MOLECULAR CLONING AND ANALYSIS OF A β-1,3-GLUCANASE FROM *ARTHROBACTER LUTEUS (OERSKOVIA XANTHINEOLYTICA)*

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by

David Mark Whitcombe BSc. (York)

Leicester Biocentre and Department of Biochemistry

University of Leicester

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

ABSTRACT

MOLECULAR CLONING AND ANALYSIS OF A β-1,3-GLUCANASE GENE FROM *ARTHROBACTER LUTEUS*.

David Mark Whitcombe

Species of *Arthrobacter luteus*, also known as *Oerskovia xanthineolytica*, can utilise yeast cells as a growth substrate. This unusual ability is due to the secretion of a battery of hydrolytic enzymes which degrade the yeast cell wall and thus lyse the cells. Although many hydrolytic enzymes are important in the degradation of the yeast cell wall, the key activities are endo-β-1,3-glucanases. In order to characterise components of the yeast lytic system and the genetic organisation of this little-understood organism, a molecular cloning approach was adopted. Large clones expressing β-1,3-glucanase were isolated from a library of *A. luteus* DNA constructed in the positive selection vector pKGW.

By a combination of subcloning, restriction mapping and Southern analysis, it was determined that the clones contained virtually the same inserts. Additional subcloning, transposon mutagenesis, deletion mapping and nucleotide sequencing were used to identify at least one glucanase gene. The predicted protein product had a molecular weight of about 46 kD. When the gene was expressed in a number of *in vivo* and *vitro* systems including *E. coli* minicells and a *Streptomyces* coupled transcription/translation system, the protein observed had a similar molecular weight. Furthermore, when the protein was produced in *E. coli* and run on activity stained gels, the β-glucanase activity co-migrated with the major glucanase of *A. luteus*. In addition the *E. coli*-produced glucanase had the ability to cause limited lysis of yeast.

FOR L.W.

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COMMUNICATIONS

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CONTENTS PAGE

ABBREVIATED RESTRICTION ENZYMES

Throughout, the following initials are used for various restriction enzymes.

B for *BamH* I

E for *EcoR* I

G for *Bgl* II

H for *Hind* III

K for *Kpn* I

P for *Pst* I

S for *Sal* I

T for *Sst* I

X for *Xho* I

Other Abbreviations Not Described in the Text

A_{123}.....Absorbance at 123 nm

dNTP ..... Deoxynucleoside 5' triphosphate

ddNTP.....Dideoxynucleoside 5' triphosphate

EDTA .....Diaminoethanetetracetic acid, disodium salt

IPTG.....Isopropyl-β-D-Thiogalactopyranoside

Q water.....Distilled water, passaged through Millipore Milli-Q ion exchange filters

SDS.....Sodium dodecyl sulphate

TEMED.....N,N,N',N','-Tetramethylethylenediamine

X-Gal.....5-Bromo-4-Chloro-3-Indolyl-β-D-Galactopyranoside

CHAPTER 1: INTRODUCTION

1.1 Aims

Simply stated, the aims of this work were:

(a) to obtain recombinant clones which carried the gene(s) encoding the yeast lytic enzyme(s) of *Arthrobacter luteus*;

(b) to obtain the nucleotide sequence of the cloned gene(s);

(c) to analyse the gene structure and other characteristics of the genetic material of this little-understood organism;

(d) to use the clones isolated above to study the relationships between the enzymes of the yeast lytic system of this organism.

1.2 The Problem

Lysis of the cells of bacteria and yeast has been considered important for a number of reasons. These include:

(a) the release of specific carbohydrates of the cell wall;

(b) the release of all the cell constituents e.g. the manufacture of yeast extracts;

(c) the release of specific endogenous enzymes which may be intracellular, or associated with the cell membrane or wall e.g invertase from *Saccharomyces cerevisiae.*

(d) the release of the products of foreign genes expressed in heterologous systems (especially in yeast).

1.21 Mechanical Disruption

Currently, breakage of microbes on an industrial scale is performed by mechanical means, usually in a high pressure homogeniser. Indeed, even on a relatively small scale such as the bench or pilot plant, bead mills are routinely used to fracture cultured cells. There are, however, disadvantages to these procedures: they require expensive equipment and high temperatures and shear stresses are often generated, which may affect the biological activity of a recovered enzyme. At present, though, more gentle procedures such as enzymic treatment of cells on this scale are prohibitively expensive.

1.22 Treatment With Enzymes

Despite the financial drawbacks of enzymes in these procedures, they have several advantages. The solubilisation of protein and saccharides in the production of yeast extract has been shown to be dramatically increased by the use of enzymes with or without mechanical treatment (Knorr *et al.*, 1979). Furthermore, many of the cloned genes which are expressed in heterologous hosts encode low yield, high value products. Therefore the cost of enzymes to improve yields would seem to be less restrictive in

these cases.

1.23 Why Yeast?

Yeast is, by far, the best studied eukaryotic microbe. It is very amenable to genetic manipulation and has been widely used in the food and drinks industries for centuries. For these reasons, it has been proposed as an appropriate host for the expression of foreign genes. An attraction of yeast in this respect is its capacity to secrete proteins into the culture medium. This process is genetically and biochemically well characterised (Novick *et al.*, 1980; 1981). Since few natural yeast proteins are secreted under normal circumstances, any heterologous products which might be exported, could be purified with relative ease. However, proteins may not be efficiently processed if the secretory pathway is overloaded. Therefore, significant amounts of a highly expressed gene product would accumulate within the cell. This may be overcome by lysis of the cells by enzymic or mechanical means.

Besides aiding recovery of intracellular products, enzymes which attack the yeast cell wall have other useful applications. In a laboratory context, they can be used in the preparation of yeast sphaeroplasts in which the cell wall is partially degraded in osmotically buffered conditions. These sphaeroplasts are routinely used in a variety of processes, most commonly in the genetic transformation of yeast cells with DNA or for "protoplast fusion" (Peberdy, 1980). Other aspects of yeast biology can be studied using these methods, for example, the controlled release of specific wall associated glycoproteins can be achieved (Valentin *et al.*, 1984), in contrast to the non-specific release obtained by mechanical means. Furthermore, yeast sphaeroplasts can regenerate their walls and this provides a means of investigating the biogenesis of the new cell wall (Necas and Kopecka, 1969).

Outside the laboratory, enzymes which attack the cell wall of yeast may find application in the treatment of insoluble yeast waste from breweries and distilleries. Much of the spent yeast is used to produce yeast extract which is often added to animal foodstuffs and used as a flavour enhancer in tinned foods. However the autolysis of these large quantities of cells leaves a significant volume of insoluble waste which is rich in carbohydrate and protein. This material is usually treated as effluent waste, incurring serious financial penalties for its disposal. In addition, it is a waste of a potentially rich growth substrate. Commercial enzyme preparations, such as "Zymolyase" (Kaneko *et al.*, 1974) or "Lyticase" (Scott and Schekman, 1980) which enhance the yield of protein and saccharides from this waste would also decrease the mass and increase the solubility of the yeast material, thereby reducing the financial penalties. However, these enzymes are prohibitively expensive for use on such a large scale.

1.24 Why Molecular Cloning?

The advent of molecular cloning has made available a wider range of approaches to many biological problems. In this case, the molecular cloning of genes encoding yeast degrading enzymes would have several advantages. For example, if the release of particular proteins from the yeast cell wall was required, it is likely that additional specificity would be achieved by using the pure products of cloned genes. Alternatively, the isolation of such genes might enable their introduction into other bacteria and so confer upon them the ability to grow upon a novel substrate, i.e. the yeast waste material. Conversely, an understanding of the structure and organisation of *A. luteus* genes might enable the introduction of genes encoding useful pathways thus enabling the conversion of a waste material (the yeast walls) to a beneficial end product. In addition, the cloned genes could be re-introduced into the original host and used to achieve higher yields of the gene products. These applications of cloning techniques would require an understanding of the DNA sequences which are important in the expression of DNA in that host and an efficient means of introducing cloned DNA back into the host.

1.3 The Substrate: the Chemical Composition of the Yeast Cell Wall

Saccharomyces cerevisiae is the most commonly used yeast, both in the laboratory, and in industry (for both brewing and baking). As a result, this work will focus entirely on *S. cerevisiae* and unless otherwise stated, the word yeast will always refer to this species.

It is useful to distinguish between "protoplast" and "sphaeroplast". Sphaeroplasts are yeast cells from which much of the cell wall has been removed (usually enzymically) while true protoplasts have no remnants of cell wall remaining. Both sphaeroplasts and protoplasts are fragile and highly susceptible to osmotic stress and must be maintained in hypertonic medium. Because lysis of unbuffered sphaeroplasts occurs readily, there is no real difference between a yeast sphaeroplasting activity and a yeast lytic activity since the distinction lies merely in the osmotic conditions.

The cell of *S. cerevisiae* is bounded by the plasma membrane which provides little or no protection from physical, chemical or biological stresses. Protection from these is conferred by the yeast cell wall which is composed largely (80-90%) of polysaccharides, with small quantities of other components e.g. proteins, lipids (Bartnicki-Garcia, 1973; Phaff, 1971).

The polysaccharides of the yeast cell wall are comprised mainly of D-glucose, N-acetylglucosamine and D-mannose: the basic components of β-glucan, chitin and mannan respectively. These three carbohydrate polymers are the important structural units of the yeast cell wall.

1.31 β-Glucan

"Glucan" is a generic term applied to a range of β-linked glucose polymers, which are heterogeneous with respect to both molecular weight and types of glycosidic bond. Homopolymeric β-1,4-glucan is better known as cellulose and is usually not described as a β-glucan. Figure 1.1 illustrates the most common linkages found in β-glucan.

The structural rigidity and protective qualities of the yeast cell wall are largely derived from its β-1,3-glucan components and hence an understanding of the chemical structures involved is essential. The biochemical structures have been reviewed many times (Wessels and Sietsma, 1981; Fleet and Phaff, 1981).

Although some cellulose is found in the yeast wall, the major glucan components (as determined by chemical extraction and defined enzymic hydrolysis) comprise β-1,3 and β-1,6-linked glucans (Manners and Masson, 1969; Manners *et al.*, 1973a). There are three main types of β-glucan in the cell wall which are usually characterised in terms of their solubility under various conditions (Manners *et al.*, 1973b).

The treatments which define the various glucans are (a) Solubilisation by extraction with hot alkali; (b) extraction with acetic acid. The bulk of the yeast glucan is the insoluble residue.

The first type of glucan is the material solubilised by the initial alkali extraction of the yeast wall material. Little is known of its structure but it is thought to consist of short chains of β-1,3-glucan having a degree of polymerisation (DP) of 50-100 (Fleet and Phaff, 1981). It is possible that this fraction is a nascent glucan molecule which has yet to be assembled into the large alkali insoluble polymer. It comprises less than 10% of the total glucan of the cell wall and is rarely considered.

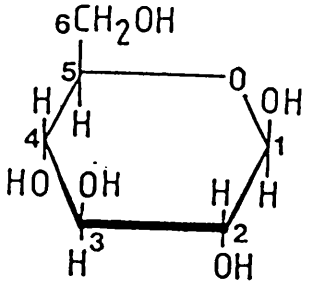
Extraction with acetic acid solubilises 10-15% of the alkali insoluble material. This fraction is composed mainly of β-1,6-linked glucose residues (Manners *et al.*, 1973b). Its molecular weight is relatively low (DP 140) and it is highly branched, having approximately 15% of the glucose residues triply substituted at the 1,3, and 6 positions. It has been proposed that the β-1,6-linked glucan and other polymers are embedded in the insoluble microfibrils of the β-1,3-linked glucan (Kopecka *et al.*, 1974).

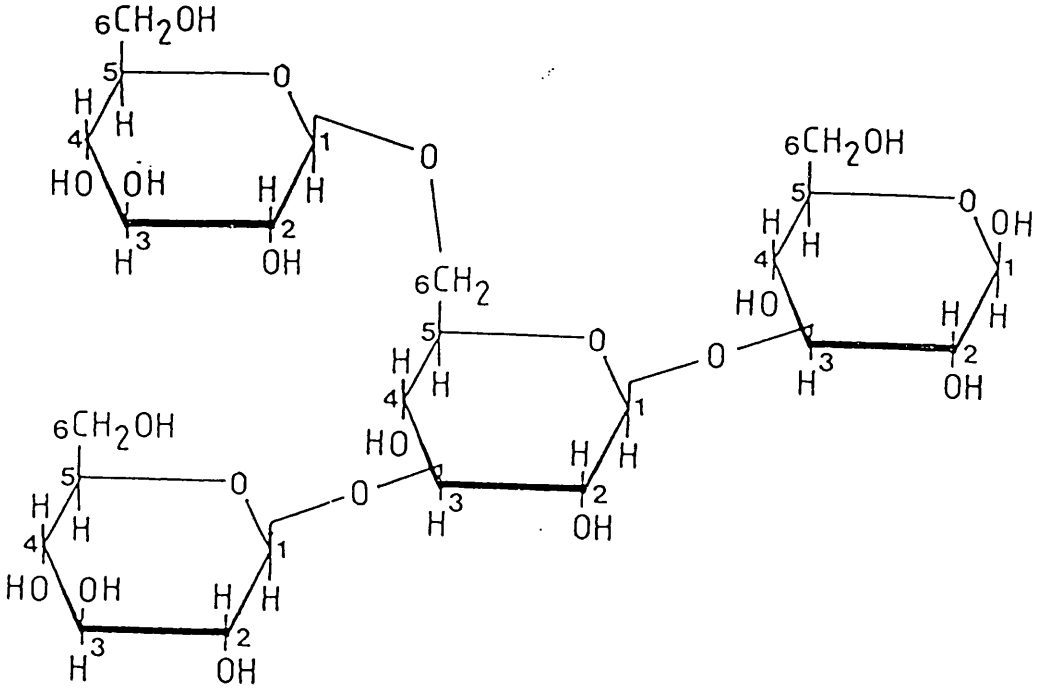
The residual glucan (85-90% of the alkali insoluble material) is the major structural determinant of the yeast cell wall. It consists of long chains of β-1,3-linked glucose residues (DP 1500) and approximately 3% of the residues are β-1,6-linked branch points. This glucan assembles into the long microfibrils to generate the essential structural strength of the wall (Necas and Kopecka, 1969; Kopecka *et al.*, 1974).

Figure 1.1: Components of Yeast Glucan.

The Upper part of the figure shows β-D-Glucose.

The Lower panel shows a β-1,3-glucan chain including a branch point: a triply-linked (at the 1,3, and 6 positions) glucose residue.





1.32 Mannan and Mannoproteins

By mass, mannoproteins are the major constituents of the yeast cell wall (Bartnicki-Garcia, 1973; Phaff, 1971) and can be divided into two classes: (a) non-enzymic proteins attached to mannose polymers, which are found in the amorphous material of the cell wall and may be structural components (Cohen and Ballou, 1981); and (b) Enzymic mannoproteins (such as invertase or acid phosphatase) found in the "periplasmic space" of yeast cells (Trimble and Maley, 1977; Barbaric *et al.*, 1980).

The mannose polymers have been found to be linked to the protein component of the molecule by two means: (a) high molecular weight polysaccharide is connected to the peptide via an N-glycosidic bond between N-acetylchitobiose and asparagine (Nakajima and Ballou, 1975); and (b) mannose oligosaccharides are connected to the peptide by O-glycosidic bonds on the hydroxyl groups of serine or threonine (Sentandreu and Northcote, 1968, 1969).

1.33 Chitin

Chitin is a homopolymer of β-1,4-linked N-acetylglucosamine residues. In *S. cerevisiae*, chitin is a very minor constituent (about 3%) of the wall, although it does play an important role in the division cycle of the yeast cell (Cabib and Bowers, 1971; Schekman and Brawley, 1979). It is the major component of the primary septum and is observed largely at the site of the bud scar found on mother and daughter cells after division (Cabib, 1975, 1981).

1.34 Summary of the Structure of the Yeast Cell Wall

The cell wall of *S. cerevisiae* is an essential barrier between the protoplast and the environment. It is also the determinant of cell size, shape and rigidity. The great bulk of the material in the cell wall is composed of carbohydrate polymers.

A small proportion is chitin which is found at the site of a bud scar. The majority of the carbohydrate is glucan and mannan. The main structural carbohydrate is a very insoluble β-1,3-glucan of high molecular weight which associates into microfibrils and is found close to wall of the protoplast. Other more soluble glucans are associated with this structure while an amorphous layer consisting of soluble glucans and structural mannoproteins can be found on the outside of the cell wall. This is shown diagrammatically in Figure 1.2.

1.4 Enzymes Which Can Degrade the Yeast Cell Wall.

The yeast cell wall is a complex substrate which may require a range of enzymes to cause complete hydrolysis of the mixture of polymers. In addition, it is likely that several activities combine so that the mixture is more effective than the sum of the individual components. However, in view of the fact that the structural strength of

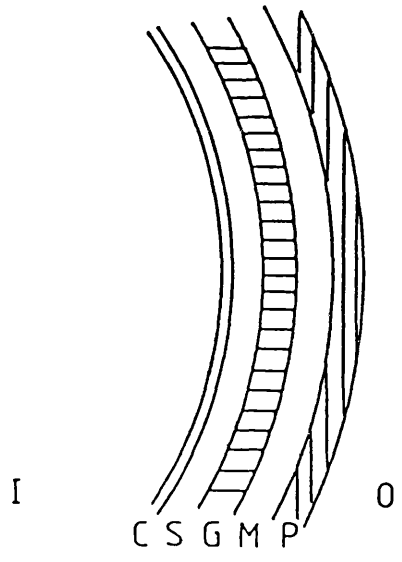


Figure 1.2: Summary of the Structure of the Yeast Cell Wall

I = Interior of the cell; S = Space; C = Cytoplasmic membrane; G = Glucan layer; M = Mixed Glucan/Glucan Layer; P = Proteomannan Layer; O = Outside of the cell.

the yeast cell wall is due to the large molecules of β-1,3-glucan, enzymes capable of acting against this substrate would be expected to play a prominent role in yeast lysis.

1.41 β-Glucanases

Since there are a range of glucans which contain β-glycosidic linkages, it is not surprising that there is a similar diversity in the enzymes described which have hydrolytic activity against these substrates. The best studied glucanases have been those capable of degrading insoluble cellulose and other related (often soluble) β-1,4-linked glucans. The battery of enzymes which are involved in the total hydrolysis of cellulose and related polymers to yield glucose are directly analogous to those concerned with the degradation of yeast glucan and related carbohydrates. Indeed, the approaches used and results gained from the analysis of cellulose degrading enzymes and their genes can be of use in the study of the β-1,3-glucanases. The activities in both cases are conveniently subdivided into endo-glucanases and exo-glucanases, although such distinctions may be arbitrary. For example, if an "exo-glucanase" releases oligosaccharides containing N glucose residues from the end of a glucan molecule, how large does N have to be before the enzyme is considered to be an endo-glucanase? This type of problem illustrates the fact that the definition of enzyme activities is often misleading.

A list of enzymes which might be expected to play a role in the complete hydrolysis of β-1,3-glucans (such as yeast glucan) to yield glucose is presented below. The Enzyme Commission (E.C.) recommended names are listed along with the enzymic activities and the E.C. numbers.

(a) Endo-1,3(4)-β-D-glucanase (E.C.3.2.1.6) catalyses the endo-hydrolysis of 1,3- and 1,4-linked β-D-glucans. The glucose of which is to be hydrolysed to generate a reducing end must also be substituted at the C3 position.

(b) Endo-1,3-β-D-glucanase (E.C.3.2.1.39) hydrolyses 1,3-β-D-glucosidic bonds in 1,3-β-D-glucans but has limited activity on mixed (1,3-, 1,4-)β-D-glucans. This enzyme is commonly known as laminarinase.

(c) Lichenase (E.C.3.2.1.73) acts in an apparently endo-manner on 1,4-β-D-glucosidic bonds in β-D-glucans containing both 1,3- and 1,4-linkages. This enzyme is particularly active on cereal glucans but will not hydrolyse glucans which contain only 1,3- or only 1,4-bonds.

(d) Exo-1,3-β-D-glucosidase (E.C.3.2.1.58) carries out successive hydrolysis of β-D-glucose units from the non-reducing end of 1,3-β-D-glucans. This enzyme has no activity on 1,3-β-D-di-glucosides although it can hydrolyse oligosaccharides.

(e) β-D-Glucosidase (E.C.3.2.1.21) is an enzyme with low specificities, capable of hydrolysing terminal non-reducing β-D-glucose residues from a range of β-D-glucosides.

In addition, other enzymes may hydrolyse the other glucosidic bonds in mixed linkage glucans. For example, cellulases (E.C.3.2.1.4) can hydrolyse the 1,4-linkages in β-D-glucans containing β-1,3- and β-1,4-bonds.

1.42 β-1,4-Glucanases

Since the study of β-1,4-glucanases (and cellulases) is at a relatively advanced stage, it is worth noting the approaches which have been taken and the information derived from this work, with a view to their application to similar problems concerning β-1,3-glucan rather than the β-1,4-linked polymer. These enzymes have been studied from a large number of organisms including *Trichoderma* (Teeri *et al.*,1983; Shoemaker *et al.*, 1983; Chen *et al.*, 1987), *Bacillus* (Robson and Chambliss, 1986), *Cellulomonas* (Whittle *et al.*, 1982; Gilkes *et al.*, 1984; Nakamura *et al.*, 1986a, b) and *Clostridium* (Cornet *et al.*, 1983a, b; Romaniec *et al.*, 1987). Understanding of these enzymes has been greatly improved by the use of a molecular cloning approach and a number of genes have been cloned from these organisms, encoding β-glucosidases and exo-glucanases as well as endo-glucanases. The availability of the nucleotide sequences and their derived protein sequences has enabled comparisons to be made and common regions to be identified (Knowles *et al.*, 1987). Furthermore, it has been possible to analyse crystals of an endo-β-1,4-glucanase of *Clostridium thermocellum* using X-ray diffraction (Joliff *et al.*, 1986). The large quantities of pure protein required for such an exercise were generated by gross over-expression of the gene encoding the glucanase in *E. coli*.

In addition, many workers have studied the β-1,3(4)-glucanases of *Bacillus* species (Cantwell and McConnell, 1982; Hinchliffe, 1984). These act against mixed (β-1,3- and β-1,4-) linkage glucans such as cereal glucan or lichenan. The study of these enzymes and their genes is of more direct relevance to the work presented here, but crystallographic data are not available for these enzymes.

The studies summarised above should lead to a greater understanding of the mode of action, substrate binding and catalytic sites of all such enzymes, including endo- and exo-acting β-1,4- ,or β-1,3-glucanases. This emphasises the usefulness of a molecular cloning approach to the study of these enzymes.

1.43 Assays for the Detection of Yeast Cell Wall Degrading Enzymes.

A large number of organisms have been characterised as being capable of producing hydrolytic enzymes which have activity on the cell wall of yeast. However, care is required when reviewing this literature since not all enzymes which have activity against yeast based substrates (e.g. isolated cell walls, or purified yeast glucan) also display activity against intact, growing yeast (Jeffries, 1976). Similarly, it is important to note that in a mixture of enzymes, the component which causes the

release of the most reducing sugars need not be the enzyme primarily responsible for the degradation of viable cells.

Assays for the detection of yeast cell wall degrading enzymes vary, but can be divided into broad groups:

1) Estimation of cell lysis either by microscopical observation (Mann *et al.*, 1972), or by measurement of changes in the optical density of suspensions of viable yeast with (Mann *et al.*, 1972) or without (Kobayashi *et al.*, 1974; Kitamura *et al.*, 1972; Scott and Schekman, 1980) osmotic support.

2) Measurement of total soluble carbohydrate or reducing sugars released into the supernatant from a suspension of viable yeast cells (Sugimori *et al.*, 1972).

3) Clearing of isolated yeast cell walls either in suspension or solidified in an agar plate (Tanaka and Phaff, 1965).

Methods 2 and 3 can lead to the identification of enzymes with apparently high activities, but which fail to show significant spaeroplasting activity. Thus, methods based on the ability to sphaeroplast viable cells are the most reliable although more difficult to perform. All three types of assay have been successfully employed to isolate organisms which secrete yeast lytic activities.

1.44 Endogenous Yeast Enzymes

Since all species of yeast must undergo cell division, it would be expected that the yeast would produce hydrolytic enzymes (including glucanases) which degrade the cell wall. However, it is also likely that such enzymes would be closely controlled with respect to quantity of enzyme and localisation to avoid their potentially lethal effects.

Nevertheless, β-glucanases associated with cell wall breakdown have been isolated from every yeast species studied to date. For example, a β-1,3-glucanase of 40 kD was identified by Abd-El-Al and Phaff (1968). Similarly endo- and exo-β-1,3-glucanases have been described from a number of *Schizosaccharomyces* species (Fleet and Phaff, 1974; Barras, 1972). It is noteworthy that an exo-glucanase of *Schizosaccharomyces versatilis* could not lyse yeast while an endo-glucanase from the same species could (Fleet and Phaff, 1974).

In summary, a number of endo- and exo- β-1,3-glucanases with molecular weights of 22-90 kD have been described. All exhibited optimal activities at pHs between 4 and 6, and many were capable of causing cell lysis.

1.45 Exogenous Enzymes

The extract from the gut of the snail *Helix pomatia* was the first exogenous sphaeroplasting enzyme described (Eddy and Williamson, 1957). However, these preparations are less than ideal since they contain a large number of contaminating

Table 1.1: Microbial Sources of Yeast Lytic Enzymes

ORGANISM	KEY ENZYME(S)	MAJOR PRODUCT(S)	REFERENCE
Hanseniaspora valbyensis	Endoglucanase,	L_4	Abd-El-Al and Phaff, (1969)
Schizosaccharomyce pombe	Endoglucanase,	L_2	Barras, (1972)
Schizosaccharomyces versatilis	Endoglucanase,	G, L_2	Fleet and Phaff, (1974)
Arthrobacter luteus	Endoglucanase,	L_5	Kitamura *et al.*, (1972)
Arthrobacter sp. YCWD3	Endoglucanase, Exo(?)glucanase,	L_2 L_5	Doi *et al.*, (1973a, b)
Arthrobacter GLM-1	Endoglucanase,	L_5	Vrsanska *et al.*, (1977b)
Bacillus circulans	Phosphomannase?	?	Nagasaki *et al.*, (1966)
Cytophaga johnsonii	Endoglucanase,	L_5	Marshall, (1973)
Flavobacterium sp.	Endoglucanase,	L_2	Bathgate *et al.*, (1974)
Micromonospora sp.	Endoglucanase,	L_2	Villanueva *et al.*, (1973)
Oerskovia xanthineolytica	Endoglucanases, Exo(?)glucanase,	L_2 L_5	Jeffries, (1976)
	Endoglucanase Protease	L_5	Scott and Schekman, (1980)

G = Glucose; L_2 = Laminaribiose; L_4 = Laminaritetraose; L_5 = Laminaripentaose.

activities, and are difficult to produce in reasonable amounts.

Microbial sources of enzyme were first described by Salton (1955), who identified *Streptomyces*, *Cytophaga* and *Myxococcus* species which could cause zones of clearing on agar plates containing yeast (*Candida pulcherrima*) walls. However, most microbial enzymes exhibiting this property fail to sphaeroplast intact viable cells (see above). Enzymes which do lyse viable cells have been isolated from a number of organisms and the properties of these enzymes are summarised in Table 1.1. In almost every case, the enzyme identified as being responsible for the yeast sphaeroplasting activity were β-1,3-glucanases, although it was suggested that the phosphomannanase of *Bacillus circulans* was important (Nagasaki *et al.*, 1966). However, "purified" phosphomannanase was found to contain β-1,3-glucanase activity and it seems more likely that the combined action of the two enzymes was responsible for the observed lytic activity (McLellan *et al.*, 1970). It is most striking that the genera *Arthrobacter* and *Oerskovia* have been frequently found to be good sources of yeast lytic enzymes and these species clearly merit further study.

1.46 The Yeast Lytic Systems of *Arthrobacters* and *Oerskoviae*

As noted above, these genera have been amongst the ones most frequently characterised as secreting yeast lytic activities. Indeed, *Oerskoviae* have been described as "true predators of yeast" (Jones and Collins, 1986). In taxonomic terms, they seem to be closely related to each other and mistakes in classification are not uncommon (Lechevalier, 1972). In comparing the various organisms which have been described, no attempt has been made to determine whether or not the bacteria are truly different from the other species described.

Arthrobacter GJM-1

Jones and Ballou (1969) isolated a species of *Arthrobacter* which was characterised by its ability to produce large quantities of α-mannanase. This species was shown to accumulate activities which could cause the lysis of viable yeast cells. The system showed some inducibility when grown on media containing yeast cell walls (Vrsanska *et al.*, 1977a). When purified using ion exchange and gel filtration chromatography, the lytic activity was shown to consist of a protease and a β-1,3-glucanase acting in concert. The molecular weights (MWs) of these proteins were 11 kD and 20 kD respectively (Vrsanksa *et al.* 1977b). A second glucanase (MW 52 kD) could enhance the lytic activity but was not found to be lytic *per se*, whilst the smaller glucanase could in fact lyse viable cells in the absence of other detectable activities.

Arthrobacter YCWD-3

Doi *et al.* (1971) isolated a species of *Arthrobacter* from the surface of rotting "Konjak". Upon culturing on media containg bakers yeast, β-glucanase activities were noted (Doi *et al.*, 1973a). A β-1,3-glucanase which caused lysis of viable yeast cells was purified (Doi *et al.*, 1973b). The molecular weight of this enzyme was 55 kD and the product of the enzyme action on yeast glucan was laminaripentaose (Doi *et al.*, 1973c). This lytic system also included a second β-1,3-glucanase of unspecified molecular weight. These authors have also attempted a molecular cloning approach and have now cloned the gene encoding the lytic glucanase (Doi and Doi, 1986).

Oerskoviae species

Several species of *Oerskovia* and *Oerkovia*-like bacteria were isolated and investigated with respect to their yeast lytic activities (Mann *et al.* 1972, 1978). *Oerskovia xanthineolytica* was identified as a good producer of lytic activity. The bacterium secreted four β-1,3-glucanases ranging in molecular weight from 18 kD to 29 kD (Jeffries, 1976; Jeffries and Macmillan, 1980). Two glucanases (MWs 26 and 27 kD) were associated with the lytic activity. The remaining two glucanases (MWs 18 and 29 kD) had a synergistic effect on the lytic activity of the combined glucanases but had no lytic activity of their own. The lytic glucanases released relatively few reducing ends from the yeast substrate and the predominant product which accumulated was laminaripentaose.

Scott and Schekman (1980) purified a lytic activity from *O. xanthineolytica*. This organism had previously been classified as *Arthrobacter luteus* strain 73-14. The lytic activity was found to be due to a synergistic combination of a β-1,3-glucanase (MW 55 kD) and a protease (MW 25 kD), although only the glucanase was capable of causing lysis on its own.

Arthrobacter luteus

A. luteus was isolated by an enrichment procedure, from brewery waste. The isolates resulting from this enrichment were almost all species of *Arthrobacter*. The strain exhibiting the highest lytic activity was designated B 111-1 and selected for further study (Kitamura *et al.*, 1972). The activity was "inducible" on media containing β-1,3-glucan, and the crude enzyme preparation thus produced was marketed as Zymolyase (see later). Initial purification of the lytic component of this mixture revealed it to be a β-1,3-glucanase of MW 21 kD (Kitamura *et al.*, 1974). The pure enzyme released laminaripentaose as the major product of its hydrolysis of β-1,3-glucans.

However, other workers reported (Funatsu *et al.*, 1978) that a protease also contributed to the yeast lytic activity in a synergistic manner. Indeed, upon

re-examination of the the enzymes of Zymolyase, a protease (MW 29 kD) was found to be an important factor in the lytic activity (Kitamura, 1982a, b). Furthermore, the glucanase component was now estimated to have a MW of 58 kD in contrast to the previous estimate of 21 kD!

Zymolyase

Zymolyase is a widely used preparation of the secreted enzymes of *A. luteus* B 111-1. It is partially purtified by adsorption to β-1,3-glucan immobilised in polyacrylamide (Kitamura, 1982c). The enriched enzyme preparation thus obtained contains a variety of activities including α-mannanase, β-1,3-glucanases, chitinase and protease (Scott and Schekman, 1980; Zymolyase data sheet), all of which would be expected to contribute to efficient degradation of the yeast cell wall.

1.47 Summary

While there are differences in the literature concerning the role of protease in yeast lytic activity, and about the size and number of the β-glucanases involved, it is clear that endo-β-1,3-glucanases play a key role. A convincing model is that the protease can disrupt the protective proteomannan layer of the cell wall allowing the lytic (and other) glucanases to penetrate to the structural glucan layer. This would explain the fact that the protease is apparently dispensable within lytic enzyme mixtures.

It is noteworthy that *Arthrobacter* and *Oerskovia* species are important sources of yeast lytic enzymes. Further characterisation of the complex enzyme systems relies heavily upon highly purified components of the mixture. The application of molecular cloning represents an ideal opportunity to obtain such pure preparations relatively easily.

The bacterium selected for further study was *A. luteus* ATCC 21606. This particular strain was chosen because:

1) the enzymes secreted by *A. luteus* are amongst the most active against viable yeast cells (Scott and Schekman, 1980);

2) Zymolyase is produced from this strain so there is already significant commercial interest in this strain (Kitamura, 1982c);

3) these enzymes are the best studied of this type (Scott and Schekman, 1980; Kitamura, 1982a, b).

1.5 *Arthrobacter luteus* ATCC 21606

The organism used throughout this work was first described by Kitamura *et al.*, (1971, 1972). These workers isolated a range of organisms from brewery waste and utilised an enrichment procedure to select for those which grew best on yeast waste.

By a variety of criteria, in particular the characteristic variation of morphology, the organism was classified as an *Arthrobacter* species and by virtue of the yellow pigmentation of its colonies, it was named *Arthrobacter luteus*. However, it has been since reclassified as *Oerskovia xanthineolytica* (Lechevalier, 1972) due to its unusual ability to hydrolyse xanthine and hypoxanthine and to a slight difference in cell wall type, compared to true *Arthrobacter* species. The taxonomic confusion means that yeast degrading *Arthrobacters* and *Oerskoviae* may be interchangeably classified, and therefore care is required when assessing the literature. Other "*Arthrobacters*" displaying similar characteristics have been isolated (reviewed above, section 1.4).

1.51 Characteristics of the Genus *Arthrobacter* and Related Organisms

Arthrobacters and *Oerskoviae* are Gram-positive Actinomycetes, commonly found in soil. They are broadly classified as "irregular, non-sporing, Gram-positive rods" (Jones and Collins, 1986) and characterised by changes in morphology from pseudomycelia, through motile rods to motile cocci. Other characteristics include a particularly high G+C content in their DNA, and an ability to degrade and utilise a variety of unusual and complex substrates (Lechevalier, 1972). Other bacteria which are taxonomically grouped with these genera include *Corynebacteria, Brevibacteria, Nocardia,* and *Cellulomonas*.

This broad group of organisms are utilised in a range of biotechnological processes including the production of nucleotides (Ogata *et al.*, 1976), amino acids (Yoshinaga and Nakamori, 1983), antibiotics (Suzuki *et al.*, 1972; French *et al.*, 1970), bioconversions of steroids (Constantinides, 1980), and degradation of complex carbohydrates (Hayward and Sly, 1976). In the case of *A. luteus*, the ability to grow on substrates composed entirely of yeast cell wall material is due to the secretion of large numbers of hydrolytic enzymes including chitinase, mannanase, protease and a variety of β-1,3-glucanases (Kitamura *et al.*, 1974).

1.52 Molecular Genetics of *Arthrobacter* and Related Actinomycetes

Like *Streptomyces* species, this group of organisms includes a large number of pathogenic, saprophytic and biotechnologically important species, though, unlike the *Streptomyces*, little is known of their molecular genetics. The sparseness of information concerning gene structure and genetic organisation is partly due to the wide variety of organisms used and the diversity of genes under study. However, it is also the case that many of the genetic studies are still in their early stages and simple genetic transformation has not been readily available for most species. The exceptions to this are the amino acid producing bacteria.

In these cases, the availability of mutants and transformation systems enables gene cloning to be carried out using the same species as both donor and host

organism. However, the absence of such strains and transformation methods need not be an obstacle, since many genes can be expressed in other bacteria. Most commonly, cloned bacterial genes are expressed in *E. coli*, but the use of other bacteria such as *Bacillus* (Hardy, 1985) or *Streptomyces* (Hunter, 1985) is not uncommon. Genes from *Streptomyces* species are often poorly expressed in *E. coli* (Thompson, *et al.*, 1984) and this could also be a problem for genes cloned from other Actinomycetes.

By far the most developed genetic system is that of the amino acid producing Actinomycetes: *Corynebacteium glutamicum* and the closely related species *Brevibacterium lactofermentum* and *Brevibacterium flavum*. Although a protoplast transformation system for *Arthrobacter* has recently been developed (Shaw and Hartley, 1986) and a large plasmid encoding enzymes involved in the degradation of nicotine isolated from *Arthrobacter oxidans* (Brandsch and Decker, 1984; Brandsch *et al.*, 1986), there are no widely available "shuttle" vectors or cloning systems in these species. It will be interesting to observe whether those systems developed for other Actinomycetes will be useful for the genetic manipulation of *Arthrobacter* or *Oerskovia*.

In the absence of such genetic systems, molecular cloning and analysis in an heterologous host is the most effective approach. This may enable the elucidation of gene structure and also the development of vectors appropriate to more complex and elegant genetics. Many different approaches have already proved useful in the molecular cloning and characterisation of genes from *Arthrobacter* and related species.

1.53 Molecular Cloning In and From the Amino Acid Producing Actinomycetes

Corynebacterium glutamicum, *Brevibacterium lactofermentum* and *Brevibacterium flavum* are used for the production of amino acids on a commercial basis and have been studied by both classical and modern genetical methods.

The availability of auxotrophic mutants, vectors and transformation procedures (Martin *et al.*, 1987) for these *Coryneforms* has enabled a variety of approaches to the molecular cloning of *Corynebacteria* genes (especially those encoding amino acid biosynthetic proteins).

E.coli auxtrophic mutants have been complemented using gene libraries of *Corynebacterium* DNA in *E.coli* vectors. Although it was not clear that the organisation of the biosynthetic pathways in the two organisms would be similar or that the heterologous genes would function well, it has been possible to isolate the genes for a variety of biosynthetic enzymes including those for tryptophan (Del Real *et al.*, 1985), lysine (Marquez *et al.*, 1985), arginine, leucine, histidine and purine (Batt *et al.*, 1985). This is encouraging evidence that the detection of cloned DNA from this type of organism by its function in *E. coli* is possible.

Direct complementation of auxtrophic mutants of *C.glutamicum* and

B.lactofermentum has been obtained using gene libraries of *Corynebacterium* DNA in vectors capable of replication in *C.glutamicum*. Using this approach the gene analogous to pheA (Follettie and Sinskey, 1986) and two other phenylalanine biosynthesis genes (Ozaki *et al.*, 1985) have been isolated. The success of this approach is entirely dependent on the availability of an adequate genetic system in which transfer of DNA into a mutant host strain is possible.

In addition, the gene encoding 2,5-diketo-D-gluconic acid reductase from *Corynebacterium sp.* has been cloned (Anderson *et al.*, 1985) using two 43 base oligonucleotide "probes" to hybridise to the DNA of interest in a gene library of that organism. The probe sequences were generated from the aminoterminal peptide sequence of the purified protein. This is a widely applicable method for the detection of cloned DNA and only relies on the availability of suitable DNA probes.

1.54 Genetics of Other Related Actinomycetes

A different method for detecting genes using DNA hybrisation has also proved effective in the cloning of the erythromycin resistance (Ery^R) gene from an erythromycin producing *Arthrobacter sp.* (Roberts *et al.*, 1985). The Ery^R gene of *Streptomyces erythreus* was used as a radio-labelled probe and used to detect the equivalent gene, firstly in genomic DNA of *Arthrobacter* and secondly in a genomic library of *Arthrobacter* DNA in the vector λ2001 (Karn *et al.*, 1984). This approach does not require any expression of the cloned gene in *E. coli*.

Detection of cloned components of the cellulase system of *Cellulomonas fimi* was accomplished by the generation of high titre, high specificity antisera to the enzyme system of *C. fimi* and screening *E. coli* transformed with a library of *C. fimi* genomic DNA carried on the plasmid vector pBR322. The genes encoding many components of the cellulolytic system of *C. fimi*, which includes endo-β-1,4-glucanases, exo-β-1,4-glucanases and β-glucosidases, have been isolated (Whittle *et al.*, 1982; Gilkes *et al.*, 1984). Similarly, genes encoding cellulolytic enzymes from *Cellulomonas uda* CB4 have been cloned in *E. coli* and their activities detected in the heterologous host (Nakamura *et al.*, 1986a, b) by direct screening on the appropriate media.

A gene encoding 6-hydroxy-D-nicotine oxidase was cloned from a plasmid of *Arthrobacter oxidans* by detection of oxidase activity (Brandsch *et al.*, 1986). The gene was expressed in either orientation with respect to the vector, indicating that its own promoter was functional in *E. coli*.

Finally a β-1,3-glucanase gene of *Arthrobacter sp.* YCWD3 has been cloned in *E.coli* (Doi and Doi, 1986). Utilising an *E. coli*/yeast "shuttle" vector based on pBR322, a recombinant clone carrying this gene was identified due to its ability to cause zones of clearing after prolonged growth on a plate containing an insoluble

yeast substrate.

Restriction analysis and subcloning localised the gene to a *Bam*H I fragment of about 8.6 kb. The gene was expressed in an orientation specific manner suggesting that vector sequences, i.e. the promoter of the tetracycline resistance (Tet^R) gene, were directing expression of the gene and showing that the expression of *Arthrobacter* DNA from its own endogenous signals was not efficient. The protein encoded by this gene was approximately 55 kD and apparently corresponded to a glucanase of *Arthrobacter sp.* YCWD3 which had been previously purified (Doi *et al.*, 1973b).

1.6 Problems and Strategies.

The multiplicity of *Arthrobacter luteus* enzymes thought to have a role in the degradation of the yeast cell wall and the difficulties in obtaining completely pure samples have contributed to the confusion which is evident from the conflicting reports in the literature (Kitamura *et al.*, 1974; Kitamura, 1982a, b). This supports the idea that a molecular cloning approach would present an ideal tool for the investigation of the inter-relationships between the various enzymes of the lytic mixture. Furthermore, such an approach would generate additional data concerning the genetic material of *A. luteus*.

A number of means for the detection of cloned DNA exist. Firstly, direct expression of the heterologous DNA could be detected, for example, by the complementation of *E. coli* auxotrophic mutants using DNA from an heterologous source such as *Brevibacterium lactofermentum* (Del Real *et al.*, 1985). Other gene products could be detected in *E. coli* by their expression of a new phenotype such as carbohydrate degrading enzymes like amylases (Kawazu *et al.*, 1987) or glucanases (Cantwell and McConnell, 1983).

However, not all gene products have easily detectable activities and it is frequently impossible to detect them in this manner. In these cases, detection of the expressed gene product might be obtained by cross-reactivity with antisera generated against the protein(s) of interest. This approach may also be useful for the detection of components of complex and poorly-characterised mixtures of proteins, or inactive subunits of holoenzymes.

Problems associated with these methods of detection are that they rely on the expression of the heterologous protein to some level, and at least limited release of the protein from the host cell. If expression is high, release of the gene product is unlikely to be a problem since a certain amount of leakage will occur, and this can be enhanced by some procedures for partial lysis of the host cells. More serious, though, is the problem of genes which are poorly expressed in the chosen host.

The most straightforward alternative to the direct detection of expressed gene products is to detect the DNA itself, using hybridisation probes. If a gene similar to

the one of interest has been previously cloned, it might be used as a radio-labelled probe to hybridise to the novel gene. Such a probe is often not available, but it may be possible to generate one by purifying the appropriate protein, determining a portion of its amino acid sequence and producing oligonucleotides which could encode such a sequence.

In the case of the yeast lytic enzymes of *A. luteus*, no appropriate, previously cloned gene was available and the complexity of the enzyme mixture made the latter approach unappealing. It was however unclear whether cloned DNA from *A. luteus* would be sufficiently well expressed in *E. coli* to allow the detection of clones encoding β-glucanase. In the case of the amino acid biosynthesis genes from the corynebacteria, the genes were expressed well enough to complement auxotrophic mutants of *E. coli* (Martin *et al.*,1986). Similarly, the cellulase gene of *Cellulomonas uda* CB4 appeared to be expressed well (Nakamura *et al.*, 1986b). It was unclear, however whether this expression was driven by vector sequences or from the gene's own promoter. The components of the cellulase system of *Cellulomonas fimi* were detected by their activity, apparently expressed from their own promoters (Gilkes *et al.*, 1984).

Only two *Arthrobacter* genes have been expressed in *E. coli*. The gene encoding a β-1,3-glucanase from *Arthrbacter* YCWD3 was only expressed in an orientation specific manner (Doi and Doi, 1986), indicating that some vector sequence, probably the promoter of the Tet^R gene, was driving the production of glucanase. In contrast, the gene encoding 6-hydroxy-D-nicotine oxidase from *A. oxidans* was well expressed in *E. coli* (Brandsch *et al.*, 1986). Indeed upon insertion of the cloned fragment into the promoter probe vector pCB192, activity of the assay gene was detected, indicating that this DNA had promoter activity in *E. coli*.

One further alternative cloning strategy remains to be considered. The *A. luteus* DNA could be cloned and expressed in a host other than *E. coli*, in particular *Streptomyces*, in the hope of ensuring better gene expression and easier detection of positive clones. *Streptomyces* strains would be expected to express the G+C-rich DNA of *A. luteus* more efficiently than would *E. coli*. Furthermore, a Gram-positive bacterium would be better able to secrete β-glucanases (which are secreted proteins in *A. luteus*) into the growth medium. However, there was no guarantee that this would be the case.

On balance, it seemed simplest and most appropriate to attempt to detect direct expression of glucanase genes in *E. coli* on test plates. If difficulties were encountered, perhaps vectors with strong, inducible promoters would be appropriate to drive the expression of the cloned genes. As a last resort, it might be necessary to attempt expression of cloned genes in a different heterologous host.

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial Strains, Growth and Genetic Transfer.

2.11 Media for Bacterial and Yeast Strains

Media (Table 2.1) were prepared essentially according to Miller (1972).

Additions

Media other than BBL were solidified by the addition of 1.5% Agar; BBL was solidified with 1% Agar, BBL soft with 0.65%.

Glucose was added to M9 and M63 minimal media at a final concentration of 0.4%, and to Yeast Peptone (YP) at 2%, to produce YPD. Media were supplemented, where appropriate, with biotin and thiamine at 1 μg/ml, thymine at 50μg/ml and casamino acids at 0.5%.

Antibiotics were added to the following concentrations: ampicillin 100 μg/ml, kanamycin 100 μg/ml, streptomycin 50 μg/ml.

Detection plates for β-glucanase and amylase activity contained barley β-glucan at 0.05% (w/v) final concentration, and 0.5% (w/v) soluble starch respectively.

Laminarin (obtained from Calbiochem or Sigma Chemical Co.), Lichenan (from Sigma), Barley β-glucan (from Biocon UK Ltd), Alkali Insoluble Glucan (AIG) from yeast, Autolysed Yeast Residue (from DCL) and Autoclaved Yeast (AY) were included as carbon sources in M63 media at a final concentration of at least 0.4% (w/v).

2.12 Preparation of Yeast-Based Substrates

(a) Autoclaved Yeast (AY): 3 kg compressed bakers yeast (DCL) was suspended in 4.5 l distilled water and autoclaved at 121°C, 15 psi. After cooling, the suspension was centrifuged at 5,000 rpm for 10 min. The pellet was retained, washed twice in ethanol and once in diethyl-ether to dry the material and stored frozen at -20°C. 300 g AY was produced from 3 kg yeast.

(b) Alkali Insoluble Glucan (AIG): 3 kg compressed bakers yeast (DCL) was suspended in 6% (w/v) NaOH (2 l), and incubated at 65°C for 2 h with shaking. The mixture was diluted 4-fold in Q water and pelleted at 5,000 rpm for 10 min. The pellet was resuspended in 6% (w/v) NaOH and the process repeated twice more. A final extraction was carried out with 3% (w/v) NaOH followed by neutralisation with HCl. The final pellet was washed twice with ethanol and once with acetone to dry the material. 120 g AIG was produced from 3 kg yeast.

(c) Autolysed Yeast Residue (AYR): The dried "waste" residue after lysis of yeast in the production of yeast extract supplied as an insoluble powder or flakes. Analysis by physical and chemical means, in particular Gas Chromatography (J. Hay, DCL, pers.

Table 2.1 Media

		g/l
Luria Broth (LB):	Tryptone	10
	Yeast Extract	5
	NaCl	5
M9 Minimal:	Na_2HPO_4	7
	KH_2PO_4	3
	NaCl	0.5
	NH_4Cl	1
	$MgSO_4$	0.246
	$CaCl_2$	0.022
2YT:	Tryptone	16
	Yeast Extract	10
	NaCl	5
BBL:	Trypticase	10
	NaCl	5
M63 Minimal:	KH_2PO_4	13.6
	$(NH_4)_2SO_4$	2
	KOH	4.2
	$MgSO_4$	0.2
	$Fe_2(SO_4)_3.6H_2O$	0.001
Yeast Peptone	Yeast Extract	10
	Peptone	20

comm.), indicated that the composition of the residue was as follows: 10% moisture, 8.7% "ash", 14.8% protein, 2.1% non-protein nitrogen (probably nucleic acids and N-acetyl-glucosamine from chitin), 3% soluble monosaccharides, 1.1% lipid, 60.6% insoluble carbohydrate matter (aproximately equally divided between mannans and β-glucans)

2.13 Strains and Plasmids

Strains and plasmids used in this work are listed in Tables 2.2 and 2.3.

Bacterial strains were routinely maintained on L-agar plates at 4°C for up to 3 weeks. For storage of strains for up to 1 year, small vials containing 4 ml of the appropriate solid medium (selective where necessary) were inoculated with a wire loop by stabbing into the medium. After overnight growth, the vial was made airtight by sealing with "Parafilm" and the cultures stored at room temperature. For permanent storage, overnight cultures were diluted 1:1 with bacterial freezing mix (LB containing glycerol at 30%). The mixture was frozen rapidly at -70°C, and duplicates were stored in different freezers at -20 or -70°C.

2.14 Bacterial Transformation

Escherichia coli were grown, well aerated at 37°C, in LB to mid-logarithmic phase (absorbance at 600 nm 0.4-0.7). The cultures were chilled on ice for 10-20 min and harvested by centrifugation in chilled, sterile pots (5,000 rpm, 10 min, 4°C). The pellets were resuspended in 1/2 the original volume of ice cold 0.1 M $MgCl_2$. The cells were immediately pelleted as above and resuspended in the same volume of ice-cold 0.1 M $CaCl_2$. After standing on ice for at least 1 h, the cells were again harvested at 4°C and resuspended in 1/20 the original volume 0.1 M $CaCl_2$. To maximise transformation freqencies, the cells were left overnight at 0-4°C before use. To store competent cells the mixture was made up to 15% glycerol by the addition of sterile, ice cold 50% glycerol/0.1 M $CaCl_2$ and frozen at -70°C.

Competent *E. coli* were transformed by incubating 100-150 µl cells with 1-10 µl DNA (ligation or supercoiled plasmid) for 30-60 min on ice. The mixture was heat shocked for 2 min at 42°C and made up to 1 ml with LB. The cells were plated on the appropriate selective media after 30-60 min "expression" period at 37°C.

2.15 Mating of *E. coli* Strains for Mutagenesis by Transposon Tn1000 (γδ)

Donor strain RB308 (bearing the plasmids to be analysed) and recipient strain CSH26 were grown in LB, with antibiotics and supplements as required, to mid-logarithmic phase. Cells were mixed at a ratio of 1 donor: 10 recipient and incubated at 37°C for at least 2 h without shaking. The mating was terminated by vortexing the mixtures and plating dilutions on selective plates containing Amp and

TABLE 2.2: Strains

SPECIES	GENOTYPE	SOURCE
Arthrobacter luteus	Wild Type	ATCC 21606
Saccharomyces cerevisiae SI50-2B	*a, his3Δ, trp1, ura3, leu2, 2.*	Lab stock
Escherichia coli K-12 derivatives		
D1210	r_k^- m_k^-, *recA, ara, proA, lacY, galK, supE, lacI*^{Q}	Lab Stock, (Kuhn *et al.*, 1986).
NM522	*Δ(lac-proAB), thi, supE, hsdΔ5, (F', proAB, lacI*^{Q}*, ZΔM15)*	Lab stock
RB308	F^+, *deoC, thyA, lacY, recA, srl*::Tn10	Lab stock
CSH26	F^-, *Δ(recA), Δ(lac-pro), thi, ara, rpsL*	Lab stock
DS410	F^-, *minA, minB, thi, ara, gal, xyl, mtl, tonA, rpsL, sup*^{0}	Lab stock (Dougan and Sherratt, 1977)

Table 2.3: Plasmids

PLASMID	FEATURES	SOURCE/REF
pKGW	Positive Selection Vector	Kuhn *et al.*, (1986)
pUC18,19	Multiple Cloning Sites, Blue/White screen for inserts	Lab stock Yanisch-Perron *et al.*, (1985)
pKM8,9	Equivalent to pUCs8 and 9, but Km resistance	G.S. Plastow Spratt *et al.*, (1986)
M13 mp18,19	Multiple cloning sites, vector for sequencing	Yannisch-Perron *et al.*,(1985)

Str, ensuring that only Streptomycin resistant CSH26 recipients which carry Amp^R plasmids can grow.

2.2 Analysis of Proteins and Sugars

2.21 Solutions.

Denaturing SDS-PAGE Gels:

Acrylamide Stock Solution: 44% (w/v) acrylamide, 0.8% (w/v) bisacrylamide, thoroughly dissolved in deionised water, deionised with Amberlite and filtered through Whatman No. 1 paper.

Separating Gel Buffer (Buffer A): 0.75 M Tris-HCl (pH 8.8), 0.2% SDS (w/v).

Stacking Gel Buffer (Buffer B): 0.25 M Tris-HCl (pH 6.8), 0.2% SDS (w/v).

Ammonium Persulphate (APS): 10% (w/v) freshly dissolved in Q water.

Electrophoresis Buffer: 0.025 M Tris, 0.192 M glycine, 0.1% (w/v) SDS (pH 8.3).

Sample Buffer (5 fold concentrate): 0.3125 M Tris-HCl (pH 8.3), 10% (w/v) SDS, 25% (v/v) β-ME, 50% (w/v) sucrose, 0.02% (w/v) bromophenol blue.

Non-Denaturing ("Native") Gels: For Native gels, the buffer recipes were identical except that SDS was omitted.

2.22 Preparation and Running of Gels

The gel methodology used was a discontinuous, denaturing system, broadly based on that of Laemmli (1970).

Routinely, 40 ml slab gels (170 mm x 200 mm x 1 mm) were prepared and the percentage of acrylamide was 10, 12 or 15%. Table 2.4 shows the recipes used for gels of these strengths and incorporates the recipe for the stacking gel which made up the top fifth of the gel. Activity gels contained barley β-glucan at 0.05% (w/v). Small scale gels (5 ml gel volume, 50 mm x 70 mm x 0.75 mm) were produced in an identical fashion with all volumes being reduced as appropriate)

Vertical gels were poured between two clean glass plates, with "Teflon" spacers at the bottom and sides of the gel. Wells were formed using "Teflon" combs and the apparatus was firmly assembled using bulldog clips.

Samples were prepared by the addition of the appropriate amount of sample buffer and, for denaturing SDS gels, each sample was boiled for 2 min. Electrophoresis was usually performed at 100 V for approximately 4 h or at 25 V overnight.

2.23 Staining

(a) Coomassie Blue Staining:

Stain: 40% Methanol (v/v), 7% Glacial acetic acid (v/v), 0.1% Coomassie Brilliant

Table 2.4: SDS-PAGE Gel Recipes

	10%	12%	15%	Stacking
Buffer A	20	20	20	-
Buffer B	-	-	-	5
44% Acryl.	9.1	10.9	13.6	1.5
Water	9.9	8.1	5.4	3.4
APS	1	1	1	0.1
TEMED	25µl	25µl	25µl	4µl

Blue R (w/v), in water;

Destain: the same mixture minus the Coomassie Blue.

Gels were soaked in stain solution for up to 3 h and destained in several changes of destain solution over 1 to 3 h.

(b) Silver Staining (Method of Wray *et al.*, 1981):

Fixing Solution: 50% Methanol (v/v) in water

Solution A: 0.8g silver nitrate in 4 ml Q water

Solution B: 21 ml 0.36% (w/v) NaOH solution plus 1 ml concentrated ammonia solution (S.G 0.880).

Solution C: Solution A added slowly to Solution B with constant mixing (additional drops of ammonia dissolved any residual brown precipitate), and made to 100 ml with Q water.

Solution D: 0.25 ml 38% (v/v) formaldehyde, 2.5 ml 1% (w/v) citric acid in 500 ml Q water (mixed freshly just prior to use).

Gels were thoroughly soaked in 3 changes of fixing solution to ensure the removal of all glycine from the gel. After washing with deionised water, the gel was immersed in freshly made solution C and stained for 15 min. The stain solution was poured off and the gel washed in a large volume of Q water for 5 min. The stain was developed by removing the water and soaking the gel in solution D for 5–20 min. The staining was stopped by rinsing the gel in many changes of water. Destaining was also possible, using a 100 fold dilution of photographic fixer (Agfa).

2.24 Paper Chromatography of Sugars

Ascending mode paper chromatography was performed as described by Block *et al.*, (1958). Samples (1–10 μl containing 0.1–1 μmoles reducing sugar) were applied to a baseline approximately 2 cm from the bottom of a piece of Whatman 1MM paper. Standards solutions were glucose (G_1), maltose (G_2), maltotriose (G_3), and a partial acid hydrolysate of laminarin or AIG. When the paper was dry, it was placed in the chromatography tank, and solvent (a mixture of 23% water, 41% ethanol and 36% nitromethane) added to cover the bottom 1 cm of the paper. Chromatography was allowed to proceed in a covered tank for 6–10 h. After drying the chromatogram, the reducing sugars were visualised with silver. The chromatogram was first briefly immersed in silver saturated acetone (1 ml saturated aqueous solution of $AgNO_3$ in 200 ml acetone) and dried. The stain was developed by rapid immersion in alkaline methanol (1 ml 40% NaOH in 100 ml methanol), followed by air drying. Finally, the stain was fixed using photographic fixer (Agfa) and washed thoroughly with water, dried and stored. The chromatogram was routinely photocopied or traced to maintain a permanent record since the silver stain was observed to fade.

2.3 Enzyme Assays

2.31 Solutions Required in Assays

Bio-Rad single reagent protein assay kit was used undiluted as rec ommended for low protein concentrations.

Azocoll (from Calbiochem) was suspended in 50 mM sodium phosphate buffer (pH 7.5) at a concentration of 6 mg/ml.

3,5-Dinitrosalicylic acid (DNS) was prepared by dissolving 1 g solid DNS (from Sigma) in 16 ml 10% (w/v) NaOH, 30 g Rochelle salt (potassium sodium tartrate tetrahydrate) and 50 ml distilled water were added and stirred with heat until all the solid was dissolved. The volume was made up to 100 ml and the liquid stored at room temperature. Carbohydrate substrates were dissolved or suspended at 5 mg/ml in 100 mM Tris-HCl (pH 7.5), 10 mM sodium azide.

Nitrocefin (Glaxo) was dissolved at 10 mg/ml in DMSO (Fisons) and diluted to 0.5 mg/ml in 100 mM sodium phosphate buffer (pH 7.0)

The β-galactoside analogue o-nitrophenyl-β-D-galactoside (ONPG) was obtained from Sigma and dissolved in 100 mM sodium phosphate buffer (pH 7.0) at 4 mg/ml. "Z-buffer" consisted of 0.75 mg/ml KCl, 0.25 mg/ml $MgSO_4.7H_2O$, 0.27% (v/v) β-mercaptoethanol in 100 mM sodium phosphate buffer (pH 7.0).

2.32 Determination of Protein Concentrations

Protein concentration was estimated using the micro-assay protocol of the Bio-Rad single reagent (Coomassie blue) method as described by Bradford (1976). The protein sample was diluted to a concentration of 1-25 μg/ml in Q water and 800 μl of sample mixed with 200 μl of Bio-Rad reagent and left at room temperature for 10 to 60 min. The A_{595} was determined and the protein concentration was estimated by reference to a calibration curve compiled with known concentrations of Bovine Serum Albumin (BSA). Allowance was made for the fact that BSA behaves anomalously in this assay in that for a given amount of protein, the A_{595} is twice that which would be obtained with an "average" protein.

2.33 Estimation of Protease Activity

The method used was essentially that of Scott and Schekman (1980). 1.3 ml of a suspension of Azocoll was incubated with 0.2 ml enzyme at 37°C for 30 min with gentle shaking, the blank contained 0.2 ml water. The remaining insoluble substrate was removed by centrifugation and the supernatant removed. The A_{520} was determined against the blank containing no enzyme. One unit of protease activity was arbitrarily defined as that amount which caused a change in A_{520} of 1, relative to the blank, in 30 min. The main limitation of this method is that an insoluble substrate

may not be equally accessible to all proteases. However, the assays were usually performed on essentially the same mixture of enzymes, hence the difficulty raised above may be irrelevant and only really apply when comparing different systems.

2.34 Determination of β-Glucanase Activity

(a) Clearing zones: the method used was an adaptation of the method of Martin and Bamforth (1983). This method relies on the fact that the dye Congo Red can bind to and stain glucans above a certain size. The minimum length of a stainable glucan chain is 6 glucose units (Wood, 1980). If this is disrupted, for example by enzymic degradation, the dye would not stain the glucan and could be washed off. Barley β-glucan is an ideal substrate for the detection of both β-1,3- and β-1,4-glucanases since it contains a high proportion of each type of glucan linkage. This is an assay which specifically measures endo-activity. An exo-type activity would not be able to disrupt the internal glucan chains and therefore would not cause significant clearing zones.

The assay was performed on plates containing 50 mM Tris-HCl (pH 7.5), 10 mM sodium azide, 0.5 mg/ml substrate (barley β-glucan or laminarin), 40 μg/ml congo red (from Sigma) and 1.5% (w/v) Difco Agar. The azide was to prevent unwanted growth on the assay plates when fresh supernatants were being assayed. Plates were poured to a depth of 5 mm, cooled and dried. Wells were bored in the agar using an 8 mm diameter cork borer (No. 5): up to 20 wells could be bored per 90 mm plate. Each well was loaded with 100 μl of enzyme mixture and the plates were incubated at 37°C for 16.5 h (overnight).

Clearing diameters (including sample well) were measured as accurately as possible and compared to a reference line, generated by measuring the clearing diameters produced by a range of dilutions of Zymolyase 100T. The equation:

$\log_{10}$(relative glucanase units) α clearing diameter (in mm) defined the line thus generated, and one unit was arbitrarily defined as that amount of enzyme which produced a clearing zone of diameter 9 mm. Very small errors in the measurement of diameters became significant above 25-30 mm and so samples were diluted, where necessary, to give clearing zones no greater than 25 mm.

(b) Reducing sugar assay: The release of reducing sugars was measured by the DNS method of Miller (1959). 100 μl carbohydrate substrate was mixed with 100 μl of enzyme; blanks contained buffer but no substrate and substrate with water replacing the enzyme. All reactions and control mixtures were incubated at 37°C for 15 min or longer and stopped by the addition of 200 μl DNS solution. The samples were boiled for exactly 5 min and cooled rapidly in iced water. The mixtures were diluted with 1 ml deionised water and thoroughly mixed. The A_{540} was determined and comparison with a reference line generated from known concentrations of reducing

sugar (glucose or maltose) enabled quantitation of the molar amounts of reducing sugar released. One unit of β-glucanase released 1 mmol reducing equivalents in 1 h.

2.35 Determination of Yeast Lytic Activity

The method of Scott and Schekman (1980) was used, with minor modifications. *Saccharomyces cerevisiae* (laboratory or commercial strains) was grown in YPD broth to logarithmic phase (A_{600}= 1 to 2). The cells were pelleted, washed in assay buffer (50 mM Tris-HCl pH 7.5) and resuspended in the same buffer to an A_{800} of 1.2. These were stored at 4°C for up to 2 weeks. Each reaction mix contained 940 µl cell suspension (well mixed to ensure equal cell densities in each tube), 50 µl enzyme (culture supernatant or control solution) and 10 µl 1 M β-mercaptoethanol. After mixing, the reactions were incubated for 30 min at 37°C. Each sample was run in duplicate and blanks contained Q water instead of enzyme. The A_{800} of each sample was measured and the decrease in absorbance relative to the blank was used to estimate the decrease in the number of intact yeast cells. By comparison with actual cell numbers as determined using a counting chamber, the A_{800} was found to be a good estimate of the number of intact cells. One unit of Lytic Activity was defined as that amount which caused a decrease in the cell density of 10% in 30 min. This was accurate only for decreases in cell density of 10-70%, so culture supernatants were diluted or incubated for longer to give figures in this range.

2.36 Determination of β-Lactamase Activity in Solution

β-Lactamase was assayed by the method of O'Callaghan *et al.*, (1972), and was measured by the spectrophotometric detection of the products of the hydrolysis of the chromogenic substrate nitrocefin.

Nitrocefin (50 µl) was added to 900 µl 100 mM sodium phosphate buffer pH 7.0 in a 1 ml cuvette. The spectrophotometer was set to measure absorbance at 490 nm and "zeroed" against the same mixture. Enzyme was added to the "reaction" cuvette and the A_{490} was monitored using the Time Scan setting on a Shimadzu UV-240 spectrophotometer. The initial rate of the reaction was calculated from the gradient of the line thus produced. Dilutions were used to facilitate accurate measurement of the gradient.

2.37 Determination of β-Galactosidase Activity

The method of Miller (1972) was used to measure β-galactosidase activity. The hydrolysis of the β-galactoside, ONPG can be monitored by the appearance of a yellow end product. Enzyme solution was adjusted to pH 7.0 and made up to a volume of 1 ml by the addition of Z-buffer. The mixture was prewarmed to 28°C in a waterbath and the reaction started by the addition of 200 µl ONPG. The reaction

was timed accurately until the mixture became yellow and 0.5 ml 1 M sodium carbonate was added to stop the reaction. The A_{420} was measured and used to determine the activity of the enzyme mixture using the formula:
$U = A_{420} \times 1{,}000/t$ (min) x V (ml). When necessary, results were adjusted to express activity relative to cell density (A_{600}).

2.4 Production of Plasmid Encoded Proteins

2.41 Release of Periplasmic Proteins from *E. coli* by Osmotic Shock

The method of Nossal and Heppel (1966) was used to release the soluble contents of the *E. coli* periplasm into the medium. Cultures grown under the appropriate selective conditions were harvested at the required cell density: equal cell numbers were harvested by centrifugation at 7,000 rpm for 10 min. Supernatants were retained and the pellet was resuspended in buffered salt (10 mM Tris-HCl/30 mM NaCl pH 7.0) before repelleting as above. The pellet was then resuspended in 6 ml buffered sucrose (30 mM Tris-HCl/20% sucrose pH 7.5) and incubated at 25°C for 10 min with gentle shaking. The cells were harvested as above, resuspended in 2 ml 0.5 mM ice-cold $MgCl_2$ and shaken gently at 0°C for 10 min. The mixture was centrifuged once more to recover the supernatant containing the soluble periplasmic proteins. The supernatants from each stage were retained as controls to ensure that no leakage and lysis had occurred.

2.42 Minicell Analysis of Plasmid Encoded Proteins

Minicells were prepared and used according to the method of Stoker *et al.*, (1984).

(a) Preparation of the minicells: 400 ml cultures of plasmid-containing DS410 were grown overnight in the appropriate selective media. The cells were chilled at 4°C for 10 min and centrifuged at 2,000 rpm in a GS3 rotor for 5 min to remove a proportion of the normal (nucleated) cells. The supernatant was transferred to a fresh centrifuge pot and the remaining whole cells and minicells were harvested by centrifugation at 8,000 rpm, 4°C for 15 min in a GS3 rotor. The supernatant was discarded and the cell pellet was resuspended in 6 ml M9 minimal medium. This was layered on to two 10-30% (w/v) sucrose gradients, (prepared by dispensing 35 ml 20% sucrose [w/v] in M9 minimal liquid medium) in a 35 ml corex tube, freezing at -70°C for at least 1 h, and slow-thawing at 4°C overnight). The gradients were centrifuged in a swing out rotor (Sorvall HB4) at 5,000 rpm for 18 min. The top portions (2/3) of the minicell band from both gradients were collected using a Pasteur pipette, pooled, diluted in an equal volume of M9 medium and pelleted in the HB4 rotor at 10,000 rpm for 10 min. The pellet was resuspended in 3 ml M9 and layered

onto a second sucrose gradient which was centrifuged as above. The top 2/3 of the minicell band was again harvested and subjected to a final centrifugation on a sucrose gradient. Again, the minicells were diluted in an equal volume of M9 and the A_{600} was determined. The minicells were recovered as before and resuspended in M9 medium containing 30% (v/v) glycerol to a density of about 2 x 10^{10} "cells"/ml (A_{600} = 2.0). The minicells were stored in glycerol at -70°C until use. An aliquot of each minicell preparation was plated on non-selective medium to ensure that there was no contamination with whole cells.

(b) Radiolabelling of the plasmid encoded proteins: 100 µl of the minicells prepared above was pelleted in a microfuge for 3 min and resuspended in 200 µl M9 minimal medium plus 3 µl 10.5% (w/v) methionine assay medium. Following incubation for 90 min at 37°C to allow degradation of chromosomally-encoded RNA, [^{35}S]-methionine (25 µCi) was added and the mixture was incubated at 37°C for 60 min, followed by the addition of 10 µl unlabelled methionine (8 mg/ml) and a further 5 min incubation. The labelled minicells were harvested by centrifugation in a microfuge for 3 min. The pellet was resuspended in 100 µl SDS-PAGE sample buffer and boiled for 2 min. Routinely, 25 µl was loaded per lane of large SDS-PAGE slab gels.

2.43 Coupled Transcription/Translation System of *Streptomyces lividans*

Plasmid encoded products were analysed by the system of Thompson *et al.*, (1984). This system is highly analogous to that of Zubay (1973) except that the extract is derived from *Streptomyces lividans* and not *E. coli*. The extracts were kindly supplied by M. Calcutt and E. Cundliffe (Department of Biochemistry, University of Leicester).

Plasmid DNA (1-6 µg in 5 µl depending on the size of the plasmid) was incubated with 11 µl S30 extract, 8 µl synthesis mix, 2.5 µl 100 mM MgOAc, 1.5 µl deionised water and 2 µl [^{35}S]-methionine (0.556 MBq/µl, 1.74 TBq/mmol). The mixture was incubated for 20-25 min at 30°C and a "chase" of 2 µl unlabelled methionine (2 mg/ml) added to each reaction. The reactions were stored frozen at -20°C when neccessary. 5-15 µl was added to 1-4 µl SDS-PAGE sample loading buffer, boiled for 5 min and run on SDS-PAGE.

Gels were either dried directly or fixed and fluorographed before drying and exposure to X-ray film. For fluorography, gels were fixed by soaking in 7% (v/v) glacial acetic acid in deionised water for 30-60 min. The gel was then soaked in scintillant e.g. "Amplify" (supplied by Amersham), for 15-30 min. The gel was removed from the scintillant and vacuum dried on to a sheet of Whatman 3MM paper, prior to exposure to X-ray film. Use of the scintillant, was found to improve the signal obtained by about 10 fold, although there was also a loss of resolution, so

where possible (i.e. when the level of incorporation of radio-isotope was high), fluorography was avoided.

2.5 Preparation of DNA

Preparation of DNA was performed generally as described by Maniatis *et al.* (1982) except where cited otherwise.

2.51 Solutions for the Preparation of DNA

The solutions used in the preparation of DNA are shown in Table 2.5.

2.52 Basic Essential Techniques

(a) Extraction with Phenol (or Phenol/Chloroform): An equal volume of phenol (phenol/chloroform) was added and the mixture mixed thoroughly (in the preparation of chromosomal DNA only gentle inversion was used to minimise shearing). The mixture was then centrifuged for 10 min at room temperature and the upper (aqueous) phase removed carefully avoiding the white material at the interface. A further extraction with chloroform/IAA was sometimes used to remove residual phenol from the aqueous phase, although it was usually assumed that ethanol precipitation was sufficient to achieve this.

(b) Precipitation with Ethanol or Isopropyl Alchohol (IPA): Nucleic acids were precipitated by the addition of 0.1 volume 3 M NaOAc (pH 5.5) and 2.5 volumes of ethanol followed by freezing in a dry ice/IMS bath. Preferential precipitation of DNA rather than RNA was performed using 0.8 volumes of IPA. The nucleic acid was recovered from each precipitation by centrifugation for 10 min at 10,000 rpm and 4°C. Where appropriate, pellets were dried under vacuum.

(c) Estimation of DNA concentration: For DNA which was prepared by Caesium chloride density gradients, or high molecular weight chromosomal DNA, the A_{260} was determined. 1 Unit of A_{260} was assumed to be equivalent to a DNA concentration of 50 μg/ml. The purity of such DNA was estimated by comparison of the A_{260} to A_{280} ratio which should approximate to 2.0 in pure DNA. DNA prepared by other methods contained too many oligonucleotides and too much RNA for such measurements to be useful, so estimation of the DNA concentration was carried out on agarose gels stained with ethidium bromide, by comparing the fluorescence of the DNA with that of a known quantity of marker DNA.

2.53 Preparation of Chromosomal DNA From *A. luteus*

Chromosomal DNA from *A. luteus* was prepared essentially by the method of Rodriguez and Tait (1983). 100–400 ml of an overnight culture of bacteria were harvested by centrifugation (6,000 rpm, 10 min at 4°C), and washed in 50 ml TEN.

Table 2.5: Solutions for DNA Preparation

SET:	20% (w/v) sucrose, 50 mM EDTA, 50 mM Tris-HCl (pH 7.6)
STET:	8% (w/v) sucrose, 50 mM Tris-HCl (pH 8.0), 50 mM EDTA, 5% Triton X-100
TE:	10 mM Tris-HCl (pH 7.6), 1 mM EDTA
TEN:	10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 10 mM NaCl
TEG:	25 mM Tris-HCl (pH 8.0), 10 mM EDTA, 50 mM glucose
Alkaline SDS:	0.2 M NaOH, 1% SDS, made up freshly each time
SDS:	25% (w/v) in water
Sodium chloride (NaCl):	5 M in water
Sodium actetate (NaOAc):	3 M NaOAc (pH 5.5),
Lysozyme:	10 mg/ml in TEN
Phenol:	Equilibrated with TE
Chloroform/Iso-amyl alcohol:	24 parts chloroform, 1 part IAA
Phenol/Chloroform:	1 part phenol, 1 part chloroform/IAA

Caesium Chloride Saturated Iso-propanol.

The cells were resuspended in SET bufffer (10 ml per 100 ml culture) and incubated with lysozyme (final concentration 0.5 mg/ml) for 30 min at 37°C. The cells were lysed by the addition of 1 volume of TE and 0.1 volumes 25% SDS, followed by gentle inversion of the tube. Repeated gentle extraction with phenol/chloroform was carried out to remove protein from the mixture, until no white precipitate was produced. All transfers of the aqueous phase were carried out carefully, using wide bore pipettes to avoid shearing the large molecular weight material. To recover the nucleic acids, the solution was transferred to a beaker and 2.5 volumes of cold ethanol were added by carefully pouring down the side of the beaker. After 10 min on ice, the precipitate, which formed at the interface, was carefully spooled out using a sterile pasteur pipette and left to resuspend in TE overnight at 4°C. RNA was removed by treatment with DNase-free pancreatic RNAse A (final concentration 50 μg/ml) for 2 h at 37°C. Residual protein was removed by incubation (1 h) with pronase (final concentration 100 μg/ml, activated by pre-incubation for 1 h at 37°C), followed by extraction with phenol/chloroform. Chromosomal DNA was recovered by precipitation with ethanol, spooling and resuspending in 2-4 ml TE.

2.54 Preparation of Plasmid DNA

Large Scale Caesium Chloride Density Gradient Method

400 ml LB, containing the appropriate antibiotic was inoculated with plasmid-containing *E. coli* and grown overnight at 37°C with shaking. The cells were harvested by centrifugation for 10 min at 8000 rpm and 4°C. The cell pellet was resuspended in 2 ml ice cold TEG (plus lysozyme, freshly added to 2 mg/ml) per 100 ml of overnight culture. After 30 min incubation on ice, the cells were lysed by the addition of 2 volumes of freshly made Alkaline-SDS and gentle inversion. Chromosomal DNA and some proteins were precipitated by the addition of 1/3 volume 3 M sodium acetate, gentle inversion and incubation on ice for 1h. The precipitate was removed by centrifugation for 10 min at 10,000 rpm and 4°C. The DNA was ethanol-precipitated from the supernatant, dried and resuspended in 4 ml sterile Q water. Caesium chloride (4 g), and ethidium bromide (to 600 μg/ml) was added to each tube. The tubes were sealed and centrifuged in a vertical rotor for 24 h at 45,000 rpm. The band representing the closed circular plasmid DNA was removed from the density gradient with a needle and syringe. Ethidium bromide was removed by repeated extraction of the plasmid DNA with caesium chloride saturated isopropanol after vigorous mixing of the 2 liquids, the phases were separated by centrifugation. The lower (aqueous) phase contained the plasmid and was removed for further extractions. The caesium chloride was removed by extensive dialysis against sterile Q water or TE.

Moderately Large (Midi) Preparation of Plasmid DNAs.

Inoculation, growth and harvesting of the cultures were carried out as above although the volumes used were either 50 or 100 ml. Cell lysis and precipitation of the chromosomal DNA were also performed as above. However, the precipitate was not removed by centrifugation: the clear portion of the lysate was carefully removed by pipette into a fresh tube (any accidentally transferred precipitate was removed by centrifugation at 10,000 rpm for 5 min) and the nucleic acid was ethanol precipitated. The resulting pellet was resuspended in 450 μl TE and extracted to extinction with phenol/chloroform. Following ethanol precipitation, the pellet was resuspended in 200 μl TE and treated with DNase-free RNase (final concentration 100 μg/ml) at 37°C for 30 min. DNA was finally recovered by ethanol extraction, vacuum dried briefly and resuspended in 500 μl TE.

Rapid Small Scale Plasmid Isolation by Boiling ("Miniprep")

The method of Holmes and Quigley (1981) was used make small amounts of DNA for rapid analysis. 1.5 ml of culture, grown overnight under appropriate selective conditions, was harvested by centrifugation, resuspended in 250 μl STET and 20 μl freshly made 10 mg/ml lysozyme added. After 5 min at room temperature, the cells were lysed by boiling in a PEG bath at 105°C for 40–45 s. The suspension was pelleted immediately by centrifugation for 10 min and the resulting gelatinous pellet removed using a sterile toothpick. Following isopropanol precipitation of the remaining supernatant, the pellet was resuspended in 100 μl TE and extracted carefully with phenol/chloroform. The DNA was recovered by ethanol precipitation and resuspended in 50–80 μl TE. 5 μl DNA solution was generally sufficient for each restriction enzyme digest.

2.6 Manipulation and Analysis of DNA

2.61 Solutions for the Manipulation and Analysis of DNA

Buffers and solutions for DNA manipulation (see Table 2.6) were prepared according to Maniatis *et al.*, (1982) or to the recommendations of the enzyme supplier.

2.62 Analysis and Manipulation of DNA

Restriction Endonuclease Digests

A typical restriction digest contained 0.1–5 μg DNA. A 20 μl digest usually contained 2 μl 10x restriction buffer (as recommended by the manufacturer), 2 μl

Table 2.6: Solutions for the Manipulation and Analysis of DNA

Restriction Enzyme and Ligation Buffers (10x Concentrates)

Low Salt: 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT

Medium Salt: 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT, 500 mM NaCl

High Salt: 100 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT, 1M NaCl

Sma I (KCl): 100 mM Tris-HCl pH 8.0, 100 mM $MgCl_2$, 10 mM DTT, 200 mM KCl

Ligation: 500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 10 mM Spermidine, 10 mM ATP, 1 mg/ml BSA.

Buffers for Exonuclease Deletion (10x Concentrates)

Exonuclease III: 660 mM Tris-HCl pH 8.0, 10 mM MgCl

Exonuclease III "Stop" Buffer: 2 M NaOAc, 133 mM EDTA

Exonuclease VII: 100 mM Tris-HCl, 300 mM KCl, 100 mM EDTA

Buffers for Southern Analysis

Depurination solution: 0.25 M HCl

Denaturing solution: 0.5 M NaOH, 1.5 M NaCl

Neutralising solution: 0.5 M Tris-HCl pH 8.0, 1.5 M NaCl

SSC (20x concentrate): 3 M NaCl, 0.3 M NaCitrate

Labelling Buffers (random hexamer priming): Solution A: 1.25 M Tris-HCl pH 8.0, 0.125 M $MgCl_2$, 1.8% (v/v) β-ME, 0.5 mM each dNTP except dCTP; Solution B: 2 M HEPES-NaOH pH 6.6; Solution C: Random Hexanucleotides at 90 OD_{260}/ml in TE; OLB buffer: solutions A, B and C mixed in the ratios 10:25:15;

"Stop" buffer: 20 mM Tris-HCl pH 7.5, 20 mM NaCl, 2 mM EDTA, 0.25% SDS, 1 μM dCTP.

"Blotto": 0.5% (w/v) dried milk (Cadbury's Marvel), 1% (w/v) SDS, 6% (w/v) polyethylene glycol 8000, 0.27 M NaCl, 1 mM EDTA, 1.5 mM sodium phosphate (pH 7.7)

Wash 1: 3xSSC, 0.1% SDS, prewarmed to 65°C

Wash 2: 0.5xSSC, 0.1% SDS, prewarmed to 65°C

(5–20 units) restriction enzyme, the remainder of the volume being made up of sterile water and DNA solution. The reactions were usually performed for 1 h at 37°C although longer incubations were sometimes used, when the concentration of DNA relative to that of the enzyme was high.

Ligations

Typical ligation reactions contained appropriate concentrations of vector and insert DNAs plus 1/10 volume of 10x ligation buffer, 0.5 Weiss units (cohesive–end ligations) or 2–4 Weiss units ("blunt" end ligations) ligase and the mixture was made up to volume with sterile water. When ligating cohesive ends, the mixture was incubated at 15°C for 3–16 h, while for "blunt" end ligations, the reactions were incubated at 25–30°C for 5–16 h.

Agarose Gel Electrophoresis

Agarose gel solutions were prepared by heating a suspension of agarose in 1x TBE (10.8 g/l Tris, 5.5 g/l boric acid, 0.9 g/l EDTA) in a microwave oven. Ethidium bromide (EtBr) was added to a final concentration of 0.5 μg/ml, gels were poured on horizontal perspex gel beds and the required number of wells were formed using "combs" of the same material. Samples were prepared by the addition of 1/10 volume of "loading buffer" (0.5% bromophenol blue, 60% sucrose, in water). Electrophoresis was performed in 1x TBE plus ethidium bromide (0.5 μg/ml), typically at 100 V constant voltage for 2–3 h. DNA was visualised and photographed under U–V light.

Electroelution of Restriction Fragments

Digested DNA was subjected to electrophoresis on an agarose gel as normal except that EtBr was excluded from both the gel and the running buffer. The gel was then briefly stained in a solution containing EtBr at 10 μg/ml. The stained gel was then observed under long wave UV light and the band of interest carefully excised from the gel with a scalpel. The gel slice was placed in dialysis tubing containing a small volume of electroelution buffer excluding all air and submerging the whole slice. The tubing was sealed, placed laterally in an electrophoresis tank containing 1xTBE and electrophoresed for 3 h at 70 V. The polarity was reversed several times to remove the fragment from the walls of the tubing and the DNA in solution was recovered by extraction with phenol/chloroform and ethanol precipitation.

2.63 Approximately Random, Size–Fractionated Fragments of Chromosomal DNA

Partial *Sau3A I* digests of *A. luteus* chromosomal DNA were produced as described by Maniatis (1978). 5 μg chromosomal DNA was mixed with 12 μl (10x) restriction buffer and made up to 120 μl with Q water. 20 μl was dispensed into a

1.5 ml eppendorf and 10 µl into 9 more tubes. 0.5 µl *Sau3A I* (diluted to 4 U/µl) was added to tube 1, and mixed well. Serial dilutions were produced by transferring and mixing 10 µl of reaction from tube (n) to tube (n+1). This leads to a dilution of the enzyme but not the DNA. The reactions were incubated for 30 min at 37°C and stopped by the addition of EDTA to a concentration of 20 mM. The conditions which generated the greatest mass of DNA fragments of the "correct" size distribution (5–15 kb), were determined by gel electrophoresis of the digested DNAs. In order to maximise the number of molecules with this size distribution, half as much enzyme was required. These conditions were then used to generate large quantities of DNA with this size distribution by scaling up of the restriction digest.

Solutions of 10 or 40% sucrose (buffered in 20 mM Tris–HCl, 5 mM EDTA, 1 M NaCl), were mixed in a two chamber gradient former and drawn into 34 ml polypropylene centrifuge tubes using a peristaltic pump. The tubes were filled from the bottom with an increasingly dense mixture of the sucrose solutions, thus producing a uniform gradient. Partially digested DNA samples were layered on the top of the gradients and after centrifugation in a swinging bucket rotor (AH627) at 26,000 rpm for 24 h at 15°C, fractions were collected from the bottom of each tube by piecing it with a sharp needle. The size of the DNA fragments in every third fraction was estimated by agarose gel electrophoresis and those of the appropriate size were pooled and ethanol precipitated.

2.64 Southern Blotting and Hybridisation Analysis

The method as described by Boulnois (1987) was used.

Chromosomal and plasmid DNAs were digested with appropriate enzymes and electrophoresed at low voltage overnight. After electrophoresis, the gel was trimmed to size and soaked in the depurination buffer for 7 min, washed in Q water and transferred to a tray containing denaturation solution. After 30 min denaturing, the gel was rinsed in Q and soaked for 30 min in neutralising solution. A piece of Whatman 3MM paper was supported above a tray of 20xSSC, with "wicks" trailing in the buffer so that the paper was always wet. The gel was placed upon the 3MM paper and a piece of "Hybond–N" (Amersham) nylon filter cut to size was wetted with 3xSSC and laid on top of the gel, excluding all air bubbles. Transfer was carried out by overlaying the nylon filter with a piece of 3MM paper cut to size and a stack of paper towels pressed down with a weighted glass plate. The transfer was carried out overnight or speeded up by frequent changes of the wet towels (every 10–15 min for 2 h).

Following transfer, the filter was washed in 3xSSC for 5 min, blotted dry on filter paper and dried thoroughly in a 65°C air incubator. The DNA was then cross–linked to the nylon filter by exposure to UV–light for 15–30 seconds on the UV

transilluminator with a single layer of Saran Wrap between the source and the filter.

The DNA for the probe was digested and electrophoresed in the absence of EtBr. It was visualised by EtBr staining and extracted from the gel by electroelution as described earlier. The purified fragment was labelled in a total volume of 25 μl, the reagents were added in the order specified: 0–15 μl Q water (depending on the volume of fragment required to give 25ng), 5 μl OLB buffer, 1 μl BSA, 16–1 μl fragment (to give 25 ng), 2.5 μl (0.925 MBq) [α-^{32}P]dCTP, 0.5 μl Klenow fragment (2 units). The labelling was incubated at room temperature for 4–20 h and stopped by the addition of 100 μl "Stop" buffer. The probe was separated from the unincorporated nucleotides on a 1 ml Sephadex G50 column, eluting with TE and collecting 100 μl fractions. The radioactivity was detected by Cerenkov counting of each fraction.

The filter was wetted briefly in 3xSSC, immersed in Blotto (preheated to 65°C) and incubated with gentle agitation for at least 2.5 h. The probe was then denatured by boiling for 8 min in a 105°C PEG bath and added to fresh Blotto (20 ml) at 65°C. The filter was transferred to the probe/Blotto mixture excluding all bubbles and incubated overnight at 65°C with gentle agitation.

All washes were carried out at 65°C with pre-warmed solutions. The hybridised filter was rinsed briefly 4 times with 150 ml of the low stringency Wash 1 (3x SSC, 0.1% SDS), prewarmed to 65°C and twice more for 10 min in the same solution. This was followed by 4 washes of 15 min in the higher stringency Wash 2 (0.5x SSC, 0.1% SDS). The filter was blotted dry, wrapped in a single layer of Saran Wrap and exposed to X-ray film.

2.65 Generation of Nested Deletions

Nested deletions were generated by the method of Henikoff (1984)

2 μg plasmid DNA was digested to completion with endonucleases *BamH I* and *Sph I* and the reactions stopped by extraction with phenol/chloroform. The DNA was precipitated, washed with ethanol, and resuspended in a mixture containing 98 μl 1x exonuclease III buffer.

The tube was pre-warmed to 37°C in a water bath and 2 μl Exonuclease III (6.4 U/μl) added (incubation continued at 37°C). Samples (10 μl) were removed every 30–60 s into tubes containing 1.5 μl exonuclease III "Stop" buffer. The samples were heated at 70°C for 10 min to destroy the enzyme activity and the DNA was ethanol precipitated and dried. Each sample was then resuspended in 10 μl 1x exonuclease VII buffer containing exonuclease VII at 100 U/ml (1 unit per tube) and incubated at 37°C for 40–60 min. Following extraction with phenol/chloroform and precipitation with ethanol, the pellets were suspended in 10 μl ligation/Klenow mix (1x ligation buffer containing each dNTP at 0.05 mM, Klenow fragment and T4 DNA ligase at

0.5 and 1 unit per tube respectively) and incubated at 30°C for 5 h. After transformation into *E. coli* NM522, plasmid DNA was prepared from a number of colonies from each time point and the size of the inserts determined by restriction analysis.

2.7 DNA Sequencing

DNA sequencing was performed using the M13-based dideoxy-nucleotide method of Sanger *et al.* (1977).

2.71 Solutions

See Table 2.7.

2.72 Preparing Template

M13 recombinants were detected as white, turbid plaques on soft agar containing IPTG and X-gal. An overnight culture of *E. coli* NM522 was diluted 1 in 100 in 2YT and aliquots (2 ml) were dispensed into fresh sterile bacteriophage tubes. Plugs of agar carrying single, well isolated plaques were picked using the narrow end of a pasteur pipette and expelled into the diluted cultures. The cells and phage were incubated at 37°C with high aeration for at least 5 h and up to 16 h (overnight).

The cultures were decanted into 1.5 ml Eppendorf tubes and the bacteria harvested by centrifugation in a micro-centrifuge for 10 min. 1 ml of phage-containing supernatant was carefully removed and the phage particles precipitated by the addition of 250 µl PEG/NaCl and incubation at room temperature for 20 min. The remainder of the supernatant was removed and stored at 4°C to be used as a stock. The precipitated phage were harvested by centrifugation for 10 min and all of the resulting supernatant was removed using a drawn out Pasteur pipette. The phage pellet was resuspended in 100 µl TE and extracted twice with phenol to remove the phage coat, before precipitation of the phage genome with ethanol. The pellet of single-stranded DNA was washed in 70% ethanol and absolute ethanol, air-dried and resuspended in 30 µl Q water.

2.73 Sequencing Reactions.

The annealing of template to primer was carried out in a 0.5 ml eppendorf tube containing 1 µl 10x TM buffer, 5 µl template, 2.5 µl primer (1 ng/µl) and 1.5 µl water. The mixture was heated for 30 min at 65°C, the liquid collected by brief centrifugation, and cooled gently on the bench for 20 min. [α-^{35}S]dATP (1 µl) was added and mixed. Aliquots (2.5 µl) were dispensed into 4 separate tubes which contained 2 µl of sequencing mix (a different mix for each reaction). The mixes were heated to 50°C in a water bath and the reactions were initiated by the addition of

Table 2.7: Solutions for Sequencing

PEG/NaCl:	20% (w/v) PEG 6000, 2.5 M NaCl
TM Buffer (10x):	100 mM Tris-HCl (pH 8.0), 100 mM $MgCl_2$
Ammonium Persulphate (APS):	10% (w/v) Ammonium persulphate prepared freshly
Acrylamide Stock:	38% (w/v) acrylamide, 2% (w/v) bis-acrylamide, stored over 20 g/l Amberlite MB1 resin at 4°C.
Gel Stock:	Urea (460 g), 100 ml 10x TBE, 150 ml Acrylamide stock made up to 1 l using Q water and filtered through Whatman No 1 paper; stored at 4°C.
Dye/Formamide:	100 ml deionised formamide, 2 ml 0.5 M EDTA, 0.1 g xylene cyanol, 0.1 g bromophenol blue.
Nucleotides:	0.5 mM solutions of dTTP, dCTP and dGTP and 10 mM solutions of ddTTP, ddCTP, ddGTP and ddATP were stored at -20°C
"Chase":	A mixture of dNTPs, each at 0.25 mM.

Mix	"T"	"C"	"G"	"A"
0.5mM dTTP	25	500	500	500
0.5mM dCTP	500	25	500	500
0.5mM dGTP	500	500	25	500
10.0mM ddTTP	50			
10.0mM ddCTP		8		
10.0mM ddGTP			16	
10.0mM ddATP				3
TE buffer	1000	1000	1000	500

1 μl (1 U) DNA polymerase I large fragment (Klenow fragment). The reactions were carried out at 50°C for 20 min and labelling was terminated by the addition of 2 μl "Chase" followed by a further 20 min incubation. All reactions were terminated by the addition of 4 μl Dye/formamide and the DNA strands were denatured by boiling for 2 min followed by rapid cooling in iced water. Reactions were occasionally stored for up to 3 d at -20°C and were re-boiled before use.

2.74 Preparing and Running the Gel

Gels (35 mm by 40 mm by 0.4 mm) were routinely used. "Plasticard" was used for spacers and the wells were formed using 6 mm spaced "sharkstooth" combs. The inner surface of the smaller gel plate was usually silicon-coated using "Sigmacote" and the plates were thoroughly taped and clamped with bulldog clips to ensure uniformity and to prevent leakage. The gels were polymerised with 500 μl fresh APS and 50 μl TEMED per 100 ml gel.

Gels were mounted on the appropriate electrophoresis apparatus and the upper and lower reservoirs filled with TBE. The gels were prewarmed by electrophoresis at 30 W for 20 min. Reacted samples for each template were loaded in 4 adjacent lanes and electrophoresed at 70 W constant power for, typically, 2 h. Usually however, only half of each sample was loaded on each gel; the remainder was loaded on a second gel which was electrophoresed for significantly longer e.g. 6 h.

Following electrophoresis, gels in the plates were laid flat and the siliconised plate removed from the gel, which was then transferred onto Whatman 3MM paper and dried under vacuum at 80°C. The dried gel was exposed to X-ray film (Kodak XAR-5 or Fuji) overnight.

CHAPTER 3: CHARACTERISATION OF THE BACTERIUM AND ITS LYTIC ENZYMES

3.1 Introduction

Although it is well documented that endo-β-1,3-glucanases play key roles within the mixtures of enzymes which cause lysis of viable yeast cells, it is unclear how many other activities (such as other glucanases or proteases) are involved. For these reasons it was decided to investigate, at a simple level, the enzymes secreted by *A. luteus*. It was also important to confirm that the strain obtained was indeed one which exhibited these activities.

3.2 The Effect of Carbon Source on the Production of Lytic Activity by *A. luteus*

In order to study the effect of carbon source on the production of lytic enzymes in batch cultures of *A. luteus*, a defined medium was used. Since *A. luteus* grew well in M63 medium, this was used for most experiments. Enzymes were also produced in cultures grown in LB, but LB is not a fully defined medium and the yeast extract which is an integral part of the medium is likely to contain small molecules derived from the yeast cell wall and these could possibly cause an induction of the expression of the yeast wall degrading proteins.

Using a defined medium, it was possible to vary the constituent minerals, carbohydrates and salts as required. The carbon sources used were: glucose and glycerol (controls), barley glucan (mixed linkage β-1,3; 1,4-glucan), laminarin (short chain β-1,3-glucan), yeast glucan (large, complex β-1,3-glucan), autolysed yeast residue and autoclaved yeast (both containing long and short yeast glucans, mannans, and other material).

Cultures of *A. luteus* were grown in M63 medium plus a single carbon source at a concentration of 0.4% (w/v). After 48 h growth, each culture was serially diluted and plated on L agar to determine the number of viable cells. The cells and remaining insoluble glucan were then removed by centrifugation. Further bacterial growth was prevented by the addition of sodium azide to a concentration of 10 mM and aliquots of the supernatant were assayed for lytic activity, protease, β-1,3-glucanase (clearing zone method or by estimation of the release of reducing sugars) and β-1,6-glucanase. The results are summarised in Table 3.1.

While it is impossible to draw far-reaching conclusions from this experiment, it does clearly show that the accumulation of yeast lytic activity in the culture supernatant of *A. luteus* is dependent upon the growth medium. The presence of glucose repressed production of these activities, but the absence of glucose was not sufficient to elicit maximal production of the lytic enzymes. Optimal production of enzymes was found in cultures grown on yeast based substrates. It is possible that

Table 3.1: Induction of Lyic Activty

Numbers express units/ml

C-source	Glucanase (plate)	Glucanase (reducing)	Protease	Lytic
Glucose	-	-	-	-
Glycerol	100	30	-	25
Laminarin	150	48	-	45
Barley Glucan	235	40	-	42
AIG	295	45	-	50
AY	379	72	3	81
AYR	345	59	3	57

small breakdown products of these substrates act as inducers of the lytic activities.

3.3 Time Course of Lytic Enzyme Production

The experiments described above give no indication of the stage of growth during which the lytic enzymes appear in the culture fluid. This is important in case the enzyme activity is manufactured early on but degrades after further growth. Thus, in order to study at what stage of the growth the enzyme was produced, cultures were sampled periodically and the supernatants assayed for the various activities.

To this end, M63 cultures were grown as described above. Since the cultures grown in yeast based media accumulated the most lytic enzyme, AYR, AY and AIG were the growth substrates used, with glucose as a control. Although the contents of these carbohydrates were complex and obscure, they were at least free of monomeric sugars. The cultures were grown for 72 h and samples (4 ml from 40 ml cultures) were periodically removed from each culture. This was replaced by 4 ml of the appropriate sterile medium (which had been prewarmed to 30°C). After dilution to determine the cell number, the supernatants were prepared and assayed as above for lytic activity, β-1,3-glucanase and protease. These assay data were plotted along with cell number. The cultures grown on yeast based substrates produced essentially the same activities at the same stage of growth, and Figure 3.1 shows a typical result for a culture grown in AYR. Lytic activity accumulated rapidly during the late logarithmic phase of growth and remained at similar levels thereafter. This correlated most strikingly with the appearance of β-1,3-glucanase activity. The glucose grown culture, as anticipated, accumulated little or no enzyme activity (not shown).

3.4 Characterisation of the Lytic Activity of the Supernatants

In order to characterise the enzyme system in more detail and to ensure that subsequent assays were performed under the appropriate conditions, the pH and temperature optima of the enzyme mixture were determined, and the endo-glucanase activities visualised using an activity stain.

3.41 Optimal Assay Conditions for the Yeast Lytic Enzymes

Lytic enzyme produced by the growth of *A. luteus* in M63 plus 0.4% (w/v) AYR was assayed at a range of pHs, the mixture being buffered with 100 mM sodium phospate or 100 mM Tris-HCl and incubated at 37°C. The lytic activity at each pH is shown in Figure 3.2a. The pH optimum was broad (pH 7-8) and this perhaps refects the multi-enzyme nature of the system.

The temperature optimum was determined with the pH constant at 7.5, but the temperature was varied using different waterbaths. Figure 3.2b shows the activities at these temperatures. The peak is again broad ranging from approximately 35°C to

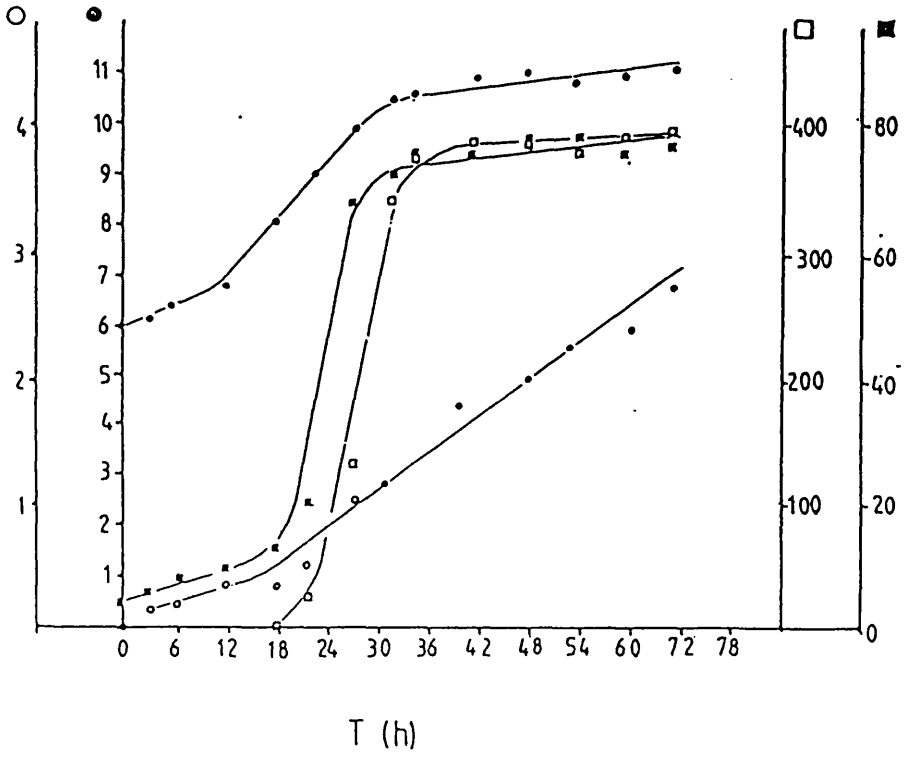


Figure 3.1: Time Course of Lytic Enzyme Production

Lines show: $\log_{10}$cells/ml (●); Units/ml Protease (O);
Units/ml lytic activity (■); Units/ml Glucanase (□)

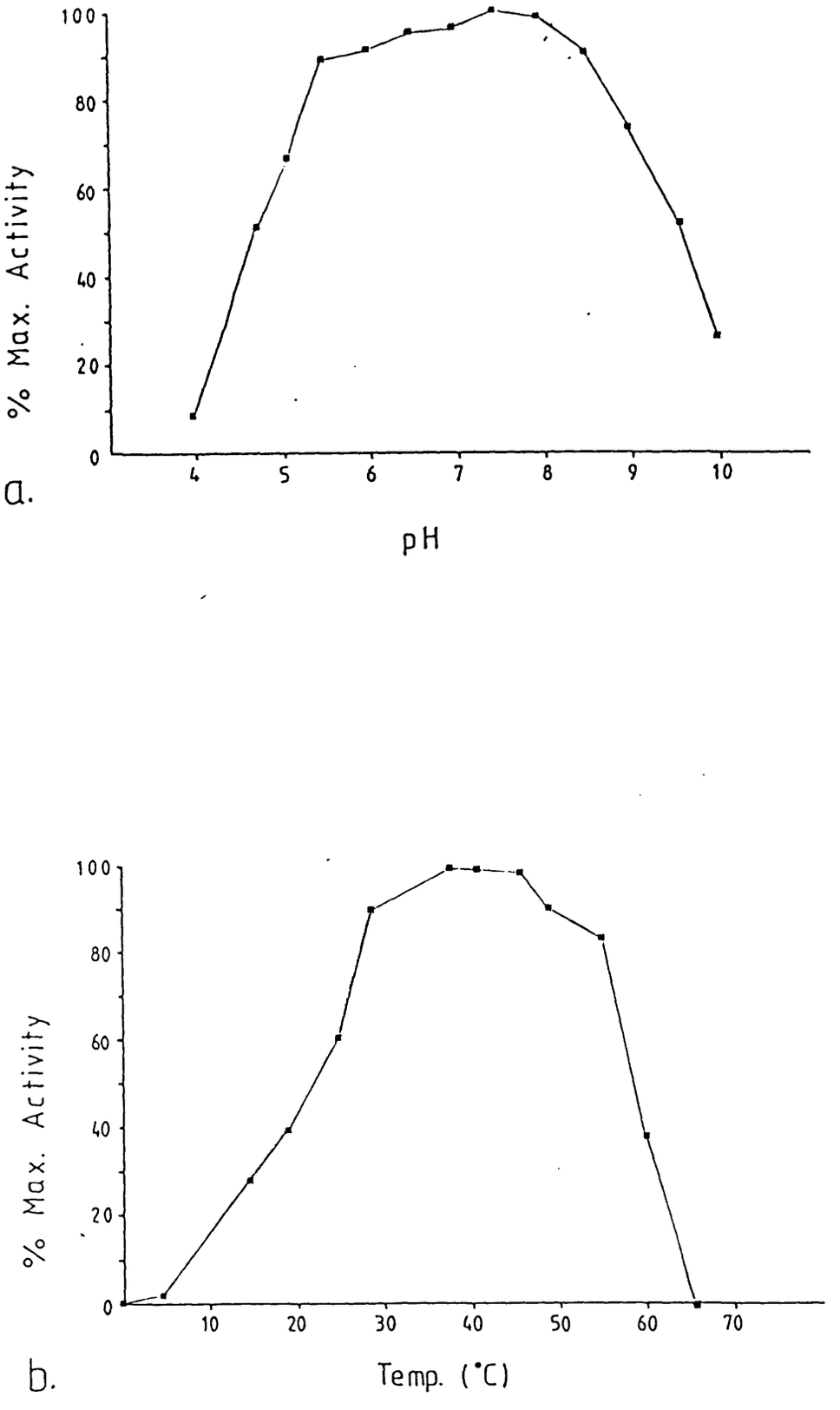


Figure 3.2: pH and Temperature Optima of the Mixture of Lytic Enzymes

45°C, with a maximum at about 43°C.

These results showed that the enzyme mixture exhibited virtually the same lytic activity over a range of conditions. It seems likely that this is due to the multiplicity of enzymes in the mixture.

3.42 Visualisation of the Endo-β-Glucanases in the Mixture

Following SDS-PAGE, it is often useful to be able to detect renatured activities *in situ*. This detection can be based upon several possible methodologies amongst which are overlaying the gel with another gel containing substrate, or washing the renatured gel with soluble substrate (Bertheau *et al.*, 1984) The method chosen here was to incorporate a soluble substrate (barley β-glucan) at a low level (0.05%) in the gel. After electrophoresis, the proteins were renatured within the gel by extensive washing with water and 50 mM Tris-HCl pH 7.5. The substrate-containing gel was incubated overnight at 37°C in buffer. Intact glucan was detected using a 0.2% solution of congo red, and regions which failed to stain represented renatured glucanases.

Cultures of *A. luteus* grown in AYR or glucose were analysed by this method as was a solution of Zymolyase (50 μg/ml). The gel was divided into two halves; one half was stained for glucanase activity and the other for proteins. The activity stained gel is shown in Figure 3.3. Clearly, the glucose grown culture produced no endo-glucanase activities while several bands were visible in both the *A. luteus* supernatant from the AYR-grown culture and in Zymolyase. The glucanase bands correspond well between these two samples and this serves to confirm that the *A. luteus* strain used in this study is indeed closely related to the production strain for Zymolyase. The major activity band of the supernatant sample is a protein of approximately 43 kD and this is also a major constituent of Zymolyase.

3.5 Summary

The organism under study has been characterised at a basic level. It has been shown that the production of lytic enzymes by cultures of *A. luteus* depends at least to some degree upon the growth medium used. The enzymes are usually produced in the greatest quantities when the culture is in the late logarithmic phase of its growth.

The lytic activity has been characterised in terms of its pH and temperature optima (pH 7-8 and 35-45°C, respectively), and a number of endo-glucanases have been visualised using activity staining of SDS-PAGE gels. The major endo-glucanase activity of the mixture has a molecular weight of around 43 kD. The glucanase activities of *A. luteus* ATCC 21606 correspond well with those found in Zymolyase, as anticipated.

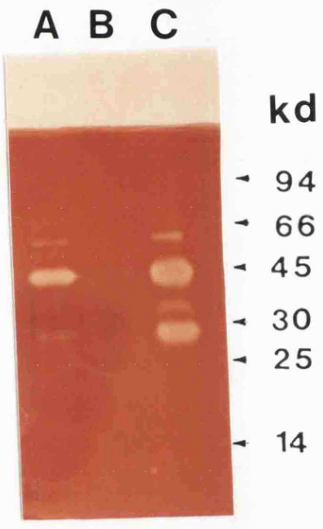


Figure 3.3: Activity Stained Gel of Extracts of *A. luteus*

A = AYR-grown culture; B = Glucose-grown; C = Zymolyase

CHAPTER 4: ISOLATION OF β-GLUCANASE POSITIVE CLONES.

4.1 Introduction

In order to obtain recombinant clones which carry the required segment(s) of DNA, a representative gene library must be constructed. This means that chromosomal DNA of the organism of interest is cloned and maintained in a vector which replicates in the chosen host, in this case, *E. coli*. In order for the library to be "representative", it should contain sufficient foreign DNA to represent several "genome equivalents". This can be achieved by obtaining as many different recombinant clones as possible and/or by maximising the size of the foreign insert in each clone. In practice, it is not possible to increase the size of the individual inserts above certain limits and a good library normally consists of a large number of recombinant clones. The formula of Clarke and Carbon (1976) allows the assessment of the probability of any given piece of DNA being represented in a gene library:

$$N = \log_n(1-P)/\log_n(1-f),$$

where N is the number of recombinant clones in the libary; P is the probability of the given piece of DNA being represented, and f is the fraction of the genome represented by an average sized insert.

The genome of *Arthrobacter luteus* may be as large as that of *Streptomyces*, at 10,000 kbp (Antonov *et al.*, 1978), or as small as that of the *Corynebacteria* (to which it is more closely related), at 2,600 kbp (Bak *et al.*, 1970; Crombach, 1978). Assuming a mean insert size of 6 kbp, the number of colonies required to give a 99% probability that the DNA of interest is represented in the library would be between 2,000 and 7,700. For a 99.9% probability, the number of clones required would be 3,000 to 11,500.

In order for these calculations to hold true, the inserts in the library must be random and must be the result of independent ligation events (it is no good having a "library" which consists of 5,000 copies of the same clone!). Of course, it is unlikely that every fragment is equally likely to be ligated and therefore represented in the library since certain inserts may be deleterious to the host and therefore under-represented in the library. However, by the use of partial digestion products and size fractionation, the "randomness" of the library can be improved.

4.11 Potential Vectors

Several types of *E. coli* vector were considered for the construction of gene libraries: plasmids, bacteriophage λ, and cosmids. On the one hand, plasmids are replicated and partitioned relatively efficiently in the host and are generally present at

high copy number. Indeed, a high copy number would be advantageous in enabling significant amounts of even a poorly expressed gene product to be manufactured in the host. However, large recombinant plasmids are less efficiently transformed into competent *E. coli*, a factor which would tend to limit the size of the library. A further disadvantage of plasmid vectors might be that any protein product produced from the insert DNA may be trapped within the host cells, hindering the detection of enzyme activities which require the release or secretion of the protein in question. However, by utilising long incubation periods or by partial disruption of the cells using surfactants or chloroform, this could be overcome.

Bacteriophage λ vectors have two advantages: firstly, they can accommodate large segments of foreign DNA (up to about 20 kb) which can be introduced into bacteria with high efficiency; and secondly, lysis of the host cells is an integral part of the 'phage life cycle so that protein products are released into the medium where they may be detected by their activity. The disadvantages of λ as a vector are worst if the cloned DNA is not expressed well in *E. coli*. In such cases, the insert DNA needs to be close to the vector expression signals. Cosmids have the advantages and disadvantages of each of the above systems. They are plasmids which also have the *cos* sites of λ. The presence of these sequences is sufficient to allow these molecules to be packaged by lambda-based systems, provided that the distance between a pair of *cos* sites is within the range 35-51 kb (Feiss *et al.*, 1977). Thus, cosmids with large inserts (up to 40 kb) may be packaged *in vitro* and *in vivo*, like λ, and introduced into bacteria in the same highly efficient way as λ. However, their size means that any given piece of cloned DNA could be very distant from vector promoters, thus expression of foreign genes may not be efficient. In addition, such large plasmids could make isolation and manipulation difficult and subcloning complex.

Using the bacteriophage vector λEMBL4 (Frischauff *et al.*, 1983) a gene library of *A. luteus* chromosomal DNA had been produced (C.Hadfield pers. comm.). In spite of extensive screening, on a variety of indicator plates, no glucanase-producing or yeast-lytic plaques were identified (DMW, not shown). It was decided that a plasmid vector with a relatively high copy number would be useful. Ideally, a strong *E. coli* promoter would be used to drive transcription through the insert DNA, in case the foreign DNA was poorly expressed in this host. Because the expression was to be driven by vector sequences, the insert size had to be relatively small to ensure the proximity of the promoter to the gene(s) of interest. This would require a large number of recombinant clones and to maximise the number of recombinants, a "positive selection vector" would be an advantage. For these reasons, the vector chosen was pKGW (Kuhn *et al.*, 1986).

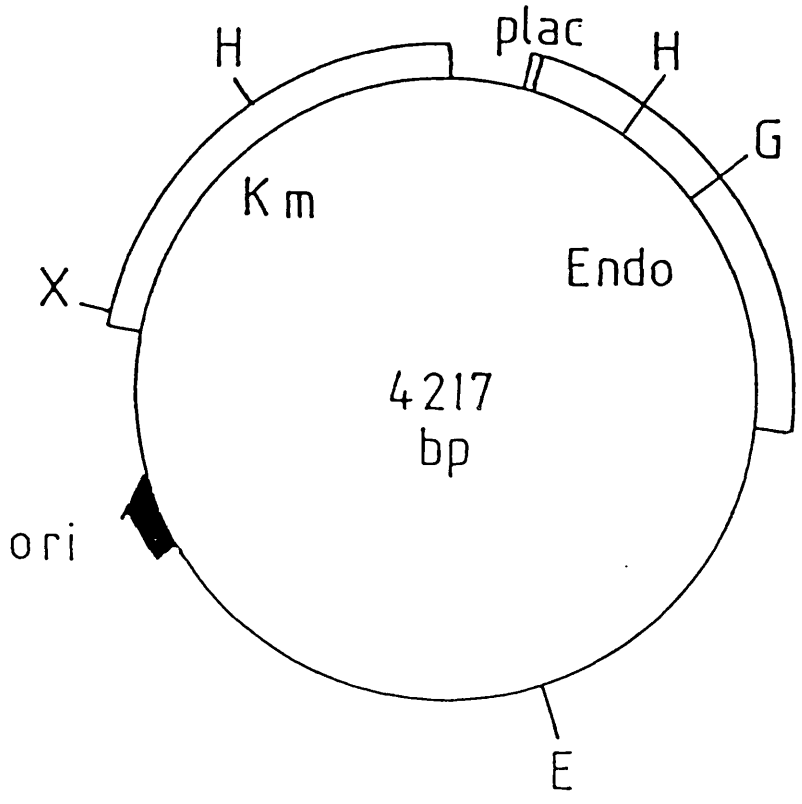


Figure 4.1: The Positive Selection Vector pKGW important restriction sites are marked. Both the kanamycin resistance gene (Km on the figure) and the endonuclease gene (Endo) are transcribed in a clockwise direction.

4.12 The Vector: pKGW

Plasmid pKGW (figure 4.1) is a vector that allows the selection of transformant clones which contain recombinant plasmids with inserts (Kuhn *et al.*, 1986). It encodes kanamycin resistance (Kan^R), thus enabling selection of cells carrying the plasmid, and carries the structural gene for the restriction endonuclease *EcoR* I. Expression of the endonuclease gene is under the control of the *lac*UV5 promoter so that, upon the addition of IPTG, the endonuclease is produced at high levels and is lethal to the cell by degrading its DNA. It is essential to choose a host strain which has no *EcoR* I methylase otherwise the DNA would be protected from degradation. In order that the cells bearing this plasmid are viable under normal growth conditions, a spontaneously arising mutant *EcoR I* gene was used. The product of this allele is still lethal when induced by IPTG, but the low level of expression under "repressed" conditions is no longer deleterious to the cell. Inserts in the *EcoR I* gene disrupt the coding sequence, thus removing this lethality and enabling cells containing recombinant plasmids to survive induction with IPTG. It is therefore an efficient method to select for plasmids with foreign DNA inserted into the *Bgl* II site of the *EcoR* I gene.

4.2 Construction of a Genomic DNA Library

4.21 Insert and Vector DNAs

Partial digestion of DNA with the restriction endonuclease *Sau3A* I generates near random DNA fragments because *Sau3A* I recognises a 4-base pair sequence (GATC) and, on average, should cut every 256 bp. If the digest proceeds to such a small degree that the DNA is significantly larger than this (e.g. 10–20 kb), only a very small subset of the possible sites will have been cleaved. Thus, those which are cleaved should be randomly distributed throughout the DNA. *Sau3A* I digests have the added advantage that they generate 5' protruding cohesive ends which can be ligated to the ends of fragments generated by a range of other commonly used restriction enzymes such as *BamH* I or *Bgl* II, which have 6 base pair recognition sequences.

Chromosomal DNA from *A. luteus* was partially digested with restriction endonuclease *Sau3A* I and size fractionated on a 10–40% sucrose gradient as described in the Materials and Methods.

Vector pKGW was prepared by digestion with *Bgl* II and following phenol extraction and ethanol precipitation, was resuspended in Q water at a concentration of 100 ng/μl.

4.22 Ligation and Transformation

A. luteus chromosomal DNA, of approximately 5–15 kbp was ligated to

Bgl II-digested pKGW. The ligations contained DNA concentrations of 30-100 μg/ml and the molar ratios of vector:insert DNAs ranged from 2:1 to 1:10. A small quantity (1/10) of each ligation mix was transformed into *E. coli* D1210 and the efficiency of the ligations estimated by plating the transformants on plates containg kanamycin, with and without IPTG. The results are shown in Table 4.1. The optimal (i.e. that which generated the most IPTG resistant colonies) molar ratio of vector:insert was approximately 1:3, and the overall concentration of DNA was 40 μg/ml. Following transformation of the remainder of the most efficient ligations, more than 8,500 transformants were obtained on kanamycin/IPTG plates.

4.23 Harvesting and Characterisation of the Library

The library was harvested by adding LB (0.5ml) to each plate and scraping the recombinant colonies off the plate, using a bent, sterile Pasteur pipette. The cell suspensions were pooled, amplified by growth for 5 h in selective media at 37°C and aliquotted into bacterial freezing mix for storage at -70°C.

Estimates of the reliability of the selection for recombinants and the size range of the inserts were obtained simple by restriction analysis of 48 small scale DNA preparations from colonies chosen at random. There were no parental type plasmids in any of the IPTG resistant colonies and the estimated size range of the inserts in the recombinant plasmids was 6-18 kb (very close to the anticipated size range). The mean insert size was approximately 9 kb. Using the equation of Clarke and Carbon (1976), this indicated that the probability of any given piece of DNA being present was greater than 99.9%, even if the genome was as complex as that of *Streptomyces*. If the genome of *Arthrobacter* was the same size as that of the *Corynebacteria*, the probability was effectively 100%.

4.3 Screening the Genomic Library

The most direct means to screen the library for genes encoding carbohydrate degrading activities, was to plate the recombinant colonies onto media containing appropriate substrates. Since *A. luteus* is known to produce amylolytic (starch degrading) activity as well as several endoglucanases, the library was tested for both endoglucanase and α-amylase (endo-amylase) activities.

Screening for amylolytic enzymes was performed as a control to assess whether or not it was feasible to detect such activities when expressed from *A. luteus* DNA in *E. coli*. The amylolytic detection procedure has advantages over that for β-glucanases: it is more sensitive and it is non-destructive, while being equally rapid to perform. Detection of amylase activity would indicate that the library was a representative one.

In order to test for α-amylase, soluble starch was incorporated in selective L agar plates and detected by its reaction with iodine vapour; zones of clearing appear

Table 4.1: Efficiency of Ligation

Numbers show the number of clones carrying inserts identified by transforming 1/10 each ligation.

DNA conc. (μg/ml)	Molar Ratio Vector:Insert	N° recombinants
30	2:1	35
	1:1	40
	1:3	65
	1:10	45
60	2:1	70
	1:1	500
	1:3	700
	1:10	175
90	2:1	210
	1:1	235
	1:3	410
	1:10	174

when the starch is enzymically degraded. For endo-β-1,3-glucanase, two slightly different substrates were appropriate: barley β-glucan or laminarin. Both substrates stain well with congo red, but the barley β-glucan is apparently a more sensitive assay (possibly due to relatively small numbers of β-1,3-bonds in the mixed glucan). It was important to remember, though, that some β-1,3-glucanases (e.g. laminarinases: E.C.3.2.1.39) have no activity on the mixed glucan. However, barley β-glucan was more cheaply available, easier to dissolve, and available in greater quantities. Therefore, this was the preferred substrate.

The method for detecting clearing of β-glucan in plates is theoretically non-destructive. However, it was often necessary to wash away the colonies in order to visualise clearing zones beneath the colonies themselves. Replicas of each plate, produced either by replica plating or picking duplicate colonies onto master plates as well as the test plates were therefore retained.

4.31 Isolation of Amylase Producing Clones

E. coli cells harbouring the library were plated at a density of approximately 500 cfu/plate on 10 plates containing starch at 0.5% (w/v), IPTG at 1 mM and kanamycin. After overnight growth at 37°C, the plates were inverted over iodine vapour for 30-60 s. Zones of clearing were visible around a number of colonies. Several of these were picked and purified by restreaking on selective media. When the amylase producing colonies were pure, plasmid DNA was prepared from them and used to transform competent *E. coli*. All the resulting colonies caused clearing on starch test plates thus confirming that the activity was plasmid-borne. Five isolates were thus demonstrated to encode amylase activity, of which four carried plasmids of different sizes and two were sibling plasmids (as determined by simple restriction analysis).

4.32 Isolation of β-Glucanase Producing Clones

Initial screening of the library on plates containing 0.05% (w/v) barley β-glucan, 1 mM IPTG and kanamycin was carried out as above (4.3.2). However, even after 48 h incubation at 37°C, no zones of clearing were observed upon flooding the plates with 0.2% (w/v) congo red solution.

Colonies were then picked onto regular arrays on test plates and duplicate selective plates, and grown for 72-96 h at 37°C. The colonies were washed from the surface of the test plates and stained with congo red solution (Teather and Wood, 1982). Beneath a number of colonies, faint clearing zones were observed. From 2,000 colonies screened 23 possible "positives" were isolated.

In order to determine whether or not these colonies represented real β-glucanase producing clones, a more sensitive method of screening was employed. After

restreaking to ensure the purity of each isolate, several representatives of each putative clone were toothpicked onto nitrocellulose discs and grown for 40 h at 37°C. The cells were then partially lysed by removing the nitrocellulose disc onto filter paper which had been wetted with Tris-HCl (50 mM), lysozyme (2 mg/ml) and 0.5% (v/v) Triton X-100 (Tsukagoshi *et al.*, 1984). After incubation at 37°C for 1 h, the disc was inverted onto a test plate containing barley β-glucan. Following overnight incubation at 37°C, the plates were developed with congo red. Clear zones of glucan hydrolysis were observed around colonies representing three different *E. coli* isolates, confirming them as true producers of β-glucanase.

4.33 Preliminary Analysis of the Putative β-Glucanase Clones

DNA was isolated from each putative β-glucanase clone by the small scale boiling method. The DNA was transformed into *E. coli* NM522 and the resulting transformants screened by the partial lysis method described above. Of these transformants, all were β-glucanase-positive, while cells transformed with other pKGW-derived clones were not. This demonstrated that the plasmid DNA from these clones conferred the ability to clear β-glucan to two different strains of *E. coli*. The three positive clones were designated pDMβ20, pDMβ40 and pDMβ60. Preliminary restriction enzyme analysis indicated that they contained inserts of foreign DNA which ranged in size from 9.5 to 13.5 kb.

4.4 Summary

A library of *A. luteus* chromosomal DNA was prepared, in the postive selection plasmid pKGW. This vector provides a means of selecting against colonies carrying non-recombinant plasmids and is therefore an excellent choice for library construction. The library contained approximately 8,500 recombinant clones with a mean size of about 9 kb which means that the probability of any given piece of DNA being represented in the library was greater than 99.9%.

Colonies which caused clearing of starch on plates were isolated, which showed that it was possible to obtain expression of *A. luteus* DNA and that sufficient enzyme could escape the cells to act on substrate in the plate.

Clones producing β-glucanase were isolated by prolonged growth of the recombinant colonies on plates containing barley β-glucan. Plasmid DNA from these clones could confer the β-glucanase activity to other strains of *E. coli*. The inserts in these plasmids ranged in size from 9.5-13.5 kb.

CHAPTER 5: CHARACTERISATION OF THE β-GLUCANASE POSITIVE CLONES

5.1 Introduction

In order to characterise more fully the DNA of these glucanase-encoding recombinant clones a number of approaches were required:

(a) the generation of a physical map (restriction map) of the three clones and the establishment of the relationships between the three loci;

(b) subcloning of the fragments carrying the glucanase gene(s) onto smaller fragments of DNA;

(c) fine scale mapping of these subclones to localise the gene coding region;

(d) confirmation, using Southern hybridisation analysis (Southern blotting), that the cloned DNA had indeed originated from *A. luteus*.

It is clear that 10-13 kb of insert DNA could encode a number of proteins and to determine which region(s) of the inserts encode glucanase(s), subclones were important. The generation of a restriction map was useful in establishing which subclonings would be appropriate, but restriction mapping of large inserts can be difficult. It is often more sensible to build up restriction maps of large fragments using maps generated for smaller subclones. Hence, subcloning and restriction analysis represent complementary approaches to the same problem.

Beyond a certain point, however, restriction mapping and subcloning may not yield sufficient information. In these cases, transposon mutagenesis is a frequently used tool for the identification of important coding regions of DNA and at a finer scale still, deletion mapping can be used to accurately map the start of the gene in question. In addition, the deletions thus generated can be used for a variety of further studies and especially in nucleotide sequencing.

Once the gene has been localised to a relatively small piece of DNA, a suitable hybridisation probe may be identified. This can be used in Southern blotting to hybridise to chromosomal DNA and cloned DNA to confirm the origins of the insert DNA in the recombinant plasmids.

5.2 Restriction Analysis and Simple Subcloning

Restriction analysis of the three β-glucanase producing clones was carried out using single digests of all the available enzymes with 6-base pair recognition sites. Those that cut infrequently were used in double digests to generate maps of the whole inserts (Figure 5.1). These maps were used as the basis for the generation of the first set of subclones. Around these skeletal maps it was then possible to map sites for enzymes which cut more frequently and further detail was provided by restriction mapping of subclones generated subsequently. Restriction enzymes with G+C-rich recognition sequences (such as *Sma* I, *Sst* I and *Sal* I) cut with such a high

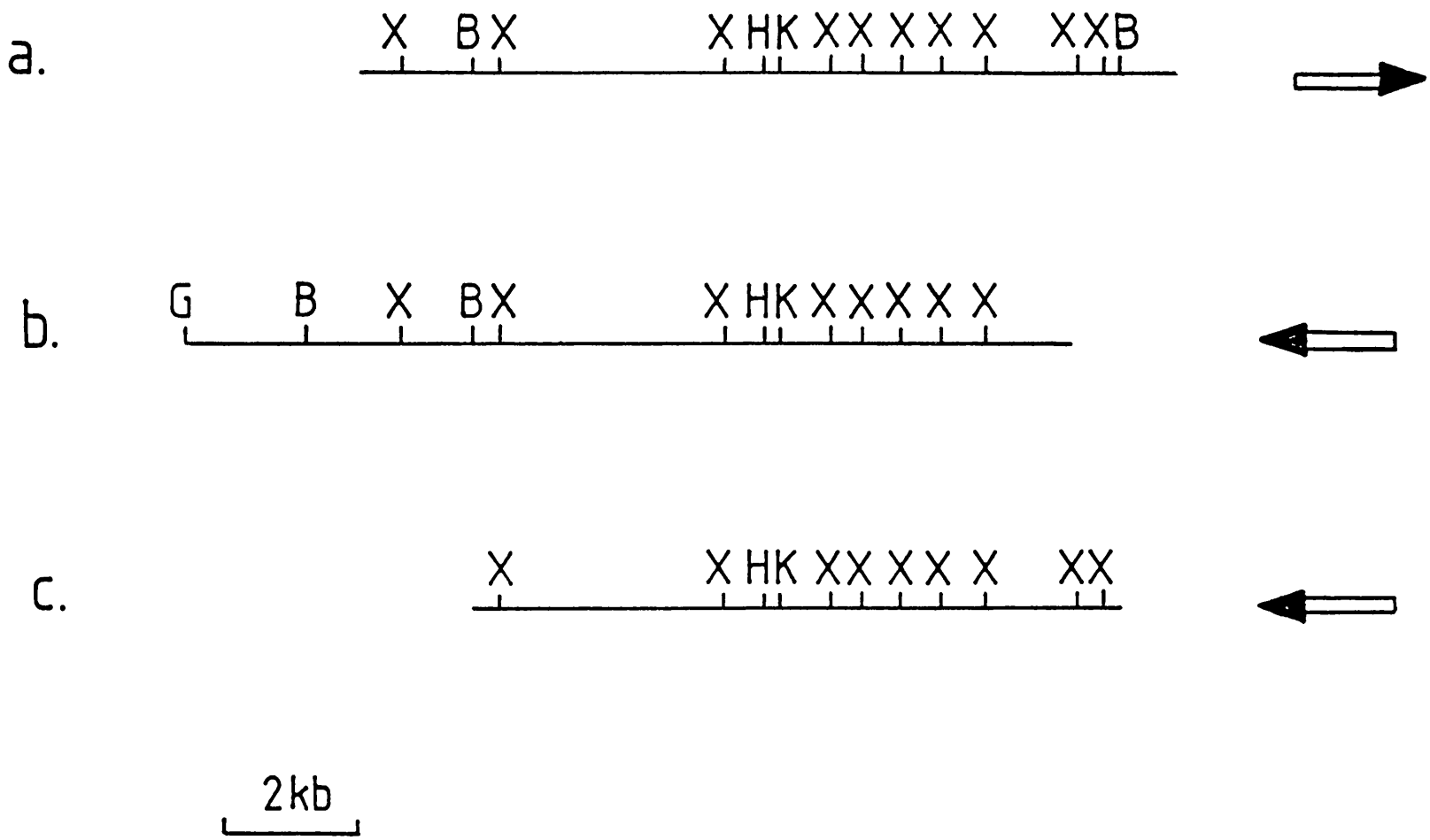
Figure 5.1: Primary Clones Encoding β-Glucanase

A shows plasmid pDMβ20

B shows plasmid pDMβ40

C shows plasmid pDMβ60

Arrows indicate the direction of vector-driven transcription


a.
X B X X H K X X X X X X X B
b.
G B X B X X H K X X X X X
c.
X X H K X X X X X X X
2kb

frequency that it was impossible to map all the sites for these enzymes on the large inserts. Others, having high A+T recognition sequences, such as *Dra* I, *EcoR* I and *Cla* I did not cut the DNA of these inserts. This is a direct reflection of the high G+C content of the cloned DNA.

There was substantial overlap between the insert DNAs of the three primary clones. However, in pDMβ20, the insert was in the opposite orientation (with respect to the vector) to that found in plasmids pDMβ40 and pDMβ60.

5.21 Simple Subcloning of pDMβ40

DNA of plasmid pDMβ40 was digested with *Kpn* I and *Bgl* II and ligated into the high copy number vectors pUC18 and pUC19 which had been digested with *BamH* I and *Kpn* I. After transformation of competent NM522, selection was performed on plates containing Amp and recombinant colonies were detected by the inclusion of X-gal; white colonies indicate a loss of complementation by the plasmid encoded α-fragment of β-galactosidase, caused by the insertion of cloned DNA in the polylinker region of the pUC vector. The resulting white colonies were tested for β-glucanase activity and significant zones of clearing were observed around a number of the pUC18-based clones after overnight growth at 37°C. Restriction analysis of the plasmid DNA carried by such transformants demonstrated that these clones contained the 9 kb *Kpn* I-*Bgl* II fragment (this subclone was designated pDMβ42; Figure 5.2). No β-glucanase activity was detectable upon similar screening of the pUC19-based subclones although restriction analysis of the plasmid DNA from these clones demonstrated the presence of the same *Kpn* I-*Bgl* II fragment. The association of β-glucanase production with orientation of this fragment suggests that the expression of the glucanase gene was being driven by the *lacZ* promoter (*plac*) of the pUC vector. Hence only the subclone with the gene orientated in the same direction as the *plac*-driven transcription led to appreciable activity being detected.

Deletion of the 2.5 kb *BamH* I fragment of pDMβ42 was carried out by simple digestion of the plasmid with *BamH* I, religation and transformation. The resulting plasmid (pDMβ43, see Figure 5.2) still encoded a β-glucanase since *E. coli* transformed with this plasmid caused clearing on glucan plates.

5.22 Subcloning of pDMβ20 and pDMβ60

A random approach to the generation of subclones from pDMβ20 and pDMβ60 was used. Several enzymes were used to digest the DNA of pDMβ20 and pDMβ60; these included *Hind* III, *Pst* I, *Sal* I and *Sma* I. Digested pDMβ20 and pDMβ60 were ligated to pUC19 linearised with the same enzyme and transformed into *E. coli* NM522 as above.

The resulting recombinants (whites) were tested on plates for the production of

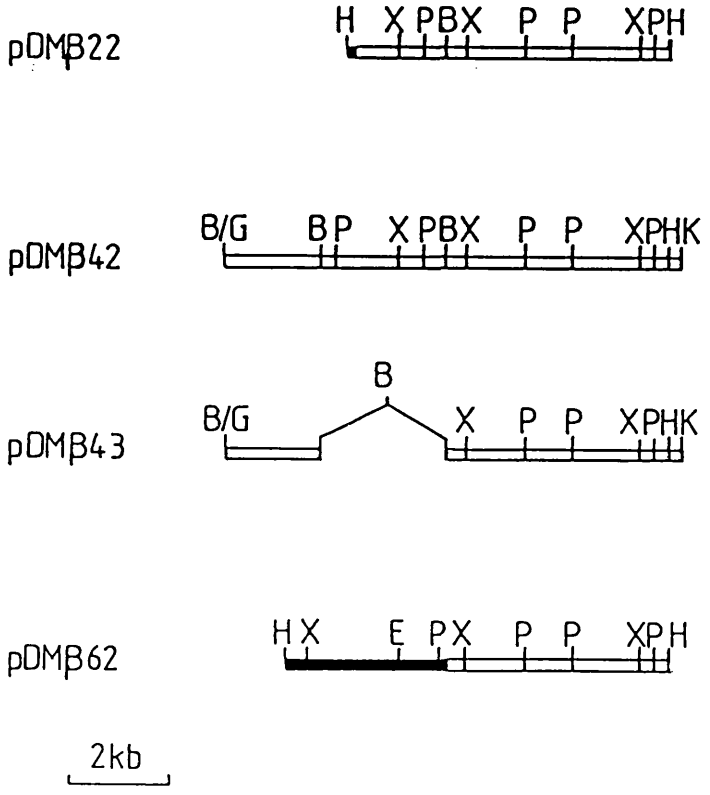


Figure 5.2: Restriction Maps of the Large pUC-Based Subclones

Transcription from the *plac* of pUC was from the right in each case.

□ *A. luteus* DNA

■ pKGW DNA.

β-glucanase. A proportion of the clones which had been generated by *Hind* III digestion of the original plasmids caused clearing on glucan plates whereas no β-glucanase producing colonies resulted from the other subcloning experiments. The *Hind* III plasmids which were glucanase positive all contained the same insert in the same orientation with respect to the *plac*. The glucanase-encoding plasmid derived from pDMβ20 was designated pDMβ22 (Figure 5.2). Similarly, the glucanase encoding derivatives of pDMβ60 were all identical and this plasmid was called pDMβ62 (Figure 5.2).

Restriction analysis demonstrated that pDMβ62 carried a segment of DNA from the original vector, pKGW, and the piece of *A. luteus* DNA was relatively small (about 4 kb), whereas plasmids pDMβ22, pDMβ42 and pDMβ43 all carried relatively large inserts (6-9 kb) of predominantly *A. luteus* DNA. The restriction maps of the pUC-based subclones confirmed that there was significant overlap between the three initial clones.

5.3 Detailed Localisation of a β-Glucanase Gene

When a fragment has been subcloned beyond a certain size, further subcloning can become tedious and time consuming. At this point, transposon mutagenesis is a commonly used technique which enables localisation of a gene on a piece of cloned DNA so long as the cloned DNA has already been restriction mapped to some extent. When a transposable element inserts into a gene coding region (or promoter), insertional inactivation usually occurs. By mapping the positions of these inactivating insertions, the position of the gene can be determined with a certain degree of confidence. It is important to be aware, though, that insertions can take place within a gene coding region without inactivating the gene product. Conversely, where expression of a gene is being driven by an heterologous promoter which may be some distance from the gene in question, insertions between the promoter and gene may also disrupt expression without being located in an essential coding region. If one keeps in mind these potential pitfalls, transposon mutagenesis is a powerful tool for the localisation of a gene within a piece of DNA. Although other transposons could be used, the system used here was Tn1000 ($\gamma\delta$) mutagenesis. The major reason for this was that all the products of the mutagenesis are insertions in the plasmid DNA, whereas other systems can lead to mutagenesis of the chromosomal DNA of the host cell.

Having localised the gene coding region through transposon mutagenesis, suitable small DNA fragments for further subclonings can be identified with a view to deletion analysis and sequencing. This allows the absolute localisation of the gene on a cloned insert to be determined. Sets of deletions were made to small subclones in order to locate the gene coding region to bring the gene under the control of the *plac* of

pUC and to facilitate sequencing at a later date.

5.31 Transposon ($\gamma\delta$) Mutagenesis

The *E. coli* sex factor F is able to co-transfer small R (resistance) plasmids at very low frequencies during conjugation (Guyer, 1978). This co-transfer has been found to be associated in the majority of cases (more than 99%) with a structural alteration in the R plasmid due to the insertion of the F attachment sequence $\gamma\delta$ (Guyer, 1978) (see restriction map, Figure 5.3). Insertion appears to be a relatively random event and is therefore a useful way of generating insertions in a non-mobilisable plasmid (Guyer, 1983). Figure 5.3 illustrates the transfer schematically. The F plasmid and the resistance plasmid combine to form a large cointegrate. This event is promoted by $\gamma\delta$ transposition resulting in duplication of the $\gamma\delta$ sequence. The hybrid plasmid contains two copies of the transposon, located at the cointegrate junctions and in the same orientation. Following conjugation this cointegrate is the substrate for a resolvase enzyme which then leads to the production of two separate plasmids, each with a copy of the $\gamma\delta$ sequence, by recombination between the two $\gamma\delta$ elements.

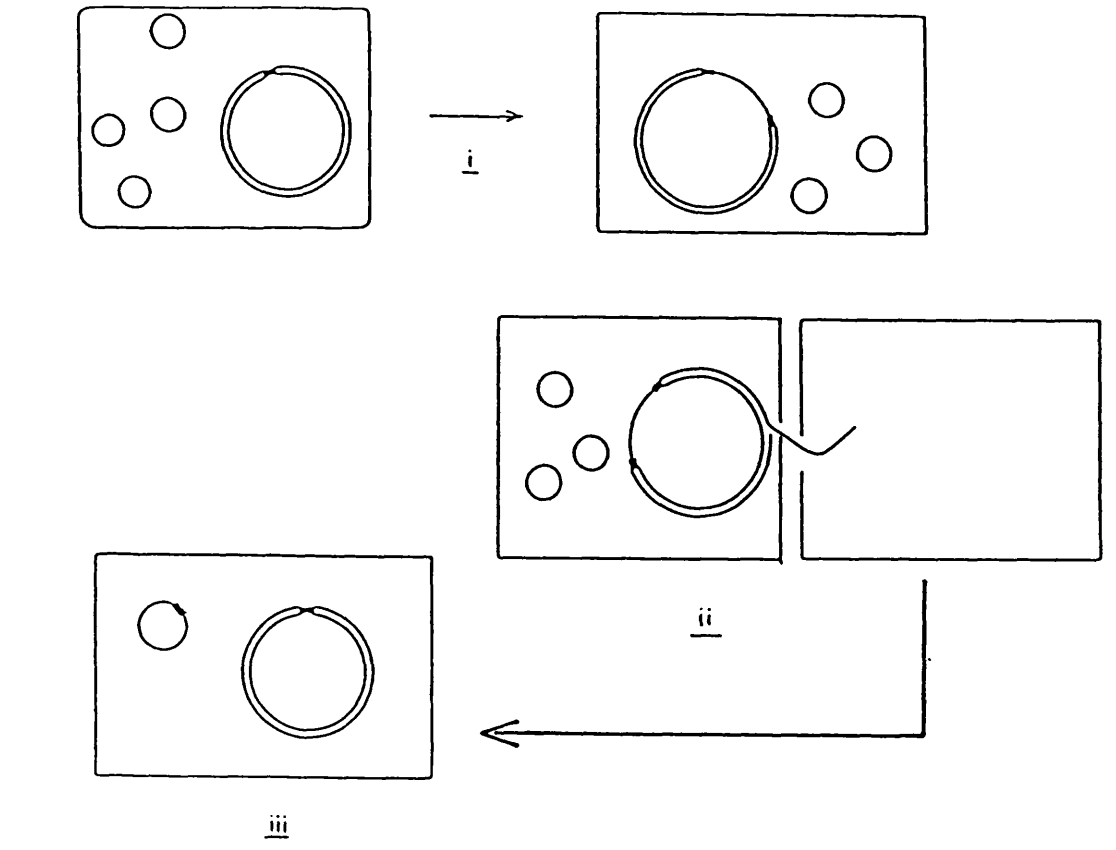
In the method used here the F^+ donor strain, RB308, is sensitive to streptomycin (Str^S) and has Ampicillin resistance conferred upon it by the pUC-derived plasmids, while the recipient strain, CSH26 is Str^R. Since the pUC plasmids are not mobilisable, the only way for the Amp^R plasmids to be transferred to the recipient strain is for a relatively rare transposition event to occur. The plasmids can then be co-transferred, leading to recipient cells which are resistant to both Str and Amp.

E. coli RB308 were transformed with pDMβ22, pDMβ42, pDMβ62 and pUC18. The tranformants were grown under selection and conjugated with CSH26 as described in the Materials and Methods. This method generated many transconjugants (table 5.1) and proved to be an efficient way for the production of relatively random insertions. 200 Amp^R/Str^R transconjugants from each mating were picked onto plates containing β-glucan and Amp which were pre-stained by the inclusion of congo red at a concentration of 0.004% (w/v). Each colony was screened for the presence or otherwise of a dye-clearing zone. Various glucanase phenotypes were observed ranging from fully active to completely inactive. Table 5.1 shows the results of this mutagenesis and subsequent screening. For simplicity, colonies exhibiting intermediate phenotypes were not analysed further. Since the pDMβ62 insert was the best defined, and a greater proportion of the $\gamma\delta$ derivatives were glucanase-negative, the insertions in this plasmid were analysed in detail.

Analysis of the $\gamma\delta$ insertions was carried out by detailed restriction digest mapping of plasmid DNA from 36 glucanase-negative and 12 glucanase-positive insertion derivatives. The position and activity of the transposon insertions is shown in

Table 5.1: Screening of γδ Transconjugants for Activity Loss.

Plasmid	Complete Activity Loss	Partial Activity	Full Activity
pDMβ22	16%	30%	54%
pDMβ42	7%	23%	70%
pDMβ62	26%	18%	56%



a.

b.

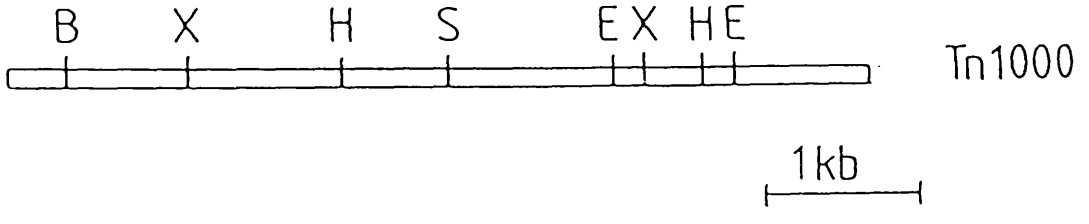


Figure 5.3: $\gamma\delta$ Mutagenesis of Resistance Plasmids.

Panel a schematically illustrates the mechanism of $\gamma\delta$ transposition and mutagenesis. First, a cointegrate plasmid froms accompanied by a duplication of the $\gamma\delta$ sequence. Secondly, conjugal transfer of the cointegrate plasmid occurs. Finally, the cointegrate is resolved into its constituent plasmids leaving an additional copy of the transposon randomly inserted within the resistance plasmid.

Panel b shows a restriction map of the $\gamma\delta$ sequence.

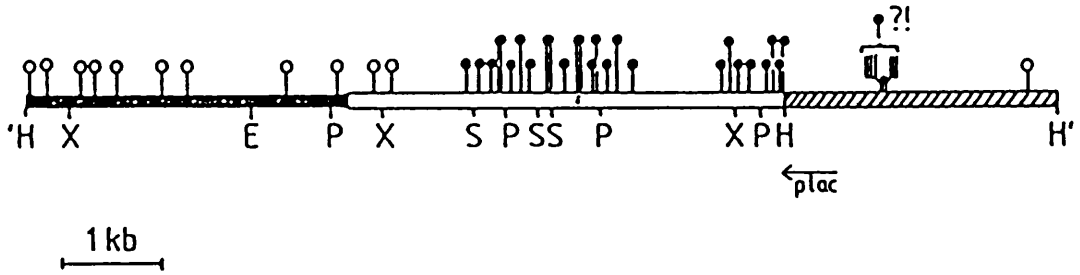


Figure 5.4: Distribution of $\gamma\delta$ Insertions in pDMβ62.

●	=	Insertions which inactivate the gene
⚲	=	Insertions which do not inactivate the gene
?!	=	Insertions which inexplicably inactivate the gene.
■		pKGW DNA
□		*A. luteus* DNA
▨		pUC DNA

Figure 5.4. A number of insertions which mapped close to the *lacZ* promoter of the pUC plasmids caused the loss of detectable β-glucanase activity. These probably represented insertions outside of the coding region of the gene which disrupt p*lac*-driven transcription. However, it was possible to map the 3'-end of the inactivating insertions and this helped to define a 3.6 kb *Xho* I fragment, which encodes an entire β-glucanase gene.

It is interesting to note that a proportion (10) of the inactivating insertions map very close to each other but within the pUC region of the hybrid plasmid. This insertion "hotspot" seems to correspond with an inverted repeat from the transposon (Tn3) which originally carried the ampicillin resistance gene. Why this should should generate β-glucanase-negative derivatives is unclear, unless other structural rearrangements had occurred which may have disrupted the β-glucanase gene or the transcription from the vector. However, no such rearrangements were evident when these plasmids were restriction mapped. In contrast, to this "hotspot", a region of approximately 700 bp with no insertions was noted, towards the 5'-end of the insert (with respect to the *plac*).

5.32 Generation of Small Subclones Which Encode β-Glucanase

The vectors pKM8 and pKM9 (Spratt *et al.*, 1986) were used for "random" subcloning from the pUC-based subclones, taking advantage of the fact that these plasmids are pUC-like, but carrying Kan^R.

The 3.6 kb *Xho* I β-glucanase encoding fragment (as defined by γδ-mutagenesis) was thus subcloned into *Sal* I digested pKM8. Of the two possible orientations of the *Xho* I fragment, only one was obtained (pDMβ90, see Figure 5.5), despite several attempts to obtain the other clone. This orientation gave production of β-glucanase at high levels.

Within pDMβ90, several *Sst* I fragments were mapped and the largest of these (2.8 kb) was subcloned into pUC19. Plasmids with the insert in both orientations were obtained (pDMβ100 and pDMβ101, Figure 5.5); around pDMβ100 transformants there were large glucan-clearing zones while for pDMβ101 (the opposite orientation), a little clearing activity was observed. This perhaps indicated that a small amount of β-glucanase was being produced from the expression signals of the *A. luteus* DNA.

5.33 Deletion Mapping

There were a number of reasons for performing deletion analysis on the cloned DNA. Firstly, a high degree of certainty in the identification of the coding region of DNA should result from such analysis. Secondly, by the deletion of the DNA between the *plac* of the pUC vectors and the coding region, it might be possible to obtain high levels of gene expression which would facilitate studies on the enzymic activity

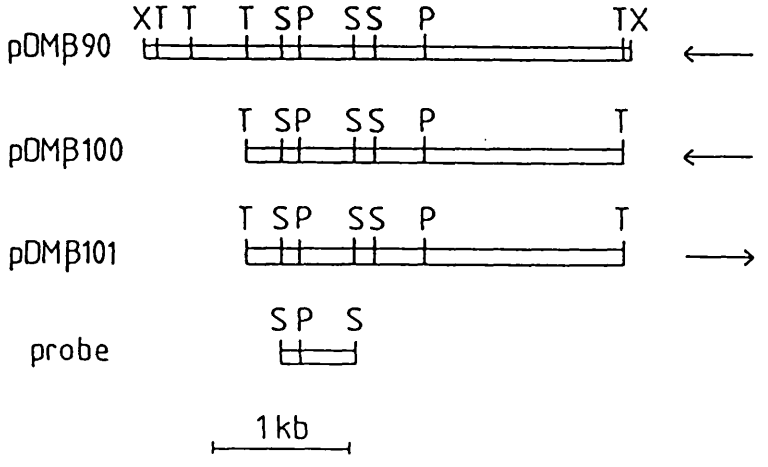


Figure 5.5: Small Subclones Which Carry a Glucanase Gene

Arrows indicate the direction of gene expression from the *plac*.

encoded by the cloned DNA. Finally, nested sets of deletions from the ends of the cloned DNA would be very useful for the sequencing of the glucanase gene (see later: Chapter 6).

Exonuclease III carries out the 3'->5' stepwise removal of 5' mononucleotides (Weiss, 1976) from a DNA molecule. This activity is specific to double-stranded DNA with either blunt or 3'-recessed ends but 3'-protruding ends are resistant. The enzyme requires Mg^{++} ions and the length of DNA deleted is roughly proportional to the incubation time providing the enzyme is in excess. After treatment with exonuclease III, the extensive 5' overhang must be removed. This can be performed using S_1 nuclease, Mung bean nuclease or exonuclease VII. In these experiments, exonuclease VII was used. This enzyme processively excises small oligonucleotides from the 3' and 5' ends of single stranded DNA. Ligation, giving closure of the deleted molecules, might be inefficient if the ends were not made completely flush by the exonuclease VII. This can be avoided by the inclusion of dNTPs and Klenow fragment in the ligation. The ligations are generally incubated at 30°C, to improve the efficiency of the ligase and the Klenow reactions.

Using the method described in Chapter 2, sets of deletions were generated from pDMβ100 and pDMβ101. Following ligation and transformation, 60-300 Amp^R colonies resulted from each sample. DNA was prepared from a representative number (5-10) from each timepoint for each orientation of the fragment. The sizes of the inserts, and hence the extents of the deletions were estimated by restriction enzyme analysis. In order to produce the larger deletions which were required to cover the whole fragment in each orientation, longer incubation times were needed. The decline in the exonuclease III activity observed in incubations of greater than 5 minutes was overcome by the addition of a second aliquot of enzyme. Figure 5.6 shows the mean size and range of sizes resulting from a typical deletion experiment.

The colonies containing pDMβ100 derived deletions were analysed to determine the effects of deletion on the β-glucanase activity (Figure 5.7). Deletions of up to 0.9 kb (Figure 5.7b, c and d) caused an increase in the amount of β-glucanase activity while deletions of 1.1 kb (Figure 5.7e) or more led to a loss of activity. The effect of deletion from the 3'-end of pDMβ100 was not studied because the suitable restriction sites for the deletion protocol were not available on this clone. Deletions were generated in pDMβ101, but expression of glucanase was poor from this clone thus preventing analysis of the 3'-end of the glucanase gene.

5.4 Southern Hybridisation Analysis

Further determination of the inter-relationships between the various inserts was derived from Southern blotting and hybridisation analysis. A small hybridisation probe (Figure 5.5) isolated from pDMβ100 was used to determine the presence or otherwise

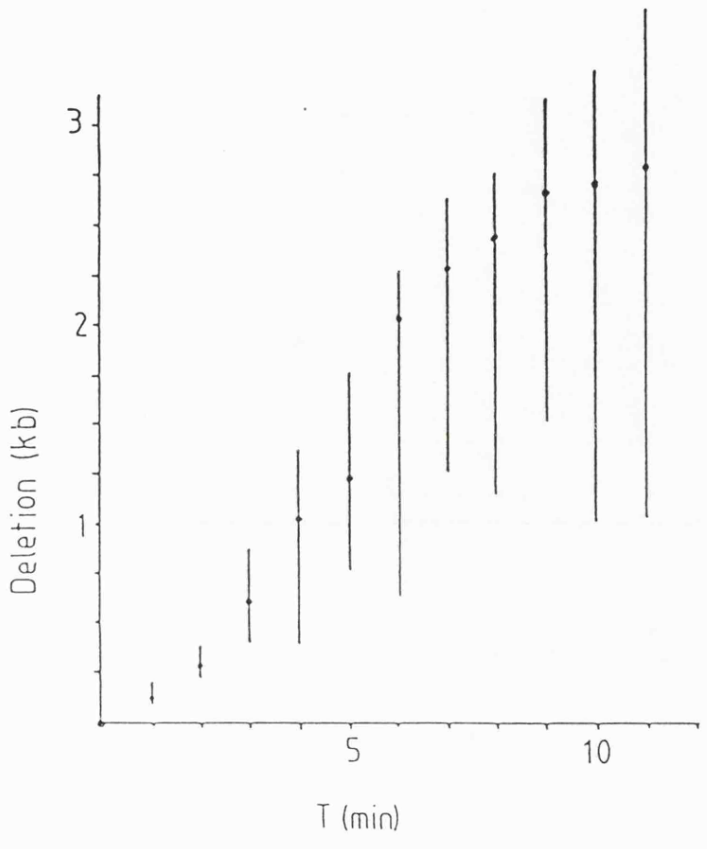


Figure 5.6: The Progress of Deletion with Time

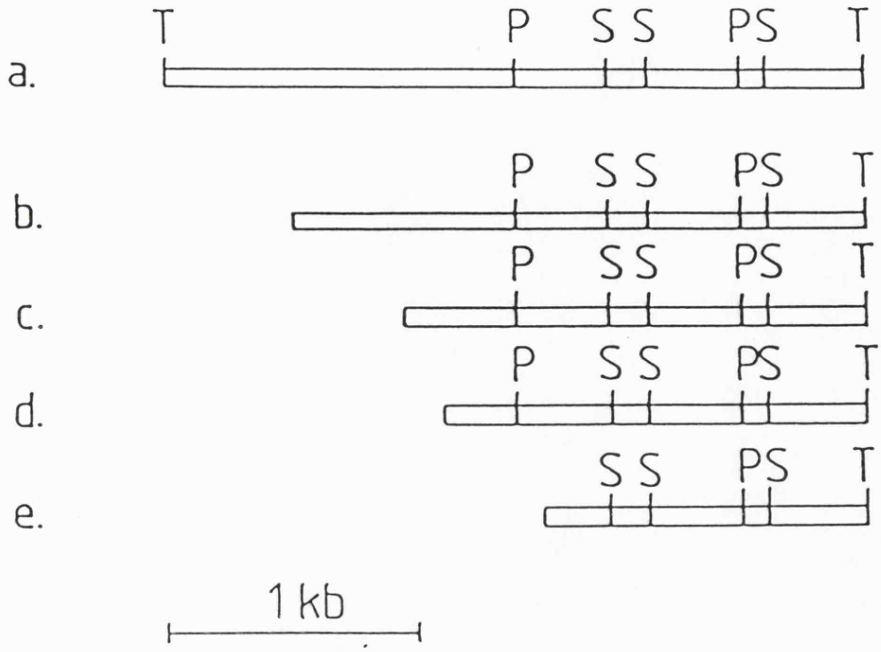
Points represent the mean extent of deletion at each time point

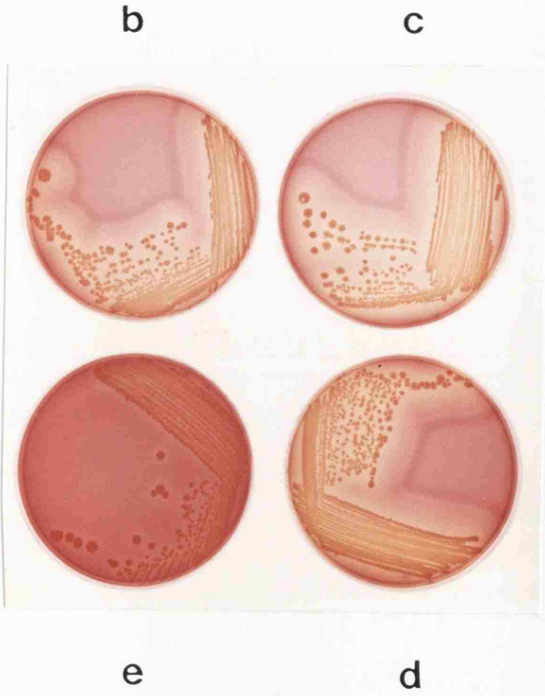
Bars represent the range of deletions isolated at each time point

Figure 5.7: Effects of Deletion on Production of Glucanase in *E. coli*

Upper Panel shows pDMβ100 (a) and the derivatives 100Δ12 (b), 100Δ24 (c), 100Δ36 (d), 100Δ31 (e)

Lower Panel shows colonies bearing these deleted plasmids plated on β-glucan. It was assumed that the largest deletion had destroyed the glucanase gene.


T P S S PS T
a.
P S S PS T
b.
P S S PS T
c.
P S S PS T
d.
S S PS T
e.
1 kb


b
c
e
d

of homologous or identical sequences in each of the primary glucanase clones. Furthermore, these analyses were extended to confirm the origin of the cloned DNA, by hybridisation to the chromosomal DNA from which the library was constructed.

By collating the results of the transposon mapping and subcloning it was possible to define a 550 bp *Sal* I fragment (see Figure 5.5), which maps towards the putative 3'-end of the β-glucanase gene but is probably largely within the coding region. This fragment was used as a probe in Southern hybridisation analysis.

Plasmids pDMβ20 pDMβ40 and pDMβ60 were digested with *Sal* I and *Xho* I and analysed by Southern blotting. The results can be seen in Figure 5.8. The three clones clearly all contained the 550 bp *Sal* I fragment from which the probe was generated (arrow A) and similarly, the 3.6 kb *Xho* I fragment which constitutes the insert DNA of pDMβ90 (arrow B). The probe also hybridised to a number of other bands within each plasmid. There were a number of possible explanations for this observation. For instance, it was possible that when isolating the probe DNA from the agarose gel, other fragments might have contaminated the preparation, but this is unlikely to have been the case since the fragment to be used as the probe was resolved well away from other digestion products. Alternatively, the additional hybridisation could have been a result of relatively non-specific cross-hybridisation due to the extremely G+C-rich nature of the DNA. However, a large number of bands were visible upon EtBr staining of the gel to which the probe did not cross-hybridise, which would indicate that this was not the case. Finally, it was possible that each clone also carried additional glucanase genes with some homology to the probe.

In Figure 5.9, chromosomal DNA from *A. luteus* and *E.coli* (negative control) plus plasmid DNA of pDMβ20 (positive control) were digested with a variety of enzymes and analysed using the same probe. The 550 bp *Sal* I fragment is clearly to be found within the DNA of *A. luteus* digested with the same enzyme (arrow A). Similarly, the 8 kb *BamH* I fragments of the cloned and the chromosomal DNAs detected by hybridisation appeared to be identical (arrow B). However the fragments generated by *Xho* I digestion were different: the plasmid-borne gene (isolated from *E. coli*) had a 3.6 kb fragment to which the probe hybridised (arrow C) whilst hybridising to a 5 kb *Xho* I fragment in the *A. luteus* DNA (arrow D). This might have been due to the *A. luteus* chromosomal DNA failing to be digested completely, but it was more likely that the *A. luteus* DNA was methylated at certain of its *Xho* I sites thus preventing the complete digestion of its DNA with this enzyme. The *Pst* I-digested DNA showed two major hybridising bands which corresponded to the two fragments which overlap the probe. Except in the case of the *BamH* I-digested DNA, additional cross-hybridising fragments were observed within the chromosomal DNA of *A. luteus*. The absence of any additional hybridising bands in the *BamH* I-cut DNA indicated all the homologous sequences detected in the other digests

Figure 5.8 Southern Analysis of the Plasmid Clones

Numbers indicate which initial glucanase clone was used (the corresponding restriction maps are also presented). Asterisks delineate the region to which the probe would be expected to hybridise. X or S indicate digestion with *Xho* I *or Sal I* respectively. Arrows indicate major cross-hybridising bands (see text).

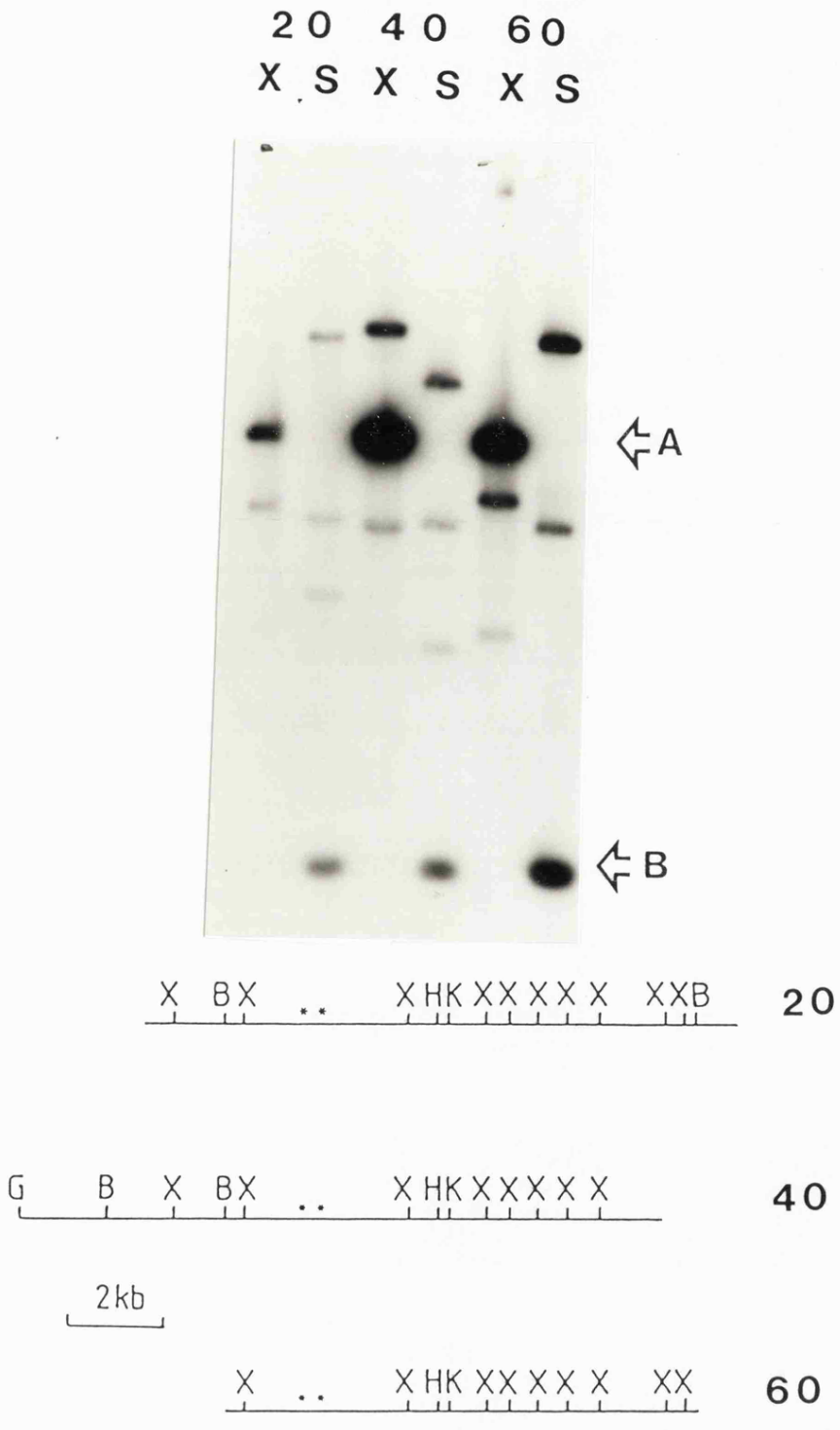
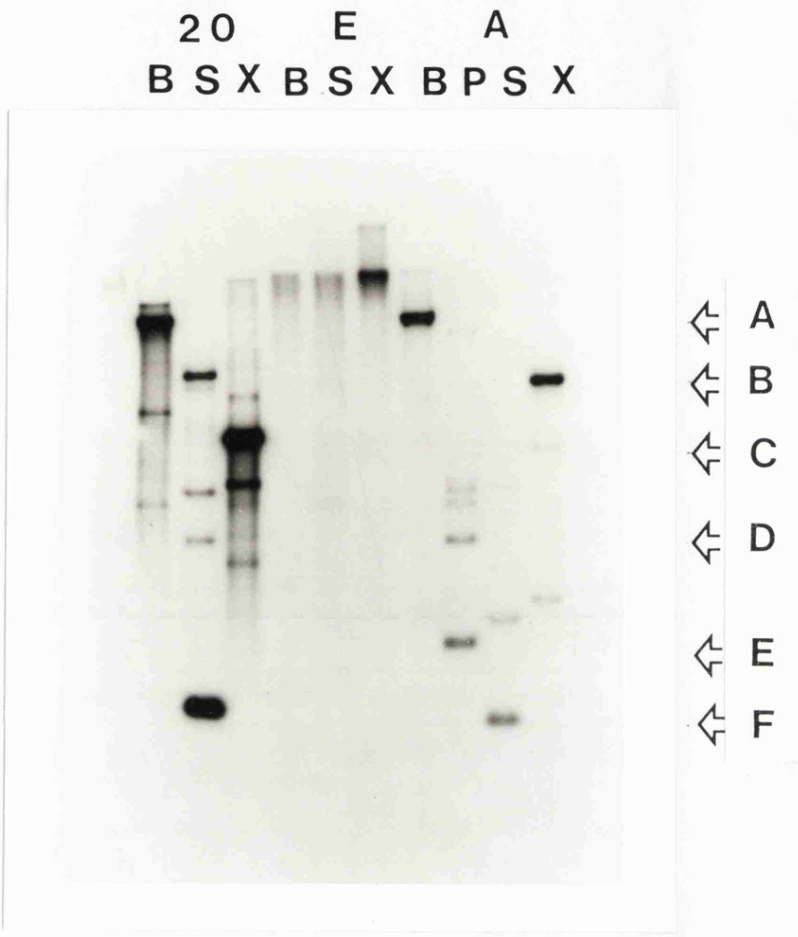

20 40 60
X S X S X S
A
B
X BX .. XHK XXXXX XXB 20
G B X BX .. XHK XXXXX 40
2kb
X .. XHK XXXXX XX 60

Figure 5.9: Southern Analysis of Chromosomal DNA.

DNA of plasmid pDMβ20 (20), *E. coli* chromosomal DNA (E) and *A. luteus* chromosomal DNA (A). DNAs were digested with the enzymes indicated: *BamH I* (B), *Pst* I (P), *Sal* I (S) or *Xho* I (X). Asterisks indicate the region of the probe. Question marks indicate the possibilty of methylation of one or other of the *Xho* I sites.


20
E
A
B S X B S X B P S X
A
B
C
D
E
F


X B X? ? X H K X X X X X X X B

were carried by a single *BamH* I fragment. Thus, either the source of the additional bands was a contaminated probe, or the large *BamH* I fragment carried several sequences homologous to the probe and it was possible that these represented additional glucanase genes.

No hybridising fragments were to be found in the digests of *E. coli* chromosomal DNA. These results demonstrated that the cloned DNA had indeed originated from *A. luteus*.

5.5 Summary

The DNA of the three primary clones was restriction mapped. The G+C bias of the insert DNA limited the information to be gained from such maps because there were many sites for enzymes (such as *Sma* I) which have recognition sequences rich in G and C, but few for enzymes with other types of recognition sites (such as *EcoR* I).

It was, however, possible to generate subclones to enable further restriction mapping of the clones. The three clones were derived from the same region of the *A. luteus* chromosome and overlapped considerably.

Expression of the glucanase activity from pUC-based subclones was markedly orientation specific, which showed the direction of transcription for this gene.

Transposon mutagenesis proved useful in identifying a region of the cloned DNA which carried the glucanase gene and facilitated further subcloning. The gene was carried on a 2.8 kb *Sst* I fragment which was then subjected to deletion analysis which localised the 5'-end of the coding region more accurately to 0.85 kb from one end of the fragment.

A 550 bp *Sal* I fragment from this subclone was used as a hybridisation probe in Southern analysis. This established that the cloned DNA fragments were the same as those found in the chromosomal DNA of *A. luteus* and confirmed the overlap between the DNA of all three initial clones.

CHAPTER 6: SEQUENCING OF A β-GLUCANASE GENE

6.1 Introduction and Strategy

In order to characterise the genetic material of *A. luteus* as fully as possible, the determination of the nucleotide sequence was undertaken. The methodology chosen was based on the "dideoxynucleotide" chain terminator method of Sanger *et al.*, (1977). For accuracy and completeness, it was necessary to determine the nucleotide sequence of both strands of the cloned DNA. This is particularly important in the case of DNA which is G+C-rich, in which a range of artifacts and ambiguities are possible.

6.2 The Approach Used

Since a number of deletion derivatives of plasmids pDMβ100 and pDMβ101 had been generated using exonuclease III (see Chapters 2 and 5), it was decided to use these nested deletions as the source of clones for the sequencing. It was considered that this would be more efficient than the generation of random subclones by restriction digestion or by sonication which would then require the analysis of many clones.

Clones carrying inserts of the appropriate sizes from each orientation were subcloned on *Hind* III-*Eco*R I fragments into M13 mp18 cut with the same enzymes. This ensured that the deleted end was always closest to the M13 primer site. The inserts were chosen to cover the relevant fragment at intervals of 200 bp to ensure that there was significant overlap between successive clones. Since the position of the gene had already been determined with some certainty, the region sequenced was a fragment which carried the expected coding region plus 4-500 base pairs at either end. Figure 6.1 schematically illustrates the clones used for the sequencing and the region of sequence which derives from each one.

To minimise any artifacts due to the high G+C content of the DNA, sequencing reactions were performed at 50°C. Another adaptation which improved the quality of the sequencing was to ensure that the gels were pre-run to heat them up. This, presumably, prevented stable secondary structures involving the labelled strand from being formed and maintained in the gel.

For each template, the completed sequencing reaction was divided into two portions. The first portion was electrophoresed until the bromophenol blue dye in the sample had reached the end of the gel (about 2 h), resolving bases 1-200. The second aliquot was run very much further allowing resolution of bases 150-300.

6.3 Computer Programs

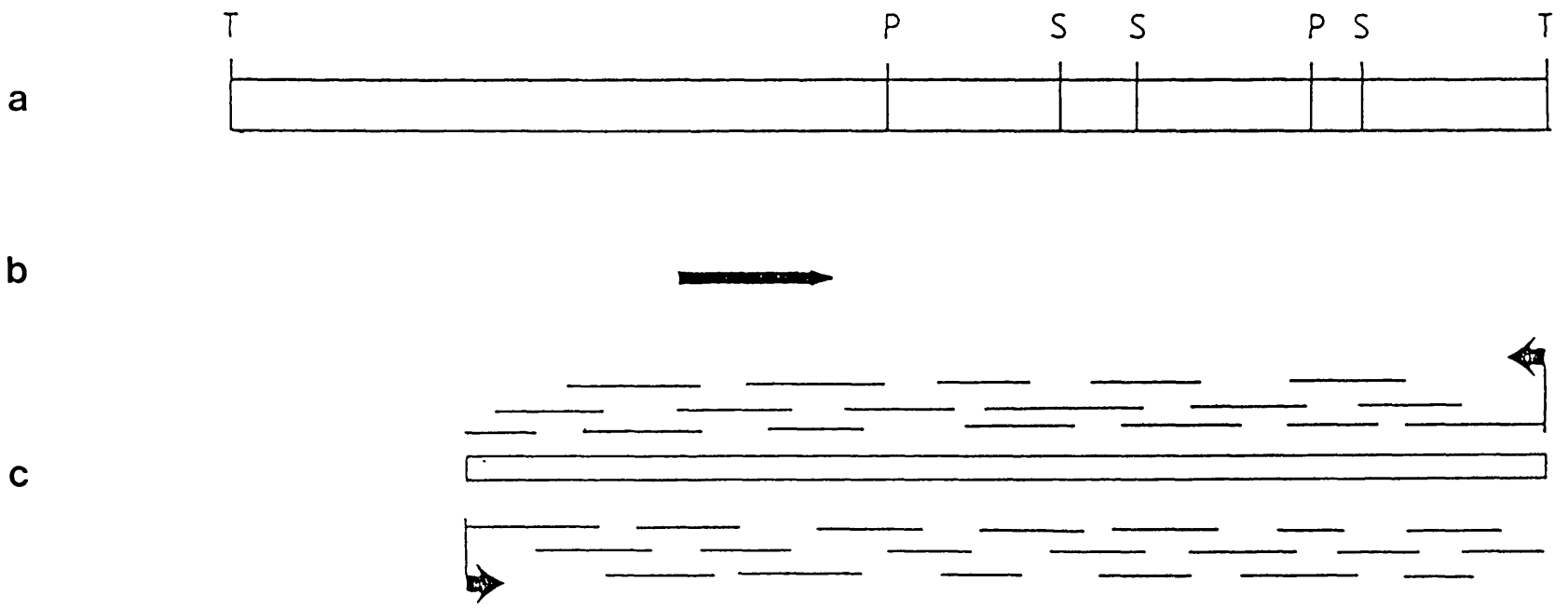
A number of computer programs were used for reading and manipulating the sequence.

Figure 6.1: Sequencing Strategy

Panel A shows the restriction map of the 2.8 kb *Sst I* fragment.

Panel B shows the region at which the gene begins (as shown by Figure 5.7)

Panel C shows the sequencing strategy: each line represents the region of sequence derived from that deletion, the arrows indicate which clones were relevant to each strand.


T
P
S
S
P
S
T
a
b
c

6.31 Reading the Sequence

The sequence was read from the autoradiographs using a Graf Bar sonic digitiser, directly onto floppy discs. Each template was read a number of times to ensure accuracy. The sequences of each individual clone were transferred to a VAX 8600 mainframe computer for further analysis.

6.32 Assembly of the DNA Sequences

All further sequence analysis utilised the programs of the University of Wisconsin Computer Genetics Group (Devereux *et al.*, 1984). The deletions in one orientation were placed in order of size and assembled by comparison with each of the adjacent clones, using the program "BESTFIT". Having determined the regions of overlap, the sequences were assembled, using the program "LINEUP" which displays the gel readings stacked above each other. The complementary strand was added to this file by similar procedures except that each gel reading was compared with its neighbours on both strands. On average, every residue was read three times in each direction because the degree of overlap was so great. The "LINEUP" program indicated points at which the sequences were not "unanimous" and these disagreements were resolved by a re-assessment of the original sequence or, in some cases, the preparation of different templates to cover that region.

6.4 Detection of Open Reading Frames (ORFs)

Several computer programs were used to determine the positions of the possible open reading frames (ORFs). Since the DNA was exceptionally G+C-rich, nearly all of the sequence was potentially an ORF. However, TESTCODE, CODONPREFERENCE, and FRAMES programs enabled quantitative assessment of the possible reading frames.

TESTCODE was used to examine the sequence for non-randomness with a periodicity of three. This particularly useful if the DNA has a biassed base composition.

Since *Streptomyces* has a similar G+C bias to that observed in *A. luteus*, a codon frequency table of *Streptomyces* genes might be used to identify regions of DNA exhibiting similar codon preferences. The CODONPREFERENCE program provided the means to accomplish this. The codon usage table was generated using the CODONFREQUENCY program in combination with the nucleotide sequences of *Streptomyces* genes deposited with the EMBL database.

FRAMES examines all six potential reading frames for "Start", "Stop" and rare codons (as determined from a codon frequency table: see above). This should show the position of the correct coding region, since, because of the G+C bias, the

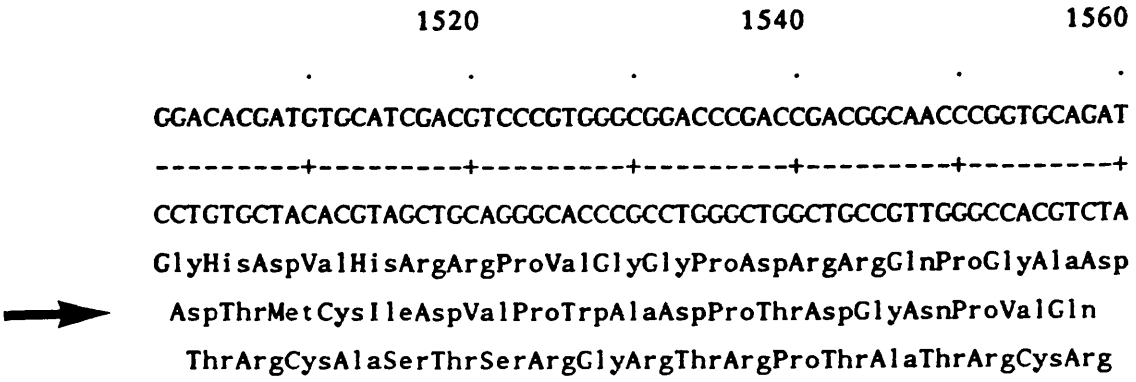


Figure 6.2: Finding Correct Reading Frames "By Eye"

The three forward reading frames translated to illustrate the abundance of arginine residues in the incorrect frames. The arrow indicates the correct reading frame.

incorrect frames would be full of "rare" codons.

An additional, qualitative test was an examination of the translations of the reading frames. Incorrect reading frames would exhibit high frequencies of arginine residues (codons CGA, CGC, CGG, CGT, AGA, AGG). Figure 6.2 shows an example from the glucanase gene sequence: the two incorrect frames have large numbers of arginines.

Figure 6.3 shows the nucleotide and putative amino acid sequences of the glucanase gene. The DNA is 2254 nucleotides in length, of which 1230 represents the putative coding region. The corresponding derived protein sequence begins at nucleotide 595 (ATG) and concludes at nucleotide 1828 (TGA). The putative protein contains 410 amino acids and has an expected molecular weight of 44,563 kD. There is also an alternative start site utilising the GTG at nucleotide 557 which would also be in the same frame and it is impossible to know which potential start codon is used *in vivo*.

6.5 Summary

The deletion clones produced in Chapter 5 proved useful for the nucleotide sequencing of the glucanase gene. The DNA was, as anticipated, extremely G+C-rich. This feature proved helpful in the identification and verification of potential ORFs. A likely coding region was identified and translated.

Figure 6.3: Sequence of a Glucanase Gene *Egl*1

The entire nucleotide sequence of the glucanase gene is presented along with the derived protein sequence of the putative coding region. Around 600 base pairs upstream and 400 downstream are also incorporated.

```
        10                  30                  50
         .         .         .         .         .         .
GTGGCCGTGCTGCGCACGACGCCGGTGGGACGAGGGGATCGCGCCAGGCCGCCGCGGGAG
---------+---------+---------+---------+---------+---------+
CACCGGCACGACGCGTGCTGCGGCCACCCTGCTCCCCTAGCGCGGTCCGGCGGCGCCCTC

        70                  90                  110
         .         .         .         .         .         .
CCACGCCCCGCACCGCCTGCTCGCGGTGTTCGACTTCTCGACAGTGGTTCGCGAGACGGA
---------+---------+---------+---------+---------+---------+
GGTGCGGGGCGTGGCGGACGAGCGCCACAAGCTGAAGAGCTGTCACCAAGCGCTCTGCCT

        130                 150                 170
         .         .         .         .         .         .
TCTCGCGGGTGCGGTTCATCACTCGTTCGGCGAGCTGGGCGCGACGTCGCGGGGTCGGCC
---------+---------+---------+---------+---------+---------+
AGAGCGCCCACGCCAAGTAGTGAGCAAGCCGCTCGACCCGCGCTGCAGCGCCCCAGCCGG

        190                 210                 230
         .         .         .         .         .         .
GCGGTCGGAGCAAAGGAGTCGTTCCAGCGGTACGCGCGACCGTCGTCCGAGGCGGGCCTG
---------+---------+---------+---------+---------+---------+
CGCCAGCCTCGTTTCCTCAGCAAGGTCGCCATGCGCGCTGGCAGCAGGCTCCGCCCGGAC

        250                 270                 290
         .         .         .         .         .         .
CCGACGACGTCGAGCTCCAGGTCCTGCTGCTCGCCGAGGGGGCGCAGACGACGCGGCCAT
---------+---------+---------+---------+---------+---------+
GGCTGCTGCAGCTCGAGGTCCAGGACGACGAGCGGCTCCCCCGCGTCTGCTGCGCCGGTA

        310                 330                 350
         .         .         .         .         .         .
CACGCAGGACCTCGACGTGCGCAGCGCCCGGGCGGCCGAGGCGATCCTCGACGGCGGTCC
---------+---------+---------+---------+---------+---------+
GTGCGTCCTGGAGCTGCACGCGTCGCGGGCCCGCCGGCTCCGCTAGGAGCTGCCGCCAGG

        370                 390                 410
         .         .         .         .         .         .
CTGCGACCGCTGACGTCCGGGGCGTTCCCGGCGGTCGGGCCGCCGCGTGCCCGGGTGCGG
---------+---------+---------+---------+---------+---------+
GACGCTGGCGACTGCAGGCCCCGCAAGGGCCGCCAGCCCGGCGGCGCACGGGCCCACGCC

        430                 450                 470
         .         .         .         .         .         .
GGGAAGCGAGATGGGAGCGATCCACGACACGCCGAAGCAGCGCTTGCTGCCGTGGTTCGC
---------+---------+---------+---------+---------+---------+
CCCTTCGCTCTACCCTCGCTAGGTGCTGTGCGGCTTCGTCGCGAACGACGGCACCAAGCG

        490                 510                 530
         .         .         .         .         .         .
GCGCCGTAGGGTCGCGGACGAGCCGAGGAACGGCCCCCGGCCCGTGCAGAGCTCGCGCCG
---------+---------+---------+---------+---------+---------+
CGCGGCATCCCAGCGCCTGCTCGGCTCCTTGCCGGGGGCCGGGCACGTCTCGAGCGCGGC
```

```
      550                 570                 590
       .   .   .   .   .   .   .   .   .   .   .   .
AGAGCTGTCCCGCACCGTGGCCGAGACGCAGGACCACCGCGGCACCTGGAGGAACATGAC
---------+---------+---------+---------+---------+---------+
TCTCGACAGGGCGTGGCACCGGCTCTGCGTCCTGGTGGCGCCGTGGACCTCCTTGTACTG
                                                      MetTh

      610                 630                 650
       .   .   .   .   .   .   .   .   .   .   .   .
CTCGCAGGTCACCGTCACCGCCCTCCTGACCCCGCCCCCGCAACCTGGCCGCCGACACCGC
---------+---------+---------+---------+---------+---------+
GAGCGTCCAGTGGCAGTGGCGGGAGGACTGGGGCGGGGCGTTGGACCGGCGGCTGTGGCG
rSerGlnValThrValThrAlaLeuLeuThrProProArgAsnLeuAlaAlaAspThrAl

      670                 690                 710
       .   .   .   .   .   .   .   .   .   .   .   .
GGGCGCCTCCGGGTCGCCGCTCGTCGCCGCGGCTCACGGCCCGCCCGCCGCGCTCGGCGT
---------+---------+---------+---------+---------+---------+
CCCGCGGAGGCCCAGCGGCGAGCAGCGGCGCCCAGTGCCGGGCGGGCGGCGCGAGCCGCA
aGlyAlaSerGlySerProLeuValAlaAlaAlaHisGlyProProAlaAlaLeuGlyVa

      730                 750                 770
       .   .   .   .   .   .   .   .   .   .   .   .
CACCGTGCCCGCGACGTCGGCCGCGCCGCACCGGCACCTCCTGTGGTCCGACGAGTTCGA
---------+---------+---------+---------+---------+---------+
GTGGCACGGGCGCTGCAGCCGGCGCGGCGTGGCCGTGGAGGACACCAGGCTGCTCAAGCT
lThrValProAlaThrSerAlaAlaProHisArgHisLeuLeuTrpSerAspGluPheAs

      790                 810                 830
       .   .   .   .   .   .   .   .   .   .   .   .
CGGCGCCGGCGGGCTCGGCGCCGAACCCCGCCGTCTGGAACCACGAGACCGGCGCGCACGG
---------+---------+---------+---------+---------+---------+
GCCGCGCCCGCCCGAGCCGCGGCTTGGGGCGGCAGACCTTGGTGCTCTGGCCGCGCGTGCC
pGlyAlaAlaGlySerAlaProAsnProAlaValTrpAsnHisGluThrGlyAlaHisGl

      850                 870                 890
       .   .   .   .   .   .   .   .   .   .   .   .
GTGGGGCAACGCGAGGCTGCAGAACTACACGGCCTCGCGCGCCAACTCCGCGCTCGACGG
---------+---------+---------+---------+---------+---------+
CACCCCGTTGCGCTCCGACGTCTTGATGTGCCGGAGCGCGCGGTTGAGGCGCGAGCTGCC
yTrpGlyAsnAlaArgLeuGlnAsnTyrThrAlaSerArgAlaAsnSerAlaLeuAspGl

      910                 930                 950
       .   .   .   .   .   .   .   .   .   .   .   .
CCAGGGCAACCTCGTCATCACGCCGCCGGAGGGCGACGGGTCGTACACGTCGGCGCGCAT
---------+---------+---------+---------+---------+---------+
GGTCCCGTTGGAGCAGTAGTGCGGCGGCCTCCCGCTGCCCAGCATGTGCAGCCGCGCGTA
yGlnGlyAsnLeuValIleThrProProGluGlyAspGlySerTyrThrSerAlaArgMe

      970                 990                1010
       .   .   .   .   .   .   .   .   .   .   .   .
GACGACCCAGGGGAAGTACCACCGCCAGTACGGGCGCATCGAGGCGCGCATCCAGATCCG
---------+---------+---------+---------+---------+---------+
CTGCTGGGTCCCCTTCATGGTGGCGGTCATGCCCGCGTAGCTCCGCGCGTAGGTCTAGGC
tThrThrGlnGlyLysTyrHisArgGlnTyrGlyArgIleGluAlaArgIleGlnIleAr
```

```
      1030                1050                1070
       .                   .                   .                   .                   .                   .
CGCGGTCAGGATCTGGCCGGCGTTCTGGATGCTCGGGCGGAGCTTCCCGGAGCGCTGGCC
---------+---------+---------+---------+---------+---------+
GCGCCAGTCCTAGACCGGCCGCAAGACCTACGAGCCCGCCTCGAAGGGCCTCGCGACCGG
gAlaValArgIleTrpProAlaPheTrpMetLeuGlyArgSerPheProGluArgTrpPr

      1090                1110                1130
       .                   .                   .                   .                   .                   .
GTCGTCGGCGGAGATCGACATCATGGAGACGTCGGTCGAGCCGACGGTGCACGGCACGTG
---------+---------+---------+---------+---------+---------+
CAGCAGCCGCCTCTAGCTGTAGTACCTCTGCAGCCAGCTCGGCTGCCACGTGCCGTGCAC
oSerSerAlaGluIleAspIleMetGluThrSerValGluProThrValHisGlyThrCy

      1150                1170                1190
       .                   .                   .                   .                   .                   .
CACGGCCGGGTACTCCGGCGGCTCCGGCATCACGGGCATGTACCAGCACCGCAGGTGGTC
---------+---------+---------+---------+---------+---------+
GTGCCGGCCCATGAGGCCGCCGAGGCCGTAGTGCCCGTACATGGTCGTGGCGTCCACCAG
sThrAlaGlyTyrSerGlyGlySerGlyIleThrGlyMetTyrGlnHisArgArgTrpSe

      1210                1230                1250
       .                   .                   .                   .                   .                   .
GTTCGCGGACACGTTCCACACGTTCGCGGTCGACTGGAACCGGGGGAATACGTGGTTCGT
---------+---------+---------+---------+---------+---------+
CAAGCGCCTGTGCAAGGTGTGCAAGCGCCAGCTGACCTTGGCCCCCTTATGCACCAAGCA
rPheAlaAspThrPheHisThrPheAlaValAspTrpAsnArgGlyAsnThrTrpPheVa

      1270                1290                1310
       .                   .                   .                   .                   .                   .
CGACGGCCAGCAGTTCCACGTCACCCCGAACGTCGCCAACGCCTGGTGTTCGACCAGCCG
---------+---------+---------+---------+---------+---------+
GCTGCCGGTCGTCAAGGTGCAGTGGGGCTTGCAGCGGTTGCGGACCACAAGCTGGTCGGC
lAspGlyGlnGlnPheHisValThrProAsnValAlaAsnAlaTrpCysSerThrSerAr

      1330                1350                1370
       .                   .                   .                   .                   .                   .
GTTCTTCCTCATCCTCAACGTCGCGGTCGGCGGGCAGTGGCCGGGCTACCCCGACGGCAC
---------+---------+---------+---------+---------+---------+
CAAGAAGGAGTAGGAGTTGCAGCGCCAGCCGCCCGTCACCGGCCCGATGGGGCTGCCGTG
gPhePheLeuIleLeuAsnValAlaValGlyGlyGlnTrpProGlyTyrProAspGlyTh

      1390                1410                1430
       .                   .                   .                   .                   .                   .
GACCCAGCTCCCGCAGCAGTTGAAGGTCGACTACGTGCGCGTCTACGACAACGGCTCGGG
---------+---------+---------+---------+---------+---------+
CTGGGTCGAGGGCGTCGTCAACTTCCAGCTGATGCACGCGCAGATGCTGTTGCCGAGCCC
rThrGlnLeuProGlnGlnLeuLysValAspTyrValArgValTyrAspAsnGlySerGl

      1450                1470                1490
       .                   .                   .                   .                   .                   .
CTCGTCGAACCCGGGGAACCCCGCGACCGGCCTGCCCACGGGGACCGGCGCGGTGGTGCC
---------+---------+---------+---------+---------+---------+
GAGCAGCTTGGGCCCCTTGGGGCGCTGGCCGGACGGGTGCCCCTGGCCGCGCCACCACGG
ySerSerAsnProGlyAsnProAlaThrGlyLeuProThrGlyThrGlyAlaValValPr
```

```
      1510                1530                1550
       .         .         .         .         .         .
GGACACGATGTGCATCGACGTCCCGTGGGCGGACCCGACCGACGGCAACCCGGTGCAGAT
---------+---------+---------+---------+---------+---------+
CCTGTGCTACACGTAGCTGCAGGGCACCCGCCTGGGCTGGCTGCCGTTGGGCCACGTCTA
oAspThrMetCysIleAspValProTrpAlaAspProThrAspGlyAsnProValGlnIl

      1570                1590                1610
       .         .         .         .         .         .
CGTCACGTGCAGCGGCACCGAGACCTGGACGCGTGGCTCGACGGACGTCCGGGTGAAGTG
---------+---------+---------+---------+---------+---------+
GCAGTGCACGTCGCCGTGGCTCTGGACCTGCGCACCGAGCTGCCTGCAGGCCCACTTCAC
eValThrCysSerGlyThrGluThrTrpThrArgGlySerThrAspValArgValLysCy

      1630                1650                1670
       .         .         .         .         .         .
CTCGACGTGCGCGACGCTCGACGCGCGGCGCGTGCAGGTGTGGACGTGCAACGGGACCGC
---------+---------+---------+---------+---------+---------+
GAGCTGCACGCGCTGCGAGCTGCGCGCCGCGCACGTCCACACCTGCACGTTGCCCTGGCG
sSerThrCysAlaThrLeuAspAlaArgArgValGlnValTrpThrCysAsnGlyThrAl

      1690                1710                1730
       .         .         .         .         .         .
GCGCGAGAAGTGGCGGTACGACGCCGGGAGCAAGGCGCTGCGCAACCCGATCCGGCTCTG
---------+---------+---------+---------+---------+---------+
CGCGCTCTTCACCGCCATGCTGCGGCCCTCGTTCCGCGACGCGTTGGGCTAGGCCGAGAC
aArgGluLysTrpArgTyrAspAlaGlySerLysAlaLeuArgAsnProIleArgLeuCy

      1750                1770                1790
       .         .         .         .         .         .
CTCGAGCCACGGCGCGCCCCTGCACGACGGCCAGCGGCTGCAGACCTGGACGTGCAACGG
---------+---------+---------+---------+---------+---------+
GAGCTCGGTGCCGCGCGGGGACGTGCTGCCGGTCGCCGACGTCTGGACCTGCACGTTGCC
sSerSerHisGlyAlaProLeuHisAspGlyGlnArgLeuGlnThrTrpThrCysAsnGl

      1810                1830                1850
       .         .         .         .         .         .
CAGACCGCCCAGCATGGACGCGCTGTGACACCGGCTGACCTGGTGACCGCGGCGACCGGC
---------+---------+---------+---------+---------+---------+
GTCTGGCGGGTCGTACCTGCGCGACACTGTGGCCGACTGGACCACTGGCGCCGCTGGCCG
yArgProProSerMetAspAlaLeuEnd

      1870                1890                1910
       .         .         .         .         .         .
CGGGGCGGGCGCCCGGGGTGCGTCACCGCGGGTGGCTGTCGGGCGGGTCGACGCGCGGGC
---------+---------+---------+---------+---------+---------+
GCCCCGCCCGCGGGCCCCACGCAGTGGCGCCCACCGACAGCCCGCCCAGCTGCGCGCCCG

      1930                1950                1970
       .         .         .         .         .         .
CCGGCTCGCCCTGCTCGCCCGGTCCGGTGGCCGACGCGGCTGCGCGGGCCGAGCGGCTCG
---------+---------+---------+---------+---------+---------+
GGCCGAGCGGGACGAGCGGGCCAGGCCACCGGCTGCGCCGACGCGCCCGGCTCGCCGAGC
```

```
      1990                2010                2030
       .                   .                   .                   .                   .                   .
ACCACGGCAGCACGATCACCGACACACGCCGCGCCGACGAGCGCCGCCCCCGACCTGGACG
---------+---------+---------+---------+---------+---------+
TGGTGCCGTCGTGCTAGTGGCTGTGTGCGGCGCGGCTGCTCGCGGCGGGGCTGGACCTGC

      2050                2070                2090
       .                   .                   .                   .                   .                   .
TCATGGGACCTGCTCGACCGCGTTCTCCGTGACGCGACGAGACGGCACCGGTCGCCGTAG
---------+---------+---------+---------+---------+---------+
AGTACCCTGGACGAGCTGGCGCAAGAGGCACTGCGCTGCTCTGCCGTGGCCAGCGGCATC

      2110                2130                2150
       .                   .                   .                   .                   .                   .
ACGCGGTCTGGACACGCTCGACCGACGGCAGCTGCGCGCCGGAACCAGCAGGAGCACCGG
---------+---------+---------+---------+---------+---------+
TGCGCCAGACCTGTGCGAGCTGGCTGCCGTCGACGCGCGGCCTTGGTCGTCCTCGTGGCC

      2170                2190                2210
       .                   .                   .                   .                   .                   .
GCCGCCCCCGCACGACCACATGAGCGCGAAGAACGTCACCGGCACAGCGGGGACGCGACCC
---------+---------+---------+---------+---------+---------+
CGGCGGGGCGTGCTGGTGTACTCGCGCTTCTTGCAGTGGCCGTGTCGCCCCTGCGCTGGG

      2230                2250
       .                   .                   .
GATGGAGTCGATGTCCAGGCCCATGCCGGAGCTC
---------+---------+---------+----
CTACCTCAGCTACAGGTCCGGGTACGGCCTCGAG
```

CHAPTER 7: PROPERTIES OF THE CLONED GLUCANASE

7.1 Enzymic Properties of the Cloned β-Glucanase Expressed in *E. coli*

Upon prolonged (3 d) growth of *E. coli* carrying glucanase-encoding plasmids, β-glucanase accumulated in the culture supernatant. This was probably due to cell death and lysis since β-galactosidase (a cytoplasmic protein) also appeared in the supernatant at the same time. The glucanase levels observed were signifcantly higher than those found in logarithmically growing cultures. Because the enzyme accumulated at elevated levels and appeared to be essentially the same protein as that manufactured in the exponentially-growing cultures (as determined on activity stained gels), the 3 d cultures were used as the source of material for the analysis of the glucanase activity.

7.11 The Optimal Reaction Conditions of the β-Glucanase

The pH and temperature optima of the glucanase produced in *E. coli* were determined by measuring reducing sugar release from AIG at pHs between 4 and 10, and temperatures between 4 and 65°C. Lytic activity was not measured since levels were relatively low. Figure 7.1 shows the pH and temperature optima of the the product of the glucanase gene. The spectra for the lytic activity of *A. luteus* supernatants are also shown. The pH and temperature optima of the *egl1* enzyme are considerably sharper than those of the enzyme mixture

All subsequent assays on the glucanase from *E. coli* were carried out at or near the pH and temperature optima, at pH 7.5 and 37°C. The slightly lower temperature was chosen for convenience and because the activity under these conditions was only marginally reduced.

7.12 The Substrate Specificity of the Glucanase Activity

The β-glucanase was prepared from *E. coli* bearing a range of subclones and assayed for yeast lytic activity and for the release of reducing sugars from yeast glucan (AIG), yeast cells, barley β-glucan, laminarin, lichenan, pustulan and CMC (carboxymethyl cellulose). Table 7.1 summarises these results.

The enzyme produced in *E. coli* was found to be active only against β-1,3 bonds. This was illustrated by the lack of activity against carboxymethyl cellulose (β-1,4-glucan) and pustulan (β-1,6-glucan). It had maximal activity against pure β-1,3-glucans such as laminarin and yeast glucan. The lower activities against barley glucan or lichenan might be explained by the fact that these glucans contain fewer β-1,3-bonds and hence the substrate may have been limiting. However, the substrate was at high concentration (1%) and when the reactions were carried out for shorter times, the activity was still greater against pure β-1,3-glucans than against mixed

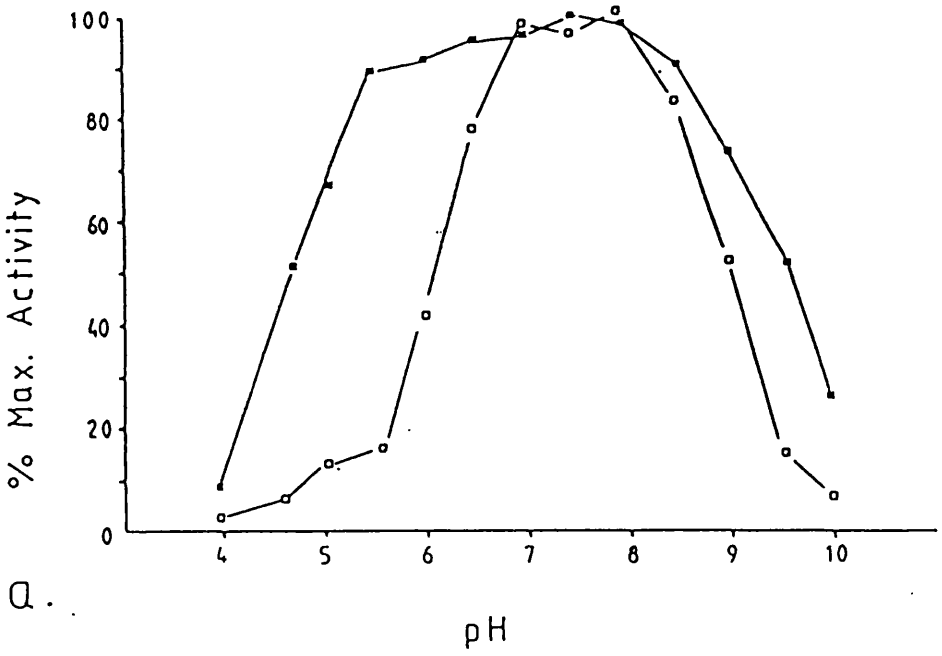
Figure 7.1: pH and Temperature Optima of the Cloned Glucanase

Panel a shows the variation of glucanase activity with pH

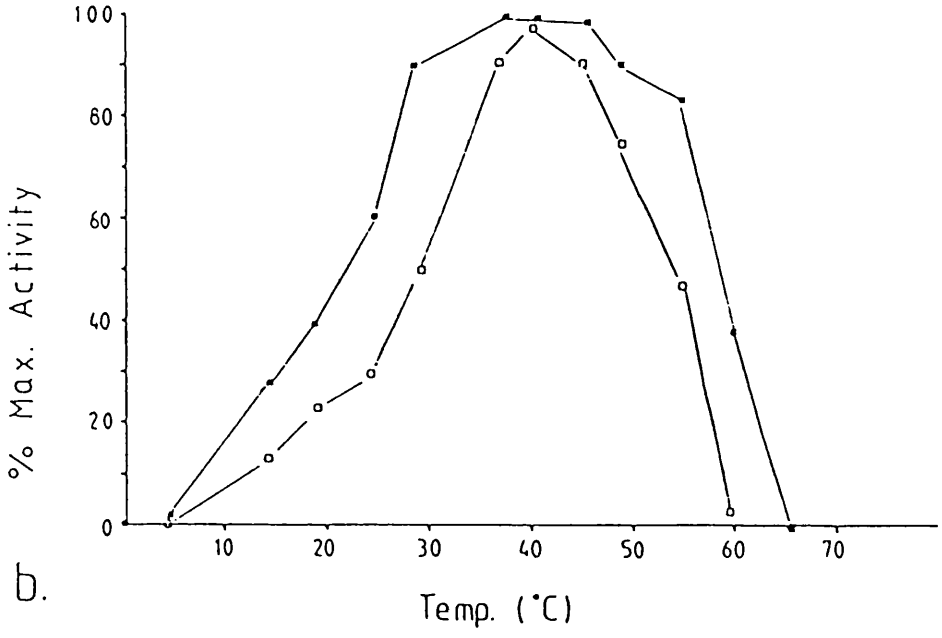
Panel b shows the variation of glucanase activity with temperature

■ = mixture of *A. luteus* enzymes

□ = *E. coli*-produced glucanase.


% Max. Activity
100
80
60
40
20
0
4
5
6
7
8
9
10
pH

a.


% Max. Activity
100
80
60
40
20
0
10
20
30
40
50
60
70
Temp. (°C)

b.

Table 7.1: Substrate Specificty of the Cloned Glucanase

	pDMβ40	pDMβ100	pDMβ101	pDMβ100Δ36
CMC	-	-	-	3
Barley Glucan	17	41	10	160
Laminarin	6	49	12	270
Yeast Glucan	20	50	5	300
Pustulan	-	-	-	-
Yeast lytic	11	15	2	30

linkage glucans. This observation, coupled with the fact that the enzyme was more active against yeast cells and AIG than against laminarin suggested that there was significant substrate preference for the higher molecular weight, less soluble β-1,3-glucans.

It is interesting to note that much reducing activity was released from viable yeast cells when incubated with the enzyme and some cell lysis was observed (equivalent to approximately 30 U/ml).

7.13 The Products of the Reactions Catalysed by the β-Glucanase

Enzyme (20 μl) was incubated with yeast glucan or laminarin (final concentration 1 mg/ml) for 18 h. The digestion products were separated by ascending mode paper chromatography and reducing sugars detected by staining with silver nitrate. Marker oligosaccharides were produced by the partial hydrolysis of laminarin using dilutions of Zymolyase. Glucose, maltose and maltotriose were also used as standard small oligo-saccharides. For comparison, the same reaction was performed using *A. luteus* supernatant instead of the *E. coli* glucanase. Figure 7.2 shows a tracing of the sugar chromatograms immediately after staining (the stain faded within weeks).

The predominant products of an overnight reaction were relatively small oligosaccharides (mainly pentamers and trimers) or glucose. The hydrolysis of laminarin was less efficient and apparently released a range of oligo-saccharides from glucose to laminaripentaose (and higher). Many of the endo-glucanases which have beeen isolated as the key components of yeast lytic systems have been shown to liberate laminaripentaose upon incubation with yeast glucan and it also seems that the *egl*1 gene product releases similar oligo-saccharides (Kitamura *et al* 1974; Scott and Schekman, 1980).

7.2 Cellular Localisation of the β-Glucanase

Many proteins are secreted into the media by gram-positive bacteria. Secretion across the bacterial cell wall is usually accompanied by a proteolytic cleavage event which removes a peptide (the signal sequence) from the N-terminal end of the protein (Pugsley, 1988). The situation in gram-negative bacteria is more complex due to the presence of an additional barrier- the outer membrane of the cell wall. Complete externalisation of proteins from these organisms is correspondingly less widespread and specialised mechanisms have been developed to carry out this function (Pugsley, 1988). However, the secretion of proteins through the inner membrane of the Gram-negative bacterial cell wall is highly analogous to the gram-positive system. Indeed when the genes for many secreted proteins of gram-positive bacteria are expressed in *E. coli*, the proteins are localised in the periplasm (Pugsley, 1988) and this event is accompanied by a proteolytic cleavage. It might therefore be expected

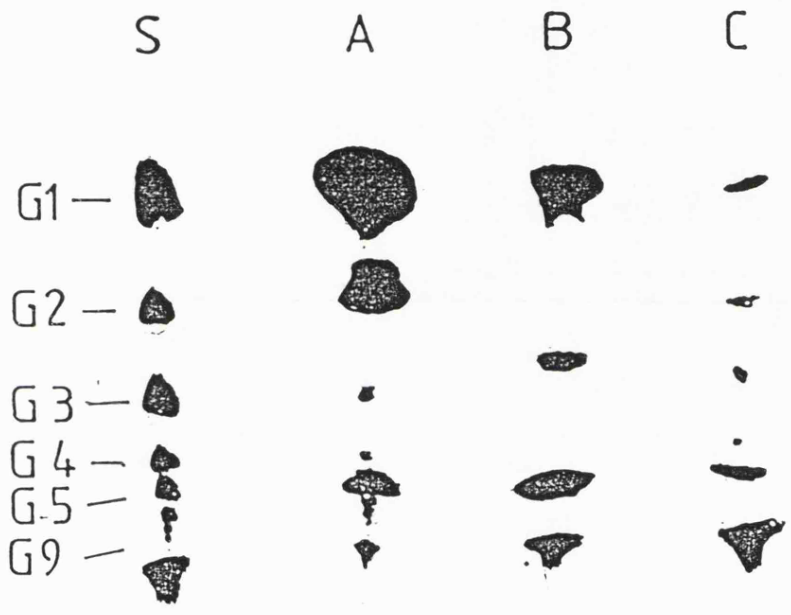


Figure 7.2: Products of Glucanase Activity

S = Oligo-saccharide markers

A = Products of *A. luteus* supernatant digestion of AIG

B = Products of *E. coli* (pDMβ100Δ36) extract digestion of AIG

C = Products of *E. coli* (pDMβ100Δ36) extract digestion of Laminarin

Table 7.2: Localisation of the Glucanase in *E. coli*.

Figures express the percentage of each activity to be found in each compartment for every culture.

Fraction	Supernatant		Periplasm		Cytoplasm	
	log	stat	log	stat	log	stat
Culture						
No plasmid						
β-galactosidase	-	2	5	15	95	83
β-lactamase	-	-	-	-	-	-
β-glucanase	-	-	-	-	-	-
pUC18						
β-galactosidase	-	7	7	4	93	89
β-lactamase	11	15	85	80	4	5
β-glucanase	-	-	-	-	-	-
pDMβ40						
β-galactosidase	-	35	10	10	90	55
β-lactamase	5	40	87	55	8	5
β-glucanase	-	39	65	30	35	31
pDMβ100Δ36						
β-galactosidase	5	40	13	23	82	37
β-lactamase	12	37	80	54	8	9
β-glucanase	13	45	60	45	27	10

that the product of this gene may also be secreted to the periplasm of *E. coli*. To determine whether this was the case, cultures (log phase or stationary phase) were fractionated into supernatants, periplasms, and cell contents and the localisation of the β-glucanase determined.

Certain plasmids (in particular, pDMβ40) could only be introduced into *E. coli* host strains which carry the *lacI*^{q} allele. This indicates that over-expression of recombinant gene(s) is deleterious to the *E. coli* cells harbouring such a plasmid. All plasmids were therefore propagated in the *lacI*^{q} host D1210; this has the added advantage of being *Lac*$^{+}$ wild type, allowing β-galactosidase to be used as a 'marker" cytoplasmic enzyme. Overnight cultures were used to inoculate L-broth which contained antibiotics as appropriate. The control strains were D1210 containing pUC18 and D1210 alone. Following growth at 37°C for 2-3 h (exponential phase) or 24 h (stationary phase), cells were harvested and fractionated into supernatant, periplasm and cytoplasm as described. Care was taken to harvest equal numbers of cells from each culture. The β-glucanase activity found in each fraction of each culture was determined by the clearing zone assay. The fractions were also assayed for β-galactosidase and β-lactamase activities as marker proteins for the cytoplasm and periplasm respectively. The presence of significant quantites of β-galactosidase in the culture supernatant was considered to be a sign of cell death and lysis, while the presence of β-lactamase alone in the supernatant indicated leakage of the periplasm. The proportion of each activity found in each fraction was calculated and the results are presented in Table 7.2.

There is clearly a significant difference between the log phase and stationary phase cultures. However, the increased presence of β-galactosidase in the supernatant of the older cultures indicates that cell lysis (or at least leakage) has occurred. With the log phase cells, however, the marker proteins are localised essentially the same as in the control cultures. The β-glucanase activity is found mainly in the periplasm. This does not appear to be dependent on the amount of glucanase produced because the percentage in each fraction is the same for cultures carrying pDMβ40 as it is for those harbouring pDMβ100Δ36.

7.3 Identification of the Protein Product of the Cloned Gene

In order to confirm that the cloned DNA encoded a discrete protein species and to relate this to the proteins observed in the yeast lytic supernatants of *A. luteus* it was decided to visualise the gene product.

SDS-PAGE analysis of the proteins of *E. coli* cells with or without the cloned DNA might reveal a difference upon staining with coomassie blue or silver. However, this would only provide indirect evidence that the cloned DNA was encoding a novel protein; the product of the cloned DNA could be causing the production of novel

host proteins. Similarly, there would be no indication of the activity carried by this protein species. A further disadvantage of this method of analysis is that there are a great many other proteins present in the cell and any differences may be obscured by these. For these reasons, other methods were used to visualise the products of the cloned DNA.

Plasmids generated by both deletion analysis and transposon mutagenesis were used. These techniques had yielded highly expressing clones in addition to derivatives which were glucanase negative. With these it was possible to analyse the proteins encoded by the various plasmids using Activity gels, *E. coli* based minicells, and a *Streptomyces lividans*–based *in vitro* coupled transcription/translation system.

7.31 *In Situ* Renaturing Activity Gels.

As described earlier, many proteins can be detected by their activity after renaturing *in situ* following SDS–PAGE. The detection of the enzymic activity was carried out at the optimal pH and temperature for the enzyme in question (as determined above).

An advantage of this type of gel is that only "active" proteins are visualised and this means that there is no masking effect of other proteins in the mixture. Furthermore, the activity encoded by the cloned DNA can be compared with the activities of Zymolyase and the enzymes produced by *A. luteus* under "inducing" conditions.

As described above (7.1), after 72 h growth in rich selective media, the greater part (70%) of the β–glucanase activity was found in the culture fluid. In the pUC–based subclones and the highly expressed deletion derivatives, the enzyme levels were high and samples of supernatant could be loaded directly onto activity gels without prior concentration. Cells of *E. coli* D1210 were transformed with pUC19, pDMβ40, pDMβ100, pDMβ101, pDMβ100Δ10, pDMβ100Δ36 and pDMβ100Δ53 (this clone has greater than half the gene coding sequence deleted). The plasmid–bearing strains were grown in 5 ml of LB + Amp for 72 h. The cells were removed by centrifugation and aliquots (30 μl) of the supernatants were prepared for electrophoresis by the addition of 3.5 μl gel loading buffer. After electrophoresis, washing, and overnight incubation, regions of β–glucanase activity were visualised using congo red to stain undegraded barley glucan.

Figure 7.3 shows a typical example of an activity gel. It can be clearly seen that cultures of *E. coli* carrying the plasmids pDMβ100, pDMβ100Δ10, and pDMβ100Δ36 produced a β–glucanase of around 44 kD which corresponded exactly to a β–glucanase protein secreted by *A. luteus* and a similar activity found in a solution of Zymolyase. However, no such activities were detected in cultures of *E. coli* carrying the plasmids pDMβ40, pDMβ101 and pDMβ100Δ53. In the first two, the

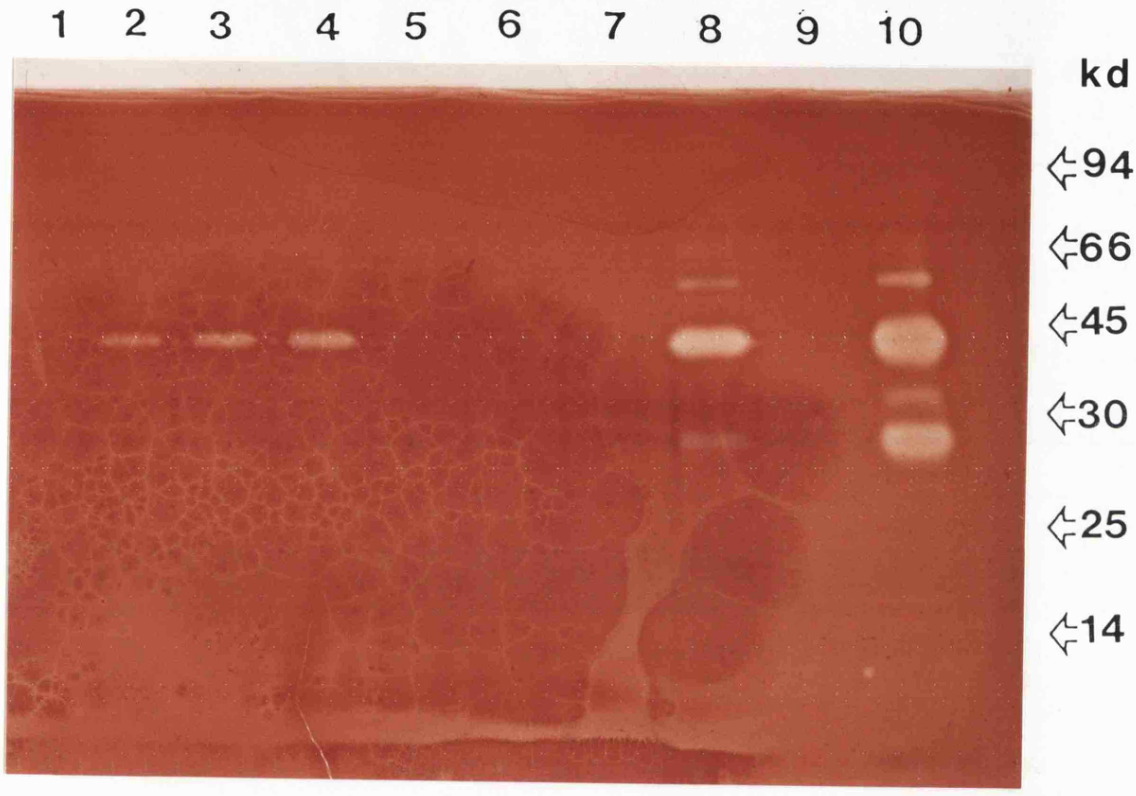


Figure 7.3: Activity Stained Gel of *E. coli*-Expressed Glucanase

1 = pUC19; 2 = pDMβ100; 3 = pDMβ100Δ10; 4 = pDMβ100Δ36; 5 = pDMβ100Δ53; 6 =p DMβ101; 7 = pDMβ40; 8 = AYR-grown *A. luteus*; 9 = Glucose-grown *A. luteus*; 10 = Zymolyase.

amount of enzyme produced was very low and may therefore have been undetectable in these cultures. In plasmid pDMβ100Δ53, the gene was significantly deleted and was therefore inactive.

These results confirmed that the cloned DNA encoded a β-glucanase. This β-glucanase co-migrated with the major β-glucanase of lytic supernatants of *A. luteus* (and Zymolyase).

7.32 Minicell Analysis of Plasmid Encoded Proteins

A method for radiolabelling plasmid-encoded proteins would formally confirm that the observed glucanase activity was not due to a plasmid specific activation of a host glucanase gene. There are two systems for the *in vivo* labeling of plasmid encoded products: "minicells" and "maxicells". Attempts were made at maxicell analysis but these were unsuccessful.

Minicells are generated by *E. coli* strains with defective division functions (*minA, minB*) such that at a certain frequency small portions of cytoplasm ("minicells") containing plasmids but no chromosome are produced. These can be purified away from the larger cells containing chromosomal DNA on sucrose density gradients. They represent "cells" which contain no chromosomal DNA but can still express plasmid encoded genes.

Following transformation of the minicell strain DS410 with pUC19, pDMβ62 and pDMβ62::γδ20 (Figure 7.4), minicells were prepared, incubated and labelled as described earlier. The protein products were electrophoresed on a 12% SDS-PAGE gel and, after fluorography and drying, were exposed to X-ray film. Figure 7.5 shows the autoradiograph from one such experiment. A great many protein species were produced from pDMβ62 and pDMβ62::γδ20, while few were observed in the pUC19 control. It seemed, therefore that the problem was not with the preparation of the minicells but lay in the nature of the cloned DNA. Indeed, control experiments indicated that the number of contaminating, viable whole cells was less than 1 in 10^6 cells/ml. However, a protein of around 44 kD, visible in the pDMβ62 lane but absent in the pDMβ62::γδ20 lane was noted (arrow). This corresponds with the data presented above (Figure 7.3), but the large number of other products observed reduced the clarity of this result. This failure to generate a significant quantity of full length recombinant protein was perhaps due to the premature termination of translation because of the presence of secondary structures in the RNA message. These might be relatively stable due to the high G+C content of the DNA.

7.33 Analysis of the Plasmid Encoded Proteins by the *In Vitro* Coupled Transcription/Translation System From *Streptomyces*

In view of the inconclusive nature of the minicell result, it appeared that there

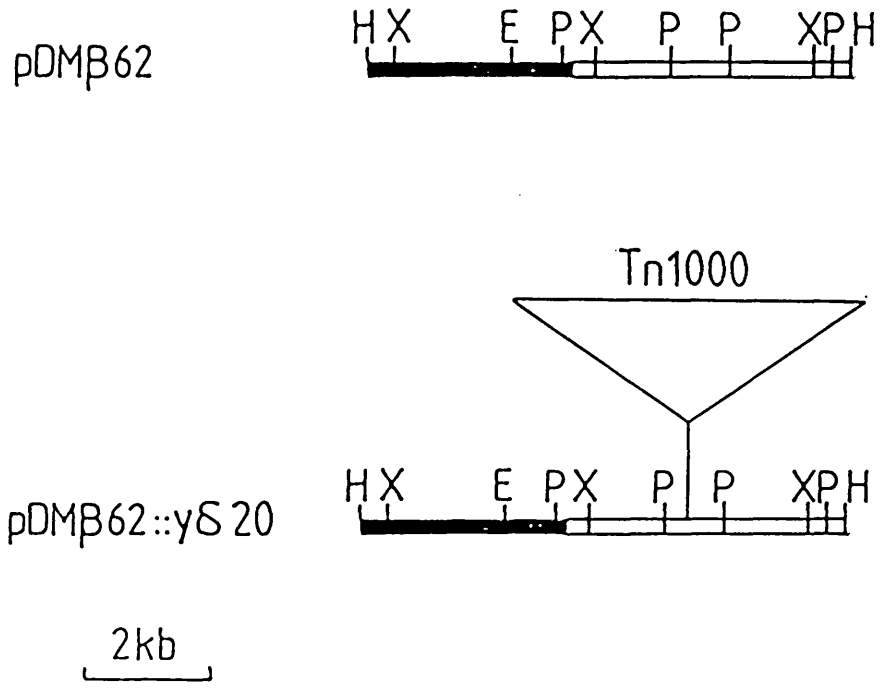


Figure 7.4: Plasmids Analysed by Minicells

pDMβ62 was expected to code for an active glucanase.

pDMβ62::$\gamma\delta$20 was expected to have no glucanase gene since it mapped directly in the coding region of the gene as determined by nucleotide sequencing

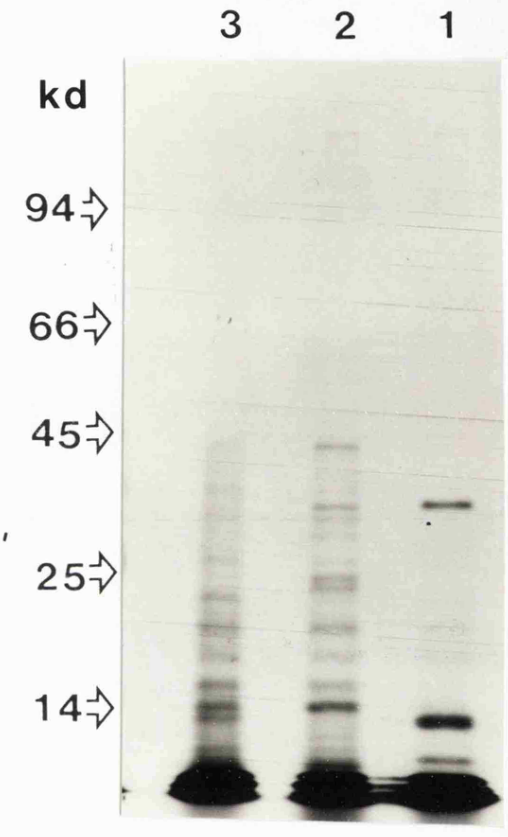


Figure 7.5: Minicell Analysis of Plasmids pDMβ62 and pDMβ62::$\gamma\delta$20

1 = pUC19; 2 = pDMβ62; 3 = pDMβ62::$\gamma\delta$20

was a problem with the expression of this cloned gene in *E. coli*. One possible alternative was the use of systems based on organisms with naturally G+C-rich DNA such as actinomycetes. *Streptomyces* are the best studied and an *in vitro* coupled transcription/translation system has been developed (Thompson *et al.*, 1984) by producing a DNA-free whole cell extract from *S. lividans*. This transcription/translation system is directly analogous to the system of Zubay (1973) except that the extract is derived from *Streptomyces lividans* and not *E. coli*. Proteins manufactured by these *in vitro* systems do not undergo post-translational processing, e.g. removal of signal sequences. Therefore many proteins are observed as larger precursors rather than "mature" sized polypeptides. The *Streptomyces* extract was considered more likely to allow identification of the plasmid encoded proteins than the minicell system which seemed to be subject to some problems. A further advantage of this system is that *E. coli* transcription and translation signals are recognised as readily as host signals (Thompson *et al.*, 1984).

DNA of plasmids pDMβ40, pDMβ100, pDMβ101, pDMβ100Δ36, pDMβ100Δ53 were analysed by this system, with pKGW and pUC19 as controls for the vector encoded polypeptides. Figure 7.6 shows an autoradiograph of a 12% SDS-PAGE gel of the proteins produced from these plasmids labelled in the *Streptomyces lividans* system. As can be seen, proteins were produced from each of the plasmids; additional products were observed in the lanes carrying recombinant plasmids. A protein of approximately 45 kD was readily detected amongst the products of plasmids pDMβ100, pDMβ100Δ36 and pDMβ101, and upon long exposure, of pDMβ40. Neither the vector controls nor pDMβ100Δ53 encoded this protein species demonstrating that the large plasmids which encode an active glucanase *in vivo*, also encode a protein of similar size when analysed *in vitro*. Clearly, though, expression from the *A. luteus* promoter was not so strong as that from the vector-borne signals. In addition, the protein species produced from pDMβ100Δ36 was slightly larger than those derived from the other plasmids, perhaps indicating a translational fusion of the coding region of *egl*1 to a small portion of vector DNA, presumably at the 5'-end of the gene.

7.4 Summary

The enzyme encoded by the cloned DNA was shown to be a β-1,3-glucanase with high specificity for long chained yeast glucan (as opposed to the shorter chains of laminarin). It could also hydrolyse the β-1,3-bonds of mixed linkage glucan.

The pH and temperature optima for the hydrolysis of yeast glucan were calculated as 7.5 and 39°C respectively.

The products of this hydrolysis were observed to be oligosaccharides equivalent to laminaripentaose, and glucose. These were assumed to be the end products of hydrolysis and no information was obtained concerning the intermediate saccharides.

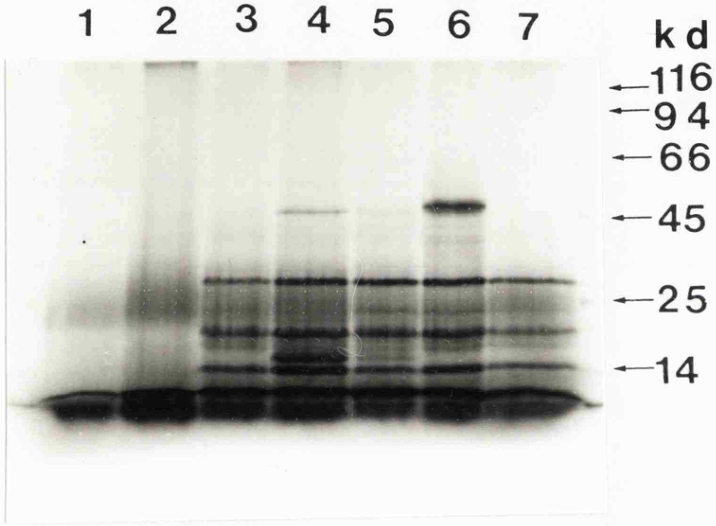


Figure 7.6: *In Vitro* Gene Expression of the Glucanase Clones

1 = pKGW; 2 = pDMβ40; 3 = pUC19; 4 = pDMβ100; 5 = pDMβ101;
6 = pDMβ100Δ36; 7 = pDMβ100Δ53.

In logarithmically growing *E. coli* carrying glucanase clones, a significant proportion of the activity was localised in the periplasm of the cells, while in stationary phase cultures, the glucanase accumulated in the supernatant. This was, however accompanied by a similar build up of β-galactosidase, indicating that cell death and lysis was responsible for this "secretion".

The transposon and deletion derived clones were used to analyse the protein encoded by the cloned DNA. This was visualised as a β-glucanase which co-migrated with the major β-glucanase of the host organism. That this protein was plasmid encoded was strongly indicated by experiments using *E. coli* minicells and (more convincingly) a *Streptomyces lividans in vitro* transcription/translation system. The sizes of the proteins thus produced were in close agreement with those predicted from the nucleotide sequence.

CHAPTER 8: DISCUSSION.

8.1 The Enzymes of the Yeast Lytic System

The enzymes secreted by *A. luteus* were found to be dependent on the carbon source in which the bacteria were grown. The presence of glucose inhibited enzyme production, while cultures grown on yeast based carbon sources (such as alkali insoluble yeast glucan) accumulated large amounts of enzyme. Intermediate results were obtained when barley glucan and laminarin were used as the carbon sources. These results are entirely consistent with all the previous data concerning yeast lytic enzymes secreted by *Arthrobacter* and *Oerskovia* species (e.g. Scott and Schekman, 1980; Vrsanska *et al.*, 1977a; Kitamura *et al.*, 1972). In addition, when the pH and temperature optima of the mixture of enzymes were determined, they also were similar to those identified by those authors. To some degree the pH optimum of around 7.5 for these enzymes is surprising, since yeast acidifies its growth medium to a pH of 6.0 or less and it might therefore be expected that the lytic enzymes would have pH optima in this range. However, subtle pH changes might affect the conformation of the protective protein–mannan layer of the cell wall, thus enabling the lytic glucanases to penetrate to the structural glucan layer and falsely elevating the observed yeast lytic activity.

Utilising gels which could be stained to detect glucanase activity after SDS–PAGE, zymolyase and a supernatant of *A.luteus* grown in "inducing" medium were compared. Both enzyme mixtures exhibited similar profiles of glucanases, although the relative abundances of the various components were found to vary slightly. This may be due to the fact that the enzymes of Zymolyase have undergone a simple affinity purification step and this may have distorted the observed quantities of the various components of the lytic mixture.

8.2 The Cloning of Genes from *A. luteus*

In Chapter 4, the cloning of genes from *A. luteus* was described. It proved relatively straightforward to clone DNA fragments which encoded enzymes capable of hydrolysing starch. These clones could be identified by overnight growth on appropriate detection plates. However, the detection of β–glucanase producing colonies was significantly more difficult, perhaps due to poor expression of the foreign DNA in *E. coli* and inefficient "secretion" of the protein to the periplasm (evidence for both of these factors was described in Chapter 7).

All three of the β–glucanase encoding clones carried approximately the same regions of *A. luteus* DNA. However, it was expected that other glucanase genes would also be identified. A number of reasons for the failure to clone any other glucanase genes can be suggested.

The gene library may not have been representative, leading to repeated isolation of certain regions of DNA. This could be due to non randomness in the insert DNA ligated to the cloning vector. This, however, seems unlikely since extreme steps including partial digestion and sucrose fractionation were utilised to optimise the randomness of the library. Other reasons for the library not being representative might be:

(a) the *Sau*3A I sites were not distributed approximately at random throughout the *A. luteus* genome (unlikely but difficult to test);

(b) certain inserts may be more deleterious (or advantageous) than others leading to under- (or over-) representation of particular fragments. These effects might be mediated by the products of expression of these genes (unlikely since gene expression levels were not high), or be factors inherent in the DNA itself.

In reality, though, none of these factors seem likely to have caused the library to be sufficiently unrepresentative. Other possibilities to explain the repeated isolation of the same clones do arise. Firstly, it may be that the DNA fragments encoding any other glucanases were so badly expressed in *E. coli* that detection using the methods described was impossible, and secondly, it might be that the clones identified carried a number of glucanase genes and that failure to identify any genes in addition to the one which was sequenced, was an artifact of the subcloning strategy.

Although levels of gene expression of other glucanase genes might have been low, steps were taken to overcome this. The colonies on the screening plates were incubated for 48 h and transcription from the *lac*UV5 promoter of the vector pKGW was maximised using high levels of IPTG. Furthermore, a large number (23) of putative clones were identified by the screening procedure and only the three clones described in detail were glucanase positive upon more sensitive retesting, utilising the triton lysis protocol described earlier.

8.21 How Many Glucanase Genes on the Primary Clones?

Since the region covered by the three clones was 15-16 kb in length, and only 1.3 kb was required to encode the *Egl*1 gene, there was clearly sufficient coding capacity for a number of other genes. Indeed, each primary clone carried at least 5 kb upstream of the *Egl*1 gene, between the promoter of the cloning vector and the 5'-end of the gene, whereas it would be anticipated that the easiest genes to identify (those which expressed glucanase at the highest levels) would be nearer to the promoter. Furthermore, pDMβ20 was found to be orientated such that expression of the *Egl*1 gene ran counter to that driven from the vector-borne promoter. This suggested two possibilities:

(a) the expression of *Egl*1 was driven by its own promoter and was sufficient to overcome the vector driven transcription;

(b) there was an additional glucanase gene running in the opposite direction to *Egl*1 and being relatively efficiently expressed from the *lac*UV5 promoter of pKGW, which would be less than 1 kb away from the putative start of such a gene. Although this is a theoretical possibility, it seems unlikely since the subcloning of *Egl*1 on a *Hind* III fragment did not lead to other glucanase clones being identified. The opposite orientation of that fragment in pUC19 should express the gene postulated above. Likewise, the opposite orientation of the *Kpn* I-*Bgl* II fragment of pDMβ40 would be expected to express that same gene and clearly does not. It can therefore be concluded that no glucanase gene, running in the opposite direction to *Egl*1 is to be found downstream from that gene.

The region defined by the *Bgl* II and the nearest *BamH* I sites of pDMβ40 is the only possible overlap between the clones isolated in this study and that described previously by Doi and Doi (1986). Figure 8.1 shows the possible comparison between the two regions. It appears that the *BamH* I-*Bgl* II fragment alone is insufficient to encode this gene, although it would be expressed in the same direction as *Egl*1. It is thus a possibility that a gene or genes are present at the 3'-end of *Egl*1 and could be expressed in the same direction as that gene.

Further circumstantial evidence for additional glucanase genes on these clones could be provided by the results of $\gamma\delta$ mutagenesis of large subclones of this region of DNA. A number of colonies expressing intermediate levels of glucanase activity were isolated. These might represent cases where a single glucanase gene was inactivated leaving at least one (presumably proximal to the pUC *lac* promoter) intact. However, these might also be as merely a normal variation in the levels of gene expression or by the possibiility that changes in DNA supercoiling, brought about by insertion of the transposon might affect the efficiency of gene expression. An extreme example of this phenomenon could explain the highly surprising finding that a large number of insertions were clustered into a small (100-200 bp) region of DNA which map within the pUC plasmid, but upstream of the *lac* promoter and which nevertheless appear to inactivate all expression of the glucanase gene. These inactivating insertions did not appear to be due to rearrangement of the plasmid DNA and another mechanism for the inactivation must be invoked.

In addition, Southern analysis suggested that more the one sequence homologous to the probe was to be found within the primary glucanase clones.

One other suggestive observation was the fact that extracts of *E. coli* cultures carrying pDMβ40 and pDMβ42 appeared to have higher levels of lytic activity relative to their glucanase activity, when compared to the levels of activity noted for the extracts derived from pDMβ100. This might imply that a low level of several different glucanase enzymes was proving more efficient in the lytic assay than would be expected from the glucanase activity alone. Alternatively, low levels of yeast cell lysis

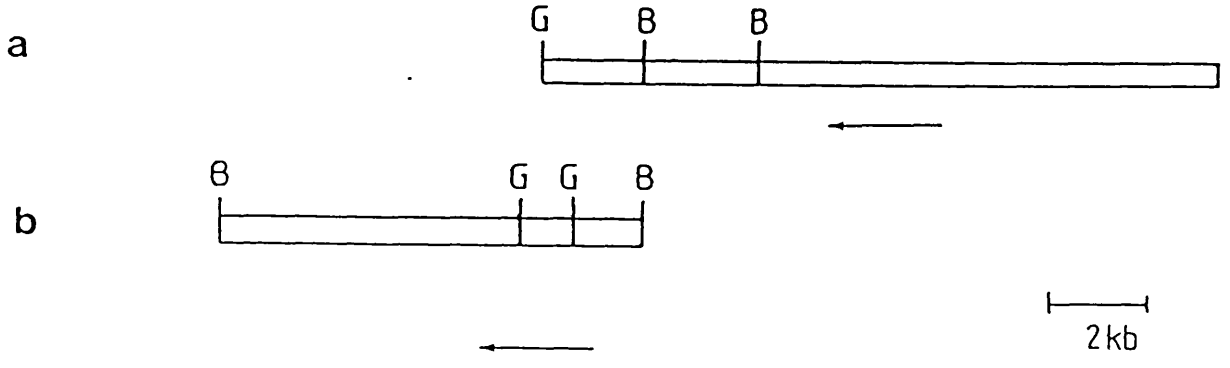


Figure 8.1: Potential Overlap Between pDMβ40 and pBX20?

Restriction maps of an area of potential overlap between two glucanases.

a. pDMβ40 showing the position and direction of transcription of a β-glucanase gene.

b. A similar map showing the approximate position and direction of transcription of a glucanase gene from *Arthrobacter* YCWD3.

might have led to increased errors in the measurement of the lytic activity.

Overall, it seems possible that other glucanase genes might be found on the cloned fragments. However, other subcloning strategies must be employed to identify other putative coding regions since the number of rare, convenient sites is highly limited. A simple way to determine the presence of other glucanase genes would be to attempt to obtain sufficient enzyme from *E. coli* grown cultures bearing large plasmids and visualise all the glucanase components on activity gels. Unfortunately, even with pDMβ42, insufficient glucanase was obtained to enable the visualisation of any activities.

8.3 Expression of the Cloned Glucanase Gene

The cloned glucanase gene was expressed in a number of systems both *in vivo* and *in vitro*. It was noted that expression in *E. coli* was poor and it was difficult to identify a significant product of this gene using the minicell system. It was possible, however, to identify a glucanase product in *E. coli* cultures by activity stained gels. A novel *in vitro* method (Thompson *et al.*, 1984), based on the Zubay system, was used to visualise the polypeptides encoded by the cloned DNA. It was apparent from the results of these coupled transcription/translation experiments that the β-glucanase gene could be expressed from its own promoter (a protein of the appropriate size was produced from pDMβ101 in which the *Egl*1 gene runs counter to the *lac* promoter of the pUC vector). However, it seemed that the expression was much higher when the glucanase gene was expressed from the vector borne promoter as well as its own. The *Streptomyces* based system was capable of recognising *E. coli* expression signals in addition to those of *A. luteus*. In this respect, it appears to be less stringent than the *E. coli* systems devised by Zubay (1973) or the *in vivo* minicell system. In addition, expression of the cloned DNA in the *Streptomyces* system was more efficient since no ladder of polypeptides was observed in this case and few additional proetin species were produced from each plasmid clone. This was in contrast to the minicell system used earlier in which a multiplicity of "extra" products were noted. Perhaps the difference is due to the fact that the gene expression apparatus of *Streptomyces* is better adapted than that of *E. coli* to G+C-rich DNA. A number of mechanisms to achieve such results can be envisaged: for example, the RNA polymerase of *Streptomyces* may be more effective at "melting" the G+C-rich DNA which would be expected to be more strongly hydrogen bonded than its *E. coli* counterpart; at the translational level, it might be the case that *E.coli* ribosomes are more prone to stalling (and hence terminating translation) due to either secodary structures formed within the mRNA or even to the fact that the population of tRNAs of *E. coli* differs substantially from those required to translate *A. luteus* DNA efficiently.

The unprocessed product generated using the *S. lividans* system had a molecular

weight of approximately 46 kD, corresponding fairly well with the size of the protein predicted from the nucleotide sequence, while the product observed on the activity stained gel was significantly smaller (44 kD). The protein tentatively identified by minicell analysis was of the smaller size although it was perhaps possible to detect an unprocessed species. These observations demonstrate that the product of the cloned gene was probably processed in *E. coli*. The fact that the 44 kD glucanase observed on activity gels was so similar in size to a protein of the *A. luteus* supernatant, indicated that the processing of this protein by *E. coli* was similar to that carried out by *Arthrobacter*. This seems to be efficiently performed in *E. coli* since little (if any) of the unprocessed material could be observed in minicells. This was associated with relatively efficient localisation of the glucanase to the periplasm in *E. coli*, which is broadly equivalent to secretion into the media by Gram-positive bacteria (Pugsley, 1988). The relatively large change in molecular weight (accounting for 20 or more amino acids) is more typical of Gram-positive signal sequences than of *E. coli*. That this could be the case is illustrated by Figure 8.2. The most likely point for signal peptide cleavage in *E. coli* is shown. This would remove amino acids 1-25 and decrease the molecular weight by approximately 2 kD.

However, it is possible that the fact that the glucanase gene product observed in the *in vivo* expression system was larger than the expected protein derived from the nucleotide sequence reflects the use of the upstream GTG as a start codon. However this is still insufficient to account for the differences in apparent molecular weights. The final alternative is that the glucanase protein electrophoresed in a slightly anomalous manner.

8.4 Features of the Nucleotide Sequence of *Egl1*.

Clearly the nucleotide sequence was highly G+C-rich. It was difficult to determine the presence of any type of promoter but the sequences upstream of the *Egl1* gene were screened for the presence of *E. coli*-like (e.g TTGACA..17bp..TAGGAT) or *Streptomyces*-like (more varied and more G+C-rich). However, no sequences strongly similar to an *E. coli* type promoter were observed while it was possible to find a large number of potential non-*E. coli*, but *Streptomyces*-like promoetrs. This might show that *A. luteus* promoters have little in common with the *E. coli* sequences and that it relies upon a more *Streptomycete*-like system. A further possibility is that the promoters of *A. luteus* do not resemble those of either *Streptomyces* or *E. coli*. Indeed, a logical continuation of the work presented here would be to map the transcripts and mRNA start points in *A. luteus* and *E. coli* and thus determine the most likely promoter region. That *A. luteus* promoters might be substantially different from those of *Streptomyces* is indicated by the poor yield of glucanase from pDMβ101 in the *in vitro Streptomyces* expression

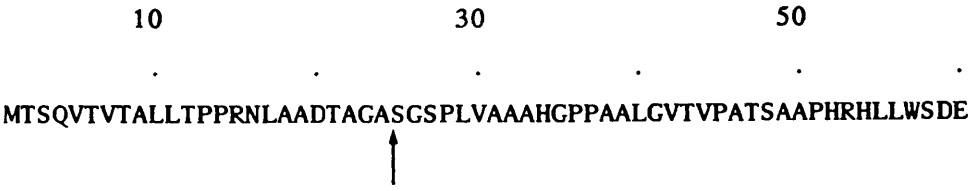


Figure 8.2: The Most Probable Point for Signal Peptide Cleavage of the *Egl1* Gene Product in *E. coli*.

The arrow indicates the most probable cleavage point.

system.

In spite of the apparently poor expression of *Egl1* in both *E. coli* and *Streptomyces* systems, there are two short sequences which could act as ribosome binding sites (AGGA or AGGAA) just upstream of the gene start at positions 570–573 and 590–594, respectively. There was no such seqeunce close to the GTG.

It is possible however that the *Egl1* gene was part of an operon and the sequence did not contain any promoters since these were located elsewhere in the operon. It is clearly important to determine the size and start point of the *Egl1* message in order to tell if this was indeed the case. The other genes of this imaginary operon should have been cloned on the regions of DNA flanking *Egl1* which were isolated on the primary glucanase encoding plasmids.

8.5 Is *Egl1* Like any Other Genes?

Both the nucleotide sequence and the derived protein sequence were compared with the sequenes in the EMBL, NBRF and SWISSPROT Databases. However, no significant homologies were found. Similarly, the gene and protein were comapared with known genes such as the *B.subtilis* β-glucanase on the data bases. Again no significant regions of homology were identified.

8.6 Conclusions

The cloning of a glucanase gene was described, which differed substantially in restriction map and molecular weight of the product, from the similar gene described by Doi and Doi (1986). It was relatively poorly expressed in a number of systems. Best expression appeared to be in the *Streptomyces*-based system of Thompson *et al.*, (1984).

The nucleotide sequence of the gene was determined and the putative translation product identified. This seemed to correspond well in molecular weight with the proteins observed *in vivo* and *in vitro*. Additional regions, flanking this DNA were also isolated and it was postulated that this contained other β-glucanase genes clustered in this region or even arranged as an operon.

Some characterisation of the gene product with respect to activity at various temperatures and pHs was carried out. In addition, the substrate specificity and the end products of its hydrolysis of glucan were investigated.

Further work was neeeded to determine the number of genes encoded by the primary glucanase clones and whether any of the additional genes encoded glucanases. More analysis was required to determine the size and start point of the *Egl1* message.

REFERENCES

Abd-El-Al A.T.H., Phaff H.J., (1968). Exo-β-glucanases in yeast. Biochem. J. **109**: 347-360.

Abd-El-Al A.T.H., Phaff H.J., (1969). Purification and Properties of Endo-β-glucanase in *Hanseniaspora valbyensis*. Can. J. Microbiol. **15**: 697-701.

Anderson S., Marks C.B., Lazarus R., Miller J., Stafford K., Seymour S., Light D., Rastetter W., Estell D., (1985). Production of 2-keto-L-gluconate, an intermediate in L-ascorbate synthesis by a genetically modified *Erwinia herbicola*. Science **230**: 144-149.

Antonov P.P., Ivanov I.G., Markov G.G., Benigni R., (1978). Reassociation analysis of DNA in studying the genome of *Stretomyces*. Studia Biophysica Berlin **69**: 67-74.

Bak A.L., Christiansen C., Stenderup A., (1970). Bacterial genome sizes as determined by DNA renaturation studies. J. Gen Microbiol. **64**: 377-380.

Barbaric S., Kozulic B., Ries B., Mildaer P., (1980). Purification and evidence for heterogeneity of acid phosphatase from *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **95**: 404-409.

Barras D.R., (1972). A β-glucan endohydrolase from *Schizosaccharomyces pombe* and its role in cell wall growth. Antonie van Leeuwenhoek J. Microbiol. Serol. **38**: 65-80.

Bartnicki-Garcia S., (1973). Fungal cell wall composition. In: CRC Handbook of Microbiology, volume II: microbial composition, pp 201-214. Eds: Laskin A.I., Lechevalier H.A., CRC Press, Cleveland.

Bathgate G.N., Palmer G.H., Wilson G., (1974). Action of endo-β-1-3-glucanases on on barley and malt β-glucans. J. I. Brewing **80**: 278.

Batt C.A., Follettie M.T., Shin H.K., Yeh P., Sinskey A.J., (1985). Genetic engineering of coryneform bacteria. Trends Biotechnol. **3**: 303-310.

Beguin P., Cornet P., Aubert J-P., (1985). Sequence of a cellulase gene of the thermophilic bacterium *Clostridium thermocellum*. J. Bacteriol. **162**: 102-105.

Bertheau Y., Madgidi-Hervan E., Kotoujansky A., Nguyen-The C., Andro T., Coleno A., (1984). Detection of depolymerase isozymes after electrophoresis or electrofocusing, or in titration curves. Anal. Biochem. **139**: 383-389.

Birnboim H.C., Doly J., (1979). A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucl. Acids. Res. **7**: 1513-1523.

Block R.J., Durrum E.L., Zweig G., (1958). A manual of paper chromatography and paper electrophoresis. pp 170-214. Academic Press Inc., New York.

Boulnois G.J., (1987). Gene cloning and analysis. Blackwell Scientific Publications Inc., Oxford.

Bradford M., (1976). A rapid and sensitive method for the detection of microgram quantities of protein, utilising the principle of protein-dye binding. Anal. Biochem. **72**: 248-254.

Brandsch R., Decker K., (1984). Isolation and characterisation of plasmid DNA from *Arthrobacter oxidans*. Arch. Microbiol. **138**: 15-17.

Brandsch R. Fuller W., Schneider K., (1986). Plasmid pAO1 of *Arthrobacter oxidans* encodes 6-hydroxy-D-nicotine oxidase: cloning and expression of the gene in *Escherichia coli*. Mol. Gen. Genet. **202**: 96-101.

Cabib E., (1975). Molecular aspects of yeast morphogenesis. Ann. Rev. Microbiol. **29**: 191-214.

Cabib E., (1981). Chitin: Structure, metabolism and regulation of biosynthesis. In: Encyclopedia of Plant Physiology: New series, vol. 13B, Plant Carbohydrates II, pp 395-415. Eds: Tanner W., Loewus F.A., Springer-Verlag, Berlin.

Cabib E., Bowers B., (1971). Chitin and yeast budding; localisation of chitin in bud scars. J. Biol. Chem. **246**: 152-159.

Cantwell B.A., McConnell D.J., (1983). Molecular cloning and expression of a *Bacillus subtilis* β-glucanase gene in *Escherichia coli*. Gene **23**: 211-219.

Chen C.M. Gritzali M., Stafford D.W., (1987). Nucleotide sequence and deduced primary structure of cellobiohydrolase II from *Trichoderma reesei*. Bio/Technology **5**: 274-278

Clarke L., Carbon J., (1976). A colony bank containing synbthetic *Col*E1 hybrid plasmids representative of the entire *Escherichia coli* genome. Cell **9**: 91-99.

Cohen R.E., Ballou C.E., (1981). Mannoprotein structure. In: Encyclopedia of Plant Physiology: New series, vol. 13B, Plant Carbohydrates II, pp 441-458. Eds: Tanner W. Loewus F.A., Springer-Verlag, Berlin.

Constantinides A., (1980). Steroid transformation at high substrate concentrations using immobilised *Corynebacterium simplex* cells. Biotechnol. Bioeng. **22**: 119-136.

Cornet P., Millet J., Beguin P., Aubert J.P., (1983a). Characterisation of two *cel* (cellulose degradation) genes of *Clostridium thermocellum* coding for endoglucanases. Bio/Technology **1**: 589-594.

Cornet P., Tronik D., Millet J., Aubert J.P., (1983b). Cloning and expression in *Escherichia coli* of *Clostridium thermocellum* genes coding for amino acid synthesis and cellulose hydrolysis. FEMS Microbiol. Letters **16**: 137-141.

Crombach W.H.J., (1978). DNA base ratios and DNA hybridisation studies of coryneform bacteria, mycobacteria and nocardiae. In: Coryneform bacteria, pp 161-179. Eds: Bousfield I.J., Callely A.G., Academic Press, London.

Dale R.M.K., M^{c}Clure B.A., Houchins J.P., (1985). A rapid single stranded cloning strategy for producing a sequential series of overlapping clones for use in DNA sequencing: application to the sequencing of corn mitochondrial 18S rRNA. Plasmid **13**: 31-40.

Del Real G., Aguilar A., Martin J.F., (1985). Cloning and expression of tryptophan genes from *Brevibacterium lactofermentum* in *Escherichia coli*. Biochem. Biophys. Res. Commun. **133**: 1013-1019.

Devereux J., Haeberli P., Smithies O., (1984). A comprehensive set of programmes for the VAX. Nucl. Acids. Res. **12**: 387-395.

Doi K., Doi A., Fukui T., (1971). Joint action of two glucanases produced by *Arthrobacter* in speroplast formation in baker's yeast. J. Biochem. **70**: 711-714

Doi K., Doi A., Ozaki T., Fukui T., (1973a). Heterogeneity of the lytic activity for yeast cell wall observed among the components of an *Arthrobacter* glucanase. J. Biochem. **73**: 667–670.

Doi K., Doi A., Fukui T., (1973b). Purification and properties of lytic β-glucanase from an *Arthrobacter* bacterium. Agric. Biol. Chem. **37**: 1619–1627.

Doi K., Doi., Ozaki T., Fukui T., (1973c). Lytic β-(1–3)-glucanase from *Arthrobacter*: patterns of action. Agric. Biol. Chem. **37**: 1629–1633.

Doi K., Doi A., (1986). Cloning and expression in *Escherichia coli* of the gene for an *Arthrobacter* β-(1–3)-glucanase. J. Bacteriol. **168**: 1272–1276.

Dougan G., Sherratt D., (1977). The transposon Tn1 as a probe for studying ColE1 structure and function. Mol. Gen. Genet. **151**: 151–160.

Eddy A.A., Williamson D.H., (1957). A method for isolating protoplasts from yeast. Nature **179**: 1252–1253.

Feinberg A.P., Vogelstein B., (1984). A technique for labelling DNA restriction fragments to high specificity. Anal. Biochem. **137**: 266–267.

Feiss M., Fisher R.A., Crayton M.A., Egner C., (1977). Packaging of bacteriophage lambda chromosomes: effect of chromosome length. Virology **77**: 281–293.

Fleet G.H., Phaff H.J., (1974). Glucanases in *Schizosaccharomyces*: isolation and properties of the cell wall associated β-glucanases. J. Biol. Chem. **249**: 1717–1728.

Fleet G.H., Phaff H.J., (1981). Fungal Glucans: Structure and Metabolism. In Encyclopedia of Plant Physiology: New series, vol. 13B, Plant carbohydrates II, pp 416–441. Eds: Tanner W., Loewus F.A. Springer–Verlag, Berlin.

Follettie M.T., Sinskey A.J., (1986). Molecular cloning and nucleotide sequence of the *Corynebacterium glutamicum pheA* gene. J.Bacteriol. **167**: 692–702.

French J.C., Howells J.D., Anderson L.E., (1970). Erythromycin Process. U.S. Patent 3,551,294. Dec 29.

Frischauf A.M., Lehrach H., Poustka A., Murray N., (1983). Lambda replacement vectors carrying polylinker sequences. J. Mol. Biol. **170**: 827–842.

Funatsu M., Hongrock O.H., Aizono Y., Shimoda T., (1978). Protease of *Arthrobacter luteus*: Properties and function in lysis of viable yeast cells. Agric. Biol. Chem. **42**: 1975–1977

Gilkes N.R., Kilburn D.G., Langsford M.L., Miller Jr. R.C., Wakarchuk W.W., Warren R.A.J., Whittle D.J., Wong W.K.R., (1984). Isolation and characterisatiion of *Escherichia coli* clones expressing cellulase genes from *Cellumonas fimi*. J. Gen. Microbiol. **130**: 1377–1384.

Guyer M.S., (1983). Transposon γδ in cloned gene analysis. Meths. Enzymol. **101**: 362–369.

Hardy K.G., (1985). Bacillus cloning methods. In DNA cloning, a practical approach, vol. II, pp 1–17. Ed: Glover D.M. IRL Press Ltd., Oxford and Washington D.C.

Hayward A.C., Sly L.I., (1976). Dextranase activity in *Oerskovia xanthineolytica*. J. Appl. Bacteriol. **40**: 355–364.

Henikoff S., (1984). Unidirectional digestion with exonuclease III creates targetted breakpoints for DNA sequencing. Gene **28**: 351-359.

Hinchliffe E., (1984). Cloning and expression of a *Bacillus subtilis* endo-1,3-1,4-b-D-glucanase gene in *Escherichia coli*. J. Gen. Microbiol. **130**: 1285-1291.

Holmes D.S., Quigley M., (1981). A rapid boiling method for the preparation of bacterial plasmids. Anal Biochem. **114**: 193-197.

Hunter I.S., (1985). Gene cloning in *Streptomyces*. In: DNA cloning, a practical approach, vol. II, pp 19-44. Ed: Glover D.M., IRL Press Ltd., Oxford and Washington D.C.

Jeffries T.W., (1975). Purification and characterisation of the yeast lytic enzyme system of *Oerskovia xanthineolytica*. PhD Thesis, Rutgers University, New Brunswick, New Jersey.

Jeffries T.W., MacMillan J.D., (1980). Action patterns of β-1,3-glucanases from *Oerskovia xanthineolytica* on laminarin, lichenan and yeast glucan. Carbohydrate Res. **95**: 87-100.

Joliff G., Beguin P., Juy M., Millet J., Ryter A., Poljak R., Aubert J.P., (1986). Isolation, crystallisation and proerties of a new cellulase of *Clostridium thermocellum*, overproduced in *Escherichia coli*. Bio/Technology **4**: 896-900.

Jones G.H., Ballou C.E., (1969). Studies on the structure of yeast mannan. II Mode of action of the *Arthrobacter* α-mannosidase on yeast mannan. J. Biol. Chem. **244**: 1052-1059.

Jones D., Collins M.D., (1986). Irregular, non-sporing Gram-positive rods. In: Bergey's manual of systematic Bacteriology volume II, Section 15, pp 1261-1491. Edited by Sneath P.H.A., Mair N.S., Sharpe M.E., Holt J.G., Williams and Wilkins, London.

Karn J., Mathes H.W.D., Gait M.J., Brenner S., (1984). A new selective phage cloning vector λ2001, with sites for *Xba I, BamH I, Hind III, EcoR I, Sst I* and *Xho I*. Gene **32**: 217-224.

Kawazu T., Nakanishi Y., Noboyuki U., Sasaki T., Yamagata H., Tsukagoshi N., Udaka S., (1987). Cloning and nucleotide sequence of the gene coding for enzymatically active fragments of the *Bacillus polymyxa* β-amylase. J. Bacteriol. **169**: 1564-1570.

Kitamura K.(1982a). Re-examination of Zymolyase purification. Agric. Biol. Chem. **46**: 963-969.

Kitamura K., (1982b). A protease which participates in yeast cell wall lysis during zymolyase digestion. **46**: 2093-2099.

Kitamura K., (1982c). Preparation of yeast cell wall lytic enzyme from *Arthrobacter luteus* by its adsorption on β-glucan. J. Ferment. Technol. **60**: 257-260.

Kitamura K., Kaneko T., Yamamoto Y., (1971). Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. Arch. Biochem. Biophys. **145**: 402-404.

Kitamura K., Kaneko T., Yamamoto Y., (1972). Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. I. Isolation of lytic strain and studies on its lytic activity. J. Gen. Appl. Microbiol. **18**: 57-71.

Kitamura K., Kaneko T., Yamamoto Y., (1974). Lysis of viable yeast cells by enzymes of *Arthrobacter luteus*. II. Purification and properties of an enzyme, zymolyase, which lyses yeast cells. J. Gen. Appl. Microbiol. **20**: 323–344.

Knorr D., Shetly K.J., Hood L.F., Kinsella J.E., (1979). An enzymatic method for yeast autolysis. J. Food Sci. **44**: 1362–1365.

Knowles J., Lehtovaara P., Teeri T., (1987). Cellulase Families and their genes. Trends Biotechnol. **5**: 255–261.

Kobayashi Y., Tanaka H., Ogasawara N., (1974). Purification and properties of F–Ia, a β–(1–3)–glucanase which is highly lytic towards the cell walls of *Pilicularia oryzae* P2. Agric. Biol. Chem. **38**: 973–978.

Kopecka M., Phaff H.J., Fleet G.H., (1974). Demonstration of a fibrillar component in the cell wall of the yeast *Saccharomyces cerevisiae* and its chemical nature. J. Cell. Biol. **62**: 66–76.

Kuhn I., Stephenson F., Boyer H.W., Greene P.J., (1986). Positive–selection vectors utilising lethality of the *Eco*RI endonuclease. Gene **44**: 253–263.

Laemmli U.K., (1970). Cleavage of structural proteins during assemby of the head of the bacteriophage T4. Nature **277**: 680–683.

Lechevalier M.P., (1972). Description of a new species *Oerskovia xanthineolytica* and emendation of *Oerskovia* Prauser *et al.* Int. J. Syst. Bacteriol. **22**: 260–264.

Maniatis T., Fritsch E.F., Sambrook J., (1982). Molecular cloning. A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mann J.W., Heintz C.E., Macmillan J.D., (1972). Yeast spheroplasts formed by cell wall degrading enzyme from *Oerskovia sp.* J. Bacteriol. **111**: 821–824.

Mann J.W., Jeffries T.W., Macmillan J.D., (1978). Production and ecological significance of yeast cell wall degrading enzymes from *Oerskovia*. Appl. Env. Microbiol. **36**: 594–605.

Manners D.J., Masson A.J., (1969). The structure of two glucans from yeast cell wall. FEBS Letts. **4**: 122–124.

Manners D.J., Masson A.J., Patterson J.C., (1973a). The structure of a β–(1–3)–D–glucan from yeast cell walls. Biochem. J. **135**: 19–30.

Manners D.J., Masson A.J., Patterson J.C., Bjorndas H., Lindberg B., (1973b). The structure of a β–(1–6)–D–glucan from yeast cell walls. J.Biochem **135**: 31–36.

Marquez G., Fernandez–Sousa J.M., Sanchez F., (1985). Cloning and expression in *Escherichia coli* of genes involved in the lysine pathway of *Brevibacterium lactofermentum*. J. Bacteriol. **164**: 379–383.

Martin H.L., Bamforth C.W., (1983). Application of a radial diffusion assy for the measurement of β–glucanase in malt. J. Inst. Brew. **89**: 34–37.

Martin J.F., Santamaria R., Sandoval H., Del Real G., Mateos L.M. Gil J.A., Aguilar A., (1987). Cloning systems in amino acid producing corynebacteria. Bio/Technology **5**: 137–146.

McLellan W.L., McDaniel L.E., Lampen J.O., (1970). Purification of

phosphomannanase and its action on the yeast cell wall. J.Bacteriol. **102**: 261-270.

Miller G.L., (19590. Use of dinitrosalicylic acid reagent for the determination of reducing sugar. Anal. Chem. **31**: 426-428.

Miller J.H., (1972). Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Nagasaki S., Newmann N.P., Arnold P., Schnable C.P., Lampen J.O., (1966). An enzyme which degrades the walls of living yeast. Biochem. Biophys. Res. Commun. **25**: 158-164.

Nakamura K., Miawa N., Kitamura K., (1986a). Cellulase genes of *Cellulomonas uda* CB4. I. Cloning and expression of β-glucosidase genes in *Escherichia coli*. J. Biotechnol. **3**: 239-246.

Nakamura K., Misawa N., Kitamura K., (1986b). Cellulase genes of *Cellulomonas uda* CB4. II. Cloning and expression of a CM-cellulase hydrolysing enzyme (endoglucanase) gene in *Escherichia coli*. J. Biotechnol. **3**: 247-253.

Nakajima T., Ballou C.E., (1975). Yeast mannoprotein biosynthesis: Solubilisation and selective assay of four mannosyl transferases. Proc. Natl. Acad. Sci. U.S.A. **72**: 3912-3916.

Necas O., Kopecka M., (1969). Synthesis of fibrillar component of regenerating cell walls in yeast protoplasts. Antonie van Leeuwenhoek J. Microbiol. Serol. **35** (supplement): B7-B8.

Nossal N.G., Heppel L.A., (1966). The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. J. Biol. Chem. **241**: 3055-3062.

Novick P., Field C., Schekman R., 91980). Identification of 23 complementation groups required for post-translational events in the yeast secretory pathway. Cell **21**: 205-215.

Novick P., Ferro S., Schekman R., (1981). Order of events in the yeast secretory pathway. Cell **25**: 461-469.

O'Callaghan C.H., Morris A., Kirby S.M., Shingle A.H., (1972). New method for the detection of β-lactamases by using a chromogenic cephalosporin substrate. Antimicrob. Agents. Chemother. **1**: 283-288.

Ogata K., Kinoshita S., Tsunoda T., Aida K., (1976). Microbial production of nucleic acid substances. Wiley, New York.

Ozaki A., Katsumata, Oka T., Furuya A., (1985). Cloning of the genes concerned in phenylalanine biosynthesis in *Corynebacterium glutamicum* and its application to breeding of a phenylalanine producing strain. Agric. Biol. Chem. **49**: 2925-2930.

Phaff H.J. (1971). Structure and biosynthesis of the yeast cell envelope. In: The yeasts, vol. II, pp 135-210. Eds: Rose A.H., Harrison S.J., Academic Press, London.

Peberdy J.F., (1980). Protoplast fusion- a tool for genetic mainpulation and breeding in industrial microorganisms. Enzyme Microb. Technol. **2**: 23-29.

Roberts A.N., Hudson G.S., Brenner S., (1985). An erythromycin-resistance gene from an erythromycin-producing strain of *Arthrobacter sp.* Gene **35**: 259-270.

Robson L.M., Chambliss G.H., (1986). Cloning of *Bacillus subtilis* DLG

β-1,4-glucanase gene and its expression in *Escherichia coli* and *Bacillus subtilis*. J. Bacteriol. **165**: 612–619.

Robyt J., French D., (1963). Action pattern and specificity of an amylase from *Bacillus subtilis*. Arch. Biochem. Biophys. **100**: 451–467.

Rodriguez R.L., Tait R.C., (1983). Isolation of Chromosomal DNA. In: Recombinant DNA techniques: An Introduction, pp 162–163. Addison-Wesley Inc., Reading, Mass.

Romaniec M.P.M., Clarke N.G., Hazlewood G.P., (1987). Molecular cloning of *Clostridium thermocellum* DNA and the expression of further novel endo-β-1,4-glucanase genes in *Escherichia coli*. J. Gen. Microbiol. **133**: 1297–1307.

Salton M.R.J., (1955). Isolation of *Stretomyces spp.* capable of decomposing preparations of cell walls from various micro-organisms and a comparison of their lytic activities with those of certain Actinomycetes and Myxobacteria. J.Gen. Microbiol. **12**: 25–30.

Sanger F., Nicklen S., Coulson A.R., (1977). DNA sequencing using chain terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**: 5463–5467.

Schekman R., Brawley V., (1979). Localised deposition of chitin on the yeast cell surface in response to mating pheromone. Proc. Natl. Acad. Sci. U.S.A. **76**: 645–649.

Scott J.H., Schekman R., (1980). Lyticase: Endoglucanase and protease activities that act together in yeast cell lysis. J. Bacteriol. **142**: 414–423.

Sentendreu R., Northcote D.H., (1968). The structure of a glycopeptide isolated from the yeast cell wall. Biochem. J. **109**: 419–432.

Sentendreu R., Northcote D.H., (1969). The characterisation of oligosaccharides attached to threonine and serine in mannan glycopeptides obtained from the cell wall of yeast. Carbohydr. Res. **10**: 584–585.

Shaw P.C., Hartley B.S., (1986). Protoplast transformation of *Arthrobacter*. Abstracts of "Microbe '86".

Shoemaker S., Schweikart V., Ladner M., Gelfand D., Kwok S., Myambo K., Innis M., (1983). Molecular cloning of Exocellobiohydrolase I derived from *Trichoderma reesei* strain L27. Bio/Technology **1**: 691–695.

Spratt B.G., Hedge P.J., Heesen S.T., Edelman A., Broome-Smith J.K. (1986). Kanamycin resistance vectors that are analogues of plasmids pUC8, pUC9, pEMBL8, and pEMBL9. Gene **41**: 337–342.

Southern E.M., (1975). Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. **98**: 503–518.

Stoker N.G., Pratt J.M., Holland I.B., (1984). *In vivo* gene expression systems in procaryotes. In: Transcription and translation, a practical approach, pp 153–157. Eds: Hames B.D., Higgins S.J., IRL Press, Oxford.

Sugimori T., Uchida Y., Tsukada Y., (1972). Distribution of yeast cell wall lytic activity among micro-organisms. Agric Biol. Chem. **36**: 669–675.

Suzuki T., Honda H., Katsuma R., (1972). Production of antibacterial compounds analogous to chloramphenicol by n-paraffin grown bacteria. Agric. Biol. Chem. **36**: 2223–2228.

Tanaka H., Phaff H.J., (1965). Enzymatic hydrolysis of yeast cell walls. I. Isolation of wall-decomposing organisms and separation and purification of lytic enzymes. J. Bacteriol. **89**: 1570-1580.

Teather R.M., Wood P.J., Use of congo red-polysaccharide interactions in enumeration and characterisation of cellulolytic bacteria from the bovine rumen. Appl. Environ. Microbiol. **43**: 777-780.

Teeri T., Salovuori I., Knowles J., (1983). The molecular cloning of the major cellulase gene from *Trichoderma reesei*. Bio/Technology **1**: 696-699.

Thompson J., Rae S., Cundliffe E., (1984). Coupled transcription-translation in extractxs of *Streptomyces lividans*. Mol. Gen. Genet. **195**: 39-43.

Trimble R.B., Maley F., (1977). Subunit structure of invertase from *Saccharomyces cerevisiae*. J.Biol. Chem. **252**: 4409-4412.

Tsukagoshi N., Ihara H., Yamagata H., Udaka S., (1984). Cloning and expression of a thermophilic α-amylase gene from *bacillus stearothermophilus* in *Escherichia coli*. Mol. Gen. Genet. **193**: 58-63.

Valentin E., Herrero E., Pastor F.I.J., Sentendreu R., (1984). Solubilisation and analysis of mannoprotein molecules from the cell wall of *Saccharomyces cerevisiae*. J. Gen. Microbiol. **130**: 1419-1428.

Vieira J., Messing J., (1982). The pUC plasmids, an M13mp7 derived system for insertion mutagenesis and sequencing with synthetic universal primers. Gene **19**; 259-268.

Vrsanska M., Kratky Z., Biely P., (1977a). Lysis of intact yeast cells and isolated cell walls by an inducble enzyme system of *Arthrobacter* GJM-1. Zeitshcrift fur Allgemeine Mikrobiologie **17**: 391-402.

Vrsanska M., Biely P., Kratky Z., (1977b). Enzymes of the yeast lytic system produced by *Arthrobacter* GJM-1 bacterium and their role in the lysis of yeast cell walls. Zeitschrift fur Allgemeine Mikrobiologie **17**: 465-480.

Weiss B., (1976). Endonuclease II of *Escherichia coliu* is exonuclease III. J. Biol. Chem. **251**: 1896-1901.

Wessels J.G.H., Sietsma J.H., (1981). Fungal cell walls- a survey. In Encyclopedia of Plant Physiology: New series, vol. 13B, Plant Carbohydrates, pp 352-394. Eds: Tanner W., Loewus F.A., Springer-Verlag, Berlin.

Whittle D.J., Kilburn D.G, Warren R.A.J., Miller Jr. R.C., (1982). Molecular cloning of a *Cellulomonas fimi* cellulase gene in *Escherichia coli*. Gene **17**: 139-145.

Wood P.J., (1980). Specificity in the interaction of direct dyes with polysaccharides. Carbohydr. Res. **85**: 271-287.

Wray W., Boulikas T., Wray V.P., Hancock R., (1981). Silver staining of proteins in polyacrylamide gels. Anal. Biochem. **118**: 197-203.

Yannisch-Perron C., Vieira J., Messing J., (1985). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33**: 103-119.

Yoshinaga F. Nakamori S., (1983). Production of amino acids. In Amino acids: Biosynthesis and genetic regulation, pp 405-429. Eds: Herrmann K.M., Somerville

R.L., Addison Wesley Publishing Co., Reading, Mass.

Zubay G. (1973). *In vitro* synthesis of protein in microbial systems. Ann. Rev. Genet. 7: 267–287.

ABSTRACT

MOLECULAR CLONING AND ANALYSIS OF A β-1,3-GLUCANASE GENE FROM *ARTHROBACTER LUTEUS*.

David Mark Whitcombe

Species of *Arthrobacter luteus*, also known as *Oerskovia xanthineolytica* can utilise yeast cells as a growth substrate. This unusual abiility is due to the secretion of a battery of hydrolytic enzymes which degrade the yeast cell wall and thus lyse the cells. Although many hydrolytic enzymes are important in the degradation of the yeast cell wall, the key activities are endo-β-1,3-glucanases. In order to characterise components of the yeast lytic system and the genetic organisation of this little-understood organism, a molecular cloning approach was adopted. Large clones expressing β-1,3-glucanase were isolated from a library of *A. luteus* DNA constructed in the positive selection vector pKGW.

By a combination of subcloning, restriction mapping and Southern analysis, it was determined that the clones contained virtually the same inserts. Additional subcloning, transposon mutagenesis, deletion mapping and nucleotide sequencing were used to identify at least one glucanase gene. The predicted protein product had a molecular weight of about 46 kD. When the gene was expressed in a number of *in vivo* and *vitro* systems including *E. coli* minicells and a *Streptomyces* coupled transcription/translation system, the protein observed had a similar molecular weight. Furthermore, when the protein was produced in *E. coli* and run on activity stained gels, the β-glucanase activity co-migrated with the major glucanase of *A. luteus*. In addition the *E. coli*-produced glucanase had the ability to cause limited lysis of yeast.