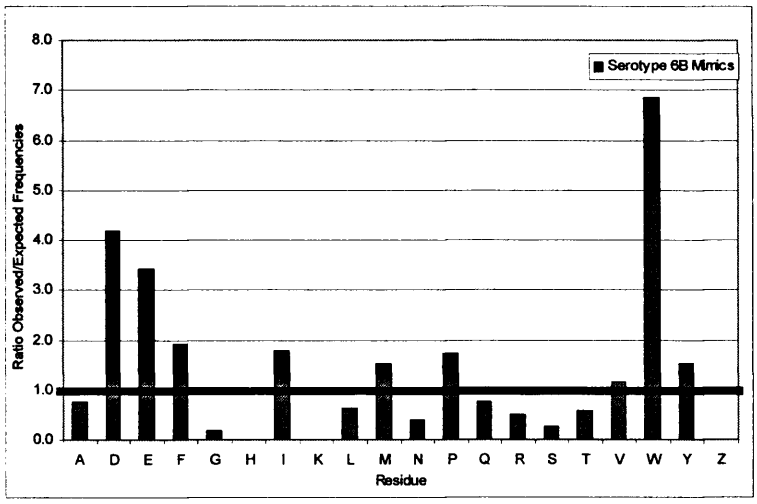

Figure 3.24. Occurrence frequencies of each residue in the peptides selected with mAb Db3G9 from the pVIII-12aa library (■). The vertical axis is the ratio between the observed frequency of each residue in the peptides and the theoretical frequency of each residue calculated based on the proportion of each codon present in the randomly generated oligonucleotides used to construct the libraries, each coding for a particular amino acid. A ratio above 1.0 indicates over-representation of a particular residue in the peptides. Cysteine has been excluded from this graph, as it is not a randomly generated residue. It is fixed in the sequences of the pVIII-12aa.Cys peptides and did not appear in the 6B mimics. The 'Z' residue corresponds to the stop codon, since all peptides are of the expected length, it is logical to observe no stop codons.

Table 3.6 Alignment and consensus identity score of serotype 6B peptide mimics

Peptide1	*Peptide 2*	*Identity (%)*
MP2	MP17	50
MP13	MP17	50
MP13	MP18	33
MP17	MP18	33
MP2	MP13	25
MP2	MP18	25
MP10	MP15	25
MP13	MP14	25
MP14	MP17	25
MP14	MP18	25
MP2	MP10	16
MP2	MP14	16
MP13	MP15	16
MP15	MP18	16
MP2	MP15	8
MP10	MP13	8
MP10	MP14	8
MP10	MP17	8
MP10	MP18	8
MP14	MP15	8
MP15	MP17	8

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MP2   ----WGPRVIFEDVT-V-- COOH
MP17  ---DW-PAVIFEDTR-A-- COOH
MP13  ---DW-PALIFDQDM-L-- COOH
MP18  ----W-PQIWFDDEEDV-- COOH
MP14  WEWDW-PRIELN----I-- COOH
MP15  ----Y--SLLVEPYT-FDP COOH
MP10  --EIY-PWYPMAERS---- COOH
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The seven serotype 6B pneumococcal polysaccharide peptide mimics were arranged using the ClustalW version 1.82 program. Each peptide was placed based on its identity to the adjacent peptides. Colour codes: Small (small+ hydrophobic (including aromatic -Y)) residues (RED), Acidic residues (BLUE), Basic residues (MAGENTA), Hydroxyl + Amine + Basic - Q (GREEN).

3.8 Immunisation with peptide mimics of serotype 6B pneumococcal polysaccharide

3.8.1 Section overview

Seven peptides had been selected from a phage display library expressing random dodecameric peptides using mAb Db3G9, a human monoclonal antibody that was raised to serotype 6B pneumococcal capsular polysaccharide (see §3.7.5). These peptides were designated MP2, MP10, MP13 MP14, MP15, MP17 and MP18.

To determine whether immunisation with a peptide mimic of serotype 6B pneumococcal polysaccharide protected mice against pneumococcal disease, mice immunised (see §2.7 for immunisation schedules) with either MP2-KLH, MP10-KLH, MP13-KLH, MP14-KLH, MP15-KLH, MP17-KLH or MP18-KLH were challenged with a dose of serotype 6B pneumococci shown to cause disease (see §3.2.2). Control mice were immunised with KLH alone and then challenged alongside the experimental groups.

3.8.2 Immunisation with MP13-KLH protected mice from serotype 6B pneumococcal disease

There was a significant difference ($P< 0.05$) observed between the survival rates of mice immunised with MP13 conjugated to KLH (MP13-KLH) compared to the mice that received KLH alone (control) following intraperitoneal challenge with 1×10^5 cfu serotype 6B pneumococci.

Kaplan-Meier survival curve analysis was performed to compare the survival data from the 30 MP13-KLH immunised mice and the 20 control mice that were challenged. The survival curves were shown to be significantly different ($P< 0.05$) with 56 % (17/30) of mice immunised with MP13-KLH surviving challenge, compared to the control mice where only 20 % (5/20) survived. These data are shown in Figure 3.25a.

Initially the decline in the number of viable animals appeared to occur at a similar rate (as seen on Figure 3.25a). Then, 145 hours after challenge, the survival of the mice immunised with MP13-KLH significantly improved with only a further 20 % decline compared to a 50 % decline in the control group. These results are reflected in the disease scores. Initially, all the mice that displayed diseases of pneumococcal disease produced a similar cumulative disease score. However, the median cumulative disease score from mice

immunised with MP13-KLH was 0, which was significantly lower (P< 0.05, T-test) than from mice that received KLH alone (12.0). This shows that once diseases appeared, the disease progressed at a similar rate for all the mice; irrespective of the vaccine they had received. These results are shown in Figure 3.25b.

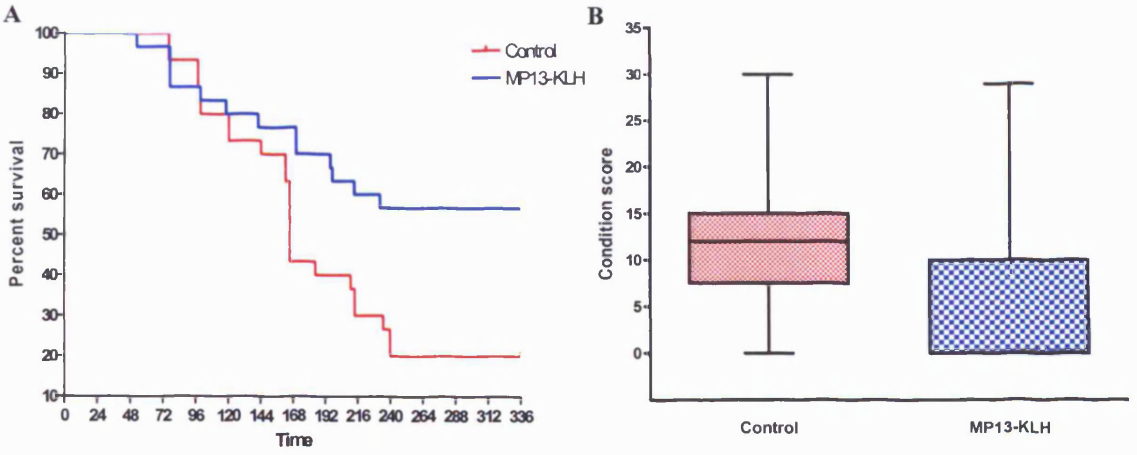


Figure 3.25. Survival of mice challenged intraperitoneally with 1x10^{5}cfu 6B pneumococci. The graph shows the Kaplan-Meier survival curves of 30 mice immunised with peptide MP13-KLH (■) compared to 20 mice immunised with KLH alone (■) prior to challenge. **(B)** The Box-plot of disease scores of mice receiving 3 doses of with peptide MP13-KLH (■) compared to mice immunised with KLH alone (■) and then challenged with 1x10^{5}cfu type 6B pneumococcus i.p. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median. The mean value of MP13-KLH is close to the 25 %.

3.8.3 Mice immunised with MP2-KLH produced the highest percent survival of serotype 6B pneumococcal disease

Seventy percent of mice (7/10) immunised with a single dose of MP2-KLH survived an intraperitoneal challenge with 1 x 10^6 cfu serotype 6B pneumococci, compared to the control mice where 40 % (4/10) survived to 15 days after challenge with the same dose of bacteria. Kaplan-Meier survival analysis determined that the difference between these survival curves was not significant (P> 0.05), this is shown in Figure 3.26.

However, mice immunised with MP17-KLH (also shown on Figure 3.26) or MP2-KLH were challenged at the same time and were administered an identical dose of pneumococci. Twenty percent (2/10) of mice immunised with MP17-KLH survived challenge. When the

survival of these mice was compared to the survival of mice immunised with MP2-KLH, a significant difference was observed ($P< 0.05$). This implies that there is some functionality to the immune response to the mimotope MP2. This deduction is reflected in the average group disease scores. These results showed the median cumulative disease score from mice immunised with MP2-KLH was 0, which was lower than from mice that received MP17-KLH or KLH alone (13.5 and 8.0 respectively). This difference is not statistically different ($P> 0.05$, ANOVA, T-test) as the range of disease scores is similar for each group. This implies that immunisation led to a greater percentage survival (decreased mortality) although the degree of diseases suffered was not affected (similar morbidity).

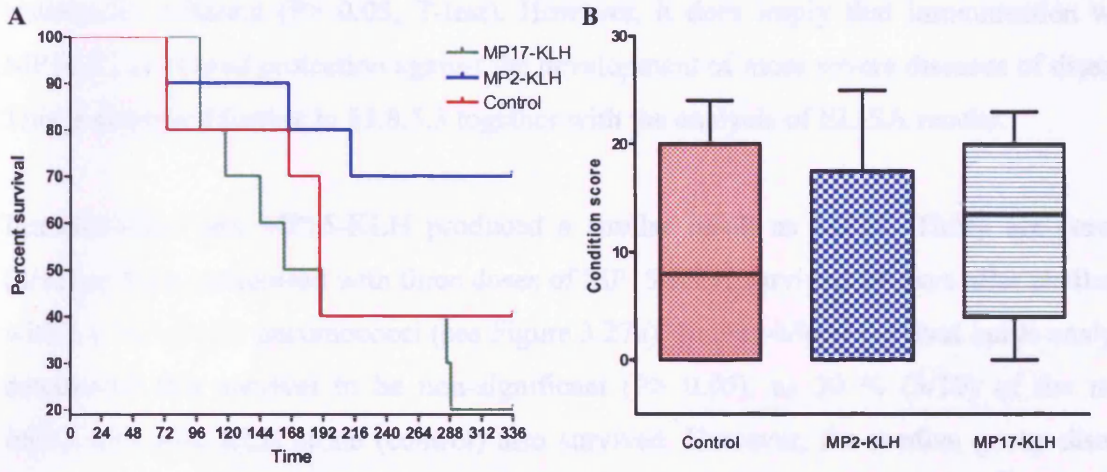


Figure 3.26. (A) Survival of mice challenged intraperitoneally with 1x10^{6}cfu 6B pneumococci. The graph shows the Kaplan-Meier survival curves of 10 mice immunised with peptide MP2-KLH (■) and 10 mice immunised with MP17-KLH (■) compared to 20 mice immunised with KLH alone (■) prior to challenge. **(B)** The Box-plot of disease scores of mice receiving 3 doses of with peptide MP2-KLH (■) compared to mice immunised with MP17-KLH (■) and mice immunised with KLH alone (■) and then challenged with 1x10^{6}cfu type 6B pneumococcus i.p. The whisker bars represent the range of diseases scores, the borders of the box are the 25% and 75% percentiles and the bar dividing the box is the median. The mean value of MP2-KLH is close to the 25 %.

3.8.4 Immunisation with MP10, MP14, MP15, MP17 or MP18 did not protect mice from serotype 6B pneumococcal disease

Immunisation with MP10-KLH, MP14-KLH, MP15-KLH, MP17-KLH and MP18-KLH conferred no protection against developing serotype 6B pneumococcal disease in mice. Figures 3.27, 3.28 and 3.29 showed that the survival of mice immunised with these peptides was not significantly different ($P> 0.05$) from the contol mice (immunised with

KLH alone) following challenge with serotype 6B pneumococci, as determined by Kaplan-Meier survival curve analysis.

Fifty percent (5/10) of mice immunised with one dose of MP14-KLH survived 18 days after challenge with 3 x 10^5 cfu 6B pneumococci. Kaplan-Meier survival curve analysis determined this survival to be non-significant (P> 0.05), as 40 % (4/10) of the mice immunised with KLH alone (control) also survived (see Figure 3.27a) However, the median group disease score was lower in the peptide-immunised mice, at 2.0, compared to 10.0 for the control group. The maximum disease score acquired for the mice receiving MP14 (19.0) was also lower than the control mice (32.0). These results were not statistically different (P> 0.05, T-test). However, it does imply that immunisation with MP14-KLH offered protection against the development of more severe diseases of disease. This is discussed further in §3.8.5.3 together with the analysis of ELISA results.

Immunisation with MP15-KLH produced a similar result as MP14. Thirty six percent (4/11) of mice immunised with three doses of MP15-KLH survived 14 days after challenge with 1 x 10^6 cfu 6B pneumococci (see Figure 3.27a). Kaplan-Meier survival curve analysis determined this survival to be non-significant (P> 0.05), as 30 % (3/10) of the mice immunised with KLH alone (control) also survived. However, the median group disease score was lower in the peptide-immunised mice, at 6.5, compared to 8.0 for the control group. The maximum disease score acquired for the mice receiving MP15 (20) was slightly lower than the control mice (24). However, this difference was not significant (P> 0.05, T-test, ANOVA).

Immunisation with three doses of MP10-KLH resulted in no difference to the survival of mice after challenge. Upon challenge with 1 x 10^5 cfu 6B pneumococci only 33 % (5/15) of the mice that received MP10-KLH survived, compared to 35 % (7/20) in the control group (see Figure 3.28a). The median disease score for the group that were immunised with MP10 was slightly lower (6.5) than those of the mice that received KLH alone (7.0) (Figure 3.28b). This concludes that immunisation with MP10-KLH does not protect mice against developing serotype 6B pneumococcal disease.

Mice immunised with one dose of MP17-KLH or MP18-KLH were challenged 98 days after administration of the vaccine. Twenty percent (2/10) of mice immunised with MP17-

KLH, and 40 % (4/10) of mice immunised with MP18-KLH survived 14 days after challenge with 1 x 10^6 cfu 6B pneumococci see Figure 3.29a). Kaplan-Meier survival curve analysis determined this survival to be non-significant (P> 0.05), as 30 % (3/10) of the mice immunised with KLH alone (control) also survived. The median disease score for mice immunised with the peptides was also higher (13.5 and 8.0 respectively) than for mice that received KLH alone (8.0) (Figure 3.29b). This concludes that immunisation with one dose of MP17-KLH or MP18-KLH does not protect mice against developing serotype 6B pneumococcal disease.

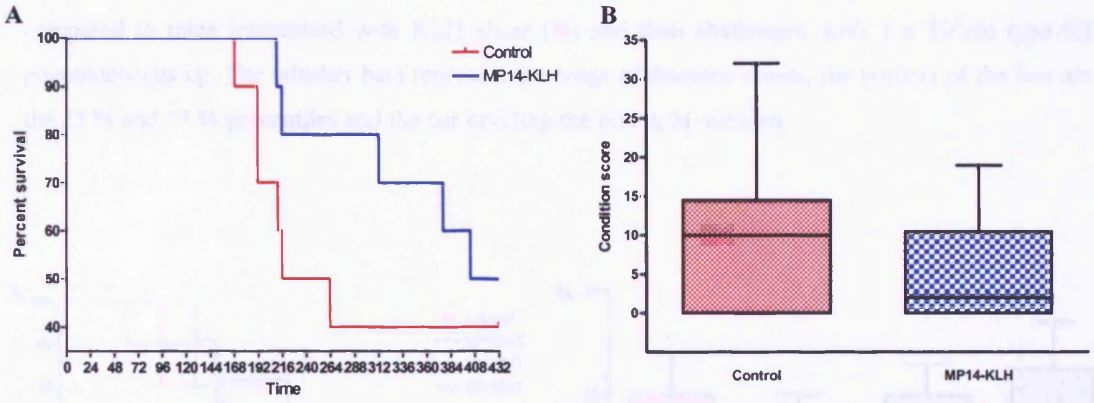


Figure 3.27. (A) Survival curve for mice immunised against type 6B pneumococcus with peptide MP14 compared to sham-immunised control mice when challenged with 3 x 10^5cfu i.p. (B) The Box-plot of disease scores of mice receiving 3 doses of with peptide MP14-KLH (■) compared to mice immunised with KLH alone (■) and then challenged with 3 x 10^5cfu type 6B pneumococcus i.p. The whisker bars represent the range of diseases scores, the borders of the box are the 25 % and 75 % percentiles and the bar dividing the box is the median.

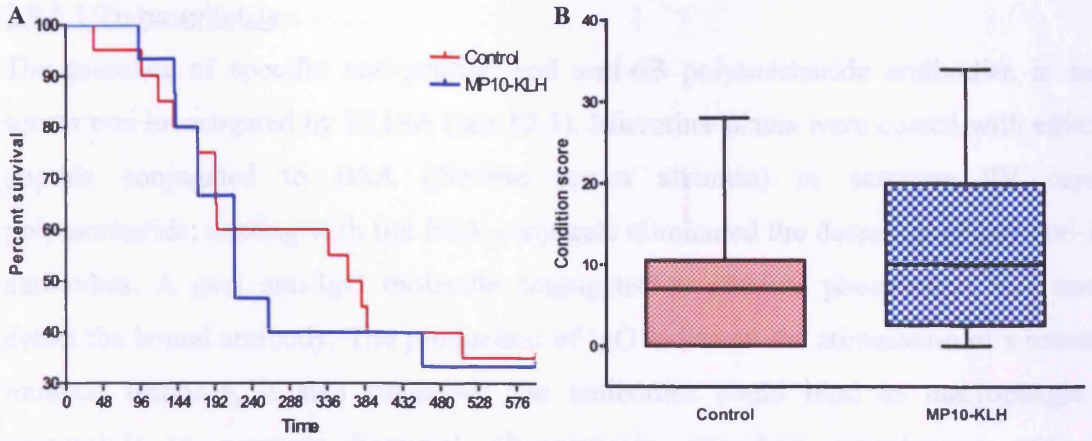


Figure 3.28. (A) Survival curve for mice immunised against type 6B pneumococcus with peptide MP10-KLH compared to mice immunised with KLH alone when challenged with 1 x 10^5cfu i.p. (B) The Box-plot of disease scores of mice receiving 3 doses of with peptide MP10-KLH (■) compared to mice immunised with KLH alone (■) and then challenged with 1 x 10^5cfu type 6B pneumococcus i.p. The whisker bars represent the range of diseases scores, the borders of the box are the 25 % and 75 % percentiles and the bar dividing the box is the median.

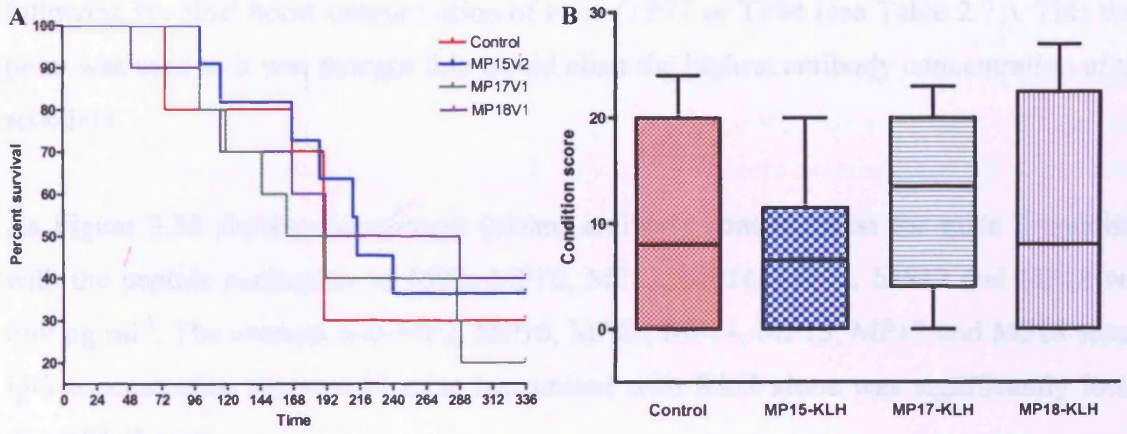


Figure 3.29. (A) Survival of mice challenged intraperitoneally with 1 x 10^6cfu 6B pneumococci. The graph shows the Kaplan-Meier survival curves of 10 mice immunised with peptide MP15-KLH (■), 10 mice immunised with MP17-KLH (■), or 20 mice immunised MP18-KLH (■) with compared to 20 mice immunised with KLH alone (■) prior to challenge. **(B)** The Box-plot of disease scores of mice immunised with peptide MP15-KLH (■), MP17-KLH (■) or MP18-KLH (■) compared to mice immunised with KLH alone (■) and then challenged with 3 x 10^5cfu type 6B pneumococcus i.p. The whisker bars represent the range of diseases scores, the borders of the box are the 25 % and 75 % percentiles and the bar dividing the box is the median.

3.8.5 The antibody response in mice immunised with serotype 6B peptide mimics

3.8.5.1 To recapitulate

The presence of specific anti-peptide and anti-6B polysaccharide antibodies in mouse serum was investigated by ELISA (see §2.3). Microtitre plates were coated with either the peptide conjugated to BSA (Bovine serum albumin) or serotype 9V capsular polysaccharide; coating with the BSA conjugate eliminated the detection of any anti-KLH antibodies. A goat anti-IgG molecule conjugated to alkaline phosphatase was used to detect the bound antibody. The production of IgG indicated the stimulation of a secondary immune response, it also suggested the antibodies could bind to macrophages and neutrophils to promote bacterial phagocytosis, stimulate complement and, most importantly, mimic the mAb Db3G9 (of the isotype IgG2) that selected the peptides.

3.8.5.2 Antibodies generated by control immunisation with KLH did not bind peptide or polysaccharide

Immunisation with the KLH alone did not elicit a significant concentration of anti-peptide antibodies compared to the mice that were immunised with KLH conjugated to the peptide mimotope. An ELISA (see §2.8.1) was performed to determine the IgG concentrations following the final boost immunisation of mice (TP77 or TP84 (see Table 2.7)). This time point was used as it was thought this would elicit the highest antibody concentration of the schedule.

As Figure 3.30 shows, the average (mean) antibody concentration for mice immunised with the peptide conjugates of MP2, MP10, MP13, MP14, MP15, MP17 and MP18 was >69 $\mu g\ ml^{-1}$. The average anti-MP2, MP10, MP13, MP14, MP15, MP17 and MP18 serum IgG concentration reported in mice immunised with KLH alone was significantly lower ($P< 0.05$, T-test).

The serum from mice immunised with KLH alone reacted to MP14, MP2 and MP10 the best (13.1 $\mu g\ ml^{-1}$, 12.0 $\mu g\ ml^{-1}$ and 8.5 $\mu g\ ml^{-1}$ IgG, respectively, but this is still significantly different ($P< 0.05$, T-test) from the sera of mice immunised with the peptide conjugate (220.1 $\mu g\ ml^{-1}$, 369.4 $\mu g\ ml^{-1}$ and 1500.5 $\mu g\ ml^{-1}$, respectively). The control serum cross-reacted far less with peptides MP13, MP15, MP17 and MP18 with a maximum concentration of 5.6 $\mu g\ ml^{-1}$, as shown on Figure 3.30. This shows that the mice

immunised with KLH alone did not develop antibodies that cross-reacted with any of the serotype 6B peptide mimics.

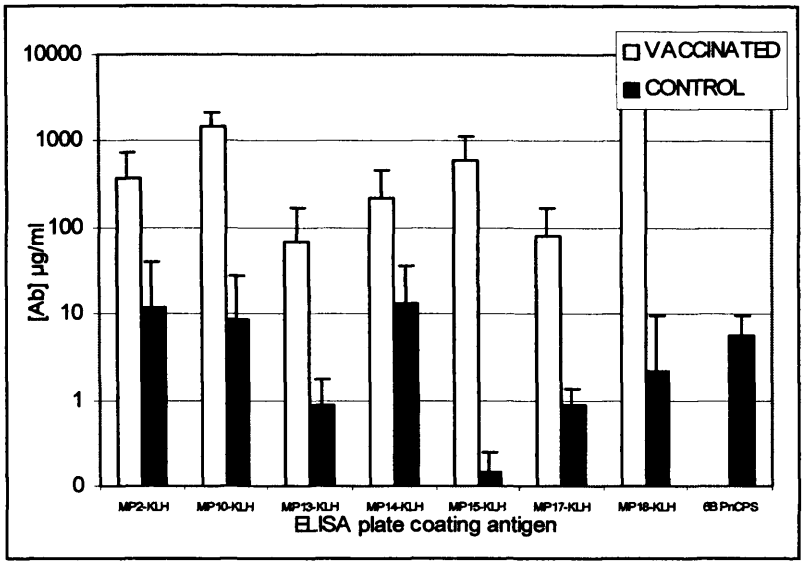


Figure 3.30. IgG titre of sera of mice immunised with peptide conjugates. The chart shows the average (mean) anti-peptide IgG concentration from the serum of mice immunised with peptide conjugates MP2 (n=10), MP10 (n=30), MP13 (n=29), MP14 (n=30), MP15 (n=30), MP17 (n=6), MP18 (n=10) from time point 84, time point 70, or time point 42 (□). The reactivity of the anti-KLH IgG antibodies to the peptide conjugates from sham-immunised mice is also shown (■). Data are from ELISAs of microtitre plates coated with 5 µg ml^{-1} peptide-BSA or 5 µg ml^{-1} 6B capsular polysaccharide, 100 µl per well. Error bars represent the standard deviation of the mean. The reactivity of the sham immunised mouse serum to 6B capsular polysaccharide of serum from mice immunised is also shown.

3.8.5.3 Antibodies generated from immunisation with all serotype 6B peptide mimics cross-reacted with serotype 6B pneumococcal capsular polysaccharide

The concentration of IgG at time point 0 was used to determine the background absorbance readings. This value was subtracted from the concentrations obtained from the other time points to indicate any increase in antibody levels after immunisation. The serum collected from mice immunised with KLH alone was also used as a negative control; these results were discussed in §3.8.5.2. Only those mice that demonstrated an increase in antibody levels were used to determine the average (mean) IgG concentration and the range (standard deviation) for each time point (see Figure 3.31).

Immunisation with MP10 elicited an anti-peptide and anti-6B polysaccharide response that was significant boosted ($P< 0.05$, repeated measures ANOVA) following administration of further doses (Figure 3.31). All 30 mice immunised with MP10-KLH elicited antibodies that reacted with MP10-BSA. The average (mean) and standard deviation of these data are shown in Figure 3.30. The average IgG concentration was 218 $\mu g\ ml^{-1}$ 14 days after the first dose of peptide, which rose nineteen-fold 14 days after the first boost (TP56) to 4.1 $mg\ ml^{-1}$. However, 17 days after the second boost (TP80) the average IgG concentration decreased to 2.8 $mg\ ml^{-1}$.

The highest concentration of anti-MP10 IgG achieved by a single mouse was 40 $mg\ ml^{-1}$, this mouse did not survive challenge. The highest anti-6B polysaccharide IgG concentration from a single mouse was 176 $\mu g\ ml^{-1}$, this mouse also did not survive challenge. The average (mean) anti-6B polysaccharide IgG concentration over the immunisation schedule followed a similar pattern to the anti-peptide antibody (see Figure 3.30). Fourteen days after the first dose of peptide the anti-6B polysaccharide IgG concentration was 0.2 $\mu g\ ml^{-1}$. This rose hundred-fold 14 days after the first boost (TP56) to 21 $\mu g\ ml^{-1}$, which increased further to 36 $\mu g\ ml^{-1}$ 17 days after the second boost (TP80).

Immunisation with MP2-KLH also produced a significant increase ($P< 0.05$, repeated measures ANOVA) in the concentration of anti-6B polysaccharide IgG over time (Figure 3.32a). Ten mice immunised with MP2-KLH elicited an average (mean) anti-6B polysaccharide IgG concentration of 241 $\mu g\ ml^{-1}$ after one dose of antigen (TP36). At the same time the concentration of anti-6B polysaccharide antibody reached 300 $\mu g\ ml^{-1}$. This is significantly higher ($P< 0.05$, T-test) than the anti-6B polysaccharide concentration generated by mice immunised with MP10-KLH at the same time point. Maybe this is why a survival rate of 70 % (7/10) was noted following the challenge of mice immunised with MP2, compared to only 32 % (8/25) in mice that received MP10.

Immunisation with either of the MP13, MP14, MP15, MP17 and MP18 peptide conjugates elicited a much lower concentration of anti-6B polysaccharide IgG compared to MP2 and MP10 (Figure 3.32bcdef). Fourteen mice, from the 30 investigated, immunised with MP13-KLH elicited an average (mean) anti-6B polysaccharide IgG concentration of 0.2 $\mu g\ ml^{-1}$ after three dose of antigen (TP77). This is a significant boost ($P< 0.05$, repeated measures ANOVA). The average ($n=30$) anti-peptide IgG concentration after three doses

of MP13 was 63 μg ml^{-1}. The highest concentration of anti-MP13 IgG achieved by a single mouse was 444 μg ml^{-1}, this mouse survived challenge. The highest anti-6B polysaccharide IgG concentration from a single mouse was 0.9 μg ml^{-1}, this mouse also survived challenge.

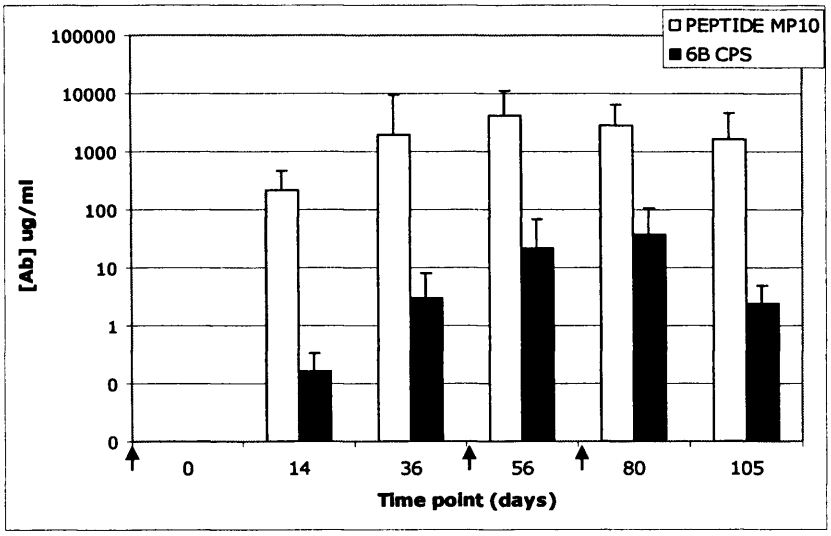


Figure 3.31. The humoral response of mice immunised with MP10-KLH. The mean (n=30) anti-MP10 response (□) and the mean (n=10) anti-6B capsular polysaccharide (PnCPS) response (■) from mice immunised with MP10-KLH. Primary and booster doses are marked on the graph by arrows (see §2.7 for more details).

Mice immunised with MP14-KLH elicited a similar immune response to those that received MP13 (see Figure 3.32c). Thirteen mice, of the 29 mice investigated gave an average (mean) anti-6B polysaccharide IgG concentration of 0.3 μg ml^{-1} after one dose of antigen (TP42). The average (n=29) anti-peptide IgG concentration at the same time point was 362 μg ml^{-1}. Both these responses significantly increased over time ($P< 0.05$, repeated measures ANOVA).

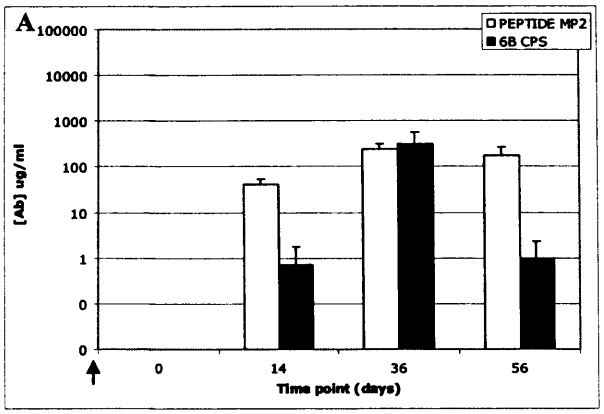
Immunisation with MP15, MP17 and MP18 peptide conjugates produced a mean (n=14, n=5, and n=5 respectively) anti-6B polysaccharide IgG concentration of 0.1 μg ml^{-1} or less. Of the mice that received MP15, the highest anti-6B polysaccharide IgG concentration recorded was 0.2 μg ml^{-1} (see Figure 3.32d), this mouse survived challenge. The highest concentration of anti-MP15 IgG achieved by a single mouse was 2.4 mg ml^{-1}. This mouse

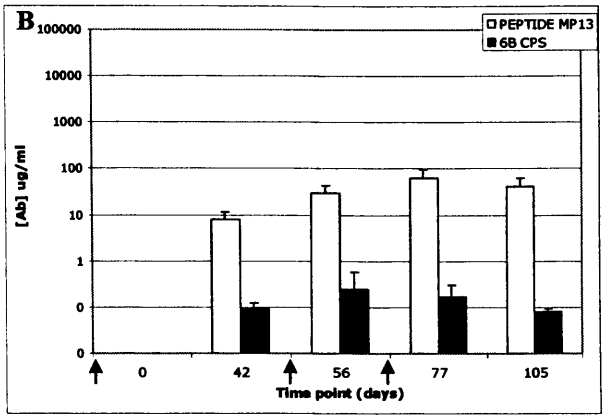
also survived challenge. Both these increases were shown to be significant ($P < 0.05$, repeated measures ANOVA).

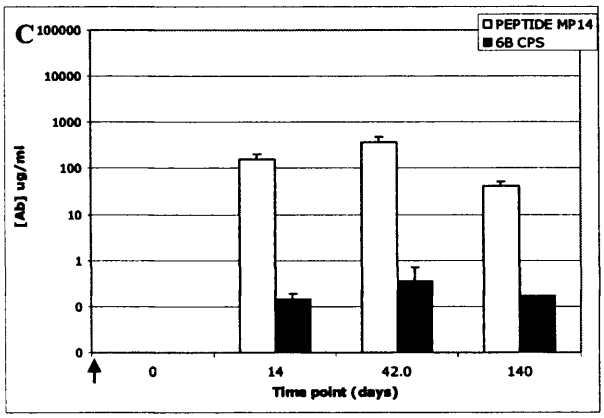
The highest anti-6B polysaccharide IgG concentration recorded from a mouse immunised with MP17-KLH was 0.7 μg ml^{-1} (see Figure 3.32e), this was not shown to be significant ($P > 0.05$, repeated measures ANOVA) and this mouse did not survive challenge. The highest concentration of anti-MP17 IgG achieved by a single mouse was 6.6 mg ml^{-1}. This is a significant increase ($P < 0.05$, repeated measures ANOVA) and this mouse did survive challenge.

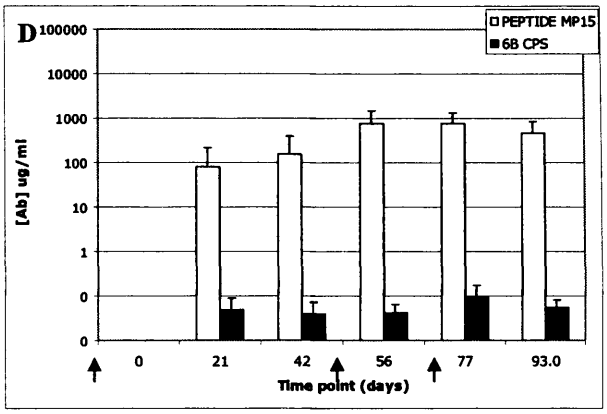
Finally, the highest anti-6B polysaccharide IgG concentration recorded from a mouse immunised with MP18-KLH was 0.2 μg ml^{-1} (see Figure 3.32f). Although this was not shown to be a significant increase ($P > 0.05$, repeated measures ANOVA), this mouse survived challenge. The highest concentration of anti-MP18 IgG achieved by a single mouse was 8.5 mg ml^{-1}. This is a significant increase ($P < 0.05$, repeated measures ANOVA) and this mouse also survived challenge.

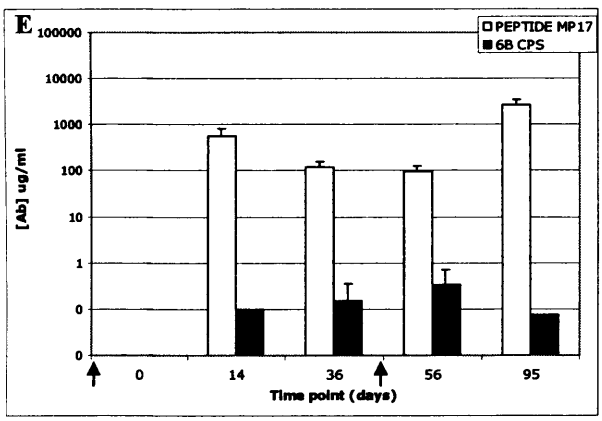
Figure 3.32 (following page) The humoral response of mice immunised with peptide conjugates MP2-KLH, MP13-KLH, MP14-KLH, MP15-KLH, MP17-KLH or MP18-KLH. **(A)** shows the mean (n=10) anti-MP2 response (□) and the mean (n=10) anti-6B capsular polysaccharide (PnCPS) response (■) from mice immunised with MP2-KLH. **(B)** shows the mean (n=30) anti-MP13 response (□) and the mean (n=14) anti-6B PnCPS response (■) from mice immunised with MP13-KLH. **(C)** shows the mean (n=29) anti-MP14 response (□) from mice immunised with MP14-KLH and the mean (n=12) anti-6B PnCPS response (■) from mice immunised with MP14-KLH (■). **(D)** shows the mean (n=30) anti-MP15 response (□) and the mean (n=14) anti-6B PnCPS response (■) from mice immunised with MP15-KLH. **(E)** shows the mean (n=10) anti-MP17 response (□) and the mean (n=10) anti-6B PnCPS response (■) from mice immunised with MP17-KLH. **(F)** shows the mean (n=10) anti-MP18 response (□) and the mean (n=5) anti-6B PnCPS response (■) from mice immunised with MP18-KLH. The IgG concentrations were determined by ELISA. Microtitre plates were coated with 5 μg ml^{-1} the BSA conjugated peptide or 5 μg ml^{-1} 6B PnCPS, 100 μl per well. The concentration of antibody at TP0 was used to determine the background level. This value was subtracted from the concentrations obtained from the other time points to indicate any increase in antibody levels after immunisation. Only those mice that demonstrated an increase in antibody levels were used to determine the mean IgG concentration and the error bars represent the standard deviation for each time point. Primary and booster doses are marked on the graphs by arrows (see §2.7 for more details).

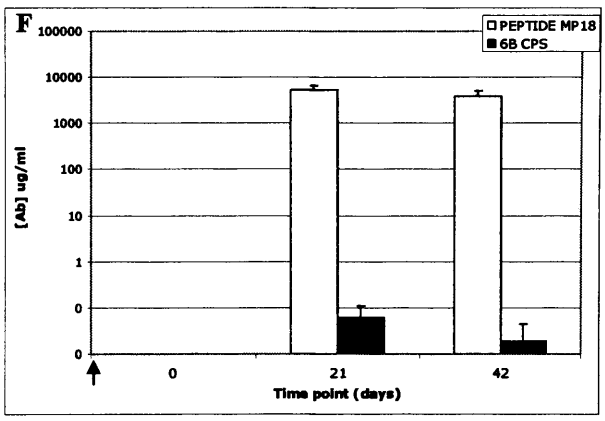












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3.8.5.4 Immunisation with MP10-KLH elicited antibodies with the highest avidity to the peptide and the strength of binding correlated to the ability to protect mice against pneumococcal disease.

Antibody avidity is a measure of the functional strength of serum antibody to bind to antigen. In this study, we compared the avidity of antibodies elicited by immunisation with seven peptide mimics of serotype 6B pneumococcal polysaccharide. The serum samples were obtained from mice immunised with MP2, MP10, MP13, MP14, MP15, MP17 or MP18 conjugated to keyhole limpet haemocyanin (KLH). Microtitre plates were coated with the respective BSA conjugated peptide. Sera were then added and bound antibodies were treated with a concentration gradient of ammonium thiocyanate (see §2.8.3) to dissociate low avidity antibody from the antigen-coated plates. The molar concentration of ammonium thiocyanate resulting in 50 % reduction in antigen binding (antibody concentration) was termed the avidity.

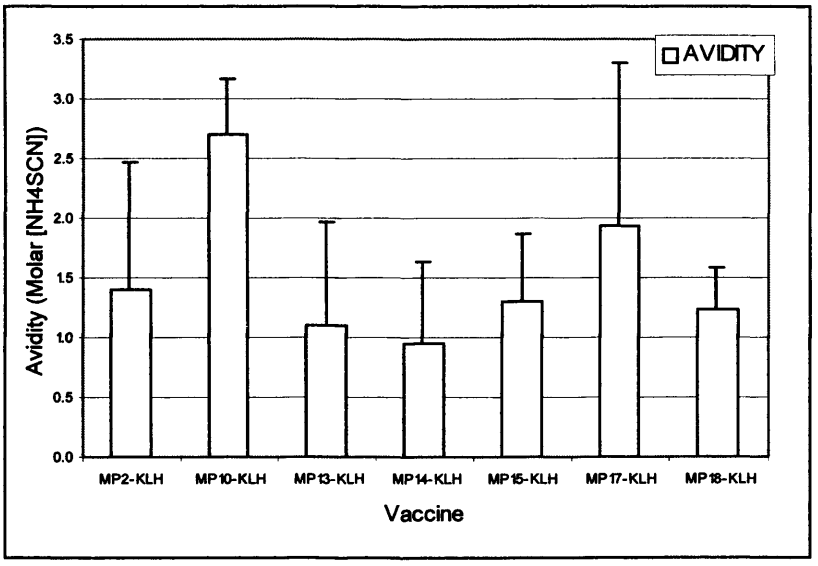

Figure 3.33. The average (mean) avidity of binding of anti-peptide specific mouse antibodies to MP2-KLH (n=10), MP10-KLH (n=30), MP13-KLH (n=30), MP14-KLH (n=18), MP15-KLH (n=30), MP17-KLH (n=10) or MP18-KLH (n=10) by treatment with ammonium thiocyanate. Serum was obtained from mice immunised with the peptide-conjugate and incubated in microtitre plates coated with peptide-BSA. The avidity of binding was determined by the addition of a concentration gradient of ammonium thiocyanate to dissociate low avidity antibody from the antigen-coated plates. The molar concentration of ammonium thiocyanate needed to dissociate 50% of the bound antibody was defined as the avidity (□). The error bars represent the standard deviation of the mean.

Figure 3.33 showed that mice immunised with MP10 evoked antibody of highest avidity of all the 6B mimics. The antibodies generated by immunisation with MP10-KLH bound to MP10-BSA with an average avidity of 2.71 M (n=30). The antibodies generated from immunisation with MP17-KLH also bound with high avidity to the peptide antigen. The average (mean) avidity was 1.93 M (n=10). The average (mean) antibody avidity to peptides MP2 (n=10), MP13 (n=30), MP15 (n=30) and MP18 (n=10) were similar at 1.17 M, 1.15 M, 1.32 M and 1.25 M respectively (see Figure 3.33).

Immunisation with MP14-KLH elicited the lowest mean antibody avidity of all the 6B mimics at 0.95 M. All the mice that received MP10-KLH developed antibody avidity greater than 1.0 M, whereas only 44 % (8/18) of the mice given MP14-KLH developed avidity values in excess of 1.0 M.

As described earlier (§3.6.4.5), previous studies have shown that the avidity of anti-pneumococcal capsular antibodies can be related to their ability to confer protection against disease (Usinger *et al* 1999). Mice immunised with MP2-KLH, MP10-KLH, MP14-KLH or MP17-KLH that generated antibodies with higher avidity showed an extended survival time compared to low-avidity antibody in survival assays. Table 4.5 shows that there was a positive correlation between a long survival time and high avidity antibodies for mice immunised with these peptides. The higher avidity antibodies generated from immunisation with MP13, MP15 and MP18 did not correlate with an extended survival time.

Table 3.7 shows the correlation coefficients for mice immunised with MP2 (n=10), MP10 (n=30), MP13 (n=29), MP14 (n=10), MP15 (n=29), MP17 (n=10) and MP18 (n=10). The positive correlation coefficients of 0.212, 0.172, 0.075 and 0.073 were observed for MP10, MP17, MP14 and MP2, respectively. The mice that produced antibodies that showed high avidity to the peptides MP13, MP15 and MP18 did not result an extended survival time.

Table 3.7 Correlation of mouse survival to antibody avidity

Vaccine	*Average avidity*	*Correlation with survival**
MP2-KLH	1.406 M	0.073
MP10-KLH	2.706 M	0.212
MP13-KLH	1.103 M	-0.112
MP14-KLH	0.951 M	0.075
MP15-KLH	1.298 M	-0.131
MP17-KLH	1.931 M	0.172
MP18-KLH	1.228 M	-0.341

* a table is shown instead of a graph (as shown in Part A) as the graph was unclear due to too many data.

3.8.5.5 Serum antibodies developed from immunisation with peptides MP2-KLH, MP10-KLH, MP13-KLH, MP14-KLH, MP17-KLH or MP18-KLH compete for binding of serotype 6B pneumococcal polysaccharide and the vaccine antigen equally.

To determine whether anti-peptide antibodies bound to serotype 6B polysaccharide, an ELISA was performed (as 3.8.5.3) except the serum was pre-incubated with either purified serotype 6B pneumococcal capsular polysaccharide, or *Escherichia coli* lipopolysaccharide (LPS) as a negative control.

Figure 3.34 shows the average (mean) percentage inhibition and standard deviation of antibodies to bind to peptides MP2-BSA, MP10-BSA, MP13-BSA, MP14-BSA, MP15-BSA, MP17-BSA or MP18-BSA by pre-adsorption to capsular polysaccharide. The display of inhibition indicated a mutual recognition of the antibody-binding site by both antigens and, therefore, the act of mimicry.

The inhibition recorded by serotype 6B pneumococcal capsular polysaccharide to all these peptides (with the exception of MP15) was significantly greater ($P < 0.05$, T-test) than when the serum was pre-absorbed to *E. coli* LPS (see Figure 3.34). Therefore, the

inhibition is specific to pneumococcal polysaccharide and MP2-BSA, MP10-BSA, MP13-BSA, MP14-BSA, MP17-BSA and MP18-BSA mimic serotype 9V pneumococcal polysaccharide. Antibodies generated by immunisation with MP17 displayed the greatest average (mean) inhibition at 52 %. The second highest was recorded for MP18 at 37 %, and then MP10 at 16 %. Antibodies generated by immunisation with MP2, MP13, MP14 and MP15 all displayed average (mean) inhibitions below that of MP10 (at 13 %, 11 %, 9 %, 2 %, respectively)

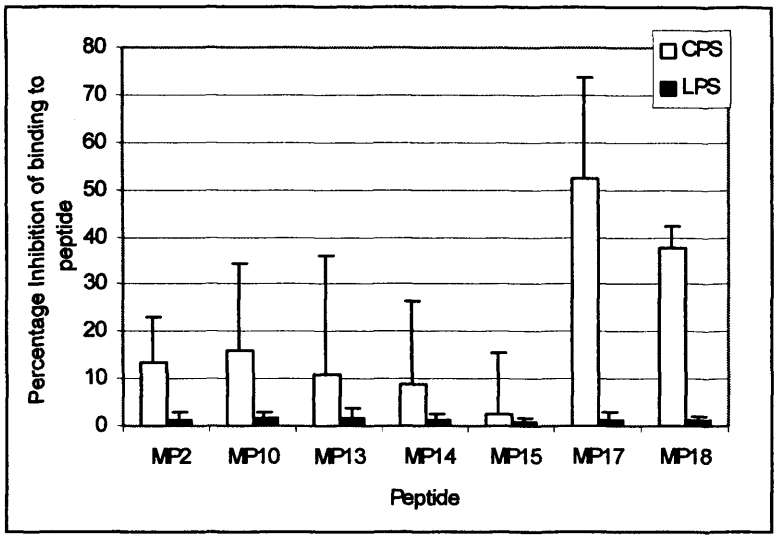

Figure 3.34. The average (mean) percentage inhibition of binding of anti-peptide specific mouse antibodies to MP2-BSA (n=10), MP10-BSA (n=30), MP13-BSA (n=30), MP14-BSA (n=18), MP15-BSA (n=30), MP17-BSA (n=10) or MP18-BSA (n=10) following pre-absorption with 6B polysaccharide. Serum was obtained from mice immunised with the peptide-conjugate and incubated with either 100 μg 6B polysaccharide (□) or *E. coli* lipopolysaccharide (■) prior to ELISA. Microtitre plates were coated with peptide-BSA to detect the presence of anti-peptide antibodies, prior to, and following the addition of polysaccharide. The percentage inhibition was determined from the difference in IgG concentrations recorded, divided by the absorbance recorded prior to addition of polysaccharide. The error bars represent the standard deviation of the mean.

3.8.6 Peptide Toxicity

3.8.6.1 Overview

This section of the thesis describes an investigation that followed an observation made during the course of immunisations with peptides MP2, MP14, MP17 and MP18. It was noticed that mice immunised with these peptides (conjugated to KLH) developed a severe reaction to the immunogen when the booster dose was administered.

To determine whether these adverse reactions were due to anaphylactic shock, we investigated the condition and immune response of mice immunised with MP2-KLH or with a modified MP2 peptide sequence. The latter peptide was identical to MP2, however the glutamic acid and aspartic acid residues were substituted for glutamine and asparagine, respectively. The reason for this decision is discussed in §5.5.1.1. The sequence of the modified peptide, known as MP2m was NH_2 –AEGEFWGPRVIF**Q**N**V**TVGDPAK – COOH (the modified residues are highlighted in boldface). The experiment was implemented using eight groups of mice, with each following a slightly different immunisation schedule (see §2.8.5 of the materials and methods). The doses received by each group and the result of anaphylaxis is recorded in Table 3.8.

Table 3.8 Groups of mice used to investigate peptide toxicity and the outcome of immunisation with two doses of antigen

Group	*1st dose*	*Treatment*	*2nd dose*	*Anaphylaxis*
V1	MP2-KLH	None	MP2-KLH	**YES**
V2	MP2-KLH	None	MP2m-KLH	NO
V3	MP2m-KLH	None	MP2m-KLH	NO
V4	MP2-KLH	None	6B PnCPS	NO
V5	MP2-KLH	Passive immunisation with mAb Db3G9	MP2-KLH	**YES**
V6	MP2-KLH	Orally dosing with cetirizine	MP2-KLH	**YES**
V7	MP2m-KLH	None	MP2-KLH	NO
V8	6B PnCPS	None	MP2-KLH	NO

3.8.6.2 Mice immunised with two doses of MP2 developed anaphylactic shock

The sera from the mice immunised with MP2-KLH were examined for the development of an IgE antibody response, and also for histamine production following injection of the second dose. Figure 3.35a shows that immunisation with MP2-KLH not only elicited a high IgG response, as was seen in §3.8.5.3, but the production of anti-peptide IgE was also provoked. Figure 3.35b shows that directly following the administration of the second dose of MP2, the serum histamine levels rise significantly ($P< 0.05$) compared to mice that received two doses of PBS alone. The histamine level was shown to peak at 11.58 nmol ml^{-1} 30 minutes after challenge compared to 3.6 nmol ml^{-1} in mice that received the control (PBS) dose. Those mice that survived displayed a significantly raised ($P< 0.05$) histamine level until at least 8 hours after the challenge was administered.

3.8.6.3 Immunisation with two doses of MP2m did not lead to anaphylaxis

To detect whether anaphylaxis resulted from the administration of the modified peptide, we looked for an increase in histamine production and a drop in body temperature in mice immunised with two doses of MP2m following the same schedule used for MP2. Figure 3.35 shows that those mice that were administered two doses of MP2 (group V1) presented increased histamine level (the highest recorded was 1312 nmol ml^{-1}) and also the lowest drop in body temperature (the lowest average difference in body temperature was -4.0°C). Those mice that received two doses of MP2m did not produce as much histamine (the mean average for groups V2, V3, V4, V7, and V8 was 94 nmol ml^{-1}) and consequently a significant difference ($P> 0.05$) in both histamine level and body temperature was recorded. The two graphs appear to mirror each other in their effects, confirming the results of the other.

3.8.6.4 Priming mice with mAb Db3G9 or the antihistamine cetirizine did not affect the onset of anaphylaxis in mice that received two doses of MP2

Figure 3.36 also shows that mice which received treatment with either the monoclonal antibody that selected MP2 from the phage library (group V5), or the antihistamine cetirizine (group V6) prior to challenge with a second dose of MP2 still developed diseases of anaphylaxis. There was no significant difference ($P< 0.05$) between the serum histamine concentration and difference in body temperatures recorded in mice from groups V5 and V6 when compared to V1. The three groups averaged a histamine level of 625 nmol ml^{-1} and a -2.8°C decrease in body temperature between them.

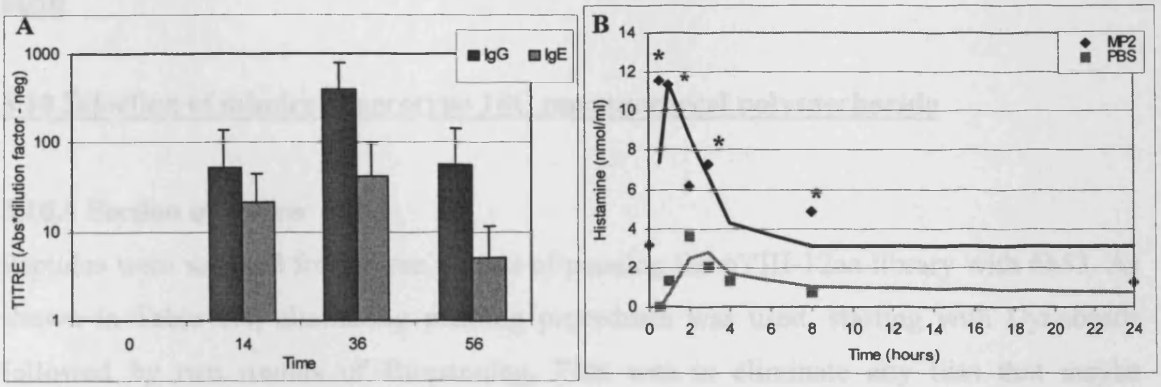

Figure 3.35 (A) The IgG and IgE antibody response in mice immunised with one dose of MP2 following the immunisation schedule outlined for MP2V4 in Table 2.7, N=10. **(B)** The histamine levels in mice immunised with MP2 following the injection of a second dose of the peptide. The graph shows that there was a significant increase (*) in the amount of histamine produced in mice that received two doses of peptide at each time point, compared to mice that recieved the immunisation solution minus the antigen, N=5.

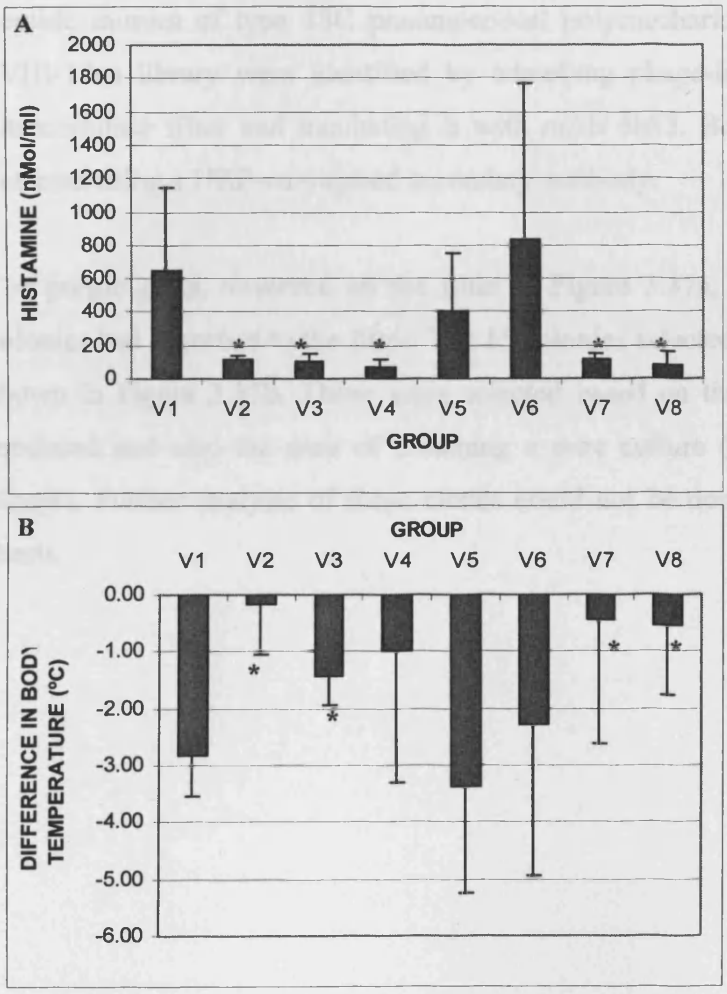

Figure 3.36. (A) The average (mean) histamine concentration in mice approximately 30 minutes after challenge with a second dose of antigen. N=5. (B) The average (mean) difference in body temperature in mice approximately 30 minutes after receiving a second dose of antigen. N=5. See Table 3.8 for details of groups. Significant differences from group V1 are shown by an asterix.

Part C: Mimics of type 18C pneumococcal polysaccharide selected using a human mAb

3.10 Selection of mimics of serotype 18C pneumococcal polysaccharide

3.10.1 Section overview

Peptides were selected from three rounds of panning the pVIII-12aa library with 6b53. As shown in Table 2.4, alternating panning procedures was used, starting with Dynabeads followed by two rounds of Biopanning. This was to eliminate any bias that maybe introduced from using a single technique (see §3.7.1 for more detail). Fifteen positive clones were identified from screening with mAb 6b53 by colony immunoscreening. Individual phage clones were purified by culture as described in §2.5.7. However, due to time restraints, no further analysis of these clones was performed.

3.10.2 Immunoscreening

Peptide mimics of type 18C pneumococcal polysaccharide that were selected from the pVIII-12aa library were identified by adsorbing phage-infected bacterial colonies to a nitrocellulose filter and incubating it with mAb 6b53. Bound antibody complexes were detected using a HRP-conjugated secondary antibody.

The purple spots, observed on the filter in Figure 3.37a, represented where the reactive colonies had adsorbed to the filter. The 15 colonies selected for further characterisation are shown in Figure 3.37b. Those were selected based on the strength of the pigment they produced and also the ease of obtaining a pure culture (i.e clearly separate from other clones). Further analysis of these clones could not be done within the time scale of this thesis.

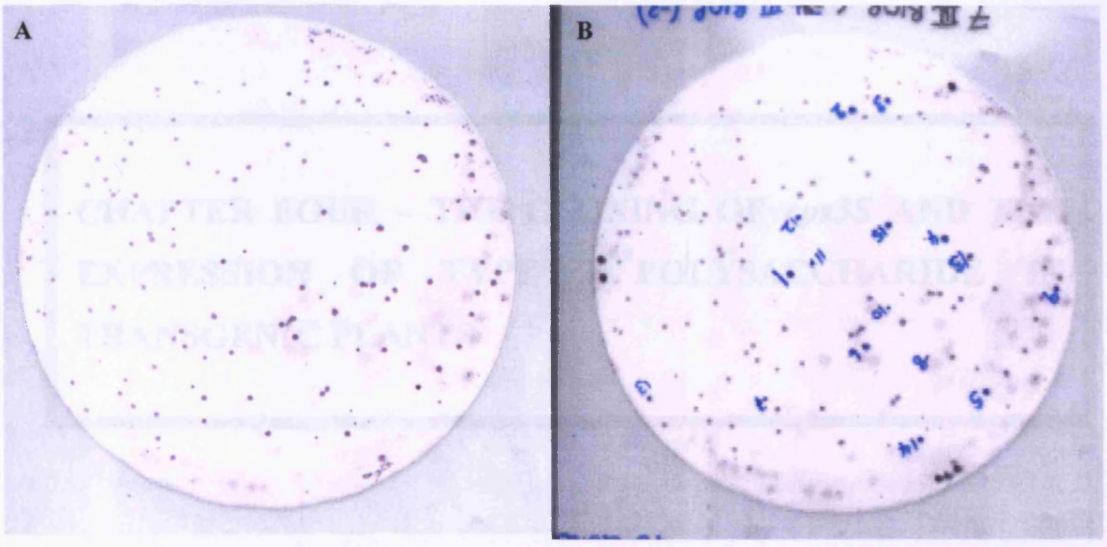

Figure 3.37. (A) shows the positive clones identified by phage infected *E. coli* colony immunoscreening with mAb 6b53. Positive clones are identified by the development of a purple pigment where the colony had adsorbed to the nitrocellulose filter. **(B)** shows the positive clones that were picked from the agar and used in subsequent experiments

CHAPTER FOUR – THE CLONING OF *cps3S* AND THE EXPRESSION OF TYPE 3 POLYSACCHARIDE IN TRANSGENIC PLANTS

4.0 Chapter Overview

This investigation aimed to reduce the cost of pneumococcal vaccine production by developing a transgenic plant capable of manufacturing pneumococcal polysaccharide. This chapter will show that we have been successful in completing our aim. Through cloning a copy of the type 3 capsule synthase gene (*cps3S*) into various bacterial and plant expression vectors we have shown the expression of the type 3 capsule synthase protein, Cps3S, by detecting specific mRNA, and we have detected the production of pneumococcal polysaccharide in transgenic plants. A list of all the plasmids made during this study was shown in Table 2.10.

4.1 Isolation of Genomic DNA and the PCR of *cps3S*

Genomic DNA from serotype 3 *S. pneumoniae* (strain WU2) was isolated as described (§2.10.1). This is shown in Figure 4.1a. This was used as a template to amplify the *cps3S* gene by PCR. Two different reactions were performed using Deep VentR DNA polymerase, a proof-reading polymerase, in the presence of either the primers CPSFOR and CPSREV, or CPSHIS and CPSREV, to amplify a 1330 bp fragment or a 1350 bp fragment respectively. The latter would incorporate a histidine-tag to the C-terminal of the expressed protein (*cps3S-HIS*). The reactions were allowed to continue for either 10 or 30 cycles, a further measure to limit the possibility of incurring errors in the final product. Those PCR reactions that were allowed to continue for 30 cycles consistently amplified a band of the correct size (~1330 bp). This is visible on lanes 3 (*cps3S*) and 6 (*cps3S-HIS*) of Figure 4.1b. Those that were allowed to run for 10 cycles (Figure 4.1b lanes 1 and 2, and 4 - 5) showed an undetectable amount of DNA after agarose gel electrophoresis. However, it was assumed that the 10-cycle reaction was successful as it was run simultaneously to the 30-cycle experiment.

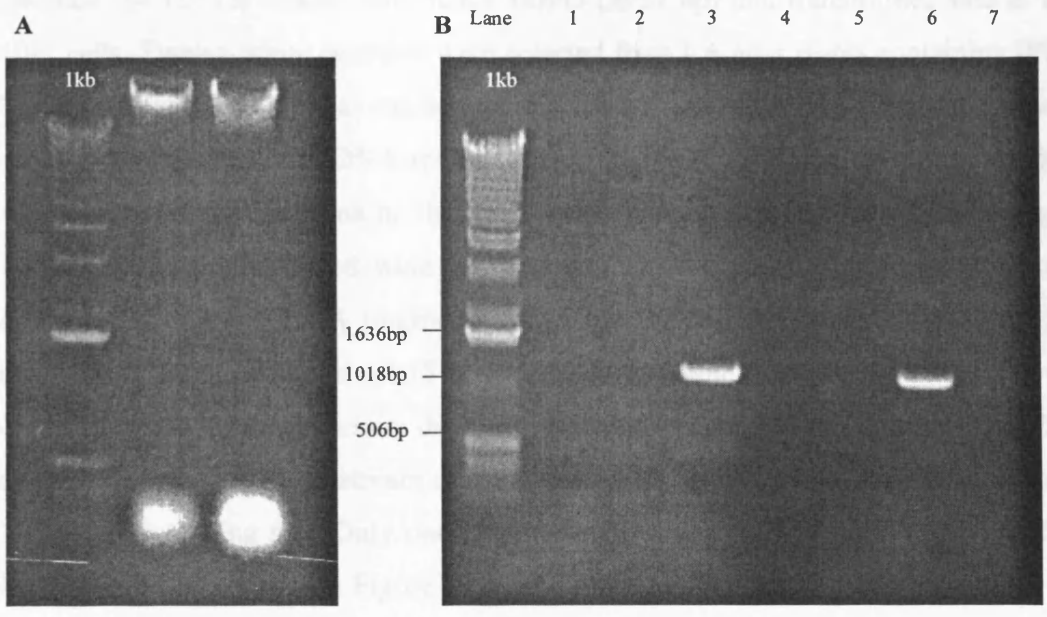

Figure 4.1 Isolation of genomic DNA and PCR of *cps3S*. (A) shows two samples of genomic DNA extracted from serotype 3 pneumococcus (strain WU2). Both lanes contain extracted genomic DNA. The DNA is shown to be intact as there is one high MW band shown and no smearing, indicating the DNA was not sheared during extraction. **(B) shows the amplified DNA fragments from different PCR reactions.** Lanes 1-3 correspond to PCR reactions in the presence of CPSFOR and CPSREV primers, to obtain a fragment of about 1330 bp. Lane 3 underwent 30 cycles and lanes 1-2, 10 cycles of the same reaction. Lanes 4-6 correspond to PCR reactions in the presence of CPSHIS and CPSREV primers, to obtain a fragment of about 1350 bp. Lane 6 underwent 30 cycles and lanes 4 and 5, 10 cycles of the same reaction. Lane 7 is the negative control, where no DNA template was used in the PCR reaction and no amplified products were observed. A 1Kb ladder (Gibco) was used as a molecular size marker in both gels.

4.2 Cloning *cps3S*

4.2.1 Cloning into pTOPO

The *cps3S* gene (~1330 bp) obtained by PCR from the genomic DNA of serotype 3 *S. pneumoniae* (§4.1), was cloned into pCR4-TOPO (3957 bp) and transformed into *E. coli* Top10F' cells. Twelve white colonies were selected from LA agar plates containing IPTG, X-Gal and ampicillin. DNA was extracted from the colonies by a small-scale preparation and the recombinant plasmid DNA (pCMS1) was digested using *Kpn* I and *Pst* I, which was expected to cut regions flanking the *cps3S* gene. The gel of this digest is shown Figure 4.2 and the fragments obtained were as expected: Lane 1 contains undigested plasmid DNA. Lane 2 shows two DNA fragments of 4 Kbp (pCR4-TOPO) and 1.3 Kbp (*cps3S*). This was because the amplified *cps3S* gene contained a unique site for *Kpn* I, which was incorporated by the CPSFOR primer during PCR. pTOPO contained a unique site for *Pst* I, which was located 20 bp downstream of the 3' end of the *cps3S* gene and derived from the pUC18 multiple cloning site. Only one clone was shown to contain both pTOPO and the *cps3S* fragments as shown in Figure 4.2. The plasmid containing the *cps3S* gene was termed pCMS1.

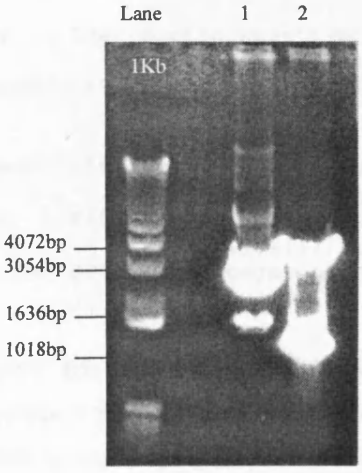

Figure 4.2. The digestion products of pCMS1 with *Kpn* I and *Pst* I. The sizes of the DNA fragments obtained on the gel confirm the cloning of *cps3S* into pCR4-TOPO. Lane 1 contains uncut pCMS1. Lane 2 contains the expect fragments of ~1330 bp (*cps3S*) and 3957 bp (pCR4-TOPO). A high concentration of DNA is observed as DNA extracted from this gel was used for sub-cloning into pCHF2. A 1Kb ladder (Gibco) was used as a molecular size marker.

4.2.2 The nucleotide sequence of the amplified *cps3S* gene

The DNA sequence of the PCR amplified *cps3S* gene was determined using pCMS1 and the commercially available primers T7 and SP6 which sequenced across the MCS of the pCR4-TOPO vector, which contained the amplified *cps3S* PCR product. The sequence obtained is shown in Figure 4.4.

The results showed a 1251 bp DNA fragment was amplified from PCR. This is 3 bp longer than the reported sequence for *cps3S*, at 1248 bp (Dillard *et al.* 1995). When the sequences were aligned mismatches in nucleotides only appeared in a region between 667 bp - 704 bp. The alignment is shown in Figure 4.3.

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cps3S: 629  aagtagggtgcttacctggtcgaacaattgcttttagaa-atatagt-ggagagagtgta 686
            |||||||||||||||||||||||||||||||||||||||| | | | |   ||||||||||
PCR  : 666  aagtagggtgcttacctggtcgaacaattgcttttagaacagagattctcagagagtgta 725
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cap3B: 629  aagtagggtgcttacctggtcgaacaattgcttttagaacagagattctcagagagtgta 688

cps3S: 687  taca-aagtttatagaagagactttcatgggatttcataaggaagtttctgatgatagaa 745
            ||||  |||||||  | ||||||||||||||||||||||||||||||||||||||||||
PCR  : 726  tacatgagtttatgaatgagactttcatgggatttcataaggaagtttctgatgatagaa 785
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cap3B: 689  tacatgagtttatgaatgagactttcatgggatttcataaggaagtttctgatgatagaa 748

cps3S: 746  gtcttacaaatttgactttnnnnnnnggctataaaactgttatgcaggatacttctgttg 805
            ||||||||||||||||||        ||||||||||||||||||||||||||||||||
PCR  : 786  gtcttacaaatttgactttaaaaaaaggctataaaactgttatgcaggatacttctgttg 845
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cap3B: 749  gtcttacaaatttgactttaaaaaaaggctataaaactgttatgcaggatacttctgttg 808

cps3S: 806  tgtatacagatgctcctacaagttggaaaaagttcattagacagcaactaaggtgggcag 865
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
PCR  : 846  tgtatacagatgctcctacaagttggaaaaagttcattagacagcaactaaggtgggcag 905
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
cap3B: 809  tgtatacagatgctcctacaagttggaaaaagttcattagacagcaactaaggtgggcag 868
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Figure 4.3. Multiple sequence alignment of the nucleotide sequence from a region of the serotype 3 *Streptococcus pneumoniae* capsular synthase gene. The alignment compares the 629 bp – 868 bp region of *cap3B* (Genbank Z47210) (Arrecubieta *et al.* 1995), the 666 bp – 905 bp region of the amplified PCR product (PCR) using primers designed against the *cps3S* sequence and the 629 bp – 865bp region of *cps3S* (Genbank U15171) (Dillard *et al.* 1995). The flanking regions of all three sequences shared 100 % identity and so are not shown in this diagram.

To eliminate the possibility that the mismatches were due to mutations introduced during PCR, several attempts at amplification were done. The products were ligated into pCR4-TOPO (as before) and the clones were sequenced. Also, an additional *cps3S* primer, CPSMID, was constructed to sequence the middle region of the *cps3S* gene. These results confirmed the sequence shown in Figure 4.3.

Variations in the nucleotide sequence of the serotype 3 capsule synthase gene have been reported (Arrecubieta *et al.* 1995; Dillard *et al.* 1995). The sequences obtained from these two studies, *cps3S* (Dillard *et al.* 1995) and *cap3B* (Arrecubieta *et al.* 1995) were taken from two different strains of serotype 3 pneumococci, WU2 and 406, respectively. In this study, we amplified the *cps3S* gene from the genomic DNA of strain WU2 and, therefore, expected the sequence to be identical to that of Dillard and colleagues. However, as shown in Figure 4.3, when the sequence was aligned to both sequences *cps3S* (GenBank accession number U15171) and *cap3B* (GenBank accession number Z47210) it displayed 100 % identity to the sequence reported by Arrecubieta *et al.* (1995) and the sequence variation from *cps3S*, reported by Arrecubieta and colleagues was consistent with our findings.

The complete DNA and predicted amino acid sequence of the Cps3S (as a single open reading frame) derived from the cloned gene is shown in Figure 4.4. The protein has been shown to share significant homology to a number of processive β-glycosyltransferases from prokaryotes and eukaryotes (see §1.7.3). These enzymes form polymers composed of β(1-4) linked repeating disaccharide units and include the cellulose synthases of plants (Dillard *et al.* 1995; Keenleyside and Whitfield 1996; Weigel *et al.* 1997). Figure 4.4 shows that the conserved domains of the processive β-glycosyltransferases were represented in the *cps3S* sequence of pCMS1. These are highlighted in boldface and those residues that are indicated by shaded boxes were 100 % conserved between the species previously described (Dillard *et al.* 1995; Keenleyside and Whitfield 1996; Weigel *et al.* 1997). All of the conserved residues described were present in our sequence and therefore, the protein was expected to function as reported previously (Dillard *et al.* 1995; Keenleyside and Whitfield 1996; Weigel *et al.* 1997). Consequently it was decided to proceed with the further cloning of *cps3S*.

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   1                                                M  Y  T  F  I  L  M  L  L  D  F  F  Q  N  H  D  F  H
   1  CTGGTACCATGTATACATTTATTTTAATGTTGTTGGATGTATACATTTATTTTAATGTTGTTGGATTTTTTTCAGAATCATGATTTTCAT
        Kpn I                                      ***

  19   F  F  M  L  F  F  V  F  I  L  I  R  W  A  V  I  Y  F  H  A  V  R  Y  K  S  Y  S  C  S  V
  91  TTCTTTATGTTGTTTTTTGTCTTTATTCTTATTCGTTGGGCGGTTATATATTTTCATGCTGTCAGATATAAGTCCTACAGTTGTAGTGTA

  49   S  D  E  K  L  F  S  S  V  I  I  P  V  V  D  E  P  L  N  L  F  E  S  V  L  N  R  I  S  R
 181  AGTGATGAGAAGTTATTTAGTTCTGTAATTATCCCTGTCGTGGATGAACCACTTAATCTTTTTGAAAGTGTACTGAATAGAATTTCCAGA

  79   H  K  P  S  E  I  I  V  V  I  N  G  P  K  N  E  R  L  V  K  L  C  H  P  F  N  E  K  L  E
 271  CATAAACCATCCGAAATTATTGTGGTTATTAACGGCCCAAAAAACGAGAGACTTGTAAAACTTTGTCATGATTTTAATGAAAAATTAGAA

 109   N  N  M  T  P  I  Q  C  Y  Y  T  P  V  P  G  K  R  N  A  I  L  F  G  L  E  H  V  D  S  Q
 361  AATAATATGACTCCAATTCAATGTTATTACACTCCTGTTCCTGGCAAGAGAAATGCTATCCTCTTTGGGCTGGAGCATGTGGATTCGCAG

 139   S  D  I  T  V  L  V  P  S  D  T  V  W  T  P  R  T  L  S  E  L  L  K  P  F  V  C  D  K  K
 451  AGTGATATTACAGTTCTAGTAGATAGTGATACAGTATGGACGCCTAGAACCTTGAGTGAGTTGCTGAAGCCTTTTGTTTGCGATAAAAAA

 169   I  G  G  V  T  T  R  Q  K  I  L  D  P  E  R  N  L  V  T  M  F  A  N  L  L  E  E  I  R  A
 541  ATAGGTGGGGTAACGACAAGACAAAAAATTCTTGACCCTGAGCGTAATCTCGTGACAATGTTTGCTAACTTGTTAGAGGAAATTAGGGCA

 199   E  G  T  M  K  A  M  S  V  T  G  K  V  G  C  L  P  G  R  T  I  A  F  R  T  E  I  L  R  E
 631  GAAGGAACTATGAAAGCAATGAGTGTGACTGGTAAAGTAGGGTGCTTACCTGGTCGAACAATTGCTTTTAGAACAGAGATTCTCAGAGAG

 229   C  I  H  E  F  M  N  E  T  F  M  G  F  H  K  E  V  S  D  P  R  S  L  T  N  L  T  L  K  K
 721  TGTATACATGAGTTTATGAATGAGACTTTCATGGGATTTCATAAGGAAGTTTCTGATGATAGAAGTCTTACAAATTTGACTTTAAAAAAA

 259   G  Y  K  T  V  M  Q  D  T  S  V  V  Y  T  P  A  P  T  S  W  K  K  F  I  R  Q  Q  L  R  R
 811  GGCTATAAAACTGTTATGCAGGATACTTCTGTTGTGTATACAGATGCTCCTACAAGTTGGAAAAAGTTCATTAGACAGCAACTAAGGTGG

 289   A  E  G  S  Q  Y  N  N  L  K  M  T  P  W  M  I  R  N  A  P  L  M  F  F  I  Y  F  T  D  M
 901  GCAGAAGGTTCTCAGTATAACAATCTAAAGATGACTCCTTGGATGATTAGAAATGCCCCTCTTATGTTTTTTATTTATTTTACAGATATG

 319   I  L  P  M  L  L  I  S  F  G  V  N  I  F  L  L  K  I  L  N  I  T  T  I  V  Y  T  A  S  W
 991  ATTTTACCTATGCTACTTATTAGCTTTGGTGTGAATATATTCCTGTTGAAAATATTAAATATAACTACAATTGTTTATACAGCTTCATGG

 349   W  E  I  I  L  Y  V  L  L  G  M  I  F  S  F  G  G  R  N  F  K  A  M  S  R  M  K  W  Y  Y
1081  TGGGAAATTATTTTATATGTTCTTTTGGGAATGATTTTTAGCTTTGGAGGAAGAAACTTTAAAGCTATGTCTAGAATGAAGTGGTATTAT

 379   V  F  L  I  P  V  F  I  I  V  L  S  I  I  M  C  P  I  R  L  L  G  L  M  R  C  S  D  D  L
1171  GTATTTCTTATTCCTGTTTTTATAATCGTTTTGAGTATAATTATGTGCCCTATTAGGCTATTAGGACTTATGAGATGTTCTGATGATTTA

 409   G  W  G  T  R  N  L  T  E  STOP
1261  GGGTGGGGAACTAGGAATTTAACAGAGTGATGAAAGGGCGAATTCGTTTAAACCTGCAG
                                ***       EcoR I        Pst I
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Figure 4.4. Nucleotide sequence of the *cps3S* gene obtained by PCR and the predicted amino acid sequence of the Cps3S protein. Important restriction sites are double underlined. The start and the stop codons are indicated by ***. The sequence prior to the *cps3S* start codon derives from the forward primer (CPSFOR) used to clone *cps3S* into pCR4-TOPO. The sequence following the *cps3S* stop codon derives from the pCR4-TOPO vector. Both sequences contain restriction sites that were used for future cloning work. The conserved residues identified in the processive β-glycosyltransferases are indicated in boldface. Residues that are 100 % conserved between the species described are indicated by shaded boxes.

The restriction sites that were used for future cloning work are also shown in Figure 4.4. The *Kpn* I restriction site at the 5' end of the *cps3S* gene was introduced by the sense primer CPSFOR. Two restriction sites at the 3' end of the *cps3S* gene were used for cloning into different expression vectors. *Pst* I was used to clone *cps3S* into pCHF2 and *EcoR* I was used to clone the gene into pRSET C. Both *Pst* I and *EcoR* I sites derived from the MCS of pCR4-TOPO.

4.2.3 Sub-cloning *cps3S* into pCHF2

To analyse the expression and function of the Cps3S protein *in planta*, the *cps3S* gene was cloned into the plant expression vector, pCHF2. This vector, as described in §2.11.2.1, contains, within the T-DNA, a duplicated CaMV35S promoter upstream of the transgene to drive strong constitutive expression of the transgene in plant cells. It also has a PR1B signal sequence-coding region to direct secretion of the transgene product via the endoplasmic reticulum to the apoplast. Also, expression of the spectinomycin resistance gene, *aadA*, allowed selection of transformed bacteria and gentamicin resistance was conferred to transformed plants.

The *cps3S* gene was cut from pCMS1 using the restriction enzymes *Kpn* I and *Pst* I. The unique *Kpn* I site of pCMS1 was 36 bases upstream of the start codon of the *cps3S* gene. The unique *Kpn* I site of the pCHF2 vector was 864 bp downstream of the 5' end of the 35S promoter sequences. This 900 bp difference ensured the promoters, PR1B sequence and *cps3S* were in the same reading frame.

To ensure the *cps3S* fragment, from pCMS1, (1331 bp) was ligated to pCHF2 in the correct orientation, the pCHF2 vector (10218 bp) was also cut with *Kpn* I and *Pst* I. The *cps3S*-containing fragment (1331 bp) was extracted from the agarose-gel (see §2.10.5.1) and ligated to the gel-extracted 10.2 Kbp linear, pCHF2 vector fragment carrying the same sticky ends. This was performed at a molar ratio of 1:3 (vector:insert). The ligation product was transformed into *E. coli* Top10F' by heat shock. Twelve spectinomycin resistant colonies were selected from LA agar plates containing spectinomycin and plasmids were isolated by a small-scale DNA extraction. To screen for positive clones, plasmid DNA was digested with *Kpn* I and *Pst* I and analysed on a 1.5 % (w/v) TAE-agarose gel. Four clones were shown to contain both pCHF2 and the *cps3S* DNA fragments following digestion. The digested DNA of one of these clones is shown in Figure 4.5.

The fragments shown in Figure 4.5 are as expected: two DNA fragments, ~1330 bp containing the *cps3S* gene and a 10.2Kbp band (lane 2) of the cloning vector. Lane 1 contains undigested plasmid DNA. The chosen plasmid was named pCMS3 (total size 11522 bp). Figure 4.6 shows the restriction sites that were used for identification and for future cloning work in the pCMS3 plasmid.

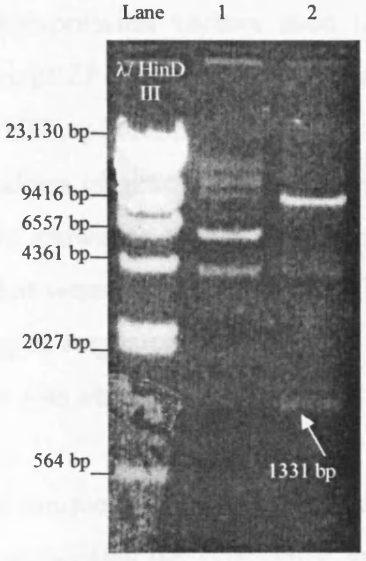

Figure 4.5. The digested products of a single transformed clone containing pCMS3 digested with *Kpn* I and *Pst* I. Lane 1 contains uncut plasmid and Lane 2 contains digested DNA. The sizes of the DNA fragments obtained on the gel confirm the cloning of *cps3S* (1331 bp) into pCHF2 (10.2 Kbp). A λ/*HinD* III ladder was used as a molecular size marker.

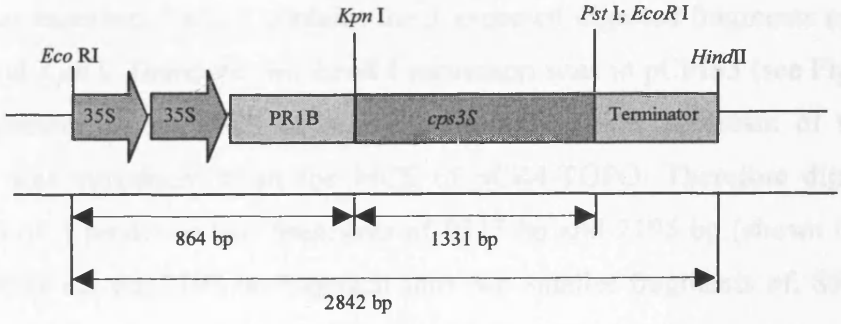

Figure 4.6. The cloning site of *cps3S* into the T-DNA region of the vector pCHF2 to form pCMS3. A duplicated CaMV35S promoter (35S), PR1B signal sequence and RBCS terminator (from the pea) are shown. The restriction endonucleases cutting sites that were used for identification and for cloning work are also displayed together with the resulting fragment sizes produced by digestion. See Figure 2.7 for a diagram of the complete T-DNA region of the pCHF2 vector.

4.2.4 Sub-cloning *cps3S* into pCambia 2301

The identification of transformed plants by kanamycin selection (expression of the *nptII* gene in pCambia 2301) is technically easier than gentamicin selection (which was conferred by transformation with pCMS3). This was thought to be because gentamicin can be toxic to plants cells (see §5.9.3.1). For this reason *cps3S* was also cloned into the plant expression vector pCambia 2301.

Both the plant expression vectors used in this study, pCHF2 and pCambia 2301, are derived from the pPZP family of *Agrobacterium* binary vectors (Hajdukiewicz *et al.* 1994). But unlike pCHF2, pCambia 2301 has a *gusA* gene within the T-DNA region, which allows easy analysis of gene function or presence in plants by glucuronidase (GUS) assay (see Figure 5.9). However, pCMS3 contained useful tools for the expression of the *cps3S* gene product that were absent in pCambia 2301, including duplicate CaMV35S promoters and a PR1B signal sequence (as discussed in §4.2.3). Therefore, the CaMV35S promoters and PR1B were also cloned into pCambia 2301.

The number of unique restriction endonuclease sites in both pCambia 2301 and pCMS3 was limited. To maintain the orientation and reading frames, pCMS3 was digested twice with either *EcoR* I and *Kpn* I, or *Kpn* I and *HinD* III. The products of these digests were analysed on a 0.7 % (w/v) TAE-agarose gel. As shown in Figure 4.7, the fragments obtained were as expected: Lane 1 contains the 3 expected digested fragments of pCMS3 using *EcoR* I and *Kpn* I. There are two *EcoR* I restriction sites in pCMS3 (see Figure 4.6); the first was present in the MCS of pCHF2, the second site, upstream of the *Pst* I restriction site was introduced from the MCS of pCR4-TOPO. Therefore digestion of pCMS3 with *EcoR* I produced two fragments of 9327 bp and 2195 bp (shown on Figure 4.7). *Kpn* I further cut the 2195 bp fragment into two smaller fragments of, 864 bp and 1331 bp, both of which are shown on Figure 4.7. The 864 bp fragment contained the promoters and signal sequence and this fragment was used for cloning into pCHF2.

In Figure 4.7, lane 2 shows the two fragments of pCMS3 produced from digestion with *Kpn* I and *HinD* III. These restriction sites are single cutters (unique) to pCMS3 and the 2 fragments obtained were as expected: 1978 bp containing the *cps3S* gene and terminator sequences, and 9544 bp of the linearised plasmid.

pCambia 2301 vector (11.5 Kbp) was cut with *EcoR* I and *HinD* III. Lane 3 shows the single band obtained, as expected. Both of these restriction sites are unique to pCambia 2301 but the smaller fragment (50 bp) produced was undetectable on the gel.

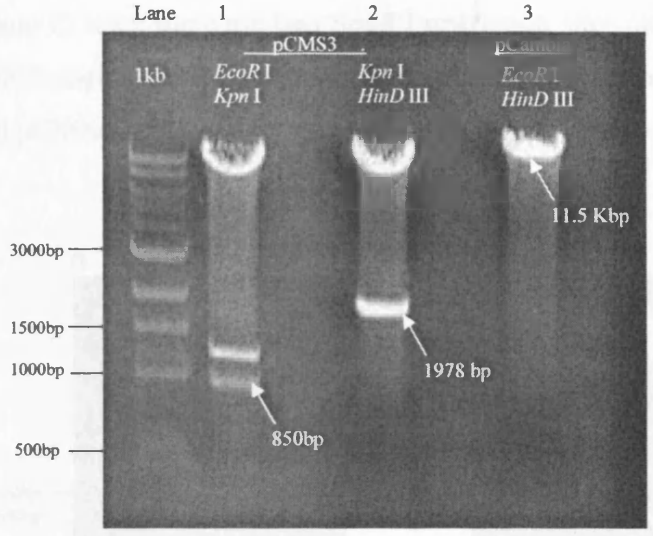

Figure 4.7. The digested products of three separate restriction digests. Lane 1 shows the expected 3 fragments of pCMS3 when digested with *EcoR* I and *Kpn* I. Lane 2 shows the expected 2 fragments produced from the digestion of pCMS3 with *Kpn* I and *HinD* III. Lane 3 shows the expected band of 11.5 Kbp when pCambia 2301 was digested with *EcoR* I and *HinD* III. The DNA fragments of 864bp (Lane 1) and 1978bp (Lane 2), indicated by the arrows, were used to reconstruct the pCMS3 T-DNA fragment containing *cps3S* and these were ligated to the 11.5Kb pCambia fragment (Lane 3). This plasmid was termed pCMS4. Undigested DNA is not shown. A 1Kb ladder (New England Biolabs) was used as a molecular size marker.

The 864 bp and 1978 bp pCMS3 fragments (from lane 1 and 2, (Figure 4.7) respectively), indicated by the arrows, were then re-ligated via the *Kpn* I site and inserted into a cut pCambia 2301, which had complementary *EcoR* I and *HinD* III sticky ends. The ligation product was transformed into *E. coli* DH5α by electroporation and selected on LA containing kanamycin. Twelve kanamycin resistant colonies were screened by colony PCR, using the CPSFOR and CPSREV primers (see §2.10.4.2) and were analysed on a 0.7 % (w/v) TAE-agarose gel. The expected band of ~1330 bp from one clone is shown in lane 1 of Figure 4.8. Lane 2 is the negative control, where no DNA template was used in the PCR reaction and no bands were identified on the gel.

The DNA from ten colonies produced a 1.3 Kbp PCR product using the CPSFOR and CPSREV primers, and plasmids from these colonies were prepared by small-scale purification (§2.10.2.1). The extracted DNA was digested with *EcoR* I and analysed on an agarose gel. The gel of one clone is shown in Fig 4.8b. Lane 1 contains un-digested DNA and lane 2 contains the 2 expected digested fragments: ~2.2kb and 11.5Kb. Two fragments were observed (lane 2) since there are two *EcoR* I restriction sites present in pCMS3, each flanking the T-DNA region. These results confirmed correct construction of the plasmid, which was named pCMS4.

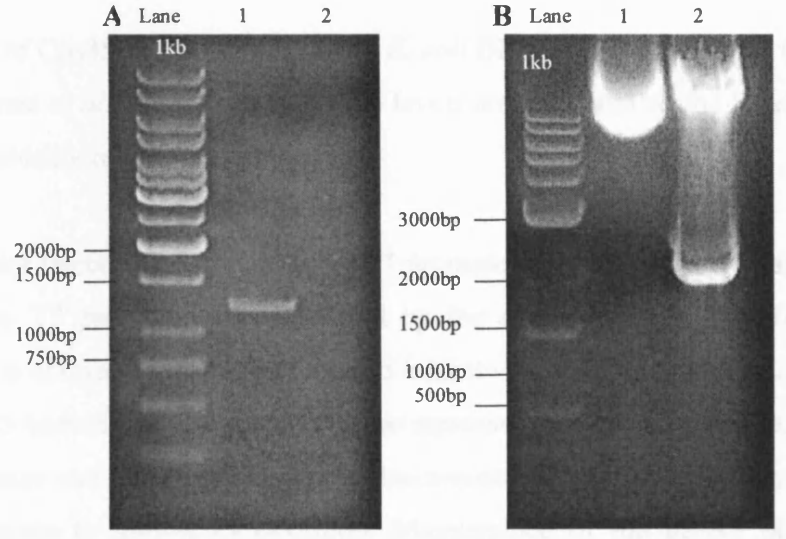

Figure 4.8. (A) The amplified product of *cps3S* by PCR using pCMS4. Lane 1 shows the amplified fragment of ~1330 bp from the DNA of a clone containing the pCMS4 plasmid. Lane 2 contains no amplified product as no template was used in the reaction. A 1 Kb ladder (Fermentas) was used as a marker. **(B) The digested products of pCMS4 cut with *EcoR* I.** Lane 1 contains uncut plasmid and Lane 2 contains digested DNA. The sizes of the DNA fragments obtained on the gel confirm the cloning of *cps3S* (~2.2 Kbp) into pCambia 2301 (11.5 Kbp). A 1 Kb ladder (NEB) was used as a molecular size marker.

The pCMS4 plasmid was prepared by large-scale DNA extraction and cloned into *A. tumefaciens* strain C58 by heat shock. pCMS4 was also cloned into *A. rhizogenes* strain LBA9402 by triparental mating. Transformed *A. tumefaciens* and *A. rhizogenes* were screened for kanamycin resistance on YEP or YMB agar respectively and by PCR using CAMFOR and CAMREV primers. Those kanamycin resistant clones containing the

pCMS4 plasmid produced an amplified product of 2500 bp (as shown in Figure 4.13b). Plant transformations with pCMS4 results are discussed later in §4.3.1.2 and §4.3.2.2

4.2.5 Sub-cloning *cps3S* into pRSET C

The *cps3S* gene was inserted into the *E. coli* expression plasmid pRSET C. As discussed in §2.11.2.3, bacterial cell cultures develop faster than whole plant culture and have greater homogeneity. Consequently, more cells are in direct contact with the antibiotic selection in the culture medium. This is a quicker, cleaner, more sensitive system for analysing protein expression.

The synthesis of Cps3S has been reported in *E. coli* BL21(DE3)pLysS cells (Arrecubieta *et al.* 1996a; Cartee *et al.* 2001), however high levels are toxic and so the level of expression of *cps3S* was closely regulated.

pRSET C vector contained a strong phage T7 promoter, which controlled expression of the transgene. The T7 promoter was regulated by the expression of T7 RNA polymerase, which in turn is controlled by a repressor and inhibitor present in *E. coli* BL21(DE3)pLysS cells. The DE3 bacteriophage contained a *lac* repressor, which repressed expression of T7 RNA polymerase and the pLysS plasmid, also present, produced T7 lysozyme that bound to T7 polymerase to inhibit transcription. Maintenance of the pLysS plasmid was by chloramphenicol selection and the addition of IPTG controlled expression of the *cps3S* gene.

pCMS4 was prepared by a large-scale DNA extraction and digested with *EcoR* I and *Kpn* I. These restriction sites flank the *cps3S* gene and avoided cloning the CaMV35S promoters into pRSET C. These were not wanted because they would drive expression of *cps3S* independently of the T7 regulators. pRSET C was digested with the same enzymes and the two fragments were ligated together. The ligation mixture was then used to transform *E. coli* BL21(DE3)pLysS cells by heat shock. Transformed cells were selected on LA containing chloramphenicol and ampicillin (to maintain pRSET C).

The plasmid DNA from 9 independent clones was prepared by small-scale purification. Clones containing the correctly constructed pCMS5 plasmid were detected by PCR using the pRSET multiple cloning site (MCS)-specific primers: PRSET-REV and T7. The PCR

results of 5 clones are shown in Figure 4.9. Only one clone was shown to contain the *cps3S* gene (lane 2).

To obtain a PCR product larger than the multiple cloning site of pRSET C (42 bp) the *cps3S* gene must have been inserted. Lanes 3 - 6 of Figure 4.9 show that these clones contain re-ligated pRSET vector, as only a very small fragment (42 bp) was amplified. However, the PCR reaction in lane 2 showed an amplified fragment of ~1.3 Kbp and therefore, *cps3S* was present. The plasmid from this clone was named pCMS5.

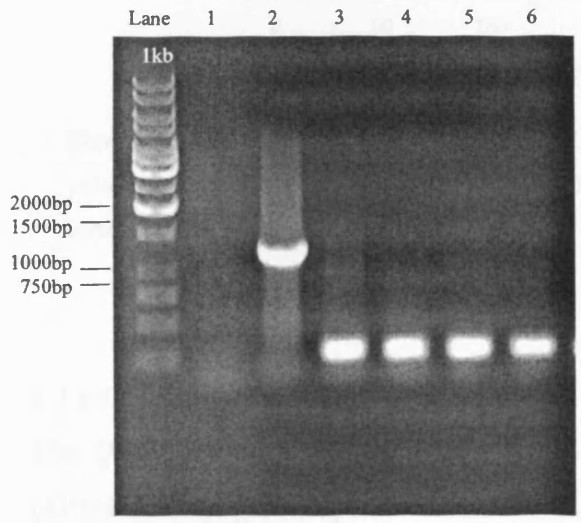

Figure 4.9. The PCR results for detecting the presence of *the cps3S* gene in pRSET C. Lane 1 shows the result of a PCR reaction using PRSET-REV and T7 (primers specific to read the MCS of pRSET C) where no DNA template was used. Lanes 2 – 6 show the results of the same PCR reaction when small-scale prepared plasmid DNA from clones transformed with a pCMS5 ligation mixture. It shows one clone contains the *cps3S* insert (Lane 2) clones in lanes 3 - 6 are only transformed with the vector. A 1Kb ladder (Fermentas) was used as a molecular size

4.2.5.1 Growth of bacteria expressing the Cps3S transgene

The growth of *E. coli* carrying pCMS5 was measured and it was found that 80 minutes after induction with IPTG, bacterial growth was significantly ($P < 0.05$) slowed. This implies that either IPTG or the synthesised protein is toxic to some *E. coli* cells. Arrecubieta and colleagues came to a similar conclusion (Arrecubieta *et al.*, 1996) although unlike these workers it has been shown here that this is not a permanent situation, as after a further 106 minutes (just over two replication cycles) of displaying a significant difference ($P < 0.05$) to the growth of the non-induced culture, the OD began to increase notably, to a level not significant from the non-induced cultures ($P > 0.05$). These results are shown in Figure 4.10.

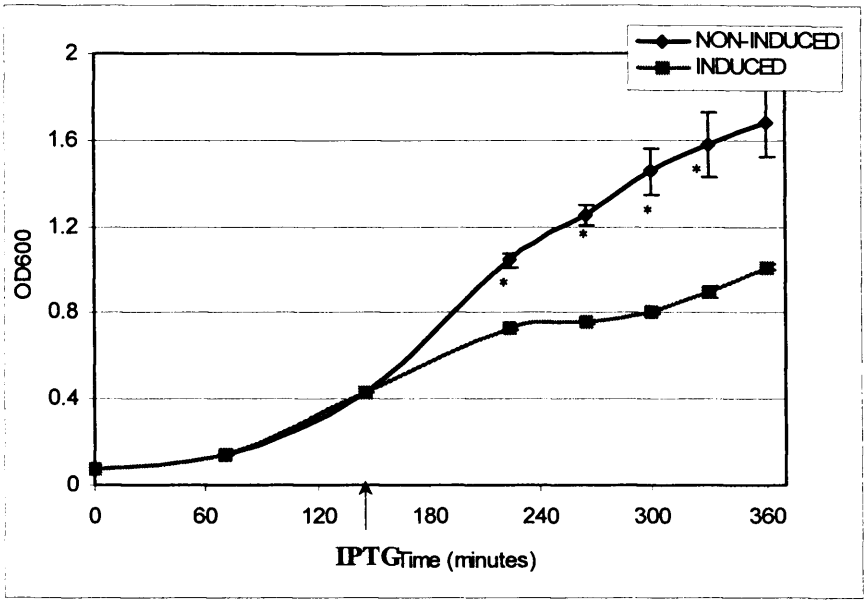

Figure 4.10. The growth curve of *E. coli* BL21(DE3)pLysS pCMS5. The asterix identify time points that show a significant difference ($P < 0.05$) between the growth of the induced and non-induced bacterial cultures.

4.2.6 Cloning *cps3S-HIS* into pGEM

The DNA encoding a His-tagged Cps3S (~1350 bp) obtained in §4.1, was cloned in pGEM-T Easy (3015 bp) and transformed into *E. coli* DH5α by heat shock. This was because the pGEM-T Easy vector is a suitable system for cloning PCR products and many useful restriction sites were present in the MCS to permit future cloning into plant expression vectors.

Ten colonies were selected from LA agar plates containing ampicillin and plasmids were isolated by a small-scale DNA extraction. To screen for positive clones, plasmid DNA (pCMS2) was digested with *EcoR* I and analysed on a 0.7 % (w/v) TAE-agarose gel. The two expected bands of 1.35 Kbp and 2.9 Kbp representing the *cps3S-HIS* gene and linearised vector DNA, respectively, are shown in lane 2 of Figure 4.11. Lane 1 is the uncut plasmid DNA.

Two bands were expected since there are two *EcoR* I restriction sites present in the pGEM multiple cloning site, into which the 'His-tagged' *cps3S* PCR product (*cps3S-HIS*) was

inserted. These results confirmed the correct construction of the plasmid, which was named pCMS2.

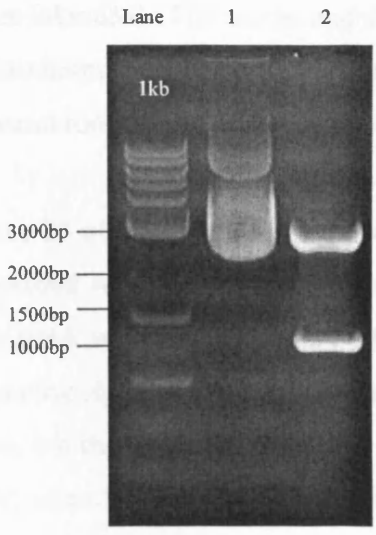

Figure 4.11. The digested products of a single clone containing pCMS2 cut with *EcoR* I. Lane 1 contains uncut plasmid and Lane 2 contains digested DNA. The sizes of the DNA fragments obtained on the gel confirm the cloning of *cps3S-HIS* (1.35 Kbp) into pGEM (2.9 Kbp). A 1Kb ladder (NEB) was used as a molecular size marker.

4.3 Plant transformation

4.3.1 *Arabidopsis thaliana*

4.3.1.1 pCMS3

pCMS3 was cloned into *A. thaliana* by *Agrobacterium tumefaciens*-mediated gene transfer. *Agrobacterium tumefaciens* containing both T_i and the binary pCMS3 plasmids were used to infect healthy whole plants by the dipping method in which the plant was submerged into a broth of *A. tumefaciens* pCMS3 (see §2.13.1.1). The T_i plasmid caused the mobilisation of the cloned DNA between the T-DNA regions of the pCMS3 vector, which contained the *cps3S* gene and PR1B signal peptide sequence, and the gentamicin resistance marker. Both DNA fragments were incorporated into the plant genome. Approximately 300 seeds were collected from the infected plants and all were plated onto selective medium. Shootlings were screened for gentamicin resistance on Murashige-Skoog (MS) agar.

Approximately 1/3 of the seeds appeared to be resistant to the antibiotic selection. Fifty of the largest and/or greenest shoots were transferred into individual universal tubes containing 10 ml of the selective (MS) medium. Transformed plants harbouring the pCMS3 plasmid are gentamicin resistant and *Agrobacterium* strains were sensitive to cefotaxime, therefore this selection prevented *Agrobacterium* contamination. After 1 - 2

weeks, 20 surviving (not displaying signs of chlorosis), uncontaminated shootlings were transferred to a small Petri dish containing MS medium supplemented with plant growth hormones (see MandM). The auxin and cytokinins added (napthaleneacetic acid (NAA) and 6-benzylaminopurine (6-BAP), respectively) stimulated the formation and growth of strong shoots and roots.

Fourteen of the 20 plantlets were transferred to soil when they reached 2 - 3 cm in height, and roots appeared well developed. After one week a single leaf was removed from each plant and the DNA isolated. PCR was performed to detect the presence of the *cps3S* gene and contaminating *Agrobacterium*, using the extracted DNA as a template. These results are not shown, but the gels confirmed that PCR using the *cps3S* specific primers, CPSFOR and CPSREV, amplified a ~1330 bp fragment from the extracted DNA from three plants (plants 3, 8 and 9). This 1.3 Kb fragment was exactly as predicted and is diagnostic of the presence of the *cps3S* gene, as discussed in §4.1. This was confirmed as the positive control, in which pCMS3 was used as a template for PCR also amplified a 1330 bp fragment. The gel also showed that this gene was specific to the transformed plant, as no bands were amplified from DNA extracted from a wild-type plant.

These results also showed that no *Agrobacterium* DNA was present in the Plant 9 DNA sample as no product was produced by PCR using the *Agrobacterium* specific primers VCF and VCR. These results show that *Agrobacterium* had been eliminated by the cefotaxime selection, since the VCF and VCR primers were designed to detect the *virC* operon located on the T_i plasmid by PCR (Sawada *et al.* 1995). If the target DNA was present a 730 bp fragment was amplified by PCR. This was shown using the positive control of *Agrobacterium* plasmid DNA. Also the DNA obtained from plants 3 and 8 amplified a fragment of 730 bp and therefore were contaminated with *Agrobacterium*. This means that the *cps3S* gene, shown to be present using *cps3S* specific primers, may not be integrated into the plant genome.

Unfortunately all fourteen *Arabidopsis* plants contracted an unrelated unidentified infection while housed in the incubator and were disposed of prior to setting seed. No further investigations could be made with these plants. Due to time shortage no further *A. thaliana* work was done with pCMS3.

4.3.1.2 pCMS4

The cloning of pCMS4 into *A. thaliana* by *A. tumefaciens* mediated gene transfer was unsuccessful. Healthy, whole *A. thaliana* plants were submerged into a broth of *A. tumefaciens* pCMS4 (see §2.13.1.1) and approximately 200 seeds were collected from the infected plants. All the seeds were plated onto selective medium and shootlings were screened for kanamycin resistance on MS agar (see §2.13.1). However none of the shootlings developed substantially. Nearly all showed significant signs of chlorosis and died, or became contaminated with fungus and were discarded. This indicated that this attempted transformation of *A. thaliana* with pCMS4 was unsuccessful.

4.3.2 Tobacco

4.3.2.1 pCMS3

The transformation of *N. tobacum* cv. Samsun plants with pCMS3 by *A. tumefaciens* mediated gene transfer was attempted. However, all the infected leaf discs developed signs of chlorosis, despite ranging the gentamicin concentration from 50 $\mu g\ ml^{-1}$ to 250 $\mu g\ ml^{-1}$. This was thought to be due to inefficient transformation or antibiotic toxicity (see §5.9.3.1)

4.3.2.2 pCMS4

pCMS4 was cloned into *Nicotinia tobacum* cv. Samsun by *A. tumefaciens* mediated gene transfer. *Agrobacterium tumefaciens* containing both T_i and the binary pCMS4 plasmids was used to infect freshly cut *N. tobacum* cv. Samsun leaf discs. The T_i plasmid caused the mobilisation of the cloned DNA between the T-DNA borders of the pCMS4 vector, which contained the *cps3S* and PR1B signal peptide sequence, and the kanamycin resistance marker (see Figure 2.7). This fragment was incorporated into the plant's own genome. Transformed plants were initially screened for kanamycin resistance on Murashige-Skoog (MS) agar (see §2.13.3).

Calluses developed from 270 individual leaf discs after 1 - 2 weeks and 24 were randomly selected and subcultured into powder rounds containing MS agar with kanamycin, cefotaxime, NAA and 6-BAP to promote shoot and root growth (see §2.13.1.1). Transformed plants harbouring the pCMS4 plasmid were kanamycin resistant and *Agrobacterium* strains were sensitive to cefotaxime, therefore the addition of cefotaxime to the media prevented *Agrobacterium* contamination. As can be seen in Figure 4.12, plantlets were transferred to soil when they reached about 5 cm in height. At that time it

was also possible to remove leaves for DNA analysis without hindering plant development. Twelve of the 24 plants were replanted and were maintained in soil for 1 - 2 months, or until they set seed. A the first filial generation (F1) was grown from the seed of six of these plants

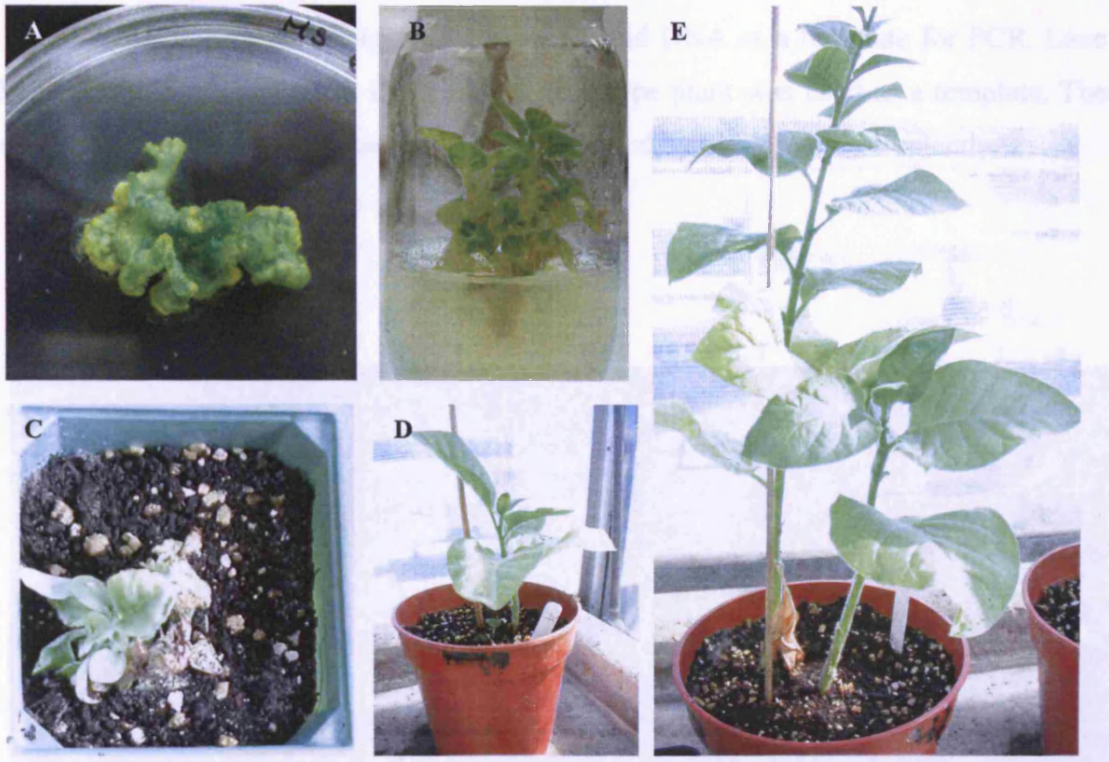

Figure 4.12 The growth stages of a parental (P) generation of transgenic *Nicotinia tobacum* cv. Samsun plants harbouring the pCMS4 plasmid. (A) a small callus in a Petri dish grown on MS agar containing kanamycin and plant growth hormones. **(B)** a large callus grown in a powder round with the same media as A. **(C)** shows a plantlet rooted in soil. **(D)** a larger plant replanted into an individual container. **(E)** a fully grown Tobacco plant just prior to setting seed

DNA was isolated from the leaf tissue of five possible transgenic plants and one wild-type plant using the DNeasy Plant Mini Kit (Qiagen) and the presence of the *cps3S* gene was detected by PCR (as described in §2.10.4.4). To eliminate the possibility that the *cps3S* gene was amplified due to the presence of residual *Agrobacterium*::pCMS4, a PCR was also performed using primers specific to the virulence region of the *Agrobacterium* T_i

plasmid (see §4.3.1) (Sawada *et al.* 1995). The results of these PCRs are shown in Figure 4.13.

Figure 4.13a showed that no *Agrobacterium* DNA was present in the any of the plant DNA samples as no product was produced by PCR using the *Agrobacterium* specific primers VCF and VCR (lanes 2 - 6). If the target DNA were present an expected fragment of 730 bp would be amplified by PCR. This is shown in the positive control (lane 8), which used a small-scale preparation of *Agrobacterium* plasmid DNA as a template for PCR. Lane 1 shows no PCR product when DNA from a wild-type plant was used as a template. These results show that *Agrobacterium* had been eliminated by the cefotaxime selection.

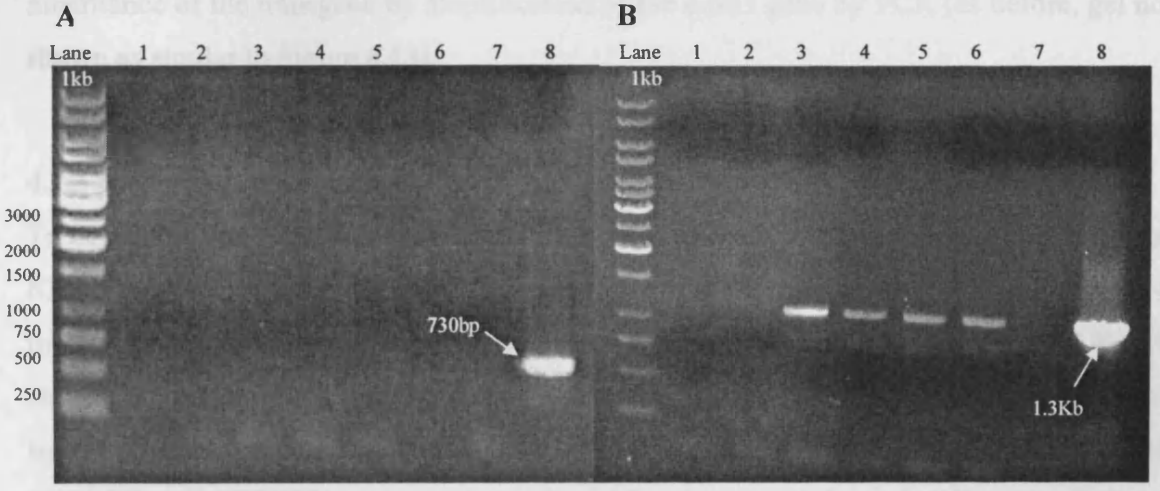

Figure 4.13. (A) PCR to detect the presence of *Agrobacterium* contamination in transgenic (pCMS4) and wild type tobacco plants. DNA was extracted from two non-transgenic (Lanes 1 and 2) and four transgenic *Nicotinia tobacum* plants carrying the pCMS4 plasmid (Lanes 3 - 6) and used as a template for PCR using *Agrobacterium* specific primers. The results show that there was no *Agrobacterium* DNA present the transgenic plant samples. The PCR reaction and Lane 8 contained *Agrobacterium* DNA as a positive control and Lane 7 contained no template. **(B) PCR to detect the presence of *the cps3S* gene in transgenic and wild type tobacco plants.** DNA was extracted from the same six *N. tobacum* plants used in the PCR shown in A. The DNA was used as a template for PCR (Lanes 1 - 6) using *cps3S* specific primers. The results show the presence of the *cps3S* gene in plants 3 – 6. The PCR reaction in Lane 8 contained purified plasmid DNA containing *cps3S* (pCMS4) as a positive control and Lane 7 contained no template DNA. A 1Kb ladder (Fermentas) was used as a molecular size marker.

Figure 4.13b showed that PCR using the *cps3S* specific primers, CPSFOR and CPSREV, amplified ~1330 bp fragment from the extracted DNA from four of the five possible transgenic plants (lanes 2 - 6). This 1.3 Kb fragment is exactly as predicted and is diagnostic to the presence of the *cps3S* gene. Lane 8 contained the positive control, in which pCMS4 was used as a template for PCR. Figure 4.13b also showed that this gene was specific to the transformed plants, as this product was not amplified from DNA sample extracted from a wild-type plant (lane 1).

The results of both PCRs showed that the *cps3S* gene was present in the transformed plants and that this presence was not due to residual *Agrobacterium* contamination. Similar results were obtained for the first filial generation (F1) of transgenic plants. Five (Plants 1, 4, 6, 11, 12) of the six plants cultivated to a F1 generation showed stable integration and inheritance of the transgene by amplification of the *cps3S* gene by PCR (as before, gel not shown as similar to Figure 4.13).

4.3.3 Reverse transcriptase PCR for the detection of *cps3S* expression *in planta*

To detect the transcription of the *cps3S* gene, reverse transcriptase PCR was performed on RNA isolated from two *Nicotinia tobacum* plants; a control wild-type plant (W) and a transgenic plant (T) containing the *cps3S,* see §2.10.4.2. The results of these RT-PCRs are shown in Figure 4.14. To eliminate the possibility that the *cps3S* gene could be amplified by the presence of residual DNA, each RNA sample was first subjected to PCR using CPSFOR and CPSREV primers (lanes 1 and 2). As expected, no bands were observed from these PCRs indicating that the samples were clear of DNA. Lanes 3 and 4 showed the PCR results of the same samples following prior treatment with reverse transcriptase. The presence of the expected fragment of 1.3 Kbp in lane 4 indicated that the transgenic plant contains *cps3S* specific RNA. However, wild-type plants do not transcribe *cps3S* specific RNA as there was no product present in lane 3. Lane 5 shows the positive control PCR results, which used purified pCMS4 DNA as a template. Here the expected fragment of 1.3 Kbp was also amplified. These results indicate that transgenic plant cells contained and produced *cps3S* specific mRNA.

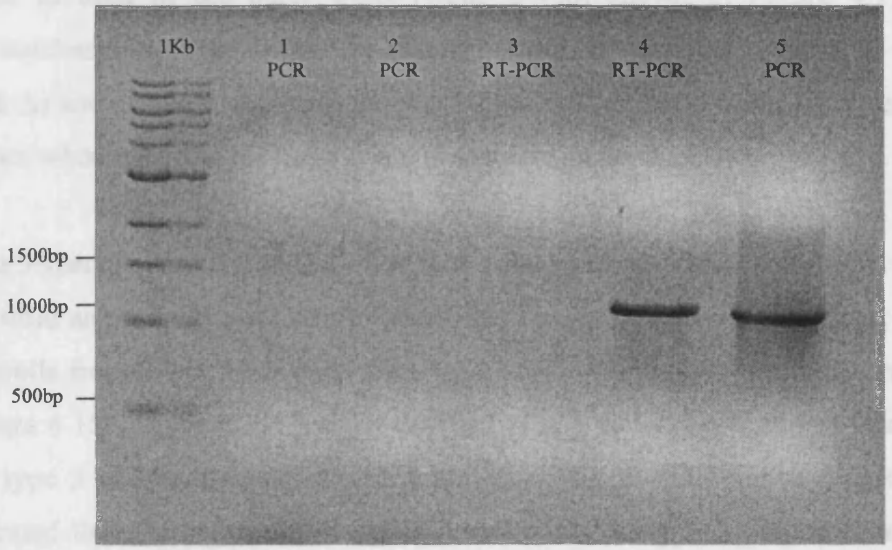

Figure 4.14. Reverse transcriptase PCR to detect the presence of mRNA from the *cps3S* gene in transgenic tobacco plants. RNA was extracted from two *Nicotinia tobacum* plants, a control or wild-type plant (Lanes 1 and 3) and a transgenic plant shown to contain the *cps3S* gene (Lanes 2 and 4). The RNA extracts were subjected to PCR using *cps3S* specific primers to detect any residual DNA (Lanes 1 and 2), which showed the absence of *cps3S* DNA. The same samples were then incubated with reverse transcriptase prior to PCR (Lanes 3 and 4) to convert mRNA to cDNA. This shows the presence of *cps3S* specific RNA from the transgenic plant (Lane 4), but not in the wild-type plant (Lane 3). Lane 5 contained purified plasmid DNA containing *cps3S* (pCMS4) that was subjected to PCR as before. A 1Kb ladder (NEB) was used as a molecular size marker.

4.3.4 Detecting pneumococcal polysaccharide production *in planta*

An Ouchterlony double immuno-diffusion, performed on sonicated transgenic plant tissue extracts, indicated the presence of type 3 specific polysaccharide.

Leaf tissue from both wild type and transgenic plants was crushed under liquid nitrogen and sonicated in water as described in §2.15.1.1. After centrifugation, the supernatant was added to each of the outer wells (wells 1 - 6) shown in Figure 4.15. Neat type 3 polysaccharide specific antiserum (Statens Serum Institute) was added to the central well (well A) and the slide was incubated at 4°C for at three days. Wells were refilled with plant extract when the previous sample had absorbed into the agar.

Type 3 specific antigen and antibody molecules migrated through the agar to bind together and form an antibody-antigen complex. This complex precipitated to form a band between the wells from which both molecules originated. This is observed between Wells 1 and A (Figure 4.15). These wells contain purified type 3 pneumococcal polysaccharide (ATCC) and type 3 polysaccharide specific antiserum. This is the positive control and the band indicated that the antiserum recognised and bound to type 3 polysaccharide under these conditions. However, it resembles more of a smear than a clear band due to the refilling of the well with pneumococcal polysaccharide throughout the incubation period. Although, it is not very clear on the Figure, this caused antibody-antigen complexes to precipitate at more than one location in the agar, as on close inspection the smear was actually a series of distinct bands. The addition of a single quantity (10 μg) of pneumococcal polysaccharide was done in future experiments.

As seen in Figure 4.15, bands of antigen antibody complex have also precipitated between well A and wells 2 and 3. Wells 2 and 3 contained sonicated plant cell extract from transgenic plants 1 and 2, respectively. This showed that these plants are producing type 3 pneumococcal polysaccharide. In contrast to the other results, no band of precipitation was observed from plants 3 - 5 (wells 3 - 5), therefore type 3 polysaccharide was not detected. This was also the case for the wild-type plant (well 6), where also no precipitation was observed. Therefore the wild-type plant does not produce an antigen that reacts with type 3 antiserum.

These results showed that the production of type 3 polysaccharide was specific to plants that contain the *cps3S* gene, although not all transgenic plants are capable of production. This will be considered in the diagram.

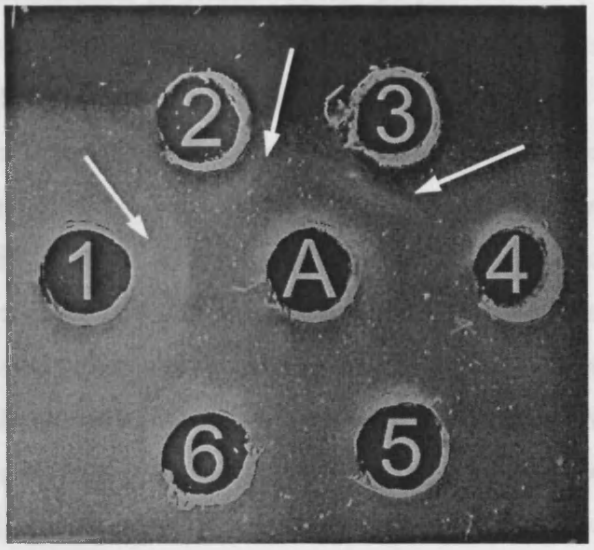

Figure 4.15. Ouchterlony immuno-double diffusion. Well 1 contained a total of 30 μg purified serotype 3 pneumococcal polysaccharide, wells 2 - 5 contained sonicated plant cell extract from tobacco plants shown to contain the *cps3S* gene. Well 6 contained sonicated plant cell extract from a wild-type, non-transgenic tobacco plant. Well A contained type 3 polysaccharide specific antiserum (Statens Serum Institute). Bands of antigen antibody complex precipitated between well A and wells 1, 2 and 3 (as indicated by the arrows). This showed that the antiserum bound to type 3 polysaccharide (well 1) under these conditions and that the plant extract from plants 1 and 2 (wells 2 and 3 respectively) contained type 3 pneumococcal polysaccharide. This is unlike the plant extracts of the transgenic plants 3 and 4 (wells 4 and 5 respectively) or the wild type plant (well 6) where no band of precipitation was observed. This indicated that these plants did not produce type 3 pneumococcal polysaccharide whereas plants 1 and 2 did.

The five plants that were shown to have stably integrated the *cps3S* gene into their genome (plants 1, 4, 6, 11, 12) were also shown to synthesise type 3 polysaccharide, but at different levels.

4.3.5 Refining the extraction of pneumococcal polysaccharide from plant tissue

As Table 4.1 shows, several methods were used to extract the type 3 specific pneumococcal polysaccharide from plant tissue. To determine the method that was most effective, the concentration of pneumococcal polysaccharide was estimated based on the amount of precipitate formed in ouchterlony immuno diffusion (see §4.3.5). We showed that the highest concentration of pneumococcal polysaccharide (~4 μg g^{-1} plant tissue – based on comparison to the precipitated band of 10 μg pneumococcal polysaccharide in Ouchterlony (§4.3.5)) was obtained after grinding plant tissue under liquid nitrogen, suspension in water (and homogenisation) and then lysing the cells by sonication (see §2.15.1.1). The concentration of pneumococcal polysaccharide could be further increased by precipitation with acetone and resuspension in a smaller volume of water. It was difficult to determine whether pneumococcal polysaccharide could be precipitated with ethanol, since the dried pellet was difficult to redissolve. Homogenisation did not yield as much polysaccharide as sonication. Cell lysis by sonication in 3M NaOH also led to the extraction of pneumococcal polysaccharide, but at a lower concentration than water. Sonication in 6M NaOH resulted in the extraction of even less pneumococcal polysaccharide. Interestingly, for both 3M and 6M NaOH extractions, when the hemicelluloses were precipitated by neutralisation with acetic acid, as described by Fry (1988), type 3 pneumococcal polysaccharide was detected in both the precipitate and the neutralised supernatant. It was thought that this method would offer a significant advantages over water extraction since type 3 pneumococcal polysaccharide is composed of some of the sugars that form the hemicelluloses of the plant and these are preferentially extracted by solubilisation in sodium hydroxide (Fry 1988). This is discussed further in §5.9.3.3.

Table 4.1 The extraction of pneumococcal polysaccharide from plant tissue

Details of extraction technique[a]	*Concentration of polysaccharide relative to control*[b]
Crude extraction by sonication in water	++
Crude extraction by sonication in water and precipitation with acetone	++
Crude extraction by sonication in water and precipitation with ethanol	+
Crude extraction by sonication in 3M NaOH	+
Crude extraction by sonication in 3M NaOH and precipitation with acetic acid (supernatant)	+
Crude extraction by sonication in 3M NaOH and precipitation with acetic acid (precipitate)	+
Crude extraction by sonication in 6M NaOH	+/-
Crude extraction by sonication in 6M NaOH and precipitation with acetic acid	+/-
Homogenisation in 3M or 6M NaOH	+/-
Homogenisation in water	-
Extraction of apoplastic fluic	-
Any method that involves heating the tissue above 60°C	-

[a]All samples were made up to 0.5 mg ml^{-1} dry weight plant tissue. [b] The grading ranged from (++++), which was equivalent to positive control (10 μg); to the lowest value (-), which means no type 3 polysaccharide was detected.

CHAPTER FIVE - DISCUSSION

5.0 Overview

This study set out to develop new strategies for the manufacture and composition of pneumococcal polysaccharide vaccines. This was because, despite the existence of three widely available commercial vaccines, diseases caused by *S. pneumoniae* remain a major cause of morbidity and mortality throughout the world, in particular among infants and the elderly (Kolberg and Jones 1998). Immunisation is considered to be an effective approach to reduce the incidence of pneumococcal disease (Andrew *et al.* 1994) and opsonisation of the polysaccharide capsule with type-specific antibody has been described as the most effective host defence against *S. pneumoniae* (see §1.2.2) (Lee *et al.* 1996).

The first pneumococcal vaccines introduced for use in humans were composed of capsular polysaccharide, which is a T-independent type 2 (TI-2) antigen. As described previously (§1.4.2.3), TI-2 antigens characteristically fail to stimulate a protective immune response in the most high-risk groups of pneumococcal disease, the elderly and in infants. This failure is partly due to their inability to promote class switching of the antibody isotype in B cells. This results in a predominantly IgM response, which does not promote immune memory and is not long-lasting (Klein 2000). In addition, TI antigens only activate mature B cells (Eales 1997) and infants, with a repertoire of immature B cells, are further restricted in their ability to efficiently raise an antibody response to polysaccharide antigens. Recently, many studies have offset these effects by conjugating the polysaccharide to a carrier protein, such as diptheria toxoid. However, the quality control issues and need for multiple conjugations (see §1.4.4) make this an expensive approach: the current 7-valent pneumococcal conjugate vaccine (PCV7) costs £49 per dose (McIntosh *et al.* 2003) and 3 doses are required to obtain complete protection. Furthermore, PCV7 has led to a reduced vaccine coverage, from 23 serotypes to 7, an issue which is thought to increase the opportunity for non-vaccine serotypes to emerge as the most prominent disease causing serotypes (Mbelle *et al.* 1999).

In this study two of the main problems associated with current pneumococcal vaccines were confronted. These were the poor immunogenicity of capsular polysaccharide and the high vaccine manufacturing costs. Firstly, the problem of immunogenicity was tackled by identifying peptide mimics of two pneumococcal serotypes, 6B and 9V, which were capable of protecting mice against developing pneumococcal disease upon immunisation.

These results were presented in chapter three and will be discussed in part A of this chapter. Secondly, the problem of high vaccine production costs was confronted by developing a transgenic plant capable of manufacturing pneumococcal polysaccharide. The outcome of this project was presented in chapter four and will be discussed in the second part of this chapter.

Part A: Peptide Mimicry

5.1 To recapitulate

To circumvent the problems associated with the TI property of a large polysaccharide, we have identified peptides that mimicked protective epitopes of serotype 6B and 9V pneumococcal capsular polysaccharide using phage display technology. Alternative methods of selection existed (see §1.5.1), all of which applied the same basic principle of molecular mimicry: an antibody raised against a specific epitope can be used to select a mimic of that epitope from a collection (library) of different molecular shapes. However, the majority of successful examples in the literature had been obtained using recombinant phage displayed peptide libraries. These examples included peptide mimics of a variety of bacterial carbohydrate epitopes including *Shigella flexneri* LPS (Phalipon *et al.* 1997) Group B streptococcal capsular polysaccharide (Pincus *et al.* 1998), *Brucella abortus* LPS (De Bolle *et al.* 1999) (see §1.5.2.4). This study has used the same technology to identify peptide mimics of both serotype 6B and 9V *S. pneumoniae* capsular polysaccharide and, furthermore, we have shown that immunisation with peptide mimics induced a protective immune response.

5.2 Summary and discussion of results

5.2.1 The identification of peptide mimics of serotype 6B and 9V pneumococcal polysaccharide

In total four peptide mimics of serotype 9V pneumococcal polysaccharide and seven mimics of serotype 6B pneumococcal polysaccharide were identified. These were selected from three different phage displayed peptide libraries, which displayed peptides of different lengths fused to the pVIII phage coat protein (pVIII-12aa, pVIII-12aa.Cys, and pVIII-15aa), using either a murine mAb (206,F-5) or a human mAb (Db3G9) (see Table 2.4). Prior to phage panning, these mAbs had been shown to protect against the

development of pneumococcal disease upon challenge (§3.3). This was to ensure the selected peptides mimicked protective epitopes on the surface of the pneumococcus. This result was confirmed by a competition ELISA between the phage displayed peptide and the native antigen. This showed that the mAbs 206,F-5 and Db3G9 could be inhibited from binding to the peptides they selected by pre-incubation with pneumococcal polysaccharide. This indicated that the peptide and native polysaccharide antigen occupied the same antibody-binding site of the mAb.

5.2.2 All the selected peptides were mimics of pneumococcal capsular polysaccharide

Ten of the eleven peptides selected from phage display libraries were shown to exhibit polysaccharide mimicry properties (excluding MP58) (§3.5 and 3.7). Also, not only did all the peptide mimics stimulate a high anti-peptide IgG antibody response upon immunisation, which was shown to be boosted upon further doses of antigen (up to a maximum of 54.6 mg ml^{-1} after three doses), they also stimulated the production of antibodies that bound to pneumococcal capsular polysaccharide (except mice immunised with MP55), although some levels were very low in comparison to the anti-peptide response (see Table 5.1). Similar results have been previously reported with non-pneumococcal polysaccharides (Phalipon *et al.* 1997; De Bolle *et al.* 1999; Moe *et al.* 1999). Phalipon and colleagues isolated a total of 19 peptide mimics of *Shigella flexneri* LPS using a phage-display library. They noted that mice immunised with four phage borne peptide mimics produced anti-LPS antibodies, but on average the titre was 100-fold less than the anti-phage titre (Phalipon *et al.* 1997). De Bolle and colleagues (1999) and Moe and colleagues (1999) isolated peptide mimics of *Brucella* sp. and *N. meningitidis* group B polysaccharide, respectively, which elicited only very weak antibacterial responses upon immunisation.

To confirm that the anti-polysaccharide response we recorded was a result of immunisation and not a non-specific immune response, the post-immunisation antibody concentrations was subtracted from that found in the pre-immune sera. A competition ELISA was also done to support these findings. These two assays vary in the manner the polysaccharide is presented to the antibody; in a direct ELISA the polysaccharide antigen is attached to a solid surface, whereas in a competition ELISA it is free in solution. The competition ELISA results showed that pre-incubation with type specific pneumococcal polysaccharide competitively inhibited the antibodies binding to all the peptide mimics with the exception

of MP15 and MP58. This could mean that some peptide-induced antibodies bind pneumococcal polysaccharide preferentially under certain conditions. For example, the antigen recognised by the predominant antibodies maybe more available for binding when the polysaccharide is free in solution. This is one limitation of this study and will be discussed in more detail later (§5.6). This may explain the difference between the outcomes of the two assays given that MP55-induced serum did not bind polysaccharide directly, but could be inhibited from binding to the peptide following pre-incubation with polysaccharide. This implied that some peptides only partially mimicked the polysaccharide epitopes. Polysaccharides are large, complex structures, therefore it was not thought that a peptide that mimicked the entire structure would be identified. However, it was important to determine whether the peptide represented the epitope recognised by the mAb, in that it bound the entire antigen-binding site. To investigate this, phage libraries displaying longer peptides could be screened (Phalipon *et al.* 1997). We only selected one peptide mimic (MP58) from a library longer than 12 amino acids, but this was not shown to be protective. Another approach, which maybe more interesting, would be to see whether the immunogenicity of the current peptides could be improved by including motifs from those peptides shown to either protect against pneumococcal disease, or stimulate a good anti-polysaccharide response in mice. The number of amino acid residues does not need to be large for a peptide to have significant biological activity (Atkins and Carey 2001). For example, leucine encephalin (a component of endorphins, which act as pain killers) is a pentapeptide (Atkins and Carey 2001). Nevertheless, the addition of just a few residues may improve the ability of peptide-induced antibodies to recognise pneumococcal polysaccharide given that it is a large structure.

5.2.3 MP7 is a' true mimic' of serotype 9V pneumococcal polysaccharide

A 'true mimic' is thought to be capable of binding native antigen specific antibodies produced in a variety of species (Grothaus *et al.* 2000). Therefore, we investigated whether sera obtained from three human volunteers immunised with pneumococcal polysaccharide could bind the peptide mimic MP7. The peptide was recognised and bound to each of the sera (§3.5.4). Furthermore, we showed that this binding was specific to a pneumococcal polysaccharide mimic, since binding could be inhibited via prior incubation with 9V polysaccharide. In particular, those individuals immunised with the polysaccharide conjugate vaccine were inhibited more than 25 %. These results indicated that MP7 could possibly be used as a human vaccine against serotype 9V pneumococcal disease.

Table 5.2 A summary of the anti-peptide and anti-polysaccharide IgG antibody response, the avidity and survival rates of mice immunised with peptide mimics of two pneumococcal serotypes selected from different phage-displayed peptide libraries.

	Vaccine	*Phage library panned*	*Average[a] total after 3 doses of antigen*		*Average[a] avidity [NH_4SCN]*	*Survival/Total (%)*
			anti- peptide antibody	*anti- polysaccharide antibody*		
9V POLYSACCHARIDE MIMICS	MP7-KLH	pVIII-12aa	603.3 μg ml^{-1}	219 μg ml^{-1}	1.567 M	12/20 (60 %)
	MP12-KLH	pVIII-12aa	12.4 mg ml^{-1}	0.6 μg ml^{-1}	0.969 M	8/20 (40 %)
	MP55-KLH	pVIII-12aa.Cys	54.6 mg ml^{-1}	-	1.827 M	5/18 (28 %)
	MP58-KLH	pVIII-15aa	12.45 mg ml^{-1}	1.0 μg ml^{-1}	1.289 M	9/19 (47 %)
6B POLYSACCHARIDE MIMICS	MP2-KLH[b]	pVIII-12aa	241.8 μg ml^{-1}	309.9 μg ml^{-1}	1.406 M	7/10 (70 %)
	MP10-KLH	pVIII-12aa	2.8 mg ml^{-1}	36.5 μg ml^{-1}	2.706 M	8/25 (32 %)
	MP13-KLH	pVIII-12aa	62.7 μg ml^{-1}	0.2 μg ml^{-1}	1.103 M	17/30 (56 %)
	MP14-KLH[b]	pVIII-12aa	362.6 μg ml^{-1}	0.3 μg ml^{-1}	0.951 M	5/10 (50 %)
	MP15-KLH	pVIII-12aa	742.9 μg ml^{-1}	0.1 μg ml^{-1}	1.298 M	4/11 (36 %)
	MP17-KLH[b]	pVIII-12aa	96.5 μg ml^{-1}	0.3 μg ml^{-1}	1.931 M	2/10 (20 %)
	MP18-KLH[b]	pVIII-12aa	3.9 mg ml^{-1}	0.1 μg ml^{-1}	1.228 M	4/10 (40 %)

[a] mean value (n values range from 2 - 30). See chapter three for more details

[b] after one dose of antigen

As a matter of interest, we also looked at whether the peptide mimics of serotype 6B pneumococcal polysaccharide could bind to another 6B PnCPS specific human mAb, DM5. All seven peptide mimics selected from the phage libraries by mAb Db3G9 also bound to DM5, in particular peptides MP2 and MP14 significantly reacted to both mAbs (see Results §3.7.3). This was suprising since these mAbs were formed with totally different V region gene families (Baxendale *et al.* 2000), and their complementarily-determining regions were found to be quite distinct when the DNA sequences of the V_L and V_H regions of the two mAbs were determined (H. Baxendale, personal communication). Shin and colleagues also described this phenomenon by identifying peptide mimotopes of serotype 6B pneumococcal polysaccharide that cross-reacted with a mAb raised to *Neisseria meningitidis* group B polysaccharide (Shin *et al.* 2002). This ability to cross react with other anti-polysaccharide antibodies could be due to the peptides mimicking a common epitope of polysaccharide antigens. However, the most likely explanation relates to the stability of the peptide mimotope. If the structure is unstable it may express multiple conformations which, over time, can rapidly switch from one conformation to another (Shin *et al.* 2002). Through NMR studies Shin and colleagues showed that a mimotope possessing two proline residues can slowly (in 10 - 100 seconds) switch between *cis* and *trans* conformations (see Figure 5.1), although one conformation was preferred over the other (Shin *et al.* 2002). This means that in certain environments, for example in ELISA or *in vivo*, the shape of the peptide could be different. This could explain why some mice in our study were protected against infection despite not being shown to develop a significant antibody response in ELISA (this is discussed further in §5.6.2).

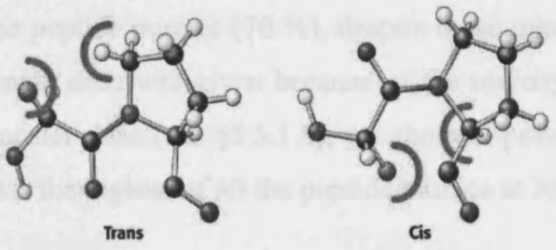

Figure 5.1. *cis* and *trans* proline structures.
Taken from Stryer (1995)

Another theory is that the stable peptide may express more than one epitope. Shin and colleagues (2002) identified a circular mimotope that expressed more than one epitope, one on the topside that could bind one mAb and another on the bottom that may bind another (Shin *et al.* 2002).

Interestingly, when we compared the results of the competitive ELISAs inhibition of Db3G9, a different conclusion was made. We found that the mAb Db3G9 was almost entirely inhibited from binding the peptide MP2 when preincubated with serotype 6B pneumococcal polysaccharide. However, the mAb DM5 did not display any sign of inhibtion. This was also observed for peptides MP10 and MP15, both of which demonstrated a lower percentage inhibition to DM5 than to Db3G9. In contrast, the binding of DM5 to peptides MP17 and MP18 displayed a greater inhibition than Db3G9. These results indicated that although both mAbs recognised and bound to the peptide mimics, they are in fact binding to different epitopes within the sequence. If they recognised the same epitopes then they would be equally inhibited.

5.2.4 Two peptides protected mice against serotype 6B and 9V pneumococcal disease following immunisation with mimics of capsular polysaccharide

The peptide mimics MP7 (a serotype 9V PnCPS mimic) and MP13 (a 6B mimic) were shown to significantly protect mice against pneumococcal disease. A summary of the survival results from mice challenged after immunisations with eleven peptide mimics is shown in Table 5.1. The most significant result came from mice immunised with peptide MP7. Sixty percent of these mice survived challenge with serotype 9V pneumococci, compared to 38 % of the control group. Another exciting result came from mice immunised with MP13. Following challenge with serotype 6B pneumococci, 56 % of these mice survived, which was significantly higher than the 20 % that survived in the control group.

Furthermore, mice immunised with MP2-KLH produced the highest percent survival of all the peptide mimics (70 %), despite these mice only receiving one dose of antigen. Only a single dose was given because of the toxicity observed following the administration of a booster dose (see §5.5.1.1), yet the anti-polysaccharide antibody concentration it elicited was the highest of all the peptide mimics at 309.9 $\mu g\ ml^{-1}$.

These results demonstrated that recognition by the mAb (through binding) alone was not a guarantee the peptide mimic would successfully elicit the desired immune response. As stated in §5.2.1, all the peptides bound the mAb that selected them, but only 5 were shown to produce > 0.5 $\mu g\ ml^{-1}$ anti-polysaccharide antibody in immunised mice (see Table 5.1 and §5.2.6). Similar results have been reported before (Harris *et al.* 2000). Harris and

colleagues found that a peptide mimic of phosphorylcholine induced IgG1 antibodies directed against the native antigen, but this did not confer protection against type 3 pneumococcal disease (Harris *et al.* 2000). Therefore, before concluding a peptide was a mimic, it was important to perform as much analysis of the immune response as possible. The results from these analyses are discussed in the following sections.

5.2.5 Immunisation with pneumococcal peptide mimics induced the production of anti-polysaccharide antibodies

The results presented in §3.6.4 and §3.8.5.3 showed that mice immunised with peptide mimics of pneumococcal polysaccharide elicited an immune response that directly bound pneumococcal polysaccharide (with the exception of MP55). We also detected cross-reactive antibody by competition ELISA (with the exception of MP15 and MP58) (see §5.2.2).

Immunisation with ten (out of the total eleven) peptide mimics stimulated the production of antibodies that bound pneumococcal polysaccharide by one or more of the methods described above, although in some cases this response was quite low. If we consider the peptides that stimulated a protective immune response, mice immunised with MP7 produced on average 219 μg ml^{-1} anti-polysaccharide IgG antibody, whereas mice immunised with MP13 produced on average 0.2 μg ml^{-1}. Although the latter is significantly lower than the former, various sources have cited protective antibody concentrations much lower, from >500 - 150 ng ml^{-1} (Mufson *et al.* 1987; Furth *et al.* 1996; Raby *et al.* 1996; Lahiri and Waltz 2001). It has also been calculated to be as low as 10 ng ml (Wood 2001). Despite this, not all the peptides that stimulated an antibody concentration greater than 150 ng ml^{-1}, protected mice from pneumococcal disease (see Table 5.1). For example, immunisation with MP10 produced an average anti-polysaccharide antibody concentration of 36.5 μg ml^{-1}, yet only 32 % of mice survived challenge. This result could also be due to the nature of the assay. The antibody concentration recorded by direct ELISA referred to the total anti-polysaccharide IgG present in mouse sera. This was because, as a whole, the presence of IgG antibodies demonstrated the ability of the peptide to stimulate a lasting immune response and the potential for protection against pneumococcal challenge. IgG antibodies (in particular IgG2) have a long half life (21 days), are known to promote immunological memory, stimulate complement and bind to macrophages and neutrophils to promote bacterial

phagocytosis (Janeway *et al.* 1999). To investigate these results further another method to assess of the protective efficacy of the antibody response was needed. The measurement of antibody avidity, the use of opsonophagocytosis assays and fluorescent labelling of bacteria will now be discussed.

5.2.6 Opsonophagocytosis and antibody avidity assays

The opsonophagocytosis assay has been regarded as the most noteworthy method for measuring the functional response to pneumococcal vaccines (Concepcion and Frasch 2001). This assay quantitates the ability of antibody to opsonise and stimulate the phagocytosis of pneumococci. By incubating the antibody with the bacterium, complement and phagocytic cells, such as HL-60 myelomonocytic cells (Usinger and Lucas 1999), the ability of the antibody to bind pneumococci and stimulate phagocytosis can be quantitated by the decline in the number of viable bacteria (cfu ml^{-1}). This method is thought to be more attractive than other measures of *in vitro* protective immunity because it more closely resemble the mechanism of natural immunity, and it does not require the use of animal models (Wenger *et al.* 1996). However, it can be labour-intensive and difficult to perform with large numbers of samples. Thus, many laboratories prefer to measure antibody avidity (Concepcion and Frasch 2001).

Antibody avidity is a measurement of the strength with which an antibody binds to an antigen. This is thought to increase over time following encounter with an antigen. Memory responses are characterised by the production of high-avidity antibody; thus, high avidity could be considered as a sign of successful priming (Goldblatt *et al.* 1998). It is thought that the ability of anti-pneumococcal capsular antibodies to confer protection against disease can be related to the strength of the bond they form with the antigen (avidity) (Usinger and Lucas 1999). Because of time restraints and that our lab was not well equipped to perform opsonophagocytosis assays, we attempted to measure the avidity of mouse antibodies to capsular polysaccharide in ELISA. However, after several attempts, which achieved varied results, the assay was deemed irreproducible. These differences may have been due to the conditions of the ELISA. For example, the concentration of antibody may not have been high enough to calculate a decrease in binding. Partidos (2000) commented that anti-peptide antibodies normally bind with high avidity to the homologous peptide and only weakly to the native protein they mimic. For these reasons, we decided to measure the avidity of mouse antibodies to the peptide mimics.

We found that those mice that produced high avidity antibody from immunisation with peptides MP2, MP10, MP12, MP14, MP17 and MP55 displayed a prolonged survival time. This was in agreement with previous findings (Usinger and Lucas 1999). However, the mice that produced high avidity antibodies to peptides MP13, MP15, MP18 and MP58 did not display a prolonged survival time (we could not calculate a correlation for MP7 as there were not enough sera available). This was not thought to be a result of the stimulation of specific antibody subclasses, as was the case with the direct ELISA, but instead due to the presence of several antibody binding sites (as a result of a polyclonal response), which exhibited differential abilities to bind capsular polysaccharide (Usinger and Lucas 1999). Therefore some antibodies would recognise and bind to pneumococcal polysaccharide better than others. Finding a method to improve the avidity of antibodies generated from peptide immunisation would pose a significant advantage to peptide mimotope research. This is especially important *in vivo* when antibody concentrations are limiting. For example, in infants which possess naive repertoires of B cells, or at times when antibody levels have declined over time after immunisation (Usinger and Lucas 1999). Furthermore, the avidity of an antibody displayed on B-cells is thought to influence which clones are activated and assume prominence during the response to infection (Usinger and Lucas 1999). Therefore, avidity could be a factor in deciding which clones enter the memory pool (Usinger and Lucas 1999). Approaches to improving the avidity of peptide mimotopes are discussed further in §5.5.

5.2.7 Anti-peptide antibodies opsonised pneumococci

Phalipon and colleagues showed that antibodies raised by immunisation with phage displayed mimics of *Shigella flexneri* LPS could bind and coat the bacterium directly (Phalipon *et al.* 1997). This is a useful technique to confirm the opsonic potential of peptide-induced antibodies. We proved this principle could also be applied to mimics of pneumococcal polysaccharide by labelling serotype 9V pneumococci with antibodies raised from immunisation with MP7. As expected, *S. pneumoniae* serotype 9V were recognised by MP7-KLH induced antibodies but not with the control (KLH) induced antibodies. These findings suggested that the peptide mimic-induced antibodies were capable of interacting with the pathogen in an *in vivo* situation and could neutralise pneumococcal infection.

5.3 Analysis of the peptide sequences

To further understand the concept of peptide mimicry, I believe it is just as valuable to consider the non-protective peptide mimics as those that induced a protective immune reponse. Previous studies have identified peptide mimics to polysaccharide antigens that were not shown to be successful immunogens (Phalipon *et al.* 1997; Mertens *et al.* 2001). We propose that this is not the end to the investigation of these peptides. For example certain sequence motifs associated with inducing a higher immune response and/or high avidity antibody to pneumococcal polysaccharide could be revealed by identifying consensus sequences amongst the peptides. Then, the outcome of immunisation could be improved by substituting these residues into the peptides that did not stimulate a high antibody response.

The first stage of this analysis was to determine whether the presence of particular residues within the peptide sequences were of some significance or simply a random event. To do this a method used by Mertens and colleagues (2001) was applied, which determined a ratio (frequency) of occurrence of each amino acid. Primarily the expected frequencies of each residue were calculated based on the number of oligonucleotides that encoded each amino acid that were used to construct the libraries. If the observed frequency was the same as the expected frequency (a ratio of 1.0) then the presence of these residues in the sequence was a random event, since the library was constructed randomly. Any ratio >1.0 indicated an over-representation of that residue (Mertens *et al.* 2001).

The results of this analysis revealed that the presence of certain residues within the peptide mimics was not random. Figures 3.9 and 3.24 showed that there appeared to be a biased proportion of some residues, as many residues present in the four 9V peptide mimics and the seven 6B peptide mimics had a value >1.0. Of particular interest were those residues that were over represented. Aspartic acid (D), phenylalanine (F), methionine (M), proline (P), tryptophan (W) and tyrosine (Y) were all over represented in both the serotype 6B and 9V peptide mimics. Additionally glycine (G), histidine (H), lysine (K), and glutamine (Q) were over represented in the 9V mimics, whereas glutamic acid (E), isoleucine (I) and valine (V) were over represented in the 6B mimics. Unlike the 9V mimics, the 6B peptides also appeared to share a consensus sequence (this will be discussed in §5.5.1).

The next question to answer was whether these residues influenced the structure of the peptide mimics, and how. This will now be discussed.

5.4 Predicting the shape of a peptide: A review

Michael Sternberg of 'Protein structure prediction: a practical approach' writes: "It is very ambitious to achieve a complete prediction of a protein structure from a sequence but it is not impossible to hypothesise" (Sternberg 1996). The physical and chemical properties of each of the twenty amino acids are well documented (Baxevanis and Ouellette 2001). Each residue is not only different in shape but also in the way they interact with other residues and their potential to form different bonds. The secondary structure of a peptide is the conformational relationship of nearest neighbour amino acids with respect to each other (Atkins and Carey 2001). Therefore, information about the interactions of amino acids could be used to predict the secondary structure of a peptide based on a residues relationship with adjacent amino acids. Hydrogen bonding between the N-H group of one amino acid residue and the C=O group of another is the interaction that plays the greatest role in the secondary structure of a protein (Atkins and Carey 2001)

5.4.1 Amino acid interactions

Sternberg helpfully produced a Venn diagram to represent common properties between residues (see Figure 5.2). This, as well as other well-documented properties has helped to make comparisons between our mimotopes. Some of the properties that are relevant to this study will now be discussed.

5.4.1.1 Aromatic amino acids

In regards to polysaccharide mimicry, probably the most important amino acids are those that contain an aromatic ring. This is because the cyclic shape, size, and (particularly in the case of tyrosine) hydrogen binding potential of aromatic amino acids are features that are said to resemble sugar moieties (Hoess *et al.* 1993). These non-covalent forces are thought to govern the interaction between an antigen and its specific receptor on lymphocytes (Phalipon *et al.* 1997). Therefore the presence of aromatic amino acids [histidine (H), phenylalanine (F), tyrosine (Y) and tryptophan (W)] in a peptide can make it more immunogenic than non-aromatic molecules (Eales 1997). Some researchers do not regard histidine as an aromatic amino acid (Stryer 1995). However, previous work on

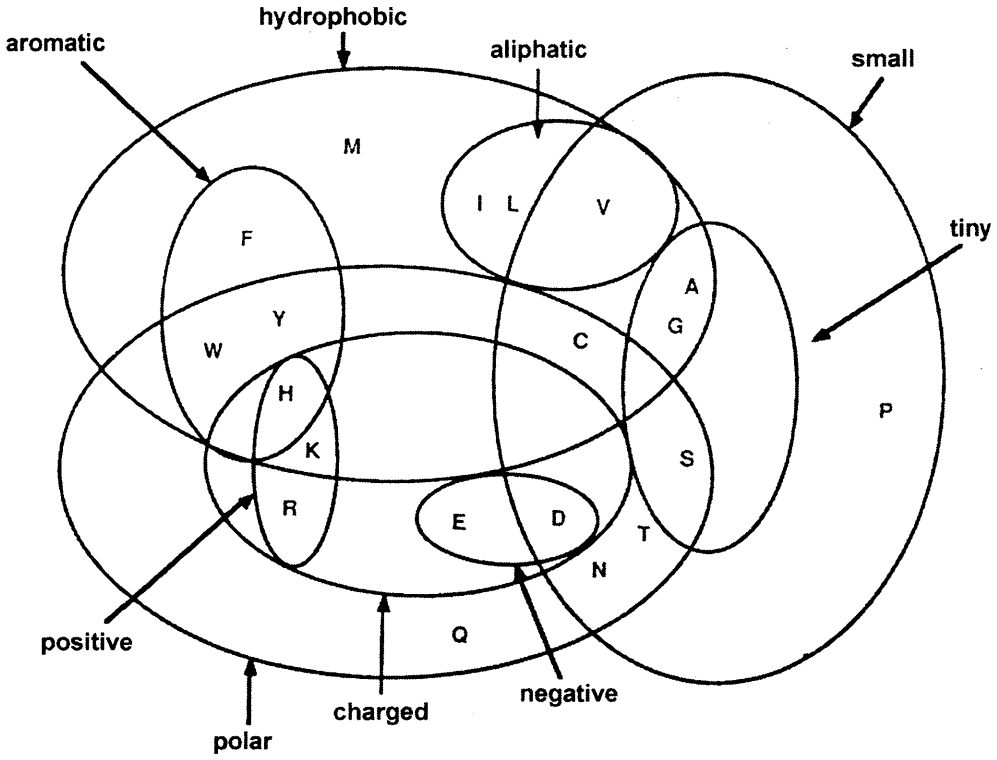

Figure 5.2. Venn diagram representation of the properties of amino acid residues (Sternberg 1996).

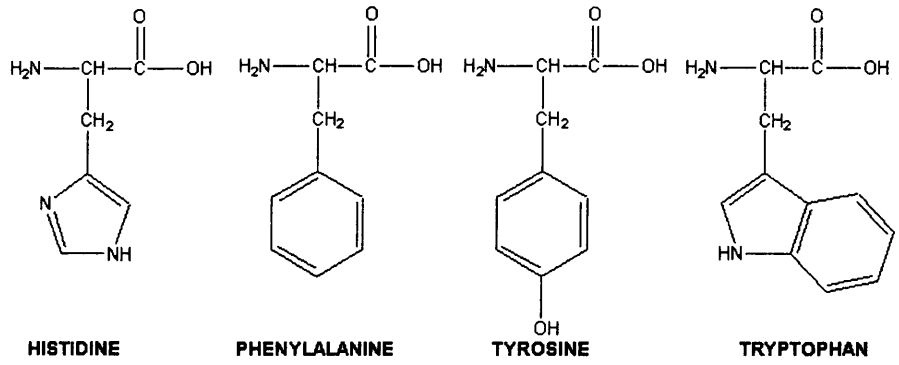

Figure 5.3. The structure of the 'aromatic' amino acids histidine, phenylalanine, tyrosine and tryptophan.

peptide mimics (Hoess *et al.* 1993; Phalipon *et al.* 1997) and protein structure (Sternberg 1996) place histidine alongside the aromatics (see Figure 5.2). This is because the electrons within the ring of an aromatic amino acid are able to resonate (delocalize). Histidine is also known to resonate when both nitrogens in the ring are protonated (with H+) (J. Grondin, personal communication) (Wolff *et al.* 2002). Therefore, histidine could share similar binding characteristics as the other three aromatic amino acids (see Figure 5.3). For these reasons, histidine will be classified as an aromatic residue in this thesis.

Interestingly, the aromatic amino acids are over-represented in our peptides. The serotype 9V mimics are particularly rich in phenylalanine and tryptophan. MP7 consists of 33 % aromatic amino acids, MP12 25 %, MP55 17 % and MP58 13 %. Only three of the four aromatic amino acids are over-represented in the 6B peptide mimics, in particular tryptophan is represented more than 6 times the expected frequency; histidine is the only one not represented. Overall MP10, MP14, MP15 and MP18 consist of 25 % aromatic amino acids, whereas MP2, MP13 and MP17 consist of 17 % aromatic amino acids. This feature of other carbohydrate mimics (Phalipon *et al.* 1997), including serotype 6B pneumococcal polysaccharide peptide mimics (Shin *et al.* 2002) has been reported previously. Shin and colleagues (2002) identified peptide mimics displaying an over-representation (>1.0) of all four aromatic amino acids (analysis was performed here based on the findings of Shin and colleagues (2002)) (Shin *et al.* 2002).

5.4.1.2 Cysteine and Proline

Cysteine (C) and Proline (P) residues create important structural features such as cyclical constraint (C) or an L-shaped bend in the linear sequence (P). As discussed in §1.5.3, two cysteines can have a disulphide bond between them, which circularises the peptide. Cysteines and prolines are extremely important to the shape of a peptide. In fact all the non-cysteine (C) containing peptides that we identified contained at least one proline (P) residue (see Figures 3.9 and 3.24). Furthermore, proline may also serve an important structural role in orientating the aromatic side chains in a manner similar to the branched nature of the carbohydrates they mimic (Phalipon *et al.* 1997). This is further discussed in §5.5.1.

5.4.1.3 Small amino acids

Alanine (A), glycine (G), serine (S), threonine (T) and valine (V) are all small residues that do not generally have a major structural role. This may explain the similarity in their occurrence. All have an observed/expected frequency below 1.0 in all the peptide mimics.

5.5 Multiple-sequence alignment

A multiple sequence alignment is the alignment of residues (either amino acid or nucleotide) of several sequences, with gaps introduced to maximise matches. Multiple sequence alignments are useful because it can identify areas of conservation among the sequences. These conserved areas can help predict the secondary and tertiary structures of new sequences (Thompson *et al.* 1994).

Many of the protein alignment programs that exist (Swiss Prot, Brookhaven Data Bank for example) are used to identify the functions of experimentally determined sequences against proteins already documented, based on sequence homology (Baxevanis and Ouellette 2001). Rarely are two sequences aligned with 100 % homology, yet the function of these proteins could in fact be similar. This is because some amino acids can be substituted by others without any alteration to the function of the protein (Thompson *et al.* 1994). Therefore if amino acids can be interchanged genetically and the protein remains functionally similar then it could be assumed the shape is maintained.

In this study, the widely available ClustalW alignment program was used to align the peptide mimics for each pneumococcal serotype. This multiple-sequence alignment program, as well as many others, utilise three steps in performing an alignment. First, pairwise alignments of all the sequences were performed to calculate a distance matrix. This was done by scoring the distance matrix that corresponded to the number of matches or runs of identical residues (1 - 2 for amino acids), minus a fixed penalty for every gap (Thompson *et al.* 1994). The second step was to generate a guide tree from the distance matrices calculated during the pairwise alignments. The third step was progressive alignment of all the sequences according to the branching order on the guide tree. The most closely related sequences were aligned first, progressing to the most distantly related sequences (Thompson *et al.* 1994). The percentage sequence identity was an estimation of the similarity of the sequences. It was thought that two sequences which share 30 – 40 %

identity over the whole sequence were generally the same (A. Stark, personal communication). This is usually relevant to proteins, but since there are no specific alignment programmes for peptides, it was thought to be just a useful threshold for this study.

The outcome of these alignments and the residues found in peptide mimics of serotype 6B and 9V pneumococcal capsular polysaccharide will now be discussed.

5.5.1 Characteristics of serotype 6B PnCPS peptide mimics

It was noticed that all the 6B peptide mimics contained a high proportion of aspartic acid (D) and glutamic acid (E) residues; both are negatively charged at neutral pH. This could imply that there is at least one positively charged residue in or near the paratope of the corresponding monoclonal antibody and that this could be an important feature of a serotype 6B pneumococcal polysaccharide mimic. These residues are of particular interest since, in aqueous solution the polypeptide chain will generally fold so that the polar, charged residues, which include D and E, are on the surface and the hydrophobic groups are clustered in the interior of the molecule where they are more stable (Stryer 1995).

All the peptide mimics contained at least one proline (P) residue, which creates an important structural feature (L-shaped bend) in a linear sequence (Simon 1993). This was discussed in §5.4.1.2 and can be seen in Figures 5.4 and 5.5, which highlight the presumed similarity between the structures of peptide mimic MP2 and 6B polysaccharide.

Additionally, all the proline residues present in these peptides were found near to aromatic amino acids. Peptide mimics MP10 (EIY**P**WY**P**MAERS) and MP15 (YSLLVE**P**YTFD**P**) both contain two proline residues, which sandwich two aromatic amino acids between them. The pairing of tryptophan and proline residues (either PW, WP or WGP) was found in six of the 7 peptide sequences (in MP15 a proline is adjacent to tyrosine, which is also an aromatic proline pairing). This could be significant, not only because the proline could orientate the tryptophan so that it is in a prominent position, but also in regards to the number of rings in close proximity. This feature has been described before (Hoess *et al.* 1993) and two hypotheses have been formed to explain this. The first is that the aromatic character of the amino acid (W, Y, or F) allows interactions with the aromatic-rich cluster often found at the bottom of the paratope of antibody structures (Hoess *et al.* 1993).

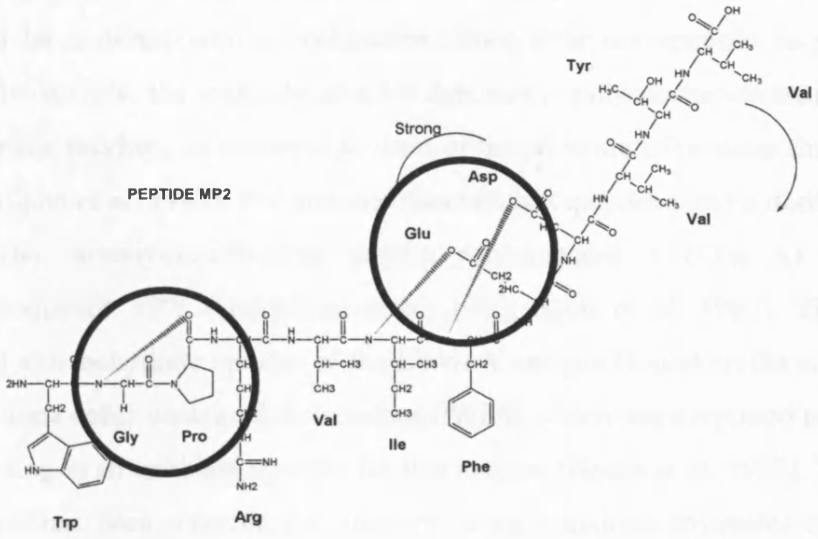

Figure 5.4 The presumed structure of the peptide mimic MP2 based on the interactions of nearest neighbour amino acids. The region highlighted by the grey box was presumed to be responsible for the toxicity effects observed after administering a booster dose of this peptide. Regions of strong H-binding potential are highlighted by arrows or grey circles.

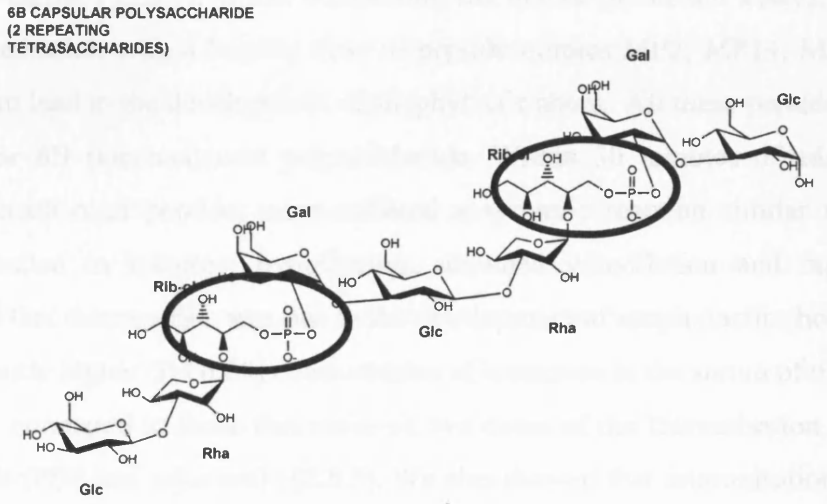

Figure 5.5 The structure of two repeating units of serotype 6B pneumococcal capsular polysaccharide based on the structure described by Kamerling (2000). The regions of high H-binding potential are highlighted by grey circles.

Therefore, aromatic residues present in the peptides could interact with aromatic residues located in the antigen binding site (hypervariable loops) of the mAb (Mertens *et al.* 2001). This would be a disappointing explanation, since it is not specific to polysaccharide mimics. Alternatively, the antibody directed against the polysaccharide structure could be rich in aromatic residues, as observed in most of the structure of proteins that interact with sugars (Phalipon *et al.* 1997). For instance, random hexapeptides and a dodecapeptide that bound to the carbohydrate-binding protein concanavalin A (Con A) contained the consensus sequence YPY (Oldenburg *et al.* 1992; Scott *et al.* 1992). The amino acid sequence of a carbohydrate epitope of the Lewis Y antigen (found on the surface of many adenocarcinoma cells) contained the residues PWLY, which were reported to be critical for peptide binding to an antibody specific for this antigen (Hoess *et al.* 1993). Interestingly, a similar result has been reported for mimicry of carbohydrate structures of anti-idiotype antibodies. Phalipon and colleagues (1997) found 4/19 peptide mimics of *S. flexneri* LPS contained either a WP or a WGP, which was regarded the same since glycine is a small residue and generally has no structural importance. Furthermore, 2/8 peptide mimics of *V. cholerae* CPS contained the same pattern (S. Falklind-Jerkerus, unpublished data). The reason for this preference for aromatic amino acids was explained in §5.4.1.1.

5.5.1.1 Peptide Toxicity

As stated earlier (§5.2.5), whilst completing the mouse protection assays, it was noticed that immunisation with a booster dose of peptide mimics MP2, MP14, MP17 and MP18 appeared to lead to the development of anaphylactic shock. All these peptides were mimics of serotype 6B pneumococcal polysaccharide. Within 30 minutes of administering the booster injection of peptide, mice suffered a systemic reaction similar to anaphylaxis, which resulted in lethargy, hypothermia, assumed vasodilation and rapid death. We confirmed that this reaction was due to the development of anaphylactic shock by detecting a significantly higher ($P < 0.05$) concentration of histamine in the serum of mice immunised with MP2 compared to those that received two doses of the immunisation solution minus the peptide (PBS and adjuvant) (§2.8.5). We also showed that immunisation with MP2 led to the production of the immunoglobulin IgE (§3.8.5.6). This antibody is indicative of an allergic response.

This phenomenon could not be due to the presence of contaminants in the immunisation solution, since the same reagents were used to prepare other peptides where this effect was

not noted (MP10 and MP13, for example). The company that manufactured the KLH conjugated peptides (CRB, Cambridge UK), confirmed that the peptides were free of toxins and that this side effect had not been reported before. Also, the presence of endotoxin was excluded by means of a LAL test (performed by A. Schaeffer, Dept of III, University of Leicester, UK), which was negative. Moreover, this result was only noted upon booster injection, whereas if toxins were present the effects would be expected after the primary dose alone. Therefore we concluded that this was a result of the peptide mimic and this itself raised several hypotheses: one hypothesis was that the peptides were mimicking a known allergen, therefore upon immunisation the mouse responds as if it encounters the allergen and goes into anaphylactic shock. However BLAST searches of the sequences of MP2, MP14, MP17 and MP18 revealed no exact matches.

To date, there are no published examples of this type of response occurring from peptide mimic vaccines. This led to the hypothesis that the peptides mimic an epitope of serotype 6B pneumococcal capsular polysaccharide that in its native state stimulates a localised allergic reaction. The basis of this hypothesis relies on previous findings that imply a role for histamine in pneumococcal infection (du Moulin *et al.* 1982; Esaki *et al.* 1991; Norn *et al.* 1994; Kjaergard *et al.* 1996; van der Ven *et al.* 1999). Van der Ven and colleagues (1999) induced pneumococcal infection in the middle ear of rats using histamine to block mucociliary beating, induce mucosal swelling and impair clearance of the eustachian tube prior to intranasal inoculation of the pneumococci (van der Ven *et al.* 1999). Du Moulin and colleagues (1982) suggested that treatment with the antihistamine cemetidine predisposed patients to the development of pneumococcal disease. Furthermore, several pneumococcal factors, including capsular polysaccharides, are known to induce the release of inflammatory mediators (Simpson *et al.* 1994). In one study, bacteria isolated from the lower respiratory tract of twelve patients with pneumococcal infection caused an IgE-mediated histamine release from the patient's own blood leukocytes, indicating that these patients were sensitised to their own bacteria (Norn *et al.* 1994). This may explain why asymptomatic colonisation can predispose to pneumococcal infection (Barthelson *et al.* 1998; Dagan *et al.* 2004) and why mast cell degranulation is observed during pneumococcal infection of the lung (Kerr *et al.* 2002). Interestingly, Kerr and colleagues detected significantly higher numbers of mast cell granules in the lungs of mice resistant to pneumococcal infection than in those of susceptible mice immediately following infection (Kerr *et al.* 2002).

Another hypothesis we considered was that certain amino acids in the sequence stimulated an antibody class switch from IgM to IgE. To investigate the latter two hypotheses we a devised a study based on the sequences of the peptide MP2. As stated earlier, we noticed that all the 6B peptide mimics contained a high proportion of glutamic acid (E) and aspartic acid (D) residues; both are negatively charged and have strong hydrogen bonding capabilities (Stryer 1995). Furthermore, as Figure 5.6 shows, the peptides that induced the allergic response presented these residues as pairs or in close proximity to each other (i.e. ED, EWD, DE). Whereas the non-allergenic peptides (MP13, MP15 and MP10) lacked this feature.

MP2	----WGPRVIF**ED**VTV-	COOH
MP17	---**D**W-PAVIF**ED**TRA-	COOH
MP18	----W-PQIWF**DDEED**V	COOH
MP14	**WEWD**W-PRI**E**LN-I---	COOH
MP13	---**D**W-PALIF**D**QDM-L—	COOH
MP15	----Y--SLLV**E**PYT-F**D**P	COOH
MP10	--**E**IY-PWYPMA**E**RS----	COOH

Figure 5.6 The sequences of the peptide mimics that induced anaphylaxis upon booster dose. Acidic amino acids are highlighted in bold. The other 6B peptide mimics that did not induce anaphylaxis are also shown. The alignment was performed by ClustalW.

To test this theory the glutamic acid (E) and aspartic acid (D) residues present in MP2 were substituted for their uncharged deivatives, glutamine (Q) and asparagine (N) respectively, which contain a terminal amide group in place of a carboxylate group. This substitution suggested that the hydrogen binding potential of these residues would be altered but the overall size of the peptide would be unchanged. The sequence of MP2 was modified since this peptide contains one glutamic acid and one asapartic acid residue and it was hypothesised that the effect of the substitution would be more pronounced. The sequence of the modified peptide, known as MP2m was NH_2 –WGPRVIF**QN**VTV – COOH (the modified residues are highlighted in *boldface*). Mice were then immunised

with either MP2-KLH or with MP2m-KLH. The experiment was done using eight groups of mice, with each having a slightly different immunisation schedule (see Table 2.8). The doses received by each group and the results of anaphylaxis were recorded in Table 3.8.

We showed that the elimination of the glutamic acid (E) and aspartic acid (D) residues from the sequence of the peptide MP2 reduced its ability to induce anaphylaxis in mice. Symptoms of anaphylaxis (raised serum histamine levels and decreased body temperature) were also significantly lower in those groups immunised with two doses of peptide. Futhermore, mice immunised with a primary dose of MP2 did not suffer anaphylaxis upon boost with MP2m. Similarly, a primary dose of MP2m did not induce anaphylaxis upon boost with MP2. This suggested that the antibodies produced in response to immunisation with MP2 were raised to the region of the peptide containing the acidic amino acids.

We also showed that mice passively immunised with the monoclonal antibody that selected MP2 from the phage library (Db3G9) prior to challenge with two doses of MP2 were equally susceptible to this peptide's ability to induce anaphylaxis. There was no significant difference between the serum histamine concentration and body temperatures recorded in these mice compared to mice that received only peptide MP2. We had hypothesised that the mAb would bind the peptide upon immunisation and counteract the effects of the boost, but this result implied that either the mAb did not recognise the allergen-causing motif, or it could mean that we administered too high a dose of peptide, so there was still free antigen.

Prophylaxis with the anti-histamine cetirizine prior to the second dose of MP2 also made no difference to the ability of the peptide to induce anaphylaxis. This could mean histamine is not the mediator or it is binding to another H receptor. There are four known histamine receptors (H1, H2, H3 and H4). Cetirizine blocks histamine binding to H1 receptors, therefore it may be useful to investigate different receptor blockers. Interestingly, the potent H2 blocker, cimetidine, has been shown not to impair pneumococcal infection of the lung (Esposito 1987). The function of the H3 receptors is not fully understood, but this could also be interesting to investigate.

5.5.2 Features of the serotype 9V PnCPS peptide mimics

The peptides selected to mimic serotype 9V pneumococcal polysaccharide share less similarity to each other than the serotype 6B mimics. This maybe because there are fewer peptides to perform an alignment. Also the four peptides are different lengths, whereas the 6B mimics were all 12 amino acids long.

Nonetheless, there are some similarities between the peptides (see Table 3.4); all three non-cysteine (C) containing peptides contained at least one proline (P) residue, both (as described before) are important structurally, and create features such as cyclical constraint or an L-shaped bend in the linear sequence. Proline may serve an important structural role in orientating the aromatic side chains in a manner similar to the branched nature of the carbohydrates they mimic (Phalipon *et al.* 1997). MP7 (KFH**P**KDI**P**YQVW) contained two proline residues, one of which was adjacent a tyrosine (Y) residue and the other adjacent a histidine (H) residue. The sequence of MP55 (RQFE**C**YTTT**C**VG) contained two cysteines, one of which is adjacent to an aromatic amino acid (tyrosine). This was not noted for MP12 (HKKDFARG**P**GWS) or MP58 (RQGEEVYMWRDSM**P**A).

5.5.2.1 Immunisation with a cyclically constrained peptide resulted in the highest antibody response

Immunisation with peptide mimic MP55, which was the only peptide that was cyclically constrained, produced antibody that had the highest avidity to the immunogen of all the peptide mimics. Other studies have reported that constrained peptides have high avidity (Brown *et al.* 1984; Mertens *et al.* 2001). Brown and colleagues found that constraining the peptide conformation successfully increased antibody binding (Brown *et al.* 1984). Mertens and colleagues (2001) hypothesised that cyclically constrained peptides would potentially give rise to better immunogenic mimics, since they allowed fewer conformations (Mertens *et al.* 2001). The strength of antibody binding to the peptide MP55 correlated to the ability to confer protection in mice since mice that produced high avidity antibody survived challenge (see §3.6.4.5). This could mean that including cysteines in the peptide sequences of less immunogenic peptides could improve the immune response. An interesting experiment might be to see whether substituting the proline residues for cysteines (since both are structurally important) increases the avidity of the antibody response. The sequence of MP7 contained two proline residues, which could be substituted for cysteines in an attempt to increase the immune response to immunisation.

5.6 The limitations of this study

The limitations of this study were present in the method of peptide selection, the stability of the peptides in different solutions, and genetic restriction in immunised individuals. These will be discussed in the following sections.

5.6.1 Method of peptide selection

Many studies have found success using phage displayed peptide libraries. These include peptide mimics of a variety of bacterial carbohydrate epitopes including *Shigella flexneri* LPS (Phalipon *et al.* 1997) Group B streptococcal capsular polysaccharide (Pincus *et al.* 1998), *Brucella abortus* LPS (De Bolle *et al.* 1999), and more recently, serotype 4 (Lesinski *et al.* 2001a) and 6B *S. pneumoniae* capsular polysaccharide (Shin *et al.* 2002). However, the success rate is difficult to determine from published work as they only report positive results. Commonly, binding peptides are missed due to overpanning. Overpanning a library usually means that a limited number of clones come to prevail in the population to the exclusion of others which have a similar or better avidity for the antibody (Barbas *et al.* 2001). It is difficult to know whether a library has been over panned because until positive clones are recovered, the user is unaware of the number of potential binders present in the library. Positive clones are only identified by immunoscreening (see §2.5.7.1). Therefore a possible solution to this is to perform immunoscreening after all rounds panning; however, if unsuccessful this could prolong the selection process.

It is also possible that the biopanning process itself may favour selection of particular sequences. For example components such as plastic, the antibody immobilisation system or blocking agents may all influence the peptides selected (Lesinski and Westerink 2001). This limitation makes it imperative that a selected peptide is evaluated with respect to its biological effect, e.g. it binds to the mAb and induces anti-carbohydrate antibodies upon immunisation. It is also important to exclude the possibility that selected phage bind epitopes outside the antigen-binding site. This can be achieved by performing inhibition assays to measure binding of selected phage to antigen specific antibodies. If pre-incubation of the mAb to the native antigen blocks binding to the peptide, the two epitopes must occupy the same location at the antigen-binding site. True peptide mimotopes should be capable of binding antigen specific antibodies and inducing a cross-reactive immune response upon immunisation (Grothaus *et al.* 2000).

In my opinion, the fact that there is no strict method to follow to obtain peptide mimics from a phage library is one of the biggest problems in using this method. For example, it is unknown what antibodies or proteins select better mimics. Of course, there is no guarantee of finding the 'ideal' vaccine candidate with any of the methods described in §1.5.1. As stated above, many studies, including this one, have found success using phage libraries, and therefore there can be no doubt it is a useful method. We identified a 'true' mimic of pneumococcal polysaccharide and two peptides that stimulated a protective immune response in immunised mice. Furthermore, as the number of published accounts grows, the technique to use will become clearer.

5.6.2 Peptide stability

The mAbs DM5 and Db3G9 were both raised to serotype 6B PnCPS, however their complementarily-determining regions were found to be quite distinct when the DNA sequences of the V_L and V_H regions of the two mAbs were determined (H. Baxendale, personal communication). This means that there was no reason to believe the peptide sequences would bind complementary regions of both mAbs. Despite this, some peptide mimics selected from the phage libraries by mAb Db3G9 also bound to DM5, in particular peptides MP2 and MP14 significantly reacted to both mAbs (see §3.7.3). As discussed previously (§5.2.3), Shin and colleagues also described peptide mimotopes of serotype 6B pneumococcal polysaccharide cross-reacting with an antibody raised to *Neisseria meningitidis* group B polysaccharide (Shin *et al.* 2002). This ability to cross react with other anti-polysaccharide antibodies could be due to the stability of the peptide, since unstable peptides could adopt more than one shape in different solutions. This will now be discussed.

Phage-borne peptides have been known to lose their ability to bind the target molecule when synthesised chemically (Felici *et al.* 1993). This is thought to be because phage displayed peptides are soluble, but free peptides may be insoluble in solution (Felici *et al.* 1993). The peptide mimics in our study were synthesised conjugated to a soluble fusion protein (KLH or BSA). The results (shown in Figure 3.16) showed that antibodies that recognised the phage borne peptide MP58 also bound the conjugate peptides. Therefore, the structure of the phage-displayed peptide was maintained after conjugation to KLH or BSA. However, this type of investigation could not be performed for the other peptides, as no other phage-borne peptides were used to immunise mice.

Structural analysis of the relationship between a phage-displayed peptide and a KLH-conjugated peptide, or an *in vivo* peptide and a peptide in solution, was thought could lead to an improvement in the immunogenicity of the peptide mimics obtained. However, due to time restraints and because the peptide needed to be much purer than was needed for immunisation this was not performed during this study. It could be achieved using X-ray crystallography, for example.

5.6.3 Genetic restriction

As Table 5.1 shows, the antibody response to immunisation with each peptide varied significantly. The highest average (mean) anti-peptide response recorded followed immunisation with peptide MP55 (at 54.6 mg ml^{-1}), whereas immunisation with MP13 only elicited an average antibody concentration of 62.7 μg ml^{-1}, nearly 1000-fold lower. These differences may be because an outbred strain of mouse was used in this study. This population contained genetically dissimilar individuals, which possessed different repertoires of MHC proteins. This was explained in §1.5.1.4. Briefly, to recapitulate, MHC class II molecules are found on the surface of antigen-presenting cells when the antigen is presented. After phagocytosis and processing, portions of the antigen bind to the MHC class II molecule, which presents the peptide on the cell surface to make it available to helper T cells. Since, the antibody response to protein antigens requires help (in the form of cytokines) provided to B cells by CD4 T cells, failure of certain antigens to bind MHC class II molecules generally reflects in a poor antibody response. Some peptides can bind to many class II alleles albeit with different avidity, implying that the same peptide can immunise individuals of diverse MHC haplotypes (see §1.5.1.4). However, some peptide antigens will not be recognised by some MHC alleles and will therefore appear invisible to the immune system of some individuals, for this reason MHC restriction is generally a big problem in vaccine development (Schuler et al. 2002).

Immunisation with MP7 stimulated the lowest concentration of antibody of all the serotype 9V peptide mimics and some immunised mice showed no antibody response. One explanation for this is that the sequence of MP7 maybe less efficient at binding the murine MHC II molecules than the other peptides (MP12, MP55 and MP58). Consequently, the MP7 sequence may not have stimulated B cell proliferation as efficiently, resulting in decreased anti-peptide antibody production. Similarly, the serotype 6B peptide mimic MP13 maybe less efficient at binding to the murine MHC II molecules than MP18. The

most likely explanation for this maybe found in the sequence of those peptides that stimulated a high antibody response, as these may contain motifs that enhance MHC recognition for those that lack a strong antibody response. All the peptides bound the mAb that selected them from the library and therefore, they must share similar binding characteristics. Replacing amino acids could enhance the binding avidity and improve MHC recognition without eliminating its ability to mimic pneumococcal polysaccharide.

Other mouse species may not respond in the same way to immunisation. Other studies have immunised BALB/c mice, however this strain is naturally more resistant to pneumococcal infection (Kerr *et al.* 2002) and therefore, may not be ideal for challenge. Furthermore, these results do not necessarily imply that the same genetic restriction would be present in humans.

5.7 Conclusion: the future of pneumococcal peptide vaccines

We have identified two peptide mimics that protect mice from developing pneumococcal disease. However, as discussed in §5.6.3, two peptides are insufficient to protect an entire population of different haplotypes. One way of improving the immune response to the other peptide mimics identified in this study, and in the future, could come from identifying structural motifs in the mimics. For example, a three-dimensional model of the interactions between the mAb-peptide or mAb-polysaccharide epitope could help identify important structural motifs in the mimics. This could be achieved by X-ray analysis of the co-crystals of these molecules (Mertens *et al.* 2001). A further method of improving the anti-pneumococcal immune response could come from conjugating the peptide mimics to an immunogenic pneumococcal protein. Four pneumococcal proteins have been shown to have potential for use as vaccines, these are PspA, pneumolysin, PsaA and PspC (Briles *et al.* 2000). Conjugation of the peptide mimics to one or more of these proteins could potentially give rise to anti-capsular and anti-protein immune responses.

Alternatively, different immunisation protocols could be tested. For example, boosting at different intervals or using an alternative adjuvant may stimulate an increased antibody response. The concentration of peptide used in each dose could also be increased. High levels of peptides are thought to generate high levels of specific MHC-peptide complexes on the antigen-presenting cell and therefore more antibody. However, in doing this, the

host in danger of developing tolerance to the antigen (Janeway *et al.* 1999). This study identified peptides that provoked different responses in mice. This could be a result of some of the limitations discussed in §5.6 and for these reasons an ideal vaccine may be a conjugate that displays different mimotopes. For example, a conjugate that displays more than one peptide sequence that mimics pneumococcal polysaccharide. This would also allow individuals of different MHC haplotypes to respond to the antigen.

Part B: Heterologous expression of pneumococcal polysaccharide *in planta*

The second part of this chapter discusses the results and impact of developing a transgenic plant capable of expressing pneumococcal polysaccharide.

5.8 To recapitulate

Prior to modern advancements in the synthetic pharmaceutical industry almost 60 years ago, mankind was dependent on plants as source of most medicinal compounds (Rao and Ravishankar 2002). Today, with the development of biotechnology, plants are reclaiming their therapeutic potential. Many investigators have already shown success using plants as vehicles for the manufacture and expression of vaccine protein antigens (Haq *et al.* 1995; Mason *et al.* 1998; Lamphear *et al.* 2002; Rigano *et al.* 2004) (see §1.6). This study has taken this concept a step further and we have shown that the recombinant expression of the type 3 capsule synthase (Cps3S) can lead to the production of type 3 pneumococcal capsular polysaccharide in plants.

The four genes involved in type 3 pneumococcal capsular polysaccharide synthesis are closely linked on the bacterial chromosome, arranged within a single locus (cassette) (Caimano *et al.* 2000). Previous work has shown that the recombinant expression of two of these proteins, $Cps3D^{+}S^{+}$, was needed for the synthesis of extra-cellular type 3 polysaccharide in *Lactococcus lactis* (Gilbert *et al.* 2000). Arrecubieta and colleagues found that expression of Cps3S alone led to the synthesis of type 3 polysaccharide in both unencapsulated pneumococci and *E. coli*, provided that the nucleotide precursors of the capsular polysaccharide (UDP-Glc and UDP-GlcA) were also present (Arrecubieta *et al.* 1996a). The two additional genes in the type 3-specific cassette, *cps3U* and *cps3M*, are not thought to be necessary for type 3 capsule production (Dillard *et al.* 1995; Caimano *et al.*

2000). We cloned a copy of the serotype 3 capsule synthase gene, *cps3S*, into plant expression vectors, which were transformed into different plant species. After cultivating a second generation of transgenic plants, we have shown the stable integration of the *cps3S* gene and production of type 3 pneumococcal polysaccharide. This discovery aims to confront the high vaccine production costs associated with pneumococcal vaccines.

5.9 Summary of results

5.9.1 The sequence of *cps3S*

A DNA fragment containing the type 3 capsule synthase gene, *cps3S,* was amplified by PCR using oligonucleotides based on the nucleotide sequence previously determined (Dillard *et al.* 1995) and cloned into a storage vector (pCR4-TOPO) (pCMS1). The DNA sequence of the MCS of this vector showed variations between the cloned nucleotide sequence of the serotype 3 capsule synthase gene and that obtained in the literature. This has been reported before as the sequences obtained from two studies (Arrecubieta *et al.* 1995; Dillard *et al.* 1995), *cps3S* and *cap3B,* showed significant mismatches in nucleotides between 667 bp - 704 bp. In their defence, the genes were obtained from two different strains of serotype 3 pneumococci, WU2 and 406, respectively. However, we amplified *cps3S* from WU2 and therefore, expected similar results to that of Dillard and colleagues. Accuracy in the sequence was maintained during PCR by only allowing the reaction to proceed for 10 cycles and by using a proofreading polymerase. Despite this, the amplified gene displayed 100 % sequence identity to that reported by Arrecubieta and colleagues (Arrecubieta *et al.* 1995). This implied that Dillard and colleagues might have reported sequencing errors and the two studies in fact used the same sequence. The quality of the sequence was important to have confidence the protein would function as expected.

5.9.2 Bacterial expression vectors

In case we detected no protein/polysaccharide expression *in planta*, we also cloned the amplified *cps3S* into a bacterial expression vector pRSET C (pCMS5). This was because the bacterial culture had many advantages over whole plant culture. Bacterial cell suspensions grow more rapidly, have greater homogeneity and, consequently, a high proportion of the cells are in direct contact with the antibiotic selection in the culture medium. This is unlike whole plant cell culture in which antibiotic selection is ceased after rooting and transfer to soil. The advantages of a bacterial culture was thought to be

beneficial to examine protein expression and/or investigate any problems that were encountered with the plant culture. pRSET C provided in-frame transcription and an upstream Shine-Delgano sequence for ribosome binding. Arrecubieta and colleagues found that expression of *cps3S* led to the synthesis of type 3 polysaccharide in *E. coli* carrying the bacteriophage DE3 (Arrecubieta *et al.* 1996a). Therefore, we transformed pCMS5 into *E. coli* BL21(DE3)pLysS. The pLysS plasmid allowed stricter control of the expression levels of the cloned protein. This was important because the use of high copy-number plasmids and/or poorly regulated promoters was thought to be the cause of previous unsuccessful attempts to clone and express pneumococcal genes involved in capsular polysaccharide synthesis in *E. coli* (Dillard and Yother 1991; Arrecubieta *et al.* 1996). All the plasmids constructed during this study were described in Table 2.10.

Due to time restraints and successful *in planta* expression (see §5.7.3.2), the full potential of the pCMS5 construct was not explored. It could be useful to look at the level and size of the protein expressed and its enzymic activity *in vitro*, for example.

5.9.3 Plant expression vectors

The small, versatile pPZP family of *Agrobacterium* binary vectors, or their derivatives, have been used on numerous occasions to transform plants (Gao *et al.* 2003; McIntosh *et al.* 2004) and have been fully sequenced (Hajdukiewicz *et al.* 1994). These vectors contain, within the T_i derived T-DNA border regions, bacterial marker genes, such as chloramphenicol (pPZP100 series) or spectinomycin resistance (pPZP200 series), a pUC18 multiple cloning site (for inserting the transgene) together with the plasmid origins for replication in *E. coli* and in *Agrobacterium* (ColE1 and pVS1, respectively) (Hajdukiewicz *et al.* 1994). The T-DNA region also contains plant marker genes, such as antibiotic resistance and ß-glucuronidase (GUS) expression (see §5.11.1).

5.9.3.1 pCMS3

In this study *cps3S* was cloned into the T-DNA region of a binary plant expression vector known pCHF2 (pCMS3), which was previously derived from pPZP222. To enhance expression of the transgene in plant cells, a duplicated CaMV35S promoter (see §2.11.2.1 for further details) had been cloned into the T-DNA region upstream of *cps3S*. Furthermore, the signal sequence known as PR1B, was used to direct secretion of the transgene. This is because unlike prokaryotic cells, plant cells are highly

compartmentalised and metabolic pathways occurring in more than one compartment have to be connected by transport processes (Kunze *et al.* 2002). A previous study investigated the secretion of different proteins using PR1B under the control of a CaMV35S promoter (Denecke *et al.* 1990). They replaced the initiation codon of the transgene with PR1B and found that the product was secreted via the endoplasmic reticulum (ER) to the apoplast (Denecke *et al.* 1990). It was thought that without a signal to direct transport, heterologous proteins would migrate via a non-specific 'default pathway' from the lumen of the ER via the Golgi apparatus to the cell surface (Denecke *et al.* 1990). However, overall protein expression was less when the signal sequence was present (Denecke *et al.* 1990). Nonetheless, it was believed that the secretion of Cps3S to the apoplast would allow easy extraction of type 3 polysaccharide in a form that could be used directly for immunisation of mice.

pCMS3 was successfully transformed into *E. coli* and *Agrobacterium tumefaciens*. Expression of the spectinomycin resistance gene, *aadA*, allowed selection of transformed bacteria, which was confirmed by DNA extraction and restriction digest. The transformed *A. tumefaciens* was then used to infect *Arabidopsis thaliana* and *Nicotinia tobacum* plants by two different, but well-established techniques for each species. The gentamicin resistance gene, *aacCI*, in pCMS3 allowed selection of transformed plants.

Arabidopsis thaliana was successfully transformed and was shown to contain the *cps3S* gene. However, many plants were discarded due to enduring *Agrobacterium* contamination and those that survived, fourteen in total, contracted a fungal infection while housed in the incubator and had to be disposed of prior to setting seed. Furthermore, several attempts to transform Tobacco with pCMS3 were unsuccessful. This was thought to be due to the method of selection. The gentamicin concentration was ranged from 50 $\mu g\ ml^{-1}$ to 250 $\mu g\ ml^{-1}$, yet all the leaf discs developed signs of chlorosis (yellowing or whitening of plant tissue). This indicated that either the cells were not transformed or the antibiotic was toxic to the eukaryotic tissue. This toxicity is not fully understood at the molecular level, but it is thought to affect various aspects of vacuolar and Golgi complex (or endoplasmic reticulum) function (Blackburn and Avery 2003). Since this is also the presumed route of polysaccharide synthesis, it was not thought to be useful to continue this route of investigation. Furthermore, gentamicin was not routinely used for selecting plant

transformants in our laboratory and, because we found success transforming plants with *cps3S* using pCMS4 (see §5.7.3.2), I would recommend abandoning using this approach.

5.9.3.2 pCMS4

The most commonly used marker genes in plants are those that provide resistance to antibiotics such as kanamycin, hygromycin and bleomycin (Draper *et al.* 1988). Kanamycin, in particular, has proved to be a very useful transformation marker (Draper *et al.* 1988) therefore, *cps3S*, along with the duplicate CaMV35S promoters, terminator and PR1B signal sequences from pCMS3 were cloned into pCambia 2301 (pCMS4).

pCMS4 was successfully cloned into *N. tobacum* by *A. tumefaciens*-mediated gene transfer. The parent generation of plants was grown from transformed leaf disc calluses using the growth hormones NAA and 6-BAP to promote shoot and root formation. A first filial (F1) generation was grown from the seed of six of the twelve plants that survived to set seed and five of these plants showed stable integration of the transgene by amplification of *cps3S* by PCR. All of these plants were also shown to synthesise type 3 polysaccharide, albeit at different levels. This could be due to the number of integrations. The site of T-DNA integration into plant DNA is apparently random and can occur several times in the same plant (Draper *et al.* 1988). This means that some plants may contain more copies of the *cps3S* gene than others, and therefore, could synthesise higher levels of type 3 polysaccharide. The number of integrations can be deduced using Mendelian genetics after counting the number of transgenic progeny that are resistant to the antibiotic selection. For example, as shown in Figure 5.7, one integration (of the T-DNA) would result in the growth of 1/4 of seeds since a ratio 1:3 would be sensitive:resistant to kanamycin. On the other hand, if there had been two integrations the ratio would increase to 1:15.

All five F1 plants displayed a 1:15 ratio or greater, and therefore contained at least two copies of *cps3S*. Growth of a second filial generation would confirm which progeny contained the most copies, but this could not be completed within the time scale of this thesis.

Figure 5.7. Punnet squares illustrating how the number of T-DNA integrations can alter the number of transformed F1 progeny

One Integration (A)

	A	a
A	AA	Aa
a	Aa	aa

1:3

Two integrations (A and B)

	AB	Ab	aB	ab
AB	AABB	AABb	AaBB	AaBb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	AaBb
ab	AaBb	AaBb	aaBb	aabb

1:15

The *cps3S* gene is present as a dominant and recessive allele since the parent strain is heterozygous. The grey boxes represent those progeny that do not contain a copy of the transgene (T-DNA).

5.9.3.3 Extraction and production of pneumococcal polysaccharide in plants

Type 3 specific pneumococcal polysaccharide was extracted from plant tissue (albeit at different levels) using several methods. The most effective method, which obtained the highest concentration of pneumococcal polysaccharide (~4 μg g^{-1} plant tissue, based on observation alone), was to grind the plant tissue under liquid nitrogen, suspend the tissue in water and lyse the cells by sonication (see §2.15.1.1). Sonication appeared to be a crucial step, since homogenisation did not yield as much polysaccharide. These results could be interpreted in two ways; the polysaccharide is present in the cytoplasm of the cell and is therefore released upon sonication, or sonication disrupts the cell wall where the type 3 polysaccharide is embedded or bound. Gilbert and colleagues found that the type 3 pneumococcal polysaccharide produced by *L. lactis* was securely (covalently) linked to the cell wall (Gilbert *et al.* 2000). Furthermore, in *E. coli* type 3 polysaccharide remained bound to the outer membrane, possibly due to a lipid anchor attached to the outer membrane (Arrecubieta *et al.* 1996). This could be what occurred in plants. Plants are known to anchor proteins to the plasma membrane using a glycosylphosphatidylinositol (GPI) lipid anchor (Fischer *et al.* 2004). Post-translational GPI-anchor modification takes place in the endoplasmic reticulum, and GPI-anchored proteins are transported via the Golgi network to the plasma membrane where they are inserted into the outer layer of the lipid bilayer (Ikezawa 2002). Hemicelluloses, which share significant similarity to type 3 polysaccharide, are also assembled in the ER and Golgi apparatus (see below). This lead to

the hypothesis that type 3 polysaccharide may also be anchored to the plasma membrane of plant cells.

As mentioned earlier, plant cells are highly compartmentalised and metabolic pathways occurring in more than one compartment have to be connected by transport processes (Kunze *et al.* 2002). Despite cloning the signal sequence PR1B we detected no type 3 polysaccharide in its destination, the apoplastic fluid. This implied that PR1B was not functioning correctly. Previous studies replaced the start codon of the transgene with PR1B (Denecke *et al.* 1990), however, we maintained the start codon and cloned an in-frame sequence of *cps3S*. This may have led to a reduction in PR1B activity. Alternatively, native plant transport processes may have interfered with polysaccharide secretion. The plant cell wall is typically composed of 30 % cellulose and about 60 % matrix polysaccharides (pectin and hemicellulose) (Fry 1988). Type 3 pneumococcal polysaccharide is composed of two of the major sugars that form the hemicelluloses (glucose and glucuronic acid) (see §1.7.3.1). These polysaccharides are assembled in the ER and Golgi apparatus by glycosyl transferases (that catalyse the transfer of a glycosyl residue from a nucleotide sugar (e.g. UDP-glucose) to an acceptor molecule) and are delivered to the cell wall by vesicles of the endomembrane system (Fry 1988). Maybe the plant cell processes type 3 polysaccharide along with the native hemicelluloses, and therefore could be discharged to the cell wall (Northcote 1974). The hemicelluloses can be extracted by harsh alkali treatments (Fry 1988). Therefore, the transgenic plant tissue was lysed by sonication in 3M NaOH. This led to the extraction of pneumococcal polysaccharide, but at a lower concentration than when water or PBS was used. Furthermore, this method also led to an increase in non-specific binding in Ouchterlony from the wild-type plant extract. Therefore, this did not appear to be a correct hypothesis.

Alternatively, Cps3S has been shown to share significant homology to a number of processive β-glycosyltransferases, which include the cellulose synthases of plants (Cartee *et al.* 2001). Many assumptions to the function of Cps3S have already been reported using comparisons to these homologs (Forsee *et al.* 2000). For example, it has been suggested that Cps3S, along with the other processive β-glycosyltransferases, could be involved in the transport of the growing polysaccharide chain to the cell surface, possibly through a pore or channel (Keenleyside and Whitfield 1996; Cartee *et al.* 2000; Forsee *et al.* 2000). This is because, to date, no export system has been found to exist for type 3 polysaccharide

in *S. pneumoniae* and no other sequences have been identified that seem likely to be involved (Keenleyside and Whitfield 1996; Cartee *et al.* 2000). The functions of Cps3S and the biosynthesis of type 3 polysaccharide were described in more detail in §1.7.1.

Continuing with the comparisons to the processive β-glycosyltransferases, it could be assumed that, *in planta*, Cps3S behaves as the native cellulose synthases. Like Cps3S, these enzymes are bound to the plasma-membrane and use sugar precursors supplied by the cytosol to spin out cellulose from the surface of the cell (Alberts *et al.* 1994; Forsee *et al.* 2000). This hypothesis agrees with the findings of Gilbert *et al.*, (2001) and Arrecubieta *et al.*, (1996). As mentioned earlier, *E. coli* synthesised type 3 polysaccharide attached to the outer membrane by a lipid anchor (Arrecubieta *et al.* 1996). *E. coli* strains have been shown to later export some cell-surface polysaccharides across the inner membrane by a transporter (i.e. group 2 and 3 K antigens and colanic acid are exported across the inner membrane by an ABC transporter) (Cartee *et al.* 2001). Therefore, maybe Cps3S is bound to the plasma membrane and secretes type 3 polysaccharide into the cytoplasm. Forsee and colleagues found that the release of polysaccharide from the membrane was temperature and pH dependant (Forsee *et al.* 2000). These could be important considerations for improving future extraction techniques.

An alternative hypothesis is that the polysaccharide remained in the cytoplasm where the precursors were found. In *E. coli* expressing Cps3S, 40 % of the type 3 specific polysaccharide accumulated in the periplasm, 40 - 50 % in the cytoplasm, and the remainder was found in the membrane fraction (Arrecubieta *et al.* 1996).

All of the presumed locations of the expressed polysaccharide *in planta* (based on the above hypotheses) are shown in Figure 5.8.

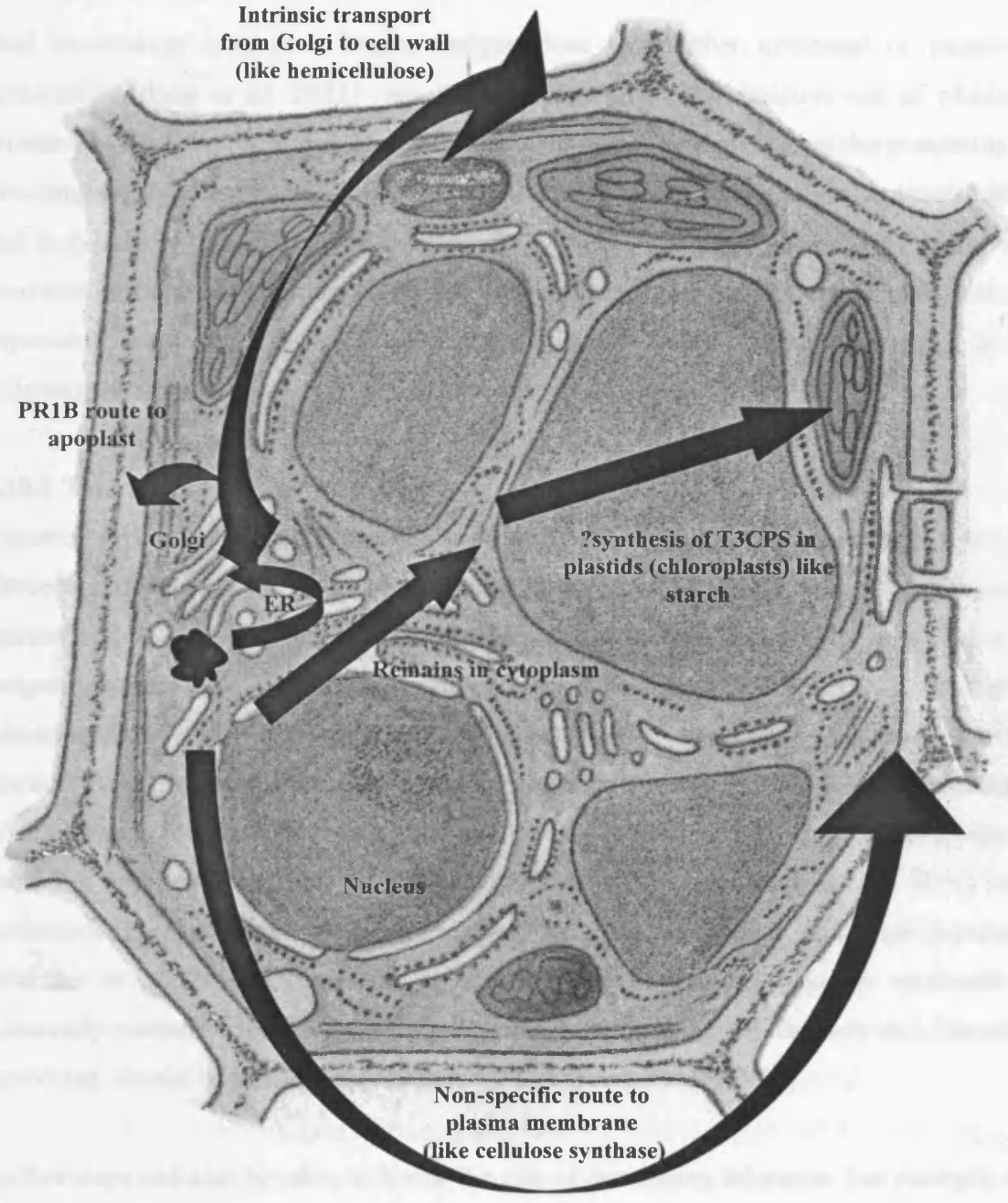

Figure 5.8. Putative routes of Cps3S and type 3 polysaccharide (T3CPS) transportation in plant cells. Five different routes have been hypothesised: 1) Cps3S is transported to the apoplast via the direction of the signal peptide PR1B, 2) Intrinsic transportation systems regard T3CPS as the hemicelluloses and transport the polysaccharide to the cell wall, 3) Cps3S remains in the cytoplasm where T3CPS is synthesised, 4) T3CPS is synthesised in plastids (chloroplasts) where the biosynthesis of starch is largely confined, 5) Cps3S is processed like the plant's own cellulose synthase and the protein is transported to the plasma membrane where T3CPS synthesis occurs. Diagram of plant cell was obtained from Alberts *et al.,* (1994)

5.10 Limitations

Oral vaccination requires a higher antigen dose than either intranasal or parenteral vaccination (Ryan *et al.* 2001) (see §5.10.1). Therefore, the effective use of plants as bioreactors for the production of a pneumococcal vaccines depends on the possibility of obtaining high levels of antigen that are stable during the life cycle of the transgenic plant and in subsequent generations (De Wilde *et al.* 2000). The limitations of this study were therefore present in the method of polysaccharide extraction, the tissue analysed for expression, gene silencing and also immune tolerance. These will be discussed in the following sections.

5.10.1 Tolerance

Repeated exposure to an oral antigen has the potential to produce immunological tolerance (Strobel and Mowat 1999). Tolerance is a useful mechanism to prevent autoimmune disease and is usually induced with repeated or continuous exposure to high doses of antigen (referred to as high zone tolerance), although some weak immunogens can induce tolerance if given in very small quantities (Strobel and Mowat 1999). This means that the immune system can fail to react to certain vaccine antigens, especially when administered in the context of food. However, the antigen dose necessary to induce protection is generally smaller than that required to produce tolerance (Tsuji *et al.* 2001) and, furthermore, induction of oral tolerance is both time-dependent and dose-dependent (Webster *et al.* 2002). Therefore, as a proviso, plant-derived vaccines expressed in commonly consumed foods should not become a component of regular diets and, like other medicines, should be administered appropriately (Webster *et al.* 2002).

Further steps can also be taken to lower the risk of developing tolerance. For example, the use of an adjuvant (such as cholera toxin) enhances immune responses at mucosal surfaces and, reduces the oral dose required to induce an immune response (Giddings *et al.* 2000). Delivering the antigen in intact plant material, rather than as a plant extract, may also enhance immunogenicity, as the tough plant cell wall and membrane compartments can provide increased protection from intestinal degradation (Modelska *et al.* 1998). High-level protein expression in seeds such as rice may also concentrate the antigen and further reduce dosing requirements. Furthermore, investigating varied immunisation schedules and strategies that combine different routes of administration, or vaccine types, are known to

result in enhanced protective immune responses (see §1.6.2.2) (Webster *et al.* 2002; Gao *et al.* 2003). For these reasons, many researchers believe it is unlikely that an edible vaccine would lead to oral tolerance (Webster *et al.* 2002).

5.10.2 Method of polysaccharide extraction

The extraction methods used to obtain type 3 polysaccharide in this study were relatively crude compared to those used previously (Gilbert *et al.* 2000; Cartee *et al.* 2001). This was due to time restraints and to avoid disposing of large quantities of the polysaccharide by mistake. It was not known where in the plant or cell polysaccharide synthesis would be the highest. Originally, the roots were predicted to express higher levels of pneumococcal polysaccharide, since the continuously growing primary cell wall was assumed to contain higher concentrations of the UDP-precursors (see §1.7.3.1). In practice, we extracted polysaccharide from leaf tissue. This was because we were working with parent and F1 generations and removal of the roots may have restricted growth of the plant and seed development. Leaf tissue was also much easier to obtain. For these reasons, the levels of polysaccharide extracted from plant tissue may not have been optimal.

For future investigations Fry (1988) describes several specific methods for purifying polysaccharides from different tissues and cell compartments.

5.10.3 Gene silencing and the problem of engineering a single metabolic step

Transgenic plants are known to suffer periods of gene silencing which leads to the inactivation or reduced transcription of a transgene (De Wilde *et al.* 2000). Napoli and colleagues reported that transformation of petunia with extra copies of the chalcone synthase gene resulted in a block in expression of both the transgene and the corresponding endogenous gene (Napoli *et al.* 1990). This was later discovered to be the result of two types of silencing that can occur in plants as well as in other eukaryotes: transcriptional gene silencing (TGS) and posttranscriptional gene silencing (PTGS) (Lessard *et al.* 2002). TGS is based on methylation of promoters and coding sequences of genes, which blocks transcription. PTGS is based on sequence-specific, targeted degradation of particular mRNAs (Lessard *et al.* 2002). Gene silencing can be associated to the development stage of the plant and the plant species used, transient expression of the transgene (not stably integrated), methylation of the gene and the transformation method (De Wilde *et al.* 2000).

If this occurred with *cps3S* then the plant would synthesise type 3 polysaccharide only in a single period of the plant's development.

Over the past few years, there has been a growing realisation that metabolic pathways must be studied in the context of the whole cell rather than at the single pathway level, and that even the simplest modifications can send ripples throughout the entire system (Capell and Christou 2004). We have metabolically engineered a plant to divert the flux of the UDP-precursors (UDP-Glc and UDP-GlcA) away from cell wall synthesis and to the manufacture of type 3 pneumococcal polysaccharide. This did not appear to effect plant growth, but in some cases the plant system has been known to reduce the effects of changing a single enzymatic step in an attempt to restore homeostasis (Capell and Christou 2004). This is discussed in §5.11.2.

5.11 The Future

5.11.1 Future experiments

With more time available, it would have been beneficial to develop a monoclonal antibody to type 3 polysaccharide. The polyclonal antibody appeared to cross-react to the wild-type plant extract, whereas a monoclonal could eliminate this background effect and make detection methods (such as immunodiffusion and immunolabelling) more sensitive.

The work so far has provided several valuable tools for future investigations. For example, the bacterial expression vector (pCMS5) could be used to examine protein expression without the time consuming task of plant culture. Furthermore, due to time restraints, the full potential of the *gusA* marker present in the plant vector pCMS4 was not explored. The GUS assay is a widely used reporter system in the study of bacteria, animals and plants (Gallagher 1992). This is mainly because of enzyme stability and sensitivity of the assay to detect ß-glucuronidase (GUS) activity by fluorometric, spectrophotometric, or histochemical techniques (Gallagher 1992). Gao and colleagues identified transformed cherry tomatillo plants by detecting the expression of GUS in pCAMBIA1301 (Gao *et al.* 2003). The tissues were immersed in X-Gluc (5-bromo-4-chloro-3-indolyl glucuronide), which stains GUS-expressing cells blue. The results (shown in Figure 5.9) demonstrated that *gusA*, and therefore the entire T-DNA region, was stably integrated into plant's DNA, since different organs all presented remarkably high GUS activity (Gao *et al.* 2003). This

useful adjuvant as it resists the gut's digestive juices (Richter and Kipp 1999). Yu and Langridge fused recombinant rotavirus fragments to CT and created a resistant vaccine that could be produced by potatoes (Yu and Langridge 2001).

5.11.2.1 Palatability

As discussed in §1.6.1, novel transformed species, such as banana (May *et al.* 1995), corn, leek (Eady *et al.* 2005), garlic (Eady *et al.* 2005), lettuce, walnut and others have been used recently to investigate *in planta* recombinant protein expression (Sala *et al.* 2003). These plants benefit from having edible, palatable tissue, which can be fed to animal subjects (such as mice) uncooked. Although fresh products are ideal, they may have short shelf life (Sala *et al.* 2003). Dried food could offer a partial solution, but the best solution (for shelf-life, stability and standardisation) could be delivery of the vaccine in the form of a dry powder, which could be mixed with food, for example. This can be achieved by using low cost food processing (Sala *et al.* 2003).

Bananas may offer the ideal oral pneumococcal vaccine delivery system. Although some researchers (Koprowski and Yusibov 2001) see a fundamental disadvantage with the banana system: They grow very slowly and it could take up to 2 years before the recombinant antigen levels can be evaluated (Johnson 1996), the advantages include, (a) ease of feeding, (b) they can be consumed uncooked so the antigen is not at risk of denaturing, (c) and they are grown in many developing countries where cheap vaccine technology is vital (Richter and Kipp 1999). This could offer an ideal solution for pneumococcal vaccines.

5.11.3 Engineering plants to synthesise all pneumococcal capsular polysaccharides

5.11.3.1 Lessons from the past

The concept of metabolic engineering in plants has been around since the 1960s when unnatural monosaccharides were fed to, and taken up by plant cells, resulting in defined alterations in the cell's biosynthetic output (Dube and Bertozzi 2003). Since then significant advances have been made, especially following the development of molecular biology techniques, and there have already been reports of transgenic plants metabolising increased levels of vitamin E (Shintani and DellaPenna 1998) and C (Smirnoff 2000; Agius *et al.* 2003), amino acids (Kishor *et al.* 1995; Hughes *et al.* 2004), biodegradable plastics (Slater *et al.* 1999; Bohmert *et al.* 2000), flavourings (Lewinsohn *et al.* 2001),

dyes, insecticides, and fragrances (Verpoorte and Memelink 2002). Metabolic engineering has also allowed plants to be exploited for their ability to manufacture polysaccharides of widely varying composition and structure. Details of the expression of some of these compounds are shown in Table 5.2.

The three basic objectives of metabolic engineering in plants are to increase the production of a specific compound, to down-regulate a specific unwanted compound, or to produce a novel compound (i.e. a molecule that is produced in nature, but not usually in the host plant, or a completely novel compound) (Capell and Christou 2004). Initially, researchers (like us) engineered single metabolic steps to increase or decrease metabolic flux to target compounds, block competitive pathways or introduce short cuts that diverted metabolic flux in a particular way (Capell and Christou 2004). In recent years plant metabolism has been successfully engineered to benefit human health (DellaPenna 2001). For example, the vitamin E activity of *Arabidopsis* seeds was increased by cloning the final enzyme in the synthesis of α-tocopherol (vitamin E), known as γ-tocopherol methyltransferase (γ- TMT) (Shintani and DellaPenna 1998). *Arabidopsis* seed, like most oilseed crops, naturally contains a high proportion of γ-tocopherol, which has 10 % of the vitamin E activity of α-tocopherol. Expression of γ-TMT resulted in the conversion of the large pool of γ-tocopherol to α-tocopherol, which corresponded to a 10-fold increase in vitamin E activity (Shintani and DellaPenna 1998).

5.11.3.2 Multi-gene engineering

As previously discussed (§5.10.2), attention is currently shifting away from single-gene engineering strategies recently and towards more complex approaches involving the simultaneous expression of multiple genes (Capell and Christou 2004). This is because methods that divert flux of a metabolic pathway in a particular way are thought to have a limited value, since the plant system could reduce these effects (by over expression of host genes) in an attempt to restore homeostasis (Capell and Christou 2004).

Already researchers have found success expressing more than one transgene in a given individual transformant, resulting in the production of completely novel compounds (Chabannes *et al.* 2001). Poly(hydroxyalkanoates) (PHAs) are natural polymers with thermoplastic properties which can be produced by bacterial fermentation (Slater *et al.* 1999). Slater and colleagues introduced four bacterial genes (an entire metabolic pathway),

Table 5.2 Examples of Metabolic Engineering in Higher Plants

	Compound	*Gene(s) or enzyme*	*Source*	*Transformed plant species*	*Product level (% normal)*	*Reference*
UPREGULATED COMPOUNDS	Mannitol	*MtlD*	*E. coli*	Tobacco, *Arabidopsis*	16	(Tarczynski *et al.* 1992)
	Proline	P5CS[a]	Mothbean	Tobacco	100–200 (10-18-fold)	(Kishor *et al.* 1995)
	Sorbitol	*Stpd1*	Apple	Tobacco	260	(Tao *et al.* 1995; Sheveleva *et al.* 1998)
	Tryptophan	TDC[b] and ASα	*Arabidopsis*	*Catharanthus roseus*	87	(Hughes *et al.* 2004)
	Vitamin C	*GalUR*[c]	Strawberry	*A. thaliana*	2-3 fold	(Smirnoff 2000; Agius *et al.* 2003)
	Vitamin E	γ-TMT[d]	*Arabidopsis*	*Arabidopsis*	10-fold	(Shintani and DellaPenna 1998)
DOWN-REGULATED	Pectic galactose	EPG[e]	*Aspergillus aculeatus*	Potato	-30	(Oxenboll Sorensen *et al.* 2000)
	Lignin	*cad*[f] and *ccr*[g]	Tobacco	Tobacco	-50	(Chabannes *et al.* 2001)
NOVEL COMPUNDS	Biodegradable plastics (PHB[h])	*Phb*ABC and TD	*Ralstonia eutropha* and *E.coli*	*Arabidopsis thaliana*	n/a	(Slater *et al.* 1999; Bohmert *et al.* 2000)
	Type 3 pneumococcal polysaccharide	Cps3S	*S. pneumoniae*	Tobacco	n/a	This study

[a]δ-pyrroline-5-carboxylate synthetase, [b]tryptophan decarboxylase, [c]NADPH-dependent D-galacturonate reductase, [d]gamma-tocopherol methyltransferase, [e]endo-polygalactanase, [f]cinnamyl alcohol dehydrogenase, [g]cinnamoyl-CoA reductase, [h]poly(3-hydroxybutyrate)

encoding the enzymes 3-ketothiolase, acetacetyl-CoA reductase, and PHA synthase from *Ralstonia eutropha* and threonine deaminase from *E. coli* into *Arabidopsis* plants which resulted in the production of poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) a useful commercial biodegradable plastic marketed under the trade name Biopol (Slater *et al.* 1999). PHBV was shown to accumulate in plant plastids in the leaves of *Arabidopsis* plants by redirecting the metabolic flow of intermediates from fatty acid and amino acid biosynthesis (Slater *et al.* 1999). When the genes for only three enzymes were cloned another biodegradable plastic, poly(3-hydroxybutyrate) (PHB), was produced and accumulated in the plastids of transgenic *A. thaliana* plants at levels exceeding 40 % dry shoot weight (Bohmert *et al.* 2000).

This is a very interesting development as it implies the expression of multiple pneumococcal genes could result in the production of all pneumococcal serotypes *in planta.* Conveniently the genes involved in pneumococcal capsular polysaccharide synthesis are closely linked on the bacterial chromosome, arranged within a single locus (cassette) (Caimano *et al.* 2000) (this was discussed further in §1.7.1). For *in planta* expression, each gene needs to be accompanied by a plant promoter (such as CaMV 35S promoter), yet this implies that cloning a vector coding for all the genes represented in the type specific cassettes within a T-DNA region could result in the production of capsular polysaccharide from all 90 pneumococcal serotypes. Furthermore, this work could provide an essential platform to explore the possibility of using the plant glycosylation machinery to conjugate bacterial polysaccharide to vaccine carrier proteins. Rigano (2004) has already reported the production of a fusion of two proteins consisting of heat labile enterotoxin (LT-B) and a 6kDa tuberculosis antigen (ESAT-6) in transgenic *Arabidopsis thaliana.* If this approach was successful, these two strategies could provide an effective, cheap, pneumococcal vaccine antigen that is simple to manufacture and would offer protection against all known serotypes of pneumococcal disease to all at risk populations.

REFERENCES

Aaberge, I. S., J. Eng, G. Lermark and M. Lovik (1995). "Virulence of *Streptococcus pneumoniae* in mice: a standardized method for preparation and frozen storage of the experimental bacterial inoculum." Microbial Pathogenesis **18**(2): 141-52.

Advisory Committee on Immunization Practices (1997). "Prevention of pneumococcal disease: recommendations of the Advisory Committee on Immunization Practices." Morbidity and Mortality Weekly Report **46**(RR-8): 1-24.

Agius, F., R. Gonzalez-Lamothe, J. L. Caballero, J. Munoz-Blanco, M. A. Botella and V. Valpuesta (2003). "Engineering increased vitamin C levels in plants by overexpression of a D-galacturonic acid reductase." Nat Biotechnol **21**(2): 177-81.

Alberts, B., D. Bray, J. Lewis, M. Raff, R. K. and J. Watson, D. (1994). Molecular Biology of the Cell. NY & London., Garland Publishing In.

Anderson, E. L., T. Bowers, C. M. Mink, D. J. Kennedy, R. B. Belshe, H. Harakeh, L. Pais, P. Holder and G. M. Carlone (1994). "Safety and immunogenicity of meningococcal A and C polysaccharide conjugate vaccine in adults." Infect Immun **62**(8): 3391-5.

Anderson, P., G. Peter, R. B. Johnston, Jr., L. H. Wetterlow and D. H. Smith (1972). "Immunization of humans with polyribophosphate, the capsular antigen of *Haemophilus influenzae*, type b." Journal of Clinical Investigation **51**(1): 39-44.

Andrew, P. W., G. J. Boulnois, T. J. Mitchell, C. J. Lee, J. C. Paton, J. T. Poolman and C. A. M. Peeters (1994). Pneumococcal Vaccines. Bacterial Protein Toxins. Freer. New York, Gustav Fischer.

Anonymous (2001). No Profit, No cure. The New Yorker. New York**:** 46.

Arditi, M., E. O. Mason, Jr., J. S. Bradley, T. Q. Tan, W. J. Barson, G. E. Schutze, E. R. Wald, L. B. Givner, K. S. Kim, R. Yogev and S. L. Kaplan (1998). "Three-year multicenter surveillance of pneumococcal meningitis in children: clinical characteristics, and outcome related to penicillin susceptibility and dexamethasone use." Pediatrics **102**(5): 1087-97.

Armitage, P., R. Walden and J. Draper (1988). Vectors for the transfomation of plant cells using *Agrobacterium*. Plant Genetic Transformation and Gene Expression. J. Draper, R. Scott and P. Armitage. Oxford, Blackwell Scientific Publications.

Arrecubieta, C., E. Garcia and R. and Lopez (1996a). "Type 3 specific synthase of *Streptococcus pneumoniae* (Cap3B) directs type 3 polysaccharide biosyntheis in *Escherichia coli* and in pneumococcal strains of different serotypes." Journal of Experimental Medicine **184**: 449.

Arrecubieta, C., E. Garcia and R. Lopez (1995). "Sequence and transcriptional analysis of a DNA region involved in the production of capsular polysaccharide in *Streptococcus pneumoniae* type 3." Gene **167**(1-2): 1-7.

Arrecubieta, C., E. Garcia and R. Lopez (1996). "Demonstration of UDP-glucose dehydrogenase activity in cell extracts of Escherichia coli expressing the pneumococcal cap3A gene required for the synthesis of type 3 capsular polysaccharide." J Bacteriol **178**(10): 2971-4.

Artz, A. S., W. B. Ershler and D. L. Longo (2003). "Pneumococcal Vaccination and Revaccination of Older Adults." Clinical Microbiology Reviews **16**(2): 308-318.

Atkins, R. and F. Carey (2001). Organic Chemistry: A brief course, McGraw-Hill Publishing.

Austrian, R. (1985). "Polysaccharide vaccines." Annales de l Institut Pasteur. Microbiology **136B**(3): 295-307.

Austrian, R. (1986). "Some aspects of the pneumococcal carrier state." Journal of Antimicrobial Chemotherapy **18 Suppl A**: 35-45.

Austrian, R. (2000). The enduring pneumococcus. *Streptococcus pneumoniae*. A. Tomasz. Larchmont, NY, Mary Ann Leibert, Inc.

Austrian, R. and J. Gold (1964). "Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia." Annals of Internal Medicine **60**: 759-76.

Avery, O. T., C. M. MacLeod and M. McCarty (1944). "Studies on the chemical nature of the substance inducing transformation of pneumococcal types: induction of

transformation by a desoxyribonucleic acid fraction isolated from pneumococcus type III." Journal of Experimental Medicine **79**: 137–158.

Bannister, B. A., N. T. Begg and S. H. Gillespie (1996). Infectious Disease. Oxford, Blackwell Science.

Baquero, F., J. Martinez-Beltran and E. Loza (1991). "A review of antibiotic resistance patterns of *Streptococcus pneumoniae* in Europe." Journal of Antimicrobial Chemotherapy **28 Suppl C**: 31-8.

Barbas, Burton and Scott (2001). Phage display: A laboratory manual. New York, Cold Spring Harbor Laboratory Press.

Barthelson, R., A. Mobasseri, D. Zopf and P. Simon (1998). "Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides." Infection and Immunity **66**(4): 1439-44.

Baxendale, H. E., Z. Davies, H. N. White, M. Spellerberg, B., F. K. Stevenson and D. Goldblatt (2000). "Immunogenetic analysis of the immune response to pneumococcal polysaccharide." European Journal of Immunology **30**: 1214-1223.

Baxevanis, A. D. and B. F. F. Ouellette (2001). Bioinformatics: A Practical Guide to the Analysis of Genes and Proteins, John Wiley and Sons.

Bechtold, N. and G. Pelletier (1998). "In planta Agrobacterium-mediated transformation of adult Arabidopsis thaliana plants by vacuum infiltration." Methods in Molecular Biology **82**: 259-66.

Bischoff, J., M. Domrachev, S. Federhen, C. Hotton, D. Leipe, V. Soussov, R. Sternberg and T. Turner (2004). Taxonomy Browser, http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id=1301.

Blackburn, A. S. and S. V. Avery (2003). "Genome-wide screening of Saccharomyces cerevisiae to identify genes required for antibiotic insusceptibility of eukaryotes." Antimicrob Agents Chemother **47**(2): 676-81.

Bohmert, K., I. Balbo, J. Kopka, V. Mittendorf, C. Nawrath, Y. Poirier, G. Tischendorf, R. N. Trethewey and L. Willmitzer (2000). "Transgenic Arabidopsis plants can

accumulate polyhydroxybutyrate to up to 4% of their fresh weight." Planta **211**(6): 841-5.

Briles, D. E., S. Hollingshead, A. Brooks-Walter, G. S. Nabors, L. Ferguson, M. Schilling, S. Gravenstein, P. Braun, J. King and A. Swift (2000). "The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection." Vaccine **18**(16): 1707-11.

Brown (1991). Essential Molecular Biology Volume 1, A Practical approach. New York, Oxford University Press.

Brown, A. (2001). "Prevnar(tm): a pneumococcal conjugate vaccine for infants and young children." Issues in emerging health technologies. **14**: 1-6.

Brown, F., G. Dougan, E. M. Hoey, S. J. Martin, B. K. Rima and A. Trudgett (1993). Vaccine Design. Chichester, John WIley and Sons, Ltd.

Brown, S. E., C. R. Howard, A. J. Zuckerman and M. W. Steward (1984). "Determination of the affinity of antibodies to hepatitis B surface antigen in human sera." J Immunol Methods **72**(1): 41-8.

Butler, J. C. and A. Schuchat (1999). "Epidemiology of pneumococcal infections in the elderly." Drugs Aging **15 Suppl 1**: 11-9.

Cadoz, M. (1998). "Potentials and limitations of polysaccharide vaccines in infancy." Vaccine **16**(14/15): 1391-1395.

Caimano, M. J., G. G. Hardy and J. Yother (2000). Capsule Genetics in Streptococcus pneumoniae and a possible role for transposition in the generation of the type 3 locus. *Streptococcus pneumoniae*. A. Tomasz. Larchmont, NY, Mary Ann Liebert Inc.

Canvin, J. R., A. P. Marvin, M. Sivakumaran, J. C. Paton, G. J. Boulnois, P. W. Andrew and T. J. Mitchell (1995). "The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus." Journal of Infectious Diseases **172**(1): 119-23.

Capell, T. and P. Christou (2004). "Progress in plant metabolic engineering." Curr Opin Biotechnol **15**(2): 148-54.

Carrillo, C., A. Wigdorovitz, K. Trono, M. J. Dus Santos, S. Castanon, A. M. Sadir, R. Ordas, J. M. Escribano and M. V. Borca (2001). "Induction of a virus-specific antibody response to foot and mouth disease virus using the structural protein VP1 expressed in transgenic potato plants." Viral Immunology **14**(1): 49-57.

Cartee, R. T., W. T. Forsee, J. W. Jensen and J. Yother (2001). "Expression of the *Streptococcus pneumoniae* Type 3 Synthase in *Escherichia coli*." Journal of Biological Chemistry **276**(52): 48831-48839.

Cartee, R. T., W. T. Forsee, J. S. Schutzbach and J. Yother (2000). "Mechanism of type 3 capsular polysaccharide synthesis in *Streptococcus pneumoniae*." Journal of Biological Chemistry **275**(6): 3907-14.

Chabannes, M., A. Barakate, C. Lapierre, J. M. Marita, J. Ralph, M. Pean, S. Danoun, C. Halpin, J. Grima-Pettenati and A. M. Boudet (2001). "Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants." Plant J **28**(3): 257-70.

Cherniak, R. and B. G. Henderson (1972). "Immunochemistry of the capsular polysaccharides from Clostridium perfringens: selected Hobbs strains 1, 5, 9, and 10." Infection and Immunity **6**(1): 32-7.

Clough, S. J. and A. F. Bent (1998). "Floral dip:a simplified method for Agrobacterium-mediated transformation of *Arabidopsis thaliana*." The Plant Journal **16**(6): 735-743.

Concepcion, N. F. and C. E. Frasch (2001). "Pneumococcal type 22f polysaccharide absorption improves the specificity of a pneumococcal-polysaccharide enzyme-linked immunosorbent assay." Clin Diagn Lab Immunol **8**(2): 266-72.

Connolly, M. and N. Noah (1996). "Surveillance of bacterial meningitis in Europe." London: King's European Meningitis Surveillance Unit, King's College School of Medicine and Dentistry, 1997.

Cummins, J., M. W. Ho and A. Ryan (2000). "Hazardous CaMV promoter?" Nat Biotechnol **18**(4): 363.

Curtiss, R. and G. A. Cardineau (1990). World Intellectual property Organisation, PCT/US89/03799.

Dagan, R., N. Givon-Lavi, J. Bar-Ziv and N. Porat (2004). The association of nasopharyngeal (NP) S. pneumoniae (Pnc) serotypes with community-acquired alveolar pneumonia (CAAP) determined by WHO standardization of interpretation of chest radiographs in children (WHO-SICR). 4th International Symposium on Pneumococci and Pneumococcal Diseases, Helsinki, Finland.

Day, L. A. (1969). "Conformations of single-stranded DNA and coat protein in fd bacteriophage as revealed by ultraviolet absorption spectroscopy." Journal of Molecular Biology **39**(2): 265-77.

De Bolle, X., T. Laurent, A. Tibor, F. Godfroid, V. Weynants, J. J. Letesson and P. Mertens (1999). "Antigenic properties of peptidic mimics for epitopes of the lipopolysaccharide from Brucella." Journal of Molecular Biology **294**(1): 181-91.

De Wilde, C., H. Van Houdt, S. De Buck, G. Angenon, G. De Jaeger and A. Depicker (2000). "Plants as bioreactors for protein production: avoiding the problem of transgene silencing." Plant Mol Biol **43**(2-3): 347-59.

DellaPenna, D. (2001). "Plant metabolic engineering." Plant Physiol **125**(1): 160-3.

Denecke, J., J. Botterman and R. Deblaere (1990). "Protein secretion in plant cells can occur via a default pathway." Plant Cell **2**(1): 51-9.

Dente, L., G. Cesareni, G. Micheli, F. Felici, A. Folgori, A. Luzzago, P. Monaci, A. Nicosia and P. Delmastro (1994). "Monoclonal antibodies that recognise filamentous phage: tools for phage display technology." Gene **148**(1): 7-13.

Dillard, J. P., M. W. Vandersea and J. Yother (1995). "Characterisation of the cassette containing genes for type 3 capsular polysaccharide biosynthesis in *Streptococcus pneumoniae.*" Journal of Experimental Medicine **181**: 973-983.

Dillard, J. P. and J. Yother (1991). "Analysis of Streptococcus pneumoniae sequences cloned into Escherichia coli: effect of promoter strength and transcription terminators." J Bacteriol **173**(16): 5105-9.

Ditta, G., S. Stanfield, D. Corbin and D. R. Helinski (1980). "Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of Rhizobium meliloti." Proceedings of the National Academy of Sciences of the United States of America **77**(12): 7347-51.

Dochez, A. and O. Avery (1917). "The elaboration of a specific soluble substance by pneumococcus during growth." Journal of Experimental Medicine **26**: 477-493.

Draper, J., R. Scott, P. Armitage and R. Walden (1988). Plant genetic transformation and gene expression : a laboratory manual. Oxford ; Boston, Blackwell Scientific Publications.

du Moulin, G. C., D. G. Paterson, J. Hedley-Whyte and A. Lisbon (1982). "Aspiration of gastric bacteria in antacid-treated patients: a frequent cause of postoperative colonisation of the airway." Lancet **1**(8266): 242-5.

Dube, D. H. and C. R. Bertozzi (2003). "Metabolic oligosaccharide engineering as a tool for glycobiology." Curr Opin Chem Biol **7**(5): 616-25.

Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers and F. Smith (1956). "Colorimetric method for determination of sugars and related substances." Analytical Chemistry **28**: 350-356.

Eady, C., S. Davis, A. Catanach, F. Kenel and S. Hunger (2005). "Agrobacterium tumefaciens-mediated transformation of leek (Allium porrum) and garlic (Allium sativum)." Plant Cell Rep.

Eales, L.-J. (1997). Immunology for life scientists., John Wiley and Sons.

Epstein, J., Q. Eichbaum, S. Sheriff and R. A. B. Ezekowitz (1996). "The collectins in innate immunity." Current Opinions in Immunology **8**(29-35).

Eriksson, M., B. Henriques and K. Ekdahl (2000). "Epidemiology of pneumococcal infections in Swedish children." Acta Paediatr Suppl **89**(435): 35-9.

Esaki, Y., Y. Ohashi, H. Furuya, Y. Sugiura, Y. Ohno, H. Okamoto and Y. Nakai (1991). "Histamine-induced mucociliary dysfunction and otitis media with effusion." Acta Otolaryngol Suppl **486**: 116-34.

Esposito, A. L. (1987). "Cimetidine does not impair lung host defense in experimental pneumococcal pneumonia." Am Rev Respir Dis **136**(2): 320-4.

Ezekowitz, R. A. (2003). "Role of Mannose-Binding Lectin in Innate Immunity." Journal of Infectious Diseases **187**(Suppl 2): S335-S339.

Felici, F., L. Castagnoli, A. Musacchio, R. Jappelli and G. Cesareni (1991). "Selection of antibody ligands from a large library of oligopeptides expressed on multivalent exposition vector." Journal of Molecular Biology **222**: 301-310.

Felici, F., A. Luzzago, A. Folgori and R. Cortese (1993). "Mimicking of discontinuous epitopes by phage-displayed peptides, II. Selection of clones recognized by a protective monoclonal antibody against the Bordetella pertussis toxin from phage peptide libraries." Gene **128**(1): 21-7.

Fenoll, A., I. Jado, D. Vicioso, S. Berron, J. E. Yuste and J. Casal (2000). "*Streptococcus pneumoniae* in children in Spain 1990-1999." Acta Pediatrica Supplement **435**: 44-50.

Finland, M. and M. W. Barnes (1977). "Changes in occurrence of capsular serotypes of Streptococcus pneumoniae at Boston City Hospital during selected years between 1935 and 1974." J Clin Microbiol **5**(2): 154-66.

Fischer, U., S. Men and M. Grebe (2004). "Lipid function in plant cell polarity." Curr Opin Plant Biol **7**(6): 670-6.

Forsee, W. T., R. T. Cartee and J. Yother (2000). "Biosynthesis of type 3 capsular polysaccharide in *Streptococcus pneumoniae*. Enzymatic chain release by an abortive translocation process." Journal of Biological Chemistry **275**(34): 25972-8.

Frasch, C. E. (1995). Meningococcal vaccines: past, present and future. Meningococcal disease. K. Cartwright. Chichester, United Kingdom, John Wiley & Sons Ltd.: 245–283.

Fry, S. C. (1988). The Growing Plant Cell Wall: Chemical and Metabolic Analysis. New York, John Wiley and Sons, Inc.

Furth, S. L., A. M. Neu, B. Case, H. M. Lederman, M. Steinhoff and B. Fivush (1996). "Pneumococcal polysaccharide vaccine in children with chronic renal disease: a prospective study of antibody response and duration." J Pediatr **128**(1): 99-101.

Galfre, G., P. Monaci, A. Nicosia, A. Luzzago, F. Felici and R. Cortese (1996). "Immunization with phage-displayed mimotopes." Methods in Enzymology **267**: 109-15.

Gallagher, S. R. (1992). GUS protocols : using the GUS gene as a reporter of gene expression, San Diego ; London : Academic Press.

Gao, C., S. Mao, C. H. Lo, P. Wirsching, R. A. Lerner and K. D. Janda (1999). "Making artificial antibodies:a format for phage display of combinatorial heterodimerix arrays." Proceedings of the National Academy of Sciences **96**: 6025-6030.

Gao, Y., Y. Ma, M. Li, T. Cheng, S. W. Li, J. Zhang and N. S. Xia (2003). "Oral immunization of animals with transgenic cherry tomatillo expressing HBsAg." World Journal of Gastroenterology **9**(5): 996-1002.

Garcia, E., C. Arrecubieta, R. Munoz, M. Mollerach and R. Lopez (1997). "A Functional Analysis of the *Streptococcus pneumoniae* Genes Involved in the Synthesis of the Type 1 and Type 3 Capsular Polysaccharides." Microbial Drug Resistance **3**(1): 73-88.

Geysen, H. M., S. J. Barteling and R. H. Meloen (1985). "Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein." Proc Natl Acad Sci U S A **82**(1): 178-82.

Geysen, H. M., R. H. Meloen and S. J. Barteling (1984). "Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid." Proc Natl Acad Sci U S A **81**(13): 3998-4002.

Geysen, H. M., S. J. Rodda and T. J. Mason (1986). "A priori delineation of a peptide which mimics a discontinuous antigenic determinant." Mol Immunol **23**(7): 709-15.

Giddings, G., G. Allison, D. Brooks and A. Carter (2000). "Transgenic plants as factories for biopharmaceuticals." Nat Biotechnol **18**(11): 1151-5.

Giebink, G. S., J. D. Meier, M. K. Quartey, C. L. Liebeler and C. T. Le (1996). "Immunogenicity and efficacy of *Streptococcus pneumoniae* polysaccharide-protein conjugate vaccines against homologous and heterologous serotypes in the chinchilla otitis media model." Journal of Infectious Diseases **173**(1): 119-27.

Gilbert, C., K. Robinson, R. W. E. LePage and J. Wells (2000). "Heterologous expression of an immunogenic pneumococcal type 3 capsular polysaccharide in *Lactococcus lactis*." Infection and Immunity **68**(6): 3251-3260.

Goebel, W. F. and O. T. Avery (1931). "Chemo-immunological studies on conjugated carbohydrate proteins VI. Synthesis of the *p*-aminobenzyl ether of the soluble specific substance of type III pneumococcus and its coupling with protein." Journal of Experimental Medicine **54**: 431-436.

Goldblatt, D., A. R. Vaz and E. Miller (1998). "Antibody avidity as a surrogate marker of successful priming by Haemophilus influenzae type b conjugate vaccines following infant immunization." J Infect Dis **177**(4): 1112-5.

Gomez, N., A. Wigdorovitz, S. Castanon, F. Gil, R. Ordas, M. V. Borca and J. M. Escribano (2000). "Oral immunogenicity of the plant derived spike protein from swine-transmissible gastroenteritis coronavirus." Archives of Virology **145**(8): 1725-32.

Granoff, D. M., S. J. Holmes, M. T. Osterholm, J. E. McHugh, A. H. Lucas, E. L. Anderson, R. B. Belshe, J. L. Jacobs, F. Medley and T. V. Murphy (1993). "Induction of immunologic memory in infants primed with Haemophilus influenzae type b conjugate vaccines." J Infect Dis **168**(3): 663-71.

Griffith, F. (1928). "The siginificance of pneumococcal types." Journal of Hygiene **27**: 113-159.

Grothaus, M. C., N. Srivastava, S. L. Smithson, T. Kieber-Emmons, D. B. Williams, G. M. Carlone and M. A. Westerink (2000). "Selection of an immunogenic peptide mimic of the capsular polysaccharide of *Neisseria meningitidis* serogroup A using a peptide display library." Vaccine **18**(13): 1253-63.

Grzych, J. M., M. Capron, P. H. Lambert, C. Dissous, S. Torres and A. Capron (1985). "An anti-idiotype vaccine against experimental schistosomiasis." Nature **316**(6023): 74-6.

Guidolin, A., J. K. Morona, R. Morona, D. Hansman and J. C. Paton (1994). "Nucleotide sequence analysis of genes essential for capsular polysaccharide biosynthesis in Streptococcus pneumoniae type 19F." Infect Immun **62**(12): 5384-96.

Gulati, S., D. P. McQuillen, J. Sharon and P. A. Rice (1996). "Experimental immunization with a monoclonal anti-idiotope antibody that mimics the Neisseria gonorrhoeae lipooligosaccharide epitope 2C7." J Infect Dis **174**(6): 1238-48.

Guttormsen, H. K., C. J. Baker, M. H. Nahm, L. C. Paoletti, S. M. Zughaier, M. S. Edwards and D. L. Kasper (2002). "Type III group B streptococcal polysaccharide induces antibodies that cross-react with Streptococcus pneumoniae type 14." Infect Immun **70**(4): 1724-38.

Hajdukiewicz, P., Z. Svab and P. Maliga (1994). "The small versatile pPZP family of Agrobacterium binary vectors for plant transformation." Plant Molecular Biology. **25**: 989.

Haq, T. A., H. S. Mason, J. D. Clements and C. J. Arntzen (1995). "Oral immunization with a recombinant bacterial antigen produced in transgenic plants." Science **268**(5211): 714-6.

Hardy, G. G., M. J. Caimano and J. Yother (2000). "Capsule biosynthesis and basic metabolism in Streptococcus pneumoniae are linked through the cellular phosphoglucomutase." J Bacteriol **182**(7): 1854-63.

Hardy, G. G., A. D. Magee, C. L. Ventura, M. J. Caimano and J. Yother (2001). "Essential role for cellular phosphoglucomutase in virulence of type 3 Streptococcus pneumoniae." Infect Immun **69**(4): 2309-17.

Harlow, E. and D. Lane (1988). Antibodies: a laboratory manual. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.

Harris, S. L., M. K. Park, M. H. Nahm and B. Diamond (2000). "Peptide mimic of phosphorylcholine, a dominant epitope found on *Streptococcus pneumoniae*." Infection and Immunity **68**(10): 5778-84.

Harrison, J. L., S. C. Williams, G. Winter and A. Nissim (1996). "Screening of Phage Antibody Libraries." Methods in Enzymology **267**: 83-109.

Haug, A. and B. Larsen (1974). Biosynthesis of Algal Polysaccharides. Plant Carbohydrate Biochemistry. J. B. Pridham. London, Academic Press, Inc.: 208-218.

Hausdorff, W. P., D. R. Feiken and K. P. Klugman (2005). "Epidemiological differences among pneumococcal serotypes." Lancet Infectious Disease **5**: 83-93.

Heineman, H. S. (1973). "Quellung test for pneumonia." N Engl J Med **288**(19): 1027.

Henrichsen, J. (1995). "Six newly recognized types of *Streptococcus pneumoniae*." Journal of Clinical Microbiology **33**(10): 2759-62.

Henrichsen, J. and S. V. Nielsen (2000). Incidence of Invasive Pneumococcal Disease in Denmark. *Streptococcus pneumoniae*. A. Tomasz. Larchmont, NY, Mary Ann Liebert Inc.

Hodges, R. G., C. M. MacLeod and W. Bernhard (1946). "Epidemic pneumococcal pneumonia. III, carrier studies." American Journal of Hygiene **44**: 207-230.

Hoess, R., U. Brinkmann, T. Handel and I. Pastan (1993). "Identification of a peptide which binds to the carbohydrate-specific monoclonal antibody B3." Gene **128**(1): 43-9.

HTML1 (2002). "(www.drugtarriff.com/index1_files/sheet015.htm)."

Hughes, E. H., S. B. Hong, S. I. Gibson, J. V. Shanks and K. Y. San (2004). "Metabolic engineering of the indole pathway in Catharanthus roseus hairy roots and increased accumulation of tryptamine and serpentine." Metab Eng **6**(4): 268-76.

Iannolo, G., O. Minenkova, R. Petruzzelli and G. Cesareni (1995). "Modifying filamentous phage capsid: limits in the size of the major capsid protein." Journal of Molecular Biology **248**(4): 835-44.

Ikezawa, H. (2002). "Glycosylphosphatidylinositol (GPI)-anchored proteins." Biol Pharm Bull **25**(4): 409-17.

Il'ichev, A. A., O. O. Minenkova, S. I. Tat'kov, N. N. Karpyshev, A. M. Eroshkin, V. A. Petrenko and L. S. Sandakhchiev (1989). "Production of a viable variant of the

M13 phage with a foreign peptide inserted into the basic coat protein." Doklady Akademii Nauk SSSR **307**(2): 481-3.

Janeway, C. A., P. Travers, M. Walport and J. D. Capra (1999). Immunobiology. USA, Garland.

Jani, D., L. S. Meena, Q. M. Rizwan-ul-Haq, Y. Singh, A. K. Sharma and A. K. Tyagi (2002). "Expression of cholera toxin B subunit in transgenic tomato plants." Transgenic Research **11**(5): 447-54.

Jennings, H. L. (1990). "Capsular polysaccharides as Vaccine Candidates." Current Opinions in Microbiology and Immunology **150**: 97-125.

Jespers, L. S., J. H. Messens, A. De Keyser, D. Eeckhout, I. Van den Brande, Y. G. Gansemans, M. J. Lauwereys, G. P. Vlasuk and P. E. Stanssens (1995). "Surface expression and ligand-based selection of cDNAs fused to filamentous phage gene VI." Biotechnology (N Y) **13**(4): 378-82.

Johnson, E. (1996). "Edible plant vaccines." Nature Biotechnology **14**: 1532-33.

Jones, C. (1998). "Capsular polysaccharides from *Neisseria meningitidis* and *Streptococcus pneumoniae*." Carbohydrate Research **184**: 279-284.

Kalin, M. (1998). "Pneumococcal serotypes and their clinical relevance." Thorax **53**: 159-162.

Kaltoft, N. Zeuthen and H. B. Konradsen (2000). "Epidemiology of invasive pneumococcal infections in children aged 0-6 years in Denmark: a 19-year nationwide surveillance study." Acta Paediatrica Supplement **89**(435): 3-10.

Kamerling, J. P. (2000). Pneumococcal polysaccharides: A chemical view. *Streptococcus pneumoniae*. A. Tomasz.

Kaplan, S. L., E. O. Mason, Jr., E. R. Wald, G. E. Schutze, J. S. Bradley, T. Q. Tan, J. A. Hoffman, L. B. Givner, R. Yogev and W. J. Barson (2004). "Decrease of invasive pneumococcal infections in children among 8 children's hospitals in the United States after the introduction of the 7-valent pneumococcal conjugate vaccine." Pediatrics **113**(3 Pt 1): 443-9.

Kayasuga, R., Y. Sugimoto, T. Watanabe and C. Kamei (2002). "Participation of chemical mediators other than histamine in nasal allergy signs: a study using mice lacking histamine H(1) receptors." Eur J Pharmacol **449**(3): 287-91.

Keenleyside, W. J. and C. Whitfield (1996). "A novel pathway for O-polysaccharide biosynthesis in *Salmonella enterica* serovar Borreze." Journal of Biological Chemistry **271**(45): 28581-92.

Kelly, D. F., R. Moxon and A. J. Pollard (2004). "*Haemophilus influenzae* type b conjugate vaccines." Immunology **113**: 163-174.

Kerr, A. R., J. J. Irvine, J. J. Search, N. A. Gingles, A. Kadioglu, P. W. Andrew, W. L. McPheat, C. G. Booth and T. J. Mitchell (2002). "Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection." Infect Immun **70**(3): 1547-57.

Kieber-Emmons, T. (1998). "Peptide mimotopes of carbohydrate antigens." Immunol Res **17**(1-2): 95-108.

Kishor, P., Z. Hong, G. H. Miao, C. Hu and D. Verma (1995). "Overexpression of [delta]-Pyrroline-5-Carboxylate Synthetase Increases Proline Production and Confers Osmotolerance in Transgenic Plants." Plant Physiol **108**(4): 1387-1394.

Kjaergard, L. L., F. O. Larsen, S. Norn, P. Clementsen, P. S. Skov and H. Permin (1996). "Basophil-bound IgE and serum IgE directed against Haemophilus influenzae and Streptococcus pneumoniae in patients with chronic bronchitis during acute exacerbations." Apmis **104**(1): 61-7.

Klein, D. L. (2000). Pneumococcal disease and the role of conjugate vaccines. *Streptococcus pneumoniae*. A. Tomasz. New York, Mary Ann Liebert: 467-478.

Knecht, J., G. Schiffman and R. Austrian (1970). "Some biological properties of pneumococcus type 37 and the chemistry of its capsular polysaccharide." Journal of Experimental Medicine **132**: 475-487.

Kolberg, J. and C. Jones (1998). "Monoclonal antibodies with specificities for *Streptococcus pneumoniae* group 9 capsular polysaccharides." FEMS Immunol Med Microbiol **20**(4): 249-55.

Kolkman, M. A., W. Wakarchuk, P. J. Nuijten and B. A. van der Zeijst (1997). "Capsular polysaccharide synthesis in *Streptococcus pneumoniae* serotype 14: molecular analysis of the complete cps locus and identification of genes encoding glycosyltransferases required for the biosynthesis of the tetrasaccharide subunit." Molecular Microbiology **26**(1): 197-208.

Koprowski, H. and V. Yusibov (2001). "The green revolution: plants as heterologous expression vectors." Vaccine **19**(17-19): 2735-41.

Kost, B., P. Spielhofer and N. H. Chua (1998). "A GFP-mouse talin fusion protein labels plant actin filaments in vivo and visualizes the actin cytoskeleton in growing pollen tubes." Plant Journal **16**(3): 393-401.

Kunze, R., W. B. Frommer and U. I. Flugge (2002). "Metabolic engineering of plants: the role of membrane transport." Metab Eng **4**(1): 57-66.

Lahiri, T. and D. A. Waltz (2001). "Preimmunization anti-pneumococcal antibody levels are protective in a majority of patients with cystic fibrosis." Pediatrics **108**(4): E62.

Lamphear, B. J., S. J. Streatfield, J. M. Jilka, C. A. Brooks, D. K. Barker, D. D. Turner, D. E. Delaney, M. Garcia, B. Wiggins, S. L. Woodard, E. E. Hood, I. R. Tizard, B. Lawhorn and J. A. Howard (2002). "Delivery of subunit vaccines in maize seed." Journal of Controlled Release **85**(1-3): 169-80.

Lee, C. J. (1987). "Bacterial capsular polysaccharides--biochemistry, immunity and vaccine." Molecular Immunology **24**(10): 1005-19.

Lee, C. J., A. Karpas, T. R. Wang, T. Kosaka and K. Koizumi (1996). "Production, Binding Characteristics and Protective Immunity of Monoclonal Antibody to Pneumococcal Type-9V Conjugate." Microbiology and Immunology **40**(11): 857-865.

Lesinski, G. B., S. L. Smithson, N. Srivastava, D. Chen, G. Widera and M. A. Westerink (2001a). "A DNA vaccine encoding a peptide mimic of Streptococcus pneumoniae serotype 4 capsular polysaccharide induces specific anti-carbohydrate antibodies in Balb/c mice." Vaccine **19**(13-14): 1717-26.

Lesinski, G. B. and J. Westerink (2001). "Novel Vaccine strategies to T-independent antigens." Journal of Microbiological Methods **47**: 137-149.

Lessard, P. A., H. Kulaveerasingam, G. M. York, A. Strong and A. J. Sinskey (2002). "Manipulating gene expression for the metabolic engineering of plants." Metab Eng **4**(1): 67-79.

Lewinsohn, E., F. Schalechet, J. Wilkinson, K. Matsui, Y. Tadmor, K. H. Nam, O. Amar, E. Lastochkin, O. Larkov, U. Ravid, W. Hiatt, S. Gepstein and E. Pichersky (2001). "Enhanced levels of the aroma and flavor compound S-linalool by metabolic engineering of the terpenoid pathway in tomato fruits." Plant Physiol **127**(3): 1256-65.

Lindsey, K. (1991). Plant tissue culture manual : fundamentals and applications. Dordrecht ; Boston, Kluwer Academic.

Luzzago, A., F. Felici, A. Tramontano, A. Pessi and R. Cortese (1993). "Mimicking of discontinuous epitopes by phage-displayed peptides, I. Epitope mapping of human H ferritin using a phage library of constrained peptides." Gene **128**(1): 51-7.

Mackett, M. and J. D. Williamson (1995). Human Vaccines and Vaccination. Oxford, BIOS Scientific Publishers.

MacLeod, C., R. Hodges, M. Heidelberger and W. Bernhard (1945). "Prevention of pneumococcal pneumonia by immunisation with specific capsular polysaccharide." Journal of Experimental Medicine **82**: 445-465.

Madigan, M., T., J. M. Martinko and J. Parker (1997). Brock Biology of Micro-organisms. New Jersey, Prentice Hall.

Magee, A. D. and J. Yother (2001). "Requirement of Capsule in Colonisation by *Streptococcus pneumoniae*." Infection and Immunity **69**(6): 3755-3761.

Maggi, T., M. Spinosa, S. Ricci, D. Medaglini, G. Pozzi and M. R. Oggioni (2002). "Genetic engineering of Streptococcus gordonii for the simultaneous display of two heterologous proteins at the bacterial surface." FEMS Microbiology Letters **210**(1): 135-41.

Malik, P., T. D. Terry, L. R. Gowda, A. Langara, S. A. Petukhov, M. F. Symmons, L. C. Welsh, D. A. Marvin and R. N. Perham (1996). "Role of capsid structure and membrane protein processing in determining the size and copy number of peptides

displayed on the major coat protein of filamentous bacteriophage." Journal of Molecular Biology **260**(1): 9-21.

Maniatis, T., E. F. Fritsch and J. Sambrook (1982). Molecular cloning: A laboratory manual. New York, Cold Spring Harbor Laboratory.

Maniatis, T., E. F. Fritsch and J. Sambrook (1988). Molecular cloning: A laboratory manual. New York, Cold Spring Harbor Laboratory.

Manning, J. T. (1976). "Life cycle patterns and their genetic control: an attempt to reconcile evolutionary and mechanistic speculation." Acta Biotheoretica **25**: 111-129.

Mason, H. S. and C. J. Arntzen (1995). "Transgenic plants as vaccine production systems." Trends in Biotechnology **13**(9): 388-92.

Mason, H. S., T. A. Haq, J. D. Clements and C. J. Arntzen (1998). "Edible vaccine protects mice against Escherichia coli heat-labile enterotoxin (LT): potatoes expressing a synthetic LT-B gene." Vaccine **16**(13): 1336-43.

May, G. D., R. Afza, H. S. Mason, A. Wiecko, F. J. Novak and C. J. Arntzen (1995). "Generation of Transgenic Banana (*Musa acuminata*) Plants via *Agrobacterium*-Mediated Transformation." Biotechnology **13**: 486-792.

Mbelle, N., R. E. Huebner and e. al (1999). "Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine." Journal of Infectious Diseases **180**: 1171-1176.

McCool, T. L., C. V. Harding, N. S. Greenspan and J. R. Schreiber (1999). "B- and T-cell immune responses to pneumococcal conjugate vaccines: divergence between carrier- and polysaccharide-specific immunogenicity." Infect Immun **67**(9): 4862-9.

McIntosh, E. D., P. Conway, J. Willingham and A. Lloyd (2003). "The cost-burden of paediatric pneumococcal disease in the UK and the potential cost-effectiveness of prevention using 7-valent pneumococcal conjugate vaccine." Vaccine **21**(19-20): 2564-72.

McIntosh, K. B., J. L. Hulm, L. W. Young and P. C. Bonham-Smith (2004). "A Rapid *Agrobacterium*-Mediated *Arabidopsis thaliana* Transient Assay System." Plant Molecular Biology Reporter **22**: 53–61.

McVernon, J., N. Andrews, M. P. Slack and M. E. Ramsay (2003). "Risk of vaccine failure after *Haemophilus influenzae* type b (Hib) combination vaccines with acellular pertussis." Lancet **361**: 1521.

Meloen, R. H., W. C. Puijk and J. W. Slootstra (2000). "Mimotopes: realization of an unlikely concept." Journal of molecular recognition **13**: 352-359.

Mertens, P., D. Walgraffe, T. Laurent, N. Deschrevel, J. J. Letesson and X. De Bolle (2001). "Selection of phage-displayed peptides recognised by monoclonal antibodies directed against the lipopolysaccharide of Brucella." International Reviews of Immunology **20**(2): 181-99.

Messing, J., B. Gronenborn, B. Muller-Hill and P. Hans Hopschneider (1977). "Filamentous coliphage M13 as a cloning vehicle: insertion of a HindII fragment of the lac regulatory region in M13 replicative form in vitro." Proceedings of the National Academy of Sciences of the United States of America **74**(9): 3642-6.

Miller, E., D. Salisbury and M. E. Ramsay (2002). "Planning, registration, and implementation of an immunisation campaign against meningococcal serogroup C disease in the UK: a success story." Vaccine **20 (suppl 1)**: S58–67.

Miller, E., P. Waight, A. Efstratiou, M. Brisson, A. Johnson and R. George (2000). "The epidemiology of pneumococcal disease in children in Europe. Epidemiology of invasive and other pneumococcal disease in children in England and Wales 1996-1998." Acta Paediatrica **89**((Suppl 435)): 17-21.

Mineo, L. (1990). Plant Tissue Culture Techniques, Proceddings of the Eleventh Workshop/Conference of the Association for Biology Laboratory Education.

Mitchell, T. J. and P. W. Andrew (1995). Vaccines against *Streptococcus pneumoniae*. Molecular and Clinical Aspects of Bacterial Vaccine Development. D. A. A. Ala'Aldeen and C. E. Hormaeche, John WIley and Sons: 93-117.

Modelska, A., B. Dietzschold, N. Sleysh, Z. F. Fu, K. Steplewski, D. C. Hooper, H. Koprowski and V. Yusibov (1998). "Immunization against rabies with plant-derived antigen." Proc Natl Acad Sci U S A **95**(5): 2481-5.

Moe, G. R., S. Tan and D. M. Granoff (1999). "Molecular mimetics of polysaccharide epitopes as vaccine candidates for prevention of Neisseria meningitidis serogroup B disease." FEMS Immunol Med Microbiol **26**(3-4): 209-26.

Moffat, A. S. (1995). "Exploring Transgenic Plants as a New Vaccine Source." Science **268**: 658-660.

Morona, J. K., R. Morona and J. C. Paton (1997). "Characterization of the locus encoding the *Streptococcus pneumoniae* type 19F capsular polysaccharide biosynthetic pathway." Molecular Microbiology **23**(4): 751-63.

Morton, D. B. and P. H. M. Griffiths (1985). "Guidelines on the recognition of pain, distress and discomfort in experimental animals and an hypothesis for assessment." The Veterinary Record **116**: 431-436.

Mounier, J., F. K. Bahrani and P. J. Sansonetti (1997). "Secretion of Shigella flexneri Ipa invasins on contact with epithelial cells and subsequent entry of the bacterium into cells are growth stage dependent." Infection and Immunity **65**(2): 774-82.

Moxon, E. R. and J. S. Kroll (1990). "The role of bacterial polysaccharide capsules as virulence factors." Current Topics in Microbiology and Immunology **150**: 65-85.

Mufson, M., H. Krause, G. Schiffman and D. F. Hughey (1987). "Pneumococcal antibody levels one decade after immunisation of healthy adults." American Journal of Medical Science **293**: 279-284.

Munoz, R., M. Mollerach, R. Lopez and E. Garcia (1997). "Molecular organization of the genes required for the synthesis of type 1 capsular polysaccharide of Streptococcus pneumoniae: formation of binary encapsulated pneumococci and identification of cryptic dTDP-rhamnose biosynthesis genes." Mol Microbiol **25**(1): 79-92.

Musoh, K., Y. Maeda, H. Tanaka, N. Inagaki and H. Nagai (2000). "Effect of a novel anti-allergic agent, HSR-609, on antigen-induced airway hyperresponsiveness in mice." Pharmacology **61**(2): 70-7.

Napoli, C., C. Lemieux and R. Jorgensen (1990). "Introduction of a Chimeric Chalcone Synthase Gene into Petunia Results in Reversible Co-Suppression of Homologous Genes in trans." Plant Cell **2**(4): 279-289.

Norn, S., L. Jensen, L. L. Kjaergaard, H. Permin, P. S. Skov and F. Espersen (1994). "Bacteria-induced IgE-mediated histamine release: examination of patients with chronic bronchitis (CB) during acute exacerbations." Agents Actions **41 Spec No**: C22-3.

Northcote, D. H. (1974). Sites of Synthesis of the Polysaccharide of the Cell Wall. Plant Carbohydrate Biochemistry. J. B. Pridham. London, Academic Press, Inc.: 165-181.

Novak, R., B. Henriques, E. Charpentier, S. Normark and E. Tuomanen (1999). "Emergence of vancomycin tolerance in *Streptococcus pneumoniae*." Nature **399**(6736): 590-3.

O'Brien, K. L. and H. Nohynek (2003). "Report from a WHO working group: standard method for detecting upper respiratory carriage of *Streptococcus pneumoniae*." Pediatric Infectious Disease Journal **22**(2): 133-40.

Ogunniyi, A. D., M. C. Woodrow, J. T. Poolman and J. C. Paton (2001). "Protection against Streptococcus pneumoniae elicited by immunization with pneumolysin and CbpA." Infect Immun **69**(10): 5997-6003.

Oldenburg, K. R., D. Loganathan, I. J. Goldstein, P. G. Schultz and M. A. Gallop (1992). "Peptide ligands for a sugar-binding protein isolated from a random peptide library." Proc Natl Acad Sci U S A **89**(12): 5393-7.

Ouchterlony, O. and L. A. Nilsson (1973). Immunodiffusion and immunoelectrophoresis. Handbook of Experimental Immunology. D. M. Weir. Oxford, Blackwell Scientific Publications: 19-21.

Oxenboll Sorensen, S., M. Pauly, M. Bush, M. Skjot, M. C. McCann, B. Borkhardt and P. Ulvskov (2000). "Pectin engineering: modification of potato pectin by in vivo expression of an endo-1,4-beta-D-galactanase." Proc Natl Acad Sci U S A **97**(13): 7639-44.

Parmley, S. F. and G. P. Smith (1988). "Antibody-selectable filamentous fd phage vectors: affinity purification of target genes." Gene **73**: 305-318.

Partidos, C. D. (2000). "Peptide mimotopes as candidate vaccines." Current Opinions in Molecular Therapeutics. **2**(1): 74-9.

Paton, J. C., P. W. Andrew, G. J. Boulnois and T. J. Mitchell (1993). "Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins." Annual Review of Microbiology **47**: 89-115.

Penn, R. L., E. B. Lewin, R. G. Douglas, Jr., G. Schiffman, C. J. Lee and J. B. Robbins (1982). "Antibody responses in adult volunteers to pneumococcal polysaccharide types 19F and 19A administered singly and in combination." Infection and Immunity **36**(3): 1261-2.

Phalipon, A., A. Foligori, J. Arondel, G. Sgaramella, P. Fortugno, R. Cortese, P. Sansonetti, J. and F. Felici (1997). "Induction of anti-carbohydrate antibodies by phage library-selected peptide mimics." European Journal of Immunology. **27**: 2620-2625.

Pincus, S. H., M. J. Smith, H. J. Jennings, J. B. Burritt and P. M. Glee (1998). "Peptides that mimic the group B streptococcal type III capsular polysaccharide antigen." Journal of Immunology **160**(1): 293-8.

Principi, N. and P. Marchisio (2000). "Epidemiology of *Streptococcus pneumoniae* in Italian children." Acta Paediatrica **435 (Suppl)**.

Prinz, D. M., S. L. Smithson, T. Kieber-Emmons and M. A. Westerink (2003). "Induction of a protective capsular polysaccharide antibody response to a multiepitope DNA vaccine encoding a peptide mimic of meningococcal serogroup C capsular polysaccharide." Journal of Immunology **110**(2): 242-9.

Raby, R., M. Blaiss, S. Gross and H. G. Herrod (1996). "Antibody response to unconjugated Haemophilus influenzae b and pneumococcal polysaccharide vaccines in children with recurrent infections." J Allergy Clin Immunol **98**(2): 451-9.

Rajadhyaksha, M. and Y. Thanavala (1995). "Delineation of the minimal hepatitis B surface antigen-specific B- and T-cell epitope contained within an anti-idiotype-derived pentadecapeptide." Proc Natl Acad Sci U S A **92**(5): 1575-9.

Rao, S. R. and G. A. Ravishankar (2002). "Plant cell cultures: Chemical factories of secondary metabolites." Biotechnol Adv **20**(2): 101-53.

Reeves, R. E. and W. F. Goebel (1941). "Chemoimmunological studies on the soluble specific substance of pneumococcus." Journal of Biological Chemistry **139**: 511-517.

Richter, L. and P. B. Kipp (1999). "Transgenic plants as edible vaccines." Curr Top Microbiol Immunol **240**: 159-76.

Richter, L. J., Y. Thanavala, C. J. Arntzen and H. S. Mason (2000). "Production of hepatitis B surface antigen in transgenic plants for oral immunization." Nature Biotechnology **18**(11): 1167-71.

Rigano, M. M., M. L. Alvarez, J. Pinkhasov, Y. Jin, F. Sala, C. J. Arntzen and A. M. Walmsley (2004). "Production of a fusion protein consisting of the enterotoxigenic *Escherichia coli* heat-labile toxin B subunit and a tuberculosis antigen in *Arabidopsis thaliana*." Plant Cell Reports **22**(7): 502-8.

Robbins, J. B., R. Austrian, C. J. Lee, S. C. Rastogi, G. Schiffman, J. Henrichsen, P. H. Makela, C. V. Broome, R. R. Facklam, R. H. Tiesjema and et al. (1983a). "Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups." Journal of Infectious Diseases **148**(6): 1136-59.

Robbins, P. W., D. Bray, B. M. Dankert and A. Wright (1967). "Direction of chain growth in polysaccharide synthesis." Science **158**(808): 1536-42.

Roberts, I. S., F. K. Saunders and G. J. Boulnois (1989). "Bacterial capsules and interactions with complement and phagocytes." Biochemical Society Transactions **17**(3): 462-4.

Ryan, E. J., L. M. Daly and K. H. Mills (2001). "Immunomodulators and delivery systems for vaccination by mucosal routes." Trends Biotechnol **19**(8): 293-304.

Sagi, L., B. Panis, S. Remy, H. Schoofs, K. De Smet, R. Swennen and B. P. A. Cammue (1995). "Gentic transformation of Banana and Plantain (*Musa* spp.) via Particle Bombardment." Biotechnology **13**: 481-486.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich (1988). "Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase." Science **239**(4839): 487-91.

Sala, F., M. Manuela Rigano, A. Barbante, B. Basso, A. M. Walmsley and S. Castiglione (2003). "Vaccine antigen production in transgenic plants: strategies, gene constructs and perspectives." Vaccine **21**(7-8): 803-8.

Sanmugalingam, D., A. J. Wardlaw and P. Bradding (2000). "Adhesion of human lung mast cells to bronchial epithelium: evidence for a novel carbohydrate-mediated mechanism." J Leukoc Biol **68**(1): 38-46.

Sawada, H., H. Ieki and I. Matsuda (1995). "PCR detection of Ti and Ri plasmids from phytopathogenic Agrobacterium strains." Applied Environmental Microbiology **61**(2): 828-31.

Saxena, I. M. and M. B. Brown (1997). "Identification of cellulose synthase(s) in higher plants: sequence analysis of processive β-glycosyltransferases with the common motif 'D, D, D35Q(R,Q)XRW'." Cellulose **4**: 33-49.

Schuler-Thurner, B., E. S. Schultz, T. G. Berger, G. Weinlich, S. Ebner, P. Woerl, A. Bender, B. Feuerstein, P. O. Fritsch, N. Romani and G. Schuler (2002). "Rapid induction of tumor-specific type 1 T helper cells in metastatic melanoma patients by vaccination with mature, cryopreserved, peptide-loaded monocyte-derived dendritic cells." J Exp Med **195**(10): 1279-88.

Scott, J. K., D. Loganathan, R. B. Easley, X. Gong and I. J. Goldstein (1992). "A family of concanavalin A-binding peptides from a hexapeptide epitope library." Proc Natl Acad Sci U S A **89**(12): 5398-402.

Scott, J. K. and G. P. Smith (1990). "Searching for peptide ligands with an epitope library." Science **249**: 386-90.

Sege, K. and P. A. Peterson (1978). "Use of anti-idiotypic antibodies as cell-surface receptor probes." Proc Natl Acad Sci U S A **75**(5): 2443-7.

Shaw, P. X., S. Horkko, M. K. Chang, L. K. Curtiss, W. Palinski, G. J. Silverman and J. L. Witztum (2000). "Natural antibodies with the T15 idiotype may act in atherosclerosis, apoptotic clearance, and protective immunity." J Clin Invest **105**(12): 1731-40.

Sheveleva, E. V., S. Marquez, W. Chmara, A. Zegeer, R. G. Jensen and H. J. Bohnert (1998). "Sorbitol-6-phosphate dehydrogenase expression in transgenic tobacco. High amounts of sorbitol lead to necrotic lesions." Plant Physiol **117**(3): 831-9.

Shields, B. (2001). "Prevnar (heptavalent pneumococcal conjugate vaccine): disease prevention in infants and children." Journal of Pediatric Health Care **15**(4): 203-8.

Shimizu, T., J. Nishihira, H. Watanabe, R. Abe, T. Ishibashi and H. Shimizu (2004). "Cetirizine, an H1-receptor antagonist, suppresses the expression of macrophage migration inhibitory factor: its potential anti-inflammatory action." Clin Exp Allergy **34**(1): 103-9.

Shin, J.-S., J. Yu, J. Lin, L. Zhong, K. L. Bren and M. H. Nahm (2002). "Peptide Mimotopes of Pneumococcal Capsular Polysaccharide of 6B Serotype: A Peptide Mimotope Can bind to Two Unrelated Antibodies." Journal of Immunology **168**: 6273-6278.

Shintani, D. and D. DellaPenna (1998). "Elevating the vitamin E content of plants through metabolic engineering." Science **282**(5396): 2098-100.

Simmons, J. S. (1926). "A culture medium for differentiating organisms of typhoid-colon aerogenes groups and for isolating of certain fungi." Journal of Infectious Diseases **39**: 209 - 241.

Simon, I. (1993). "Predictions from the regularities of the primary structure of proteins." Peptide Research **6**: 260.

Simpson, S. Q., R. Singh and D. E. Bice (1994). "Heat-killed pneumococci and pneumococcal capsular polysaccharides stimulate tumor necrosis factor-alpha production by murine macrophages." Am J Respir Cell Mol Biol **10**(3): 284-9.

Singleton, J. A., S. M. Greby, K. G. Wooten, F. J. Walker and R. Strikas (2000). "Influenza, pneumococcal, and tetanus toxioid vaccination of adults--United States, 1993-7." MMWR CDC Surveill Summ **49**(9): 39-62.

Slater, S., T. A. Mitsky, K. L. Houmiel, M. Hao, S. E. Reiser, N. B. Taylor, M. Tran, H. E. Valentin, D. J. Rodriguez, D. A. Stone, S. R. Padgette, G. Kishore and K. J. Gruys (1999). "Metabolic engineering of Arabidopsis and Brassica for poly(3-hydroxybutyrate-co-3-hydroxyvalerate) copolymer production." Nat Biotechnol **17**(10): 1011-6.

Smirnoff, N. (2000). "Ascorbic acid: metabolism and functions of a multi-facetted molecule." Curr Opin Plant Biol **3**(3): 229-35.

Smit, P., D. Oberholzer, S. Hayden-Smith, H. J. Koornhof and M. R. Hilleman (1977). "Protective efficacy of pneumococcal polysaccharide vaccines." JAMA **238**: 2613-6.

Smith, G. P. (1985). "Filamentous fusion phage: novel expression vectors that displayed cloned antigens on the virion surface." Science **228**: 1315-1317.

Smith, G. P. and J. K. Scott (1993). "Libraries of peptides and proteins displayed on filamentous phage." Methods in Enzymology. **217**: 228-257.

Snape, M. D. and A. J. Pollard (2005). "Meningococcal polysaccharide - protein conjugate vaccines." Lancet Infectious Disease **5**: 21-30.

Spanjaard, L., A. van der Ende, H. Rumke, J. Dankert and L. van Alphen (2000). "Epidemiology of meningitis and bacteraemia due to Streptococcus pneumoniae in The Netherlands." Acta Paediatr Suppl **89**(435): 22-6.

Stein, M. P., J. C. Edberg, R. P. Kimberly, E. K. Mangan, D. Bharadwaj, C. Mold and T. W. Du Clos (2000). "C-reactive protein binding to FcgammaRIIa on human monocytes and neutrophils is allele-specific." J Clin Invest **105**(3): 369-76.

Sternberg, G. M. (1881). "A fatal form of septicaemia in the rabbit, produced by subcutaneous injection of human saliva. An experimental research." National Board Health Bulletin **2**: 781-783.

Sternberg, M. (1996). Protein Structure Prediction: a practical appraoch. Oxford, UK, Oxford University Press.

Strobel, S. and A. Mowat (1999). "Immune responses to dietary antigens: oral tolerance." Immunology Today **19**: 173-181.

Stryer, L. (1995). Biochemistry. New York, W.H. Freeman & Company.

Syriopoulou, V., G. L. Daikos, K. Soulis, A. Michos, H. Alexandrou, I. Pavlopoulou, I. P. A. Pagali, C. Hadjichristodoulou and M. Theodoridou (2000). "Epidemiology of invasive childhood pneumococcal infections in Greece." Acta Paediatr Suppl **89**(435): 30-4.

Takeuchi, Y., M. Dotson and N. T. Keen (1992). "Plant transformation: a simple particle bombardment device based on flowing helium." Plant Molecular Biology **18**(4): 835-9.

Tao, R., S. L. Uratsu and A. M. Dandekar (1995). "Sorbitol synthesis in transgenic tobacco with apple cDNA encoding NADP-dependent sorbitol-6-phosphate dehydrogenase." Plant Cell Physiol **36**(3): 525-32.

Tarczynski, M. C., R. G. Jensen and H. J. Bohnert (1992). "Expression of a bacterial mtlD gene in transgenic tobacco leads to production and accumulation of mannitol." Proc Natl Acad Sci U S A **89**(7): 2600-4.

Thompson, J. D., D. G. Higgins and T. J. Gibson (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." Nucleic Acids Res **22**(22): 4673-80.

Thompson, K. M., J. Sutherland, G. Barden, M. D. Melamed, I. Randen, J. B. Natvig, V. Pascual, J. D. Capra and F. K. Stevenson (1991). "Human monoclonal antibodies against blood group antigens preferentially express a VH4-21 variable region gene-associated epitope." Scandinavian Journal of Immunology **34**(4): 509-18.

Ting, L. P. and J. H. Wang (1982). "Functional groups at the catalytic site of BF1 adenosinetriphosphatase from Escherichia coli." Biochemistry **21**(2): 269-75.

Travella, S., S. M. Ross, J. Harden, C. Everett, J. W. Snape and W. A. Harwood (2005). "A comparison of transgenic barley lines produced by particle bombardment and Agrobacterium-mediated techniques." Plant Cell Rep **23**(12): 780-9.

Tsuji, N. M., K. Mizumachi and J. Kurisaki (2001). "Interleukin-10-secreting Peyer's patch cells are responsible for active suppression in low-dose oral tolerance." Immunology **103**(4): 458-64.

Tuboly, T., W. Yu, A. Bailey, S. Degrandis, S. Du, L. Erickson and E. Nagy (2000). "Immunogenicity of porcine transmissible gastroenteritis virus spike protein expressed in plants." Vaccine **18**(19): 2023-8.

Tuomanen, E. I. and H. R. Masure (2000). Molecular and Cellular Biology of Pneumococcal infection. Streptococcus pneumoniae. A. Tomasz.

Twumasi, P. A., Jr., S. Kumah, A. Leach, T. J. O'Dempsey, S. J. Ceesay, J. Todd, C. V. Broome, G. M. Carlone, L. B. Pais, P. K. Holder and et al. (1995). "A trial of a group A plus group C meningococcal polysaccharide-protein conjugate vaccine in African infants." J Infect Dis **171**(3): 632-8.

Usinger, W. R. and A. H. Lucas (1999). "Avidity as a determinant of the protective efficacy of human antibodies to pneumococcal capsular polysaccharides." Infect Immun **67**(5): 2366-70.

Valadon, P., G. Nussbaum, L. F. Boyd, D. H. Margulies and M. D. Scharff (1996). "Peptide libraries define the fine specificity of anti-polysaccharide antibodies to *Cryptococcus neoformans*." Journal of Molecular Biology **261**: 11–22.

Valentine, L. (2003). "Agrobacterium tumefaciens and the plant: the David and Goliath of modern genetics." Plant Physiol **133**(3): 948-55.

van der Ven, L. T., G. P. van den Dobbelsteen, B. Nagarajah, H. van Dijken, P. M. Dortant, J. G. Vos and P. J. Roholl (1999). "A new rat model of otitis media caused by Streptococcus pneumoniae: conditions and application in immunization protocols." Infect Immun **67**(11): 6098-103.

Van Oss, C. J. and C. F. Gillman (1973). "Phagocytosis as a surface phenomenon. 3. Influence of C1423 on the contact angle and on the phagocytosis of sensitised encapsulated bacteria." Immunological Communications **2**(4): 415-9.

Verpoorte, R. and J. Memelink (2002). "Engineering secondary metabolite production in plants." Curr Opin Biotechnol **13**(2): 181-7.

Viti, F., F. Nilsson, S. Demartis, A. Huber and D. Neri (2000). "Design and use of Phage display libraries for the selection of antibodies and enzymes." Methods in Enzymology. **326**(29): 480-505.

Webb, K. J., S. Jones, M. P. Robbins and F. R. Minchin (1990). "Characterisation of transgenic root cultures of Trifolium repens, Trifolium pratense and Lotus corniculatus and transgenic plants of Lotus corniculatus." Plant Science **70**: 243-54.

Webster, D. E., M. C. Thomas, R. A. Strugnell, I. B. Dry and S. L. Wesselingh (2002). "Appetising solutions: an edible vaccine for measles." Med J Aust **176**(9): 434-7.

Weigel, P. H., V. C. Hascall and M. Tammi (1997). "Hyaluronan synthases." Journal of Biological Chemistry **272**(22): 13997-4000.

Wenger, J. D., J. DiFabio, J. M. Landaverde, O. S. Levine and T. Gaafar (1999). "Introduction of Hib conjugate vaccines in the non-industrialized world: experience in four 'newly adopting' countries." Vaccine **18**(7-8): 736-42.

Wenger, J. D., S. R. Steiner, L. B. Pais, J. C. Butler, B. Perkins, G. M. Carlone and C. M. Broome (1996). Laboratory correlates for protective efficacy of pneumococcal vaccines: how can they be identified and validated? Program and abstracts of the 36th Interscience Conference on Antimicrobial Agents and Chemotherapy, American Society for Microbiology, Washington, D.C.: 150.

Westerink, M. A., A. A. Campagnari, M. A. Wirth and M. A. Apicella (1988). "Development and characterization of an anti-idiotype antibody to the capsular polysaccharide of Neisseria meningitidis serogroup C." Infection and Immunity **56**(5): 1120-7.

Westerink, M. A. and P. C. Giardina (1992). "Anti-idiotype induced protection against Neisseria meningitidis serogroup C bacteremia." Microb Pathog **12**(1): 19-26.

Westerink, M. A., P. C. Giardina, M. A. Apicella and T. Kieber-Emmons (1995). "Peptide mimicry of the meningococcal group C capsular polysaccharide." Proceedings of the National Academy of Sciences of the United States of America **92**: 4021-4025.

Willis, B. C., S. M. Ndiaye, D. P. Hopkins and A. Shefer (2005). "Improving influenza, pneumococcal polysaccharide, and hepatitis B vaccination coverage among adults aged <65 years at high risk: a report on recommendations of the Task Force on Community Preventive Services." MMWR Recomm Rep **54**(RR-5): 1-11.

Wolff, N., C. Deniau, S. Letoffe, C. Simenel, V. Kumar, I. Stojiljkovic, C. Wandersman, M. Delepierre and A. Lecroisey (2002). "Histidine pK(a) shifts and changes of

tautomeric states induced by the binding of gallium-protoporphyrin IX in the hemophore HasA(SM)." Protein Sci **11**(4): 757-65.

Wood, P. (2001). Understanding Immunology. Harlow, England, Pearson Education Ltd.

World Health Organisation (1999). "Pneumococcal vaccines." Weekly Epidemiological Record **74**(23): 177-83.

World Health Organisation, G. (1996). "Report of the Scientific Group of Experts (SAGE) of the Children's Vaccine Initiative and the Global Programme for Vaccines and Immunization." Global Programme for Vaccines and Immunization.

Yu, J. and W. H. Langridge (2001). "A plant-based multicomponent vaccine protects mice from enteric diseases." Nat Biotechnol **19**(6): 548-52.