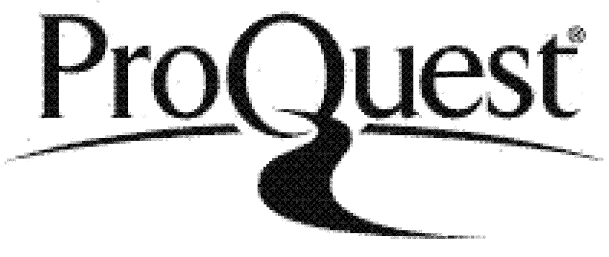
Genetic Studies on *Porphyromonas gingivalis* W83

Thesis submitted for the degree of
Doctor of Philosophy at the University
of Leicester.

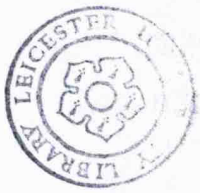
by

Janette Maley BSc
Department of Microbiology and Immunology
University of Leicester

September 1994



ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346


LEICESTER
LIBRARY

Statement

The accompanying thesis submitted for the degree of Doctor of Philosophy entitled " Genetic Studies on *Porphyromonas gingivalis* W83" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester between October 1989 and September 1992.

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

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

Signed.....  ..... Date.....Sept 15th 1994

Acknowledgements

For his supervision and advice throughout the duration of this thesis, I would like to sincerely thank Dr Ian Roberts.

I am also grateful to Dr Nadja Shoemaker for her generous donations of bacterial strains and plasmids.

I am very grateful to numerous members of the department past and present who have assisted me in a number of ways. In particular, my sincere gratitude goes to Andrea Mitchell (nee Shepherd), Dr Tim Mitchell and Dr Mark Coleman for their help and advice. Thanks also to Andrew Wallace, Anna Ostrowski and Archie Lovatt for their good-humoured support and advice.

I am deeply indebted to Dr Julie Eastgate and John Sutherland for their unfailing support throughout the difficult times.

Finally, I would like to thank my family and Jonathan Stephens for the continued support over the years without which I would never have made it.

Abstract

Until recently molecular biological approaches to the study of putative virulence factors of the oral pathogen *Porphyromonas gingivalis* were limited. This was due to the absence of a genetic system which could facilitate the production of isogenic mutant derivatives. Among the many components produced, the capsule is believed to be of particular importance in the pathogenicity of *P. gingivalis*.

In order to identify mutants lacking capsular polysaccharide, purified capsular material from *P.gingivalis* W83 was used to generate capsule-specific antisera. However, these antisera contained antibodies which recognised lipopolysaccharide. The use of polymyxin B-agarose was found to be useful in the removal of LPS-reactive material. Capsular polysaccharide was purified further and shown to be free of detectable LPS.

A system for conjugal transfer of plasmid DNA from *Escherichia coli* to *P. gingivalis* was developed. This method was then used for the introduction of the suicide-vector R751::Tn*4351*Ω4 into *P. gingivalis*. Examination of a number of *P. gingivalis* Tn*4351* mutants demonstrated the presence of multiple copies of Tn*4351* in the chromosome of the mutants.

Recovery of plasmid pNJR12 from *P. gingivalis* transconjugants led to the isolation of IS*1126*. This insertion sequence was present in the chromosome of all strains of *P. gingivalis* examined but absent from other members of the genus. The amino acid sequence of the major open reading frame (ORF1) was derived from the nucleotide sequence of IS*1126*. ORF1 contained the conserved motifs displayed by the transposase proteins of IS-elements belonging to the IS*4* family.

List of Abbreviations

APS	ammonium persulphate
bp	base pair
BSA	bovine serum albumin
cfu	colony forming unit
EDTA	disodium ethylene diaminetetra - acetic acid
H_2O_2	hydrogen peroxide
IPTG	isopropylthiogalactoside
kb	Kilobase pair
kDa	Kilodaltons
OPD	O-phenylene diamine
PBS	phosphate buffered saline
PEG	polyethylene glycol
PMN	polymorphonuclear leukocytes
rpm	revolutions per minute
SDS	sodium lauryl sulphate
SSC	standard sodium citrate
TE	Tris - EDTA buffer
TEMED	N, N, N', N' - tetramethylethylenediamine
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside

Contents

1 Introduction

2 Materials and Methods

3 Purification of Capsular Polysaccharide from *P. gingivalis* W83 and the Generation of Anticapsular Antibodies

Chapter 1

Introduction

Human periodontal diseases are recognised as a group of inflammatory disorders of connective tissue and bone which are related to the accumulation of a subgingival microbial plaque around the tooth. This may lead to the destruction of the collagen fibre attachment to the tooth and the resorption of the supporting alveolar bone leading to the loss of the tooth.

From a microflora which may often contain in excess of three hundred different bacterial species, only a small group of organisms have been specifically implicated in the pathogenesis of such diseases. Among them, *Porphyromonas gingivalis* is generally regarded as an important agent in the aetiology of periodontitis as well as a variety of other oral diseases.

1.1 Historical Overview

In 1921, Oliver and Wherry isolated a small anaerobic Gram-negative rod from a variety of human sites. When grown on blood agar plates, these bacteria produced brown or black-pigmented colonies. On the assumption that the pigment melanin was responsible for the colour, they named the organism *Bacterium melaninogenicum.*

Since the growth of this organism was found to be enhanced on solid medium by inclusion of the growth factors X and V, a characteristic of the genus *Haemophilus*, the organism

was described in the third edition of Bergey's Manual of Determinative Bacteriology as *H. melaninogenicus* (Bergey *et al.*, 1930). In 1939, the generic name *Bacteroides* was adopted, and *H. melaninogenicus* was reclassified as *Bacteroides melaninogenicus* (Roy and Kelly, 1939). Despite extensive biochemical heterogeneity (Courant and Gibbons, 1967; Sawyer *et al.*, 1962), all strains remained in a single species until 1970. The fermentative activity of the species, as measured by the ability to reduce the pH of media containing glucose when compared to growth in the same media without glucose differed among isolates. Some strains (asaccharolytic) did not reduce the pH and were therefore classified as non-fermentative. The saccharolytic strains could be further divided into two groups, weakly or strongly fermentative. The species was redefined to form three subgroups. *Bacteroides melaninogenicus* subsp. *melaninogenicus* contained strains which were strongly fermentative, whilst weakly fermentative strains and nonfermentative strains were classified as *B. melaninogenicus* subsp. *intermedius* and *asaccharolyticus*, respectively (Holdeman and Moore, 1970).

Many differences in characteristics were found between *B. melaninogenicus* subsp. *asaccharolyticus* and the other subspp. of the genus. In particular, different DNA base composition, differences in the ability to utilise glucose and the production of different fermentation products led to *B. asaccharolyticus* being classified as a separate species (Finegold and Barnes, 1977). Examination of the electrophoretic mobilities of malate dehydrogenase (MDH) and the G+C composition of the DNA of different *B. asaccharolyticus* strains demonstrated that it was possible to separate oral from non-oral isolates (Shah *et al.*, 1976). Oral strains were found to have slower MDH mobility and a lower G+C content (46-48%) than the strains of non-oral origin (52-54%) (Shah *et al.*, 1976). SDS-PAGE polypeptide patterns also revealed two distinct groups (Swindlehurst *et al.*, 1977). It was proposed that asaccharolytic black-pigmented *Bacteroides* (BPB) isolated from oral sites be assigned to the genus *B. gingivalis* and that the species *B. asaccharolyticus* be retained for the nonfermentative strains recovered from nonoral sites (Coykendall *et al.*, 1980). Asaccharolytic strains isolated from monkeys, which had

belonged to *B. melaninogenicus* subsp. *macacae* (Slots and Genco, 1980) were also elevated to species level as *B. macacae* (Coykendall *et al.*, 1980). A third group of human asaccharolytic strains that were isolated from infected dental root canals were genetically, serologically and physiologically different from the other asaccharolytic species, were placed in a third species of human isolates, *B. endodontalis* (van Steenbergen *et al.*, 1984; van Winkelhoff *et al.*, 1985).

The saccharolytic BPB were also genetically heterogeneous and were also split into a number of species. *Bacteroides melaninogenicus* subsp. *melaninogenicus* was revised to form the three species: *B. denticola* (Shah and Collins, 1981), *B. melaninogenicus* and *B. loeschii* (Holdeman and Johnson, 1982). Strains of *B. melaninogenicus* subsp. *intermedius* were also reclassified as *B. intermedius* or *B. corporis* (Johnson and Holdeman,1983). *Bacteroides melaninogenicus* subsp. *levii*, originally isolated from bovines, was elevated to the species *B. levii* (Johnson and Holdeman, 1983).

Asaccharolytic BPB resembling *Bacteroides* strains had also been isolated from a number of different animal species, with some organisms isolated from cats being described as members of the species *B. salivosus* (Love *et al.*, 1987) or as *B. gingivalis*. Asaccharolytic BPB resembling *B. gingivalis* and *B. asaccharolyticus* have also been identified in sheep (McCourtie and Poxton, 1990) and dogs (Karjalainen *et al.*, 1993; Yamasaki *et al.*, 1990).

Major differences, including glucose metabolism (Holdeman *et al.*, 1984) and cellular fatty acid composition (Miyagawa *et al.*, 1979; Shah and Collins, 1980), between BPB and the type species of the genus, *B. fragilis*, have resulted in BPB being assigned to new genera. Asaccharolytic organisms now belong to the genus *Porphyromonas* (Shah and Collins, 1988) whilst saccharolytic species have been placed in the genus *Prevotella* (Shah and Collins, 1990). However, *B. levii* and *B. macacae* remain in the genus *Bacteroides*. A second species has been proposed for some of the asaccharolytic cat strains, *P. circumdentaria* (Love *et al.*, 1992). Recently, a new species, *P. canoris*, has been proposed

for black-pigmented asaccharolytic strains isolated from dogs (Love *et al.*, 1994). Changes in nomenclature are shown in Table 1.1.

1.2 General Characteristics of the genus *Porphyromonas*

Members of the genus *Porphyromonas* are characterised by the production of porphyrin pigments (Shah *et al.*, 1979). The major porphyrin produced is a dark brown/black pigment, which has been identified as protohaeme and the light brown, protoporphyrin (Shah *et al.*, 1979). The genus belongs to the family *Bacteroidaceae*, a large family of Gram-negative, obligately anaerobic, nonsporing, nonmotile rods or coccobacilli. *Porphyromonas* can be readily distinguished from other members of the family by the distinctive black- pigmented colonies on blood agar plates. In contrast to the saccharolytic black-pigmented Gram-negative anaerobic species which produce rough, dry colonies, *Porphyromonas* species form smooth, slightly mucoid, wet, shiny, convex colonies, which are 1-3mm in diameter. The colonies darken progressively from the edge of the colony towards the centre after 6-10 days. Growth in liquid medium is not significantly affected by the addition of carbohydrate and the terminal pH remains near neutral irrespective of its presence. The major metabolic end products are acetic and butyric acids, together with lower levels of propionic, isobutyric, and isovaleric acids. The amino acids aspartate and asparagine are fermented but these species generally possess only a limited ability to ferment amino acids.

Porphyromonas gingivalis

Porphyromonas gingivalis cells grown in a liquid medium are typically coccobacilli or rods (0.5 by 1.0-2.0μm) with the optimum temperature for growth being 37°C. Growth is markedly enhanced by the presence of protein hydrolysates, trypticase or proteose peptone and yeast extract (Shah and Williams, 1987). *Porphyromonas gingivalis* can be easily differentiated from the other two human species in the genus by DNA base composition, its

Table 1.1 Recent changes in nomenclature of black-pigmented *Bacteroides*

Previous Designation		**Present Designation**
Black-pigmented *Bacteroides*		Black-pigmented Gram-negative anaerobic rods
B. melaninogenicus subsp.	*Bacteroides* sp.	
macacae	*macacae*	*Bacteroides macacae*
levii	*levii*	*Bacteroides levii*
melaninogenicus	*melaninogenicus*	*Prevotella melaninogenica*
	loeschii	*Pr. loeschii*
	denticola	*Pr. denticola*
intermedius	*intermedius*	*Pr. intermedia*
		Pr. nigrescens
	corporis	*Pr. corporis*
asaccharolyticus	*asaccharolyticus*	*Porphyromonas asaccharolytica*
	gingivalis	*P. gingivalis*
	endodontalis	*P. endodontalis*
	salivosus	*P. salivosa*
		P. circumdentaria
		P. canoris

ability to haemagglutinate sheep erythrocytes, and by its trypsin-like activity (Slots, 1981; Slots and Genco, 1979).

Porphyromonas gingivalis requires a number of growth factors, including haemin, and for some strains, vitamin K (Gibbons and MacDonald, 1960). Vitamin K is a precursor for menaquinone biosynthesis which is required as an electron carrier in the electron transport system of this organism (Shah and Collins, 1980), and haemin has also been suggested to be involved in this system (Gibbons and MacDonald, 1960). Haemin has been shown to be important in both the physiology and virulence of this organism (Carman *et al.*, 1990). *Porphyromonas gingivalis* cells transferred to a haemin-deficient environment prior to inoculation into mice were avirulent, whereas, cells grown in haemin-excess conditions caused 100% mortality (McKee *et al.*, 1986). In conditions of excess haemin, *P.gingivalis* produces a black-pigment which has been shown to be a derivative of haemin, protohaeme (Shah *et al.*, 1979). This is accumulated by the organism when grown in media with a high haemin concentration and stored for use when the haemin in the environment has been depleted (Rizza *et al.*,1968). It was suggested that the lipopolysaccharide may be the haemin binding component of this organism (Grenier, 1991). However, a 26 kDa iron-repressible outer membrane protein (OMP) has been identified, and implicated in haemin-binding (Bramanti and Holt, 1992a). Expression of this protein resulted in increased haemin uptake (Yu and Bramanti, 1993) and, therefore, like similar proteins in other pathogenic bacteria, may play an important role in the sequestration of iron *in vivo*. This protein was found in all seven strains of *P. gingivalis* examined (Bramanti and Holt, 1992b).

Porphyromonas gingivalis, has been implicated as a major aetiologic agent in the pathogenesis of human and animal periodontal disease (Laliberte and Mayrand, 1983; McCourtie and Poxton, 1990; Slots, 1982; Slots and Genco, 1984; Yamasaki *et al.*, 1990). Despite their many similarities, human strains can generally be distinguished from animal strains by their being catalase-negative (*P. gingivalis* biotype 1), whereas animal strains are

usually catalase-positive (biotype 2) (Laliberte and Mayrand, 1983; Karjalainen *et al*, 1993). Some catalase-negative strains have been isolated from experimental infections in monkeys but it is possible that they were infected by their handlers.

A number of studies by Loos and coworkers have demonstrated that *P. gingivalis* strains exhibit extensive genetic heterogeneity. Examination of 33 *P. gingivalis* isolates from a variety of worldwide sources by restriction endonuclease analysis yielded 29 distinct patterns. *Porphyromonas gingivalis* strains W50 and W83 exhibited identical profiles as did strains 2561 and 381, suggesting that either these strains were identical, or, extremely closely related (Loos *et al.*, 1990; van Steenbergen *et al.*, 1989). The extent of heterogeneity was also highlighted by restriction fragment length polymorphism (RFLP) analysis of the fimbrillin locus among 39 human and animal strains. Nine RFLP groups were found but 25 unique fingerprints were identified (Loos and Dyer, 1992). Recently multi-locus enzyme electrophoresis (MLEE) studies were extended to include 100 different human isolates of *P. gingivalis* and related strains from other animals. The strains were tested for the presence of catalase activity and the electrophoretic mobilitity of 16 metabolic enzymes. These experiments showed the existence of 78 distinctive electrophoretic types (ETs) which clustered in three major phylogenetic divisions. All 88 human isolates were contained in division I along with 4 out of 7 monkey isolates. Division II contained the remainder of the monkey isolates and the other animal strains belonging to biotype 2. The sheep isolates, like division I members, were catalase-negative but different enough to be placed in a third group, division III. It was suggested that the name *P. gingivalis* be limited to human isolates and that divisions II and III represent distinct species of *Porphyromonas* (Loos *et al.*, 1993). These studies have not produced any evidence to suggest that genetic structure is related to invasiveness, nor did strains isolated from healthy individuals form a single genetically distinct group.

1.3 The Association of *P. gingivalis* with Periodontal Disease

Periodontal disease encompasses a family of highly prevalent, inflammatory infections which affect the periodontal tissues i.e. the supporting structure of the teeth. The bacterial flora associated with healthy gingival tissue is frequently comprised primarily of Gram-positive organisms (Page, 1986; Socransky *et al.*, 1977; Slots, 1979). The gingival crevice has a low bacterial density and the bacteria present are continuously eliminated by the gingival exudate and through desquamation of the epithelium at the bottom of the crevice. However, when oral hygiene is neglected, the accumulated bacteria soon initiate an inflammatory reaction in the gingivae and this often causes a change in the types of bacteria present (Page, 1986; Theilade *et al.*, 1967; Moore *et al.*, 1982). Gram-positive organisms usually decrease in numbers and Gram-negative forms become prominent (Dzink *et al.*, 1985; Moore *et al.*, 1983; Slots, 1979; Syed and Loesche, 1978). After 1-2 weeks, gingivitis may occur where there is severe inflammation when the gingivae are swollen and red, and bleed easily when probed, there is also measurable exudate from the pocket. The nature of the resulting disease is determined by the interaction of the bacteria and host. The bacteria may produce disease directly by producing enzymes which may degrade the host tissues (Slots, 1981; van Steenbergen *et al.*, 1986) or indirectly by inducing responses from the host (Bom-van Noorloos *et al.*, 1989). Although the host defence mechanisms against the bacteria are primarily protective, the reaction that occurs may enhance the disease. PMN, in particular, are believed to be active participants in the tissue destruction of periodontal disease (Altman *et al.*, 1992).

Periodontitis is a term that covers a number of periodontal diseases that affect the gingivae, the supporting connective tissue, and alveolar bone, which together anchor the teeth in the jaw and is the most common form of periodontal disease. This type of infection is initiated and sustained by the dental plaque. Adult periodontitis is one of the most common forms of periodontitis and is a progressively destructive inflammatory process arising from the host

reaction. The reaction extends into the deeper structures of the periodontium and causes loss of attachment and bone which leads to pathological pocket formation. In more advanced instances, the tooth becomes increasingly mobile and eventually results in the loss of the tooth.

Periodontitis may be either generalised or site-specific. Elevated levels of a limited number of anaerobic Gram-negative species and spirochaetes are associated with the process. However the bacterial composition alters markedly between different sites in one individual as well as between individuals. *Porphyromonas gingivalis*, in particular, is among the limited number of putative periodontopathogens that have been associated with periodontitis in adults, periodontal abscesses (Moore, 1987; Slots and Listgarten, 1988) and is sometimes also associated with generalised forms of juvenile periodontitis (Moore *et al.*, 1985).

The first report implicating black-pigmented organisms with periodontal disease was in 1928 by Burdon who noted that such organisms were present in the oral cavity of man and that their numbers increased significantly in individuals with periodontitis. Recently, a variety of studies have shown that *P. gingivalis* is usually absent or present in very small numbers in healthy gingival tissue and the subgingival flora of individuals with gingivitis (White and Mayrand, 1981; Zambon *et al.*, 1981). In patients with advanced periodontitis *Porphyromonas gingivalis* may comprise in excess of 35% of the subgingival microflora (Dahlen, 1992; Spiegel *et al.*, 1979; White and Mayrand, 1981). Some researchers have shown that eradication of *P. gingivalis* from patients correlates with the disappearance of disease (Loesche *et al.*, 1981: van Dyke *et al.*, 1988). There is also an increased probability of finding *P. gingivalis* associated with disease with an increase in the age of the patient (Savitt, 1991).

Porphyromonas gingivalis is isolated from many natural infections as part of a mixed flora (Socransky *et al.*, 1988). It is probable that organisms present in the microflora may serve as growth substrate providers for each other. *Porphyromonas gingivalis* is known to have a

beneficial effect on the growth of *Treponema denticola* (Nilius *et al.*, 1993). A number of studies have demonstrated that the presence of other bacterial species is important for the growth of *P. gingivalis* (Gharbia *et al.*, 1989). Among them, *Corynebacterium* and *Wolinella recta* have been shown to significantly enhance the growth of *P. gingivalis* (Grenier and Mayrand,1986; Takazoe *et al.*, 1971). Mixed culture studies have also demonstrated the importance of interactions with other organisms for the ability of *P. gingivalis* to cause disease. An inoculum containing a mixture of oral organisms only produced progressive and transmissible disease in guinea pigs when *P. gingivalis* was included in the mixture (MacDonald *et al.*, 1963). Another study demonstrated that *P. gingivalis* was noninfectious when injected into guinea pigs as a pure culture, whereas the same strain, when co-inoculated with haemin, succinate, or a succinate-producing organism, caused a rapid and often fatal infection (Mayrand and McBride, 1980).

More recently, it was concluded that the rapid progression of periodontitis in macaque monkeys could be directly correlated with the implantation of *P. gingivalis* into the animals (Holt *et al.*, 1988). Similarily, immunisation of squirrel monkeys with killed whole-cells of *P. gingivalis* decreased the amount of colonisation and the degree of alveolar bone loss when the animals were later challenged with viable cells (Clark *et al.*, 1992).

Other evidence has been obtained from the results of immunological studies. Bacterial infections are often accompanied by a specific immune response to the pathogen. Periodontally healthy individuals have low levels of specific antibodies to *P. gingivalis*, whilst IgG levels to various components of *P. gingivalis* are considerably higher in subjects with advanced periodontitis (Zafiropoulos *et al.*, 1992). These include antibodies to lipopolysaccharide (LPS), fimbriae and other protein antigens (Ismaiel *et al.*,1988; Kurihara *et al.*,1991; Naito *et al.*, 1987).

Porphyromonas gingivalis appears to colonise solely subgingival sites of the oral cavity but can also be sporadically isolated from the tongue, tonsils, saliva, and rarely from

supragingival dental plaque (Shah *et al.*, 1976). Since *P. gingivalis* is recovered almost exclusively from diseased sites, its primary means of infection remains unknown. It is generally believed that this organism is not present in the healthy oral cavity before development of permanent teeth but may become important with the onset of puberty (Slots, 1982). However, since it occurs in prepubescent mixed anaerobic infections e.g., odontogenic abscesses and periodontitis (Moore *et al.*, 1985; Sweeney *et al.*, 1987), there is reason to believe that *P. gingivalis* is either present in undetectable numbers, or at least occurs frequently in the transient flora of the child.

1.4 Virulence Factors

Although *P. gingivalis* has been the subject of intense investigation for a considerable length of time, the pathogenic mechanisms involved in the initiation and development of periodontitis are still not fully understood. Information regarding the contribution of individual virulence factors to the pathogenicity of this organism is far from complete. Since it is an inflammatory disease, one of the main characteristics of periodontitis is that as the disease progresses there is an influx of PMN into the site of infection. To survive and replicate in crevicular fluid *P. gingivalis* must possess a range of factors that will allow evasion of immune defences.

Some researchers have used spontaneous mutants and chemically induced mutants to investigate the possible role of different factors in virulence (Hoover *et al.*, 1992a; McKee *et al.*, 1988; Shah *et al.*, 1989). However the spontaneous mutants are known to be defective in a number of properties (Shah *et al.*, 1989) and it is not known whether chemically induced mutants carry more than one mutation. As a result, most of the information obtained has come from the purification of individual components and their subsequent analysis in a variety of *in vitro* assay systems. Additional information on virulence comes from subcutaneous infections of mice and guinea pigs (Baumgartner *et al.*,

1992; Kastelein *et al.*, 1981), the use of gnotobiotic rats (Heijl *et al.*, 1980) and subcutaneous implanted chambers (Genco *et al.*, 1991).

Porphyromonas gingivalis is known to produce a variety of putative virulence determinants. No individual factor has been shown to be solely responsible for virulence and it is generally accepted that virulence is multifactorial.

1.4.1 Adhesins

A prerequisite for many bacterial infections is that the organism must attach to, and subsequently colonise the host tissue before infection can occur. In the gingival crevice, there is a continual flow of crevicular fluid which will wash away any bacterial cells which are unattached.

Porphyromonas gingivalis can adhere to, and then be internalised by human epithelial cells (Duncan *et al.*, 1993). Studies of the microflora of the gingival pocket have shown that *P. gingivalis* is part of the microbiota attached to epithelial cells (Dzink *et al.*, 1989). This organism can also adhere to saliva-coated hydroxyapatite (Cimasoni *et al.*, 1987). However as both of these adherence properties are inhibited by serum and crevicular fluid (Okuda *et al.*, 1981; Slots and Gibbons, 1978), it has been postulated that the first stage of colonisation could also be through the attachment of *P. gingivalis* to other bacteria already attached to the gingival tissue as dental plaque, or to the plaque on the dorsum of the tongue (Slots and Gibbons, 1978).

Porphyromonas gingivalis has been shown to exhibit a specific, nonrandom affinity for coaggregation with other oral bacteria (Eke *et al.*, 1989; Kolenbrander and Anderson, 1989). *Porphyromonas gingivalis* strains have been shown to coadhere in varying degrees to a number of oral streptococcal species (Lamont *et al.*, 1992). Binding among the different strains can be influenced by sugars, saliva or proteases. Using a turbidimetric

assay it was found that binding to one strain of *S. mitis* was inhibited by arginine and lysine and that plasma and serum inhibited the binding in a dose-dependent manner (Nagata *et al.*,1991). Fibrinogen was the most potent inhibitor of the plasma-derived proteins tested. The adherence of a *P. gingivalis* strain to a strain of *S. sanguis* immobilised on agarose beads was greatly inhibited by bathing the streptococci in human serum beforehand or by pretreatment of *P. gingivalis* with a number of enzymes (Stinson *et al.*, 1991). This suggested that the *P. gingivalis* adhesin was protein in nature. However, examination of the interactions between five strains of *P. gingivalis* and a number of *Streptococcus* species revealed that, with only one exception, binding was unaffected or even enhanced by pretreatment of the streptococcal strains with saliva (Lamont *et al.*, 1992). Adherence was not altered by pretreatment with pronase or by the presence of sugars whereas periodate treatment of *P. gingivalis* reduced binding significantly, suggesting the involvement of a carbohydrate receptor. *Porphyromonas gingivalis* adheres to *Actinomyces viscosus* more avidly than other black-pimented Gram-negative anaerobes (Li and Ellen, 1989) and this function is inhibited by trypsin (Ellen *et al.*, 1992) and increased in conditions which enhance trypsin-like activity (Li *et al.*, 1991). The adherence of *P. gingivalis* to *Fusobacterium nucleatum* is inhibited by lactose (Kolenbrander and Anderson, 1989). It would therefore seem likely that in a variety of different conditions, any of a number of surface structures of *P. gingivalis* could be involved in its adherence to a number of different bacterial species. It is also possible that experimental procedures could be in part responsible for the range of different results, e.g. coaggregation between some species can be detected by filter assays but not those relying on turbidity.

The haemagglutinin and fimbriae produced by *P. gingivalis* are believed to be important in adhesion and will now be discussed.

Haemagglutinin

Porphyromonas gingivalis is known to be capable of agglutinating erythrocytes (Okuda and Takazoe, 1974) which is inhibited by arginine and lysine (Okuda *et al.*, 1986). The

ability of an organism to cause haemagglutination is often indicative of its ability to adhere to various animal cell types. It would seem that *P. gingivalis* may produce more than one haemagglutinin. Three major proteins were present in haemagglutination preparations (Inoshita *et al*, 1986) whilst the use of anti-haemagglutinin antisera detected two major protein antigens in immunoblots of *P. gingivalis* cell-surface extracts (Mouton *et al*., 1989). Two haemagglutinin genes (*hag*A and *hag*B) were cloned from *P. gingivalis* 381 whose products reacted with polyclonal antibodies raised against whole cells and reduced their ability to inhibit haemagglutination. The genes encoded proteins of 125 and 50 kDa (Progulske-Fox *et al*., 1989a). Antisera specific to the 125 kDa protein reacted strongly to two bands of 43 and 38 kDa in cell lysates of *P. gingivalis* and also to a number of minor bands (Progulske-Fox *et al*., 1989a). The gene encoding the 50 kDa protein has been expressed in a vaccine strain of *Salmonella typhimurium* with the intention of evaluating the immunogenicity of the haemagglutinin in a murine model (Dusek *et al*., 1993). A third haemagglutinin gene (*hag*C) has been identified which has homology to *hag*B and isogenic mutants deficient in either protein have been produced (Lepine and Progulske-Fox, 1993). Since all *hag*B$^-$ and *hag*C$^-$ transconjugants were capable of agglutinating erythrocytes then it would seem that both of these genes are expressed in *P. gingivalis* (Lepine and Progulske-Fox, 1993).

Fimbriae

Many *P. gingivalis* strains possess fimbriae which differ in their antigenic specificities (Suzuki *et al*., 1988). Twenty of 23 strains examined in one study contained material which was cross-reactive in the 41-49 kDa molecular mass range with polyclonal antiserum raised against the 43 kDa fimbrillin protein purified from dissociated fimbriae of *P. gingivalis* 2561 (Lee *et al*., 1991). Of the reactive strains 15 also reacted in varying degrees to polyclonal antiserum raised against purified fimbriae of the same strain whereas only 9 reacted to anti-fimbrial monoclonal antibodies (Lee *et al*., 1991). This suggests that

although common epitopes exist in most strains, the fimbriae of *P. gingivalis* have different antigenic components.

Fimbriae were originally believed to be responsible for haemagglutination (Okuda *et al.*, 1981) but various researchers have produced conflicting results about their involvement in this process (Mouton *et al.*, 1989; Yoshimura *et al.*, 1984). Highly purified fimbrial preparations lacked any detectable haemagglutinating activity (Yoshimura *et al.*, 1984) and such preparations were found to be incapable of inhibiting the binding of anti-haemagglutinin antibodies to cell-surface antigens of *P. gingivalis* (Mouton *et al.*, 1989). The isolation of a *fim*A mutant of *P. gingivalis* which was unable to produce fimbriae but still had the ability to haemagglutinate (Hamada *et al.*, 1994) would suggest that fimbriae are not involved in haemagglutination.

Instead, it would seem that fimbriae may function in the attachment of *P. gingivalis* to both host and bacterial cells (Grenier and Mayrand, 1987a). Immunization with purified fimbriae protects rats from the onset of experimental periodontal disease (Evans *et al.*, 1992), and synthetic peptide analogues of the fimbrillin protein inhibited the adherence of *P. gingivalis* to saliva-coated hydroxyapatite (Lee *et al.*, 1992). *FimA* mutants have a diminished capacity to adhere to fibroblasts and epithelial cells (Hamada *et al.*, 1994)Also, antibodies generated against purified fimbrial preparations inhibit coadhesion of *P. gingivalis* to *A. viscosus* (Goulbourne and Ellen, 1991).

However, the exact importance of fimbriae remains unclear as noninvasive strains have been shown to bind to oral tissues more avidly than the usually less fimbriated invasive strains, and there appears to be no correlation between the ability to colonise tissue and the ability to invade (Naito *et al.*, 1993).

Studies have suggest that fimbriae may contribute to the overall hydrophobicity of the cell, whereby invasive strains were less hydrophobic and less fimbriated than the noninvasive strains (Naito *et al.*, 1993; Watanabe *et al.*, 1992). The fimbriae of *P. gingivalis* strain may

be involved in the pathology of the disease as purified preparations have been shown to induce expression of neutrophil chemotactic factors and of IL-1 (Hanazawa *et al.*, 1991; 1992), which may be important for PMN infiltration into the gingival tissues and play a part in bone destruction.

The gene encoding the fimbrial subunit protein of *P. gingivalis* 381 was cloned and sequenced (Dickinson *et al.*, 1988). However, the authors could not detect expression of the predicted 35.9 kDa protein in *E. coli*. No homologous sequence was found in other black-pigmented Gram-negative anaerobes examined (Dickinson *et al.*, 1988) whereas, all thirty-nine *P. gingivalis* strains examined contained the *fimA* allele (Loos and Dyer, 1992).

The fimbrillin gene from *P. gingivalis* 2561 has been cloned recently and shown to encode a 41 kDa protein when expressed in *E. coli* The recombinant protein reacted with antibodies to fimbrillin and synthetic fimbrial analogues and was also capable of inhibiting the binding of *P. gingivalis* to saliva-coated hydroxyapatite (Sharma *et al.*, 1993).

Given the adhesive properties of both fimbriae and haemagglutinins, it is possible that they may function in the colonisation by *Porphyromonas gingivalis*.

1.4.2 Lipopolysaccharide (LPS)

The LPS produced by *P. gingivalis* is biochemically atypical when compared to the LPS produced by other Gram-negative bacteria. Originally it was believed that the sugars heptose and 2-keto-3-deoxyoctonate (KDO) were absent from *P. gingivalis* LPS (Mansheim and Kasper, 1977; Mansheim *et al.*, 1978). It has been recently shown that KDO is present in a phosphorylated form (Fujiwara *et al.*, 1990; Johne *et al.*, 1988; Kumada *et al.*, 1993) and that this phosphorylation may have previously masked its detection.

LPS of *P. gingivalis* can be separated into at least two serological types depending on whether they react with monoclonal antibodies raised against the LPS of *P. gingivalis* strain 381 or 6/26 (Fujiwara *et al.*, 1990). Strains which react to monoclonal antibodies raised against *P. gingivalis* 381 are classified as serogroup I whereas those that react to anti-LPS monoclonals directed at *P. gingivalis* 6/26 are serogroup II (Fujiwara*et al.*, 1990).

In comparison to enterobacterial LPS (i.e. *E. coli, S.typhimurium*), the LPS from *P. gingivalis* has relatively low endotoxicity as determined by Schwartzman reaction in rabbits (Koga *et al.*, 1985). It has been suggested that this may be due to lack of phosphorylation of the lipid A moiety (Johne *et al.*, 1988) which is essential for LPS to exhibit full endotoxic activity. Despite this, LPS from *P. gingivalis* has been demonstrated to have a number of pharmocological properties. It is a stimulator of interleukin-1 (IL-1) production in macrophages (Hanazawa *et al.*, 1985). IL-1 is an important regulator of immunological and inflammatory reactions and is a potent mediator of alveolar bone loss being capable of both inhibiting bone formation (Dinarello, 1989) and stimulating collagenase production by fibroblasts (Postlethwaite *et al.*, 1983). LPS from *P. gingivalis* also stimulates macrophages to secrete tumour necrosis factor (TNF) (Garrison *et al.*, 1988; Hamada *et al.*, 1988; Sismey-Durrant and Hopps, 1991) which is also capable of inducing bone-resorption (Bertolini *et al.*, 1986). These features could contribute to the *in vivo* alveolar bone loss which is a characteristic displayed in patients with chronic inflammatory periodontal disease.

Unlike enterobacterial LPSs, the LPS from *P. gingivalis* has also been shown to be capable of direct inducement of interleukin-8 (IL-8) production from human gingival fibroblast cultures, a known neutrophil chemotactic factor which also causes an increase in the expression of receptors for complement proteins on PMN (Tamura *et al.*, 1992).

It has been demonstrated that *P. gingivalis* LPS is strongly mitogenic for mice spleen cells (Koga *et al.*, 1985) This activity was not inhibited by the addition of polymyxin B (Hamada *et al.*, 1988; Koga *et al.*, 1985) which specifically binds LPS via the lipid A

moiety (Morrison *et al.*, 1976). This would suggest that the mitogenicity may be due to a factor other than LPS or that the lipid A component of *Porphyromonas gingivalis* LPS binds poorly to polymyxin B.

1.4.3 Proteolytic Activities

The association between bacterial plaque and soft tissue destruction in periodontal disease was suggested by Frostell and Soder (1970). Plaque contains a variety of types of proteolytic enzymes and the proteolytic activity increases with increasing amounts of plaque (Soder, 1972).

Porphyromonas gingivalis is strongly proteolytic and is capable of degrading a number of substrates (Hinode *et al.*, 1991). Some of the proteolytic activities displayed by *P. gingivalis* strains will now be discussed.

Porphyromonas gingivalis proteases can degrade iron transport proteins albumin, haemopexin and haptoglobin (Carlsson *et al.*, 1984a) which probably provide a source of haemin. Immunoglobulin M, IgA and IgG, which interact with the complement system are degraded, as well as complement proteins C3, C4, C5 and factor B (Kilian, 1981; Schenkein *et al.*, 1988; Sundqvist *et al.*, 1985). These features may assist in evasion of phagocytic defence but it is most likely that these activities are concerned with the liberation of nutrients and any contribution to virulence may be purely coincidental (Grenier *et al.*, 1989; Kilian, 1981). In addition it is capable of degrading a number of plasma proteinase inhibitors (Carlsson *et al.*, 1984b) which may enhance tissue destruction through host proteases and cause rapid progression of the disease.

SDS-PAGE analysis in the presence of BSA-conjugated polyacrylamide has demonstrated the presence of 8 proteolytic bands (Grenier *et al.*, 1989). Seven of these were present in

vesicle and membrane preparations, three of the seven were also found in the supernatant and one was exclusively extracellular.

Some activities have been studied extensively and will now be discussed.

Collagenase activity

Porphyromonas gingivalis produces collagenolytic activity (Mayrand and Grenier, 1985; van Steenbergen and de Graff, 1986; Sundqvist *et al.*, 1987). This includes: a cell-associated collagenase (Birkedal-Hansen *et al.*, 1988); a trypsin-like protease with collagenase activity (Bedi and Williams, 1994); and, an extracellular thiol-protease exhibiting collagenase activity (Smalley *et al.*, 1988).

This activity is commonly believed to be important in the virulence of the organism by the degradation of collagen, the major constituent of gingival tissue. However, the precise contribution to the virulence of *P. gingivalis* has yet to be conclusively established. Although it was demonstrated that the degree of virulence in guinea pig infections could be correlated to collagenolytic activity (Grenier and Mayrand, 1987a), other workers have been unable to find such differences in the level of activity between invasive and noninvasive strains (Neiders *et al.*, 1989; Birkedahl-Hansen *et al.*, 1988). The collagenolytic activity of cells of *P. gingivalis* strain W50 grown under conditions that increase virulence, i.e. haemin-excess, was one-third of that when grown under haemin-limitation (Marsh *et al.*, 1988). It is probable that this enzyme functions to provide nutrients for the organism.

Kuramitsu and coworkers have cloned and sequenced a gene expressing collagenase activity (*prt*C) from *P. gingivalis* ATCC 53977 which had a molecular weight on SDS-PAGE of 35 kDa. Since it is believed to exist in its native form as a dimer of 70 kDa this may be the same enzyme isolated by Sorsa *et al.* (1987). It is believed to be membrane-associated and a sequence homologous to the gene was present in all strains examined and all three serotypes (Kato *et al.*, 1992; Takahashi *et al.*, 1991).

Trypsin-like activity

The digestive enzyme trypsin acts on lysyl and arginyl bonds of peptide chains. *Porphyromonas gingivalis* can degrade many of the synthetic substrates that are degraded by trypsin. This activity is therefore referred to as trypsin-like activity. Some of this is found in the supernatant of spent culture media (Fujimura and Nakamura, 1987; Hinode *et al.*, 1991; Ono *et al.*, 1987; Sorsa *et al.*, 1987) and some in cell-fractions (Fujimura and Nakamura, 1987; 1990; Nishikata and Yoshimura, 1991; Shah and Gharbia, 1989). In the region of 80% of the extracellular trypsin-like activity in batch cultures is associated with the vesicle fraction of *P. gingivalis* (Smalley *et al.*, 1987). The major secreted protease of *P. gingivalis* is a thiol-activated cysteine proteinase, gingivain (Roberts *et al.*, 1990; Shah *et al.*, 1990). This enzyme is almost always referred to in the literature as a trypsin-like enzyme due to its ability to cleave synthetic trypsin substrates. It has a MW of 43 kDa on SDS-PAGE (Wallace *et al.*, 1992) and may exist as an aggregate of several monomer units (Fujimura *et al.*, 1992). This enzyme possesses haemolytic activity (Shah and Gharbia, 1989; Gharbia *et al.*,1993) which is capable of liberating haeme from erythrocytes and could therefore contribute to fulfilling the organism's haem requirement.

Trypsin-like enzyme activity is found in *P. gingivalis* W50 wildtype cells at three times the level found in mutant derivatives (McKee *et al.*, 1988; Marsh *et al.*, 1989; Shah *et al.*, 1989; Smalley *et al.*, 1989). Furthermore, when grown under haemin limitation *P. gingivalis* strain W50 has low virulence (McKee *et al.*, 1986) and this was associated with a marked decrease in trypsin-like activity but not in other enzymes (Marsh *et al.*, 1994).

A number of possible roles have been suggested for the trypsin-like activity of this organism. A 80 kDa trypsin-like enzyme isolated from the outer membrane vesicles, has been shown to be capable of protecting*Capnocytophaga ochracea* from the bactericidal effects of serum by its degradation of IgG, IgM and complement protein C3 (Grenier 1992). It is probable that this protein is responsible for one of the eight enzymatic

activities described previously (Grenier *et al.*, 1989) and could also be the same as that purified by Yoshimura *et al* (1984). Analysis of culture supernatants of *P. gingivalis* also implicated trypsin-like activity in the degradation of serum proteins, whereby the level of degradation correlated with the level of trypsin-like activity (Fishburn *et al.*, 1991).

There is also evidence to suggest that haemagglutination could be mediated by a trypsin-like enzyme since nitrosoguanidine mutants which are defective in trypsin-like activity are also defective in haemagglutination (Hoover *et al.*, 1992a). In addition, arginine inhibits both haemagglutination and trypsin-like activity of total membrane preparations (Nishikata *et al.*, 1989) therefore it is possible that the two activities may be present on the same protein. It is also possible that this activity is involved in the attachment of *P. gingivalis* to Gram-positive organisms as enhancers and inhibitors of trypsin-like proteases cause an increase and a decrease, respectively in the coaggregation of *P. gingivalis* to *A. viscosus* and mutants defective in trypsin-like activity do not coadhere (Li *et al.*, 1991). The inclusion of trypsin in the assay produced a concentration-dependent inhibition of coadhesion (Ellen *et al.*, 1992).

There may be a role for a trypsin-like enzyme in attachment to host epithelial cells since the binding of *P. gingivalis* to these cells is enhanced by trypsin-like enzymes and papain (Childs, 1990). These enzymes cleave arginine containing peptide bonds, therefore, they could increase the number of arginine residues exposed and increase the adhesion of *P. gingivalis*. Therefore the protease may expose cryptic receptors and thereby allow the attachment of *P. gingivalis*.

A gene (*prt*T) encoding a 53.9 kDa protein expressing trypsin-like activity and gelatinase activity has been recently cloned (Otogoto and Kuramitsu, 1993). The gene product demonstrated strong hydrolytic activity on a synthetic trypsin substrate but has not yet been fully characterised. In contrast to most of the trypsin-like enzymes from *P. gingivalis*, this was not strongly inhibited by sulfhydryl reagents. Park and McBride (1992) have cloned a gene whose product also exhibits trypsin-like activity, from *P. gingivalis* W83. They

found the same 3.2kb *Hin*dIII fragment carrying the sequence in all of the strains they examined with the exception of ATCC 33277 which contained a homologous sequence on a 5.0kb fragment. This sequence was absent in *P. asaccharolytica* and six other black-pigmented Gram-negative anaerobic species examined. In the absence of 2-mercaptoethanol, Western blot analysis revealed the recombinant protein to be 80 kDa. However, antiserum reacted with bands of 50 kDa and 38 kDa when the samples were prepared in the presence of 2-mercaptoethanol. The authors suggested that this was the same as an extracellular and vesicle-associated trypsin-like enzyme described by other authors (Smalley and Birss, 1991). An isogenic mutant derivative deficient in this protease has been produced but the effect of the mutation on virulence has not yet been reported (Park and McBride, 1993).

Neuraminidase

Neuraminidases cleave N-acetyl neuraminic acid from oligosaccharides, glycolipids or glycoproteins. These enzymes can modify the host's ability to respond to bacterial infection. Desialidation of immunoglobulin G decreases its ability to bind complement. Leukocytes and erythrocytes, when incubated with such enzymes, lose their glycoprotein receptors, which, *in vivo*, leads to their removal from circulation. *Porphyromonas gingivalis* produces a neuraminidase (Moncla *et al.*, 1990). Neuraminidase treatment enhances binding of *P. gingivalis* to oral epithelial cells (Childs, 1990) and this enzyme could function to expose cryptic receptors on the host tissue which could then react with the bacterial adhesins.

N-acetyl-hexosaminidase

N-acetyl-hexosaminidase is involved in the degradation of mucopolysaccharides. *Porphyromonas gingivalis* produces an N-acetyl-hexosaminidase and this may be involved in the breakdown of host tissue and therefore may play a role in the pathology of the

disease. The gene encoding this enzyme has been cloned and sequenced from *P. gingivalis* strain W83 and is absent from other members of the genus (Lovatt and Roberts, 1994).

1.4.4 Vesicles

Electron microscopy of *P. gingivalis* broth grown cells show the presence of extracellular 'blebs' or vesicles. These vesicles are a direct outgrowth of the outer membrane and have the capacity to entrap the contents of the periplasmic space, LPS and other outer membrane proteins and may act as a virulence factor. It has been suggested that their small size (approximately 20-150nm) may allow them to cross epithelial barriers which cannot be penetrated by whole cells (Deslauriers *et al.*, 1990; Grenier and Mayrand, 1987b). Vesicles may be regarded as packages of enzymes and may deliver potent hydrolytic enzymes into subgingival sites with the potential to destroy the integrity of the underlying connective tissue. Furthermore, vesicle-bound enzymes may function to release peptides (from host tissue and secretions) to support the nutritional requirements of these species *in vivo*. This can be supported by their *in vitro* effects on human serum. Pretreatment of human serum with these vesicles completely inhibits its bactericidal effect on other oral organisms. Since this effect was time and temperature sensitive, it was implied that this involved a protease(s) (Grenier and Belanger, 1991), and could be achieved through the degradation of immunoglobulins and complement proteins.

Vesicles promote bacterial attachment between homologous cells and can facilitate the attachment to normally non-coaggregating bacterial species such as *A. viscosus* and *A. naeslundii* (Ellen and Grove, 1989; Grenier and Mayrand, 1987b).

LPS is a major constituent of the *P. gingivalis* vesicle and the vesicle may serve as a vehicle for the transport of the LPS into gingival connective tissue which would then be in a position to interact with host immune cells and influence the inflammatory responses that contribute to the destruction of the periodontium (Bramanti *et al.*, 1989). Vesicles also

have the ability to competitively bind antibody directed at intact bacteria that share the same or similar antigenic properties.

The observation that a Tn*4351* insertional mutant of *P. gingivalis* A7436 which expresses increased numbers of vesicles has a higher level of virulence than the parent strain (Genco *et al.*, 1993) might suggest an important role for vesicles in the pathology of periodontal disease.

Alternatively it is possible that vesicle production is a response to nutritional stress and that any virulence efffects are coincidental. This may be supported by the observation that in conditions of haemin-limitation large numbers of vesicles are released whereas, in conditions of excess haemin, few are seen (McKee *et al.*, 1986).

Whether vesicles function as a virulence factor or as the liberator of nutrients, they have the potential of making important contributions to the survival of *P. gingivalis*.

1.4.5 Capsule

The capsular material of *P. gingivalis* can be visualised as an electron dense layer external to the outer membrane of the cell with a diameter of 16-32nm (Haapasalo *et al.*, 1990). The chemical composition of capsular polysaccharide varies among strains. The major components of polysaccharide from *P. gingivalis* strain A7A1-28 include glucosamine, galactosamine and glucose (Schifferle *et al.*, 1989). The capsular material of *P. gingivalis* strain 382 is composed of galactose, glucose and glucosamine (Mansheim and Kasper, 1977), whilst rhamnose, glucose, galactose, mannose, and methylpentose have been found in *P. gingivalis* strain 381 (Okuda *et al.*, 1987).

It has been shown recently that *P. gingivalis* strains can be characterised according to the presence and immunoreactivity of their capsular antigen (van Winkelhoff *et al.*, 1993). A number of strains characterised as either virulent or non-virulent in animal models were

examined. A reactive heat-stable non-somatic antigen, believed to be the capsule (K) antigen, was found only in virulent strain. Four virulent strains analysed represented three distinct serogroups of K antigens.

The capsule is believed to have a role in the virulence of *P. gingivalis*. Invasive strains of *P. gingivalis* have visibly thicker capsules and have been shown to be more resistant to serum killing and have an increased resistance to killing by PMN whereas nonvirulent strains are easily killed (van Steenbergen *et al.*, 1987). It has also been suggested that the invasive capacity of this organism is related to the extent of encapsulation whereby strains with thicker capsules demonstrate a higher level of invasion (Reynolds *et al.*, 1989).

The strain specificity of capsular polysaccharide could probably account for the observation that phagocytosis of virulent strains of *P. gingivalis* requires opsonisation by strain-specific IgG, whereas an avirulent strain could be opsonised and phagocytosed by normal serum (Cutler *et al.*, 1991). This would suggest that C3b accumulation was masked by the capsule or did not occur at the cell wall. The importance of the capsule in disease is also highlighted by the ability to reduce the severity of disease in mice by immunization with capsular polysaccharide conjugated to BSA (Schifferle *et al* ., 1992).

Other biological properties have been suggested for the capsular material. It has been suggested that this structure is directly involved with bone resorption (Wilson *et al.*, 1993) and that this can be inhibited by anti-capsule antisera from periodontitis patients (Meghji *et al.*, 1993).

A Tn*4351* mutant derivative of *P. gingivalis* A7436 was isolated and found to have a much thicker capsule than the parent but the effect on virulence was not reported (Genco *et al.*, 1993).

A more detailed analysis of capsules as virulence factors will be discussed in Section 1.5.

1.5 Bacterial Capsules: General Features

The majority of bacteria isolated from natural habitats produce EPS (Costerton *et al.*, 1981). EPS often constitutes the outermost layer of the cell exposed to the environment, therefore its properties affect the interactions of the bacterium. EPS affects dehydration, ionic interaction, adhesion and colonisation of inert surfaces, infection by bacteriophage and virulence of the bacterium (Costerton *et al.*, 1981). Upwards of 95% of EPS is reported to be water (Sutherland, 1972). Bacterial polysaccharides are linear macromolecules composed of repeating mono- or oligosaccharides which may be substituted with short side chains. The exact number of repeats in any one molecule is variable. Monosaccharides represented include neutral sugars, polyols, uronic acids and amino sugars. In addition, non-sugar substitutions including phosphate, formate, succinate, pyruvate and acetyl groups are common although sulphation has not been observed (Dudman, 1977; Sutherland, 1972, 1977). These macromolecules can exhibit greater diversity by varying not only the components but also the linkage. Consequently, a wide range of structurally different bacterial polysaccharides have been identified (Kenne and Lindberg, 1983). EPS can exist as an amorphous slime layer around the producing cell and those nearby, or may form a complete capsule around each individual cell (Ørskov *et al.*, 1977). It is not entirely clear how the capsule is maintained on the cell surface but in Gram-negative organisms it appears that capsular polysaccharide is attached either to phospholipid presumed to reside in the outer membrane (Gotschlich *et al.*, 1981; Kuo *et al.*, 1985) and in others, to core lipid A (Jann and Jann, 1990).

1.5.1. Capsules as Virulence Factors

Bacterial encapsulation has been recognised as a virulence determinant in pathogenic bacteria. Encapsulated bacteria are responsible for some of the most serious invasive infections to which man is susceptible. It has been known since the first half of this

century that encapsulated organisms are more virulent than their unencapsulated variants. Enzymatic removal of capsular polysaccharide from *Streptococcus pneumoniae* caused the lethal inoculum to rise by a factor of 10^6 (Avery and Dubose, 1931). It is believed that the mechanism for enhanced virulence is related to the ability of encapsulated organisms to evade normal host defence mechanisms.

1.5.1.1 Interactions of capsules with complement

Certain immunoglobulins and complement proteins can act as opsonins which modify the surface of capsulate bacteria, but the cleavage product of the third complement component, C3b, is of particular importance (Winkelstein, 1981).

Upon exposure to serum, bacteria may cause the generation of C3b through the activation of either the alternative (AP) or the classical complement pathway (CP). In contrast to the CP, activation of the AP does not depend upon antibodies to recognise specific molecules on the target cell-surface, instead it relies on molecular structures on the target cell to upset the delicate balance of the proteins involved so that their activation and deposition are focused on its surface. Activated AP enzymes assemble themselves on the target membrane and cleave a C3b fragment from C3. The small amounts of C3b that are deposited on the bacterial surface may be amplified by the formation of a complex between C3b and factor B of the AP to give C3bBb, which acts as a C3 convertase. C3bBb fixes more C3b to the activator surface so that more B binding sites are exposed and the feedback continues. C3b accumulation on the surface of Gram-negative organisms can lead to the generation of the membrane attack complex (MAC) which displaces lipid molecules thus disrupting the phospholipid bilayer of target cells leading to osmotic cell lysis. The central controlling element is factor H which competes for surface bound C3b with factor B thereby blocking the formation of C3bBb (Fearon, 1978). The binding of H

to C3b facilitates termination of the C3b amplification loop and limits the deposition of this important opsonin on the bacterial surface.

There are other potentially significant immune functions which result from the formation of C3b. If lysis of the micro-organism does not occur the bacteria will nevertheless become heavily coated with C3b which can act as a ligand for specific receptors on PMN or macrophages. This will promote C3b-mediated opsinophagocytosis of the opsinised bacteria. Capsules may interfere with MAC formation by steric mechanisms. Some pneumococci bind C3b on the cell surface under the capsule such as the capsule acts as a mechanical barrier to recognition of C3b by the phagocytic cells (Winkelstein, 1981; Brown *et al.*, 1983).

Certain bacterial capsules can take advantage of the competition between factor B and factor H. The *Escherichia coli* K1 capsule and type III group B streptococci contain N-acetyl neuraminic acid which increase the affinity of factor H for cell bound C3b resulting in the breakdown of the amplification loop (Edwards *et al.*, 1982; Stevens *et al.*, 1978). Other capsules, for example, type 7 and 12 pneumococci have a decreased affinity for factor B resulting in a relative increase in binding of factor H (Brown *et al.*, 1983).

1.5.1.2 Interactions of capsules with phagocytes

The PMN, whose function is to ingest and dispose of unwanted cell debris, immune aggregates, bacteria and other micro-organisms, is an important part of the host's defence. The outcome of the interaction between PMN and microbe often determining whether dissemination of the infection and perhaps host death will occur.

Phagocytes can only fulfil their function if they can recognise and distinguish micro-organisms from host cells. The host defence therefore labels the targets with antibody and

complement fragments and the phagocyte recognises these labels by the specific receptors on their membrane surface.

Most capsular polysaccharides are hydrophilic and confer a negative charge on the bacterial cell, characteristics which are intrinsically antiphagocytic in their effect by reducing the surface tension at the interface between the phagocyte and the bacterium (Van Oss and Gillman, 1973). This impairs the uptake and subsequent killing by polymorphonuclear leukocytes (Horwitz and Silverstein, 1980).

1.5.1.3 Interactions of capsules with the cellular immune system

Bacterial capsules may affect the response of lymphocytes or macrophages by directly mimicing the activity of a cytokine. The capsule of the periodontal pathogen *Actinobacillus actinomycetemcomitans* has been shown to have many biological activities of IL-1 such as, bone-resorbing activity, stimulation of collagenase and prostaglandin E_2 activity. These are not inhibited by polymyxin B and cannot therefore be attributed to LPS (Harvey *et al.*, 1987). These features have been supposed to be important in the pathogenesis of the disease caused by this organism.

1.6 Genetic Approaches to the study of *P. gingivalis* Virulence Factors

To date, the majority of research using molecular biology to dissect the contribution of individual factors to the virulence of *P. gingivalis* have relied on cloning and expression of putative virulence determinant genes in *E. coli* (Progulske-Fox *et al.*, 1989a; Roberts *et al.*, 1990). Genetic approaches have been severely limited by the inability to produce isogenic mutant derivatives of *P. gingivalis* either through transposon mutagenesis or, by the

reintroduction of cloned DNA on plasmid vectors. This has been due to the lack of any suitable system for introducing foreign DNA into this organism.

A number of cryptic plasmids have been found in *P. asaccharolytica* and other black-pigmented Gram-negative anaerobes, but despite intense searches no plasmids or bacteriophages have been found in *P. gingivalis* (Hohne *et al.*, 1993; Yoshimoto and Umemoto, 1990). Construction of vectors based on these plasmids has only been initiated fairly recently (Hoover *et al.*, 1992b). Therefore, there are presently no vectors available specifically for use in this group of organisms.

A system of plasmid shuttle vectors and transposon suicide vectors has been developed for use in colonic *Bacteroides* species (Shoemaker *et al.*, 1986; 1989; 1991). Such organisms are taxonomically closely related to *P. gingivalis* in comparison to *E. coli* (Woese, 1987). The vectors have been constructed from naturally occurring *Bacteroides* cryptic plasmids and antibiotic resistance transposons and *E. coli* plasmids (Shoemaker *et al.*, 1986; 1989; 1991). As a result a number of groups have attempted to exploit such vectors for use in *Porphyromonas* species.

Numerous plasmids have been identified in a number of *Bacteroides* species, some of which are cryptic and widely distributed among these organisms (Callihan *et al.*, 1983; Mary *et al.*, 1986; Nagy *et al.*, 1990). Plasmid encoded resistance to a variety of antibiotics is one of two distinct mechanisms of antibiotic transfer which exist within this genus with the other being through the transfer of mobilizable chromosomal resistance elements. Plasmid associated transfer of clindamycin/erythromycin resistance has been studied in detail for three plasmids, pBF4 (41kb), pBFTM10 (14.6kb), and pBI136 (81kb). The plasmids are unrelated except for the clindamycin/erythromycin resistance determinant (*erm*F) which is highly homologous among them (Guiney *et al.*, 1984; Smith and Gonda, 1985) and belongs to a family of *erm* genes widely distributed among Gram-positive bacteria (Rasmussen *et al.*, 1986). The clindamycin/erythromycin resistance gene is flanked by homologous 1.2kb direct repeats which represent transposons in these plasmids:

Tn*4351* (pBF4); Tn*4400* (pBFTM10); and Tn*4551* (pBI136). In addition to clindamycin/erythromycin resistance, Tn*4351* and Tn*4400* also encode a tetracycline resistance gene which functions only in *E. coli* grown in an aerobic environment (Guiney *et al.*, 1984; Robillard *et al.*, 1985).

The majority of clindamycin/erythromycin resistance strains do not have the *erm*F gene on a plasmid but have transposons located on their chromosomes encoding for clindamycin/erythromycin resistance (Callihan *et al.*, 1984; Marsh *et al.*, 1983). These transposons are conjugative and can transfer clindamycin/erythromycin resistance via conjugation in the absence of plasmid DNA (Macrina *et al.*, 1981; Mays *et al.*, 1982). In addition, transferable tetracycline resistance is common in *Bacteroides*. Although a tetracycline resistance plasmid has been identified in related ruminal strains of *B. ruminicola* (Flint *et al.*, 1988), in most instances no plasmid has been identified. The transfer of tetracycline resistance is associated with Tn*4399*, which is another conjugative transposon and is also capable of mobilising nonconjugal plasmids into recipient strains (Hecht and Malamy, 1989).

A large number of shuttle-vectors have now been constructed for use in this group of organisms (Pheulpin *et al.*, 1988; Shoemaker *et al.*, 1989). The origin of replication for *Bacteroides* in the shuttle-vectors is usually provided by small cryptic *Bacteroides* plasmids, some of which also contain a mobilisation region recognised by IncP plasmids, such as R751. The antibiotic resistance marker is derived from *Bacteroides* resistance genes, which, if derived from pBF4 or pBFTM10, also provide a Tc^r marker for *E.coli*. The *E. coli* replication origin and a transfer origin are provided by *E. coli* plasmids. Such vectors are transferred to *Bacteroides* recipients by conjugal transfer from *E. coli*.

The first successful report of the introduction of foreign DNA into *P. gingivalis* was by Progulske-Fox *et al* (1989b). In these experiments *E. coli* was used as the donor in conjugation experiments which resulted in the transfer of plasmid pE5-2 into *P. gingivalis* strains I372 and 381, as well as *Pr. intermedia*. Recently, this vector has been introduced

into *P. gingivalis* strains by electroporation but only when the plasmid DNA used was purified from homologous strains (Yoshimoto *et al.*, 1993). Another *Bacteroides* vector, pVal1 was used to demonstrate that it was possible to achieve conjugal transfer of this plasmid into a variety of *P. gingivalis* strains (Dyer *et al.*, 1992). A number of insertion replacement isogenic mutants have recently been produced by reintroducing cloned DNA into *P. gingivalis* using the broad host range RSF1010-based cosmid, pJRD215, which can be mobilised at high frequencies by IncP plasmids (Joe *et al.*, 1994; Park and McBride, 1993; Lepine and Progulske-Fox, 1993).

Transposon mutagenesis

The transposon Tn*4351* was originally identified as part of the *Bacteroides* plasmid pBF4. The IncP plasmid R751 does not replicate in *Bacteroides* species and can therefore be used as a suicide-vector (Shoemaker *et al.*, 1986). Transposon Tn*4351* was cloned into R751 to create R751::Tn*4351*Ω4 (Shoemaker *et al.*, 1986) which has been used to create transposon mutants defective in starch and mucopolysaccharide utilisation in *B. thetaiotaomicron* (Anderson and Salyers, 1989; Salyers *et al.*, 1988).

Plasmid R751::Tn*4351*Ω4, has also been used in *P. gingivalis*. It has been demonstrated that Tn*4351* insertion into the chromosome of *P. gingivalis* is random and often only one insertional event occurs (Dyer *et al.*, 1992; Hoover *et al.*, 1992c).

1.7 Aims of Investigation

The final objective of this study was to produce acapsular transposon mutants of *P. gingivalis*W83 and to examine the effects of such mutations on virulence and identify the gene(s) involved in capsule production. In order to produce such mutants it was necessary to develop a transfer system to provide a means of introducing foreign DNA into this organism. Once this had been achieved, suicide-vectors carrying transposons would be

used to generate transposon mutants. To identify mutants, which were acapsular or defective in capsule expression required the generation of specific anti-capsular antibodies. This meant that an efficient method for recovery of highly purified capsular material from the organism was required.

Chapter 2

Materials and Methods

2.1 Bacterial strains and plasmids

The bacterial strains and plasmids which were used in this study are listed in Tables 2.1.a, b.

2. 1. 1 Growth conditions and Media

All anaerobes were maintained on blood agar base number 2 (OXOID) supplemented with 7% (v/v) horse blood. Broth grown cells were cultured in *Bacteroides* medium (Shah *et al.*, 1976) containing haemin (50μg/ml) and menadione (5μg/ml). Cultures were incubated at 37°C in an anaerobic chamber with an atmosphere of N_2: H_2: CO_2 (80: 10: 10). Unless otherwise stated, *E. coli* strains were grown in Luria broth (L-broth) at 37°C, in an aerobic environment, with the addition of 1.5% agar as required. B-agar (0.1% peptone, 0.8% NaCl, 1.5% agar) was used where stated. Antibiotics were added to the growth medium where necessary at the following concentrations: ampicillin at 100μg/ml, trimethoprim at 100-200μg/ml, clindamycin at 0.5μg/ml, tetracycline at 10μg/ml, kanamycin at 50μg/ml, and gentamicin at 50-200μg/ml. Antibiotics were obtained from Sigma chemical company Ltd.

Bacterial strains were routinely harvested by centrifugation (2800g) at 4°C for 10 mins.

Table 2. 1. a Bacterial Strains

Strain	Characteristics	Reference or source
E. coli		
DH5α	F^-, *Rec*A, *hsd*R17, *lac*$^-$	Sambrook *et al.*, 1989
JM101	*sup*E *thi* (*lac-pro*AB) F [*traD*36*pro*AB$^+$]*lac*^{q} *lacZ* M15	Sambrook *et al.*, 1989
SF8	*Rec*B *Rec*C Sm^r	N. B. Shoemaker
HB101	F^-, *hsd20*, (r^-_B,m^-_B), *Rec*A13, *ara-14*, *proA2*, *lacY1*, *galK2*, *rpsL20* (Sm^r), *xyl-5*, *mtl-1*, *supE44*, λ^-	Sambrook *et al.*, 1989
DS410	*min*A, *min*B, *ara*, *xyl*, *mtl*, *azi*, *thi*,	Reeve (1977)
P. gingivalis		
11834	clinical specimen	H. N. Shah
HG1241	clinical specimen	A. J. van Winkelhoff
W50	clinical specimen	H. N. Shah
W83	clinical specimen, Gen^r, Cc^s, Em^s	H. N. Shah
WpH35	clinical specimen	H. N. Shah
P. endodontalis HG370	clinical specimen	A. J. van Winkelhoff
P. asaccharolytica NCTC 9337		NCTC
B. uniformis 1001	spontaneous Rif^r mutant of *B. uniformis* 0061	Shoemaker *et al.*, 1985

Table 2. 1. b Plasmids

Plasmid	Characteristics	Reference or source
pACYC184	Cm^r, Tc^r, cloning vector	Chang and Cohen, 1978
pBR328	Ap^r, Tc^r, cloning vector	Bolivar *et al.*, 1977
pGB110	carries the K5 capsule gene cluster	Roberts *et al.*, 1986
pJM1	pUC18 with the 1.3kb *EcoRI/SstI* fragment from pNJR12-1	This study
pNJR5	*E. coli-Bacteroides* shuttle-vector Kn^r Rep^+ Mob^+ (Em^r Rep^+ Mob^-)	Shoemaker *et al.*, 1989
pNJR12	*E. coli-Bacteroides* shuttle-vector Kn^r Rep^+ Mob^+ (Tc^r Rep^+ Mob^-)	Stevens *et al.*, 1992
pNJR12-1	pNJR12 containing IS*1126*	This study
pUC18	cloning vector	Yanisch-Perron*et al.*, 1985
R751	IncP Tp^r Tc^r $traA^+B^+$mobilises vectors from *E. coli* to *Bacteroides*	Shoemaker *et al.*, 1985
R751::Tn4351Ω4	R751 with a tandem partial duplication of Tn*4351*	Shoemaker *et al.*, 1985
M13mp18/19	bacteriophage cloning/sequencing vector	Yanisch-Perron *et al.*,1985

Mob^+ and Mob^-, can and cannot be mobilised respectively; Rep^+ and Rep^-, can and cannot replicate respectively. Plasmid phenotypes within parentheses are expressed in *Bacteroides* species, and those outside are expressed in *E. coli*.

2.2 Transformation of *E. coli*

2. 2. 1 Production of competent cells

Competent cells were prepared essentially as described by Sambrook *et al* (1989). L-broth (10ml) was inoculated with 100µl of an overnight culture and grown to mid-log phase (OD_{600} 0.5). The cells were harvested, washed in 10mM NaCl, and resuspended in 4ml of ice-cold $CaCl_2$ (100mM). The cells were placed on ice for 30 mins and collected by gentle centrifugation (1800g) at 4°C for 5 mins. The cell pellet was resuspended in 1ml ice-cold $CaCl_2$ and transformed immediately.

2. 2. 2 Transformation with Plasmid DNA

Competent cells (100µl) and up to 25µl of DNA (in water) to be transformed were mixed and placed on ice for 1h. The cells were heat-shocked at 42°C for 3 mins. L-broth (500µl) was added to the cells which were then incubated for 1h at 37°C and plated onto L-agar plates (100µl per plate) which contained the appropriate antibiotic(s). The plates were then incubated overnight at 37°C.

2. 2. 3 Transformation with Bacteriophage DNA

Competent *E. coli* JM101 cells were incubated on ice for 1h with the construct DNA and heat-shocked at 42°C for 3-5 mins and then reincubated on ice for 1h. The transformed cells together with 200µl mid-log phase JM101 were added to 2.5ml molten soft top B-agar (held at 42°C) containing 10µl IPTG (100mM), and 20µl X-gal (2% in dimethyl formamide).The suspension was mixed and poured immediately onto a B-agar plate, rocked to disperse, left to set for 15 mins and then incubated overnight at 37°C.

2.3 Conjugal Transfer of Plasmid DNA from *E. coli* to *P. gingivalis* W83

A number of published methods previously used for *P. gingivalis* and colonic *Bacteroides* were originally used to try to introduce plasmid DNA into *P gingivalis* W83.

2.3.1 Progulske-Fox *et al* (1989b)

For each mating both the donor and recipient cells were grown to early log phase ($1X10^8$-$2x10^8$ cfu/ml). 0.5ml of *E. coli* donor culture was pelleted by centrifugation in a microfuge for 2 mins. 1ml of an overnight *P. gingivalis* W83 culture was added to the *E. coli* cell pellet and the centrifugation was repeated. The pellet was then resuspended in 200µl Todd Hewitt broth (THB) and the entire suspension spotted on blood agar plates. Controls of donor and recipient cells were treated similarly. All plates were then incubated at 37°C for 24h anaerobically. Bacterial cells were then recovered from the plates by swabbing the surface of the plates with a sterile cotton swab and resuspended in 3ml of THB. After vortexing the tube, 0.5 to 1ml of the cell suspension was streaked onto the appropriate selection plates. However, since *P. gingivalis* strain W83 does not grow as quickly as the strains used in their study, the plates were incubated in an anaerobic chamber for 7 days rather than the 4 days described in the original method.

2. 3. 2 Shoemaker *et al* (1986)

Donors and recipients were grown separately to optical density at 650nm of about 0.2 ($1x10^8$-$2x10^8$ cfu/ml). Donors and recipients were mixed at ratios between 0.1 and 1.0 (donor to recipient), pelleted by centrifugation, suspended in 0.1ml of THB and spotted on a nitrocellulose filter that had been placed on a blood agar plate. The plates were incubated aerobically at 37°C for a variety of time lengths. The bacteria were collected from the filters as described above, resuspended and plated on selective medium. Plates were then incubated for 7 days in an anaerobic chamber.

2.3.3 Shoemaker *et al* (1985)

Donors and recipients were grown to early log phase ($1x10^8$ to $2x10^8$ cfu/ml). One ml of each was mixed and collected by centrifugation. The pellet was resuspended in 100µl of BM and spotted on nitrocellulose filters (Millipore HAWG) on blood agar plates. The filters were incubated at 37°C for 10-12h in GasPak jars. After incubation, the cells were resuspended in BM by vortexing and plated on selective medium and incubated anaerobically for 1 week at 37°C.

2.4 Procedures for DNA Extraction

DNA extraction used the following solutions:

Solution I (ice-cold)
50mM glucose
25mM Tris-HCl pH8.0
10mM EDTA

Solution II
0.2M NaOH
1% SDS

Solution III (ice-cold)
5M acetic acid
3M potassium acetate

2. 4. 1. Extraction of chromosomal DNA

The method used was based on that of Saito and Miura (1963). Bacterial cells from 10ml stationary phase cultures were resuspended in 10mls of solution I and placed on ice for 30 mins. SDS was added to a final concentration of 1% and EDTA to 50mM. After gentle mixing, the preparation was left at room temperature until the solution was clear. An equal volume of phenol:chloroform (Section 2.4.5) was added and thoroughly mixed by gentle inversion. Phenol:chloroform extractions were repeated until there was no protein remaining at the interface. Chromosomal DNA was retrieved from the aqueous phase by gently

pouring 2 volumes of ethanol (chilled to -20°C) down the side of the tube. Precipitated DNA was spooled out using the rounded end of a Pasteur pipette, resuspended in distilled water and stored at -20°C.

2. 4. 2. Small Scale Extraction of Plasmid DNA

Stationary phase bacterial cultures (1.5ml) were used. Cells were resuspended in 100µl of solution I and placed on ice for 10 mins. 200µl of solution II was added and the tube was carefully mixed and placed on ice for a further 5 mins. Solution III (150µl) was added, mixed and placed on ice for 5 mins. The preparation was centrifuged in a bench top microfuge for 5 mins (13000g) and the supernatant retained. Protein was removed by phenol extraction. Two volumes of ethanol were added and DNA precipitated at room temperature for 10 mins. The pellet was obtained by centrifugation at 13000g for 5 mins and then resuspended in 25µl of distilled water.

2. 4. 3. Large Scale Extraction of Plasmid DNA

Stationary phase bacterial cultures (400ml) were used according to the method of Birnboim and Doly (1979). The cell pellet was resuspended in 10ml of solution I and placed on ice for 30 mins. 20ml of solution II was added, mixed and then left on ice for 10 mins. 15ml of solution III was added and after incubation on ice for 10 mins the preparation was centrifuged at 4°C at 28500g for 20 mins to remove cell debris. DNA was precipitated by the addition of 0.6 volumes of isopropyl alcohol and allowed to stand at room temperature for 15 mins. The precipitate was collected by centrifugation for 30 mins at 20°C (6000g). The DNA pellet was dried and resuspended in sterile distilled water to give a final volume of 17ml. Caesium chloride and ethidium bromide were added to a final concentration of 1g/ml and 50µg/ml respectively. Chromosomal and plasmid DNA were separated at 40000rpm using a Sorvall TV850 rotor in a Sorvall OTD60 ultracentrifuge for 18h at 20°C. DNA was visualised under UV light and the lower band of plasmid DNA collected. Ethidium bromide was removed by the addition of an equal volume of caesium chloride saturated isopropanol.

The contents were then mixed and the two phases allowed to separate at room temperature. The upper phase containing ethidium bromide was discarded and the lower phase retreated twice with caesium chloride saturated isopropanol or for as many times as required to remove any remaining visible ethidium bromide. Caesium chloride was removed by exhaustive dialysis against distilled water at 4°C. The dialysate was stored at -20°C.

2. 4. 4. Extraction of M13mp18/mp19 Template DNA

White plaques obtained after transformation with construct DNA (Section 2. 2. 3) were picked into 2ml of L-broth containing 50µl of an overnight JM101 culture and incubated at 37°C with vigorous agitation for 4-4$^1/_2$h. 1.5ml of culture was centrifuged in a bench top microfuge for 2 mins. The cell pellet was used to extract replicative form DNA to confirm the identity of the insert (using the small scale plasmid extraction method) whilst the supernatant containing phage particles was used to isolate single-stranded, template DNA. The supernatant (800µl) was mixed with 200µl of freshly prepared 2.5M NaCl, 20% PEG 6000 and incubated for 30 mins at room temperature. The phage were collected by centrifugation in a microfuge for 5 mins, the pellet was resuspended in 100µl of 1.1M Sodium acetate pH7.0. After extractions with phenol:chloroform, then chloroform:isoamyl alcohol (50:1, v:v), the DNA was precipitated with ethanol -20°C, collected by centrifugation and resuspended in 10µl of sterile distilled water. 7µl were used in a sequencing reaction (Section 2. 9).

2. 4. 5. Phenol Extraction and Ethanol Precipitation of DNA

One volume of phenol:chloroform (1:1, w/v) equilibrated in Tris-HCl pH7.5 was added to the DNA sample and mixed carefully. The aqueous phase was separated at 20°C by centrifuging for 20 mins at 2730g or in a benchtop microfuge for 5 mins. One volume of chloroform was added to the aqueous phase and the phases again separated by centrifugation. The DNA was then precipitated by the addition of $^1/_{10}$th the volume of 3M Sodium acetate and 2 volumes of absolute ethanol. The sample was placed at -20°C for a

minimum of 30 mins and the DNA collected by centrifugation at 13000g or for 5 mins in a benchtop microfuge.

2. 5. Techniques used in routine DNA manipulation

Restriction endonucleases and DNA modifying enzymes were purchased from Pharmacia Biochemicals Inc or Life Technologies Ltd (GIBCO/BRL).

Restriction endonuclease cleavage of DNA was performed according to the manufacturer's recommendations typically in 20µl reactions with one unit of enzyme at 37°C. T4 DNA ligase was used at 14°C overnight.

DNA fragments were separated by agarose gel electrophoresis using 0.7-1.0% Seakem agarose in TAE buffer (40mM Tris-acetate, 1mM EDTA). The electrophoresis was conducted in TAE containing 0.5µg/ml ethidium bromide with 1-5V/cm between the electrodes. Prior to loading, the samples were mixed with the appropriate volume of 6x loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol, 15% w/v ficoll). 1 kb ladder (GIBCO/BRL) was used as the size markers. DNA was visualised using a long wave UV transilluminator and gels photographed using Polaroid 667 film.

2. 6. Transfer of DNA to Nylon Filters by Southern Blotting

DNA was transferred to filters using the method described by Southern (1975). After separating DNA samples by agarose gel electrophoresis, the gel was photographed along side a linear rule. The DNA was de-purinated by soaking the gel in 0.25M HCl for 7 mins. After briefly rinsing in distilled water, the gel was placed in denaturing solution (0.5M NaOH, 1.5M NaCl) for 30 mins with occasional shaking. The gel was again rinsed in

distilled water and submerged in neutralising solution (0.5M Tris-HCl pH7.5, 3M NaCl) with occasional shaking for another 30 mins. The gel was then placed on 10 sheets of Whatman paper (3MM), presoaked with 20xSSC (1XSSC: 0.15M sodium chloride, 0.015M trisodium citrate, pH7.0), without trapping any air bubbles. A pre-wetted (6xSSC) sheet of nylon membrane (Hybond-N, Amersham International PLC) was placed on the gel with a pre-wetted sheet (6xSSC) of Whatman paper on top, again taking care to avoid air bubbles. Four sheets of dry Whatman paper were placed on top. A stack of dry paper towels were placed above this. The whole assembly was weighted down with a glass plate with a 0.5kg weight on top. The lower sheets of Whatman were regularly soaked with 20xSSC and the paper towels on top regularly changed for the initial 2 hours. The apparatus was then left overnight. After dismantling, the filter was rinsed in 3xSSC, airdried and exposed to UV light from a longwave transilluminator for 5 mins to fix the DNA to the filter. Filters were stored in the dark at room temperature until required for DNA hybridisation (Section 2. 8).

2. 7. Preparation of Filters for Colony Hybridisation

Bacteria were grown at 37°C for the appropriate length of time on a nylon filter (Hybond-N) which had been placed on the surface of an agar plate containing the appropriate antibiotics. The filter was removed from the plate and placed on Whatman paper (3MM), which had been soaked in denaturing solution, for 5 mins. The filter was then transferred to Whatman paper soaked in neutralising solution, for a further 5 mins. After airdrying, the DNA was fixed to the filter (Section 2.6). Cell debris was removed from the surface of the filters by gentle scrubbing in 5xSSC using polymer wool. The filters were then airdried in preparation for DNA hybridisation (Section 2. 8).

2. 8. DNA Hybridisation Procedures

2. 8. 1. Production of a Radiolabelled DNA Probe

Plasmid DNA was cleaved with the appropriate restriction endonucleases and the fragments separated by agarose gel electrophoresis on a 1% low melting point agarose gel (BRL). The required DNA fragment was excised from the gel and added to sterile water (1.5ml water per gram agarose). The sample was placed in a boiling waterbath for 7 mins and then stored at -20°C. Prior to use the sample was placed in a boiling waterbath for 5 mins. DNA was radiolabelled using random hexanucleotide primers as described by Feinberg and Vogelstein (1983). Nucleotides and hexanucleotides were obtained from Pharmacia and [α-^{32}P] dCTP from Amersham International PLC.

2. 8. 2. Hybridisation of DNA immobilised on filters with the probe

Filters were incubated at 65°C in 20ml of prehybridisation solution (see below) for 2h in a Hybaid hybridisation oven. This solution was then discarded and replaced by an equal volume of hybridisation solution (see below) containing the probe. The filter was incubated overnight at 65°C.

prehybridisation solution	hybridisation solution
3xSSC	3xSSC
2xDenhardts	5xDenhardts
200µg/ml salmon sperm DNA	200µg/ml salmon sperm DNA
0.1% SDS	0.1% SDS
6% PEG 6000	6% PEG 6000

Both solutions were stored at -20°C without the salmon DNA. Salmon DNA was sheared by forcing it through a narrow gauge syringe needle and denatured by boiling prior to use.

50xDenhardts
1% ficoll
1% BSA
1% polyvinylpyrrolidone

After hybridisation, the filters were washed four times for 15 mins at 65°C in 200ml 0.1%SDS containing 1XSSC. To permit identification of as little as 40% homology, hybridisations were conducted in solution containing 30% formamide at 42°C. Washes in such experiments used 0.1%SDS and 6xSSC and were also conducted at 42°C. The filters were wrapped wet if reuse was plannned or dried briefly if not.

The filters were wrapped in Saran wrap for autoradiography and placed in a cassette carrying intensifying screens. Kodak X-Omat AR film was exposed to the filters at -70°C. Films were developed in an Agfa-Geveart automatic film processing machine.

2. 8. 3 Removal of bound probe from a filter

The probe was stripped from some filters following autoradiography and the filter was re-used with another probe. The probe was removed according to the manufacturer's instructions: the filters were shaken in 0.4M NaOH at 45°C for 30 mins and then transferred to prewarmed 0.1xSSC, 0.1%SDS, 0.2M Tris-HCl pH7.5 and shaken at 45°C for a further 30 mins. Successful removal of the probe was confirmed by reduction of deflection of a Geiger counter to background levels.

2. 9 DNA Sequencing

DNA was sequenced by the chain termination method described by Sanger *et al* (1977) in which DNA synthesis from deoxynucleotide triphosphates is terminated by the addition of dideoxynucleotide triphosphates. The M13 cloning vectors, M13mp18 and M13mp19 were used to generate single stranded DNA templates (Section 2. 4. 4).

Sequence reactions were performed using the Sequenase version 2.0 kit produced by United States Biochemical Corporation. The protocol recommended by the manufacturer was followed using the universal primer (-40) or oligonucleotide primers. DNA fragments were radiolabelled by incorporating [α-^{35}S]dATP in the extension reactions. The radiolabelled DNA fragments were separated by gradient gel electrophoresis (Biggin *et al.*, 1983). Preparation of the gel used the following solutions:

gel solution 1
7ml 5xTBE acrylamide
45µl 10% ammonium persulphate
2.5µl TEMED

gel solution 2
40ml 0.5xTBE acrylamide
180µl 10% ammonium persulphate
7.5µl TEMED

5xTBE acrylamide(per litre)
430g Urea
150ml 10xTBE
150ml 40% acrylamide
50g sucrose
50mg bromophenol blue

0.5xTBE acrylamide(per litre)
430g Urea
50ml 10xTBE
150ml 40% acrylamide

40% acrylamide(per litre)
380g acrylamide
20g bisacrylamide
deionised with 50g amberlite

10xTBE
0.089M Tris
0.089M Boric acid
0.002M EDTA

Electrophoresis grade ammonium persulphate was purchased from Sigma Chemical company Ltd, acrylamide and bisacrylamide (NN'-methylene bisacrylamide) from BDH.

To prepare the gel, clean plates (20cmx50cm) were taped together separated by 0.4mm spacers. 10ml of gel solution 1 followed by 15ml of gel solution 2 were drawn up into a 25ml pipette. Four air-bubbles were introduced to form a rough gradient. The liquid was run between the gel plates and the cavity filled with the remainder of gel solution 2. The comb was positioned and the plates clamped along each side. Gels were routinely left to set overnight.

A vertical electrophoresis system was used. The top tank contained 0.5xTBE running buffer and the lower contained 1xTBE. Prior to loading, the gel wells were rinsed with running buffer. Electrophoresis was conducted at 40W for $2^1/_2$h to visualise the smallest DNA fragments and for increasingly longer periods of 4h, $6^1/_2$h or 8h to separate larger fragments.

Following electrophoresis the gel was soaked in fixing solution (10% acetic acid) for 10 mins. It was transferred to pre-wetted filter paper, covered with Saran wrap and dried under vacuum at 80°C for $1^1/_2$h. Autoradiography used Dupont Cronex film and took place at room temperature.

Nucleotide sequences were analysed using the University of Wisconsin Genetics Computer Group sequence analysis software (Devereux *et al.*, 1984) on the Leicester University IRIX service and on the Daresbury Laboratory system. The program FASTA was used for sequence comparisons (Pearson and Lipman, 1988).

2. 10. Minicells

Anucleate cells of *E. coli* DS410 were prepared essentially as described by Reeve (1977).

The solutions used were as follows:

M9 salts (10x) (per litre)
60g Na_2HPO_4
30g KH_2PO_4
5g NaCl
10g NH_4Cl

Sucrose gradient (20ml)
4g sucrose
1xM9 to 20ml
Frozen at -20°C. Thawed at 4°C
overnight before use

D-cycloserine
11mg D-cycloserine
5ml of deionised water
Filter sterilised

Methionine assay medium (MAM)
525mg MAM (Difco Labs., Michigan, USA)
5ml distilled water
boiled for 5 mins

Glucose in M9 salts (GM)
16mg glucose
4ml 1xM9

Glucose and MAM in M9 salts (GMM)
60µl MAM
16mg glucose
400µl 10xM9
3540µl distilled water

Methionine
10mg methionine in 5ml distilled water. Filter sterilised

Plasmids pUC18 and pJM1 were transformed into the *E. coli* minicell strain, DS410. Cells harbouring each plasmid type were grown in brain heart infusion broth (OXOID) (800ml) and harvested by gentle centrifugation (443-997g) for 5 mins at 4°C. The supernatant was removed and recentrifuged at 7089g for 15 mins at 4°C. The pellet was resuspended in 3ml of ice-cold 1xM9 salts and loaded on a sucrose gradient before centrifugation at 4260g for 20 mins at 4°C. The top two-thirds of the diffuse cell-layer formed was collected and the cells pelleted by centrifugation at 7100g for 10 mins at 4°C. The pellet was again resuspended in 3ml of 1xM9 salts and centrifuged through a second sucrose gradient. The cells were removed and harvested as before, and again resuspended in 1xM9 salts. The absorbance of the cells at λ_{650nm} was adjusted to 2.0 OD units. Cells in 50µl of the suspension were collected in a microfuge for 5 mins. The cells were resuspended in 100µl of GMM, added to 10µl of D-cycloserine and incubated for 90 mins at 37°C. Cells were pelleted in a microfuge and resuspended in pre-warmed (37°C) GMM. 12µCi of ^{35}S-methionine was added and the mixture incubated at 37°C for 45 mins. The cells were collected by centrifugation in a microfuge for 2 mins and resuspended in 100µl of GMM containing 200µg/ml of unlabelled methionine. The mixture was incubated at 37°C for 15 mins, collected by centrifugation in a microfuge for 2 mins and resuspended in 25µl of SDS-PAGE loading buffer. After 5 mins in a boiling waterbath, 8µl were run on a mini-PROTEAN gel (Section 2. 11). Following electrophoresis the gel was fixed in 10% acetic acid, 40% methanol for 20 mins, rinsed briefly with distilled water and transferred to

Amplify solution (Amersham) for 1h. The gel was dried under vacuum at 80°C for 30 mins before autoradiography overnight at -70°C.

2. 11. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis

SDS-PAGE was conducted using a mini-PROTEAN II vertical slab system (BIORAD) using discontinuous gels as described by Laemmli (1970). The following solutions were used in the preparation of gels:

separating gel solution (10%)
4.02ml distilled water
2.5ml 1.5M Tris-HCl pH8.8
100µl 10%SDS
3.33ml 30% acrylamide
50µl 10%APS
5µl TEMED

stacking gel solution (4%)
6.1ml distilled water
2.5ml 0.5M Tris-HCl pH6.8
100µl 10%SDS
1.3ml 30% acrylamide
50µl 10%APS
10µl TEMED

30% acrylamide(per 100ml)
29.2g acrylamide
0.8g bisacrylamide
filtered

The separating gel solution was poured into the assembled gel sandwich using a Pasteur pipette and immediately overlaid with isobutanol. The gel was left for 1h to polymerize. Once set, the gel was rinsed thoroughly with distilled water to remove the alcohol and the surface blotted dry using Whatman paper (3MM) before pouring in the stacking gel solution. The comb was placed in position and allowed to set for 45 mins.

Prior to loading, the wells were rinsed with running buffer. After assembly of the central buffer chamber, running buffer was poured until it reached a level halfway between the short and long plates. Buffer was poured into the outer chamber so that at least 1cm of the

gel was covered. Samples were combined with loading buffer and then placed in a boiling waterbath for 5 mins and then loaded into the wells. Gels were run at 120V for 1h.

Loading buffer
4ml distilled water
1ml 0.5M Tris-HCl, pH6.8
800µl glycerol
1.6ml 10%SDS
400µl 2-β-mercaptoethanol
200µl 0.05% bromophenol blue

5x Running buffer (per litre)
15g Tris base
72g glycine
5g SDS

After electrophoresis, the assembly was dismantled and the gel treated with one of a number of stains or used in Western blot experiments.

2. 11. 1 Staining for proteins

The gel was stained for 30 mins with 0.5% Coomassie blue R-250 (BIORAD) in 40% methanol, 10% acetic acid before destaining for 1-3h in 40% methanol, 10% acetic acid to remove background.

2. 11. 2 Visualisation of LPS

Gels were stained using a silver staining kit (BIORAD) which was used in accordance with the manufacturer's instructions.

2. 11. 3 Periodic acid - Schiffs stain for carbohydrates

The gel was fixed in 30% methanol, 10% acetic acid for 30 mins. The remainder of the steps were conducted at 4°C in the dark. Chilled Periodic acid solution (0.5%) was added to the gel which was rocked for 20 mins. The gel was rinsed for 30 s in deionised water before Schiffs reagent (Sigma) was added and the gel rocked in this for 30 mins. The gel was then washed with 10% acetic acid.

2. 12 Western Blotting

Proteins were electrophoretically transferred from SDS-PAGE gels to nitrocellulose sheets (Hybond-N) using the Towbin buffer system (Towbin *et al.*, 1979).

The solutions used were as follows:

Transfer buffer
0.025M Tris base
0.192M glycine
20% methanol

Tris-Nacl buffer (TN) (per litre)
1.21g Tris base
8.7g NaCl
pH to 7.4 with 8M HCl

Blocking buffer
3% dried milk in TN

Developing solution
15mg 4-chloro-1-napthol in 5ml of methanol
TN to 30ml
15µl 6% hydrogen peroxide

SDS-PAGE gels were equilibrated for 2-3 mins in transfer buffer along with one sheet of nitrocellulose (Hybond-N) and 2 sheets of Whatman filter paper (3MM). The nitrocellulose was placed on one side of the gel and smoothed with a test tube to remove airbubbles. A sheet of filter paper was placed ontop, with the other being placed on the gel side, both were smoothed as before. The sandwich was then placed in a gel holder from a mini-Transblot apparatus (BIORAD). The tank was half-filled with transfer buffer and blotted overnight at 26V at 4°C.

The apparatus was dismantled and the nitrocellulose transferred to blocking buffer and rocked for 3h at 37°C. The filter was washed four times in TN. The whole filter, or strips cut out, were soaked in blocking buffer containing anti-capsular antisera at a dilution of 1:200. This was rocked for 3h at 37°C before being washed in the same manner as before. The filter was then transferred to blocking buffer containing secondary antibody at either 1:500 or 1:1000. This was again rocked for 3h at 37°C. Following a final series of washes,

the filter was placed in developing solution until bands appeared. The filter was rinsed briefly with distilled water and airdried on Whatman paper (3MM).

2. 13 Procedures for polysaccharide analysis

2. 13. 1 Phenol/sulphuric acid assay for carbohydrate determination

This was conducted according to the method of Dubois *et al* (1956), which detects simple sugars, oligosaccharides and polysaccharides.

200µl of 5% phenol (w/v) in distilled water was added to 200µl of sample. 1ml of concentrated sulphuric acid was added in a fast stream directly to the surface of the liquid. This was left at room temperature for 15-30 mins. A standard curve was constructed by measuring rhamnose in a number of standards, the concentrations routinely used were 0, 10, 50, 100, 200, 400, 600, 800 and 1000 µg/ml (rhamnose in distilled water, w:v). The absorbance was measured at λ_{488nm} and a standard curve constructed by plotting the absorbance value obtained for each of the standards against it's rhamnose concentration. The rhamnose concentration (rhamnose equivalents) of capsule preparations was determined by conversion from absorbance to rhamnose concentration using the standard curve.

2. 13. 2 Assay for protein determination

Protein determination was conducted using a protein assay dye (BIORAD) according to the manufacturer's instructions. Dye reagent was diluted 1 in 5 in distilled water. Samples were used neat or diluted up to 1 in 100 in distilled water. 120µl of sample was added to 1ml of diluted dye reagent and the tube mixed by vortexing. After 15 mins at room temperature the absorbance was read at λ_{595nm} and the concentration of protein determined from a standard curve. The standard curve was constructed in the manner described in section 2.13.1 except that lysozyme was used as the standard.

2. 13. 3 Determination of LPS concentration

The amount of LPS in samples was determined using a Limulus amoebocyte lysate (LAL) assay kit (KABI Diagnostics) which was used according to the manufacturer's instructions.

2. 13. 4 Removal of LPS from capsule preparations

LPS was removed by affinity chromatography with polymyxin B agarose (Sigma) using the method described by Issekutz (1983). All materials used were purchased endotoxin free or autoclaved prior to use. All steps were conducted in a Laminar flow cabinet.

A 5ml plastic syringe case was plugged with a small amount of polymer wool. The agarose was packed into a column by pouring the slurry into the syringe. The agarose was washed by running 10ml of acetate buffer pH 4.0 (41ml 0.2M acetic acid; 9ml 0.2M sodium acetate; 50ml distilled water) and 10ml of 0.1M borate buffer pH 8.0 (50ml 0.2M boric acid; 4.9ml 0.05M sodium borate; 145.1ml distilled water) containing 0.5M NaCl for three successive alternating cycles. The column was then washed extensively with PBS pH7.3 (8g sodium chloride; 0.34g monobasic potassium phoshate; 1.21g dibasic potassium phosphate; made to 1L with distilled water) and stored at 4^{o}C.

An adjustable screw on clip regulated the flow rate through siliconised tubing. The affinity chromatography was conducted at room temperature with a flow rate of 6ml/h. Endotoxin content was monitored using the LAL assay (Section 2. 13. 4).

2. 14 Production of Polyclonal antiserum

2. 14. 1 Immunization procedure

Serum was raised in a 100 day rabbit. Injection and bleeding of the animal was performed behind closed doors by trained staff.

A preliminary test bleed (1-2ml) was taken from the rear marginal ear vein prior to immunization. Polysaccharide (600µl) containing 150 µg/ml of rhamnose equivalents (see section 2.13.1) was emulsified in an equal volume of Freund's complete adjuvant. This was injected subcutaneously at 2 week intervals for 14 weeks. The last injection was conducted with Freund's incomplete adjuvant. Blood was taken prior to injections and one month after the final injection.

2. 14. 2 Harvesting serum

Erythrocytes were removed from heparinised blood by centrifugation at 3900rpm for 10 mins at 4°C. The supernatant was removed and mixed with 250µl of thrombin. The tube was inverted 2 or 3 times, if a large clot had not appeared then a similar amount of thrombin was added. The clot was pelleted as before and the serum removed, aliquoted and stored at -20°C. Serum was tested for anti-capsular antibodies by ELISA (Section 2. 15) and for anti-LPS antibodies by Western blotting (Section 2. 12).

2. 15 ELISA

Enzyme-linked immunosorbent assays were conducted as described by Voller (1980)

The solutions used were as follows:

PBS pH7.3

PBST
0.05% Tween 20 in PBS

BSA-PBST
3% BSA in PBST

Coating buffer(L)
1.59g Na_2CO_3
2.93g $NaHCO_3$
to 1L with dH_2O

substrate
10mg OPD
10µl 30% H_2O_2
4.9ml 0.1M citric acid pH5.0
5.1ml 0.2M Na_2HPO_4

100µl of whole cells were harvested by centrifugation and the cell pellet resuspended in 100µl of PBS. The centrifugation was repeated and the pellet resuspended in 100µl of PBS. Cells were diluted in coating buffer (1:100) and 100µl aliquots placed in wells of a flat-bottomed microtitre plate (Nunc Gibco, Uxbridge, UK) and left overnight at 4°C to allow binding to occur. The wells were washed extensively with PBST to remove cells which had not bound. The wells were then filled with BSA-PBST and incubated at 37°C for 30 mins. The solution was removed and 100µl of rabbit antiserum, diluted in BSA-PBST (1:500, v:v), was added. Following incubation for 1h at 37°C, the wells were again washed thoroughly with PBST. Diluted anti-rabbit antiserum conjugated to horseradish peroxidase, diluted in PBST (1:200, v:v), was added and the plate returned to 37°C for 1h. Wells were washed with PBST and developed by the addition of 100µl of substrate solution. Once the colour had developed sufficiently the reaction was terminated by the addition of 50µl of 4M sulphuric acid.

Chapter 3

Purification of capsular polysaccharide from *P. gingivalis* W83 and the generation of anticapsule antibodies

3.1 Introduction

The capsular polysaccharide of many pathogenic bacterial species is often a major virulence determinant in these organisms (Robbins *et al.*, 1974; Vermeulen *et al.*, 1988). Because of this, the capsule is often one of the most obvious candidates for examination when trying to decipher the role of virulence determinants in the pathogenicity of a particular species. Many strains of *P. gingivalis* produce a capsule, which is believed to be extremely important in the virulence of this organism (van Steenbergen *et al.*, 1985; 1987). The presence of anti-capsular antibodies in periodontally diseased individuals would also indicate its involvement (Naito *et al.*, 1987). However, such sera are not capsule-specific, containing antibodies to a number of other cell components.

Porphyromonas gingivalis strains W83 and HG184 are among the most virulent of the species in a murine model and exhibit the highest resistance to phagocytosis *in vivo* (van Steenbergen *et al.*, 1987). Given that one of the major features of many bacterial capsules is that they confer resistance to killing by phagocytes, then it would seem possible that the capsule of these strains would also contribute to this.

The most effective way of examining the contribution of any potential virulence factor is by producing isogenic mutants defective in a single virulence factor. The only way to ensure that only one mutation occurs is through the reintroduction of a disrupted copy of the gene, or by transposon mutagenesis.

With bacterial capsules, unless the organism produces sufficient polysaccharide to allow the capsule to be viewed by eye, detection of mutants lacking a capsule usually involves the screening of cells with antisera specific to the capsule or by resistance to capsule-specific bacteriophage (Echarti *et al.*, 1983; Silver *et al.*, 1981). Bacteriophages have not as yet been identified in *P. gingivalis* strains (Sandmeier *et al.*, 1993) meaning that the only method appropriate for the identification of acapsular mutants was the use of antisera raised against the capsule antigen. To obtain such antibodies, highly purified capsular polysaccharide is required.

This chapter describes approaches taken to purify polysaccharide from *P. gingivalis* W83 and generate antiserum specific to this material.

3. 2 Methods and Results

3. 2. 1 Capsule purification

The first method used in attempts to generate purified polysaccharide was similar to that of Wilson *et al* (1985) and was obtained from H. N. Shah (pers. comm.).

Method1

Cells from a 4-5 day-old stationary phase liquid culture of *P. gingivalis* W83 were harvested by centrifugation for 10 mins at 11080g. The cell pellet was resuspended in 1/5 the volume of 0.85% PBS pH7.3 and agitated at 4°C overnight to dissociate the outer membrane complex from the cells. The cells were again collected by centrifugation at

11080g for 15-20 mins and the supernatant retained. The cell pellet was again resuspended in PBS pH7.3 and the supernatant again retained. Surface associated material was then precipitated from the supernatant by the addition of two times the volume of chilled acetone which was stored overnight at -70°C. The extract was concentrated in a rotary evaporator by the evaporation of the acetone under vacuum at 50°C. The dried extract was resuspended in 1/50 the original volume of distilled water and dialysed overnight against distilled water and then freeze-dried. A 100mg portion of the extract was resuspended in 2ml of PBS and loaded onto a Sepharose 4B column at a flow rate of 1.4ml/min with 2 min fractions collected to separate high MW capsule from lower MW LPS and proteins. Fractions were examined to determine the polysaccharide content by measuring the O.D. at λ-215nm. Proteinase K was added (28U per starting litre of culture) and the extract incubated overnight at 37°C. The extract was centrifuged in a Sorvall ultracentrifuge at 27krpm using a AH627 for 4h to pellet any remaining LPS and then concentrated by centrifugation at 2556g for 10-20 mins in an amicon filter unit with a 30,000 kDa molecular mass cut off

The yield of polysaccharide as determined by estimation of neutral sugar content of the final extract was found to be 800mg per 10L of starting culture . However, silver staining of capsule extract after SDS-PAGE revealed ladder-like bands characteristic of LPS (fig 3. 1). Other investigators in the laboratory were working on capsules therefore it was decided to employ an alternative method for the production of capsular material which had been successfully used for *E. coli* capsules (K. Jann, pers. comm.).

Method 2

Cells from one litre of stationary phase culture were dehydrated by resuspension of the cell pellet in 1/5 the volume of absolute ethanol and then collected by centrifugation and the pellet again resuspended in ethanol. After harvesting the pellet was resuspended in1/30 volume of ethanol and again collected by centrifugation at 11080g for 10 mins. The pellet was finally resuspended in 1/20 volume of acetone, and then centrifuged at 11080g for 20

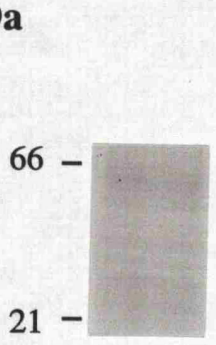


Figure 3. 1

Silver stained SDS-PAGE gel showing polysaccharide material extracted from *P. gingivalis* W83 using capsule purification method 1.

mins. The resultant pellet was airdried. Negatively charged molecules such as polysaccharides, DNA etc were solubilised by resuspension in 1/100 volume of 1M $MgCl_2$. Following incubation for 4h at 37°C, the solution was centrifuged at 11080g for 10 mins. The supernatant was removed and the polysaccharide precipitated by the addition of five times the volume of ethanol and placed at -20°C for at least 4h. The extract was collected at 10krpm for 10 mins and allowed to airdry. The pellet was resuspended in 1/200 of the original volume with 0.2M Tris-HCl pH 7.5. To this was added, 1mg of DNAase (1300KU) and 2mg of RNAase (100KU) per litre of starting culture and the solution was incubated at 37°C. After 4h incubation, 1mg (28U) of proteinase K was added and the preparation incubated overnight at 37°C. The sample was ultracentrifuged and then concentrated as described in method 1.

The neutral sugar content of material acquired in this manner was routinely found to be 1.2mg per 10L of starting culture. SDS-PAGE analysis revealed that this method generated capsule material which was apparently free of LPS after specific silver staining. These preparations were used to raise polyclonal antibodies in a rabbit as described in the materials and methods section. An ELISA was used to monitor antibody production and showed an increase in antibody titre following various booster injections (Table 3.1). However, when some of this high titre antiserum was used in Western blot analysis of *P. gingivalis* W83 cells, ladder-like bands characteristic of LPS were found (fig 3. 2).

Method 3

To try to overcome the problem of LPS contamination a number of steps were added to the procedure. The $MgCl_2$ solubilisation step was repeated in order to maximise the yield of polysaccharide. Just prior to ultracentrifugation, EDTA was added to a final concentration of 300mM, which was believed to enhance separation of capsule and LPS (M. Coleman, pers. comm.), and the sample ultracentrifuged twice. The resultant supernatant was concentrated using an ultrafiltration unit which allowed continuous agitation of the extract in

Table 3.1

ELISA using antisera raised against capsular material produced using method 2. Whole cells of *P. gingivalis* were used as the antigen

Serum sample	Absorbance λ490nm
Test	0
1	0.012
2	0.448
3	0.862
4	1.033
5	0.843
6	0.502
7	1.770

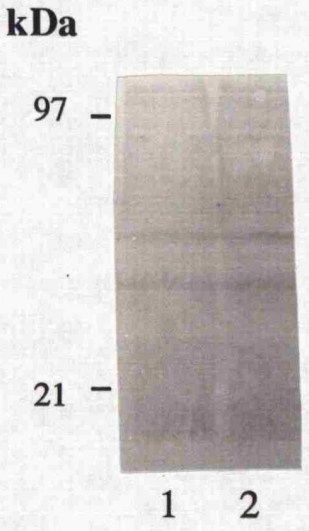


Figure 3. 2.

Western blot analysis of *P. gingivalis* W83 whole cells (lanes 1 and 2) following SDS-PAGE. The filter was probed with antiserum raised against capsular material extracted using method 2.

order to minimise loss through binding to the membrane of the filter. Following concentration, the extract was loaded on a Polymyxin B-agarose column to remove any remaining LPS. The removal of LPS and protein, as well as the neutral sugar content were monitored throughout this procedure. At the end of the procedure the extract was shown to be free of any detectable protein and contained levels of LPS similar to those found in blank controls in the LAL assay (Table 3. 2). 0.29% of the total neutral sugar component remained. However, this is not to say that only 0.29% of EPS remained at the end of the procedure. The neutral sugar assay did not specifically measure capsular polysaccharide but measured all neutral sugars regardless of the cellular component they were part of. At the start, most of the neutral sugar would not have been due to capsular material but would have been composed of other cellular components.

Use of this method generated capsule preparation which contained endotoxin at the same level as background as determined by the LAL assay. The yield of polysaccharide was also much higher than the previous two methods containing some 2.1mg of neutral sugars per 10L of starting culture (Table 3.3).

3. 2. 2 Removal of anti-LPS antibodies from antisera

In order to remove anti-LPS antibodies from the antisera so that only anti-capsular antibodies would remain, total antiserum was loaded onto a polymyxin B agarose column which had previously been overloaded with a crude LPS preparation from *P. gingivalis* W83. This was then washed extensively with PBS. The remaining components of the antiserum eluted in two fractions as determined by assaying protein levels of the fractions, and both of these were used in Western blot analysis of whole cells of *P. gingivalis*. The antibodies which eluted in fraction 1 still contained significant amounts of reactivity against LPS but this was greatly reduced compared to untreated antiserum. Fraction 2 contained much less LPS reactive material (fig 3. 3).

Table 3. 2

LPS, protein and neutral sugar content throughout the various stages of capsule purification using method 3.

Extraction stage	% component remaining		
	LPS	Protein	Neutral sugar
Original cell pellet	100	100	100
First $MgCl_2$ step	100	13	27
After first ultracentrifugation	6.6	1.25	5.04
After second ultracentrifugation	3	0.1	1.69
Extract before polymyxin B agarose	0.25	0	0.38
Final extract	0	0	0.29

Table 3. 3

The yield of polysaccharide obtained from each purification method, as determined by estimation of the neutral sugar content.

Capsule purification Method	Neutral sugar content mg
1	0.8
2	1.2
3	2.1

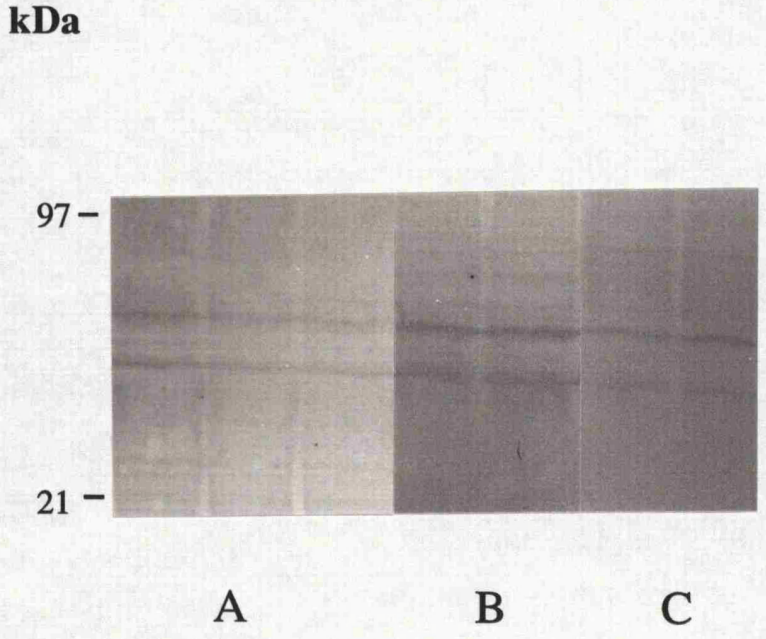


Figure 3. 3

Western blot of *P. gingivalis* W83 whole cells following SDS-PAGE analysis using: antiserum raised against capsular material extracted using method 2 (A); antiserum raised against the same material following the removal of anti-LPS antibodies using Polymyxin B agarose affinity chromatography, Fraction 1 (B) or Fraction 2 (C).

3. 2. 3. Examination of *P. gingivalis* W83 chromosomal DNA for homology to *E. coli* K5 capsule genes

The capsules produced by *E. coli* group II, *H influenzae* and *N. meningitidis* share some biochemical characteristics. In addition, the gene clusters encoding these structures are organised in similar modular fashion and it has been suggested that these genes share a common ancestor (Boulnois and Jann, 1990; Frosch *et al.*, 1991). To determine whether *P. gingivalis* W83 contained any homologous sequences, chromosomal DNA from this strain was cleaved with the restriction endonucleases *Bam*HI and *Bgl*II in a double-digest. The two endonucleases digest pGB110 such that the K5 regions I and III are excised such that one fragment contains region I whilst another contains region III. The DNA was then analysed by Southern blot analysis using radiolabelled probes from the *E. coli* K5 capsule gene cluster. No hybridisation occurred, with each region binding only to itself, even when conditions used would have permitted hybridisation between sequences with as little as 40% homology (fig 3. 4).

3. 3. Discussion

Varying degrees of success were achieved using the different capsule purification techniques. Use of method 1 for the production of capsular material from this strain of this organism was particularly unsuccessful due to the presence of visible LPS in the final preparations. For this reason, a second method was then used which removed all visible LPS from the extract as determined by specific silver-staining following SDS-PAGE. The finding that antisera raised against preparations of material produced by this method contained anti-LPS antibodies indicated that the LPS of this organism was typical to other Gram-negatives being extremely immunogenic. The recent observation that antisera raised against heat-killed *P. gingivalis* W83 and HG184 elicited a poor immune response in rabbits and required additional boosts to give reasonable amounts of anti-capsular

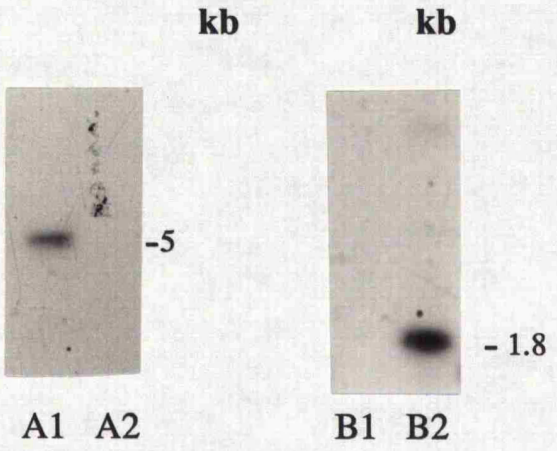


Figure 3. 4

Southern blot analysis of *P. gingivalis* W83 chromosomal DNA probed with radiolabelled regions of the *E. coli* K5 capsule gene cluster which are conserved in a number of bacterial species. Plasmid pGB110 (A1, B2) and chromosomal DNA from *P. gingivalis* W83 (A2, B1) were cleaved with the restriction endonucleases *BamH*I and *Bgl*II and probed with region I (A) or region III (B). pGB110 was cleaved with the endonuclease indicated and the fragments containing regions I and III excised following electrophoresis in low-melting point agarose, radiolabelled and used as probes.

antibodies (van Winkelhoff *et al.*, 1993) might suggest that the immunogenicity of the capsular material of these strains is low. A similar situation exists for capsular polysaccharide of a number of human pathogens (Finne *et al.*, 1983; Kaijser and Jodal, 1984) The K1 and K5 antigens of *E. coli* mimic host factors and are therefore not recognised as foreign it is possible that the K-antigens of some *P. gingivalis* strains could function in a similar fashion. *Porphyromonas gingivalis* strains W50 and W83 produce K1 polysaccharide (van Winkelhoff *et al.*, 1993). The low immunogenicity of K1 antigen could explain why other researchers were unable to identify the capsular antigen of *P. gingivalis* W50 (Schifferle *et al.*, 1989).

The failure to detect LPS in the material produced using method 2 by means of silver staining SDS-PAGE gels could be explained by the finding that the minimum amount of LPS required for detection by this method is in the region of 0.5μg (Kido *et al.*, 1990). Because of this a more efficient method was required for complete removal of LPS. This involved the use of a method which exploited the biochemical differences between polysaccharide and LPS.

Polysaccharide produced by method 3 contained LPS at the same level as the negative controls in the LAL assay and it was probably justifiable to assume that such preparations were in reality free of any LPS contamination following final purification of the extract on the polymyxin B-agarose column. This of course was not proven conclusively as the LAL assay system was extremely sensitive, with the limit of detection being 0.0125ng/ml. However, the fact that the use of such a column was extremely effective in removing anti-LPS antibodies from the antisera and the fact that this method was previously shown to remove essentially completely endotoxin from a solution containing 1mg/ml of LPS (Issekutz, 1983), reinforce the belief that method 3 is an effective way of removing the very small amounts of LPS present in preparations produced using method 2. However, the purpose of generating anti-capsular antibodies was to allow the identification of acapsular transposon mutants. Since Tn*4351* was found to be inappropriate as a means for

generating such mutants, polysaccharide preparations obtained using method 3 were never used to raise antisera.

Although purification of the antibodies was not taken to completion in this study, it would appear that this is an acceptable means of generating specific anti-capsular antibodies under such circumstances. It has been shown previously that it is possible to produce antisera specific to a particular cell-component by the removal of anti-LPS antibodies. These were removed from antisera raised against *P. gingivalis* fimbrial preparations by the use of autoclaved sonicates of whole cells (Goulbourne and Ellen, 1991). Similarily, specific antibodies were generated against *E. coli* Pap pilin by the pretreatment of antisera raised with Pap pilin mutants. These served to remove contaminating LPS reactive material (Salit *et al.*, 1988). Given that the different K antigens of *P. gingivalis* are not cross-reactive then it should also be possible to remove anti-LPS antibodies from the antiserum by pre-absorption with a strain producing either a different K antigen or no K antigen at all.

Presently, researchers are still using methods for the purification of capsular polysaccharide from *P. gingivalis* which contain small amounts of LPS. Provided any role of LPS in these studies can be completely discounted then this is acceptable. For instance, it was demonstrated unequivocally that bone-resorbing activity was due to the capsular structure of *Actinobacillus actinomycetemcomitans* and not LPS, as the presence of polymyxin B in the assay had no effect on the results obtained (Harvey *et al.*, 1987). However, some researchers have obtained results which they have attributed to capsular material without totally refuting the possibility of involvement of LPS. High titre antisera from six patients suffering from periodontitis was used to demonstrate that this could inhibit bone destruction in a murine calvarial assay caused by the surface associated material of *P. gingivalis* strain W50 (Meghji *et al.*,1993). The authors suggested that bone-resorption was mediated by the capsular material of this strain. However, although the surface-associated material only contained low levels of LPS (Meghji *et al.*, 1993), it is possible that the inhibition produced by the antisera could have been through the

neutralisation of surface-associated material by anti-LPS antibodies and not by anti-capsular antibodies. The authors did not indicate whether or not the sera were selected at random or chosen specifically because of their reactivity with *P. gingivalis* W50 and whether the sera was reactive with other cell components (Meghji *et al.*, 1993).

The three different serotypes of capsule antigen produced by *P. gingivalis* K1, 2 and 3 are not cross-reactive (van Winkelhoff *et al.*, 1993). It has been shown that phagocytosis of virulent *P. gingivalis* strains required specific IgG raised against each strain, whereas a non-virulent strain was phagocytosed in the absence of antibody. Only 3 of 18 patients in the study produced antiserum which enhanced phagocytosis of the virulent strain *P. gingivalis* A7436 (Cutler *et al.*, 1991). Such findings imply that different individuals harbour not only different strains of *P. gingivalis*, but strains which exhibit different K-antigens.

That different individuals harbour different strains has been shown by other investigators. DNA analysis demonstrated that inter-individual differences occur (except in the cases of partners) with one group of researchers finding 9 different strains in 9 individuals and the other 7 different strains in 7 patients (Saarela *et al.*, 1993; van Steenbergen *et al.*, 1993). Given this, and the specificity of antisera to the K-antigens, it would be surprising if 6 different individuals would harbour 6 different strains which all produced the same K-antigen. Interestingly, a monoclonal antibody raised against the LPS of *P. gingivalis* strain ATCC 33277 reacted with 96% of fresh *P. gingivalis* isolates from a total number of 108 (Shelburne *et al.*, 1993).

The use of nonspecific antisera for serological studies on the capsule is also acceptable provided that the anti-capsular antibodies can be shown as a separate entity. It is possible to demonstrate the presence of 2 heat-stable antigens (LPS and K-antigen) by double immunodiffusion and immunoelectrophoresis (van Winkelhoff *et al.*, 1993). However, when the main objective of a study requires identification of acapsular mutants by the detection of a negative reaction then clearly such antibodies are of little use as the only

convenient method for screening for such mutants at present is through the use of immunoblotting.

Although Southern blot analysis revealed *P. gingivalis* to have no significant DNA sequence homology with capsule genes of *E. coli*, this does not mean that there would be no homology at the amino acid level between the proteins involved in polysaccharide expression. Such findings were obtained for the genes of thiol-activated bacterial toxins but the proteins are highly homologous (Mengaued *et al.*, 1988). However, this is not to imply that such homologies exist between *P. gingivalis* and *E. coli* gene products.

Chapter 4

Introduction of foreign DNA into *P. gingivalis* W83

4.1 Introduction

Until recently, the genetic manipulation of *P. gingivalis* was extremely limited by the lack of any genetic transfer system for the (re)introduction of DNA into this organism. No plasmid or bacteriophage DNA has ever been detected in any strain of *P. gingivalis* although a number of cryptic plasmids have been identified in other species of the genus (Sako, *et al.*, 1988; Yoshimoto and Umemoto, 1990). Because of this, investigators have attempted to exploit vectors constructed for use in the colonic *Bacteroides*. These vectors can be transferred from *E. coli* to *Bacteroides* by conjugal transfer (Shoemaker *et al.*, 1986; 1989; Stevens *et al*, 1992).

(A more detailed summary of background information is given in Section 1. 6).

The transposon Tn*4351* was originally identified as part of the *B. fragilis* plasmid pBF4 (Shoemaker *et al.*, 1985) and encodes resistance to clindamycin/erythromycin in *Bacteroides* hosts and tetracycline resistance in aerobically grown *E. coli* (Guiney *et al.*, 1984). This transposon has been exploited for use in a number of *E. coli/Bacteroides* shuttle-vectors (Shoemaker *et al.*, 1985; Smith and Gonda, 1985). The whole of the transposon has also been cloned into suicide-vectors (Shoemaker *et al.*, 1986).

A number of plasmid vectors have been constructed using the broad host-range *E. coli* plasmid RSF1010, which provides maintenance and kanamycin resistance in *E. coli* and the cryptic *Bacteroides* plasmid pB8-51 which allows maintenance in *Bacteroides* hosts. The *Bacteroides* resistance markers are derived from a number of naturally occurring *Bacteroides* antibiotic resistance elements (Shoemaker *et al.*, 1985, 1989; Stevens *et al.*, 1992).

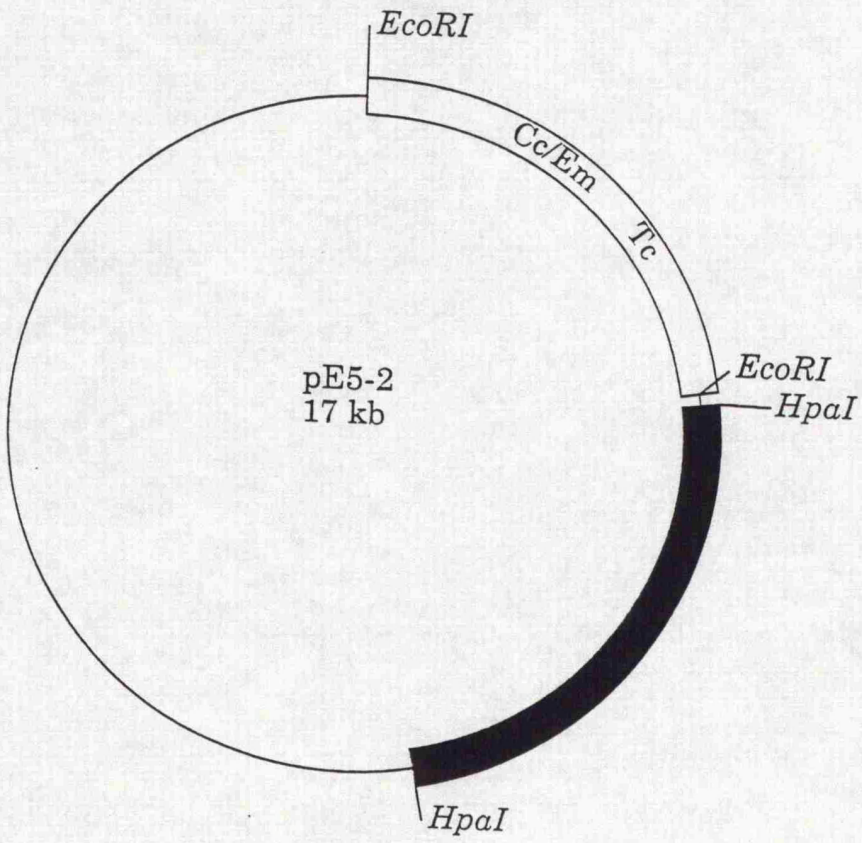
Plasmid pE5-2 (fig. 4. 1) carries the clindamycin/erythromycin resistance gene from Tn*4351* allowing selection in *Bacteroides* species (Shoemaker *et al.*, 1985). This plasmid was transferred to *P. gingivalis* by conjugal transfer from *E. coli* demonstrating that *Bacteroides* plasmids can be transferred to and maintained in *P. gingivalis* strains and may therefore have potential for use in this organism (Progulske-Fox *et al.*, 1989b). However, this vector was transferred to *P. gingivalis* strains with low efficiency generating $2x10^{-7}$ transconjugants per recipient and does not possess many unique restriction endonuclease sites. The introduction of more versatile vectors with higher transfer frequencies may be more useful in the genetic manipulation of this organism.

Plasmid pNJR5 (fig. 4.2) is a shuttle-cosmid vector and contains the clindamycin/erythromycin resistance determinant from Tn*4400* (Shoemaker *et al.*, 1989). This vector has the highest mobilisation frequency of all of the vectors constructed by this group (Shoemaker, pers. comm.) as well as containing a number of useful cloning sites. This vector also contains the *cos* site from bacteriophage Lambda.

Plasmid pNJR12 (fig. 4. 3) has a multiple cloning site and carries a tetracycline resistance gene from the chromosomal element of *B. thetaiotaomicron* DOT allowing selection of *Bacteroides* species (Stevens *et al.*, 1992).

All of these vectors are mobilised from the *E. coli* donor to *Bacteroides* recipients by R751.

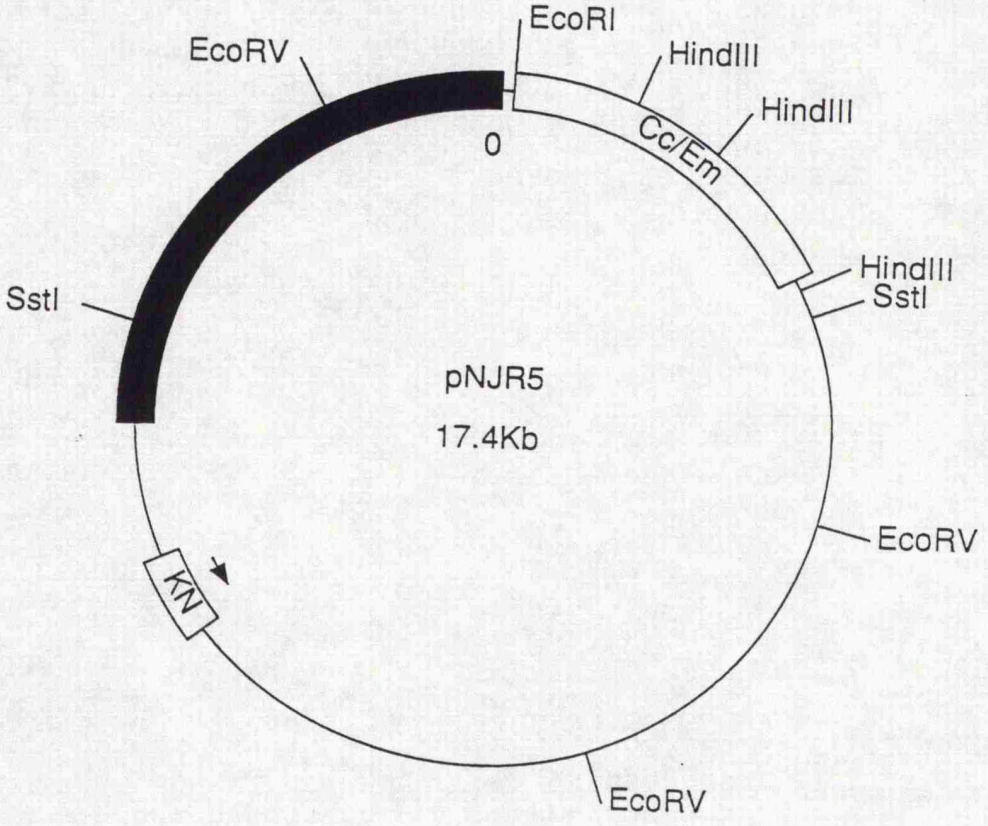
Figure 4.1 Plasmid pE5-2



The filled box is the cryptic *Bacteroides* plasmid pB8-51. The box labelled Cc/Em Tc is derived from the *Bacteroides* plasmid pBF4 which encodes clindamycin resistance in *Bacteroides* hosts and tetracycline resistance in aerobically grown *E. coli*. The remainder of the vector is from RSF1010.

Shoemaker *et al*., 1985.

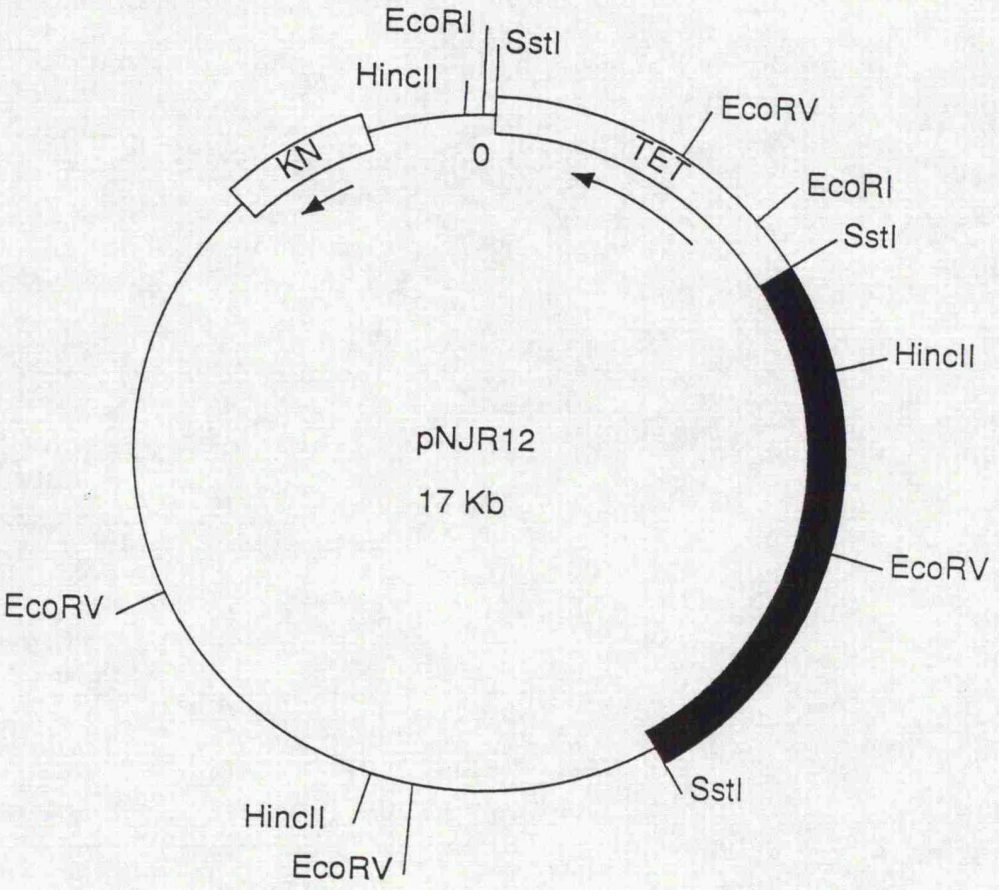
Figure 4.2 Plasmid pNJR5



The box labelled Cc/Em is derived from the *Bacteroides* transposon Tn*4400*. The filled box represents plasmid pB8-51. The kanamycin gene (KN) and the remainder of the plasmid are from RSF1010.

Shoemaker *et al*., 1989

Figure 4.3 Plasmid pNJR12



The box labelled TET denotes the tetracycline resistance gene from a *Bacteroides* transferable chromosomal element. The filled box represents pB8-51. The remainder of the vector including the kanamycin resistance gene is from RSF1010.

Stevens *et al.*, 1992

Plasmid R751 from the IncP incompatibility group is not maintained in *Bacteroides* species (Shoemaker *et al.*, 1986). This property allows it to be used as a suicide vector for the delivery of transposons into the genome of recipient cells from this group of organisms. R751: :Tn*4351*Ω4 (fig. 4.4) contains a partial tandem duplication of Tn*4351* in R751 and is self-mobilising from *E. coli* encoding resistance to trimethoprim and tetracycline in *E. coli* and clindamycin/erythromycin resistance in *Bacteroides* (Shoemaker *et al.*, 1986).

This chapter details the use of plasmids pNJR5 and pNJR12 in attempts to establish a reliable and efficient system for plasmid transfer into *P. gingivalis* W83. Once achieved this system would then be used for the production of Tn*4351* transposon mutants.

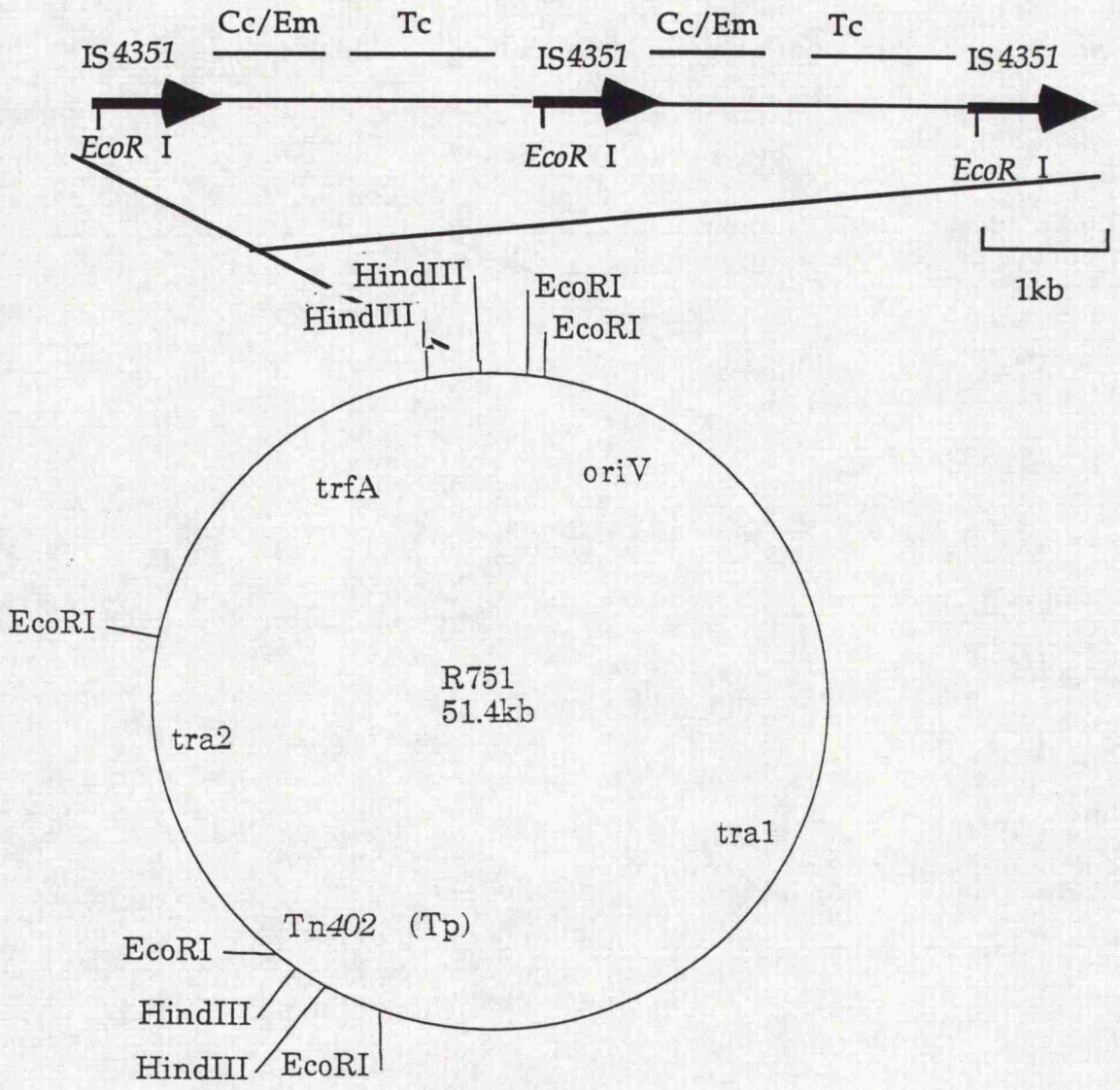
4.2 Results

4. 2. 1. Introduction of plasmid DNA into *P. gingivalis* W83

Because of its high mobilisation frequency, pNJR5 was used in initial mating experiments. Despite numerous attempts, no successful transfer of this vector was recorded using the method used previously to introduce pE5-2 into *Porphyromonas* species (Progulske-Fox *et al.*, 1989b). Nor did the method used to transfer such vectors into colonic *Bacteroides* (Shoemaker *et al.*, 1985; 1986b) produce any transconjugants. This indicated that a variety of conditions would have to be altered to try to achieve successful transfer. This involved conducting matings which differed in a number of variables, including:

a. the starting cfu/ml of the recipient cells
b.donor:recipient ratio
c. aerobic/anaerobic mating
d. the duration of the mating
e. length of incubation on selective media

Figure 4.4 R751::Tn*4351*Ω4



Transposon Tn*4351* inserted into R751 to give R751::Tn*4351*Ω4. Tp refers to trimethoprim resistance. Cc/Em and Tc denote resistance to clindamycin/erythromycin and tetracycline, respectively. The origin of replication is indicated by oriV. The self-transfer genes are represented by tra1 and tra2. An additional gene required for replication of R751, trfa is also highlighted.

Meyer and Shapiro, 1980; Shoemaker and Salyers, 1987

Initial mating experiments used erythromycin as the selective agent for recovery of *P. gingivalis* transconjugants. However, the *E. coli* donor strain had high background resistance to 10μg/ml of erythromycin. As a result, clindamycin at 10μg/ml was subsequently used.

A mating experiment produced a number of clindamycin resistant transconjugants which, when transferred to fresh medium containing clindamycin at 10μg/ml, could not be maintained. Since low expression of the clindamycin resistance gene was known to occur in some *Bacteroides* species (Shoemaker, pers. comm.) it was possible that this was also true for *P. gingivalis* and was responsible for the poor growth of the transconjugants and problems with their propagation. Therefore the MIC of the parent strain for clindamycin was determined and found to be 0.1μg/ml. All remaining experiments using pNJR5 used clindamycin at a concentration of 0.5μg/ml. To overcome the problem of maintaining clindamycin resistant transconjugants, a second plasmid pNJR12, which encodes tetracycline resistance, was also used in mating experiments.

Matings involving the transfer of plasmids pNJR5 and pNJR12 to *B. uniformis* were conducted as controls. The transfer frequencies obtained (Tables 4. 1. a,b) were similar to those obtained previously in this species (Shoemaker *et al.*, 1985).

In the first group of successful mating experiments into *P. gingivalis* using pNJR5 and pNJR12 few variables were altered to try to determine the most critical conditions necessary for successful transfer of the vectors into *P. gingivalis* W83. This involved mixing 1ml of a recipient culture with one of two different volumes of donor. Plates were then incubated anaerobically for 24, 48 or 72h before cells were collected and spread onto selective media (Tables 4. 1.c, d). The influence of the various parameters on the transfer frequency was not completely clear. However, on the basis of these results an important parameter for the recovery of transconjugants of *P. gingivalis* W83 with either plasmid

Table 4. 1. a

Conjugal Transfer of plasmid pNJR5 from *E. coli* to *B. uniformis*

Starting cfu/ml		Donor:Recipient	Mating	Transfer †
Donor	Recipient	Ratio	Time (h)	Frequency
$2.00x10^8$	$9.00x10^6$	140:1	72	$1.18x10^{-5}$
$2.00x10^8$	$9.00x10^6$	14:1	72	$1.78x10^{-6}$
$1.85x10^8$	$5.85x10^7$	10:1	24	$3.90x10^{-5}$
$1.85x10^8$	$5.85x10^7$	100:1	24	$5.00x10^{-6}$
$1.78x10^9$	$8.00x10^6$	14:1	72	$4.20x10^{-3}$
$1.78x10^9$	$8.00x10^6$	1:17	72	$4.50x10^{-5}$

Table 4. 1. b

Conjugal transfer of pNJR12 from *E. coli* to *B. uniformis*

Starting cfu/ml		Donor:Recipient	Mating	Transfer †
Donor	Recipient	Ratio	Time (h)	Frequency
$5.40x10^7$	$2.10x10^7$	10:1	72	$8.80x10^{-3}$
$5.40x10^7$	$2.10x10^7$	1:10	72	$3.90x10^{-5}$
$6.50x10^7$	$9.00x10^6$	12:1	72	$2.23x10^{-4}$
$6.50x10^7$	$9.00x10^6$	120:1	72	$2.92x10^{-4}$
$1.15x10^8$	$5.85x10^7$	10:1	24	$1.50x10^{-5}$
$1.00x10^7$	$1.00x10^4$	100:1	96	$1.70x10^{-5}$

†Transfer frequencies are expressed as the number of transconjugants per recipient cell at the end of the mating.

Table 4. 1. c

Conjugal transfer of pNJR5 from *E. coli* DH5α to *P. gingivalis* W83

Starting cfu/ml Donor	Recipient	Donor:Recipient Ratio	Mating Time (h)	Transfer † Frequency
$1.95x10^6$	$4.00x10^7$	1:1026	24	0
$1.95x10^6$	$4.00x10^7$	1:103	24	0
$1.95x10^6$	$1.50x10^8$	1:3846	24	$3.30x10^{-7}$
$1.95x10^6$	$1.50x10^8$	1:385	24	0
$1.85x10^8$	$1.71x10^8$	1:46	24	0
$1.85x10^8$	$1.71x10^8$	1:5	24	0
$1.85x10^8$	$5.80x10^8$	1:157	24	0
$1.85x10^8$	$5.80x10^8$	1:16	24	0
$4.80x10^7$	$1.00x10^4$	12:1	48	$2.00x10^{-6}$
$4.80x10^7$	$1.00x10^4$	120:1	48	0
$3.00x10^7$	$1.36x10^9$	1:2266	48	0
$3.00x10^7$	$1.95x10^8$	1:33	48	$5.13x10^{-9}$
$0.90x10^7$	$5.40x10^7$	1:240	72	$2.20x10^{-7}$
$0.90x10^7$	$5.40x10^7$	1:24	72	$7.40x10^{-8}$
$4.80x10^7$	$1.00x10^4$	12:1	72	$4.50x10^{-4}$
$4.80x10^7$	$1.00x10^4$	120:1	72	$2.50x10^{-4}$
$1.40x10^7$	$7.60x10^6$	1:2	72	$1.30x10^{-7}$

†Transfer frequencies are expressed as the number of transconjugants per recipient cell at the end of the mating.

Table 4. 1. d

Conjugal Transfer of plasmid pNJR12 from *E. coli* SF8to *P. gingivalis* W83

Starting cfu/ml Donor	Recipient	Donor:Recipient Ratio	Time (h)	Transfer † Frequency
$1.15x10^8$	$4.40x10^8$	1:19	24	0
$1.15x10^8$	$5.80x10^8$	1:252	24	0
$1.15x10^8$	$5.80x10^8$	1:25	24	0
$1.07x10^8$	$1.36x10^9$	1:636	48	0
$1.07x10^8$	$1.36x10^9$	1:64	48	0
$1.07x10^8$	$7.50x10^8$	1:350	48	0
$1.07x10^8$	$7.50x10^8$	1:35	48	0
$1.07x10^8$	$1.95x10^8$	1:91	48	0
$1.07x10^8$	$1.95x10^8$	1:9	48	0
$1.07x10^8$	$1.00x10^4$	27:1	48	$2.50x10^{-5}$
$1.07x10^8$	$1.00x10^4$	270:1	48	$2.20x10^{-4}$
$6.00x10^7$	$7.60x10^7$	1:5	48	$1.30x10^{-8}$
$6.00x10^7$	$7.60x10^7$	1:51	72	0
$0.40x10^7$	$5.40x10^7$	1:540	72	$2.20x10^{-7}$
$0.40x10^7$	$5.40x10^7$	1:54	72	$9.60x10^{-7}$
$2.00x10^8$	$1.45x10^8$	1:36	72	0
$2.00x10^8$	$1.45x10^8$	1:4	72	0
$2.00x10^8$	$1.86x10^8$	1:47	72	0
$2.00x10^8$	$1.86x10^8$	1:5	72	0
$1.07x10^8$	$1.00x10^4$	27:1	72	0
$1.07x10^8$	$1.00x10^4$	270:1	72	0

†Transfer frequencies are expressed as the number of transconjugants per recipient cell at the end of the mating.

were determined to be that the recipient cells be between $1x10^4$-$1x10^6$cfu/ml at the very start of the mating procedure. Increasing the cfu/ml of the recipient caused a decrease in the transfer frequency. The highest frequencies obtained for pNJR12 occurred when the mating time was 48h whereas 72h were required for pNJR5 since these generated transconjugants at frequencies of up to $2.2x10^{-4}$ and $2.5x10^{-4}$ respectively. Although the best frequencies were obtained when the cfu/ml of the donor was between $4.8x10^7$-$1.0x10^8$, the age of the donor culture did not appear to be as critical a factor as that of the recipient. In these experiments an excess of recipient to donor usually had a detrimental effect on the efficiency of transfer. Decreasing the donor in relation to recipient reduced the transfer efficiency with both plasmids.

Under these conditions, transfer frequencies of approximately $1x10^{-4}$ - $5.1x10^{-9}$ were achieved. Higher frequencies were comparable to those obtained for *B. uniformis*.

After the initial isolation of transconjugants harbouring either plasmid the time for the appearance of colonies was comparable to that of the parent strain on solid media. However, pNJR5 transconjugants grew very poorly in liquid media containing even slightly more clindamycin than the MIC of the parent strain and also on plates containing only $0.5\mu gml^{-1}$, in relation to transconjugants containing pNJR12. Since pNJR5 transconjugants also required additional incubation time, it was assumed that expression of the clindamycin resistance gene was extremely poor in this strain. Because of this, pNJR12 was used in the remainder of the experiments to attempt to characterise more fully other factors influencing the conjugation process (Table 4. 2). Again no definite conclusions could be drawn regarding the contribution of each of the factors. The optimum length of mating which gave best results was 37-44h. The likelihood of isolating transconjugants prior to this was very small and increasing mating times beyond this caused a fall in the number of transconjugants recovered. Continued mating caused even greater falls in the frequencies produced. However, this was not always true for individual mating mixtures but was the general trend (Table 4. 2). The transfer frequencies in this

Table 4. 2.

Effect of mating time and donor:recipient ratio on the conjugal transfer of plasmid pNJR12 from *E. coli* SF8 to *P. gingivalis* W83

Starting cfu/ml: donor- 7.50×10^6; recipient- 9.00×10^6

Mating Time (h)	Transfer Frequency † Donor:Recipient Ratio					
	1:5	1:6	1:9	1:10	1:12	1:16
16	0	0	0	0	0	0
21	0	0	4.30×10^{-8}	0	0	0
37	3.60×10^{-8}	3.60×10^{-8}	1.40×10^{-7}	3.70×10^{-8}	2.77×10^{-8}	0
43	9.10×10^{-9}	9.10×10^{-9}	2.30×10^{-8}	0	7.90×10^{-9}	1.11×10^{-8}
71	3.2×10^{-10}	5.40×10^{-9}	7.2×10^{-10}	3.2×10^{-10}	7.25×10^{-9}	4.8×10^{-10}

Starting cfu/ml: donor-3.20×10^7; recipient-9.00×10^6

Mating Time(h)	Transfer Frequency † Donor : Recipient Ratio						
	1.2:1	1:1.1	1:1.4	1:2	1:3	1:4	1:6
19	0	0	0	0	0	0	0
26	0	3.0×10^{-8}	0	1.5×10^{-8}	3.8×10^{-7}	0	7.6×10^{-8}
42	0	7.2×10^{-9}	1.4×10^{-8}	2.5×10^{-8}	4.6×10^{-6}	7.2×10^{-9}	0
66	0	5.1×10^{-10}	3.3×10^{-9}	1.9×10^{-9}	1.6×10^{-8}	7.9×10^{-9}	0

†Transfer frequencies are expressed as the number of transconjugants per recipient cell at the end of the mating.

Table 4. 2. continued

Effect of mating time and donor:recipient ratio on the conjugal transfer of pNJR12 from *E. coli* SF8 to *P. gingivalis* W83

Starting cfu/ml: donor-1.7×10^7; recipient-2×10^5

Mating Time(h)	Transfer Frequency † Donor : Recipient Ratio							
	43:1	28:1	21:1	17:1	12:1	11:1	8.5:1	6:1
20	0	0	0	0	0	0	0	0
32	0	0	0	0	0	0	0	0
44	0	0	0	0	0	0	0	0
60	3.7×10^{-7}	6.3×10^{-7}	0	0	0	0	0	0
68.5	0	0	0	0	0	0	0	0
86	0	0	0	0	1.7×10^{-5}	0	3.3×10^{-9}	2.7×10^{-8}

†Transfer frequencies are expressed as the number of transconjugants per recipient cell at the end of the mating.

group of experiments was much lower than those in Table 4. 1. d. This may have been due to the use of recipient cultures with slightly higher cfu/ml. Alternatively, the cfu/ml of the donor was lower in the latter experiments and it may be that the influence of the age of the donor culture was greater than previously thought.

The length of incubation on selective media after the mating necessary to identify transconjugants was much greater than those previously reported for *P. gingivalis* and *Bacteroides* species (Progulske-Fox *et al.*, 1989b; Shoemaker *et al.*, 1985). This could explain the failure of initial experiments to generate transconjugants. No single pNJR12 transconjugant was isolated before 10 days of incubation on selective media. In order to recover pNJR5 transconjugants, the length of incubation had to be increased by almost 50% to 14 days.

4. 2. 2. Recovery of plasmid DNA from *P. gingivalis* transconjugants

Colony blot analysis was conducted on *P. gingivalis* transconjugants carrying each plasmid. The signals generated by *P. gingivalis* transconjugants were significantly weaker than those of *E. coli* and *B. uniformis* controls (fig. 4. 5).

Attempts were made to recover plasmid DNA from each type of transconjugant. However, even large volumes, of up to 3 litres of starting culture failed to yield any visible plasmid DNA. This has been reported by another group (Dyer *et al.*, 1992). This problem was not encountered with *B. uniformis*. Because of this it was decided that total DNA lysates extracted from *P. gingivalis* transconjugants be used to transform *E. coli* and plasmid DNA be isolated from *E. coli* transformants.

Initially a total lysate from one of each of a *P. gingivalis* pNJR5 or pNJR12 transconjugant was made and used to transform *E. coli*. Plasmids pNJR5 and pNJR12 recovered from *E. coli* transformants were digested with the restriction enzymes *EcoR*V

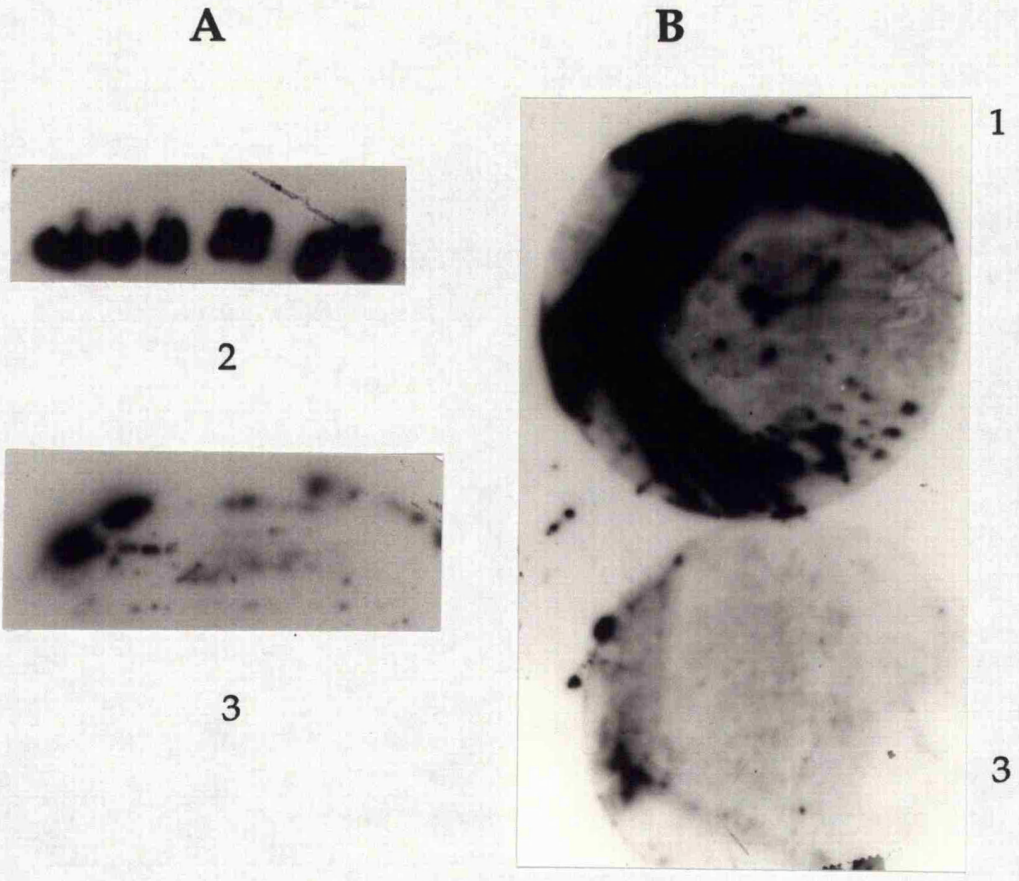


Figure 4. 5

Colony hybridisations of cells containing either plasmid pNJR5 (A) or pNJR12 (B) from *E. coli* (1), *B.uniformis* (2) or *P. gingivalis* W83 (3). *E. coli* and *P. gingivalis* cells were streaked on agar plates whereas *B. uniformis* colonies were picked onto plates.

or *Sst*I, respectively. The restriction pattern of pNJR12 which had originally been isolated from *P. gingivalis* was found to have a fragment which had increased in size from 2.7 kb in pNJR12 to 3.5 kb in the recombinant plasmid (pNJR12-1). The restriction pattern of pNJR5 did not differ at all to that expected (fig 4. 6).

Total DNA preparations from other *P. gingivalis* pNJR12 transconjugants were examined to determine the frequency of occurence of pNJR12-1. Nine different transconjugants were digested with *Hinc*II and then examined by Southern blotting using pNJR12 as the probe. Close examination revealed that seven were another type of recombinant pNJR12 which contained an insert of only 0.7 kb in size. This was in a different location to the insert in pNJR12-1, and this second type were termed pNJR12-2. Two transconjugants did in fact contain pNJR12 (fig 4. 7).

Restriction enzyme patterns of the three plasmid types were compared (fig. 4. 8). Plasmid pNJR12-1 contained an additional site for each of the restriction endonucleases, *Cla*I, *Sma*I, and *Sst*I but pNJR12-2 did not contain any extra sites for any of the enzymes used. Cleavage of pNJR12-1 with the restriction enzyme *Sst*I revealed that, in addition to the fragment of increased size, there was also an additional 0.5 kb fragment (fig. 4. 8, table 4.3). Plasmid pNJR12-1 contained an additional 1.3 kb of DNA which had inserted into a 0.6 kb *EcoR*I/*Sst*I fragment of the tetracycline resistance gene (fig. 4. 9), whereas pNJR12-2 contained an additional 0.7 kb of DNA located within a 2.4kb *EcoR*V/*Sst*I fragment derived from pB8-51 (fig. 4. 9).

To determine whether the inserts in pNJR12-1 and pNJR12-2 were derived from *P. gingivalis*, chromosomal DNA was extracted from the parent strain, cleaved with a number of restriction enzymes and then probed with one of three probes (fig. 4. 9). The probes were the 2.7 kb *Sst*I fragment of pNJR12 (probe A), the corresponding 3.5 kb fragment from pNJR12-1 (probe B). The 4.8 kb *Sst*I fragment from pNJR12-2

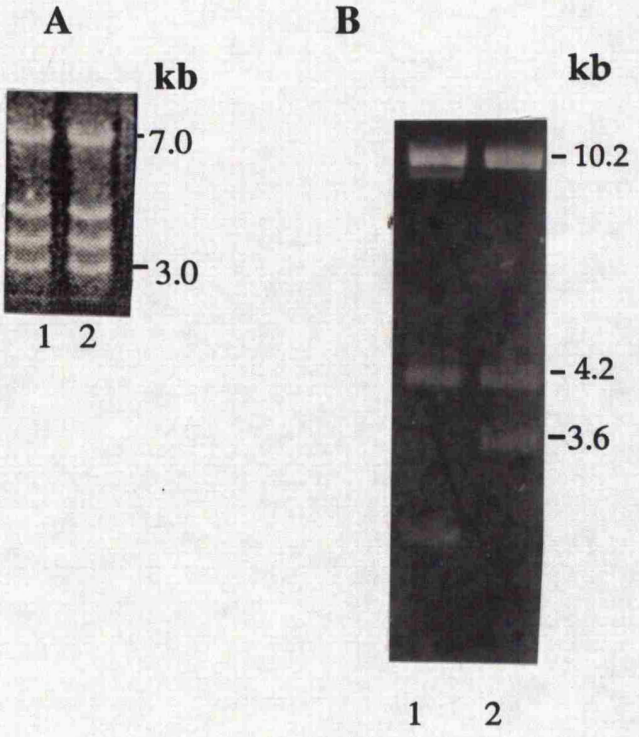


Figure 4. 6

Agarose gel electrophoresis of plasmid pNJR5 (panel A) isolated from *E. coli* (lane 1) or from *E. coli* via a *P. gingivalis* transconjugant (lane 2) cleaved with the restriction endonuclease *EcoR*V. Plasmid pNJR12 (panel B) from *E. coli* (lane 3) or from *E. coli* via a *P. gingivalis* transconjugant (lane 4) was cleaved with the restriction endonuclease *Sst*I The sizes in kb are indicated to the right of the panels.

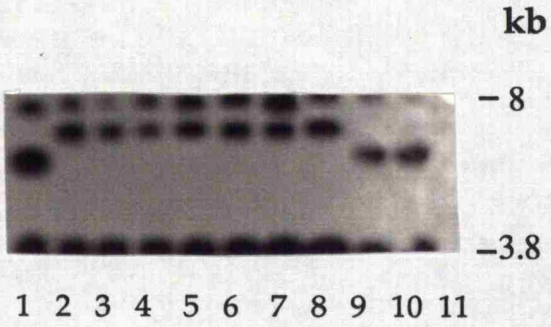


Figure 4. 7

Total DNA extracted from nine *P. gingivalis* pNJR12 transconjugants digested with restriction enzyme *Hinc*II, probed with radiolabelled pNJR12. Track1: pNJR12; Tracks 2-10: *P. gingivalis* pNJR12 transconjugants; Track 11: *P. gingivalis*. The sizes are given in kb.

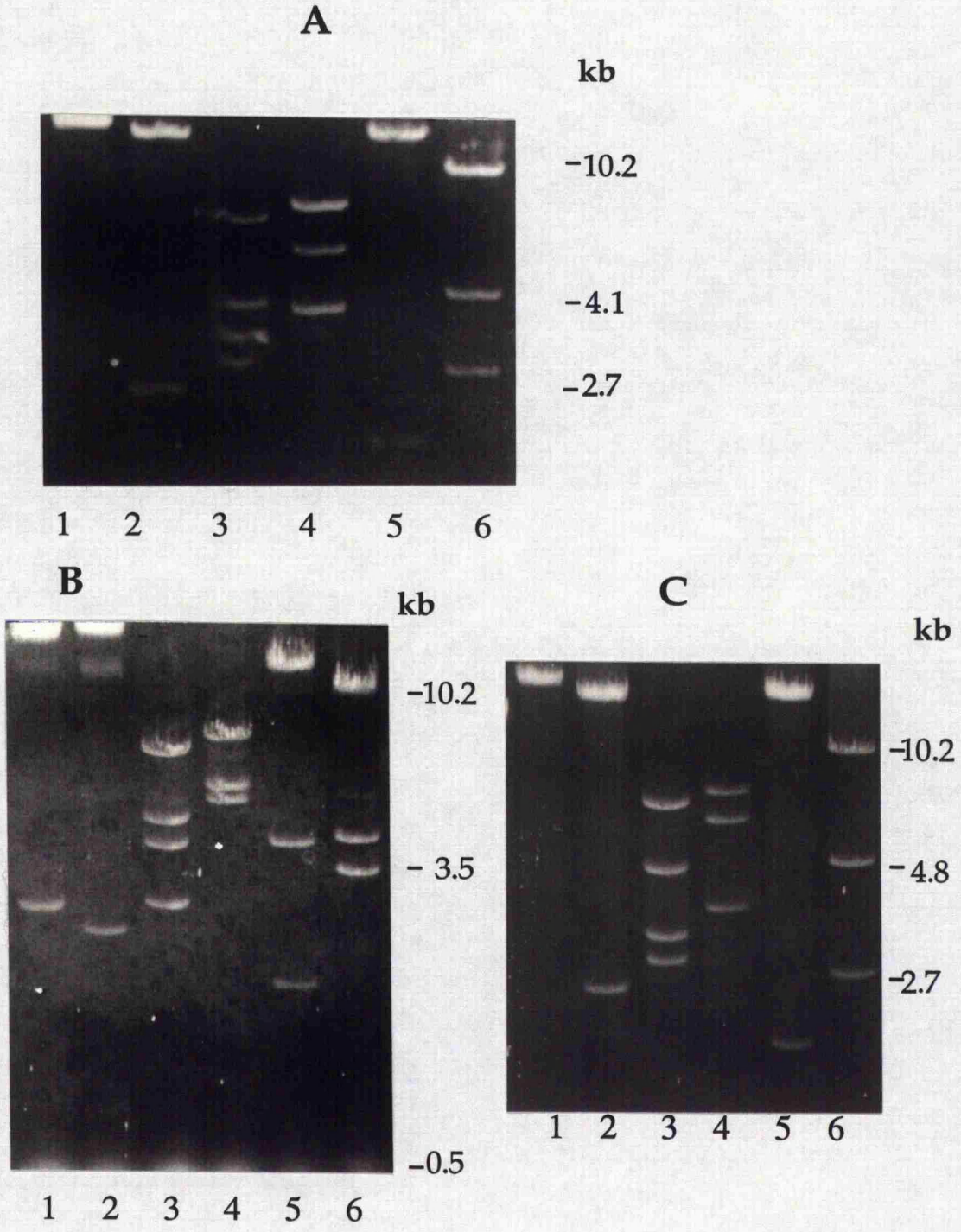

A
kb
-10.2
-4.1
-2.7
1 2 3 4 5 6
B
kb
-10.2
- 3.5
-0.5
1 2 3 4 5 6
C
kb
-10.2
-4.8
-2.7
1 2 3 4 5 6

Figure 4. 8.

Plasmids pNJR12 (A), pNJR12-1 (B) and pNJR12-2 (C) cleaved with restriction endonucleases *Cla*I (track 1), *EcoR*I (track 2), *EcoR*V(track 3), *Hinc*II (track 4), *Sma*I (track 5), or *Sst*I (track 6). Numbers to the right indicate molecular sizes in kb.

Table 4.3

DNA fragments generated by the cleaved of plasmid pNJR12, pNJR12-1 or pNJR12-2 by restriction endonucleases

Restriction endonuclease	Size of fragments (kb) generated following cleaved of plasmid DNA with restriction endonucleases		
	pNJR12	pNJR12-1	pNJR12-2
*Cla*I	17	2.7, 15.6	17.7
*EcoR*I	2.5, 14.5	2.5, 15.8	2.5, 15.2
*EcoR*V	2.8, 3.5, 4.0, 6.7	2.8, 4.0, 4.8, 6.7	2.8, 3.5, 4.7, 6.7
*Hinc*II	3.8, 5.8, 7.4	5.1, 5.8, 7.4	3.8, 6.5, 7.4
*Sma*I	1.5, 15.5	1.5, 3.9, 12.9	1.5, 16.2
*Sst*I	2.7, 4.1, 10.2	0.5, 3.5, 10.2	2.7, 4.8, 10.2

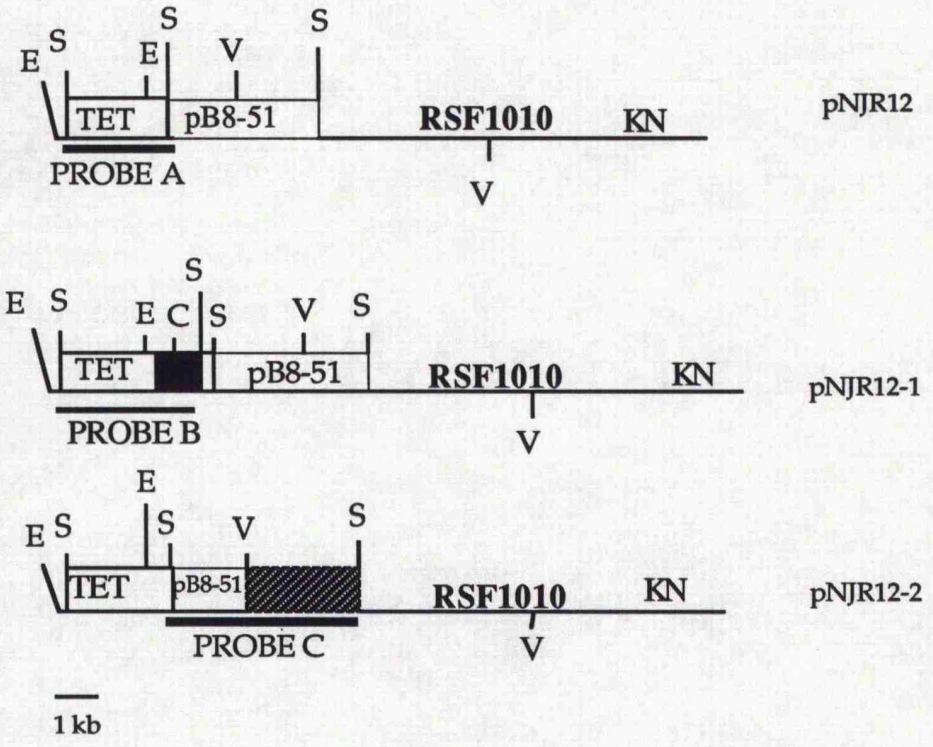


Figure 4. 9

Partial restriction maps of plasmids pNJR12, pNJR12-1 and pNJR12-2. The restriction endonuclease sites shown are: C, *Cla*I; E, E*coR*I; S, *Sst*I and V, *EcoR*V. The hatch-filled box represents the fragment of pNJR12-2 which carries the *E. coli* insertion sequence. The filled box represents IS *1126*. TET indicates the tetracycline resistance gene from a *Bacteroides* transferable chromosomal element. KN denotes kanamycin resistance. Probes A, B and C were used in Southern blot analysis of chromosomal DNA from *E. coli* and *P. gingivalis* to determine the origin of the two IS-elements (fig 4.10).

containing the 0.7 kb insert was also used (probe C).

When probe A was used it bound to the 2 positive controls (pNJR12 and pNJR12-1) but to neither *P. gingivalis* nor *E. coli* chromosomal DNA (fig 4. 10). However, when the same samples were probed with probe B a large number of hybridising fragments in the digested *P. gingivalis* DNA were found whereas there was no hybridisation of probe B to the chromosomal DNA of *E. coli* (fig. 4. 10). This indicated that the additional DNA in pNJR12-1 originated from the chromosome of *P. gingivalis*. Since, bacterial IS-elements are often found in multiple copies on the host chromosome (Galas and Chandler, 1989) the binding patterns obtained were characteristic of an IS-element. Therefore, the additional 1.3 kb of DNA present in pNJR12-1 was designated IS*1126*. Probe C did not hybridise to *P. gingivalis* W83 chromosomal DNA but to that from *E. coli* and also gave a pattern characteristic of an IS-element (fig. 4. 10) and must therefore have been acquired either prior to transfer of the vector from *E. coli* to *P. gingivalis* or after the plasmid was transformed into *E. coli*.

4. 2. 3. Effects of IS*1126* on transfer frequency

Mating experiments were conducted using pNJR12 and pNJR12-1 to determine whether the presence of IS*1126* in pNJR12 would alter the transfer of the vector to *P. gingivalis* recipients. Attempts were made to use donor cultures at similar cfu/ml. No difference was found in the frequency of transfer (Table 4. 4).

Total DNA from eight *P. gingivalis* transconjugants recovered following conjugation using pNJR12 and five from experiments which used pNJR12-1 were cleaved with *Sst*I and then probed with pNJR12. Five of the eight pNJR12 transconjugants contained pNJR12 whilst three had a plasmid with a restriction enzyme profile typical of pNJR12-2

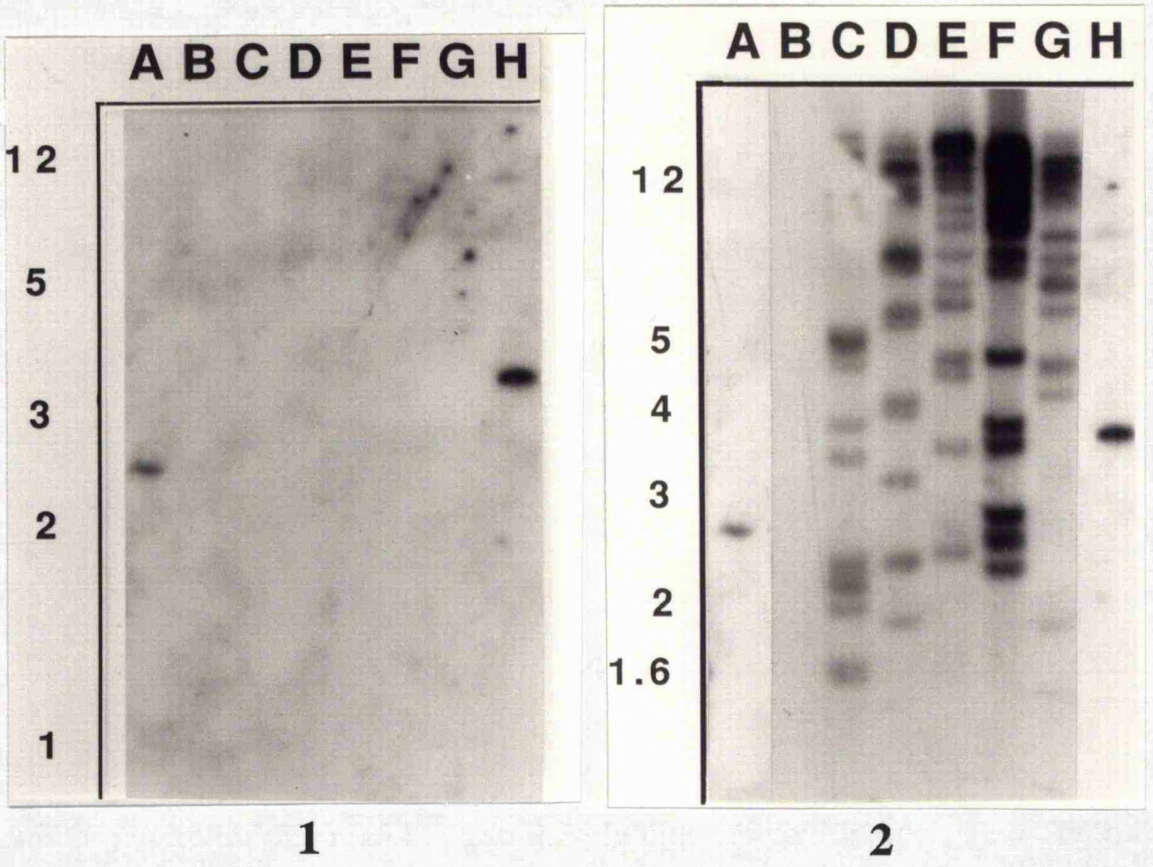


Figure 4. 10

Southern blot analysis of chromosomal DNA from *P. gingivalis* and *E. coli* probed with radiolabelled probe A (1) or probe B (2). Plasmids pNJR12 (lane A) and pNJR12-1 (lane H) were digested with the restriction endonuclease *Sst*I. *E. coli* chromosomal DNA was digested with the restriction endonuclease *BamH*I (lane B). *P. gingivalis* W83 chromosomal DNA was cleaved with the following restriction endonucleases *Sst*I (lane C), *Hinc*II (lane D), *EcoR*V (lane E), *EcoR*I (lane F) or *BamH*I (lane G). The sizes are given in kb.

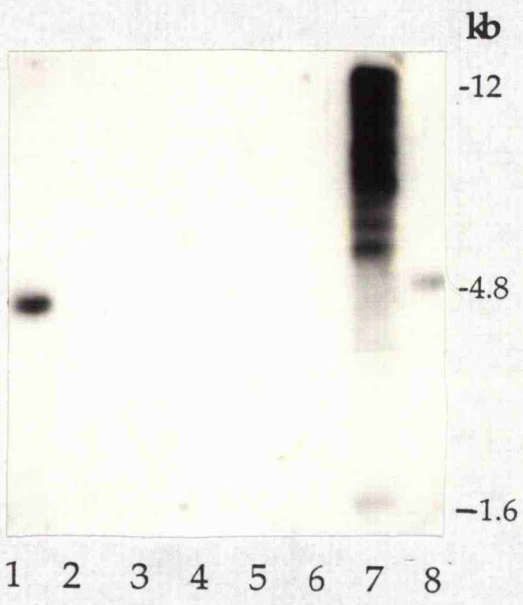


Figure 4. 10

Southern blot analysis of chromosomal DNA from *P. gingivalis* W83 and *E. coli* K54 probed with radiolabelled probe C. Plasmids pNJR12 (lane 1) and pNJR12-1 (lane 8) were cleaved with the restriction endonuclease *Sst*I. *P. gingivalis* W83 chromosomal DNA was cleaved with the restriction endonucleases *BamH*I (lane 2), *EcoR*I, (lane 3) *EcoR*V (lane 4), *Hinc*II (lane 5), or *Sst*I (lane 6). *E. coli* chromosomal DNA was cleaved with the restriction endonuclease *Sst*I (lane 7). The sizes are given in kb.

Table 4. 4.

Effect of IS *1126* on the transfer frequency of pNJR12 into *P. gingivalis*.

Starting cfu/ml: pNJR12 *E. coli* SF8-2.76x10^9
pNJR12-1 *E. coli* JM101-9.2x10^7
P. gingivalis W83- 5.00x10^7

Mating Time (h)	Donor :Recipient Ratio 1.1:1		1:2.7		1:1.8		1:5.4		1:3.6		1:11	
	pNJR12	pNJR12-1	pNJR12	pNJR12-1	pNJR12	pNJR12-1						
19	0	4.3x10^{-11}	0	0	0	0						
41	0	7.0x10^{-10}	0	2.9x10^{-10}	0	2.9x10^{-10}						
46	7.8x10^{-10}	3.1x10^{-9}	3.3x10^{-10}	5.2x10^{-11}	1.1x10^{-10}	1.2x10^{-9}						
65	0	4.7x10^{-10}	0	1.2x10^{-9}	0	2.5x10^{-9}						

Starting cfu/ml: pNJR12 donor-2.76x10^8
pNJR12-1 donor 9.2x10^7
recipient - 9x10^7

Mating Time (h)	Donor : Recipient Ratio 1:1.6		1:5		1:3.3		1:10		1:6.5		1:20	
	pNJR12	pNJR12-1	pNJR12	pNJR12-1	pNJR12	pNJR12-1						
19	0	5.9x10^{-11}	0	2.0x10^{-9}	0	9.4x10^{-10}						
41	1.7x10^{-11}	5.2x10^{-11}	1.5x10^{-10}	1.1x10^{-10}	2.6x10^{-10}	1.1x10^{-11}						
46	9.2x10^{-10}	2.1x10^{-10}	1.1x10^{-9}	6.0x10^{-10}	6.0x10^{-10}	6.0x10^{-10}						
65	1.0x10-9	2.1x10^{-10}	ND	3.6x10^{-10}	5.4x10^{-10}	4.9x10^{-10}						

Table 4. 4 continued

Starting cfu/ml : pNJR12 donor - 4.1×10^8
pNJR12-1 donor - 9×10^7
recipient - 8.1×10^6

Mating Time (h)	Donor : Recipient Ratio 1:1	1:1	5:1	5:1	10:1	2:1
	pNJR12	pNJR12-1	pNJR12	pNJR12-1	pNJR12	pNJR12-1
16	0	0	0	0	0	0
23	0	3.1×10^{-8}	0	0	0	0
44	1.2×10^{-8}	0.9×10^{-7}	6.0×10^{-9}	2.1×10^{-8}	3.1×10^{-10}	5.5×10^{-9}
56	0.7×10^{-8}	2.3×10^{-8}	0	4.2×10^{-8}	0	8.1×10^{-10}

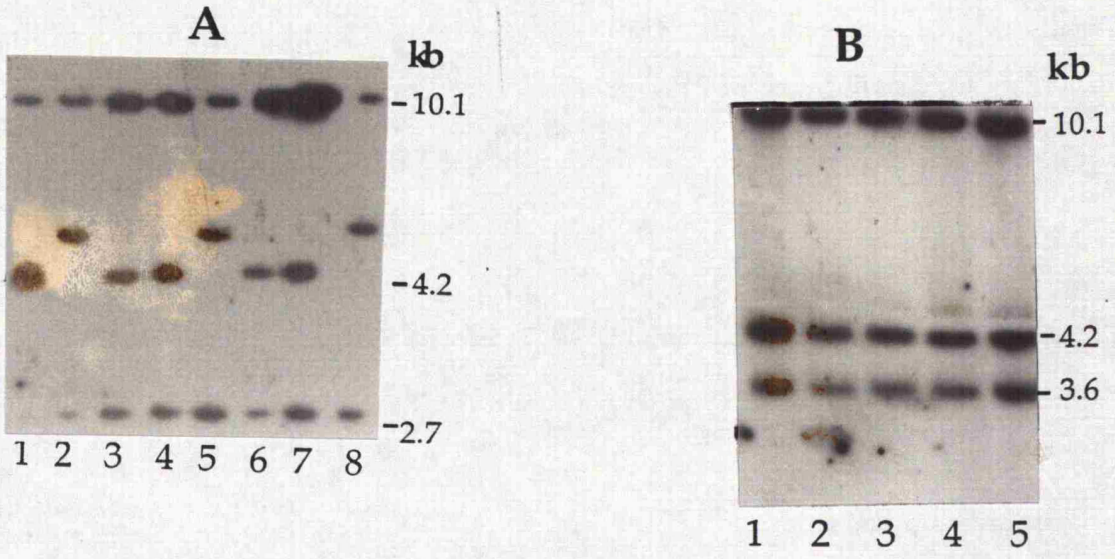


Figure 4. 11

Southern blot analysis of total DNA lysates purified from *P. gingivalis* W83 transconjugants following conjugal transfer of pNJR12 (A) or pNJR12-1 (B). The DNA was digested with the restriction endonuclease*Sst*I and probed with radiolabelled pNJR12. Panel A includes transconjugants harbouring pNJR12 (tracks 1, 3, 4, 6, and 7) or pNJR12-2 (tracks 2, 5 and 8). In panel B, all transconjugants contain pNJR12-1. Sizes are given in Kb.

(fig 4. 11). All five of the pNJR12-1 transconjugants produced pNJR12-1 profiles suggesting that no further recombination or insertions had occurred (fig 4. 11).

DNA from the transconjugants was also probed with probe B (fig. 4.9). All five transconjugants containing pNJR12-1 exhibited the same IS*1126* pattern as wildtype *P. gingivalis* W83 with the exception of a 3.5 kb fragment which had increased in intensity and represented the 3.5 kb fragment of pNJR12-1 (fig. 4.12). All transconjugants which had been shown to contain pNJR12 or pNJR12-2 contained an additional 2.7 kb fragment which represented the smallest *Sst*I fragment of pNJR12. However, the transconjugants containing pNJR12 also contained a 4.2 kb fragment which was not found in any transconjugants harbouring any other type of plasmid (fig 4.12). Transconjugants containing pNJR12-1 and pNJR12-2 did not contain any other band which had increased in intensity.

4. 2. 4. Tn*4351* transposon mutagenesis of *P. gingivalis*

On the basis of the results from the mating experiments using pNJR12, the conditions required for the introduction of foreign DNA into *P. gingivalis* had been established. Therefore it was decided that the main objective of this study, the production of *P. gingivalis* transposon mutants, be pursued. The suicide vector R751::Tn*4351*Ω4 was also transferred to *P. gingivalis* W83 by conjugation from *E. coli*. Once again the efficiency of recovery of clindamycin resistant colonies was greatly affected by the starting cfu/ml of the recipient cells whereby higher transfer frequencies were obtained when younger recipient cultures were used in matings (Table 4. 5). Increasing the recipient culture from $1.7x10^5$ cfu/ml to $1.2x10^8$ cfu/ml caused the frequency to drop from $2.8x10^{-4}$ to $8.5x10^{-9}$. However, since the use of a recipient culture with $4.5x10^5$ cfu/ml resulted in a frequency of only $5.2x10^{-7}$ transconjugants/recipient at the end of the mating then clearly other factors such as the duration of mating time, donor cfu/ml and donor:recipient ratio must also have influence on the conjugation process. Chromosomal DNA from nine *P.*

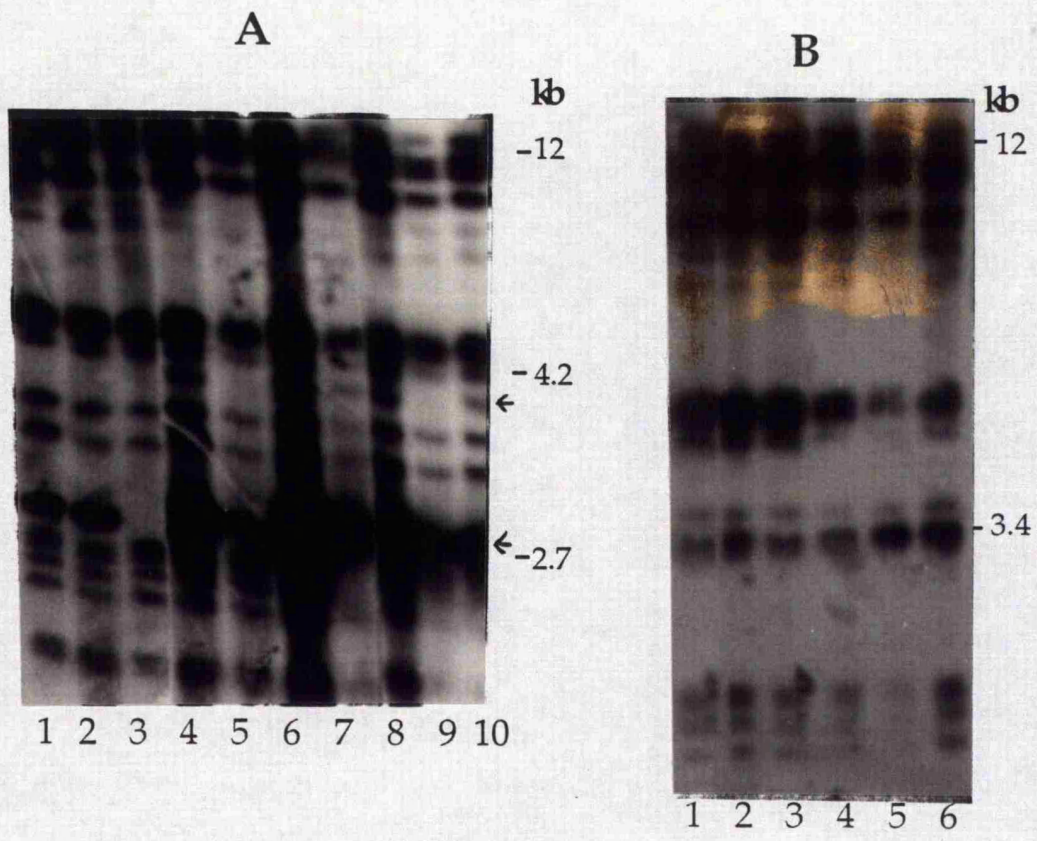


Figure 4. 12

Southern blot analysis of total DNA lysates from *P. gingivalis* W83 transconjugants following conjugal transfer of pNJR12 (A) or pNJR12-1 (B). The DNA was digested with the restriction endonuclease *Sst*I and then probed with probe B. The arrows indicate additional bands. Samples in panel A tracks 1, 2, 5 and 9 correspond to pNJR12-2. Samples in panel A tracks 4, 6, 7, 8 and 10 represent cells containing pNJR12. Tracks in panel B contain pNJR12-1 transconjugants (B2-6). *P. gingivalis* W83 chromosomal DNA is in tracks A3 and B1. The sizes are in kb.

Table 4. 5

Conjugal Transfer of R751::Tn*4351*Ω4 into *P. gingivalis* W83

Starting cfu/ml		Donor:Recipient	Mating	Transfer †
Donor	Recipient	Ratio	Time (h)	Frequency
$1.7x10^7$	$1.2x10^8$	1:34	72	$8.5x10^{-9}$
$2.8x10^7$	$4.5x10^5$	12:1	48	$5.2x10^{-7}$
$1.4x10^7$	$1.7x10^5$	16:1	72	$4.2x10^{-4}$
$1.4x10^7$	$1.7x10^5$	1.6:1	48	$2.8x10^{-4}$
$1.4x10^7$	$5.0x10^7$	1:18	48	$4.0x10^{-8}$

† Transfer frequenies are expressed as the number of transconjugants per recipient cell at the end of the mating procedure.

gingivalis Tn*4351* mutants was extracted and digested with *Hind*III and then probed with the 3.8 kb *EcoR*I fragment from the R751::Tn*4351* Ω4 which is specific for Tn*4351* (fig. 4. 14). If Tn*4351* had inserted only once into the chromosome of *P. gingivalis* then two bands would be expected. The bands would be at least 1.2 kb and 3.6 kb depending on their location in the chromosome. However, in all cases, multiple bands were present and three of these, 3.2, 3.8, and 4.8 kb *Hind*III fragments, were found in all nine mutants examined (fig 4. 13).

In about 50% of *Bacteroides* Tn*4351* insertional mutants there is also cointegration of R751 (Shoemaker *et al.*, 1986). In order to determine whether the insertion of Tn*4351* also resulted in the cointegration of R751, in *P. gingivalis* W83, R751 was used as a radiolabelled probe. Chromosomal DNA from a number of *P. gingivalis* W83 Tn*4351* mutants was digested with *EcoR*I. All samples were found to contain a faint band of 3.6 kb (fig 4. 13).

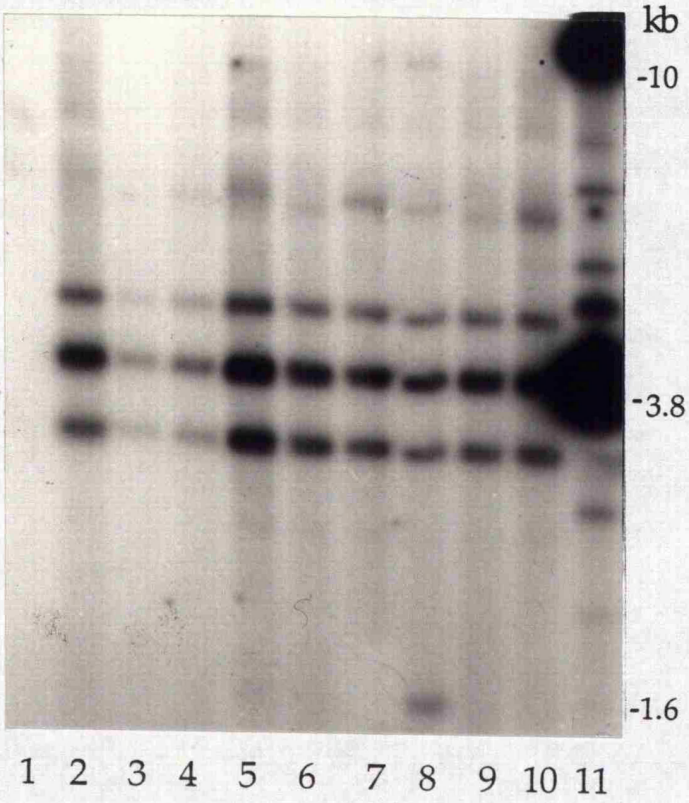
4.3 Discussion

4. 3. 1. Introduction of plasmid DNA into *P. gingivalis* W83

Conditions which allowed for reproducible transfer of plasmid DNA into *P. gingivalis* W83 appeared to be when the recipient cells be at very early growth phase of no more than $1x10^6$ cfu/ml. Using such cells meant that the optimal mating time was around 40h before transferring the mixture to selective media. Plates should then be incubated for 10-14 days depending on the vector used. However, there appear to be a number of variables which can influence the efficiency of transfer of vectors into *P. gingivalis* W83. Altering one variable may require changing others to compensate for this.

However, this study was never intended to provide a definitive examination of the mechanisms involved in plasmid transfer to *P.gingivalis* W83. Rather, the aim was to

A



B

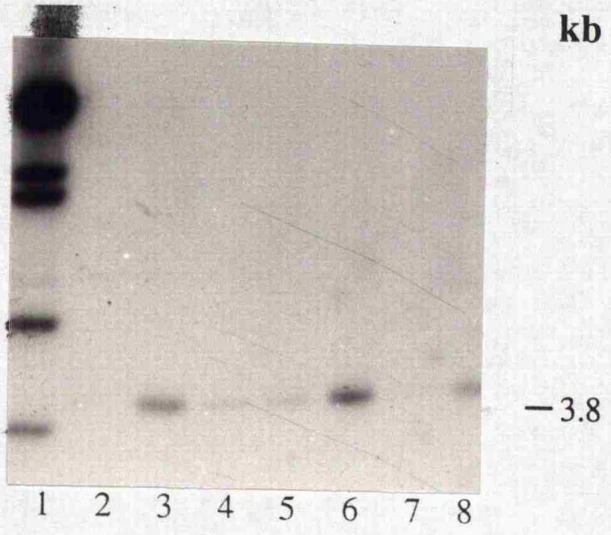


Figure 4. 13

Southern blot analysis of chromosomal DNA from *P. gingivalis* W83 Tn*4351* mutants probed with radiolabelled Tn*4351* specific probe (A) or R751 (B). Chromosomal DNA from *P. gingivalis* W83 wildtype (1) and nine Tn*4351* mutants (2-10) and plasmid R751::Tn*4351*Ω4 (11) were cleaved with the restriction enzyme *Hind*III. Plasmid R751::Tn*4351*Ω4 (1); chromosomal DNA from six *P. gingivalis* Tn*4351* mutants (3-8) and *P. gingivalis* W83 wildtype (2) were digested with restriction endonuclease *EcoR*I. Sizes are given in kb.

provide a reliable system for the introduction of plasmid DNA.

The highest frequencies obtained in this study were comparable to those routinely achieved in *Bacteroides* (Shoemaker *et al.*, 1985) and higher than those found in other studies using *P. gingivalis*. Other studies have isolated 10^{-7}-10^{-11} *P. gingivalis* transconjugants per recipient (Dyer *et al.*, 1992; Progulske-Fox *et al.*, 1989). The differences are probably a combination of the development of vectors with higher transfer frequencies and the use of different methods. Other studies have conducted mating experiments using recipient cells at later growth phases or cells scraped from plates as well as conducting matings for shorter times.

Transfer frequency was growth phase dependent with the cfu/ml of *P. gingivalis* W83 recipient cells apparently being the most influential factor in determining the conjugation frequency. On only two occasions did the use of cultures with greater than $1x10^8$ cfu/ml give rise to the appearance of any transconjugants. Generally speaking, lower cfu/ml cultures required slightly longer mating times. The effect of growth phase on transfer frequency in *P. gingivalis* ATCC 33277 was not studied (Hoover *et al.*, 1992b). However, other researchers have not found any difference (Dyer *et al.*, 1992) but the fact that it is possible to conjugate efficiently into other strains using recipient cells at a cfu/ml which is not usually successful in *P. gingivalis* W83 ($1x10^8$) (Progulske-Fox *et al.*, 1989b; Yoshimoto *et al*, 1993) would indicate that strain variation does exist.

Cell ratio of the donor and recipient cultures at the start of the mating procedure was also important. Plasmid pVal-1 was transferred in conditions where the cell ratio of donor to recipient was 1 (Dyer *et al.*, 1992). The optimum ratio for use in *P. gingivalis* strain ATCC 33277 was between 0.25 and 0.5, if the ratio was increased to 1.0 the transfer frequency was adversely affected by a decrease in the viability of recipient cells (Hoover *et al.*, 1992b). The reverse was found to be true for *P. gingivalis* strain W83 whereby excess donor to recipient increased transfer frequency. In colonic *Bacteroides* species, donor to recipient ratios of 0.1 to 4.0 are most effective (Shoemaker *et al.*, 1986).

Decreasing the number of donor cells relative to recipient decreased the transfer frequency but once the recipient cells were in excess of the donor cells, further increase had little additional effect on frequency.

In colonic *Bacteroides* exposure to oxygen during matings enhances the transfer efficiency (Shoemaker *et al.*, 1986). Matings involving these species are conducted in an aerobic environment (for 18-20h) and cells are not exposed to an anaerobic environment until transferred to selective media (Shoemaker *et al.*, 1986). Incubation of *P. gingivalis* W83 for 24h in an aerobic environment resulted in the total loss of viability, even when a culture of $4x10^8$ cfu/ml was used. Therefore, the effect of oxygen on the frequency of conjugal transfer of plasmids in *P. gingivalis* W83 was not examined. It has been shown that short aerobic incubation prior to anaerobic mating does increase the efficiency of transfer into other *P. gingivalis* strains (Dyer *et al.*, 1992; Hoover *et al.*, 1992b) and this would probably also be true for *P. gingivalis* W83. However, differences in aerotolerance among different strains exist since *P. gingivalis* strain 381 was found to remain viable after 24h in an aerobic environment (Dyer *et al.*, 1992).

The most important factor required for the detection of conjugation in *P. gingivalis* W83 was that the final selective plates be incubated for 2-3 times longer than the normal incubation for the routine growth of *P. gingivalis* W83 on non-selective media (4-5 days). Although some researchers have now succeeded in introducing foreign DNA into a range of *P. gingivalis* strains, they have not reported any difference among strains with respect to this (Dyer *et al.*, 1992; Hoover *et al.*, 1992b).

It has been seen that an individual strain varies in its ability to maintain closely related plasmid vectors. In the case of *P. gingivalis* W50, pE5-2 cannot be maintained (Yoshimoto *et al.*, 1993). However, this strain can be used as a recipient for another vector, pVal-1 (Dyer *et al.*, 1992). Although these two vectors contain the same *Bacteroides* antibiotic resistance gene and cryptic plasmid, they are clearly not similar enough to allow transfer of both into the same strain. However, there could be other

reasons why this should be so. The latter study involved 7-21 days incubation on selective media (Dyer *et al.*, 1992) as opposed to only 7 days when pE5-2 was used (Yoshimoto *et al* 1993). It is possible that apparently conjugation negative strains may have required longer incubation time in order for transconjugants to be recovered. Alternatively, it may be that the mating conditions used were just not suitable for use in some strains. It is also possible that *P. gingivalis* strain W50 is conjugative positive for both plasmid types only occasionally and that more than five mating experiments would have to be conducted in order to be sure of isolating any transconjugants.

The results presented here show quite clearly that for either of the two vectors used, much longer incubation times are required for *P. gingivalis* W83. Colony blot analysis of *P. gingivalis* transconjugants produced very weak signals when compared to *E. coli* and *B. uniformis* cells containing the same vectors. This could indicate low copy numbers of each plasmid in *P. gingivalis*. The delay in the appearance of resistant colonies may be a reflection of this low copy number, whereby the cells at first grow very badly due to insufficent production of the resistance gene products. *Porphyromonas gingivalis* W83 transconjugants containing pNJR12, pNJR12-1 or pNJR12-2 are resistant to 60μg/ml of tetracycline on solid media but do not grow well in broth cultures containing more than 5μg/ml. Similarily, although some pNJR5 containing transconjugants were originally isolated on 10μg/ml of clindamycin on agar, they would not grow in over 0.3μg/ml in broth media. No reference has ever been made with respect to the copy number of pE5-2 in strains of *P. gingivalis* (Progulske-Fox *et al.*, 1989b; Yoshimoto *et al.*, 1993) but difficulties in isolating pVal-1 from *P. gingivalis* transconjugants have been reported and the authors also suggested that this could be due to low copy number of this vector in this strain (Dyer *et al.*, 1992). The problem of plasmid purification was encountered with *P. gingivalis* W83 regardless of the plasmid used and this would suggest that such vectors are not that suitable for use in *P. gingivalis*. Plasmids pNJR5 and pNJR12 exist in *E. coli* at about 15 copies per cell. The difference in the signals from *P. gingivalis* transconjugants and *E. coli* or *B. uniformis* cells in the colony blot experiments would

indicate that these are present in *P. gingivalis* strain W83 in extremely low levels, with maybe even as little as one or two copies per cell. It may be that the creation of vectors based on the naturally occurring plasmids in other members of the genus may be of greater use for the genetic manipulation of *P. gingivalis* (Hoover *et al.*, 1992b).

Another factor which must be taken into account when conducting mating experiments in this species is that different strains demonstrate different antibiotic sensitivity. None of the strains used in the experiments with pVal-1 grew on media containing 100μg/ml of gentamicin (Dyer *et al.*, 1992). However, this concentration has been used to counterselect for *P. gingivalis* transconjugants (Hoover *et al.*, 1992b; Progulske-Fox *et al.*, 1989b; Yoshimoto *et al.*, 1993). Likewise *P. gingivalis* W83, in this study, grew well on 200μg/ml of gentamicin. Similar findings occurred with respect to erythromycin resistance. The MIC of the different strains varies with some being resistant to 0.2-0.3μg/ml (Dyer *et al.*, 1992), whilst *P. gingivalis* ATCC 33277 has background resistance to 10μg/ml and was therefore selected on 20μg/ml of erythromycin (Hoover, pers. comm). As such one should be cautious about generalising about the antibiotic sensitivity of different strains of *P. gingivalis*. Each strain should be checked prior to attempting any mating experiment.

Conjugation and electroporation studies on a number of *P. gingivalis* strains using pE5-2 as the vector demonstrated that the strains could be divided into three conjugation groups, low, high or negative depending on the number of transconjugants isolated. *Porphyromonas gingivalis* W83 was contained in the low conjugation group (Yoshimoto and Umemoto, 1993). However, the frequencies were expressed in terms of donor and the cfu/ml of the donor cells at the end of the mating was not indicated which makes it difficult to relate their findings to those presented here. Differences could be because the mating incubation only lasted for 24h, whereas here, at least 26h, but usually greater than 37h were required in order for the highest frequency to be achieved. Experiments using *P. gingivalis* ATCC 33277 as the recipient used mating times of 36-48h (Hoover *et al.*,

1992b) whilst 17-21h matings were used for studies involving the transfer of pVal-1 (Dyer *et al.*, 1992). However, since the latter study used cells scraped from solid media, it is difficult to compare their findings to those presented by other groups who all used liquid cultures.

Recently, the first successful transformation of *P. gingivalis* strains with plasmid DNA was conduted via electroporation (Yoshimoto *et al*, 1993). However, strain-specificity occurred and most strains could only be transformed with plasmid purified from the homologous strain indicating that as with many other bacterial species, differences in restriction/modification systems may be occurring. Because of the difficulties in obtaining sufficient amounts of plasmid DNA from *P. gingivalis* (Dyer *et al.*, 1992) very large amounts of starting culture would be required to isolate the concentration of plasmid DNA required for cloning. Coupled with the strain-specificity, the potential of electroporation for use in this species at present would appear to be very limited. As a result of this, conjugation remains the most successful way of introducing DNA into this organism and the use of bacterial conjugation has produced the first well-defined mutants of this species (Joe *et al.*, 1994; Park and McBride, 1993).

As more genetic studies are conducted on *P. gingivalis* strains, it is becoming clear that some strains produce identical genetic profiles as determined by RFLP analysis and MLEE. The ability and competence to accept plasmid DNA could be used to differentiate strains that exhibit identical profiles. For example, *P. gingivalis* strains W83 and W50 cannot be differentiated genetically (Loos *et al.*, 1993) however, as it was possible to introduce pE5-2 into *P. gingivalis* W83 but not into *P. gingivalis* W50 (Yoshimoto *et al.*, 1993) then clearly the two strains cannot be identical. *Porphyromonas gingivalis* strains ATCC 33277 and 381 cannot be separated on genetic profiles (Loos *et al.*, 1993). Although both strains were contained in the high conjugation group, plasmid DNA purified from *P. gingivalis* ATCC 33277 produces $7x10^3$ transformants/μg of DNA in

this strain but did not produce any in *P. gingivalis* 381 (Yoshimoto *et al.*, 1993) which would again suggest that these strains are different.

Purification of plasmid DNA from a *P. gingivalis* pNJR12 transconjugant revealed a recombinant form of pNJR12, pNJR12-1, which contained an additional 1.3 kb of DNA. When used as a radiolabelled probe this additional segment of DNA hybridised to multiple bands in *P. gingivalis* W83 restriction endonuclease cleaved chromosomal DNA. This pattern of binding was characteristic of an IS-element and the DNA which induced this pattern was termed IS*1126*. The presence of this element on pNJR12 probably occurred through the transposition of IS*1126* from the chromosome of *P. gingivalis*. Plasmid pNJR12-1 was isolated on only one occasion but to determine whether or not this event was rare would require analysis of many more transconjugants. The fact that it was possible to recover pNJR12 and the second type of recombinant plasmid, pNJR12-2 would indicate no functional requirement of IS*1126* for maintenance of the plasmid in *P. gingivalis*. Plasmid pNJR12-2 contained an *E. coli* insertion sequence, which was about the same size (700 bp) as IS*1*. This element must have originated from the donor chromosome. As multiple plasmid preparations made using the *E. coli* donor, never harboured any plasmid other than pNJR12, it would appear that this insertion sequence inserted into pNJR12 immediately before transfer to *P. gingivalis*. or after transforming the plasmid from *P. gingivalis* to *E. coli.*

The significance of the presence of both the *E. coli* insertion sequence and IS*1126* is unknown. The presence of IS*1126* in the vector did not enhance its frequency of transfer to *P. gingivalis* cells. The lack of any difference in frequency between the two plasmids may have been due to different donor cfu/ml. In all cases, the pNJR12 donor cultures were slightly older than the pNJR12-1 donors. Southern blot analysis revealed that the reintroduction of IS*1126* into *P. gingivalis* did not produce any other visible rearrangements in either the plasmid or the genome. All transconjugants harbouring pNJR12 exhibited an IS*1126* profile which contained an additional 4.2 kb SstI fragment

which could not be accounted for. The fragment could not be pNJR12 derived as the probe used was the 3.5 kb fragment containing IS*1126* which only hybridises with the 2.7 kb fragment found in pNJR12 and pNJR12-2. Also, no additional band was observed in the profile of transconjugants containing pNJR12-2, nor did these exhibit any fragment which had increased in intensity. The only conclusion would seem to be that this fragment is the product of some form of relocation or rearrangement of IS*1126* on the chromosome induced by the introduction of pNJR12 into the cell.

The characterisation of IS*1126* is discussed in detail in Chapter 5.

4. 3. 2. Transposon Mutagenesis

Tn*4351* integrates into the chromosome of *P. gingivalis* W83 but exactly what occurred remains unclear. Single random insertion of this element into this strain was not observed but has been found in *P. gingivalis* strains ATCC 33277 and 381 (Dyer *et al.*, 1992; Hoover *et al.*, 1992b). To determine more precisely the pattern of Tn*4351* insertion in *P. gingivalis* such studies may have to be conducted which include the use of a variety of different strains.

Like the matings using pNJR12, frequency of transfer in matings involving R751::Tn*4351*Ω4 varied with different conditions. Again the cfu/ml of the recipient culture was found to be important with an increase in cfu/ml associated with a decrease in transfer frequency. However, some recipient cultures which had been expected to produce higher frequencies did not. This again highlighted the fact that other variables may also affect the transfer frequency. Genomic DNA containing only one copy of Tn*4351*, cleaved with *Hind*III and probed with the Tn*4351* specific radiolabelled probe will generate two fragments of at least 1.2 and 3.6 kb (fig. 4. 14). If insertion occurred randomly then the size of the two fragments would differ among the mutants. However, insertion of the entire Tn*4351* duplication would generate an additional fragment of 3.8 kb in size (fig. 4. 14). The intensity of the 3.8 kb fragment in all of the mutants examined

suggests that the transposon had rearranged such that two complete copies of Tn*4351* and an almost complete copy had inserted site-specifically (fig 4.14). This could be supported by the presence of at least 5 bands in total in each mutant. The other bands could represent an additional, single copy of IS*4351*. Since the additional bands differ in size, then random insertion does occur but since the 3 kb and 4.8 kb fragments are present in all mutants then clearly the main insertional event is site-specific. Multiple insertions have been reported in some *P. gingivalis* 381 mutants (Dyer *et al.*, 1992). The authors suggested that such patterns could have been produced through multiple insertion of the transposon or by some rearrangement following insertion.

In *B. uniformis* 1001 some insertions of Tn*4351* and R751::Tn*4351*Ω4 produced auxotrophs most of which required methionine. The authors could not decide whether this was due to the existence of a transposition hot spot or, was a reflection of the organisation of the gene(s) involved in methionine biosynthesis. However, methionine auxotrophs were not predominant in Tn*4351* auxotrophs in *B. ovatus* 0038 or *B. thetaiotaomicron* 5482 (Shoemaker *et al.*, 1986). The *B. uniformis* 1001 auxotrophs were unstable and displayed high reversion frequencies. However, since most of the revertants remained resistant to erythromycin it was concluded that either there were multiple Tn*4351* insertions or secondary transpositions from the primary site (Shoemaker *et al.*, 1986).

In about 50% of *Bacteroides* Tn*4351* mutants, R751 integrated along with the transposon into the chromosome. However this integration usually entails the whole of the vector (Shoemaker *et al.*, 1986; Hoover *et al.*, 1992b). A number of integration-restriction schemes exist for R751::Tn*4351*Ω4 inserting into the chromosome of *Bacteroides* (Shoemaker pers. comm.) but since each involves the integration of the whole of R751, then none of these explain what occurred here. The fact that the band was faint would

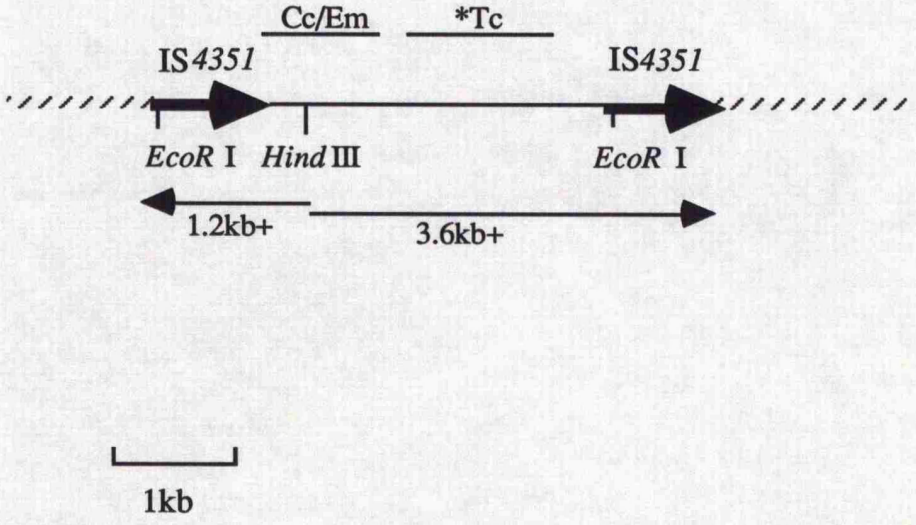


Figure 4. 14. a

Insertion scheme for one copy of Tn*4351* integrating into the recipient chromosome. The slashed lines represent the chromosome. Cc/Em and *Tc refer to clindamycin/erythromycin and tetracycline resistance respectively.

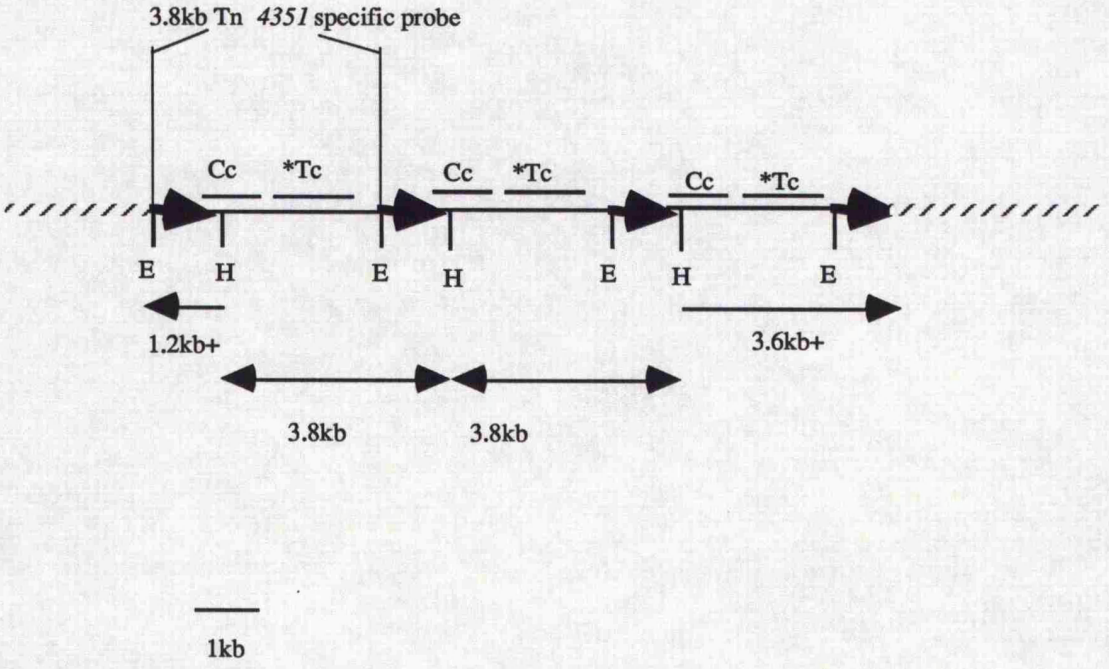


Figure 4. 14. b

Insertion scheme for rearranged copies of Tn*4351* integrating into the recipient chromosome. The slashed lines represent the chromosome. Copies of IS*4351* are indicated by the thick arrows Cc/Em and *Tc refer to clindamycin/erythromycin and tetracycline resistance respectively. The restriction endonucleases *EcoR*I and *Hind*III are represented by E and H respectively. The Tn*4351* specific probe used in Southern blot analysis of *P. gingivalis* Tn*4351* mutants (fig 4. 13) is also highlighted.

suggest that only a small amount of R751 had integrated. Although no attempt was made to determine precisely which region of R751 had inserted, it is likely that a small fragment immediately adjacent to the outside end of one of the copies of IS*4351* was carried into the chromosome by transposition of Tn*4351*.

In summary it would appear that Tn*4351* is inappropriate as a means for generating transposon mutants in *P. gingivalis* W83.

Chapter 5

Characterisation of IS*1126*

5. 1. Introduction

Bacterial transposable elements have been found in all genera in which they have been looked for (Cirillo *et al.*, 1991; van der Zee *et al.*, 1993). These elements may play a central role in evolution by providing mechanisms for the generation of diversity, and, in conjunction with DNA transfer systems, for its rapid dissemination to other bacteria (Ajioka *et al*; 1989; Terai *et al.*, 1991). Insertion sequence (IS) elements are the smallest form of transposable elements found in bacteria. These are segments of DNA capable of moving themselves into different positions and orientations in the host genome (Galas and Chandler, 1989). They can mediate DNA rearrangements that affect genetic organisation, gene expression and regulation and were originally identified in *E. coli* through the mutations they induced in the galactose and lactose operons (Malamy, 1970; Shapiro, 1969). Bacterial IS-elements are found in naturally occurring plasmids (Welch and Macrina, 1981; Smith and Gonda, 1985) and are present in various copy numbers on chromosomes throughout the bacterial kingdom (Mazel *et al.*, 1991; Coucheron, 1993). They range in size from 700-2500 bp and do not carry any detectable phenotypes but instead encode only the proteins required for the transposition process (Galas and Chandler, 1989).

Transposition is independent of homology between the sequences of the target DNA and the IS-element (Galas and Chandler, 1989). IS-elements are characterised by the presence

of inverted repeats at either end of the element which provide the active site for the activity of the transposase and are therefore essential for transposition (Derbyshire and Grindley, 1992). On insertion into the host these elements cause duplication of target site DNA as a consequence of staggered cleavage of target sequences during transposition with the length of the duplication being characteristic for each element (Mazel *et al.*, 1991). IS-elements can be found flanking DNA which is non-transposable in the absence of the IS-elements (Berg *et al.*, 1989; Kleckner, 1989). Such structures are transposons, the central region of which usually carries a detectable phenotypic trait, which is often resistance to an antibiotic (Smith and Gonda, 1985). Often the elements encodes one major ORF which often spans almost the entire length of the element, encoding the transposase enzyme (Galas and Chandler, 1989). One or more smaller ORFs overlapping the large ORF may also be present but their function is not always known (Rak *et al.*, 1982). Many IS-elements are species-specific due to coevolution with their host and this quality makes them extremely useful targets for the detection of pathogens in clinical specimens by means of PCR.

Bacterial transposable elements have become indispensable tools in molecular biology, with their properties being exploited for genetic analysis where they are used for insertional mutagenesis and the delivery of genes and regulatory sequences (for review see Berg *et al.*, 1989). A number of families of IS-elements have been described where the similar genetic organisation and sequence homologies between the members from different bacterial hosts suggest that the family members may share a common evolutionary origin. Until recently the largest family was the IS*3* family (Fayet *et al.*, 1990) whose members transposases most conserved domain is also shared with a number of retroviruses (Kulkosky *et al.*, 1992). However, a new family, the IS*4* family, has recently been identified (Reszohazy *et al.*, 1993). This family contains in excess of 40 IS-elements which are derived from a wide variety of different bacteria (Reszohazy *et al.*, 1993). The amino acid sequence of the transposase protein of members belonging to this family exhibits two highly conserved domains. Each domain has a conserved core motif.

There is an amino acid C-terminal region which can be up to 60 amino acids long (C1) whilst the second motif is located in a 29 amino acid sequence at the N-terminal (N3) and these regions correspond to the domain shared by the IS*3* family and retroelements. The family is divided into two subgroups depending on the location of the domains in relation to each other. The IS*4* subgroup contains elements where the distance between the two domains is 20-110 amino acids in length. IS-elements who have the N3 and C1 immediately adjacent to one another are contained in the IS*5* subgroup (Reszohazy *et al.*, 1993).

In this chapter the nucleotide sequence of IS*1126* was determined and the distribution of the element among the species and genus examined.

5. 2. Results

5. 2. 1. Attempts to clone IS*1126* in *E. coli*

In order to determine whether IS*1126* could cause cointegrate formation and therefore, transposition in *E. coli*, attempts were made to clone the *Hin*cII fragment of pNJR12-1, containing the whole of IS*1126* (fig. 4.9) into a variety of *E. coli* vectors including pUC18, pACYC184 and pBR328. In all cases no recombinants could be isolated. However, it was possible to clone both the 3.5 kb *Sst*I fragment and the 1.3kb *EcoR*I/*Sst*I fragment into pUC18 which contained all but 128 bp of IS*1126* (Section 5. 2. 4).

5. 2. 2. Nucleotide sequence of IS*1126*

The sequencing strategy (fig 5.1) involved cloning various fragments in both M13mp18 and M13mp19. A number of oligonucleotides were also obtained which allowed priming on fragments too large to sequence using the universal primer.

IS*1126* was found to be located 171 bp upstream of the start codon for the tetracycline resistance gene in pNJR12. As IS-elements were originally identified through their ability to alter expression of genes located downstream of the insert site, it was thought that pNJR12-1 containing W83 transconjugants might have increased resistance to tetracycline when compared to pNJR12 containing cells. However, no difference was found nor, when transferred, did it confer resistance in *E. coli*.

The DNA sequence of IS*1126* was found to be 1338 bp in length with 12 bp perfect inverted repeats at the termini of the element (fig 5. 2) with the entire element being flanked by direct repeats of the sequence GTAGG which represents duplication of the target site in pNJR12. The G and C content of the nucleotide sequence of IS*1126* was calculated to be 47.4% which is comparable to that found in this species (46-48%) suggesting that this element is of *Porphyromonas* origin.

Various host proteins are known to influence the transposition process (Galas and Chandler, 1989) and a number of binding sites for host proteins were searched for. A sequence from 1115-1125 bp had only one mismatch with the consensus sequence $AA(N)_4TTGAT$ of the histone-like protein IHF. Two mismatches were found for the sequence TTATCCACA of DnaA protein from position 1160-1170 bp. Dam methylation sites were found at positions 231-234 bp, 243-246 bp and 1183-1186 bp (fig 5. 2).

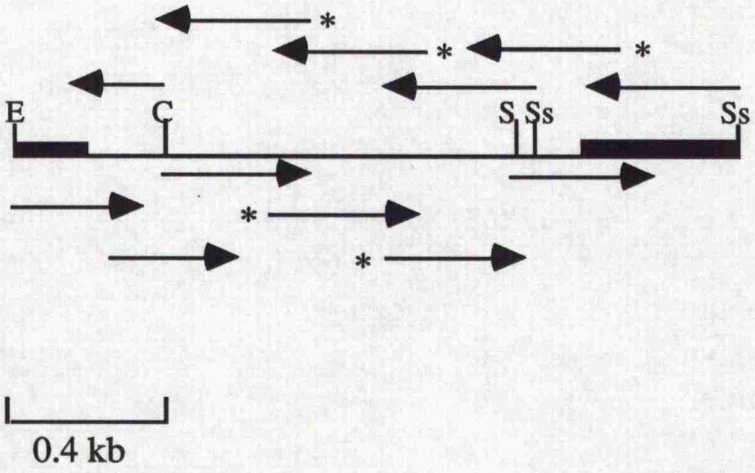


Figure 5. 1 Sequencing strategy

Restriction enzyme cleavage map of IS*1126* in pNJR12-1. The thick and thin lines represent pNJR12 and IS*1126* DNA respectively. The plain arrows indicate data obtained using the universal primer. The arrows marked with a star represent reactions primed with oligonucleotides. Restriction enzyme target sites: C, *Cla*I; E, *EcoR*I; S, *Sma*I; Ss, *Sst*I.

```
GAGACCTTTGCAcggcgattggcgtatattttgtttgttaattcattgtataataggaag
        10                  30                  50

              -35               -10          M  A  Y  Q  S
ttatttcgtatattttgagtattaaaaacagcataatattcctcccatggcataccaatcc
        70                  90                  110

K  N  T  D  E  H  V  T  F  A  D  A  L  L  S  K  R  Y  R  K
aagaataccgatgagcatgtaacatttgcagacgcactcctttcaaagcgttatcgcaaa
       130                 150                 170

A  Q  N  D  F  L  N  Q  V  E  R  L  I  D  W  R  P  I  R  D
                                      ClaI
gcacaaaacgacttcctcaatcaggttgagaggcttatcgattggcgtccgatcagggac
       190                 210                 230 Dam

V  I  N  K  K  Y  T  K  R  Q  N  A  M  R  A  P  A  Y  D  V
gtgatcaacaagaaatacacgaagcgacaaaatgccatgcgcgccccggcttatgacgtg
   Dam 250                 270                 290

I  L  L  F  K  M  L  L  L  E  T  W  Y  N  L  S  D  C  A  L
attctcttattcaagatgttgcttttggagacatggtacaacctcagtgattgtgctttg
       310                 330                 350

E  E  R  I  N  D  S  I  T  F  S  R  F  L  G  L  K  M  E  E
gaggagcgcatcaatgattcaatcacctttcccgattcttgggactgaagatggaagag
       370                 390                 410

V  S  P  D  H  S  T  I  S  R  F  R  S  A  L  T  E  L  G  S
gtatctcccgaccacagcaccatcagtcgatttcgttcggcactgacagagttgggctca
       430                 450                 470

W  T  N  Y  W  R  S  L  T  N  N  F  P  A  I  T  F  R  S  G
tggacaaactattggcgcagtttaacaaacaactttcccgccatcacatttcggtcaggg
       490                 510                 530

K  G  C  L  S  M  Q  A  L  W  R  R  H  I  K  P  N  G  T  I
aaggggtgcttgtcgatgcaagccttgtggagacgccacataaagcccaacggaaccatt
       550                 570                 590

T  I  E  V  A  A  N  R  E  D  N  R  S  E  A  E  K  E  D  E
acgattgaagtcgcagcgaacagagaagacaatcggagcgaggcggaaaaagaggacgag
       610                 630                 650

E  D  Y  Q  K  Q  V  V  R  R  R  K  G  T  D  E  E  A  V  G
gaggattatcaaaaacaggttgtccgccggcgtaaagggacggatgaagaagccgttggg
       670                 690                 710

C  T  N  K  S  V  I  T  T  D  T  K  S  I  V  L  T  N  V  Q
tgtacaaacaaaagcgttatcactacggatacaaaaagcattgtcttgaccaatgttcaa
       730                 750                 770

G  I  V  Q  K  V  I  T  T  A  A  N  R  S  D  T  K  E  F  I
ggcattgttcaaaaggtgataacgacagcagcgaaccgcagtgacacgaaggagtttatt
       790                 810                 830


P  L  L  Q  G  A  N  I  P  Q  G  T  A  V  L  A  D  K  G  Y
ccgctattgcagggtgcaaacatacctcaaggcacagccgtcttggcggacaaaggatat
       850                 870                 890
```

```
A  C  G  E  N  R  S  Y  L  Q  T  H  H  L  Q  D  G  I  M  H
gcttgcggggaaaatcgttcctacctgcaaacccatcaccttcaagacggcatcatgcac
       910                 930                 950

K  A  Q  R  N  R  A  L  T  E  E  E  K  Q  G  N  K  A  I  S
aaggcacaacgcaacagggcattgaccgaggaagagaagcaaggaaacaaagcaatcagt
       970                 990                1010

P  I  R  S  T  I  E  R  T  F  G  S  I  R  R  W  F  H  G  G
ccgatacggagcaccatcgaacgcacctttggcagtattcgccggtggtttcatggcgga
      1030                1050                1070

R  C  R  Y  R  G  L  A  K  T  H  T  Q  N  I  L  E  S  I  A
cgatgtcgataccggggacttgccaagacccatactcaaaacattcttgaaagcatcgcc
      1090                1110      IHF       1130

F  N  L  Y  R  T  P  G  I  I  M  S  S  S  L  G  *
                 SmaI
tttaatttatacagaaccccggggataattatgtcctcatctctaggataaggcataacc
      1150          dnaA  1170              Dam1190

          SstI
ccccttgaggagctcgtgcaagtacgtccgcaaggggggatttacaactactttcactcc
      1210                1230                1250

ttactgccacccttttcactcgctccttttttgccaagaactcctcttccctccatctcc
      1270                1290                1310

ttatttTGCAAAGGTCTC
      1330
```

Figure 5. 2.

Complete nucleotide sequence of the 1,338 bp insertion element IS*1126* and deduced amino acid sequence of ORF1. The 12 bp terminal inverted repeats are shown in uppercase typeface. Underlined nucleotides correspond to putative -35 and -10 promoter regions and transcription terminators. Putative binding sites for host proteins are also highlighted. Selected restriction endonuclease sites are shown in italics.

DNA database searches were conducted using the FASTA program (Pearson and Lipman, 1988) and IS*1126* was found to have nucleotide homology with other IS-elements. In a 530 bp overlap from the reverse of position 132 bp to 638 bp, there was 51.7% identity with the sequence of IS*5* from *E. coli* (fig 5.3.a). A similar amount of homology was found against IS*1106* from *Neisseria meningitidis*, which contained 58.2% identity and stretched from position 698 bp over a length of 306 bp to 1002 bp (fig 5.3.b). The inverted repeats of IS*1126* display the three external nucleotides GAG characteristic of sub-group 5 of the IS*4* family of IS-elements. Sequences immediately beside the repeats also have homology to the ends of IS-elements contained within the IS*5* subgroup (fig 5.4).

5. 2. 3. Presence and analysis of open reading frames (ORFs) on IS*1126*

IS*1126* nucleotide sequence was examined for the presence of ORFs. Only 2 ORFs were found which were greater than 50 amino acids in length. One of these, ORF1 extended from position 106 to 1191 bp on IS*1126* (fig. 5.2). ORF 2 was much smaller and overlapped ORF1 covering the reverse of positions 844 to 1113 bp (not shown). ORFs 1 and 2 could probably encode for proteins of 361 and 90 amino acids in length, with the theoretical isoelectric points being 10.26 and 8.34, respectively. The potential molecular weights were 41 kDa for ORF1 and 11 kDa for ORF2. Putative *E. coli* promoter sequences were identified for ORF1 as was a potential transcription termination stem loop structure (fig 5.2).

The predicted amino acid sequence of each ORF was used in searches of protein databases using the FASTA program (Pearson and Lipman, 1988). Homology was only found for ORF1. The predicted protein of ORF1 had homology to putative transposases

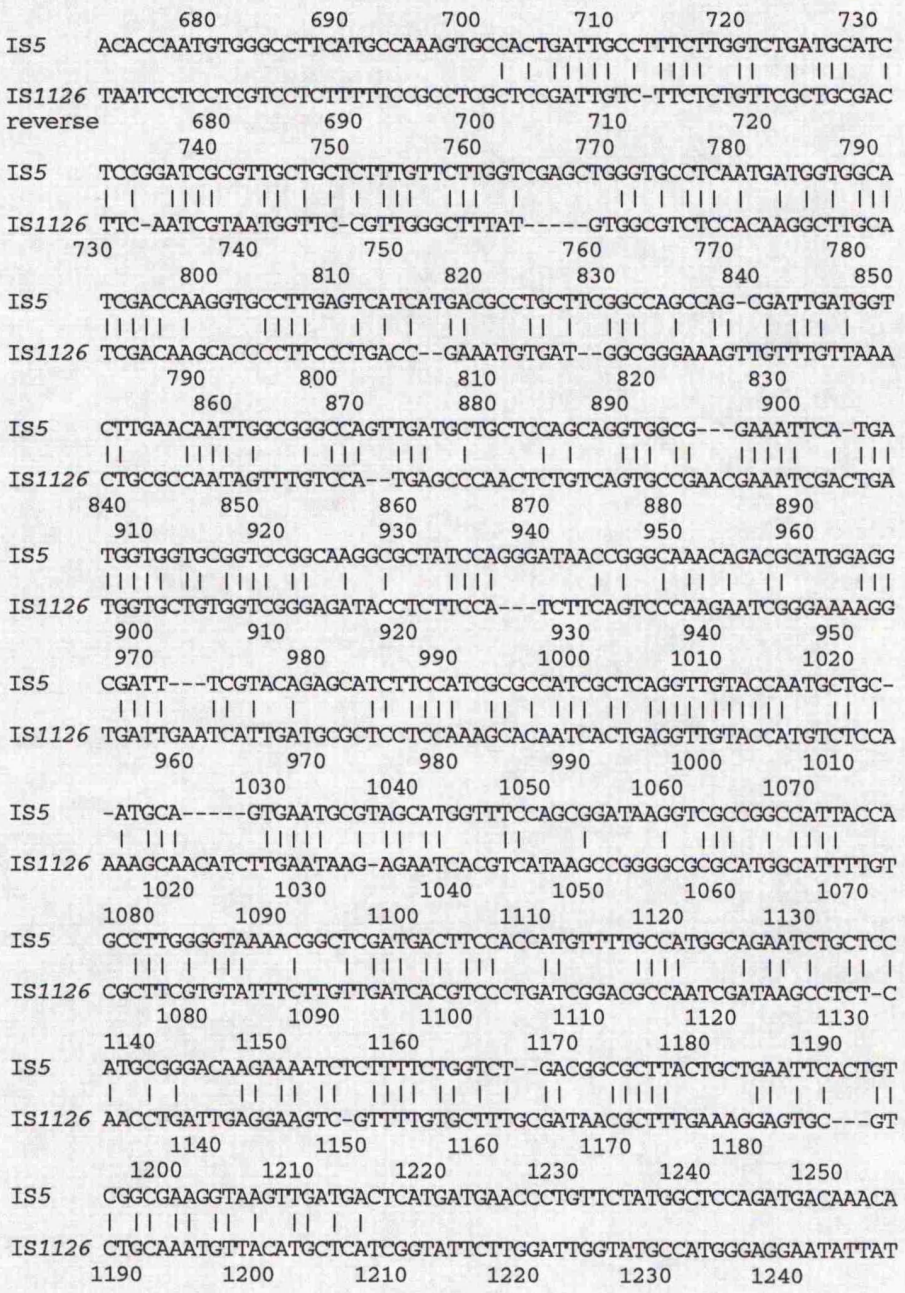


Figure 5. 3a

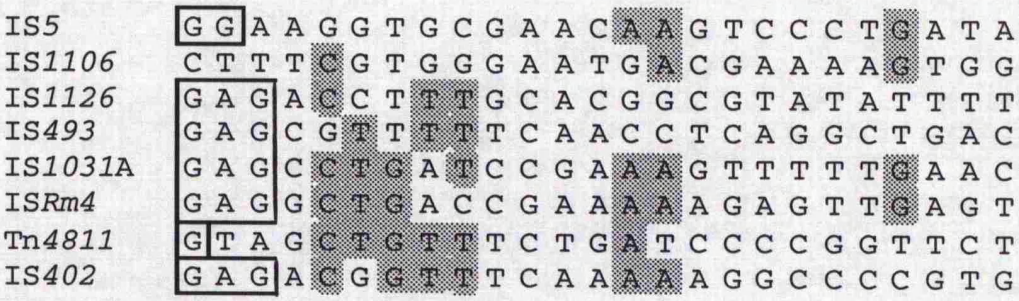
Comparison between the nucleotide sequence of IS1126 and IS5. A match line indicates identical nucleotides. The sequences are numbered according to their position in the complete sequence.

```
       670        680        690        700        710        720
IS1126 ATCAAAAACAGGTTGTCCGCCGGCGTAAAGGGACGGATGAAGAAG-CCGTTGGGTGTACA
                                        | | |||  |||| | |||||||  |  | |
IS1106 AAGGACAAATCAGCGGCCAAACCACACCGAGTAAGGACAAAGATGCCCGTTGGATAAAGA
             910        920        930        940        950        960

       730        740        750        760        770        780
IS1126 AACAAAAGCGTTATCACTACGGATACAAAAAGCATTGTCTTGACCAATGTTCAAGGCATT
       || |    |  ||  |   ||| |||||| | |||   | | ||| |||   |||||  |
IS1106 AAAACGGCCTCTACAAACTCGGTTACAAACAACATACCCGT-ACCGATGCGGAAGGCTAT
             970        980        990       1000       1010       1020

       790        800        810        820        830        840
IS1126 GTTCAAAAGGTGATAACGACAGCAGCGAACCGCAGTGACACGAAGGAGTTTATTCCGCTA
           | ||  ||   |  ||  | || ||   |  |||    ||  |  |    ||| |
IS1106 ACCGAGAAACTGCACATTACCCCCGCCAATGCCCATGAGTGCAAACACCTGCCGCCGTTG
             1030       1040       1050       1060       1070       1080

       850        860        870        880        890        900
IS1126 TTGCAGGGTGCAAACATACCTCAAGGCACAGCCGTCTTGGCGGACAAAGGATATGCTTGC
       |||  |  ||      ||  |  ||  |||| ||  ||||||  || ||||||||| ||||   |
IS1106 TTGGAAGG-----AC-TGCCCAAAGGTACGACCGTCTATGCCGACAAAGGCTATGACAGT
                  1090       1100       1110       1120       1130

       910        920        930        940        950        960
IS1126 GGGGAAAATCGTTCCTACCTGCAAACCCATCACCTTCAAGACGGCATCATGCACAAGGCA
       |  |||||| ||      |  |||  ||   |||||  |  ||  |||||||| |||| |||  ||
IS1106 GCGGAAAACCGGCAACATCTGAAAGAACATCAGTTGCAGGACGGCATTATGCGCAAAGCC
         1140       1150       1160       1170       1180       1190

       970        980        990       1000       1010       1020
IS1126 CAACGCAACAGGGCATTGACCGAGGAAGAGAAGCAAGGAAACAAAGCAATCAGTCCGATA
         ||||||  |   |  |||| ||   | |   || |||
IS1106 TGCCGCAACCGTCCGCTGACGGA-AACGCAAACCAAACGCAACCGATATTTGTCGAAGAC
         1200       1210       1220       1230       1240       1250
```

Figure 5. 3b

Comparison between the DNA sequences of IS*1126* and IS*1106*. Identical nucleotides are indicated by a match line. The sequences are numbered according to their position in the complete sequence.

Left



Right

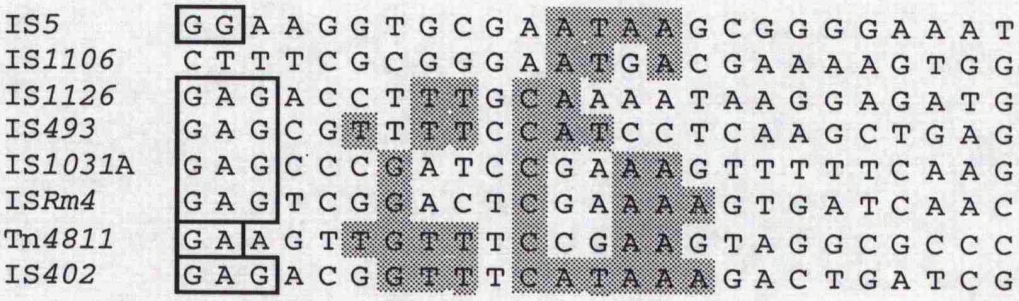


Figure 5. 4.

DNA sequence conservation among the IS-ends of elements belonging to the IS*5* subgroup of the IS*4* family. Boxed nucleotides show the external conserved trinucleotide GAG. Shaded nucleotides are conserved in more than 50% of the sequences.

of other IS-elements. In particular, the predicted amino acid sequence of ORF1 was 25.4% identical over 327 amino acids with the major ORF of the transposase of IS*1186* from *Bacteroides fragilis* (fig 5.5.a). Also, 29.4% identity was found in a 201 amino acid overlap with the transposase of IS*1106* (fig 5.5.b). Due to the extensive homology found with IS*5* at the DNA level, it was perhaps surprising that only 30.9% identity over 55 amino acids was found when ORF1 was compared to the transposase of IS*5* (fig 5.5 c).

IS*5* and IS*1106* belong to the recently identified IS*4* family of IS-elements which is based on the conservation of the amino acid sequence of their transposases. ORF1 of IS*1126* contains both the N3 and C1 motifs which are found in these proteins (fig 5.6.a, b) and, as with IS*1186*, because these motifs are immediately adjacent to one another then IS*1126* should also be considered a member of the IS*5* subgroup.

5. 2. 4. Expression of ORF1 in *E. coli* minicells

The 1386 bp *EcoR*I/*Sst*I fragment of pNJR12-1 (containing 1215 bp of IS*1126*) was cloned into pUC18 which had previously digested with *EcoR*I and *Sst*I, to give plasmid pJM1 (fig 5.7) in which ORF1 was immediately downstream of the *lac* promoter. Both pJM1 and pUC18 were used in minicell analysis which showed that pJM1 encoded for a non-vector protein of approximately 35 kDa which was absent from the vector-only control (figure 5.8).

5. 2. 5. Distribution of IS*1126* in other porhyromonads

To determine the incidence of IS*1126* among the genus, chromosomal DNA from a number of *P. gingivalis* strains, *P. endodontalis* HG370 and *P. asaccharolytica* NCTC 9337 was extracted and cleaved with *BamH*I which has no site in IS*1126*. These

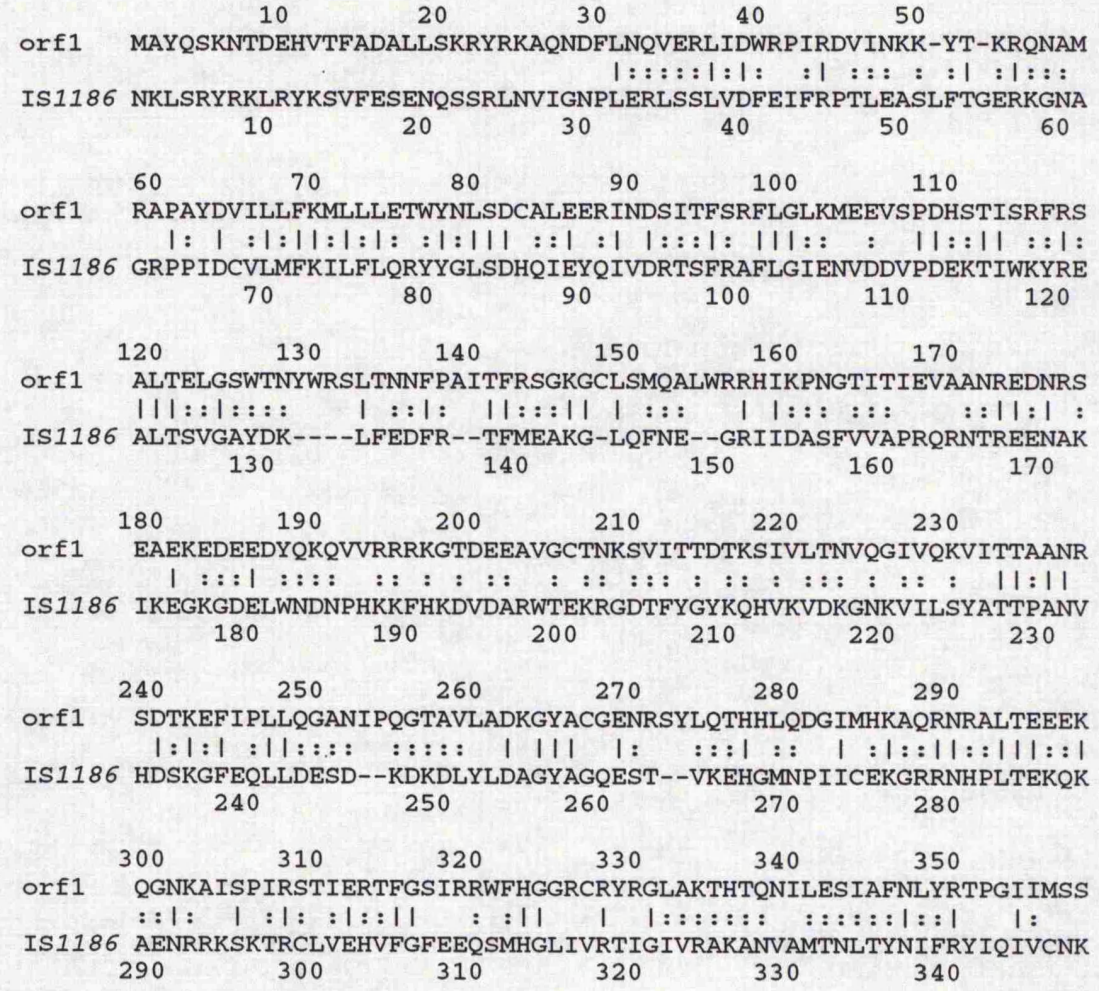


Figure 5. 5a

Comparison between the predicted proteins encoded by ORF1 and the major ORF of IS*1186*. A horizontal line indicates identity, a dotted line similar.

```
       130       140       150       160       170       180
orf1   YWRSLTNNFPAITFRSGKGCLSMQALWRRHIKPNGTITIEVAANREDNRSEAEKEDEEDY
                                        :: ::: ::::: :::::::: :  : ::
IS1106 MRYRNWLAQDDTLSELLELINRQLTEKGLKVEKASAAVVDATIIQTAGSKQRQAIEVDEE
         60        70        80        90       100       110

       190       200       210       220       230       240
orf1   QKQVVRRRKGTDEEAVGCTNKSVITTDTKSIVLTNVQGIVQKVITTAANRSDTKEFIPLL
        :   :   ::|::|   ::::: : : |  : |:::| ::|: :|:||  : |:: |||
IS1106 GQISGQTTPSKDKDARWIKKNGLYKLGYKQHTRTDAEGYTEKLHITPANAHECKHLPPLL
        120       130       140       150       160       170

       250       260       270       280       290       300
orf1   QGANIPQGTAVLADKGYACGENRSYLQTHHLQDGIMHKAQRNRALTEEEKQGNKAISPIR
       :|  :|:||:| |||||:::||| :|::|:||||||:|| |||:|||:::: |: :| :|
IS1106  EG-LPKGTTVYADKGYDSAENRQHLKEHQLQDGIMRKACRNRPLTETQTKRNRYLSKTR
          180       190       200       210       220       230

       310       320       330       340       350       360
orf1    STIERTFGSIRRWFHGGRCRYRGLAKTHTQNILESIAFNLYRTPGIIMSSSLGX
         ::|::||:::| |: :|  | || |: :|: |::: :|| :::: : :::
IS1106  YVVEQSFGTLHRKFRYARAAYFGLIKVSAQSHLKAMCLNLLKAANRLSAPASAX
          240       250       260       270       280
```

B

```
              10        20        30        40        50        60
IS5    MFVIWSHRTGFIMSHQLTFADSEFSSKRRQTRKEIFLSRMEQILPWQNMVEVIEPFYPKA
                  :::|||||: :|:: |:::|: ||:::|::: |: : :|||:  |:|
orf1         MAYQSKNTDEHVTFADALLSKRYRKAQND-FLNQVERLIDWRPIRDVINKKYTKR
                     10        20        30        40        50

                  70        80        90       100       110
IS5    GNGRR--PYPLETMLRIHCMQHWYNLSDGAMEDALYEIASMRLFARLSLDSALPDRTTIM
        |::|  :| :  :::: :: |||||| |:|: : :  ::: |  |:::::: ||::||
orf1   QNAMRAPAYDVILLFKMLLLETWYNLSDCALEERINDSITFSRFLGLKMEEVSPDHSTIS
           60        70        80        90       100       110

      120       130       140       150       160       170
IS5    NFRHLLEQHQLARQLFKTINRWLAEAGVMMTQGTLVDATIIEAPSSTKNKEQQRDPEMHQ
       :||  |::
orf1   RFRSALTELGSWTNYWRSLTNNFPAITFRSGKGCLSMQALWRRHIKPNGTITIEVAANRE
          120       130       140       150       160       170
```

C

Figure 5. 5

Comparison between the predicted proteins of ORF1 and IS1106 (B) or IS5 (C). Identical amino acids are indicated by a horizontal line, a dotted line denotes similarity.

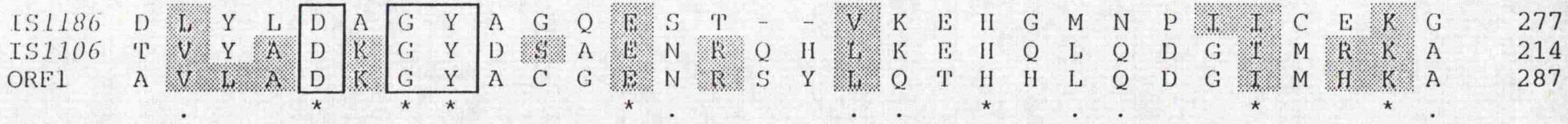


Figure 5. 6. a

The N3 region of the IS4 family of IS-elements. The core motif is boxed. Functionally related amino acids conserved in the sequences of members of the family are shaded. Co-ordinates on the right correspond to the last residue of the region. An asterisk denotes identical amino acids, a dot similarity.

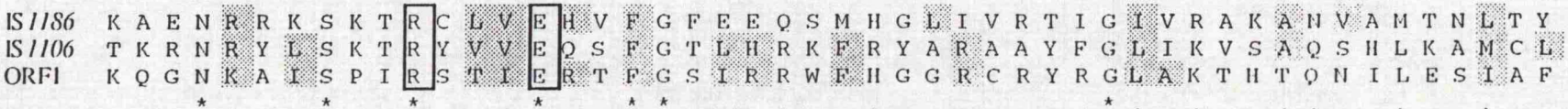


Figure 5. 6. b

The C1 conserved region of the transposases belonging to the IS*4* family of elements. Amino acids found in the core motif are boxed. Functionally related amino acids conserved among the transposases of the family are shaded. An asterisk denotes identical amino acids, a dot similarity.

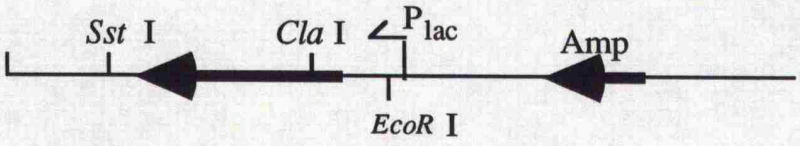


Figure 5. 7

Partial restriction map of plasmid pJM1.

The putative transposase of IS*1126* is indicated by the thickest arrow. P_{lac} denotes the β-galactosidase promoter. The thinnest arrow represents the ampicillin resistance gene.

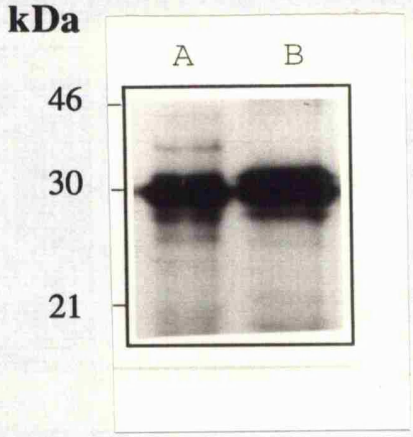


Figure 5. 8

Expression of the protein encoded by ORF1 from IS*1126* in *E. coli* minicells. Both tracks are *E. coli* DS410 containing either pJM1 (A) or pUC18 (B).

were analysed in a Southern blot with the 1.3 kb EcoRI/SstI fragment from pNJR12-1, containing most of IS*1126*, used as the radiolabelled probe. IS*1126* was present in only the *P. gingivalis* strains with each strain exhibiting a different profile. The copy number of IS*1126* among the strains was also different with *P. gingivalis* strains W83 and 11834 containing approximately 12 copies whereas other strains such as *P. gingivalis* HG 241 containing as few as 8 copies (fig. 5.9).

5. 3. Discussion

IS*1126* is the first transposable element to be found in any species of *Porphyromonas*. A number of cryptic plasmids have been identified in other members of the genus but the discovery of IS*1126* represents the first evidence of any mechanism for genetic variability in *P. gingivalis*. The nucleotide homology exhibited between IS*1126* and IS*5* and IS*1186* could suggest that these elements have evolved from a common ancestor. The homology of IS*1126* with each of these elements occurred over different parts of the sequence. Almost half of the element contained homology to the DNA sequence of IS*5* whereas the homology to IS*1106* occurred over 3/4 of IS*1126* and was in a different region to the homology with IS*5*. IS-elements and transposons can dramatically affect the expression of downstream genes. This can involve the abolishment of gene expression or can place the expression of the gene under the control of the element. In the *Bacteroides* transposons, Tn*4351* and Tn*4551*, transcription of the Cc/Emr gene (*Erm*F) is initiated within one of the two copies of the IS-element. The start codon for *Erm*F is located 26 bp downstream of the end of IS*4351* (Rasmussen *et al.*, 1987). In Tn*4551*, one IS-element is situated 17 bp from the start codon of the resistance gene (Smith, 1987). IS*1126* was inserted 171bp upstream of the start codon for the tetracycline resistance gene. It was possible that the insertion of IS*1126* close to the promoter for the tetracycline resistance gene in pNJR12 might have some effect on the level of resistance to tetracycline due to altered transcription but no increase or decrease was observed

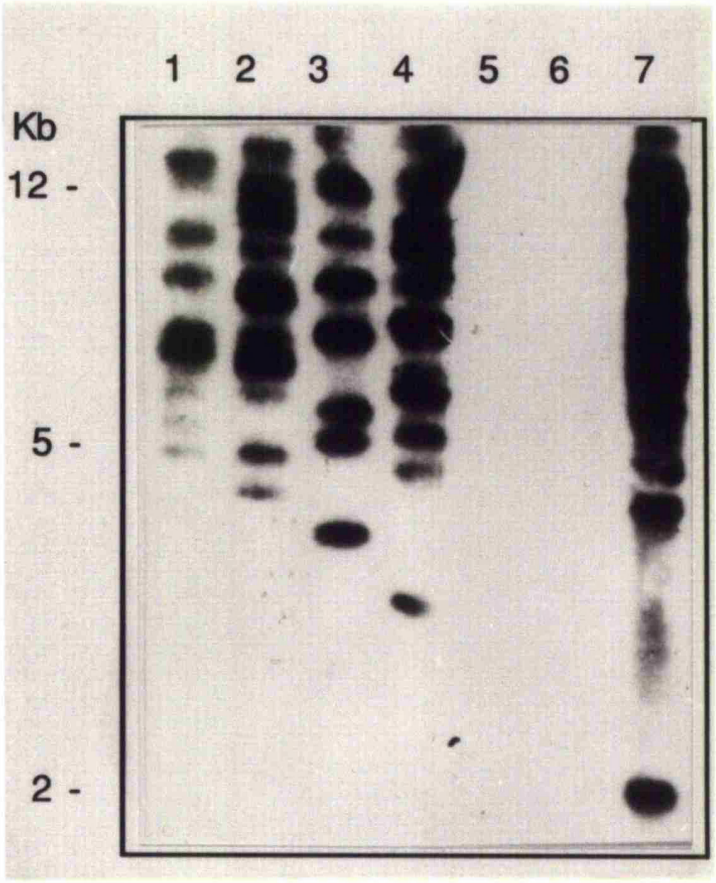


Figure 5. 9

Southern blot analysis of chromosomal DNA from a number of *Porphyromonas* strains using IS*1126* as a radiolabelled probe. Chromosomal DNA from: *P. gingivalis* strains HG241 (lane 1), WpH35 (lane 2), 23A-3 (lane 3), 11834 (lane 4), W83 (lane 7); *P. asaccharolyticus* NCTC 9337 (lane 5) and *P. endodontalis* HG370 (lane 6) were all cleaved with the restriction endonuclease *BamH*I. Sizes are given in kb.

indicating transcription of the *tet*Q gene was not under the control of IS*1126*.

Attempts were made to clone the whole of IS*1126* into *E. coli* vectors to allow the visualisation of protein products in minicells and also to conduct cointegrate formation studies to determine whether or not this element could transpose in *E. coli*. The reasons for the inability to clone the entire element are not clear. It was possible to clone almost all of the element but not the whole entity. The *Hinc*II fragment used also contained the *Bacteroides* tetracycline resistance gene. Even if IS*1126* had affected expression of the tetracycline gene, this would have been mediated by the end of the element closest to the gene. As it was possible to clone a 1,209 bp fragment of IS*1126* and the tetracycline gene downstream of it, then it can only be assumed that failure to transform *E. coli* was a result of inactivation of plasmid gene expression following the insertion of IS*1126* in *E. coli* vectors. Plasmid pNJR12, and probably pNJR12-1, is present in about 15 copies in *E. coli*. It is possible that IS*1126* may not be maintained above a certain copy number. The region which caused the problem was from position 1210 bp to the end of the element and was 19 bp after the end of ORF1.

A common characteristic of IS-elements is that upon transposition into the target DNA of the host chromosome a direct duplication of the target site is generated through the filling in of cleavage sites by the host DNA repair mechanisms. In the case of IS*1126* 5 bp direct repeats of the sequence GTAGG were found. The specificity for a particular sequence is variable among different IS-elements and even the length of the sequence that is duplicated can vary between 2 insertion events for a given IS-element (Galas and Chandler, 1989). Detailed analysis of a number of IS*1126* insertion events would be necessary to establish to what extent the duplicated target sequence GTAGG is specific.

Another main feature of IS-elements is that each end of the sequence contains inverted repeats which also vary according to the element and these may or may not be perfect copies of one another. In the IS*4* family of IS-elements, the sequence at either end of the element are important in determining which of the two subgroups of the family the

element will be placed in. The sequences at the termini of IS*1126* suggest similarities with the IS*5* group of elements. However the most compelling evidence for the inclusion of IS*1126* in this group is from the strong homology exhibited between ORF1 and the transposase enzymes of IS*1106* and IS*1186*. Greatest conservation is found at the carboxy terminus of the proteins which contain the C1 and N3 regions. This homology is also present in the transposase of IS*5* but most of the homology between the 35 kDa protein and the IS*5* transposase is found at the amino terminus. Comparison of the amino acid sequence of these proteins with each other as well as with other members of the IS*5* subgroup using FASTA analysis confirmed the closeness of these four proteins, with all of them being extremely similar compared to the other IS*5* members. This would suggest that these proteins have a common ancestral link which is considerably more recent than that between the other members of the group and family. Unlike most of the other IS*4* family members, IS*1126*, IS*5* and IS*1106* do not display the Y and K residues of the C1 region motif [Y-(2)-R-(3)-E-(6)-K]. Instead of a tyrosine (Y), all of the latter 3 transposases have a serine (S) residue. In IS*10*R, site-directed mutagenesis of the Y to an S reduced transposition to an undetectable level (Haniford *et al.*, 1989). It had been hypothesized that the presence of a Y residue at that position was required in an early step in transposition as the S-mutant did not accumulate excised transposon fragment. Clearly this cannot be the case with IS*1126*, IS*5* and IS*1106*.

The putative transposase had a molecular mass of 35 kDa on SDS-PAGE as opposed to the predicted protein of 41 kDa. This discrepancy could be simply due to aberration on SDS-PAGE and this was also found with the transposase of IS*10* which has a predicted MW of 46 kDa but was only 42 kDa on SDS-PAGE (Kleckner, 1989). This may be due to the extremely basic nature of these proteins.

Given the homology to other transposases, it would appear that ORF1 also encodes such a protein and it is likely that it would be responsible for target site recognition, cleavage of host DNA and then the ligation of the element. The expected ability of the protein to

bind DNA is given further credence by the extremely basic nature of the predicted product. This is a feature common to transposases (Galas and Chandler, 1989).

Although IS-elements produce the proteins required for transposition, it is known that a number of host proteins can influence the process. Dam methylation in particular has been shown to have a detrimental affect on the transposition of other members of the IS*4* family with both IS*10* and IS*50* affected through its ability to interfere with DNA-protein interactions (Berg, 1989; Kleckner, 1989). Integration host factor (IHF) is known to change the structure of DNA and is required for the activity of IS*10* (Kleckner, 1989). Transposition of IS*50* has been shown to require *E. coli* DnaA protein (Berg, 1989), whilst other elements have no DnaA consensus sequence and are probably not affected. Given the presence of putative sites on IS*1126* it would seem likely that such factors could also act to regulate the transposition of IS*1126*.

IS-elements have often co-evolved with their host and are therefore species-specific. As a result of their mobile nature, these elements can often be found at different locations in the chromosome and often variable copy numbers are found in different strains (Coucheron, 1993). This was found to be true of IS*1126*, with this sequence being absent from other species in the genus. Different strains of *P. gingivalis* exhibit restriction fragment length polymorphism and contain different numbers of the element in their genomes. *Porphyromonas gingivalis* strains exhibit extensive genetic heterogeneity with some 78 different genotypes being found in 100 isolates (Loos *et al.*, 1993). Insertion sequences can act as portable regions of homology which serve as substrates for the host recombination system. It is possible that IS*1126* could have contributed to this heterogeneity by its likely ability to induce a variety of DNA rearrangements as homologous recombination between two IS*1126* elements could probably generate deletions and inversions (fig. 5. 11).

Porphyromonas gingivalis is usually absent from the gums of healthy individuals and is virtually always recovered from diseased sites. It is believed to be absent from the

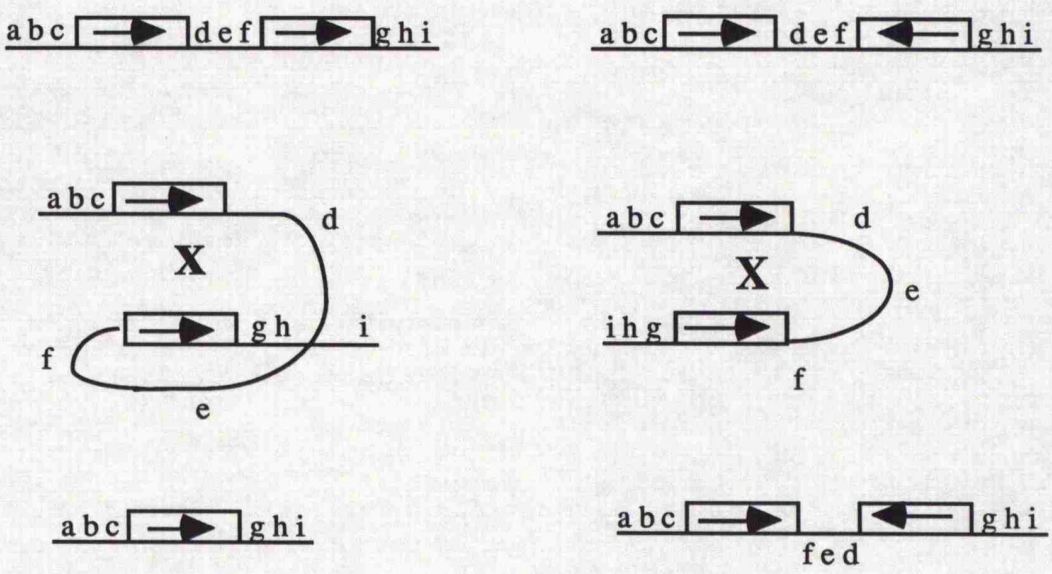


Figure 5. 11

Models for homologous recombination between two IS*1126* elements located on the chromosome as direct repeats (A) or inverted repeats (B).

healthy oral cavity before the development of permanent teeth (Slots, 1982). However, as it does occur in mixed infections and prepubertal periodontitis (Moore *et al.*, 1985; Sweeney *et al.*, 1987), it is possible that it may be present in numbers too small to be detected by usual methods. PCR procedures using primers based on the sequence found in IS*1126* could overcome this difficulty. Over one hundred strains of *P. gingivalis* have been isolated, however, with the application of molecular biology to the study of this organism, it is becoming clear that some strains exhibit identical genetic profiles as determined by RFLP analysis (Loos *et al.*, 1990) and MLEE (Loos *et al.*, 1993) and are therefore extremely similar or identical. IS*1126* would be extremely useful in discriminating among different strains.

The presence of this element in all strains of *P. gingivalis* examined suggest that the potential to exploit it for use in genetic studies of this organism would be limited. However, as IS*1126* is absent in the remainder of the genus, then it should be possible to use it in the genetic manipulation of these organisms. Vectors constructed for use in colonic *Bacteroides* are present in extremely low copy numbers in strains of *P. gingivalis* (Chapter 4). It is likely that this will also be true for the other members of the genus. Considering the limitations of heterologous systems, it would be advantageous to construct vectors based on naturally-occurring *Porphyromonas* DNA transfer mechanisms. It should be possible to construct a variety of tools for the genetic manipulation of these organisms as has been done for other groups of organisms. These could include construction of transposons by sandwiching a phenotypic marker between two copies of the element (Romero and Klaenhammer, 1991). Once this was achieved it would be possible to generate fusion to promoterless reporter genes to permit analysis of transcription regulation and some genes such as *pho*A (alkaline phosphatase) could also be used to identify surface proteins (Berg *et al.*, 1989).

Chapter 6

Summary

The aim of the work presented in this thesis was to produce transposon mutant derivatives of *P. gingivalis* W83 defective in the production of capsular polysaccharide and to then assess the effects of such mutations on the virulence of the organism and thereby establish the role of the capsule in the pathogenicity of this organism.

There is growing evidence that, as with other bacterial species, the presence of a capsular structure enhances the virulence potential of *P. gingivalis*. Not all strains of *P. gingivalis* produce a capsule, instead, exopolysaccharide production is limited to only those strains which cause severe infections in a murine model (van Winkelhoff *et al.*, 1993). It has been a long held belief that the increased virulence demonstrated by such strains was due to capsular structures (van Steenbergen *et al.*, 1987) and the finding that different virulent strains are only susceptible to phagocytosis in the presence of specific antisera would seem to confirm this (Cutler *et al.*, 1991).

A variety of methods were used for the production of polysaccharide. The removal of contaminating LPS required the use of polymyxin-B affinity chromatography and yielded preparations containing no more LPS than found in negative controls. However, confirmation of the purity of the EPS produced would require that antibodies be raised against it and the reactivity of these then tested against LPS. Antisera raised against polysaccharide generated by a previous method contained anti-LPS antibodies. This was probably a result of both the high immunogenicity of LPS and the low immunogenicity of PS. Since LPS reactive material was present in the antiserum, it was not demonstrated

whether the antiserum actually contained any anti-capsular antibodies but this was assumed to be the case. Immunoelectrophoresis and immunodiffusions would have to have been conducted in order to determine this conclusively. Alternatively, further treatment of the serum, resulting in complete removal of LPS reactive material could be generated. This would be best achieved by pre-absorption of the serum with heat-killed *P. gingivalis* cells which lacked the K1 capsule antigen.

In order to produce transposon mutants, an efficient and reliable system for the introduction of foreign DNA into this organism was required. At the outset of this investigation, genetic manipulation of *P. gingivalis* was restricted to one study which involved the introduction of plasmid pE5-2 (Progulske-Fox *et al.*, 1989). However, this did not include any analysis of any of the factors which may have affected the reproducibility of conjugal transfer of plasmid DNA into *P. gingivalis*. Although this study is not a definitive account of conjugation in *P. gingivalis,* it has highlighted a number of variables which can affect the conjugation process. Since 1992, there have been numerous accounts of the transfer of foreign DNA into this organism. Comparison of the findings presented in this study to those of other researchers demonstrate that conditions vary significantly from strain to strain and also differ when different vectors are used. Conditions suitable for one strain may be entirely inappropriate for another. Consequently, the conditions necessary for transfer into each strain would have to be determined in order to conduct transfer as efficiently as possible. A number of factors were found to affect the transfer of plasmid DNA into *P. gingivalis* W83, one of the most important appeared to be the cfu/ml of the recipient culture. The lower the cfu/ml, the higher the transfer frequency obtained, with cultures of $1x10^8$ cfu/ml rarely generating transconjugants. The cfu/ml of recipients also determined what the mating time should be with lower cfu/ml recipients requiring 12-24h longer matings than higher cfu/ml cultures. The length of time required for the growth of transconjugants on selective media was much longer than the length of time required for wildtype cells on solid media and could be a reflection of low copy number of each plasmid type in *P. gingivalis* W83. The

presence of the vectors in *P. gingivalis* at low copy numbers could be a major limiting factor on the usefulness of these vectors in this species. Purification of plasmid DNA from this organism was extremely difficult and such problems have been encountered in other strains (Dyer *et al.*, 1992) and this may prove to be a universal problem when using *Bacteroides* vectors in *P. gingivalis*. However, the method outlined in this study has been successfully employed to generate the first *Porphyromonas gingivalis* gene-replacement mutants (Joe *et al.*, 1994; Park and McBride, 1993) and therefore could make an important contribution to genetic studies of this organism.

If there is limited information about a particular gene then a random approach to generating mutants, such as transposon mutagenesis may be required. Tn*4351* inserted site-specifically into all *P. gingivalis* W83 Tn*4351* mutants examined. Multiple copies of the transposon were present in each of the mutants examined implying multiple Tn*4351* insertion or rearrangement following the insertion of one copy of the transposon. To determine the exact percentage of multiple insertion would require the analysis of a much greater number of mutants but it appears unlikely that Tn*4351* would be suitable for use in *P. gingivalis* W83. Unlike what has been found in Tn*4351* mutants of *B. uniformis* and other strains of *P. gingivalis*, not all of R751 cointegrated with the transposon. Instead, only a very small fragment of R751 was present in the mutants.

IS*1126* was found in multiple copies in all strains of *P. gingivalis* examined, the differing RFLP obtained indicate the usefulness of this element in identifying different strains. The acquisition of IS*1126* by pNJR12 was only detected on one occasion indicating no absolute requirement for the element in terms of maintenance of the vector. IS*1126* did not have any effect on the tetracycline resistance of the cells. The reintroduction of IS*1126* on pNJR12-1 did not appear to affect the transfer frequency nor did it induce any detectable rearrangement of the plasmid or the distribution of IS*1126* on the chromosome of *P. gingivalis*. The reason for failure to clone the whole of IS*1126* in *E. coli* vectors is

not clear but may be due to the inactivation of plasmid genes by IS*1126* insertion. As a result, it was not possible to demonstrate the transposition of IS*1126*.

Whether IS*1126* resides on the host chromosome only as an IS-element or as part of a transposon was not examined. However, since IS*1126* is absent from other species of the genus it should prove invaluable in the development of more suitable vectors for *Porphyromonas* which may allow easier manipulation of these organisms.

The homology found between the amino acid sequence of the 35 kDa protein of IS*1126* and transposases of the IS*5* group of elements would indicate that this protein has a similar function and is therefore assumed to be responsible for the transposition of IS*1126*. Although *P. gingivalis* is quite distantly related to the hosts of these elements, there is clearly extensive similarity among this group of proteins which may reflect the conservation of functional domains in the transposase enzymes.

In different bacterial species, the complete nucleotide sequence of IS-elements is rarely 100% identical among the different strains, instead homologies in the range of 75-95% are common. Isolation and analysis of the sequences homologous to IS*1126* present in other strains of *P. gingivalis* could perhaps give some insight into how related the different strains are to one another and how the species has evolved.

REFERENCES

Ajoika, J.W. and Hartl, D.L. (1989) Population dynamics of transposable elements. *In:* "Mobile DNA". (D.E. Berg and M.M. Howe, eds.), pp. 939-958. Am. Soc. Microbiol. Washington D.C.

Altman, L.C., Baker, C., Fleckman, P. Luchtel, D. and Oda, D. (1992) Neutrophil-mediated damage to human gingival epithelial cells. J. Periodont. Res. **27:** 70-79.

Anderson, K.L. and Salyers, A.A. (1989) Genetic evidence that outer membrane binding of starch is required for starch utilization by *Bacteroides thetaiotaomicron*. J. Bacteriol. 3194-3204.

Avery, O.T. and Dubose, R. (1931) The protective action of a specific enzyme against type III pneumococcus infection in mice. J. Exp. Med. **54:**73-89.

Baumgartner, J.C., Falkler, W.A. Jr. and Beckerman, T. (1992) Experimentally induced infection by oral anaerobic micro-organisms in a mouse model. Oral Microbiol. Immunol. **7:** 253-256.

Bedi, B.S and Williams, T. (1994) Purification and characterization of a collagen degrading protease from *Porphyromonas gingivalis*. J. Biol. Chem. **269:** 5919-606.

Berg, C.M., Berg, D.E. and Groisman, E.A. (1989) Transposable genetic elements and the genetic engineering of bacteria. *In* Mobile DNA (D.E. Berg and M.M. Howe, Ed.), pp. 109-162. A.S.M., Washington D.C.

Berg, D.E. (1989) Tn*5*. *In* Mobile DNA (D.E. Berg and M.M. Howe, Ed.), pp. 185-210. A.S.M., Washington D.C.

Bergey, D.H., Harrison, F.C., Breed, R. S., Hammer, B.W. and Huntoon, F.M. (1930) p.314. Bergey's manual of determinative microbiology. 3rd ed., Williams and Wilkins, Baltimore.

Bertolini, D.R., Nedwin, G.E., Bringan, T.S., Smitz, D.D. and Mundy, G.R. (1986) Stimulation of bone resorption and inhibition of bone formation in vitro by human tumor necrosis factor. Nature. **319:** 516-518.

Biggin, M.D., Gibson, T.J. and Hong, G.F. (1983) Buffer gradient gels and ^{35}S label as an aid to rapid DNA sequence determination. P.N.A.S. **80:** 3963-3965.

Birnboim, H.C. and Doly, J. (1979) A rapid extraction procedure for screening recombinant plasmid DNA. Nuc. Acid Res. **1:** 1513-1523.

Birkedahl-Hansen, H., Taylor, R. E., Zambon, J.J., Barwa, P.K. and Neiders, M.E. (1988) Characterisation of collagenolytic activity from strains of *Bacteroides gingivalis*. J. Periodontal Res. **23:** 258-264.

Bolivar, F., Rodriguez, R.L., Greene, P.J., Betlach, M.C., Heynecker, H.L., Boyer, H.W., Crosa, J.H. and Falkow, S. (1977) Construction and characterisation of new cloning vehicle II. A multipurpose cloning system. Gene **2:** 95-99.

Bom-van Noorloos, A.A., van Steenbergen, T.J.M. andBurger, E.H. (1989) Direct and immune-cell-mediated effects of *Bacteroides gingivalis* on bone metabolism in vitro. J. Clin. Periodontol. **16:** 412-418.

Boulnois, G.J. and Jann, K. (1990) Bacterial polysaccharide capsule synthesis, export and evolution of diversity. Mol. Microbiol. **3:** 1819-1823.

Bramanti, T.E. and Holt, S.C. (1992a) Localization of a *Porphyromonas gingivalis*: 26-kilodalton heat-modifiable, hemin-regulated surface protein which translocates across the outer membrane. J. Bacteriol. **174:** 5827-5839.

Bramanti, T.E. and Holt, S.C. (1992b) Effect of porphyrins and host iron transport proteins on outer membrane expression in *Porphyromonas (BActeroides) gingivalis*: identification of a novel 26 kDa hemin-repressible surface proteins. Microb. pathogen. **13:** 61-73.

Bramanti, T.E., Wong, G.G., Weintraub, S.T. and Holt, S.C. (1989) Chemical characterization and biologic properties of lipopolysaccharide from Bacteroides gingivalis strains W50, W83 and ATCC 33277. Oral Microbiol. Immunol. **4:** 183-192.

Brown, E.J., Joiner, K.A., Gaither, T.A., Hammer, C.H. and Frank, M.M. (1983) The interaction of C3b bound to pneumococci with factor H (beta 1H globulin), factor I (C3b/C4b inactivator) and properdin factor B of the human complement system. J. Immunol. **131:** 409-415.

Burdon, K. L. (1928) *Bacterium melaninogenicum* from normal and pathologic tissues. J. Inf. Dis. **42:** 161-171.

Callihan, D.R., Young, F.E. and Clark, V.L. (1983) Identification of three homology classes of small, cryptic plasmids in intestinal *Bacteroides* species. Plasmid. **9:** 17-30.

Callihan, D.R., Young, F.E. and Clark, V.L. (1984) Presence of two unique genes encoding macrolide-lincoside-streptogramin resistance in the members of the *Bacteroides fragilis* group as determined by DNA-DNA homology. J. Antimicrob. Chemother. **14:** 329-338.

Carlsson, J., Herrmann, B.F., Hofling, J. F. and Sundqvist, G. K. (1984b) Degradation of the human proteinase inhibitors alpha-1-anti-trypsin and alpha-2-macroglobulin by *Bacteroides gingivalis*. Infec. Immun. **43:** 644-648.

Carlsson, J., Hofling, J. F. and Sundqvist, G. K. (1984a) Degradation of albumin, hemopexin, haptoglobin and transferrin, by black-pigmented *Bacteroides* species. J. Med. Micro. **18:** 39-46.

Carman, R. J., Ramakrishan, M. D. and Harper, F. H. (1990) Hemin levels in culture medium of *Porphyromonas (Bacteroides)* and trypsin-like protease production. Infec. Immun. **58:** 4016-4019.

Chang, A.C.Y. and Cohen, S.N. (1978) Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J. Bacteriol. **134:** 1141-1156.

Childs, W.C. and Gibbons, R.J. (1990) Selective modulation of bacterial attachment to oral epithelial cells by enzyme activities associated with poor oral hygiene. J. Periodont. Res. **25:** 172-178.

Cimasoni, G., Song, M. and McBride, B.C. (1987) Effect of crevicular fluid and lysosomal enzymes on the adherence of streptococci and bacteroides to hydroxyapatite. Infect. Immun. **55:** 1484-1489.

Cirillo, J.D., Barletta, R.G., Bloom, B.R. and Jacobs, Jr, W.R. (1991) A novel transposon trap for mycobacteria: isolation and characterization of IS*1096*. J. Bacteriol. **173:** 7772-7780.

Clark, W.B., Beem, E., Magnusson, I., Marks, R.G. and McArthur, W.P. (1992) Effect of immunization on *Porphyromonas gingivalis* (Pg) and alveolar bone loss (ABL). J. Dental Res. **71:** special issue abstr. 1486.

Coucheron, D.H. (1993) A family of IS*1031* elements in the genome of *Acetobacter xylinum:* nucleotide sequence and strain distribution. Mol. Microbiol. **9:** 211-218.

Courant, P.R. and Gibbons, R.J. (1967) Biochemical and immunological heterogeneity of *Bacteroides melaninogenicus*. Arch. Oral Biol. **12:** 1605-1613.

Costerton, J.W., Irvin, R.T. and Cheng, K.J. (1981) The bacterial glycocalyx in nature and disease. Ann. Rev. Microbiol. **35:** 299-324.

Coykendall, A.L., Kaczmarek, F.S. and Slots, J. (1980) Genetic heterogeneity in *Bacteroides asaccharolyticus* (Holdeman and Moore, 1970): Finegold and Barnes, 1977; Approved lists, 1980 and proposal of *Bacteroides gingivalis* sp. nov. and *Bacteroides macacae* (Slots and Genco) comb. nov. Int. J. Syst. Bacteriol. **30:** 559-564.

Cutler, C.W., Kalmar, J.R. and Arnold, R.R. (1991) Phagocytosis of virulent *Porphyromonas gingivalis* by human polymorphonuclear leukocytes requires specific Immunoglobulin G. Infect. Immun. **59:** 2097-2104.

Dahlén, G., Manji, F., Baelum, V. and Fejerskov, O. (1992) Putative periodontopathogens in "diseased" and "non-diseased" persons exhibiting poor oral hygiene. J. Clin. Periodontol. **19:** 35-42.

Derbyshire, K.M. and Grindley, N.D.F. (1992) Binding of the IS*903* transposase to its inverted repeat. P.N.A.S. **84:** 8049-8053.

Deslauriers, M., ni Eidhin, D., Lamonde, L. and Mouton, C. (1990) SDS-PAGE analysis of protein and lipolysaccharide of extracellular vesicules and Sarkosyl-insoluble membranes from *Bacteroides gingivalis*. Oral Microbiol. Immunol. **5:** 1-7.

Devereux, J., Haeberli, P. and Smithies, O. (1984) A comprehensive set of sequence analysis programs for the VAX. Nucl. Acid Res. **12:** 387-395.

Dickinson, D.P., Kubiniec, M.A., Yoshimura, F. and Genco, R.J. (1988) Molecular cloning and sequencing of the gene encoding the fimbrial subunit protein of *Bacteroides gingivalis*. J. Bacteriol. **170:** 1658-1665.

Dinarello, C. A. (1989) Interleukin-1 and its biologically related cytokines. Adv. Immunol. **44:** 153-205.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A. and Smith, F.(1956) Colorimetric method for determination of sugars and related substances. Anal. Biochem. **28:** 350-356.

Dudman, W.F. (1977) The role of surface polysaccharides in natural environments. In: Surface carbohydrates of the prokaryotic cell. Sutherland, I.W. (ed.) pp.357-414.c Academic, New York.

Duncan, M.J., Nakao, S., Skobe, Z. and Xie, H. (1993) Interactions of *Porphyromonas gingivalis* with epithelial cells. Infect. Immun. **61:** 2260-2265.

Dusek, D.M., Progulske-Fox, A., Whitlock, J. and Brown, T.A. (1993) Isolation and characterization of a cloned *Porphyromonas gingivalis* hemagglutinin from an avirulent strain of *Salmonella typhimurium*. Infect. Immun. **61:** 940-946.

Dyer, D.W., Bilalis, G., Michel, J.H. and Malek, R. (1992) Conjugal transfer of plasmid and transposon DNA from *Escherichia coli* into *Porphyromonas gingivalis*. Biochem. Biophys. Res. Comms. **186:** 1012-1019.

Dzink, J.L., Gibbons, R.J., Childs, W.C. and Socransky, S.S. (1989) The predominant cultivable microbiota of crevicular epithelial cells. Oral Microbiol. Immunol. **4:** 1-5.

Dzink J. L., Tanner A. C. R., Haffajee A. D. and Socransky S. S. (1985) Gram-negative species associated with active destructive periodontal lesions. J. Clin. Periodontol. **12:** 648-659.

Echarti, C.E., Hirschel, B., Boulnois, G.J., Varley, J.M., Waldvogel, F. and Timmis, K.N. (1983) Cloning and analysis of the K1 capsule biosynthesis genes of *Escherichia coli*: lack of homology with *Neisseria meningitidis* group B DNA sequences. Infect. Immun. **41:** 54-60.

Edwards, M.S., Kasper D.L., Jennings, H.J., Baker C.J. and Nicholson-Weller A. (1982) Capsular sialic acid prevents activation of the alternative complement pathway by type III group B Streptococci. J. Immunol. **128:** 1278-83.

Eke P. I., Rotimi V. O. and Langhon B. E. (1989) Coaggrgation of black-pigmented *Bacteroides* species with other oral bacteria. J. Med. Microbiol. **28:** 1-4.

Ellen , R.P., Song, M. and Buivids, I.A. (1992) Inhibition of *Actinomyces viscosus - Porphyromonas gingivalis* coadhesion by trypsin and other proteins. Oral Microbiol. Immunol. **7:** 198-203.

Ellen, R.P. and Grove, D.A. (1989) *Bacteroides gingivalis* vesicles bind to and aggregate *Actinomyces viscosus.* Infect. Immun. **57:** 1618-1620.

Evans, R.T., Klausen, B., Sojar, H.T., Bedi, G.S., Sfintescu, C., Ramamurthy, N.S., Golub, L.M. and Genco, R.J. (1992) Immunization with *Porphyromonas (Bacteroides) gingivalis* fimbriae protects against periodontal destruction. Infect. Immun. **60:** 2926-2935.

Fayet, O., Ramond, P., Polard, P., Prer, M.F. and Chandler, M. (1990) Functional similarities between retroviruses and the IS*3* family of bacterial insertion sequences ? Molec. Microbiol. **4:** 1771-1777.

Fearon D. T. (1978) Regulation by membrane sialic acid of b-1-H-dependent decay dissociation of amplification C3 convertase of the alternative complement pathway. P. N. A. S. USA. **75:** 1971-1975.

Feinberg, A.P. and Vogelstein, B. (1983) A technique for radiolabelling restriction endonuclease fragments to a high specific activity. Anal. Biochem. **132:** 6-13.

Finegold, S.M. and Barnes, E.M. (1977) Report of the ICSB taxonomic subcommittee on Gram-negative anaerobic rods. Proposal that the saccharolytic and asaccharolytic strains at present classified in the species *Bacteroides melaninogenicus* (Oliver and Wherry) be

classified in two species as *Bacteroides melaninogenicus* and *Bacteroides asaccharolyticus*. Int. J. Syst. Bacteriol. **27:** 388-391.

Finne, J., Leinonen, M. and Mäkelä, P.H. (1983) Antigenic similarities between brain components and bacteria causing meningitis, implications for vaccine development. Lancet. **2:** 355-357.

Fishburn, C.S., Slaney, J.M., Carman, R.J. and Curtis, M.A. (1991) Degradation of plasma proteins by the trypsin-like enzyme of *Porphyromonas gingivalis* and inhibition of protease activity by a serine protease inhibitor of human plasma. Oral Microbiol. Immunol. **6:** 209-215.

Flint, H.J., Thomson, A.W. and Bisset, J. (1988) Plasmid-associated transfer of tetracycline resistance in *Bacteroides ruminicola.* Appl. Env. Micobiol. **54:** 855-860.

Frosch, M., Edwards, U., Bousset, K., Krau*b*e, B. and Weisberger, C (1991) Evidence for a common molecular origin of the capsule gene loci in Gram-negative bacteria expressing group II capsular polysaccharides. Mol. Microbiol. **5:** 1251-1263.

Frostell, G. and Söder, Ö. (1970) The proteolytic activity of plaque and its relation to soft tissue pathology. Int. Dent. J. **20:** 436-450.

Fujimura, S. and Nakamura, T. (1987) Isolation and characterization of a protease from *Bacteroides gingivalis*. Infect. Immun. **55:** 716-720.

Fujimura, S. Shibata, Y. and Nakamura, T. (1992) Comparative studies of three proteases of *Porphyromonas gingivalis*. Oral Microbiol. Immunol. **7:** 212-217.

Fujiwara, T., Ogawa, T., Sobue, S. and Hamada, S. (1990) Chemical, immunobiological and antigenic characterisations of lipopolysaccharides from *Bacteroides gingivalis* strains. J. Gen. Microbiol.**136:** 319-326.

Galas, D.J. and Chandler, M. (1989) Bacterial Insertion sequences. *In* Mobile DNA (D.E. Berg and M.M. Howe, Ed.), pp. 109-162. A.S.M., Washington D.C.

Garrison, S.W., Holt, S.C.and Nichols, F.C. (1988) Lipopolysaccharide stimulated PGE_2 release from human monocytes: comparison of lipopolysaccharides prepared from suspected periodontal pathogens. J. Periodontol. **59:** 684-687.

Genco, C.A., Cutler, C.W., Kapczynski, D., Maloney, K. and Arnold, R.A. (1991) A novel mouse model to study the virulence of and host response to *Porphyromonas (Bacteroides) gingivalis*. Infect. Immun. **59:** 1255-1263.

Genco, C.A., Lassiter, M., Forng, R.Y. and Basil, M.O. (1993) Characterization of Tn*4351* generated mutants of *Porphyromonas gingivalis*. J. dental Res. **72:** special issue abstr. 123.

Gharbia, S.E., Shah, H.N. and Welsh, S.G. (1989) The influence of peptides on the uptake of amino acids in *Fusobacterium*; predicted interactions with *Porphyromonas gingivalis*. Curr. Microbiol. ***19***: 231-235.

Gharbia, S.E., Shah, H.N., Sreedharan, S. and Brocklehurst, K. (1993) Physiological properties of gingivain, a cysteine proteinase, isolated from *Porphyromonas gingivalis*. FEMS Immunol. Med. Microbiol. **6:** 157-158.

Gibbons R. J. and MacDonald J. B. (1960) Hemin and vitamin K compounds as required factors for the cultivation of certain strains of *Bacteroides melaninogenicus*. J. Bacteriol. **80:** 164-170.

Gotschlich, E.C., Fraser, B.A., Nishmura, O., Robbins, J.B. and Liu, T.-Y. (1981) Lipid on capsular polysaccharides of Gram-negative bacteria. J. Biol. Chem. **256:** 8915-8921.

Goulbourne, A.P. and Ellen, R.P. (1991) Evidence that *Porphyromonas gingivalis* fimbriae function in adhesion to *Actinomyces viscosus*. J. Bacteriol. **173:** 5266-5274.

Grenier, D. (1991) Hemin-binding property of *Porphyromonas gingivalis* outer membranes. FEMS Microbiol. Letts. **77:** 45-50.

Grenier, D. (1992) Inactivation of human serum bactericidal activity by a trypsin-like protease isolated from *Porphyromonas gingivalis*. Infect. Immun. **60:** 1854-1859.

Grenier, D. and Bélanger, M. (1991) Protective effect of *Porphyromonas gingivalis* outer membrane vesicles against bactericidal activity of human serum. Infect. Immun. **59:** 3004-3008.

Grenier, D., Chad, O. and McBride, B. C. (1989) Characterisation of sodium dodecyl sulfate-stable *Bacteroides gingivalis* proteases by polyacrylamide gel electrophoresis. Infec. Immun. **57:** 95-99.

Grenier, D. and Mayrand, D. (1986) Nutritional relationships between oral bacteria. Infect. Immun. **53:** 616-620.

Grenier, D. and Mayrand, D. (1987b) Functional characterization of extracellular vesicles produced by *Bacteroides gingivalis*. Infect. Immun. **55:** 111-117.

Grenier, D. and Mayrand, D. (1987a) Selected characteristics of pathogenic and nonpathogenic strains of *Bacteroides gingivalis*. J. Clin. Micro. **25:** 738-740.

Grenier, D., Mayrand, D. and McBride, B.C. (1989) Further studies on the degradation of immunoglobulins by black-pigmented *Bacteroides* Oral Microbiol. Immunol. **4:** 12-18.

Guiney, Jr, D.G., Hasegawa, P. and Davis, C.E. (1984) Expression in *Escherichia coli* of cryptic tetracycline resistance genes from *Bacteroides* R plasmids. Plasmid. **11:** 248-252.

Haapasalo, M., Kerosuo, E. and Lounatmaa, K. (1990) Hydrophobicities of human polymorphonuclear leukocytes and oral *Bacteroides* and *Porphyromonas* spp., *Wolinella recta*, and *Eubacterium yurii* with special reference to bacterial surface structures. Scand. J. Dental Res. **98:** 472-481.

Hamada, N., Watanabe, K., Sasakawa, C., Yoshikawa, M., Yoshimura, F. and Umemoto, T. (1994) Construction and characterization of a *fimA* mutant of *Porphyromonas gingivalis*. Infect, Immun. **62:** 1696-1704.

Hamada, S., Koga, T., Nishihara, T., Fujiwara, T. and Okahashi, N. (1988) Characterization and immunobiologic activities of lipopolysaccharides from periodontal bacteria. Adv. Dental Res. **2:** 284-291.

Hanazawa, S., Murakami, Y., Hirose, K., Amano, S., Ohmori, Y., Higuchi, H. and Kitano, S. (1991) *Bacteroides (Porphyromonas) gingivalis* fimbriae activate mouse peritoneal macrophages and induce gene expression and production of interleukin-1. Infect. Immun. **59:** 1972-1977.

Hanazawa, S., Murakami, Y., Takeshita, A., Kitami, H., Ohta, K., Amano, S. and Kitano, S. (1992) *Porphyromonas gingivalis* fimbriae induce expression of the neutrophil chemotactic factor KC gene of mouse peritoneal macrophages: role of protein kinase C. Infect. Immun. **60:** 1544-1549.

Hanazawa, S., Nakada, K., Ohmori, Y., Miyoshi ,T., Amano, S. and Kitano, S. (1985) Functional role of interleukin 1 production by *Bacteroides gingivalis* lipopolysaccharide in peritoneal macrophages from C3H/HeN and C3H/HeJ mice. Infect. Immun. **50:** 262-270.

Haniford, D.B., Chelouche, A.R. and Kleckner, N. (1989) A specific class of IS*10* transposase mutants are blocked for target site interactions and possible formation of an excised transposon fragment. Cell. **59:** 385-394.

Harvey, W., Kamin, S., Meghji, S. and Wilson, M. (1987) Interleukin-1 like activity in capsular material from *Haemophilus actinomycetemcomitans*. Immunology. **60:** 415-418.

Hecht, D.H. and Malamy, M.H. (1989) Tn*4399*, a conjugal mobilizing transposon of *Bacteroides fragilis*. J. Bacteriol. **171:** 3603-3608.

Heijl, L., Wennström, J., Lindhe, J. and Socransky, S.S.(1980) Periodontal disease in gnotobiotic rats. J. Periodont. Res. **15:** 405-419.

Hinode, D., Hayashi, H. and Nakamura, R. (1991) Purification and characterization of three types of proteases from culture supernatants of *Porphyromonas gingivalis*. Infect. Immun. **59:** 3060-3068.

Holdeman, L. V. and Johnson, J.L. (1982) Description of *Bacteroides loeschii* sp. nov., emendation of the description of *Bacteroides melaninogenicus* (Oliver and Wherry) Roy and Kelly 1939 and *Bacteroides denticola* Shah and Collins 1981. Int. J Syst. Bacteriol. **32:** 399-409.

Holdeman, L. V., Kelley, R.W. and Moore, W.E.C. (1984) Genus I. *Bacteroides* Castellani and Chalmers 1919. *In*: Kreig, N.R. and Holt, J.G. (eds). Bergey's manual of systematic bacteriology. Williams and Wilkins Co. Baltimore pp 604-631.

Holdeman, L.V. and Moore, W.E.C. (1970) Outline of clinical methods in anaerobic bacteriology. 2nd ed, Virginia polytechnic Institute and State University Anaerobic Laboratory. Blacksburg, VA.

Holt S.C., Ebersole J., Felton J., Brunsvold M. and Kornman K.S. (1988) Implantation of *Bacteroides gingivalis* in nonhuman primates initiates progression of periodontitis. Science. **239:** 55-57.

Hoover, C.I., Abarbarchuk, E., Ng, C.Y. and Felton, J.R. (1992c) Transposition of Tn*4351* in*Porphyromonas gingivalis*. Plasmid. **27:** 246-250.

Hoover, C.I., Kapila, Y.L., Ng, C.Y. and Felton, J.R. (1992b) Restriction mapping and subcloning of a plasmid from *P. endodontalis*. J. Dental Res. **71:** special issue abstr. 1498.

Hoover, C.I., Ng, C.Y. and Felton, J.R. (1992a) Correlation of haemagglutination activity with trypsin-like protease activity of *Porphyromonas gingivalis*. Archs. oral Biol. **37:** 515-520.

Horwitz M. A. and Silverstein S. C. (1980) Influence of the *Escherichia coli* capsule on complement fixation and on phagocytosis and killing by human phagocytes. J. Clin. Invest. ***65*:** 82-94.

Inoshita, E., Amano, A., Haniska, T., Tamagawa,H., Shizukishi, S. and Tsunemitsu, A. (1986) Isolation and some properties of exohemagglutinin from the culture medium of *Bacteroides gingivalis* 381. Infect. Immun. **52:** 421-427.

Ismaiel, M.O., Greenman, J. and Scully, C. (1988) Serum antibodies against the trypsin-like protease of *Bacteroides gingivalis* in periodontitis. J. Periodont. Res. **23:** 193-198.

Issekutz A. C. (1983) Removal of Gram-negative endotoxin from solutions by affinity chromotography. J. Immunol. Methods. **61:** 275-281.

Jann, K. and Jann, B. (1990) Structure and biosynthesis of the capsular antigens of *Escherichia coli*. Curr. Topics Microbiol. Immunol. **150:** 19-42.

Joe, A., Murray, C.S. and McBride, B.C. (1994) Nucleotide sequence of a *Porphyromonas gingivalis* gene encoding a surface-associated glutamate dehydrogenase and construction of a glutamate dehydrogenase-deficient isogenic mutant. Infect. Immun. **62:** 1358-1368.

Johne, B., Olsen, I. and Bryn, K. (1988) Fatty acid and sugars in lipopolysaccharides from *Bacteroides intermedius*, *Bacteroides gingivalis* and *Bacteroides loeschii*. Oral Microbiol. Immunol. **3:** 22-27.

Johnson, J.L. and Holdeman, L.V. (1983) *Bacteroides intermedius* comb. nov. and descriptions of *Bacteroides corporis* sp. nov. and *Bacteroides levii* sp. nov. Int. J. Syst. Bacteriol. **33:** 15-25.

Kaijser, B. and Jodal, U. (1984) *Escherichia coli* K5 antigen in relation to various infections in healthy individuals. J. Clin. Microbiol. **19:** 264-266.

Karjalainen, J., Kanervo, A., Vaisanen, M-L., Forsblom, B., Sarkiala, E. and Jousimies-Somer, H. (1993) *Porphyromonas gingivalis*-like Gram-negative rods in naturally occurring periodontitis in dogs. FEMS Microbiol. Immunol. Med. Microbiol. **6:** 207-212.

Kastelein, P., van Steenbergen, T.J.M., Bras, J.M. and de Graaff, J. (1981) An experimentally induced phlegmonous abscess by a strain of *Bacteroides gingivalis* in guinea pigs and mice. Anton. van Leeuwen. **47:** 1-9.

Kato, T., Takahashi, N. and Kuramitsu, H.K. (1992) Sequence analysis and characterization of the *Porphyromonas gingivalis* prtC gene, which expresses a novel collagenase activity. J. Bacteriol. **174:** 3889-3895.

Kenne, L. and Lindberg, B. (1983) Bacterial polysaccharides. In: Aspina, G.O. (ed.) The polysaccharides. pp.287-360. Academic Press, New York.

Kido, N., Ohta, M. and Kato, N. (1990) Detection of lipopolysaccharides by ethidium bromide staining after sodium dodecyl sulfate-polyacrylamide gel electrophoresis. J. Bact. *172*: 1145-1147.

Kilian, M. (1981) Degradation of immunoglobulins A1, A2, and G by suspected principal pathogens. Infec. Immun. **34:** 757- 765.

Kleckner, N. (1989) Transposon Tn*10*. *In* Mobile DNA (D.E. Berg and M.M. Howe, Ed.), pp. 227-268. A.S.M., Washington D.C.

Koga, T., Nishihara, T., Fujiwara, T., Nishizawa, T., Okahashi, N., Noguchi, T. and Hamada, S. (1985) Biochemical and immunobiological properties of lipopolysaccharide (LPS) from *Bacteroides gingivalis* and comparison with LPS from *Escherichia coli*. Infec. Immun. **47:** 638-647.

Kolenbrander, P.E. and Anderson, R.N. (1989) Inhibition of coaggregation between *Fusobacterium nucleatum* and *Porphyromonas (Bacteroides) gingivalis* by lactose and related sugars. Infec. Immun. **57:** 3204-3209.

Kulkosky, J., Jones, K.S., Katz, R.A., Mack, J.P.G. and Skalaka, A.M. (1992) Residues critical for retroviral integrative recombination in a region that is highly conserved among retroviral/retrotransposon integrases and bacterial insertion sequence transposases. Mol. Cell.Biol. **12:** 2331-2338.

Kumada, H., Haishima, Y., Konodo, S., Umemoto, T. and Hisatsune, K. (1993) Occurrence of 2-keto-3-deoxyoctonate (KDO) and KDO phosphate in lipopolysaccharides of *Bacteroides* species. Curr. Microbiol. **26:** 239-244.

Kuo, J.S.-C., Doelling, V.W., Graveline, J.F. and McCoy, D.W. (1985) Evidence for covalent attachment of phospholipid to the capsular polysaccharide of *Haemophilus influenzae*. J. Bacteriol. **163:** 769-773.

Kurihara, H., Nishimura, T., Nakagawa, M., Tanimoto, I., Nomura, Y., Kokeguchi, S., Kato, K. and Murayama, Y. (1991) Humoral immune response to an antigen from *Porphyromonas gingivalis* 381 in periodontal disease. Infect. Immun. **59:** 2758-2762.

Laemmli U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

Laliberte, M. and Mayrand, D. (1983) Characterisation of black-pigmented *Bacteroides* strains isolated from animals. Appl. Microbiol. **55:** 247-252.

Lamont, R.J., Hersey, S.G. and Rosan, B. (1992) Characterization of the adherence of *Porphyromonas gingivalis* to oral strptococci. Oral Microbiol. Immunol. **7:** 193-197.

Lee, J-Y., Sojar, H.T., Bedi, G.S. and Genco, R.J. (1991) *Porphyromonas (Bacteroides) gingivalis* fimbrillin: size, amino-terminal sequence, and antigenic heterogeneity. Infect. Immun. **59:** 383-389.

Lee, J-Y., Sojar, H.T., Bedi, G.S. and Genco, R.J. (1992) Synthetic peptides analogous to the fimbrillin sequence inhibit adherence of *Porphyromonas gingivalis*. Infect. Immun. **60:** 1662-1670.

Lepine, G. and Progulske-Fox, A. (1993) Isogenic mutations in two hemagglutinin genes of *Porphyromonas gingivalis*. J. Dent. Res. **72:** special issue abstract 120.

Li, J. and Ellen, R.P. (1989) Relative adherence of *Bacteroides* species and strains to *Actinomyces viscosus* on saliva-coated hydroxyapatite. J. Dent. Res. **68:** 1308-1312.

Li, J., Ellen, R.P., Hoover, C.I. and Felton, J.R. (1991) Association of proteases of *Porphyromonas (Bacteroides) gingivalis* with its adhesion to *Actinomyces viscosus*. J. Dent. Res. **70:** 82-86.

Loesche, W.J., Syed, S.A., Morrison, E.C., Langhon, B. and Grossman, N.S. (1981) Treatment of periodontal infections due to anaerobic bacteria with short term treatment with metronidazole. J. Clin. Periodontol. **8:** 29-44.

Loos, B.G., Mayrand, D., Genco, R.J. and Dickinson, D.P. (1990) Genetic heterogeneity of *Porphyromonas (Bacteroides) gingivalis* by genomic DNA fingerprinting. J. Dent. Res. **69:** 1488-1493.

Loos, B.G. and Dyer, D.W. (1992) Restriction fragment length polymorphism analysis of the fimbrillin locus, *fim A*, of *Porphyromonas gingivalis*. J. Dent. Res. **71:** 1173-1181.

Loos, B.G., Dyer, D.W., Whittam, T.S. and Selander, R.K. (1993) Genetic structure of populations of *Porphyromonas gingivalis* associated with periodontitis and other oral infections. Infect. Immun. **61:** 204-212.

Lovatt, A. and Roberts, I.S. (1994) The cloning and expression of the β-N-acetyl-hexosaminidase gene *(nahA)* from *Porphyromonas gingivalis* in *Escherichia coli*: a lipoprotein enzyme associated with the outer membrane. Microbiol. in press.

Love, D.N., Bailey, G.D., Collings, S. and Briscoe, D.A. (1992) Description of *Porphyromonas circumdentaria* sp. nov. and reassignment of *Bacteroides salivosus* (Love, Johnson, Jones, and Calverley 1987) as *Porphyromonas* (Shah and Collins 1988) *salivosa* comb. nov. Int. J. Syst. Bacteriol. **42:** 434-438.

Love, D.N., Johnson, J.L., Jones, R.F. and Calverley, A. (1987) *Bacteroides salivosus* sp. nov., an asaccharolytic black-pigmented *Bacteroides* species from cats. Int. J. Syst. Bacteriol. **37:** 307-309.

Love, D.N., Karjalainen, J., Kanervo, A., Forsblom, B., Sarkiala, E., Bailey, G.D., Wigney, D.I. and Jousimies-Somer, H. (1994) *Porphyromonas canoris* sp. nov., an asaccharolytic, black-pigmented species from the gingival sulcus of dogs. Int. J. Syst. Bacteriol. **44:** 204-208.

MacDonald, J. B., Socransky, S. S. and Gibbons, R. J. (1963) Aspects of the pathogenesis of mixed anaerobic infections of mucous membranes. J. Dent. Res. **42:** 529-544.

McCourtie, J. and Poxton, I. R. (1990) Characterization of *Bacteroides* from sheep periodontal disease by SDS-PAGE of outer membrane proteins. FEMS Microbiol. Letts. **71:** 5-10.

McKee, A. S., McDermid, A. S., Baskerville, A., Dowsett, A. B., Ellwood, D. C. and Marsh, P. D. (1986) Effect of hemin on the physiology and virulence of *Bacteroides gingivalis* W50. Infec. Immun. **52:** 349-355.

McKee, A.S., McDermid, A.S., Wait, R., Baskerville, A. and Marsh, P.D. (1988) Isolation of colonial variants of *Bacteroides gingivalis* with a reduced virulence. J. Med. Microbiol. **27:** 59-64.

Macrina, F.L., Mays, T.D., Smith, C.J. and Welch, R.A. (1981) Non-plasmid associated transfer of antibiotic resistance in *Bacteroides*. J. Antimicrob. Chemother. **8:** 77-86.

Malamy, M.H. (1970) Some properties of insertion mutations in the lac operon, p. 359-373. *In* J.R. Beckwith and D. Zipser (ed.), *The lactose operon.* Cold Spring Harbor laboratory, Cold Spring Harbor, N.Y.

Mansheim, B.J. and Kasper, D.L. (1977) Purification and immunochemical characterization of the outer membrane complex of *Bacteroides melaninogenicus* subspecies *asaccharolyticus*. J. Inf. Dis. **135:** 787-799.

Mansheim, B.J., Onderdonk, A.B. and Kasper, D.L. (1978) Immunochemical and biologic studies of the lipopolysaccharide of *Bacteroides melaninogenicus* subspecies *asaccharolyticus*. J. Immunol. ***120***: 72-78.

Marsh, P.D., McKee, A.S. and McDermid, A.S. (1994) The effect of growth rate and haemin on the virulence and proteolytic activity of *Porphyromonas gingivalis* W50. Microbiol. **140:** 861-865.

Marsh, P.D., McKee, A.S., McDermid, A.S. and Dowsett, A.B. (1989) Ultrastructure and enzyme activities of a virulent and an avirulent variant of *Bacteroides gingivalis* W50. FEMS Micro. Letts. **59:** 181-186.

Marsh, P.K., Malamy, M.H., Shimell, M.J. and Tally, F.P. (1983) Sequence homology of clindamycin resistance determinants in clinical isolates of *Bacteroides* spp. Antimicrob. agent. chemother. **23:** 726-730.

Mary, P., Pheulpin, P., Bechet, M. and Guillaume, J.B. (1986) Plasmids and antibiotic resistances in clinical isolates of *Bacteroides* spp. *In* Biology of Anaerobic Bacteria (Dubourguier, H.C., Ed.), pp. 165-173.

Mayrand, D. and Grenier, D. (1985) Detection of collagenase activity in oral bacteria. Can. J. Microbiol. **31:** 134-138.

Mayrand, D. and McBride, B.C. (1980) Ecological relationships involved in a simple mixed anaerobic infection. Infec. Immun. **27:** 44-50.

Mays, T.D., Smith, C.J., Welch, R.A., Delfini, C. and Macrina, F.L. (1982) Novel antibiotic resistance transfer in *Bacteroides*. Antimicrob. Agents Chemother. **21:** 110-118.

Mazel, D., Bernard, C., Schwarz, R., Castets, A.M., Houmard, J. and de Marsac, N.T. (1991) Characterization of two insertion sequences, IS*701* and IS*702*, from the cyanobacterium *Calothrix* species PCC 7601.Mol. Microbiol. **5:** 2165-2170.

Meghji, S., Henderson, B. and Wilson, M. (1993) High-titer antisera from patients with periodontal disease inhibit bacterial capsule-induced bone breakdown. J. Periodont. Res. **28:** 115-121.

Mengaued, J., Vicent, M-F., Chenevert, J., Pereira, J.M., Geoffrey, C., Gicquel-Sanzey, B., Baquero, F., Perez-Diaz, J-C. and Cossart, P. (1988) Expression in *Escherichia coli* and sequence analysis of the listeriolysin O determinant of *Listeria monocytogenes*. Infect. Immun. **56:** 766-772.

Miyagawa, E., Azuma, R. and Suto, T. (1979) Cellular fatty acid composition in Gram-negative obligately anaerobic rods. J. Gen. Appl. Microbiol. **25:** 41-51.

Moncla, B.J., Braham, P. and Hillier, S.L. (1990) Sialidase (neuraminidase) activity among gram-negative anaerobic and capnophilic bacteria. J. Clin. Micro. 422-425.

Moore, W.E.C. (1987) Microbiology of periodontal disease. J. Periodontal Res. **22:** 335-341.

Moore, W.E.C., Holdeman, L.V., Smibert, R.M., Good, I.J., Burmeister, J.A. and Hanney, R.R. (1982) Bacteriology of experimental gingivitis in young adult humans. Infec. Immun. **38:** 651-667.

Moore, W.E.C., Holdeman, L.V., Cata, E.P., Smibert, R.M., Burmeister, J.A. and Hanney, R.R. (1983) Bacteriology of moderate (chronic) periodontitis in mature adult humans. Infec. Immun. **42:** 510-515.

Moore, W.E.C., Holdeman, L.V., Cata, E.P., Smibert, R.M., Burmeister, J.A., Palcanis, K.G. and Hanney, R.R. (1985) Comparative bacteriology of juvenile periodontitis. Infect. Immun. **48:** 507-519.

Morrison, D.C. and Jacobs, D.M. (1976) Binding of polymyxin B to the lipid A portion of bacterial lipopolysaccharides. Immunochem. **13:** 813-818.

Mouton, C., Bouchard, D., Deslauriers, M. and Lamonde, L. (1989) Immunological identification and preliminary characterisation of a non-fimbrial haemagglutinin adhesin of *Bacteroides gingivalis*. Infect Immun. **57:** 566-573.

Nagata, H., Murakami, Y., Inoshita, E., Shizukuishi, S. and Tsunemitsu, A. (1991) Inhibitory effect of human plasma and saliva on coaggregation between *Bacteroides gingivalis* and *Streptococcus mitis*.J. Dent. Res. **69:** 1476-1479.

Nagy, E., Dányi, É. and Földes, J. (1990) Plasmid analysis of clinical isolates of *Bacteroides fragilis* group strains. Acta Microbiol. Hung. **37:** 367-373.

Naito Y., Okuda K. and Takazoe I. (1987) Detection of specific antibody in adult human periodontitis sera to surface antigens of *Bacteroides gingivlais*. Infec. Immun. **55:** 832-834.

Naito, Y., Tohda, H., Okuda, K. and Takazoe, T. (1993) Adherence and hydrophobicity of invasive and noninvasive strains of *Porphyromonas gingivalis*. Oral Microbiol Immunol. **8:** 195-202.

Neiders, M.E., Chen, P.B., Suido, H., Reynolds, J.J., Zambon, J.J, Shlossman, M. and Genco, R.J. (1989) Heterogeneity of virulence among strains of *Bacteroides gingivalis*. J. Periodontal Res. **24:** 192-198.

Nilius, A.M., Spencer, S.C. and Simonson, L.G.(1993) Stimulation of in vitro growth of *Treponema denticola* by extracellular growth factors produced by *Porphyromonas gingivalis*. J. Dental Res. **72:** 1027-1031.

Nishikata, M. and Yoshimura, F. (1991) Characterization of *Porphyromonas (Bacteroides) gingivalis* hemagglutinin as a protease. Biochem. Biophys. Res. Commun. **178:** 336-342.

Nishikata, M., Yoshimura, F. and Nodasaka, Y. (1989) Possibility of *Bacteroides gingivalis* hemagglutinin possessing protease activity revealed by inhibition studies. Microbiol. Immunol. **733:** 75-80.

Okuda, K. and Takazoe, I. (1974) Hemagglutinating activity of *Bacteroides melaninogenicus*. Archs. Oral Biol. **19:** 415- 416.

Okuda, K., Slots, J. and Genco, R. J. (1981) *Bacteroides gingivalis, Bacteroides asaccharolyticus*, and *Bacteroides melaninogenicus* subspecies: cell surface morphology and adherence to erythrocytes and human buccal epithelial cells. Curr. Microbiol. **6:** 7-12.

Okuda, K., Fukumoto, Y., Takazoe, I., Slots, J. and Genco, R. J. (1987) Capsular structures of black-pigmented *Bacteroides* isolated from human. Bull. Tokyo dent. Coll. **28:** 1-11.

Okuda, K., Yamamoto, A., Naito, Y., Takazoe, I., Slots, J. and Genco, R.J. (1986) Purification and properties of haemagglutinin from culture supernatant of *Bacteroides gingivalis*. Infect. Immun. **54:** 659-665.

Oliver, W.W. and Wherry, W.B. (1921) Notes on some bacterial parasites of the human mucous membranes. J. Infec. Dis. **28:** 341-345.

Ono, M., Okuda, K. and Takazoe, I. (1987) Purification and characterization of a thiol-protease from *Bacteroides gingivalis* strain 381. Oral Microbiol. Immunol. **2:** 77-81.

Ørskov, I., Ørskov,F., Jann, B. and Jann, K. (1977) Serology, chemistry and genetics of O and K antigens of *Escherichia coli*. Bacteriol. Rev. **41:** 667-710.

Otogoto, J.-I. and Kuramitsu, H.K. (1993) Isolation and characterization of the *Porphyromonas gingivalis prfT* gene, coding for protease activity. Infect. Immun. **61:** 117-123.

Page, R.C. (1986) Gingivitis. J. Clin. Periodontol.**13:** 345-355.

Park, Y. and McBride, B.C. (1992) Cloning of a *Porphyromonas (Bacteroides) gingivalis* protease gene and characterization of its product. FEMS Microbiol. Letts. **92:** 273-278.

Park, Y. and McBride, B.C. (1993) Characterization of the *tpr* gene product and isolation of a specific protease-deficient mutant of *Porphyromonas gingivalis* W83. Infect. Immun. **61:** 4139-4146.

Pearson, W.R. and Lipman, D.J. (1988) Improved tools for biological sequence comparison. P.N.A.S. **85:** 2444-2448.

Pheulpin, P., Tierny, Y., Béchet, M. and Guillaume, J.-B. (1988) Construction of new shuttle plasmid vectors for *Escherichia coli* - *Bacteroides* transgeneric cloning. FEMS Microbiol. Letts. **55:** 15-22.

Postlethwaite, A.E., Lachman, L.B., Mainardi, C.L. and Kang, A.H. (1983) Interlekin 1 stimulation of collagenase production by culture fibroblasts. J. Exp. Med. **157:** 801-806.

Progulske-Fox, A., Tumwasorn, S. and Holt, S.C. (1989a) The expression and function of a *Bacteroides gingivalis* hemagglutinin gene in *Escherichia coli.* Oral. Microbiol. Immunol. **4:** 121-131.

Progulske-Fox, A., Oberste, A., Drummond, C. and McArthur, W.P. (1989b) Transfer of plasmid pE5-2 from *Escherichia coli* to *Bacteroides gingivalis* and *B. intermedius*. Oral Microbiol. Immunol. **4:** 132-134.

Rak, B., Lusky, M. and Hable, M. (1982) Expression of two proteins from overlapping and oppositely oriented genes on transposable DNA insertion sequence element IS*5*. Nature. **297:** 124-128.

Rasmussen, J.L., Odelson, D.A. and Macrina, F.L (1986) Complete nucleotide sequence and transcription of *ermF*, a macrolide-lincosamide-streptogramin B resistance determinant from *Bacteroides fragilis*. J. Bacteriol. **168:** 523-533.

Rasmussen, J.L., Odelson, D.A. and Macrina, F.L (1987) Complete nucleotide sequence of insertion element IS*4351* from *Bacteroides fragilis*. J. Bacteriol. **169:** 3573-3580.

Reeve, J.N. (1977) Bacteriophage infection of minicells. A general method for identification of 'in vivo' bacteriophage directed polypeptide biosynthesis. Mol. Gen. Genet. **158:** 73-79.

Reynolds, H. S., van Winkelhoff, A. J., Schifferle, R. E., Chen, P. B. and Zambon, J. J. (1989) Relationship of encapsulation of *Bacteroides gingivalis* to invasiveness. J. Dent. Res. **68:** special issue: abstr. 1171.

Rezsöhazy, R., Hallet, B., Declour, J. and Mahillon, J. (1993) The *IS*4 family of insertion sequences: evidence for a conserved transposase motif. Mol. Microbiol. **9:** 1283-1295.

Rizza, V., Sinclair, P.R., White, D.C. and Courant, P.R. (1968) Electron transport system of the protoheme-requiring anaerobe *Bacteroides melaninogenicus*. J. Bacteriol. **96:** 665-671.

Robbins, J.B., McCracken, G.H., Gotschlich, E.C., Ørskov, I and Hanson, L.A. (1974) *Escherichia coli* K1 capsular polysaccharide associated with neonatal meningitis. N. Engl. J. Med. **290:** 267-271.

Roberts, I.S., Gharbia, S., Arnott, M.A., Wallace, A., Shah, H.N. and Williams, D.M. (1990) Purification of the secreted thiol-activated protease of *Porphyromonas (Bacteroides) gingivalis* and the cloning and expression of the gene in *Escherichia coli*. *In* Clinical and molecular aspects of anaerobes ed. Borriello, S.P. pp. 227-233. Petersfield: Wrightson Biomedical Publishing Ltd.

Roberts, I.S., Mountford, R., High, N., Bitter-Suerman, D., Jann, K., Timmis, K.N. and Boulnois, G.J. (1986) Molecular cloning and analysis of the genes for the production of the K5, K7, K12 and K92 capsular polysaccharides of *Escherichia coli*. J. Bacteriol. **168:** 1228-1233.

Robillard, N.J., Tally, F.P. and Malamy, M.H. (1985) Tn*4400*, a compound transposon isolated from *Bacteroides fragilis*, functions in *Escherichia coli*. J. Bacteriol. **164:** 1248-1255.

Romero, D.A. and Klaenhammer, T.R. (1991) Construction of an IS*946*-based composite transposon in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **173:** 7599-7606.

Roy, T.E. and Kelley, C. D. (1939) Genus VIII. *Bacteroides* Castellani and Chalmers p556 569. In: D.H. Bergey, R.S. Breed, E.G.D. Murray, and A.P. Hitchens (ed.), Bergey's manual of determinative bacteriology, 5th ed. Williams and Wilkins Co., Baltimore.

Saarela, M., Stucki, A.-M., von Troil-Lindén, B., Alaluusua, S., Jousimies-Somer, H. and Asikainen, S. (1993) Intra- and inter- individual comparison of *Porphyromonas gingivalis* genotypes. FEMS Immunol. Med. Microbiol. 99-102.

Saito, H. and Miura, K.I. (1963) Preparation of transforming deoxyribonucleic acid by phenol treatment. Biochim. Biophys. Acta. **72:** 619-629.

Sako, K., Takazoe, I. and Okuda, K. (1988) Isolation and characterization of plasmid DNA from *Bacteroides* strains isolated from the human oral cavity. Oral Microbiol. Immunol. **3:** 72-76.

Salit, I.E., Hanley, J., Clubb, L. and Fanning, S. (1988) Detection of pilus subunits (pilins) and filaments by using anti-P pilin antisera. Infect. Immun. **56:** 2330-2335.

Salyers, A.A., Pajeau, M. and McCarthy, R.E. (1988) Importance of mucopolysaccharides as substrates for *Bacteroides thetaiotaomicron* growing in intestinal extracts of exgermfree mice. Appl. Env. Microbiol. **54:** 1970-1976.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) Molecular cloning: a laboratory manual, 2nd ed, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Sandmeier, H., Bar, K. and Meyer, J. (1993) Search for bacteriophages of black-pigmented Gram-negative anaerobes from dental plaque. FEMS Immunol. Med. Microbiol. **6:** 193-194.

Sanger, F., Nicklen, S. and Coulson, A.R. (1977) DNA sequencing with chain termination inhibitors. PNAS. **74:** 5463-5467.

Savitt, E.D. and Kent, R.L. (1991) Distribution of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis* by subject age. J. Periodontol. **62:** 490-494.

Sawyer, S.J., MacDonald, J.B. and Gibbons, R.J. (1962) Biochemical characteristics of *Bacteroides melaninogenicus*. A study of thirty-one strains. Arch. oral Biol. **7:** 685-691.

Schenkein, H.A. (1988) Failure of *Bacteroides gingivalis* W83 to accumulate bound C3 following opsonization with serum. J. Periodontal Res. **24:** 20-27.

Schifferle, R.E. , Reddy, M.S., Zambon, J.J., Genco, R.J. and Levine, M.J. (1989) Characterization of a polysaccharide antigen from *Bacteroides gingivalis*. J. Immunl. **143:** 3035-3042.

Schifferle, R.E., Chen, P.B., Aguirre, A., Genco, R.J. and Levine, M.J. (1992) Polysaccharide-protein conjugate modifies experimental *Porphyromonas gingivalis* infection. J. Dental Res. **71:** special issue abstr. 1485.

Shah, H.N., Bonnet, R., Mateen, B. and Williams, R.A.D. (1979) The porphyrin pigmentation of subspecies of *Bacteroides melaninogenicus*. Biochem. J. **180:** 45-50.

Shah, H.N. and Collins, M.D. (1980) Fatty acid and isoprenoid quinone composition in the classification of *Bacteroides melaninogenicus* and related taxa. J. Appl. Bacteriol. **48:** 75-87.

Shah, H.N. and Collins, M.D. (1981) *Bacteroides buccalis*, sp. nov., *Bacteroides denticola*, sp. nov., and *Bacteroides pentosaceus*, sp. nov., new species of the genus *Bacteroides* from the oral cavity. Zentralblatt fur Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene, Abteilung I Originate, Reihe C2, 235-241.

Shah, H.N. and Collins, M.D. (1988) Proposal for reclassification of *Bacteroides asaccharolyticus*, *Bacteroides gingivalis*, and *Bacteroides endodontalis* in a new genus, *Porphyromonas*. Int. J. Syst. Bacteriol. **38:** 128-

Shah, H.N. and Collins, M.D. (1990) *Prevotella*, a new genus to include *Bacteroides melaninogenicus* and related species formerly classified in the genus *Bacteroides*. Int. J. Syst. Bacteriol. **40:** 205-208.

Shah, H.N. and Gharbia, S. (1989) Lysis of erythrocytes by the secreted cysteine protease of *Porphyromonas gingivalis* W83. FEMS Microbiol. Lett. **61:** 213-218.

Shah, H.N., Gharbia, S.E., Kowlessur, D., Wilkie, E. and Brocklehurst, K. (1990) Isolation and characterization of gingivain, a cysteine proteinase from *Porphyromonas gingivalis* strain W83. Biochem. Soc. Trans. **18:** 578-579.

Shah, H.N., Seddon, S.V. and Gharbia, S.E. (1989) Studies on the virulence properties and metabolism of pleiotropic mutants of *Porphyromonas gingivalis* (*Bacteroides gingivalis*) W50. Oral Immunol. Microbiol. **4:** 19-23.

Shah, H.N. and Williams, R.A.D. (1987) Utilisation of glucose and amino acids by *Bacteroides intermedius* and *Bacteroides gingivalis*. Curr. Microbiol. **15:** 241-246.

Shah, H.N., Williams, R.A.D., Bowden, G.H. and Hardie, J.M. (1976) Comparison of the biochemical properties of *Bacteroides melaninogenicus* from human dental plaque and other sites. J. Appl. Bacteriol. **41:** 473-492.

Shapiro, J.A. (1969) Mutations caused by the insertion of genetic material into the galactose operon of *Escherichia coli*. J. Mol. Biol. **40:** 93-105.

Sharma, A., Sojar, H.T., Lee, J.-Y. and Genco, R.J. (1993) Expression of a functional *Porphyromonas gingivalis* fimbrillin polypeptide in *Escherichia coli*: purification, physicochemical and immunochemical characterization, and binding characteristics. Infect. Immun. **61:** 3570-3573.

Shelburne, C.E., Sanberg, G.P., Binsfeld, C.A., Wolff, L.F. and Curry, R.A. (1993) Monoclonal antibodies to lipopolysaccharide of four oral bacteria associated with periodontal disease. J. Periodont. Res. **28:** 1-9.

Shoemaker, N.B., Anderson, K.L., Smithson, S.L., Wang, G.-R. and Salyers, A.A. (1991) Conjugal transfer of a shuttle vector from the human colonic anaerobe *Bacteroides uniformis* to the ruminal anaerobe *Prevotella (Bacteroides) ruminicola* B_14. Appl. Env. Microbiol. **57:** 2114-2120.

Shoemaker, N.B, Barber, R.D. and Salyers, A.A. (1989) Cloning and characterization of a *Bacteroides* conjugal teteracycline-erythromycin resistance element by using a shuttle cosmid vector. J. Bacteriol. **171:** 1294-1302.

Shoemaker, N.B., Guthrie, E.P., Salyers, A.A. and Gardner, J.F. (1985) Evidence that the clindamycin-erythromycin resistance gene of *Bacteroides* plasmid pBF4 is on a transposable element. J. Bacteriol. **162:** 626-632.

Shoemaker, N.B., Getty, C., Gardner, J.F. and Salyers, A.A. (1986) Tn*4351* transposes in *Bacteroides* and mediates the integration of R751 into the *Bacteroides* chromosome. J. Bacteriol. **165:** 929-936.

Shoemaker, N.B. and Salyers, A.A. (1987) Facilitated transfer of IncPβ R751 derivatives from the chromosome of Bacteroides uniformis to Escherichia coli recipients by a conjugal Bacteroides tetracycline resistance element. J. Bacteriol. **169:** 3160-3167.

Silver, R.F., Finn, C.W., Vann, W.F., Aaronson, W., Schneerson, R., Kretschmer, P.J. and Garon, C.F.(1981) Molecular cloning of the K1 capsular polysaccharide genes of *Escherichia coli.* Nature. **289:** 696-698.

Sismey-Durrant, H.J. and Hopps, R.M. (1991) Effect of lipopolysaccharide from *Porphyromonas gingivalis* on prostaglandin E_2 and interleukin-1b release from rat peiosteal and human gingival fibroblasts *in vitro.* Oral Microbiol. Immunol. **6:** 378-380.

Slots, J. (1979) Subgingival microflora and periodontal disease. J. Clin. Periodontol. **6:** 351-382.

Slots, J. (1981) Enzymatic characterization of some oral and non-oral Gram-negative bacteria with the API ZYM system. J. Clin. Microbiol. **14:** 288-294.

Slots, J. (1982) Importance of black-pigmented *Bacteroides* in human periodontal disease. In: Host-parasite interactions in periodontal disease. R. J. Genco and S. E. Mergenhagen, Eds., Washington D. C.: A. S. M. pp27-45.

Slots, J. and Genco, R.J. (1979) Direct hemagglutination technique for differentiating *Bacteroides asaccharolyticus* oral strains from non-oral strains. J. Clin. Microbiol. **10:** 371-373.

Slots, J. and Genco, R.J. (1980) *Bacteroides melaninogenicus* subsp. *macacae*, a new subspecies from monkey periodontopathic indigenous microflora.Int. J. Syst. Bacteriol. **30:** 82-85.

Slots, J and Genco, R. J. (1984) Black-pigmented *Bacteroides* species, *Capnocytophaga* species, and *Actinobacillus actinomycetemcomitans* in human periodontal disease: virulence factors in coloniation, survival, and tissue destruction. J. Dent. Res. **63:** 412-421.

Slots, J. and Gibbons, R. J. (1978) Attachment of *Bacteroides melaninogenicus* subspecies *asaccharolyticus* to oral surfaces and its possible role in colonization of the mouth and of periodontal pockets. Infec. Immun. **19:** 254- 264.

Slots, J. and Listgarten, M.A. (1988) *Bacteroides gingivalis, Bacteroides intermedius* and *Actinobacillus actinomycetemcomitans* in human periodontal diseases. J. Clin. Periodontol. **15:** 85-93.

Smalley, J.W. and Birss, A.J. (1987) Trypsin-like enzyme activity of the extracellular membrane vesicles of *Bacteroides gingivalis* W50. J. Gen. Microbiol. **133:** 2883-2894.

Smalley, J.W. and Birss, A.J. (1991) Extracellular vesicle-associated and soluble trypsin-like enzyme fractions of *Porphyromonas gingivalis* W50. Oral Microbiol. Immunol. **6:** 202-208.

Smalley, J.W., Birss, A.J. and Shuttleworth, C.A. (1988) The degradation of type I collagen and human plasma fibronectin by the trypsin-like enzyme and extracellular membrane vesicles of *Bacteroides gingivalis* W50. Arch. oral Biol. **33:** 323-329.

Smalley, J.W., Shuttleworth, C.A and Birss, A.J. (1989) Collagenolytic activity of the extracellular vesicles of *Bacteroides gingivalis* W50 and an avirulent mutant W50/BE1. Arch. Oral Biol. **34:** 579-583.

Smith, C.J. (1987) Nucleotide sequence analysis of Tn*4351*: use of *ermFS* operon fusions to detect promoter activity in *Bacteroides fragilis*. J. Bacteriol. **169:** 4589-4596.

Smith, C.J. and Gonda, M.A. (1985) Comparison of transposon-like structures encoding clindamycin resistance in *Bacteroides* R plasmids. Plasmid.**13:** 182-192.

Socransky, S.S., Manganiello, A.D., Propas, D., Oram, V. and van Houte, J. (1977) Bacteriological studies of developing supragingival dental plaque. J. Periodont. Res. **12:** 90-106.

Söder, P.-Ö. (1972) Proteolytic activity in the oral cavity: proteolytic enzymes from human saliva and dental plaque material. J. Dent. Res. **51:** 389-393.

Sorsa, T., Uitto, V.-J., Suomalainen, K., Turto, H. and Lindy, S. (1987) A trypsin-like protease from *Bacteroides gingivalis*: partial purification and characterization. J. Periodont. Res. **22:** 375-380.

Southern, E. (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98:** 503-

Spiegel, C.A., Hayduk, S.E., Minah, G.E. and Krywolap, G.N. (1979) Black-pigmented *Bacteroides* from clinically characterized periodontal sites. J. Periodontal Res. **14:** 376-382.

Stevens, A.M.. Sanders, J.M., Shoemaker, N.B. and Salyers, A.A. (1992) Genes involved in production of plasmidlike forms by a *Bacteroides* conjugal chromosomal element share amino acid homology with two-component regulatory systems. J. Bacteriol. **174:** 2935-2942.

Stevens, P., Huang, S.N.H., Welch, W.D. and Young, L.S. (1978) Restricted complement activation by*Escherichia coli* with the K-1 capsular serotype: a possible role in pathogenicity. J. Immunol. **121:** 2174-2180.

Stinson, M.W., Safulko, K. and Levine, M.J. (1991) Adherence of *Porphyromonas gingivalis* to *Streptococcus sanguis* in vitro. Infect. Immun. **59:** 102-108.

Sundqvist, G.K., Carlsson, J. and Hanstrom, L. (1987) Collagenolytic activity of black-pigmented *Bacteroides* species. J. Periodontal Res. **22:** 300-306.

Sundqvist, G.K., Carlsson, J., Herrmann, B.F. and Tarnvik, A. (1985) Degradation of human immunoglobulins G and M and complement factors C3 and C5 by black-pigmented *Bacteroides*. J. Med. Micro. **19:** 85-94.

Sutherland, I.W. (1972) Microbial exopolysaccharides. Adv. Microbial Phys. **8:** 143-213.

Sutherland, I. W. (1977) Bacterial exopolysaccharides - their nature and production. In: Surface carbohydrates of the prokaryotic cell. Sutherland, I.W. (ed.). pp.27-96. Academic Press Inc. (London) Ltd.

Suzuki, Y., Yoshimura, F., Tani, H. and Suzuki, T. (1988) Fimbriae from the oral anaerobe *Bacteroides gingivalis*: a screening of clinical isolates from various places. Adv. Dental Res. **2:** 301-303.

Sweeney , E.A., Alcoforado, G.A.P., Nyman, S. and Slot, J. (1987) Prevalence and microbiology of localized prepubertal periodontitis. Oral Microbiol. Immunol. **2:** 65-70.

Swindlehurst, C.A., Shah, H.N., Parr, C.W. and Williams, R.A.D. (1977) Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of polypeptides from *Bacteroides melaninogenicus*. J. Appl. Bacteriol. **43:** 319-324.

Syed, S.A. and Loesche, W.J. (1978) Bacteriology of human experimental gingivitis; effect of plaque age. Infect. Immun. **21:** 821-829.

Takahashi, N., Kato, T. and Kuramitsu, H.K. (1991) Isolation and preliminary characterization of the *Porphyromonas gingivalis prt*C gene expressing collagenase activity. FEMS Microbiol. Letts. **84:** 135-138.

Takazoe, I., Nakamura, T., Homma, T., Yamaoto, A., Hashimoto, Y., and Okuda, K. and Tanaka, M. (1971) Growth promoting effect of *Corynebacterium* upon *Bacteroides*. Bull. Tokyo dent. Coll. **12:** 95-97.

Tamura, M., Tokuda, M., Nagaoka, S. and Takada, H. (1992) Lipopolysaccharides of *Bacteroides intermedius (Prevotella intermedia)* and *Bacteroides (Porphyromonas) gingivalis* induce interleukin-8 gene expression in human gingival fibroblast cultures. Infect. Immun. **60:** 4932-4937.

Terai, A., Baba, K., Shirai, H., Yoshida, O., Takeda, Y. and Nishibuchi, M. (1991) Evidence for insertion sequence-mediated spread of the thermostable direct hemolysin gene among *Vibrio* species. J. Bacteriol. **173:** 5036-5046.

Theilade, E., Wright, W. H., Jensen, S. B. and Loe, H. (1967) Experimental gingivitis in man. II. A longitudinal, clinical, and bacteriological investigation. J. Periodont. Res. **1:** 1-13.

Trinks, K., Habermann, P., Beyreuther, K., Starlinger, P. and Ehring, R. An IS*4* encoded protein is synthesized in minicells. Molec. Gen. Genet. **182:** 183-188.

Towbin, H., Staehelin, T. and Gordon, J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. P.N.A.S. **76:** 4350-4354.

van der Zee, A., Agterberg, C., van Agterveld, M., Peeters, M. and Mooi, F.R. (1993) Characterization of IS*1001*, an insertion sequence element of *Bordetella parapertussis*. J. Bacteriol. **175:** 141-147.

van Dyke, T.E., Offenbacher, S., Place, D., Dowell, V.R. and Jones, J. (1988) Refractory periodontitis: mixed infection with *Bacteroides gingivalis* and other *Bacteroides* species. J. Periodontol. **59:** 184-189.

van Oss, C.J. and Gillman, C.F. (1973) Phagocytosis as a surface phenomenon: influence of C1423 on the contact angle and on the phagocytosis of sensitized encapsulated bacteria. Immunol. Commun. **2:** 415-

van Steenbergen, T.J.M. and de Graff, J. (1986) Proteolytic activity of black-pigmented *Bacteroides* strains. FEMS Microbiol. Lett. **33:** 219-222.

van Steenbergen, T.J.M., Namavar, F. and de Graff, J. (1985) Chemiluminescence of human leukocytes by black-pigmented *Bacteroides* strains from dental plaque and other sites. J. Periodontal Res. **20:** 58-71.

van Steenbergen, T.J.M., Delemarre, F.G.A., Namavar, F. and de Graff, J. (1987) Differences in virulence within the species *Bacteroides gingivalis*. Antonie van Leeuwenhoek. **53:** 233-244.

van Steenbergen, T.J.M., Menard, C., Tijhof, C.J., Mouton, C. and de Graaff, J. (1993) Comparison of three molecular typing methods in studies on transmission of *Porphyromonas gingivalis*. J. Med. Microbiol. **39:** 416-421.

van Steenbergen, T.J.M., van Winkelhoff, A.J., Mayrand, D., Grenier, D. and de Graff, J. (1984) *Bacteroides endodontalis* sp. nov., an asaccharolytic black-pigmented *Bacteroides* species from infected dental root canals. Int. J. Syst. Bacteriol. **34:** 118-120.

van Steenbergen , T.J.M., van Winkelhoff, A.J., van der Velden, U. and de Graaff, J. (1989) Taxonomy, virulence and epidemiology of black-pigmented *Bacteroides* species in relation to oral infections. Infection. **17:** 194-196.

van Winkelhoff, A.J., Appelmelk, B.S., Kippuw, N. and de Graaf, J. (1993) K-antigens in Porphyromonas gingivalis are associated with virulence. Oral Microbiol. Immunol. **8:** 259-265.

van Winkelhoff, A.J., van Steenbergen, T.J.M., Kippuw, N. and de Graaff, J. (1985) Further characterization of *Bacteroides endodontalis*, an asaccharolytic black-pigmented *Bacteroides* species from the oral cavity. J. Clin. Microbiol. **22:** 75-79.

Vermeulen, Cross, A., Byrne, W.R. and Zollinger, W. (1988) Quantitative relationship between capsular content and killing of K1-encapsulated *Escherichia coli*. Infect. Immun. **56:** 2723-2730.

Voller, A., (1980) Enzyme linked immunosorbent assay. In: Manual of clinical immunology, Washington D. C. Am. Soc. Microbiol.

Wallace, A., Rigg, G., Hyman, S.C., James, R. and Roberts, I.S. (1992) Generation and characterization of a monoclonal antibody specific for the major thiol-activated cysteine proteinase of *Porphyromonas gingivalis* W83. Letts. Appl. Microbiol. **15:** 202-206.

Watanabe, K., Yamaji, Y. and Umemoto, T. (1992) Correlation between cell-adherent activity and surface structure in *Porphyromonas gingivalis*. Oral Microbiol. Immunol. **7:** 357-363.

Welch, R.A. and Macrina, F.L. (1981) Physical characterization of *Bacteroides* R plasmid pBF4. J. Bacteriol. **145:** 867-872.

White, D. and Mayrand, D. (1981) Association of oral *Bacteroides* with gingivitis and adult periodontitis. J. Periodontal Res. **16:** 259-265.

Wilson, M., Kamin, S. and Harvey, W. (1985) Bone resorbing activity of purified capsular material from *Actinobacillus actinomycetemcomitans*. J. Periodont. Res. **20:** 484-491.

Wilson, M., Meghji, S., Barber, P. and Henderson, B. (1993) Biological activities of surface-associated material from *Porphyromonas gingivalis*. FEMS Immunol. Med. Microbiol. **6:** 147-156.

Winkelstein, J. A. (1981) The role of complement in the host's defence against *Streptococcus pneumoniae*. Rev. Infect. Dis. **3:** 289-

Woese, C.R. (1987) Bacterial evolution. Microbiol. Rev. **51:** 221-271.

Yamasaki, T., Nagata, A., Kiyoshige, T., Sato, M. and Nakamura, H. (1990) Black-pigmented, asaccharolytic *Bacteroides* species resembling *Porphyromonas gingivalis* (*Bacteroides gingivalis*) from beagle dogs. Oral Microbiol. Immunol. **5:** 332-335.

Yanisch-Perron, C., Viera, J. and Messing, J. (1985) Improved M13 phage cloning vectors and host strains: nucleotide sequences of M13mp18 and pUC19 vectors. Gene. **33:** 103-119.

Yoshimoto, H. and Umemoto, T (1990) Characterization and physical mapping of plasmids of black-pigmented *Bacteroides*. Oral Microbiol. Immunol. **5:** 208-212.

Yoshimoto, H., Takahashi, Y., Hamada, N. and Umemoto, T. (1993) Genetic transformation of Porphyromonas gingivalis by electroporation. Oral Microbiol. Immunol. **8:** 208-212.

Yoshimura, F., Nishikata, M., Suzuki, T., Hoover, C.I. and Newbrun, E. (1984) Characterization of a trypsin-like protease from the bacterium *Bacteroides gingivalis* isolated from human dental plaque. Arch. Oral Biol. **29:** 559-564.

Yoshimura, F., Takahashi, K., Nodasaka, Y. and Suzuki, T. (1984) Purification and characterization of a novel type of fimbriae from the oral anaerobe *Bacteroides gingivalis*. J. Bacteriol. **160:** 949-957.

Yu, H.S.K. and Bramanti, T.E. (1993) Hemin uptake in *Porphyromonas gingivalis* is mediated by Omp26. J. Dent. Res. **72:** special issue abstr. 382.

Zafiropoulos, G-G.K., Flores-de-Jacoby, L., Hungerer, K-D. and Nisengard, R.J. (1992) Humoral antibody responses in periodontal disease. J. Periodontol. **63:** 80-86.

Zambon, J.J., Reynolds, H.S. and Slots, J. (1981) Black-pigmented *Bacteroides* spp. in the human oral cavity. Infect. Immun. **32:** 198-203.