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THE CLONING AND EXPRESSION OF AMYLOLYTIC GENES IN *Escherichia coli* AND THEIR ROLE IN STARCH UTILIZATION.

by

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Thesis submitted to the University of Warwick
and
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To Mom and Dad

Joyce and Stan Taylor

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DECLARATION.

I hereby declare that this thesis has been composed by myself and has not been accepted in any previous application for a degree. The work of which it is a record has been done by myself, under the supervision of Dr Mark Edwards. All sources of information have been specifically acknowledged by means of reference.

P. Taylor.

SUMMARY

The structural genes for the K.pneumoniae maltohexaose (G6)-producing amylase, the K.pneumoniae pullulanase and the B.licheniformis α-amylase were cloned and expressed in E.coli. Plasmids carrying the G6-producing amylase, conferred starch utilization on E.coli, but this growth was much slower than growth on glucose. On SDS-PAGE gels, the putative G6-producing amylase had an M_r 67,000. Strains carrying the pullulanase plasmid (pPT14) were able to utilize pullulan as their sole carbon source. On SDS-PAGE the cloned pullulanase comigrates with purified pullulanase (M_r 120,000+). Certain insertions and deletions in the cloned pullulanase fragment reduce growth rate on pullulan. The phenotypes expressed by malB strains and mutants which reduce plasmid copy number suggested that at least one other gene product was required in addition to the pullulanase gene, for effective utilization of pullulan by E.coli. A possible candidate for one of these extra gene products was a protein (M_r 94,000) which was expressed in maxicells. Possible mechanisms of pullulan utilization are discussed.

Strains carrying the B.licheniformis α-amylase plasmids (pPT80, pPT81, pPT83, and pPX2) could all grow on starch. A copy number of approximately 50 was required for efficient growth (i.e. as good as growth on glucose). When expressed in E.coli the α-amylase is found almost entirely in the periplasmic space. The evidence suggests that the α-amylase is poorly expressed in E.coli and only relatively small amounts of enzyme are required for starch utilization. A model for starch utilization in E.coli is discussed. The whole of the 3.466 kb insert carrying the B.licheniformis α-amylase gene was sequenced. The insert had three open reading frames, only one of which formed a complete gene. This open reading frame corresponded to the B.licheniformis α-amylase gene, coded for a protein of M_r 58,492 and had a 29 amino-acid putative signal sequence. The protein was very homologous to both the B.amyloliquefaciens and B.stearothermophilus α-amylases. The B.licheniformis α-amylase was put under tac promoter control on a high copy number plasmid. Induction with IPTG caused lethality. Uninduced α-amylase levels were sufficient to allow E.coli to grow on starch. The mRNA start points for both the wildtype gene and the tac construct were determined. The effect of removing the major part of the α-amylase signal sequence was studied. This was achieved by a combination of site-directed and linker mutagenesis of the α-amylase DNA. This construct was poorly expressed in E.coli and did not exhibit a lethal phenotype.

ABBREVIATIONS

ATP	(Adenosine triphosphate)
CIP	(Calf intestinal phosphatase)
Cb	(Carbenicillin, 200 ug/ml)
cAMP	(Cyclic adenosine monophosphate)
^oC	(Degrees centigrade)
DNA	(Deoxyribonucleic acid)
Dex	(Maltodextrin)
EDTA	(Ethylenediaminetetra-acetic acid)
g	(Gramme)
G1, G2, G3, etc.	(Glucose, maltose, maltotriose, etc.)
hr	(Hour)
kd	(Kilodalton)
l	(Litre)
M	(Molar)

min	(Minute)
ml	(Millilitre)
mM	(Millimolar)
uM	(micromolar)
uCi	(Microcurie)
M9	(M9 minimal medium)
M_r	(Relative molecular mass)
nm	(Nanometre)
OD	(Optical density)
PAGE	(Polyacrylamide gel electrophoresis)
Pi	(Orthophosphate)
SDS	(Sodium dodecyl sulphate)
RNA	(Ribonucleic acid)
Tc	(Tetracycline, 10 ug/ml)
TEMED	(N,N,N',N'-tetramethylethylenediamine)

Tris	(2-amino-2hydroxymethylpropane 1,3-diol)
VB	(Vogel and Bonner minimal medium)
Δ	(Deletion)
ϕ	(Phi)
λ	(Bacteriophage lambda)

CHAPTER 1

INTRODUCTION.

1.1 Background.

With the advent of recombinant DNA technology, *Escherichia coli* has become a very important industrial micro-organism. Many genes have been cloned and expressed in *E.coli*, mainly because it has a well defined genetic system making gene manipulation a relatively simple task. The growth medium is an important consideration when using *E.coli* to over-produce proteins and small molecules on an industrial scale. Usually, the most expensive constituent of the medium is the carbon source and, for *E.coli*, glucose (in the form of crude glucose syrups) is the most convenient choice. However, on an industrial scale glucose tends to be the most expensive constituent of the medium and its price is prone to market fluctuations. Glucose syrups are usually produced by either chemical (Whistler, 1984) or enzymatic (Hausser *et al*., 1983; Lloyd and Nelson, 1984) degradation of starch. It would therefore be more cost effective if starch could be used, instead of glucose. However, *E.coli* can not utilize starch, even though it does have cytoplasmic enzymes (amylomaltase and maltose phosphorylase) which can break down starch degradation products (small maltodextrins) to glucose. Therefore, it

seems likely that *E.coli* can not grow on starch simply because the substrate is too large to be transported into the cytoplasm. Bacteria which can grow on starch invariably have extracellular amylolytic enzymes, which can break down the starch to maltodextrins which are small enough to be transported into the cell.

It was the intention of this project to produce a strain of *E.coli* which could grow efficiently on starch. The strategy for this was to clone amylolytic enzyme genes into *E.coli* and study their effects on starch utilization.

1.2 The Chemical Structure of Starch.

Starch is the major storage polysaccharide of plants. It is a gluco-polymer of high molecular weight and is a mixture of two distinct forms, amylose and amylopectin. Amylopectin is usually the major of the two components, making up 70-80% of the total mass of starch.

1.2.1 The Structure of Amylose.

Amylose consists of long unbranched chains of α-1,4 linked α-D-glucopyranose units (figure 1.1). It has one reducing end and one non-reducing end and takes the form of polydisperse chains, varying in M_r from a few thousand to over five hundred thousand. Although amylose is not soluble in water, it forms hydrated micelles in which the chain exists as a loose, flexible helix (Rees and Scott, 1971). Hydrated molecules with a degree of polymerisation (DP) of >40 residues, give a blue colouration with iodine. Shorter chain lengths give red, brown and yellow complexes (Bailey and Whelan, 1961). This reaction has been the basis of many amylase assays and has been used to differentiate between linear and branched polysaccharides.

1.2.2 The Structure of Amylopectin.

Amylopectin consists of short amylose chains (12-50 glucose residues) linked by α-1,6 linkages (figure 1.2). Like amylose it has one reducing end, but because

Non-reducing end

Amylose chain

Reducing end

Figure 1.1

Diagramatic representation of an amylose chain. The symbolic representation is shown below the structural representation.

Figure 1.2

A branch point in amylopectin. The symbolic representation is shown below the structural representation.

of its branched structure, has many non-reducing ends. Unlike amylose, amylopectin is soluble in water and can have a M_r as high as 100 million. In a hydrated solution, amylopectin gives a red violet-colour with iodine. This reaction can be used to distinguish between linear and branched molecules as amylose absorbs 19-20% of its weight in iodine from a dilute solution of I_2 and KI, whereas, at the same concentration, amylopectin absorbs very little (Lansky et al., 1949).

1.2.3 Physical Properties of Starch.

Native, unprocessed starch exists as tight packed crystalline granules, which are resistant to chemical and enzymatic reactions such as acid hydrolysis and amylolytic cleavage. In order to make starch susceptible to such reactions, it must first be gelatinised. Gelatinisation is effected by heating the granules in water to $100^{o}C$. On heating, the granule undergoes a series of irreversible changes. First the granule swells, then at a critical temperature it undergoes a rapid gelatinisation with a concomitant increase in size and then loses all of its native organisation. Eventually the granule disperses into a hydrated colloidal sol. "Solutions" containing more than 3% starch absorb all the available water, resulting in a semi-solid paste which, upon cooling, sets to a gel (Olkku and Rha, 1978). This can be a problem when using high concentrations of starch in fermentations, so in practice partially hydrolysed (soluble starch) is used.

Soluble starch can be used at much greater concentrations, due to its increased solubility and fluidity.

Another important reaction of starch is retrogradation which is the return of a hydrated "solution", to an insoluble aggregate (Olkku and Rha, 1978). This is an undesirable phenomenon because in this state the starch is resistant to amylolytic hydrolysis. Retrogradation is encouraged when the starch concentration is high, the temperature is low and when there is a preponderance of short linear molecules (Whistler and Johnson, 1948; Lansky *et al*., 1949).

1.3 Amylolytic Enzymes.

Amylolytic enzymes are ubiquitous in nature, being found in animals, plants and microorganisms. There are four main classes of amylolytic enzymes, namely α-amylase, β-amylase, glucoamylase, and debranching enzymes. All of these enzymes catalyze the hydrolysis of either α-1,4 or α-1,6 linkages (or both in the case of glucoamylase) by the transfer of a glucosyl residue to water.

1.3.1 α-amylases.

α-amylases cause the endo-hydrolysis of α-1,4 linkages in starch. They are termed α-amylases, because the newly formed reducing end groups have an α-anomeric conformation or downward mutorotation. They have no

activity against α-1,6 glycosidic linkages, but because they have an endo-action, can bypass α-1,6 bonds to produce a mixture of α-1,4 linear and α-1,4, α-1,6 branched maltodextrins. α-amylases also show a high degree of multiple attack, i.e. they cleave the substrate chain repeatedly without dissociation from the substrate (Robyt and French, 1970).

Within the α-amylases there are two distinct types, namely the liquefying and the saccharifying enzymes. The two types can be distinguished by their action pattern on starch for the increase in reducing power produced by the saccharifying enzyme is about twice that of the liquefying enzyme. This is because the saccharifying enzymes cleave preferentially near the non-reducing end of the starch molecules.

The list of bacterial α-amylases is extensive and the majority are found in Bacillus spp. (for a comprehensive list see Fogarty, 1983). Relatively few bacterial α-amylases genes have been cloned and expressed in E.coli and all of them have been obtained from Bacillus spp. (Table 1.1) and have a range of end product specificities. All of the α-amylases so far found produce end products which are small enough to be transported and utilized by E.coli (i.e. smaller than maltoheptaose i.e. seven glucose residues; G7).

Table 1.1

Cloned Bacterial and Fungal Amylolytic Enzymes.

Organism	Enzyme	Reference
B.amyloliquefaciens	α-amylase	Palva *et al*., 1981.
B.coagulans	α-amylase	Cornelis *et al*., 1982.
B.licheniformis	α-amylase	Ortlepp *et al*., 1983; Palva *et al*., 1984.
B.stearothermophilus	α-amylase	Mielenz, 1983.
B.subtilis	α-amylase	Yang *et al*., 1983.
K.pneumoniae	Pullulanase	Takizawa and Murooka, 1984, 1985.
Aspergillus niger	Glucoamylase	Boel et al., 1984a and 1984b.
Aspergillus awormori	Glucoamylase	Nunberg *et al*., 1984.

1.3.2 β-amylases.

β-Amylases occur widely in plants and, to a much lesser degree, in microorganisms. β-amylases catalyse the exohydrolysis of alternate α-1,4 glucosidic bonds, from the non-reducing end of the molecule to form β-maltose (downward mutorotation). β-Amylase can not hydrolyse the α-1,6 bonds of amylopectin or pass these branch points. Therefore, the result of prolonged digestion of amylopectin by β-amylase is a high molecular weight, branched limit dextrin (Banks and Greenwood, 1967). For complete digestion to maltose the combined action of a debranching enzyme (α-1,6 glucosidase) is required. There are few bacterial β-amylases reported and they are found mainly in *Bacillus* spp., *Pseudomonas* spp. and *Streptomyces* spp. None of the relevant structural genes have been cloned to date. There are also some novel β-type, exo-acting amylases, which do not yield maltose as their end product. *Pseudomonas stutzeri* produces an exo-acting amylase yielding maltotetraose as its final product . It can not pass α-1,6 branch points and thus forms a high molecular weight limit dextrin when amylopectin is used as substrate (Robyt and Ackerman, 1971). *Aerobacter aerogenes* yields maltohexaose from amylopectin by exo-attack, but can bypass α-1,6 linkages even though it can not hydrolyse them (Kainuma *et al*., 1972, 1975).

1.3.3 Glucoamylase (Amyloglucosidase).

Glucoamylase is found mainly in starch degrading moulds such as *Aspergillus* and *Rhizopus* spp. (Pazur, 1967 and 1972). Only two bacteria containing this enzyme has been reported in the literature. One is an obscure bacterium of unknown species (Caceres and Martinez-Peinado, 1980). The other is a *Flavobacterium* and its glucoamylase is active against cyclodextrins but has reduced activity against linear polysaccharides (Bender, 1981).

The action pattern of glucoamylase is similar to that of β-amylase, in that it catalyses the exohydrolysis of α-1,4 bonds from the non-reducing end of the molecule, but forms β-glucose rather than β-maltose (Reese *et al*., 1967). However glucoamylase has an unusual property, in that it can also hydrolyse α-1,6 bonds (albeit at a much slower rate than α-1,4 bonds). In theory glucoamylase should be able to completely degrade starch to glucose. However, studies with purified enzyme have shown incomplete hydrolysis, which is probably due to structural irregularities and modifications in the starch molecules (Abdullah *et al*., 1963). Recently, the structural genes for the glucoamylases from *A.niger* (Boel, *et al*., 1984a & 1984b) and *A.awormori* (Nunberg *et al*., 1984) have been cloned and sequenced from cDNA.

1.3.4 Debranching Enzymes (α-1,6 glucosidase).

α-1,6 glucosidases catalyse the exohydrolysis of α-1,6 bonds of amylopectin. There are three main types namely plant R-enzyme, bacterial pullulanase and isoamylase. Pullulanase is the bacterial equivalent of plant R-enzyme and has the same specificity. They both hydrolyse pullulan (an α-1,6 linked polymer of α-1,4 maltotriose; Figure 1.3) to liberate maltotriose, and the α-1,4 links of amylopectin to liberate α-1,4 maltodextrin. Pullulanase action on glycogen (a very highly branched form of amylopectin) is negligible, presumably due to steric hinderance. However when pullulanase is used in conjunction with β-amylase, complete digestion of glycogen can be obtained (Abdullah and French, 1970; Wober, 1976).

Isoamylase is similar to pullulanase in its specificity for α-1,6 linkages, but it can not degrade pullulan. It does however, debranch amylopectin faster than pullulanase and can also debranch glycogen (Harada, _et al_., 1968; Yokobayoshi _et al_., 1969 and 1970).

Pullulanase is found mainly in _Klebsiella_, _Streptomyces_ and _Bacillus_ spp. (Fogarty, 1983) while isoamylase has been found in _Escherichia_, _Pseudomonas_, and _Cytophaga_ spp.(Fogarty, 1983). Reports of pullulanase being found in _Escherichia_ spp. (Palmer, _et al_., 1973), have been proved to be erroneous (Dessein and Schwartz, 1974; Konishi _et al_., 1979). However, _E.coli_

Figure 1.3

Symbolic representation of a chain of pullulan, showing how it is cleaved to give maltotriose (G3) and G3 oligomers.

does contain a debranching enzyme (Jeanningross _et al_., 1976) which completely debranches amylopectin, but has little activity on glycogen. _E.coli_ also contains enymes involved in the intracellular breakdown of glycogen, including a glycogen debranching enzyme (Preiss, 1984).

1.4 The Transport of Proteins and Nutrients in E.coli.

In this section I would like to discuss the areas of protein and nutrient transport which are particularly relevent to this project. There are two intrinsic problems in engineering _E.coli_ to utilize starch;

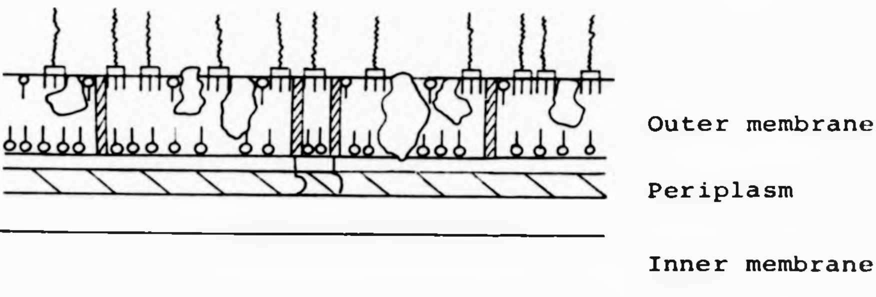
1) The amylolytic enzymes must be able to act on the substrate so, in this case, they must be at least extra-cytoplasmic (exported at least into the periplasm or) or might even need to be extra-cellular (secreted into the culture medium). The term "export" will be used to define protein transport from the cytoplasm to the periplasm and the term "secretion" will be used to define protein transport from the periplasm into the culture medium (Pugsley and Schwartz, 1985).

2) Once produced, the breakdown products must be transported into the cell, where they can be utilized as a carbon source.

Both of these functions involve transport across the *E.coli* cell envelope of both enzyme(s) and reaction products.

1.4.1 Structure of the E.coli Cell Envelope.

The cell envelope of *E.coli* (a Gram negative bacterium) consists of three main layers namely the cytoplasmic membrane, the periplasm and the outer membrane (Figure 1.4). The cytoplasmic membrane consists of a phospholipid bilayer and is involved in nutrient transport, energy coupling, DNA replication and synthesis of lipopolysaccharide, lipids and peptidoglycan (Costerton *et al*., 1974; Lugtenberg and Van Alphen, 1983). The space between the inner and outer membranes is called the periplasm and contains many proteins and chemicals involved in catabolic and transport functions. It also contains the peptidoglycan layer which is resposible for maintaining cell shape. The outer membrane is much simpler than the inner membrane and is firmly anchored to the peptidoglycan by covalently linked lipoprotein. The outer membrane is more permeable than the cytoplasmic membrane, carrying the porins (OmpF and OmpC) which act as a coarse molecular sieve (cut off $\approx M_r$ 650; Nakae, 1976). The outer membrane is also the site of the receptor proteins and antigenic determinants (for reviews see: Costerton *et al*., 1974; Inouye, 1979; Osborn and Wu, 1980; Lugtenberg and Van Alphen, 1983).



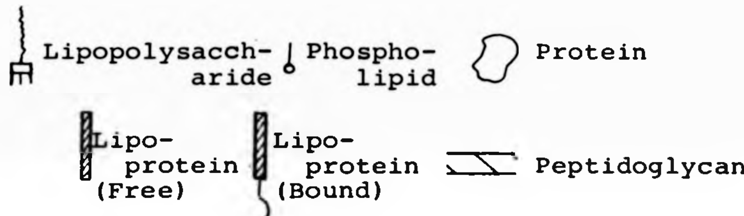


Figure 1.4

The E.coli Cell Envelope.

Simplified, diagramatic representation of the E.coli cell envelope showing its trilayer structure.

1.4.2 Protein Transport in E.coli

There are two major problems which must be overcome for a protein to be secreted by E.coli. Firstly the protein has to be exported across the inner membrane and into the periplasm. Secondly it must traverse the periplasm then cross the outer membrane. Although there is some evidence for secretion via "Bayers junctions" (Bayer, 1975) which would preclude the secreted protein from crossing the periplasm. Very few proteins are completely transported out of the cell in E.coli (i.e. across both membranes and into the extra-cellular environment). Those that are have a complicated export machinery which enables them to traverse the outer membrane and be released into the medium, e.g. hemolysin (Goebel and Hedgpeth, 1983) and colicins, such as cloacin DF13 (Oudega et al., 1982). With hemolysin there is very strong evidence that at least two other protein products (structural genes $hylB_a$ and $hylB_b$; Wagner et al., 1983) are required for translocation and release from the outer membrane of E.coli. The colicins are somewhat different in that it is thought the combined action of phospholipase A and "lysis protein" causes localised areas of increased outer membrane permeability which allow colicin and other periplasmic proteins to be released outside the cell, without releasing cytoplasmic proteins (Pugsley and Schwartz, 1985).

The generally accepted model for protein transport (Benson et al., 1985) is as follows. Translation of the exported protein is initiated by cytoplasmic ribsomes. Translation then proceeds until the N-terminal "signal sequence" emerges from the ribosome and this is then recognised by the signal recognition particle (SRP) which arrests further translation. The complex then interacts with a docking protein to form an export site in the cytoplasmic membrane; translation then resumes, docking protein and SRP are released, and the protein is transported through the membrane and the signal sequence is cleaved off by signal peptidase (Zwizinski and Wickner, 1980). Many aspects of this hypothesis have not yet been proven in prokaryotes and, in fact, the process is even more complex in that it has been shown that additional intragenic information plays a role in correctly locating proteins in the membranes (inner and outer) and periplasm of E.coli (Emr et al., 1980a & b; Bremer et al., 1982; Silhavy et al., 1983). Most of the evidence in favour of this model for protein export has come from a series of elegant genetic studies which have made extensive use of gene fusions and extragenic suppressor mutations (for reviews see: Halegoua and Inouye, 1979; Silhavy et al., 1979; Kreil, G., 1981 ;Silhavy et al., 1983; Benson, S.A., 1985; Pugsley and Schwartz, 1985).

Many mutations which affect protein localization in E.coli are suppressors of signal sequence mutations; prlA (secY), prlB and prlC (Emr et al., 1981; Emr and

Bassford, 1982; Shultz et al., 1982; Ito et al., 1983; Shiba et al., 1984). prlA is thought to code for a protein which is involved in signal peptide recognition (Pugsley and Schwartz, 1985). Other mutations having pleiotropic effects on protein export, perA/enzZ (Hall and Silhavy, 1981), expA (Dassa and Boquet, 1981) and dye (Buxton and Drury, 1984) affect the cell's ability to synthesise and export several different proteins, but how they do so is unknown. More is known about other pleiotropic mutations such as secA (Oliver and Beckwith, 1981, 1982), secB (Kumanamoto and Beckwith, 1983), secC (Ferro-Novick et al., 1984), and prlF (Kiino and Silhavy, 1984). These mutations affect protein export and synthesis, and are conditionally lethal. A number of mutants carrying mutations which are suppressors of secA have been isolated (Benson et al., 1985) and all of these confer a cold sensitive phenotype (i.e can not grow at 23°C). One of these mutatations maps in prlA and there is evidence that secA and prlA interact with each other (Brickman et al., 1984). Another mutation maps in secC (Ferro-Novick et al., 1984) and appears to be allelic with the rpsO gene (ribsomal protein S15; Benson et al., 1985) and the evidence suggests that the secC gene product is involved in the coupling of translation and export.

One aspect of protein transport that is very relevant to this project and has been intensively studied is the role of the "signal sequence" (Blobel and Dobberstein, 1975a; 1975b). It has been noted, with very

few exceptions (Buchel et al., 1980; Fried et al., 1981; Froshauer and Beckwith, 1984; Innis et al., 1984; Wolfe and Wickner, 1984; Yu et al., 1984), that both extra-cellular and extra-cytoplasmic proteins (i.e. inner membrane, periplasmic and outer membrane proteins) are synthesised with an extra 15-36 amino acid extension at their N-terminus, termed the signal sequence. The signal sequences of different extra-cellular proteins are generally not conserved at the DNA or amino acid level (von Heijne, 1985). However, they do share structurally similar domains namely, a positively charged hydrophilic N-terminus, a central hydrophobic region and a more polar C-terminus defining the cleavage site (Perlman and Halvorson, 1983; von Heijne, 1983). Many export-deficient mutants carry mutations which alter the signal sequence, especially in the hydrophobic region, showing its importance in the early stages of the export process (Emr et al., 1978; Bassford and Beckwith, 1979; Emr, et al., 1980a, 1980b; Bedouelle et al., 1980; Michaelis et al., 1983). For example, signal sequence mutations have been studied using lacZ gene fusions with the LamB protein. Some of these fusions lead to a Mal^{s} phenotype when grown on maltose due to the high level of expression of the hybrid protein blocking the cell's membrane export sites, resulting in cell death (Silhavy et al., 1977). Mal^{r} mutants can be selected which no longer export the hybrid protein. Export deficient mutations in the lamB gene can be selected by isolating Mal^{r} revertants from a Mal sensitive lamB-lacZ fusion strain (Emr and Silhavy, 1980).

The mutations in these revertants fall into three catagories:-

I) Mutations which introduce a charged residue into the hydrophobic core of the signal sequence. Mutations of this type block export of the LamB protein by altering the conformation of the signal sequence (Emr *et al*., 1980b).

II) Mutations which lead to a Mal^r phenotype by blocking export, but which have little effect when recombined into a wild type *lamB* background (Emr and Silhavy, 1982). These mutations also map in the hydrophobic core of the signal sequence.

III) Mutations which reduce expression of the hybrid protein by ≈80%. These mutations map in the N-terminal hydrophilic region of the signal sequence (Hall *et al*., 1983).

These results suggest that the structure of the central hydrophobic core is important in the initiation of protein export and that the N-terminal hydrophilic region may be involved with the coupling of export and translation (Benson *et al*., 1985).

1.4.3 Maltose/Maltodextrin Utilization in E.coli K-12.

The maltose/maltodextrin transport regulon of E.coli K12 is clustered in two regions of the chromosome, called malA and malB (Schwartz, 1966). The malB region, situated at 91 min on the genetic map (Bachmann, 1983) consists of two divergently transcribed operons named the malEFG and malK-lamB-molA operons (Hofnung, 1974; Raibauld, et al. 1979; Silhavy, et al., 1979). These two operons are specifically involved in the active transport of maltose and maltodextrins across the bacterial envelope.

MalF, G and K proteins are associated with the inner membrane (Silhavy et al., 1979; Shuman et al., 1980; Shuman and Silhavy, 1981). MalE is the periplasmic maltose binding protein [MBP](Kellerman and Smzelcman, 1974). Mutations in any of these mal genes lead to a Mal^- Dex^- phenotype (can not grow on maltose or maltodextrins). The molA gene was discovered by DNA sequencing of the downstream region of lamB (Clement and Hofnung, 1981). The molA gene codes for a periplasmic protein, but its function is unknown (M. Hofnung personal communication). LamB is an outer membrane protein which functions as the phage Lambda receptor and in the transport of maltodextrins (Randall-Hazelbauer and Schwartz, 1973; Szmelcman and Hofnung, 1975). Mutations in the lamB gene lead to a Dex^- phenotype and, under certain conditions a Mal^- phenotype (see 1.4.4).

The *malA* region is situated at 75 min on the genetic map and consists of the *malPQ* operon and the *malT* gene. The *malPQ* operon codes for amylomaltase and maltose phosphorylase respectively (Hofnung *et al*., 1971; Debarbouille *et al*., 1978; Debarbouille and Schwartz, 1979). These two proteins function in the intra-cellular utilization of maltose. The *malT* gene is the positive regulator of the whole system (Hofnung *et al*.,1971; Debarbouille and Schwartz, 1979) and is itself regulated by the catabolite activator protein [CAP](Chapon, 1982). The *malEFG* and *malK-lamB-molA* operons are controlled by *malT* and also require the action of CAP, whereas *malPQ* function is CAP independant and requires only *malT*.

1.4.4 Involvement of the LamB Protein in Maltose/Maltodextrin Transport.

When grown on maltose under normal laboratory physiological growth conditions (1-10mM maltose), *lamB* mutants have a Dex^{-} Mal^{+} phenotype (i.e. they can not grow on maltodextrins but, can grow on maltose). However, under conditions where the maltose concentration is low (less than 10uM) they have a Dex^{-} Mal^{-} phenotype (unable to grow on maltodextrins or maltose: Szmelcman and Hofnung, 1975), thus implicating the LamB protein in maltose transport at low substrate concentrations.

The LamB protein is also essential for transport of maltodextrins larger than maltotriose (M_r 504.5: Szmelcman and Schwartz, 1976; Wandersman *et al*., 1979)

which is just below the upper exclusion limit of the non-specific porins (Decad and Nikaido, 1976; Nakae, T., 1976; Nikaido and Nakae, 1979). In contrast, the upper exclusion limit for efficient maltodextrin utilization in wild type cells is maltoheptaose (M_r 1,152: Wandersman _et al_., 1979; Ferenci, 1980). Although maltodextrins up to M_r 2,500 (G15) can be transported via the LamB protein into the periplasm, _E.coli_ can not metabolise them (Ferenci, 1980). This is presumably because they are too large to be transported across the cytoplasmic membrane where they could be degraded by amylomaltase and maltose phosphorylase.

The specificity of maltose/maltodextrin transport is not a function of the LamB protein alone for there is a great deal of evidence for a LamB-MBP interaction which allows maltose oligosaccharides to be specifically transported (Wandersman _et al_., 1979; Heuzenroeder and Reeves, 1980; Ferenci, 1980; Ferenci _et al_.,1980a and 1980b; Bavoil and Nikaido, 1981; Bavoil _et al_. 1983). For example, one _malE_ containing mutant (_malE88_) which is defective in its interaction with the LamB protein, can not utlize maltodextrins or grow on maltose at low concentrations. It can grow on maltose even at high concentrations, but can not grow on maltodextrins even though it still has a high maltodextrin binding affinity (Wandersman _et al_., 1979). It has been suggested that the MBP alters the LamB protein from a non-specific pore to a specific maltodextrin pore. That the LamB protein can act as a non-specific pore has been shown

conclusively (Luckey and Nickaido, 1980; Heuzenroeder and Reeves, 1980), but recently it has been shown that the MBP has no influence on the transport of non-maltodextrin sugars (Brass et al., 1985). However, this does not disprove the theory that the MBP-LamB interaction leads to specificity for maltodextrin transport. The general theory is that the MBP-LamB interaction causes the maltoporin channel to open (Shuman, 1982; Hengge and Boos, 1983; Brass et al., 1985), thus explaning the phenotype of the malE88 containing mutant.

1.4.5 Interaction of Amylose and Amylopectin with the LamB Maltoporin.

Although the LamB maltoporin has low affinity for maltose, it does have a high affinity for maltodextrins and starch polysaccharides (amylose and amylopectin) (Ferenci, 1980; Ferenci, et al., 1980a, 1980b). The binding of amylose and amylopectin is specific for the LamB protein, for no binding is observed in lamB mutants and binding increases as the maltodextrins get larger. This binding has been shown to be MBP independent. However starch polysaccharides competively inhibit the binding of maltose and maltodextrins to the MBP (Ferenci, 1980a, 1980 b). These polysaccharides must therefore pass through the LamB maltoporin and into the periplasm where they interact with the MBP. The fact that E.coli can not utilize these polysaccharides is presumably because of the inability of the rest of the system to transport them to the cytoplasm.

1.4.6 lamB-independent Growth on Maltodextrins.

Recently, three outer membrane permeability mutants of *E.coli* K-12 have been isolated (Benson and Decloux, 1985). The relevant mutations allow *E.coli* to grow on maltodextrins with a M_r greater than 1,000 in the absence of the *lamB* gene. Two of these mutations map in the structural gene for the outer membrane protein OmpF (Lugtenberg and van Alphen, 1983) and the remaining mutation maps in the gene coding for the other major outer membrane protein, OmpC (Lugtenberg and van Alphen, 1983). Maltose transport was also greatly increased in strains carrying these mutations. The evidence suggests that the structure of the porins are altered so that the exclusion limit is increased thus allowing the cell to utilize maltodextrins with a M_r greater than 1,000. However it is not known if starch can gain access to the periplasm in these mutants and, even if it could, the starch could not be transported to the cytoplasm where it could be utilized (see section 1.4.4)

1.4.7 Transport of Maltose and Maltodextrins across the Cytoplasmic Membrane.

The MBP is also essential for transport across the cytoplasmic membrane. Evidence for this comes from studies on a mutant with a polar mutation in *malE* (Shuman, 1982). This strain sythesises all of the other components of the system constitutively, but can not grow

on maltose (even when the concentration is as high as 25mM). However, if the MBP is added back to the periplasm after calcium treating the cells, transport is restored (Brass et al., 1981). The current view on translocation (Schuman, 1982; Hengge and Boos, 1983) is that the MBP forms a complex with the substrate and this MBP-maltose/maltodextrin complex then interacts with the membrane bound components (MalF, G, and K) which effect the transfer into the cytoplasm. It is thought that this system may be analagous to the histidine transport system where MalF and MalG form the "pore" (eqivalent to hisQ and hisM) and MalK is possibly an ATPase (equivalent to hisP, Ardeshir, et al., 1981; Higgins et al., 1982). However the evidence is still somewhat circumstantial and remains to be proven.

1.4.8 The malP-Q Operon - Its Function in the Catabolism of Maltose and Maltodextrins.

After transport to the cytoplasm the maltose/maltodextrins can be utilized in intermediary metabolism. This is carried out by the combined action of amylomaltase and maltose phosphorylase (Wiesmeyer and Cohn, 1960a & 1960b; Schwartz and Hofnung, 1967; Hofnung et al., 1971).

Amylomaltase catalyses the reaction (I):-

(I) maltose + $(\alpha\text{-}1,4 \text{ D glucosyl})_n$

$\uparrow\downarrow$

glucose + $(\alpha\text{-}1,4 \text{ glycosyl})_{n+1}$

The glucose produced is then phosphorylated at the expense of ATP, to glucose-6-P (G6P). The smallest maltodextrin that can take part in this transglucosylation is maltotriose. Further maltose is an excellent donor , but a poor acceptor for the transglucosylation (Palmer et al., 1968). Thus the reaction (II) below does not occur:-

(II) maltose + maltose

$\uparrow\downarrow$

maltotriose + glucose

Maltose phosphorylase takes the maltodextrin produced in reaction (I) through to glucose-1-P (G1P):-

(III) $(\alpha\text{-}1,4 \text{ D glucosyl})_n$ + Pi

$\uparrow\downarrow$

(α-1,4 D glucosyl)n-1 + glucose-1-P

The G1P produced is then taken through to G6P and enters intermediary metabolism. Short, linear, α-1,4 linked maltodextrins are very active subtrates. The smallest maltodextrin which can be efficiently phosphorylated is maltopentaose (G5) so glucose and maltose can not act as substrates. Although maltotriose (G3) and maltotetraose (G4) are phosphorylated, this occurs at a 100 fold slower rate than when maltopentaose (G5), maltohexaose (G6) and maltoheptaose (G7) are used as substates by the maltose phosphorylase. (Schartz and Hofnung, 1967).

As discussed above, E.coli has all the machinery for efficient transport and catobolism of α-1,4 linked maltodextrins within the size constraints described. However, it is not known if α-1,6, α-1,4 linked maltodextrins are transported by the maltose/maltodextrin transport system. If they are, E.coli does possess cytoplasmic enzymes which can hydrolyse α-1,6, α-1,4 linked maltodextrins (see 1.3.4) to glucose.

1.5 Choice of Genes for Cloning in E.coli: General Considerations.

It is clear from the discussion above that there are a number of important points to consider when choosing enzymes which might enable E.coli to grow on starch:-

1) An enzyme which can hydrolyse α-1,4 linkages in starch is essential. For complete utilization of starch, an α-1,6 hydrolysing activity is probably needed, but not essential, i.e. if the maltodextrins produced by an α-1,4 digestion contained α-1,6 linkages and could be transported, they could be metabolised.

2) A liquefying α-amylase would be the best choice, as it will quickly reduce the viscosity of starch, thus making fermentation easier. This requirement excludes exo-acting β-amylases, which would also definitely require the action of a debranching enzyme for efficient utilization of starch.

3) The genes must be expressed in E.coli and therefore should preferably be of bacterial origin (most eukaryotic genes are much more difficult to clone and express in E.coli).

4) The end products of hydrolysis must be smaller than maltoheptaose for efficient transport and catabolism.

5) The enzymes must be transported out of the cell so they can act on the substrate. At the start of the project it was assumed that complete secretion from the cell and into the culture medium would be required.

For these reasons the initial approach was to clone the amylolytic enzymes from K.pneumoniae. This organism is very closely related to E.coli and can grow on starch. Also, it contains a debranching enzyme which is exported (pullulanase) and two amylases (see Chapter 3 and 4).

It was also decided, to clone a Bacillus spp. α-amylase. Although this would probably not be exported into the medium, the Bacillus amyloliquefaciens α-amylase cloned into E.coli is transported across the cytoplasmic membrane into the periplasm (Cornelis et al., 1982). The B.licheniformis α-amylase was chosen because the enzyme had been well characterised (see chapter 5). The enzyme has a high specific activity, its breakdown products would be efficiently transported/catabolised by E.coli and at the time, the structural gene had not been cloned.

CHAPTER 2

MATERIALS AND METHODS

2.1 Chemicals.

General reagents used in this work were supplied by BDH chemicals. Amino acids, carbon sources and specialist reagents were from Sigma. Ultra pure acrylamide and phenol was supplied by Bethesda Research Laboratories.

2.2 Radiochemicals

All radiochemicals were supplied by Amersham International.

2.3 Enzymes.

All restriction endonucleases were supplied by New England Biolabs or Bethesda Research Laboratories (BRL). DNA Polymerase (Klenow fragment), used in DNA sequencing and site directed mutagenesis, was obtained from Boehringer Mannheim. Polynucleotide Kinase was obtained from P.L. Laboratories and T4 DNA Ligase, AMV Reverse Transcriptase and S1 nuclease were from Biolabs. Ribonucleases A and T1 were obtained from Sigma.

2.4 Bacterial Strains.

The following bacterial strains were used.

E.coli K-12

HB101 - *leu*, *pro*, *thi*, *lacY*, *recA*, $r_k^- m_k^-$, *rpsL*, *endA* (Boyer and Roulland-Dussoix, 1969).

JM101 - (*lac-pro*)Δ, *supE*, *thi1*/F'*proA*$^+$,*B*$^+$, *lacI*^{q}, *lacZ*Δ*M15*, *tra*Δ*36* (Messing *et al*., 1981).

CSR603 - *recA1*, *uvrA6*, *phr-1* (Sancar *et al*. 1979)

MC4100 - *araD139*, Δ(*argF-lac*)*205*, *flbB5301*, *ptsF25*, *relA1*, *rpsL150*, *deoC1*, λ^- (Casadaban, 1979).

pop3208 - *araD139*, Δ(*argF-lac*)*205*, *flbB5301*, *ptsF25*, *relA1*, *rpsL150*, *deoC1*, *lamB204*am, λ^- (M. Schwartz).

TST1 - *araD139*, Δ(*argF-lac*)*205*, *flbB5301*, *ptsF25*, *relA1*, *rpsL150*, *deoC1*, *malE52*::Tn*10* λ^- (T.J. Silhavy).

TST6 - araD139, Δ(argF-lac)205, flbB5301, ptsF25, relA1, rpsL150, deoC1, malF55::Tn10, λ^- (T.J. Silhavy).

TST2 - araD139, Δ(argF-lac)205, flbB5301, ptsF25, relA1, rpsL150, deoC1, malG53::Tn10, λ^- (T.J. Silhavy).

pop3295 - Δ(argF-lac)205, flbB5301, ptsF25, relA1, rpsL150, deoC1, malK62am, λ^- (M. Schwartz).

MH70 - araD139, Δ(argF-lac)205, flbB5301, ptsF25, relA1?, rpsL150, bglR15, deoC1, malQ63am, λ^- (T.J. Silhavy).

HW2 - metE (M. Edwards).

HW14 - met, r_k^-, trpR, supE, supF (J-I. Flock).

HW87(WT217) - araD139(ara-leu)Δ7697, (lacIPOZY)Δ74, galU, galK, r_k^-, rpsL, srl, recA56 (Nugent et al., 1983).

HW1111 - tyrAΔ, $lacI^q$::Tn10 (N. Grinter).

WT264 - araD139(ara-leu)Δ7697 (lacIPOZY)Δ74 galU, galK, r_k^-, rpsL, srl, recA56 chromosomal low copy number (Nugent et al., 1983).

With the exception of JM101 all strains are F^-,

B.licheniformis

NCIB6346 - his (Rogers and Forsberg, 1971).

K.pneumoniae

HW205 - Unknown genotype (S. B. Primrose).

HW206 - Unknown genotype (S. B. Primrose).

2.5 Culture Media.

All recipes are for 1 litre of media.

Luria broth (LB) 10g tryptone, 5g yeast extract, 5g NaCl, 2g glucose; adjusted to pH 7. L-Agar plates were made by adding 15g/l agar.

10 x M9 Salts 60g Na_2HPO_4, 30g KH_2PO_4, 5g NaCl and 10g NH_4Cl.

M9-minimal media (M9) 100ml M9 salts, 2g sugar (as appropriate), 0.25g $MgSO_4.7H_2O$, 21.9mg $CaCl_2.6H_2O$ and 1mg thiamine. Plates were made by adding 15g/l agar.

M9-casamino acids minimal media as above with the addition of 5g/l casamino acids.

50 x Vogel and Bonner salts 10g $MgSO_4$, 100g citric acid, 500g K_2HPO_4 and 174g $NaNH_4HPO_4.4H_2O$.

Vogel and Bonner minimal medium (VB) 20ml VB salts, 2g sugar (as appropriate), 1mg thiamine and 1ml 0.01 $FeCl_3$. Plates were made by adding 15g/l agar.

Hershey salts 5.4g NaCl, 3g KCl, 1.1g NH_4CL, 22mg $CaCl_2.6H_2O$, 0.2mg $FeCl_3.6H_2O$, 87mg KH_2PO_4 and 7.1g Tris-HCl adjusted to pH 7.4.

Hershey Minimal Medium 2g maltose, 100mg threonine, 100mg leucine, 100mg proline, 200mg arginine and 1mg thiamine in one litre of Hershey salts.

NoPi minimal media (NoPi) 10g Bactopeptone, 2g maltose, 1.21g Tris-base, 2.03g $MgCl_2$, 2g NH_4Cl, 1g Na_2SO_4, 0.5g $CaCl_2$, 0.5g NH_4NO_3, 1g KCl, 0.05 proline, 0.05 leucine and antibiotic as appropriate; adjusted to pH 7.4.

NZCYM agar 10g N-Zamine, 5g NaCl, 5g yeast extract, 1g casamino acids, 2g $MgSO_4.7H_2O$ and 15g of agar, adjusted to pH 7.5.

NZCYM soft agar as above with 7g/l agar.

2 x YT broth 16g tryptone, 10g yeast extract and 5g NaCl.

YT agar 8g tryptone, 5g yeast extract, 2.5g NaCl and 15g of agar.

YT soft agar as above with 7g/l agar.

Carbenicillin (Cb) at 200 ug/ml and tetracycline (Tc) at 10 ug/ml was added as required.

2.6 Growth of bacteria.

For general maintenance of strains L-agar plates were used (plus antibiotic as appropriate) incubated at $37^{o}C$. Liquid cultures were incubated at $37^{o}C$ with shaking. All optical densities (OD) were measured at 670 nm unless stated otherwise. Cultures used for producing amylase or pullulanase enzyme extracts were grown in M9 + maltose liquid minimal medium.

2.7 Cloning.

2.7.1 Restriction fragment isolation.

All restriction endonucleases were used according to manufacturers recommendations. Analytical agarose gels were 0.8%, 1%, or 1.5% and were run in 1 x TBE buffer. Restriction fragment recovery from polyacrylamide gels was by electroelution. Restriction fragment isolation from agarose gels was carried out by extraction from low gelling temperature agarose (BRL) (Maniatis *et al*.,

1982). Fragments were visualised by long wave UV irradiation following staining by ethidium bromide (EtBr, 100ng/ml) and excised in as small a volume as possible (typically 100ul). The sample was then melted at 65°C in a total volume of 500ul in 300mM NaAc, 10mM Tris pH 8, 1mM EDTA. Agarose was then removed by three extractions with phenol (v/v 1:1), and traces of phenol removed by 2 extractions with ether (v/v 1:1). Fragments were then precipitated by addition of 2.5 volumes of ethanol and were then held for 1hr at -20°C, then recovered by centrifugation for 10 minutes in an Eppendorf microfuge. Fragments were washed once in 70% ethanol and taken up in 20ul of TE (10mM Tris pH 8, 1mM EDTA).

2.7.2 Fragment ligations.

Ligation of fragments was carried out typically in a volume of 20ul containing 100mM Tris pH7.5, 10mM $MgCl_2$, 10mM B-mercaptoethanol, 2mM ATP. Ligations of fragments possessing complementary ends were carried out for 4 hrs at room temperature. Blunt end ligations were carried out overnight at 25°C.

2.7.3 Preparation of plasmid DNA.

Plasmid DNA was prepared by the alkali denaturation method (Birnboim and Doly, 1979). For large scale preparations, (50 ml of cells), the use of ultracentrifugation was replaced by sephacryl gel filtration. Final precipitates of DNA prepared by the

alkali denaturation method were taken up in a total volume of 200ul containing 10mM Tris pH8, 1mM EDTA, 100ug/ml RNase A, 20ug/ml Rnase T1, and incubated for 30 mins at 37°C. To this was then added 20ul of 100% glycerol and the total volume was loaded onto a 30cm x 15mm Sephacryl S300 (Sigma) column. Column buffer of 10mM Tris pH8, 1mM EDTA, 150mM NaCl was pumped at a flow rate of 2ml/min. Detection of nucleic acid was by an in-line LKB Uvicord detector linked to a Kipp and Zonen BD40 chart recorder. Plasmid DNA yields of 50-200ug could typically be collected over a period of 30 min in a volume of 5-7ml, entirely free of contaminating cellular RNA, and the DNA was precipitated in 2.5 volumes of ethanol at -20°C for 1 hr and resuspended in TE.

2.7.4 Transformation.

Transformation of all strains of E.coli was carried out by the Ca^{++} method (Cohen *et al*., 1972).

2.7.5 Preparation of λ DNA.

Rapid λ DNA isolation was by the method of Davis *et al*., (1980). Large scale CsCl λ DNA preparations were by the method of Maniatis *et al*., (1982).

2.7.6 Construction of the *B.licheniformis* λ Charon 28 Genomic Library.

A genomic library of the *B.licheniformis* genome was constructed in λ Charon 28 (BRL) essentially as described in Maniatis *et al*., (1982). The internal *Bam*H I stuffer fragment was replaced with 15-20 kb partial *Sau*3A I fragments from *B.licheniformis* NCIB 6346. *In-vitro* packaging was by the method of Collins and Brunig, (1978).

2.7.7 Preparation of Chromosomal DNA.

K.pneumoniae chromosomal DNA was prepared by the method of Marmur, (1961). A much quicker method was employed for the preparation of chromosomal DNA from *B.licheniformis*. A 10ml overnight culture (L-broth) was pelleted (10,000 rpm 5 sec) and the cells were resuspended in 1ml of solution 1 (50 mM D-glucose, 25 mM Tris-HCL pH 8, 10 mM EDTA and 2 ug/ml lysozyme). After incubation for 5 min at room temperature 10 volumes of lytic mix was added (0.2% sarcosyl, 0.5 mM EDTA and 100 ug/ml proteinase K) followed by incubation at 50°C for 30 min. The lysate was made up to a density of 1.6 g/ml with CsCl, EtBr was added to 500 ug/ml and the total mixture was centrifuged for 17 hrs at 40,000 rpm in a Beckman VTi 50 rotor. DNA was collected by side puncture using a 21 guage syringe needle. Two volumes of H_2O were added and the EtBr was extracted with TE saturated

butanol. The DNA was precipitated with 2 volumes of ethanol, washed once in 70% ethanol, lyophilised and resuspended in 0.5 ml TE. This yielded ≈ 1 mg of chromosomal DNA which was pure enough to be digested with restriction endonucleases.

2.7.8 Restriction Mapping.

All restriction mapping was carried out as described in Maniatis *et al*., 1982.

2.8.1 DNA Gel Electrophoresis.

All poly-acrylamide gels used other than in DNA sequencing were 20cm x 15cm x 1mm. They were prepared from 30% stock (29%-Acrylamide, 1% Bis-acrylamide) and run in TBE buffer (90mM Tris HCL pH 8.3, 90mM boric acid, 2.5mM EDTA). Gels were polymerised by addition of 400ul of 10% ammonium persulphate and 30ul TEMED to 100ml of acrylamide/TBE solution. Electrophoresis was typically carried out for 16 hours at 300V. Denaturing gels were as native gels but included 7M urea. Gels used in DNA sequencing were 30cm x 20cm x 0.4mm. They were prepared as 6% (5.8%-Acrylamide, 0.2%-Bis-acrylamide) stock solutions containing 1 x TBE and 7M urea. Gels were polymerised by addition of 300ul of 10% ammonium persulphate and 40ul TEMED to 50ml of acrylamide/TBE/ urea. Electrophoresis was carried out for 2-6 hours at a constant power of 40W, depending on the extent of sequencing required.

Agarose gels were run on flatbed systems of 20cm x 15cm or 10cm x 8cm submerged in TBE buffer. For visualisation or extraction of specific fragments less than 200 bp in length, 2% gels were used. All other gels were of 1% agarose. For extraction of fragments, low gelling temperature agarose was used. Marker fragments of phage λ DNA digested with *Hind* III and phage ϕX174 digested with *Hae* III were used on all gels (Appendix 2). Single colony gels were run as described by Kado and Liu, (1981) in gels containing E buffer (40 mM Tris-acetate pH 7.9, 2 mM EDTA).

2.8.2 Protein Gel Electrophoresis.

Proteins were separated by SDS-PAGE as descibed by Laemmli, 1970. The upper stacking gel was 5%, the lower gel ranged from 12.5-8%. Samples were lysed in SDS sample buffer (10% glycerol, 0.01% bromophenol blue, 5% mercaptoethanol, 3% SDS, and 62.5 mM Tris pH 8) by boiling for 2 min. Gels were run for 2-3 hrs at 200-250 volts, stained for 1 hr in 25% methanol, 10% glacial acetic acid, 0.05% Coomassie brilliant blue then destained in 25% methanol, 10% glacial acetic acid and dried under vacuum at 80°C (Bio-Rad gel drier).

2.9 DNA Sequencing.

All DNA sequencing was carried out by the dideoxy chain termination method (Sanger *et al*.,1977). Shotgun

DNA sequencing was carried out as described by Bankier and Barrell, (1983) and primers were annealed for 30-60 min at 55°C in microtitre dishes. Sequencing primers were made on an ABS 380B DNA synthesiser, diluted to 2.5 ug/ml and used in the microtitre protocol instead of universal primer. Sequencing data was processed using the Staden computer programs (Staden, 1982 & 1984a, 1984b, 1984c).

2.10 Site Directed Mutagenesis.

Site directed mutagenesis of the B.licheniformis α-amylase promoter was carried out using the method of Zoller and Smith, (1982).

2.11 Synthesis of the oligonucleotides.

All oligonucleotides used in this work were synthesised by the oligonucleotide synthesis group of the organic chemistry department at G.D.Searle or by myself on an Applied Biosystems 280B DNA synthesiser, using solid phase phosphotriester chemistry (Ito et al., 1982).

2.12 Oligonucleotide primed reverse transcription.

2.12.1 RNA preparation.

Total cell RNA was prepared from E.coli as follows: a fresh colony was used as the inoculum for an

overnight culture, grown in M9 + maltose under antibiotic selection. The following morning 50 ml of culture in a 1 litre baffle flask were inoculated at OD_{670} of 0.01. When the culture reached an OD_{670} of 0.5 10 ml of culture was centrifuged at 10,000 rpm for 1 minute. For the tac/α-amylase strain the culture was induced at OD 0.4 and incubated for a further 30 min. Cells were washed in 10 ml buffer A (10mM Tris pH 7.5, 5mM $MgCl_2$, 10mM KCl,), and re-pelleted for 1 minute. The supernatant was removed and cells were taken up in 2 ml of buffer A plus 300 ug/ml T4 lysozyme and cells were immediately frozen in liquid nitrogen and then placed in a 65°C bath for 3 minutes. 220ul of 10% SDS was then added, at which point the solution became totally clear. To this solution was added 80ul of 3M NaAc pH 5.2 and an equal volume of phenol pre-heated to 65°C. The mix was then vortexed for 2 minutes and centrifuged for 5 min at 10,000 rpm. The aqueous layer was removed along with any interface and the process repeated until little or no interface was evident (typically 4 or 5 times). The aqueous layer was then extracted twice with an equal volume of ether. NaCl was then added to 2M and the nucleic acid precipitated by addition of 1.5 volumes of EtOH followed by storage at -20°C for 30 min. The nucleic acid was then centrifuged at 10,000 rpm for 10 min, lyophilised and resuspended in 1ml H_2O. When dissolved, NaCl was added to 3M and the solution was left overnight at 4°C to selectively precipitate the RNA. This was then centrifuged for 30 min at 10,000 rpm, washed 3x in 70% EtOH, lyophilised, taken up in 200ul H_2O and stored at -70°C. Yields were

between 100-400ug from 10 ml of cells.

2.12.2 Reverse Transcription to Identify Transcription Initiation Point.

Reverse transcription of messenger RNA prepared as described above was carried out in a micro-titre plate. Approximately 2ul of RNA (approximately 2ug) was annealed to approximately 2.5ng of oligonucleotide primer in a 10ul solution containing 10mM Tris pH7, 5mM $MgCl_2$, by heating to 85°C followed by slow cooling. To this was then added 1ul of dNTP mix (1 mM each of dGTP, dCTP and dTTP), 1ul of ^{35}S dATP, and 1ul of reverse transcriptase, and the mixture was incubated for 30-60 minutes at 37°C. After a 15 min chase with dATP (1 ul of a 1mM solution), 5ul of formamide dye mix (100% deionised formamide, 20mM EDTA, 0.03% xylene cyanol FF and bromophenol blue) was added and the mixture heated to 80°C for 15 minutes. This was then electrophoresed on a normal sequencing gel along with appropriate sequence reactions as indicators.

2.13 Enzyme Assays.

2.13.1 Preparation of Cells for Assay.

For amylase and pullulanase assays cells were grown in 50 ml M9 minimal medium (sugar as appropriate), pelleted, washed twice in 20 ml Tris pH 7 + 1 mM $CaCl_2$ buffer and resuspended in 1 ml of the same buffer. Cells were broken by sonication (2 x 30 sec at full power; MSE

Soniprep 150) and centrifuged for 5 min at 10,000 rpm to remove the whole cells. Extracts to be assayed for alkaline phosphatase and α-amylase cells were grown in NoPi + maltose medium then fractionated into cytoplasmic and periplasmic fractions (Willis et al., 1974). Extracts for β-galactosidase assay were grown in M9 + maltose minimal medium + 0.5 mM IPTG and fractionated as described above.

2.13.2 Amylase and Pullulanase Assay.

Amylase and pullulanase activity was measured by a 10-fold scale down of the dinitrosalicylic acid (DNSA) reducing end method (Bernfeld, 1955). An equal volume of substrate (2% starch or 5% pullulan) and suitably diluted enzyme (both in 50 mM Tris pH 7, 1 mM $CaCl_2$) were incubated at 37°C for 0-30 min. Samples (0.1ml) were taken at 5 min intervals and mixed with 0.1 ml of DNSA solution (1g DNSA, 0.4 M NaOH, 30g potassium sodium(+)-tartrate in 100 ml H_2O). After heating to 100°C for 5 min the tubes were cooled on ice, 1 ml of H_2O was added and the OD_{492} was determined. Specific activity was expressed as umoles maltose or maltotriose/min/mg protein by calibration with standard mixtures of maltose and maltotriose. Protein estimation was by the Bio-Rad protein assay (Bio-Rad Lab. Ltd.) against BSA standards.

2.13.3 B-Galactosidase assay.

B-Galactosidase activity was measured essentially as described by Miller, (1972).

2.13.4 Alkaline Phosphatase Assay.

Alkaline phosphatase was assayed as described by Glenn and Mandelstam, (1971).

2.14 Detection of Amylase Clones by Iodine Staining.

Amylase positive clones (plaques and colonies) were screened by the iodine staining method. Starch was added to the normal plating media (0.4%) after growth colonies or plaques containg amylase activity were detected by staining the plates with iodine vapour (iodine cystals heated to 60°C). Positive clones were identified as clear areas against a dark blue background (figure 2.1).

2.15 Expression of Plasmid Proteins in Maxicells.

The maxicell labelling technique used was essentially as described by Sancar *et al*., (1979). 0.1 ml of a plasmid containing culture of CSR603 was inoculated into 10 ml M9 + maltose + casamino acids supplemented with Cb. The culture was incubated at 37°C to an OD of 0.8-1. The culture was then transferred to a 2 inch 'Nucalon' petri dish and irradiated for 5 min

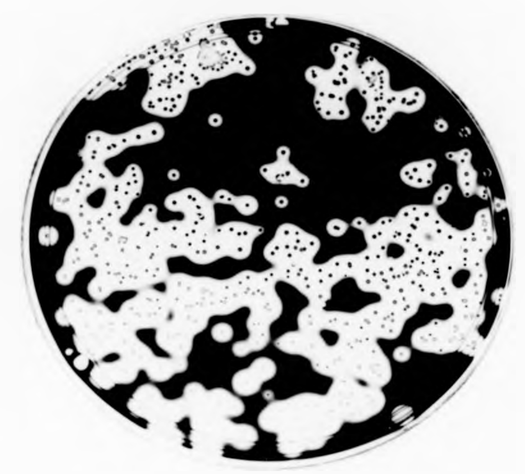


Figure 2.1

Detection of α-amylase clones by Iodine Staining.

HW87/pPT81 plated out on L-agar + starch plates after overnight incubation at 37°C followed by iodine staining. Clear halos around the cells are caused by amylolytic breakdown of the starch.

(with stirring) with a 254nm UV lamp placed ≈4 inches above the dish. Cycloserine was added (200 ug/ml) and the culture was incubated for a further 16 hrs. The culture was pelleted, washed in M9 salts and resuspended in 5 ml Hershey minimal medium (see 2.5). After incubation for 1 hr proteins were labelled by adding 25 uCi of ^{35}S-methionine followed by incubation for 1 hr. The cells were then pelleted, resuspended in SDS sample buffer, lysed, and run on a 12.5% SDS gel as described previously. After drying, the gel was subjected to autoradiography.

2.16 Two-dimensional Electrophoresis.

Two-dimensional electrophoresis was as described by O'Farrel, (1975). The first dimension was isoelectric focusing to equilibrium (7500 V hr) in 1.6% pH 5-7 and 0.4% pH 3-10 ampholine mixture. The second dimension was 10% SDS-PAGE as described previously. Proteins were labelled with ^{35}S-methionine.

2.17 Copy Number Determination.

Plasmid containing strains were lysed and electrophoresed by the method of Kado and Liu, (1981). An equivalent of 1ml of OD 0.5 culture was taken for each analysis. A strain carrying pBR322 was used as a standard and was assumed to have a copy number of 50.

Gels were scanned using a Joyce Loebl gel scanner and the relative copy number was calculated using the area of the pBR322 peak as the standard.

CHAPTER 3

CLONING AND CHARACTERISATION OF THE K.pneumoniae MALTOHEXAOSE PRODUCING AMYLASE GENE.

3.1 Introduction.

Klebsiella spp. are known to contain an α-amylase and a novel maltohexaose (G6) producing amylase (Kainuma, et al., 1972). Little is known about the α-amylase, but the maltahexaose exo-amylase has been purified and characterised (Kainuma, et al., 1975). It forms maltahexaose from starch and has the ability to bypass α-1,6 branch points, but not hydrolyse them; it has an M_r of 54,000 as determined by gel filtration; and 80% of its activity is retained after 15min at 50°C. The sub-cellular location of this enzyme is unknown.

3.1.1 Cloning Strategy.

Forty strains of K.pneumoniae were tested for their ability to utilize starch. All of the strains tested were able to grow on starch, but some grew faster than others. Chromosomal DNA was prepared from two of the fastest growing strains, HW205 and HW206. Unfortunately, during the preparation, they were accidently pooled together. As the DNA isolation protocol used at this time was quite lengthy, it was

decided to carry on and attempt to clone the amylase/amylases from the mixed chromosomal preparation.

The chromosomal DNA was cut with four restriction endonucleases with hexanucleotide recognition sequences; *Eco*R I (GAATTC); *Hind* III (AAGCTT); *Bam*H I (GGATCC); and *Bgl* II (AGATCT). These enzymes were chosen because they will cut infrequently within the chromosomal DNA, thus giving fragments of suitable size for a direct "shotgun cloning" into a plasmid vector. More than one enzyme was used in order to increase the chances of getting a complete and functional gene (i.e. there may be intergenic sites for some of these enzymes). The fragments generated from the digests were ligated into the plasmid vector pAT153 (Twigg and Sherratt, 1980) cut with *Eco*R I, *Hind* III, and *Bam*H I respectively (*Bgl* II has a four base 3' end complementary to *Bam*H I and can therefore be ligated into a *Bam*H I site). The vector-to-insert ratio was 1:50. *E.coli* HW87 (*recA*, *leu* strain with a high transformation frequency) was transformed with the ligation products and recombinants were selected on L-agar + Cb.

Approximately 3000 Cb^r colonies were obtained from each ligation, with only 20-30 on the control plates (vector without insert), thus indicating a recombination frequency of around 99%. All of the colonies were patched onto L-agar + carb and VBA + leu and incubated at 37^oC. After two days incubation 16 amylase positive clones were obtained from the *Hind* III library and two

from the Bgl II library. No amylase positive clones were obtained from either the BamH I or the EcoR I libraries.

Of the 16 clones obtained from the Hind III library, 5 grew slowly on amylose. Two explanations were, that either two different amylases had been cloned from the same strain (K.pneumoniae has at least two different amylases (Kainuma et al., 1975)) or two amylases had come from different strains and had different specificities. However, the four fast growing clones also grew on VBP + leu. This seemed to indicate that the clones must also have pullulanase activity. It was subsequently realised that Vogel and Bonner medium was not the ideal medium for selecting amylase clones. This medium contains citrate, which, ordinarily, E.coli can not utilise, because it does not have a citrate permease. Citrate chelates Ca^{++} and as amylases in general require Ca^{++} for activity VB medium was a bad choice.

All 16 clones were plated on M9 + leu containing either amylose (M9A) or pullulan (M9P) and VB + leu with the same carbon sources. All clones were also plated on VB + leu without any carbon source. The results in Table 3.1 show that the 11 fast growing clones (2-5, 7, 8, 10, 11, 13, 14 and 16) all grew on VB without an additional carbon source. However, they failed to grow on unsupplemented M9. Presumably the clones were growing on the citrate in VB and this probably indicates that the citrate permease gene from K.pneumoniae had been cloned.

Table 3.1

a) Growth of Putative *K.pneumoniae* Amylase Clones on Vogel and Bonner Minimal Media Containing Amylose or Pullulan.

Clone	VB + Amy	VB + Pull	VB
1	+	-	-
2	+	+	+
3	+	+	+
4	+	+	+
5	+	+	+
6	+	-	-
7	+	+	+
8	+	+	+
9	+	-	-
10	+	+	+
11	+	+	+
12	+	-	-
13	+	+	+
14	+	+	+
15	+	-	-
16	+	+	+
17*	+	-	-
18*	+	-	-

---

b) Growth of Putative *K.pneumoniae* Amylase Clones on M9 Minmal Media Containing Amylose and Pullulan.

Clone	M9 + Amy	M9 + Pull	M9
1	+	-	-
2	-	-	-
3	-	-	-
4	-	-	-
5	-	-	-
6	+	-	-
7	-	-	-
8	-	-	-
9	+	-	-
10	-	-	-
11	-	-	-
12	+	-	-
13	-	-	-
14	-	-	-
15	+	-	-
16	-	-	-
17*	+	-	-
18*	+	-	-

---

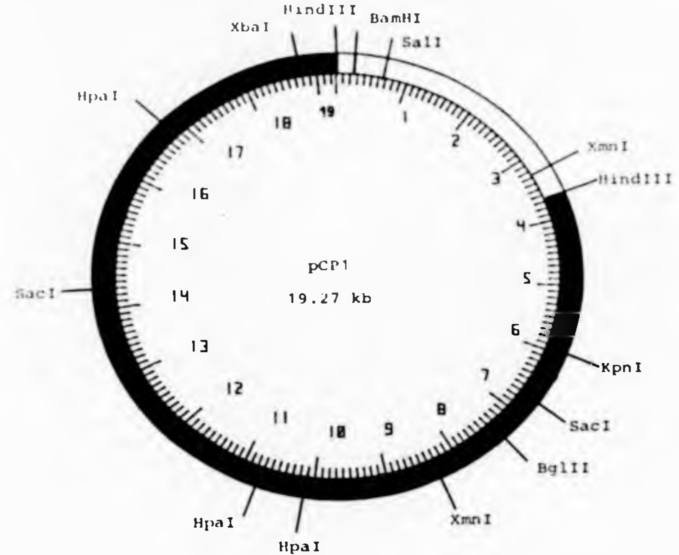
*: *Bgl* II clones, others are *Hind* III clones.
+: Growth at 37°C on agar plates after 48 hrs.
-: No growth, even after 7 days.
Apart from amylose (Amy) or pullulan (Pull) no carbon source was added to the VB or M9 media. All plates were supplemented with leu.

It was presumed that all the slow-growing clones (1, 6, 10, 12, 15, 17 and 18) were carrying the putative amylase gene(s). Restriction analysis subsequently confirmed that the putative citrate permease clone (pCP1, Fig. 3.1) was not related to the putative amylase clone (pPT1, Fig. 3.4). These results highlight the need for caution when deciding on an appropriate selection procedure in gene cloning experiments.

A gene bank for each enzyme was constructed by pooling all the colonies off each plate into 50 ml of L-broth, incubating overnight and isolating the plasmid DNA.

3.1.2 Restriction Analysis of the Hind III and Bgl II Clones.

Plasmid DNA was prepared from the 5 *Hind* III (1, 6, 9, 12 and 15; Figure 3.1 and 2 *Bgl* II (17 and 18; Figure 3.1) amylase clones. In the first instance they were analysed by digestion with *Pvu* II (CAGCTG), (Figure 3.2). The results suggest that the *Bgl* II and *Hind* III clones are related and there are two related clones of different sizes within each restriction class. These clones are probably carrying the same amylase and the difference in size could be due to the clones coming from two different strains (due to the mixing of the chromosomal DNA from HW205 and HW206). If this assumption is correct then the chromosomal organisation must be slightly different in the two strains. One of



Enzyme	Sites
BamH I	5
Cla I	5
Eco RI	6
Eco RV	5
Pst I	14
Pvu I	0
Pvu II	14
Sal I	5
Sma I	8
Sph I	4

Figure 3.1

Restriction Analysis of pCP1 (citrate utilization).

Restriction mapping was carried out as described in Chapter 2. The plasmid vector is pAT153 and the 15.6 kb insert is shown in black. Also listed are the enzymes which were used in the analysis, but the target sites of which were not accurately mapped. The number of sites refers to those sites found only within the cloned insert.

the Hind III clones (clone 1; figure 3.2) was named pPT1 and subjected to more detailed restriction anylysis. The other clones were not further characterized. Figure 3.3 shows the restriction enzymes used in this analysis and the restriction map of pPT1 is shown in figure 3.4.

3.1.3 Deletion Mapping of pPT1

To determine the exact location of the amylase gene within the insert, pPT1 was subjected to deletion analysis (see Chapter 2). Briefly, the 8.09 Kb insert was isolated, partially cut with Sau3A and the fragments up to 2.0 Kb were isolated and cloned into BamH I cut pAT153. The clones obtained were then screened for growth on starch. The 23 amylase positive clones obtained from this screen were analysed by single colony agarose gel electrophoresis (Kado and Liu, 1981; figure 3.5). On the basis of size, 8 amylase positive clones were chosen for restriction analysis (figure 3.6.1 & 3.6.2) The result of this analysis, and a diagrammatic summary of it, is shown in figure 3.7. It was concluded that the amylase gene could be sub-cloned on a 3.9 Kb EcoR I/Sal I fragment. This fragment was ligated into EcoR I/Sal I cut pAT153 (pPT11). To make the manipulation of the amylase insert easier and to help in future constructions, the Sal I site was made blunt by PolI filling the 3' overhang and an EcoR I linker was inserted to make pPT12 (figure 3.8).

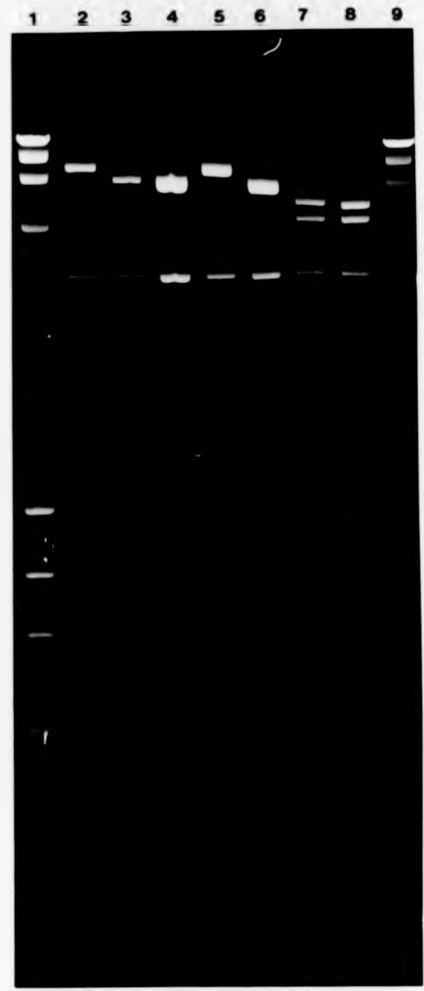


Figure 3.2

Pvu II Digestion of the Hind III and Bgl II Amylase Clones.

Plasmid DNA after digestion with *Pvu* II and electrophoresis in 1% agarose/T.B.E. The tracks are: 1 & 9, λ *Hind* III/ϕX174 *Hae* III; 2, *Hind* III clone 1; 3, *Hind* III clone 2; 4, *Hind* III clone 3; 5, *Hind* III clone 4; 6, *Hind* III clone 5; 7, *Bgl* II clone 1; 8, *Bgl* II clone 2.

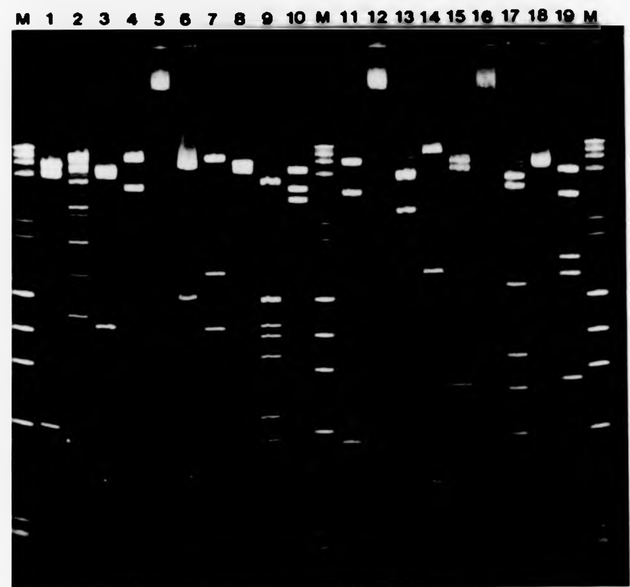


Figure 3.3

Enzymes used in the Restriction Analysis of pPT1.

Track No.	Enzyme	Sites in Insert
1	*Cla* I	2
2	*Ava* I	n
3	*Bam* HI	2
4	*Hind* III	2
5	*Bgl* II	0
6	*Xmn* I	2
7	*Hpa* I	3
8	*Kpn* I	2
9	*Pst* I	8
10	*Pvu* I	2
11	*Pvu* II	5
12	*Sac* I	0
13	*Sal* I	2
14	*Sma* I	3
15	*Sph* I	2
16	*Xba* I	0
17	*Eco* RV	6
18	*Eco* RI	1
19	*Nru* I	4

Plasmid DNA was digested with the restriction enzymes shown above and run on a 1.4% and 0.8% agarose/T.B.E. slab gel as described in Chapter 2. The 1.4% gel is shown above. M - λ *Hind* III/ϕX174 *Hae* III DNA markers. n: indeterminate number of sites.

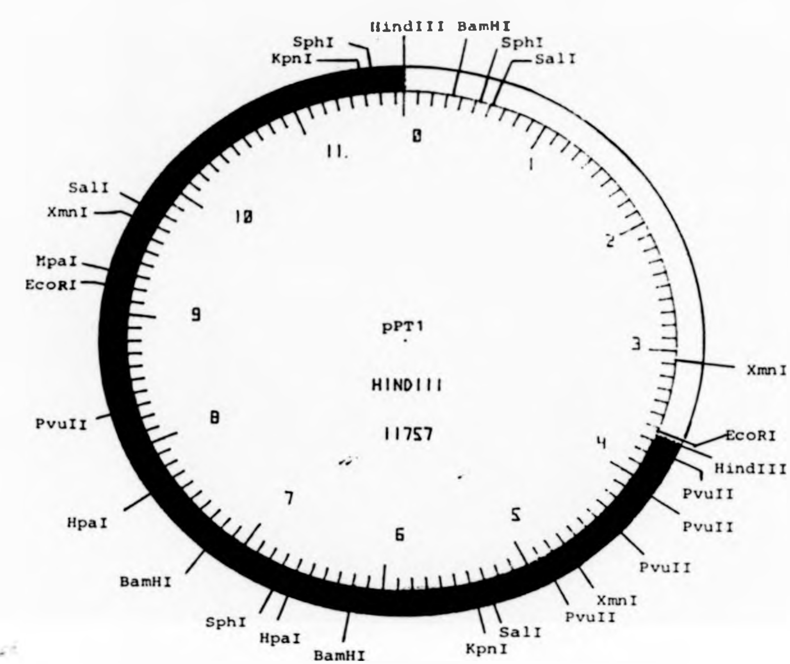
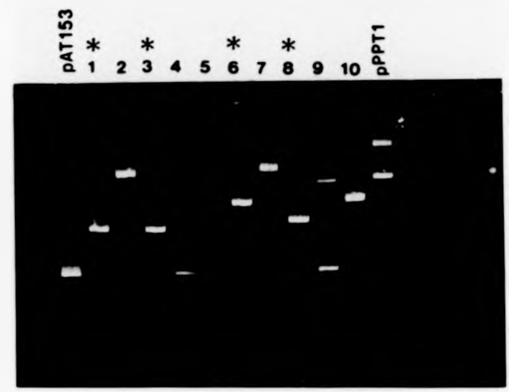


Figure 3.4

Restriction Map of pPT1.

pPT1 was restriction mapped as described in Chapter 2. The plasmid vector is pAT153 and the 8.09 kb Hind III insert is shown in black.



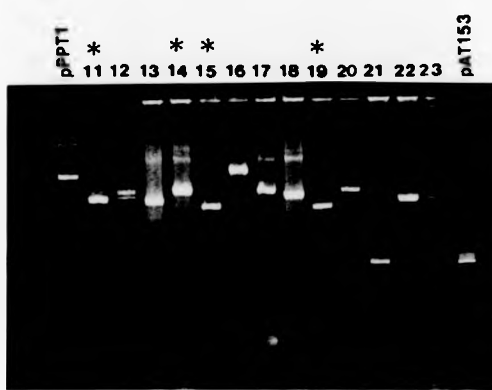


Figure 3.5

Single Colony Gel Electrophoresis of the Amylase-Positive pPT1 Sau3A I Deletion Derivatives.

Samples were prepared as described in Chapter 2 and run on a 0.8% agarose/T.B.E. slab gel at 150V for 3 hrs. Samples were flanked with size markers of pAT153 and pPT1. Samples 4, 9 and 21 are the same size as pAT153 and were discarded Samples 2, 7 and 16 are the same size as pPT1 and were also discarded.

*: deletion derivatives chosen for restriction analysis.

Figure 3.6.1

Restriction Analysis of Amylase Positive, pPT1 Sau3A I Deletion Derivatives.

Plasmid DNA was digested with restriction endonucleases, Sal I, Xmn I, Hpa I, Bam HI, Pvu II and run on a 1% agarose/T.B.E slab gel. The numbers denote plasmids selected after single colony gel analysis (Figure 3.5). M - λ Hind III/ ϕX174 Hae III DNA markers.

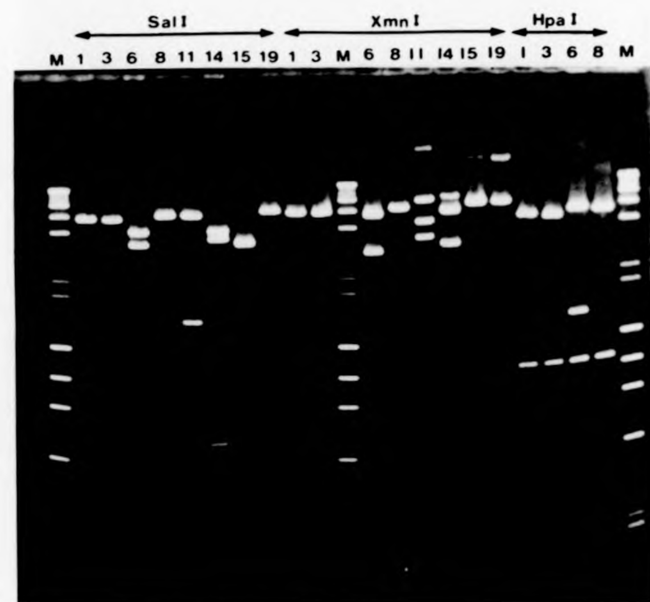
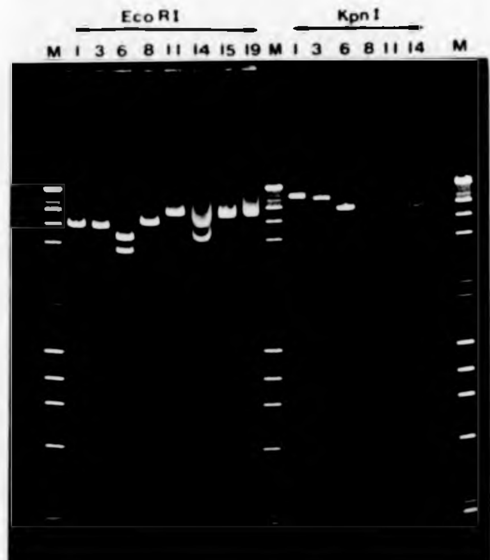
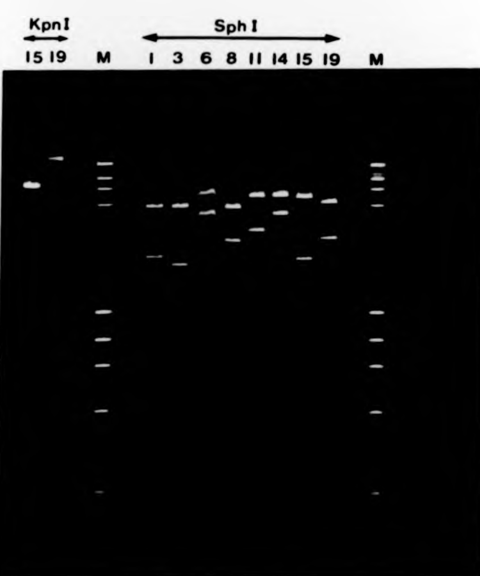


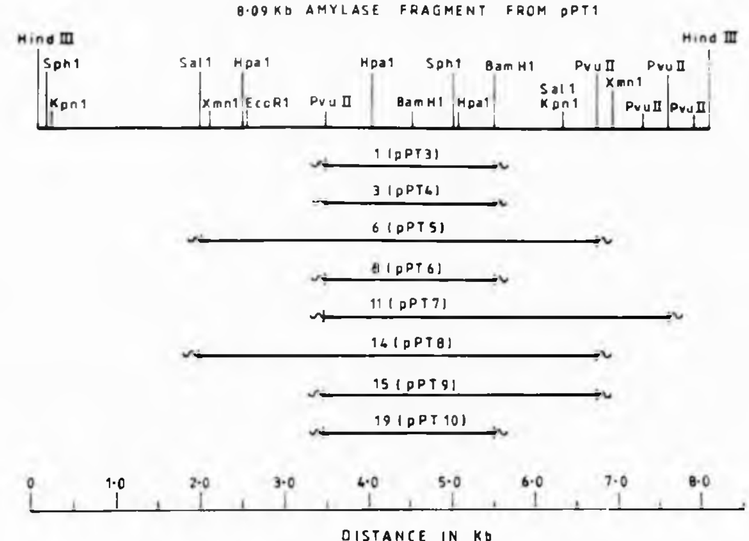
Figure 3.6.2

Restriction Analysis of Amylase Positive, pPT1 Sau3A I Deletion Derivatives.

Plasmid DNA was digested with restriction endonucleases, EcoR I, Kpn I, Sph I and run on a 1% agarose/T.B.E slab gel. The numbers denote plasmids selected after single colony gel analysis (figure 3.5). M: λ Hind III/ ϕX174 Hae III DNA markers.







Sites in Insert.

Plasmid	Sal I	Xmn I	Hpa I	Pvu II	Eco RI	Kpn I	Sph I
1 (pPT3)	0	0	2	1	0	0	1
3 (pPT4)	0	0	2	1	0	0	1
6 (pPT5)	2	1	3	2	1	1	2
8 (pPT6)	0	0	2	1	0	0	0
11 (pPT7)	1	2	2	4	0	1	1
14 (pPT8)	2	1	3	2	1	1	1
15 (pPT9)	1	0	2	2	0	1	1
19 (pPT10)	0	0	2	1	0	0	1

Figure 3.7

Deletion Analysis of pPT1 Deletion Derivatives.

Diagramatic representation of the restriction analysis shown in figure 3.6.

⌐ - DNA of unknown length, but deleted before the next restriction site along the insert.

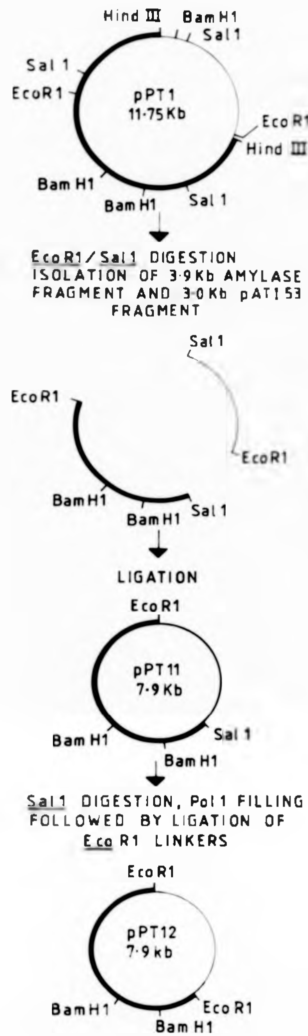


Figure 3.8

Construction of pPT12.

All manipulations were carried out as described in Chapter 2. The vector is pAT153 and the bold line represents the amylase insert.

3.2 Some Properties of the K.pneumoniae Amylase Expressed in E.coli.

3.2.1 Specific Activity in Crude Extracts.

All cultures were grown to stationary phase in M9M (maltose) + leu medium; washed in 50mM Tris pH 7.0, 1mM $CaCl_2$; resuspended in the same buffer and sonicated. After centrifugation (5,000 rpm for 5 min) to remove any whole cells, the extracts were used directly in a scaled down 3,5-Dinitrosalicylic acid (DNSA) reducing end assay (see Chapter 2).

Table 3.2

The Specific Activity (crude extracts) of the Cloned K.pneumoniae Amylase compared with that of HW205 and HW206.

Strain	µmoles maltose/min/mg protein
HW87/pAT153	<0.001
HW87/pPT12	0.08
HW205	0.02
HW206	0.098

Assays were carried out at 37°C at pH 7.0 with a substrate of 1% soluble starch.

Table 3.2 shows a comparison of the activity of the cloned amylase against that of the donor strains. It is clear from this that there is a five fold difference in the enzyme activity/expression between the wild type amylases in HW205 and HW206. The activity of the amylase in E.coli is also a lot lower than would be expected from

a clone on a high copy number plasmid (≈100 per genome). However, this does not take into account the fact that HW205 and HW206 have other amylolytic activities (e.g. pullulanase and possibly other amylases), which will also contribute to the overall activity. A small amount of residual activity was consistently obtained in E.coli HW87 extracts not containing the clone. This activity is presumably due to the intracelluar maltodextrin/glycogen degrading enzymes.

3.2.2 Action Pattern of the K.pneumoniae Amylase on Starch.

While determining the action pattern of this enzyme using crude extracts a problem was encountered. It appeared that the breakdown products produced by the amylase were also hydrolysed by enzyme(s) present in the crude extract, thus making end-product analysis difficult. This problem was particularly noticeable when looking at the breakdown products produced by the action of pullulanase (see Chapter 4). This hydrolysis of the end-products was presumed to be caused by the action of amylomaltase, therefore a malQ strain MH70 was used for the analysis. MH70 was transformed with pPT12 and grown under inducing conditions in tryptone maltose broth (TMB) then a crude extract was prepared. The extract was incubated with starch (1% final conc.) at 37°C and pH 7, samples were taken at various time intervals and subjected to T.L.C. (Hansen, 1975a & b). The T.L.C. chromatogram (figure 3.9) shows that initially (5-60min)

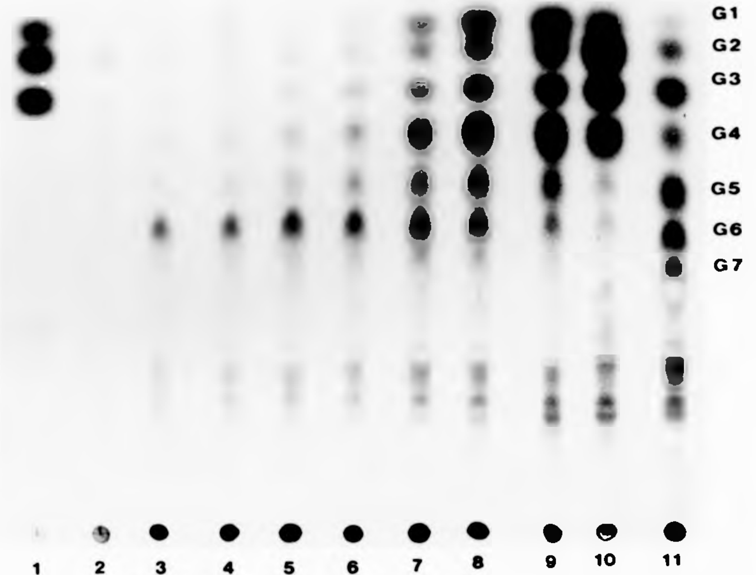


Figure 3.9

Action Pattern of the Cloned K.pneumoniae Amylase on Soluble Starch.

Samples were prepared as described in the text and subjected to T.L.C. as described in Materials and Methods (Chapter 2). Samples are 2 ul of: 1) glucose (G1), maltose (G2), maltotriose (G3) standard mixture (0.2%); 2) 0 time digest; 3) 5 min; 4) 10 min; 5) 15 min; 6) 30 min; 7) 1hr; 8) 2 hrs; 9) 4 hrs; 10) 24 hrs; and 11) starch hydrolysate (partial acid hydrolysis of soluble starch).

maltohexaose (G6) is the predominant end product which is characteristic of exo-acting amylase. However, smaller breakdown products (G1-G5) are also formed. After 24hrs the major products are maltotetraose (G4), maltotriose (G3), maltose (G2) and glucose (G1). As the maltohexaose is the predominant breakdown product of the highly purified enzyme, the rapid formation of smaller maltodextrins may be due to enzymes already present in E.coli.

3.2.3 The Effect of Glucose Maltose and Starch on Amylase Expression by HW87 carrying pPT1 and pPT12.

HW87/pPT1 and HW87/pPT12 were grown 16 hrs in 10 ml M9 + casamino acids + cb containing either, glucose, maltose or starch. Casamino acids medium was used because of the need to analyse mainly expression effects caused by the carbon source and not growth effects (i.e. the medium will support growth without an added carbon source). Samples containing 0.4 OD equivalents were analysed by SDS/PAGE Figure 3.10 shows that a protein of $\approx M_r 67,000$. is induced with maltose and starch. This protein is repressed by glucose and maltose is a much better inducer than starch. The protein is absent in the controls without pPT1 or pPT12 and as the gel results mirror the enzyme data (table 3.3) it is probably the K.pneumoniae amylase.

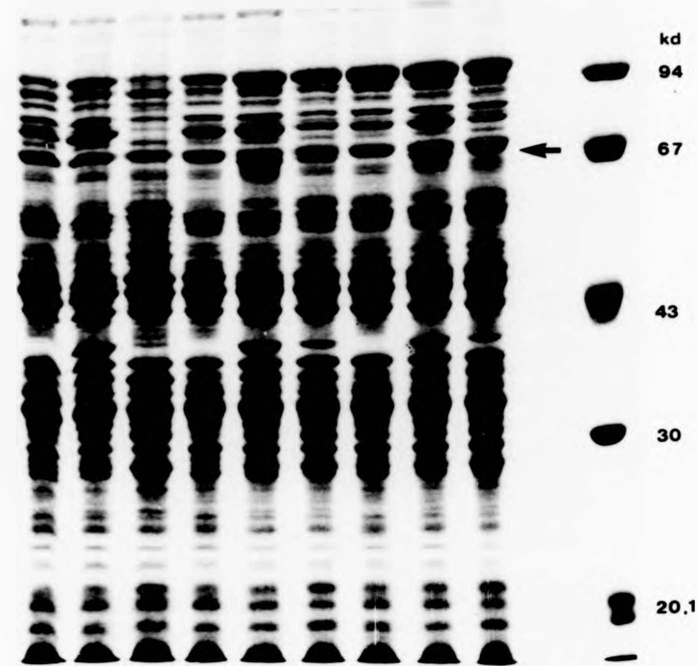


Figure 3.10

SDS-P A G E Analysis of Proteins from HW87/pPT1 and HW87/pPT12 When Cultured in Glucose, Maltose, and Starch Minimal Medium.

Samples were prepared as descibed in the text and run on a 0.8% SDS-P A G E as described in Chapter 2. The tracks are: 1) HW87 glucose; 2) HW87 maltose; 3) HW87 starch; 4) HW87/pPT1 glucose; 5) HW87/pPT1 maltose; 6) HW87/pPT1 starch; 7) HW87/pPT12 glucose; 8) HW87/pPT12 maltose; 9) HW87/pPT12 starch; 10) Protein markers. The arrow indicates a protein band which is induced by maltose and starch in strains carrying pPT1 and pPT12, but is absent in the plasmid free controls.

It is of note that when one compares the controls grown on maltose and starch (figure 3.10; tracks 2 & 3), that starch does not induce the proteins involved in maltose/maltodextrin transport and utilization. For example a protein of M_r39,000. (probably the MBP) is clearly induced by maltose, but not by starch. This is indirect evidence that the starch can not cross the inner membrane. Both the protein of M_r39,000 and the putative amylase are induced in HW87 carrying both of the amylase clones (figure 3.10; tracks 6 & 9) grown in the presence of starch, but to a lesser extent than when the same strains are grown on maltose. This shows that the starch is being broken down into units which are small enough to be transported across the membrane thus inducing the maltose operons, but suggests that either the end product of breakdown (maltohexaose, G6) is a poor inducer of the system or that it is produced in low concentrations (thus explaining the slow growth rate on starch). However, it is known that E.coli can grow as well on maltodextrins up to maltaheptaose (G7) as on glucose (Wandersman et al., 1979), and so the latter of the two possibilities is probably the correct interpretation. This does not rule out the possiblity that the amylase, when induced, is toxic to the cell causing a slower growth rate. There is a certain amount of evidence for this (Chapter 4, figure 4.13), but even taking this into account the clones still grow much more slowly on starch than they do on maltose (table 3.4).

Table 3.3

Effect of Glucose, Maltose and Starch on Amylase Activity in Crude Extracts.

Strain	Glucose	Maltose	Starch
	umoles maltose/min/mg protein.		
HW87/pPT1	0.00	0.066	0.026
HW87/pPT12	0.00	0.052	0.027

Cells were grown to stationary phase in 50 ml of M9 + casamino acids + appropriate sugar, then processed as described in Chapter 2. The reaction time was 5 min at 37°C, pH 7 with starch as substrate (1%).

3.2.4 Two-Dimensional Electrophoresis of HW87/pPT12.

The proteins from HW87/pPT12 were subjected to two-dimensional electrophoresis as described in Chapter 2. The results (figure 3.11 (B)) show a spot of M_r67,000. which is absent in the HW87 control and therefore is plasmid encoded. This spot comigrates with the band seen on the SDS-PAGE gel in section 3.2.3. This is additional evidence in favour of the hypothesis that this protein is due to the cloned *K.pneumoniae* amylase since no other major plasmid encoded products (apart from the *tet* and *bla* gene products) can be seen. However, it is worth noting that comparisons of two-dimensional protein gels can be very misleading and many minor differences can be seen between the HW87 control gel and the HW87/pPT12 gel.

Figure 3.11

Two-Dimensional Electrophoresis of Proteins from HW87/pPT12.

Cells were labelled with ^{35}S-methionine and processed as described in Chapter 2. The first dimension isoelectric focusing to equilibrium (7500 V hr) was in 1.6% pH 5-7 and 0.4% pH 3-10 ampholine mixture; the second dimension was in 10% SDS-P.A.G.E. A) HW87 without plasmid; B) HW87/pPT12 (amylase clone). a - putative amylase protein.

3.3 Starch Utilization.

3.3.1 Growth of E.coli on Starch using the K.pneumoniae Amylase.

Although pPT12 allows E.coli to grow on starch, this growth is slower than on glucose or maltose (table 3.4). The doubling time in liquid M9 minimal media + starch for this strain is 184 min (Chapter 4; figure 4.14).

Table 3.4

Comparative Growth of HW87/pAT153 and HW87/pPT12 on Glucose, Maltose and Starch.

Strain	Carbon Source	48 hrs	96 hrs
HW87/pAT153	glucose	+++	+++
HW87/pAT153	maltose	+++	+++
HW87/pAT153	starch	-	-
HW87/pPT12	glucose	+++	+++
HW87/pPT12	maltose	+++	+++
HW87/pPT12	starch	+	++

Comparative growth on solid medium (M9 + leu).
- (no growth) to +++ (full growth).

3.3.2 Effect of Reducing Plasmid Copy Number on Starch Utilization.

To study the effect of lowering the copy number and thus the expression of the *K.pneumoniae* amylase in *E.coli*, WT264 (a chromosomal copy number mutant of HW87; Nugent *et al*., 1983) was transformed with pPT1 and pPT12. WT264 maintains the copy number of ColE1 derived plasmids at ≈one copy per chromosome. No growth was observed with the low copy derivatives (table 3.5).

Table 3.5

Effect of Low Plasmid Copy Number on Starch Utilization.

Strain	Growth on Starch.
HW87/pPT1	+
WT264/pPT1	-
HW87/pPT12	+
WT264/pPT12	-

WT264 derivatives were incubated for one week at 37°C without significant growth.

3.3.3 Effect of Mal Mutations on Starch Utilization.

A a number of *mal* strains were transformed with pPT1 and pPT12. All of these strains have an isogenic background except for the *malK* strain, which does not have the *araD139* mutation and the *malQ*, which has one extra mutation, *bglR15*. The ability of these strains to grow on starch and maltose was examined. Table 3.6 shows

the result of this experiment. The mutations give rise to the expected phenotype with respect to maltose, in that the only mutation which does not lead to a Mal^- phenotype is the lamB mutation, because mutations in malE, F, G, K and malQ, all have a Mal^- phenotype and lamB mutations only lead to a Mal^- phenotype at low maltose concentrations (see Chapter 1). Growth on starch shows a different phenotype. Like maltose malE, F, G, K all lead to a Dex^- phenotype (i.e. can not grow on maltodextrins), but the lamB mutation also causes a Dex^- phenotype. It is interesting that the malQ lesion has a Dex^+ phenotype, although the growth rate is reduced when compared to that of the wild type strain (MC4100/pPT12), which suggests that the cell can utilize maltodextrins via maltose phosphorylase alone. It has previously been shown that in vitro maltose phosphorylase has no activity against maltose (Schwartz and Hofnung, 1967). This is evidence that in vivo the maltose reaction can not occur, but maltodextrins larger than maltose can participate in the reaction.

Table 3.6

Effect of Maltose Mutations on Starch Utilization by *E.coli* carrying the *K.pneumoniae* Amylase.

Strain*	Growth: Maltose	Growth: Starch
lamB$^+$/pPT1(MC4100)	+++	++
lamB/pPT1(pop3208)	+++	-
malE/pPT1(TST1)	-	-
malF/pPT1(TST6)	-	-
malG/pPT1(TST2)	-	-
malK/pPT1(pop3295)	-	-
malQ/pPT1(MH70)	-	+
lamB$^+$/pPT12	+++	++
lamB/pPT12	+++	-
malE/pPT12	-	-
malF/pPT12	-	-
malG/pPT12	-	-
malK/pPT12	-	-
malQ/pPT12	-	+

+ to +++ relative growth after 48 hrs.
- no significant growth after 6 days. All plates incubated at 37°C. * - see strain list for complete genotype.

Discussion.

From the results described in this chapter it can be concluded that the maltohexaose (G6) producing amylase has been cloned from K.pneumoniae. It is also clear that this enzyme has a low specific activity especially when it is compared to both the cloned K.pneumoniae pullulanase (Capter 4) and the B.licheniformis α-amylase (Chapter 5). Assuming that the protein of M_r 67,000 does in fact correspond to the amylase it is quite highly expressed in E.coli (1-2% of total cell protein under inducing conditions).

The amylase gene, cloned on a high copy number plasmid does, allow E.coli to utilize starch, but the growth rate is extremely slow. Because the enzyme has a low specific activity this slow growth rate is most likely due to the enzyme not being able to provide maltohexaose quickly enough. This hypothesis is supported by the observation that reduction in plasmid copy number leads to a Dex^- phenotype. The amylase is also an exo-amylase, so α-1,6 branch points will have to be hydrolysed otherwise limit dextrins will be formed (see Chapter 1). Although this amylase can bypass α-1,6 branch points it does so at a much reduced rate (Kainuma et al., 1975). Because E.coli can utilize starch when it contains this enzyme must mean that the amylase is actually reaching the substrate, therefore it is very interesting that lamB mutants can not grow on

starch. The conclusion drawn from this is that the amylase must be exported, at the very least, into the periplasm (because starch can gain access to the periplasm but not the cytoplasm, Chapter 1: section 1.4.1. A model for the mechanism of starch utilization by E.coli will be discussed later (see discussion, Chapter 5).

Another point which became obvious when trying to sort out the action pattern of this amylase is that E.coli contains enzymes which can very efficiently break down maltodextrins. Even in the absence of amylomaltase which was presumed to be the main degradative enzyme, the maltohexaose produced was quickly degraded to smaller dextrins (predominantly G1-G4). Some of this degradation is due to the action of the amylase itself as it can hydrolyse maltohexaose to maltose and maltotetraose (G4), but at a very slow rate (Kainuma et al., 1975). Therefore the bulk of this activity must come from E.coli. This is unlikely to be due to the action of maltose phophorylase, because there was no added phosphate source, but there is the possiblity that the malQ mutation is leaky. This point could be easily checked out by carrying out similar experiments in a malQΔ strain.

From the above discussion it can be concluded that the K.pneumoniae maltohexaose amylase can not fulfil the main aim of this project i.e. be used to allow E.coli to utilize starch as sole carbon and energy as well as it

can use glucose, although it does demonstrate the feasibility of the general approach.

CHAPTER 4

CLONING AND CHARACTERISATION OF THE K.pneumoniae PULLULANASE GENE.

4.1 Introduction.

The *K.pneumoniae* pullulanase has been well characterized and plays an important role in the breakdown and efficient utilization of starch by this organism (Wober, 1976; Wohner and Wober, 1978; Ueda and Marshall, 1980). It is a large protein in the region of M_r 145,000 (Wallenfels et al., 1966; Wohner and Wober, 1978; Eisele et al., 1972). In *Klebsiella* it exists in two forms, bound to the outer membrane and free in the culture medium (Wallenfels et al., 1966; Wohner and Wober, 1978). The extracellular form occurs in late logarithmic growth and release can be prevented by specific culture methods (Wallenfels et al., 1966; Hope and Dean, 1975). There is some evidence for a factor (called inductive effector, Ohba and Ueda, 1982) which causes the release of cell bound pullulanase into the media.

Pullulanase is induced by pullulan, maltodextrins, maltotriose and maltose and repressed by glucose (Wallenfels *et al*., 1966; Hope and Dean, 1974; Wohner and Wober, 1978; Ohba and Ueda, 1982). Recently it has been

shown that the cloned Klebsiella pullulanase gene (pulA) (Michaelis et al., 1985) is positively regulated by the malT gene product in E.coli. The promoter for pulA has now been sequenced and it has similarities with other malT controlled promoters (Chapon and Raibaud, 1985), but does not have a recognisable CAP binding site.

When pullulanase is expressed in E.coli it is not localised in the same way as in K.pneumoniae, in that it is found mainly associated with the inner and outer membranes and is not released into the culture medium and thus is not accessible to its substrate. (Takizawa and Murooka, 1984; Takizawa and Murooka, 1985; Michaelis et al., 1985). Of great interest is the recent discovery that pullulanase is a lipoprotein (Pugsley et al., 1986). This is the first report of secretion of a lipoprotein by a Gram negative organism and may have important implications for protein export. The evidence also suggests that pullulanase is modified and processed in E.coli in the same way as it is in K.pneumoniae. The fact that E.coli can not correctly localise pullulanase (see above) in the outer membrane, or secrete it into the medium, suggests that E.coli lacks at least one other gene product which functions in the secretion of this enzyme.

4.1.1 Cloning Strategy.

The pullulanase gene was selected by direct screening of the *Eco*R I, *Hin*d III, *Bam*H I, and *Bgl* II *K.pneumoniae* plasmid gene banks (see chapter 3). HW87 was transformed with DNA from each gene bank and selection was for growth on pullulan. The *Eco*R I gene bank produced 108 clones by this method, but no clones were obtained from the rest. Eight of the colonies from the *Eco*R I library were further characterised by digestion with *Pvu* II and all showed the same gel pattern indicating that they were duplicates of the same clone (figure 4.1). One of these, pPT14, was selected for more detailed restriction analysis.

4.1.2 Restriction Analysis of pPT14.

Plasmid pPT14 was digested with 19 restriction enzymes which have hexanucleotide recognition sites (figure 4.2). A restriction map was constructed for enzymes having up to three sites within the insert (figure 4.3) the rest were not mapped.

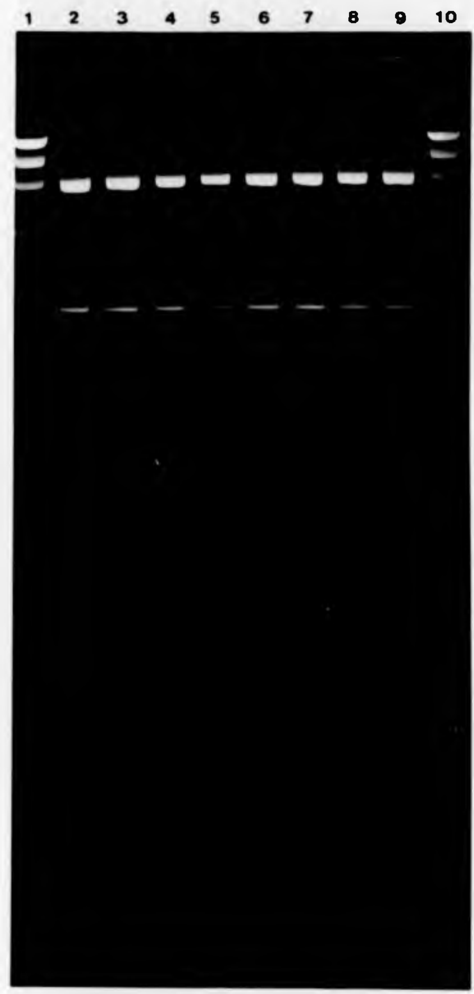


Figure 4.1

Digestion of Putative Pullulanase Clones with Pvu II.

Plasmid DNA from the eight putative pullulanase clones were digested with Pvu II and run on a 1% agarose/T.B.E. slab gel as described in Chapter 2. The tracks are: 1) & 10) λ Hind III/ϕX174 Hae III M.W. markers; 2-9) putative pullulanase clones 1-8. All of the clones give the same restriction pattern with this enzyme.

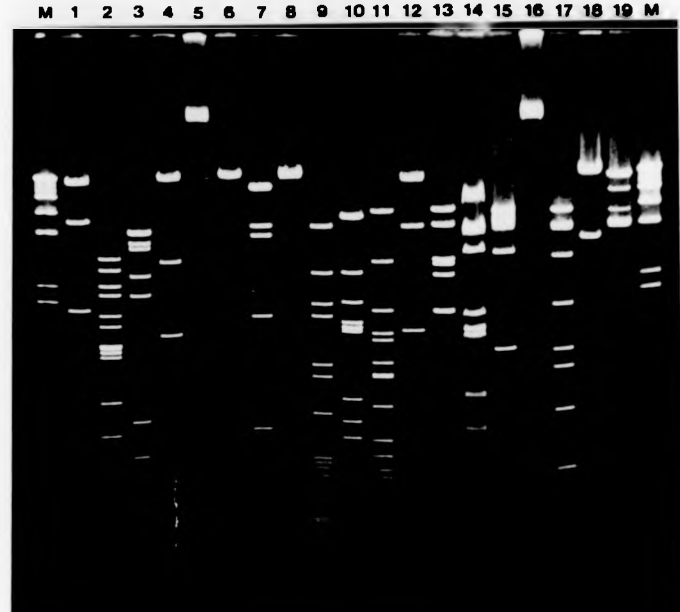


Figure 4.2

Enzymes used in the Restriction Analysis of pPT14.

Track No.	Enzyme	Sites in Insert
1	*Cla* I	2
2	*Ava* I	14
3	*Bam* HI	8
4	*Hind* III	2
5	*Bgl* II	0
6	*Xmn* I	1
7	*Hpa* I	6
8	*Kpn* I	1
9	*Pst* I	n
10	*Pvu* I	8
11	*Pvu* II	16
12	*Sac* I	3
13	*Sal* I	7
14	*Sma* I	10
15	*Sph* I	5
16	*Xba* I	0
17	*Eco* RV	8
18	*Eco* RI	2
19	*Nru* I	2

Plasmid DNA was digested with the restriction enzymes shown above and run on a 1% agarose/T.B.E. slab gel as described in Chapter 2. M - λ *Hind* III DNA markers. n: indeterminate number of sites.

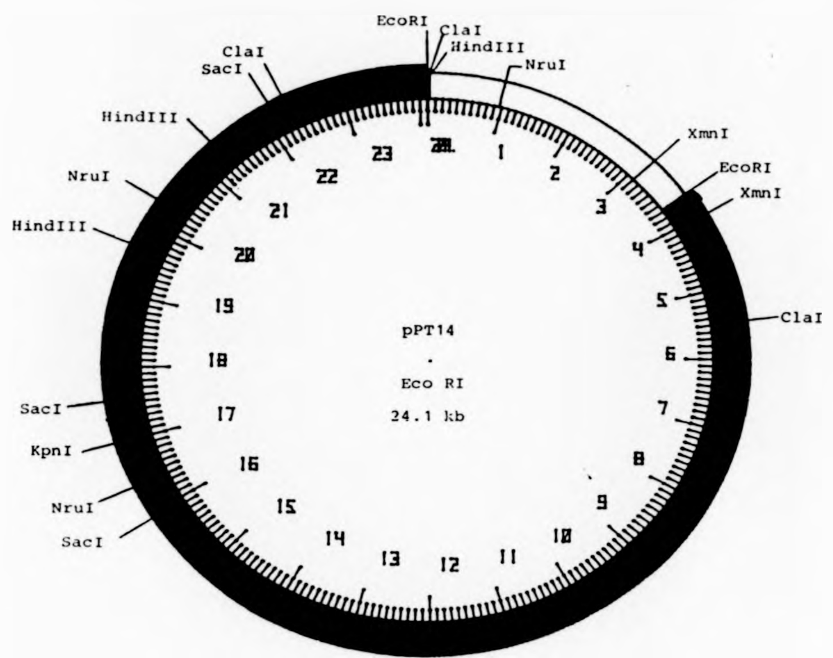


Figure 4.3

Restriction Map of pPT14

Restriction mapping was carried out as described in Chapter 2. The plasmid vector is pAT153 and the 24.45 kb EcoR I insert is shown in black.

4.1.3 Location of the Pullulanase Gene within the Insert.

Two main approaches were taken to determine the exact location of the gene within the insert:

a) insertional inactivation of the gene using Bgl II linkers (there are no Bgl II sites in the insert or vector).

b) the subcloning of specific DNA fragments and screening for pullulanase activity.

4.1.4 Insertional Inactivation by Bgl II Linkers.

pPT14 was linearised by partial digestion with Rsa I (GTAC) subjected to LGT agarose gel electrophoresis and the linear band was isolated (figure 4.4) . Rsa I has a tetranucleotide recognition site which cuts many times within the insert, but only twice within the vector, giving DNA with flush ends. Bgl II linkers (GAGATCTC) were ligated into the linear fragments at a 50 fold excess to the available ends, to prevent recircular-isation of the linear plasmid. Full size clones were selected by single colony gel electrophoresis (figure 4.5). By this method 52 full sized clones were selected then checked for growth on pullulan and their Bgl II sites were mapped.

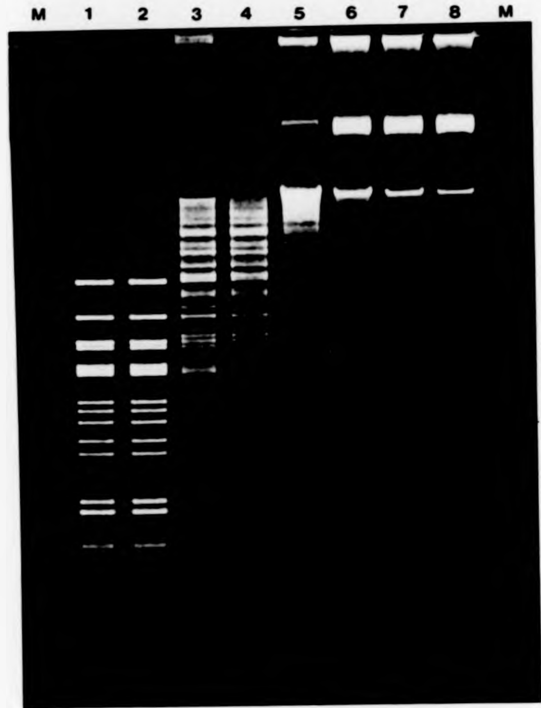


Figure 4.4

Isolation of Linear, Cut pPT14 using Rsa I.

Plasmid DNA (1ug) was digested with doubling dilutions of Rsa I then loaded onto a 1% LGT agarose slab gel as described in Chapter 2. The tracks are: 1) neat enzyme; 2) 1/2 dilution; 3) 1/4 dilution; 4) 1/8 dilution; 5) 1/16 dilution; 6) 1/32 dilution; 7) 1/64 dilution; 8) 1/128 dilution; M - λ Hind III markers. Amount of enzyme was 1ul of each dilution in a 20ul reaction volume. The 24.1 kb linear band from tracks 5-8 was extracted from the agarose and Bgl II linkers (GAGATCTC) were ligated into the blunt sites created.

Figure 4.5

Single Colony Gel of Pullulanase Clones with Bgl II Insertions.

Samples were prepared as described in Chapter 2 and run on a 0.8% agarose/E-buffer gel at 150V for 3 hrs. pPT14 prepared in the same way was used as a size marker. Tracks are: 1) pPT14; 2) - 24) Putative full sized clones. All the clones which were the same size as pPT14 were selected for further analysis. Mixed preparations (more than one plasmid band, due to cross-contamination) which had a full size Bgl II insertion were purified to single colonies and rescreened.

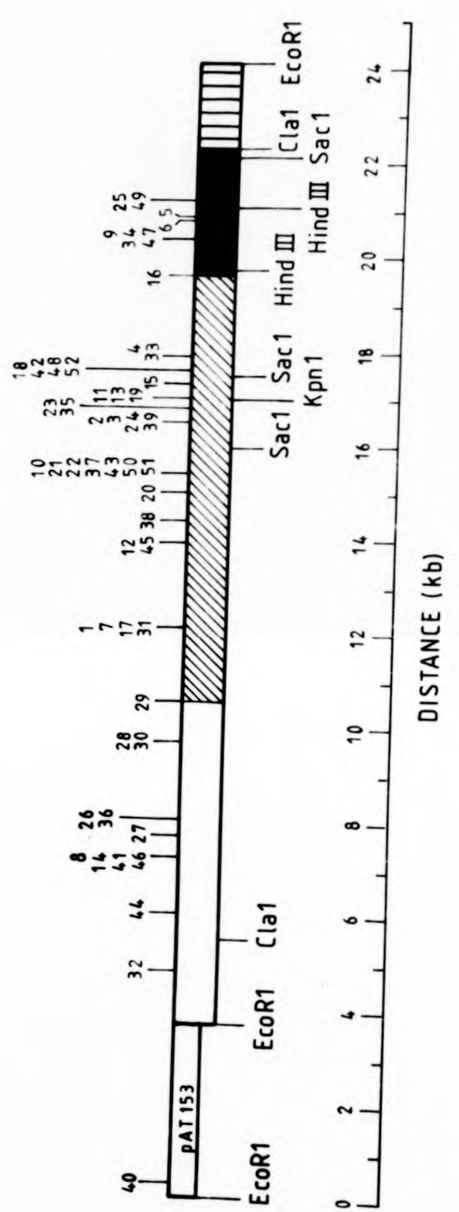


Figure 4.6

Restriction Map of Bgl II Insertions in pPT14: Their Effect on Growth on Pullulan in E.coli HW87.

The 52 Bgl II insertions were mapped by a combination of EcoR I/Bgl II, Kpn I/Bgl II, and Xmn I/Bgl II double digests. The effect on growth on pullulan was determined (table 4.1) and is diagramatically represented as follows: The open box shows the insertions which had no effect on growth; Cross hatch shows insertions which result in a slow growth phenotype; The filled box shows insertions which result in a no growth phenotype (Pul$^-$); Vertical bars (between Cla I and EcoR I) depicts an area where it is unknown if insertions affect growth, but it is known that deletion at this Cla I site leads to a Pul$^-$ phenotype. The numbers refer to individually selected full-size clones.

As can be seen in figure 4.6 and table 4.1 the insertions caused three phenotypes namely, clones which did not grow on pullulan, clones which grew on pullulan as well as the parental plasmid and clones which had a reduced growth rate on pullulan.

All of the clones showing insertional inactivation were clustered between 18.75 and 22.2 kb in the insert (figure 4.6) indicating that the structural gene for pullulanase was situated in this area. This was also confirmed by specific deletions (see below). The insertions which gave a slow growth phenotype were spread over a wide area (10.3 to 18.0 kb, figure 4.6). The rest of the insertions (0 to 8.75 kb) grew as well as the parental plasmid. As expected an insertion at one of the *Rsa* I sites within the vector was obtained, the other *Rsa* I site is within the Ap^r gene so would not be isolated because the initial selection was for Ap^r. As this insertion mutant also grew as well as the parental plasmid containing strain on pullulan it acted as an internal control for the comparative growth effects of the insertions between 10.3 and 18.0 kb.

Although this method of mapping is initially more cumbersome than traditional methods, such as transposon inactivation, it does offer some advantages:

1) The linker can be mapped exactly in the insert.

Table 4.1

Effect of Bgl II Plasmid Insertions on the Growth of HW87/pPT14 on Pullulan.

Insert No.	48hrs	72hrs	96hrs
1	+	++	+++
2	+	++	+++
3	+	++	+++
4	+	++	+++
5	-	-	-
6	-	-	-
7	-	-	-
8	+	++	+++
9	-	-	-
10	+	+	++
11	+	++	+++
12	+	++	+++
13	+	++	+++
14	+	++	+++
15	+	++	+++
16	-	-	-
17	+	++	+++
18	+	++	+++
19	++	+++	+++
20	+	++	+++
21	+	++	+++
22	+	++	+++
23	+	++	+++
24	-	+	+++
25	-	-	-
26	+++	+++	+++
27	+++	+++	+++
28	+++	+++	+++
29	+	++	+++
30	+++	+++	+++
31	+	++	+++
32	+++	+++	+++
33	++	+++	+++
34	-	-	-
35	+	++	+++
36	+++	+++	+++
37	+	++	+++
38	-	+	++
39	-	+	++
40	+++	+++	+++
41	-	+	++
42	-	+	++
43	-	+	++
44	+++	+++	+++
45	-	+	++
46	+++	+++	+++
47	-	-	-
48	-	+	++
49	-	-	-
50	-	+	++
51	-	+	++
52	-	+	++
pPT14 control	+++	+++	+++

The medium was M9 + pullulan + leu. - (no significant growth) to +++ (full growth).

2) It enables the subcloning of specific fragments without having large amounts of "foreign" DNA; i.e. it allowed the subcloning of the pullulanase gene on an EcoR I/Bgl II fragment (see below).

3) Extra internal restriction sites are formed which makes sequencing by specific fragments easier (Fotheringham et al., 1986).

There are a few disadvantages which may give false results:-

1) Small deletions may occur where Rsa 1 sites are close together.

2) There is no direct selection for the insertion event (e.g. antibiotic), so the only way to confirm an insertion event is by a restriction digestion analysis.

4.1.5 Deletion Analysis of pPT14.

A number of specific restriction fragments were subcloned into pAT153 and their ability to confer growth on pullulan was determined. Figure 4.7 shows the results of this, confirming the position of the pullulanase gene somewhere on the 7.0 kb EcoR I/Kpn I fragment.

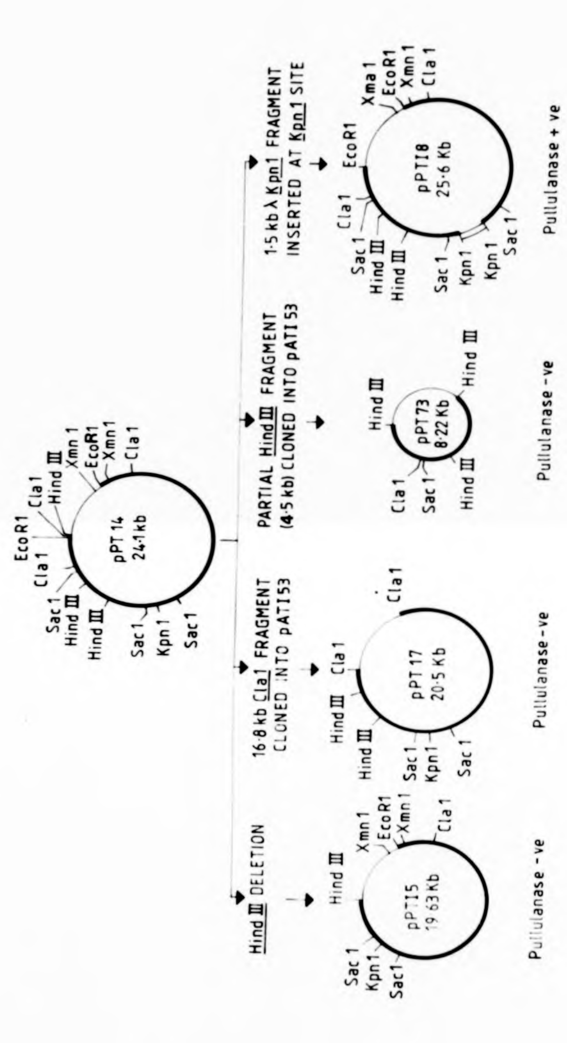


Figure 4.7

Determination of the Location of the Pullulanase Gene on pPT14.

All manipulations were carried out as described in Chapter 2. The Hind III and Cla I sites interrupt the structural integrity of the pullulanase gene, but the λ 1.5 kb fragment does not. From the above information it can be concluded that all the information required for pullulan utilization is found on the DNA fragment clockwise of the Kpn I site. However, it should be noted that the λ insertion, like the Bgl II insertions (figure 4.6) leads to a slow growth phenotype.

A 1.5 kb *Kpn* I fragment was also inserted into the *Kpn* I site of pPT14 resulting in a pul$^+$ phenotype (figure 4.7). However, as with the *Bgl* II insertions which mapped in this area, clones with this insertion grew much more slowly than the parental plasmid (pPT14). In fact a *Kpn* I site also has an *Rsa* I within it thus some of the *Bgl* II insertions would map in the same place.

4.1.6 Subcloning of the Pullulanase Gene.

The subcloning of the gene was relatively simple, as the clones containing the *Bgl* II insertions could be used. Three clones were chosen with *Bgl* II insertions at different distances from the nearest *Hind* III site, namely clones 3, 18 and 19 (figure 4.6). The subclones were named pPT76, pPT77 and pPT78 respectively. As pAT153 does not contain a *Bgl* II site one was created by inserting a *Bgl* II linker at its *Nru* I site. The full cloning procedure for pPT77 is shown in figure 4.8 pPT76 and pPT78 were also constructed in the same way.

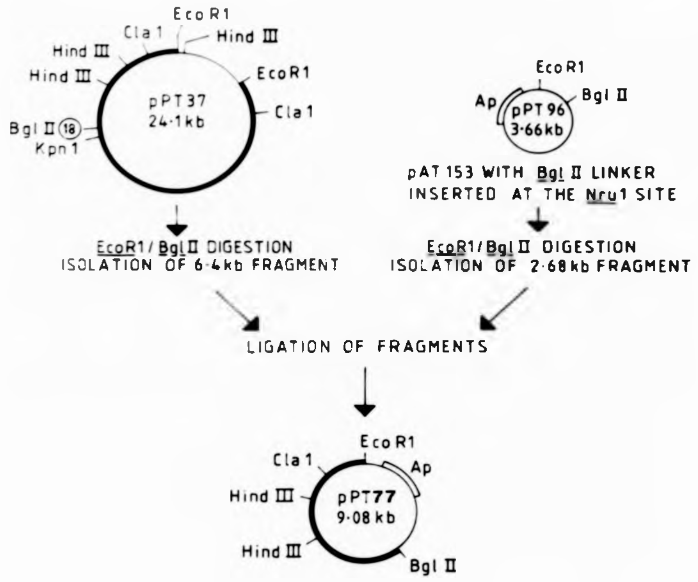


Figure 4.8

Subcloning of the Pullulanase Gene.

All manipulations were carried out as described in Chapter 2. pPT37 is the Bgl II No. 18 shown in figure 4.7. Similar contructs were made using Bgl II insertions 3 (pPT36) and 19 (pPT38) to give pPT76 and pPT78 respectively. This gave three different sized clones containing the pullulanase gene.

4.2 Properties of the Pullulanase Gene Expressed in E.coli.

4.2.1 Specific Activity in Crude Extracts of HW87 carrying pPT14, pPT76, pPT77 and pPT78.

The pullulanase clones were grown to stationary phase in M9 maltose + leu + Cb medium, extracts were prepared and the specfic activity was determined by the DNSA assay using pullulan as subtrate. Specific activity is expressed as umoles maltotriose/min/mg protein. The pullulanase activity of the K.pneumoniae donor strains was also determined (table 4.2).

Table 4.2

Specific Activity of Pullulanase Expressed in E.coli HW87

Strain	μmoles maltotriose/min/mg protein.
HW87/pAT153	0.00
HW87/pPT14	0.92
HW87/pPT76	3.96
HW87/pPT77	3.86
HW87/pPT78	3.47
HW205	0.98
HW206	0.28

Assays were carried out at 37°C at pH 7.0, substrate 5% pullulan.

HW87/pPT14 has a specific activity equal to the best *K.pneumoniae* strain (HW205) and only threefold higher than HW206. This is surprising when one considers that the cloned gene is on a multicopy plasmid. All of the subclones show a fourfold increase in activity over the full sized clone. This increase in activity is expected as the reduction in size of the plasmid is likely to lead to an effective increase in the copy number. No activity was found in HW87 extracts which did not contain the pullulanase gene, showing that *E.coli* does not contain an enzyme which can degrade pullulan.

4.2.2 Effect of Glucose, Maltose and Pullulan on Expression.

HW87 carrying pPT14, pPT76, pPT77 or pPT78 was grown to stationary phase in VB casamino acids medium containing either glucose, maltose or pullulan. Samples containing 1ml 0.4 O.D. were taken then analysed by SDS-P A G E. As can be seen in figure 4.9 the pullulanase gene is repressed by glucose and induced by both maltose and pullulan, but maltose is a marginally better inducer than pullulan. The cloned pullulanase is also the same M_r as that of the purified enzyme (Sigma Chemical Co.). The enzyme data (table 4.3) also confirm the gel observations.

The gel also shows quite clearly that pullulan itself does not induce elements of the maltose/maltod-extrin utilization system. The M_r 39,000 band

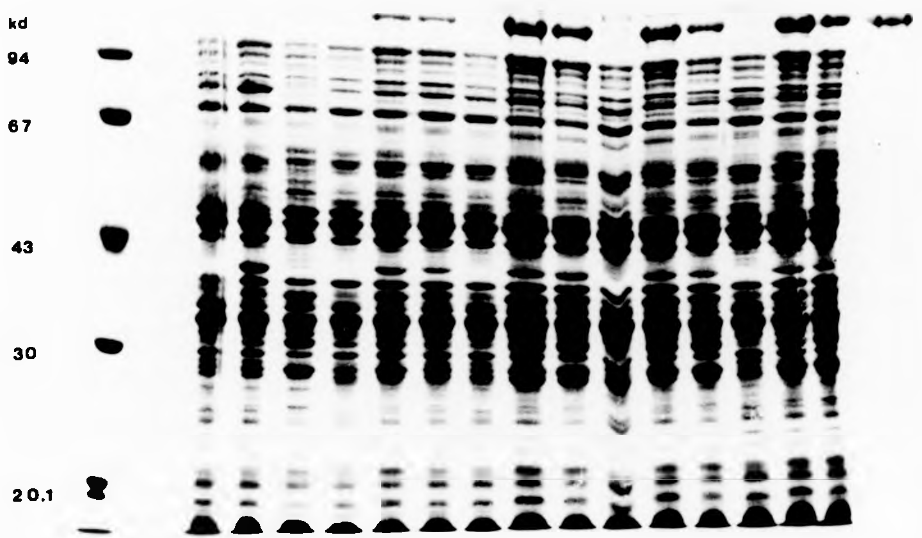


Figure 4.9

SDS-P A G E Analysis of Proteins from HW87 Pullulanase Derivatives, Cultured on Glucose, Maltose or Pullulan Minimal Medium.

All cultures were grown in M9 + casamino acids + Cb containing either glucose, maltose or pullulan. Samples containing 0.4 OD_{670} equivalents were run on a 0.8% SDS-P A G E as described in Chapter 2. The tracks are: 1) HW87 glucose; 2) HW87 maltose; 3) HW87 pullulan; 4) HW87/pPT14 glucose; 5) HW87/pPT14 maltose; 6) HW87/pPT14 pullulan; 7) HW87/pPT76 glucose; 8) HW87/pPT76 maltose; 9) HW87/pPT76 pullulan; 10) HW87/pPT77 glucose; 11) HW87/pPT77 maltose; 12) HW87/pPT77 pullulan; 13) HW87/pPT78 glucose; 14) HW87/pPT78 maltose; 15) HW87/pPT78 pullulan; 16) Purified pullulanase (Sigma Chemical Co.). M - protein markers.

corresponding to the MBP is absent in the HW87 control grown in the presence of pullulan (figure 4.9, track 3), whereas it is clearly evident in tracks containing the pullulanase clones (figure 4.9, tracks 6, 9, 12 & 15). This is analogous to what was found with the amylase clones growing on starch (Chapter 3). However, in the case of the pullulanase clones the induction is much better, being about the same as maltose. This is probably a reflection of the much higher specific activity of the pullulanase.

Table 4.3

Effect of Glucose, Maltose and Pullulan on Enzyme Activity in Crude Extracts Prepared from HW87/pPT14 and HW87/pPT76.

	μmoles maltotriose/min/mg protein		
Strain	Glucose	Maltose	Pullulan
HW87/pPT14	0.063	0.72	0.60
HW87/pPT76	0.29	3.15	2.83

Cells were grown to stationary phase in 50 ml of M9 + casamino acids + appropriate sugar, then processed as described in Chapter 2. Reaction time was for 5 min at 37°C, pH 7 using pullulan (5%) as substrate.

4.2.3 Maxi-cell Expression of pPT14 Derived Plasmids.

Strain CSR603 (Sancar et al., 1979) was transformed with the following plasmids: pPT14; subclones pPT76-78; pPT15, Hind III deleted pPT14 (figure 4.10); and pPT73, 9.5 kb partial Hind III band (figure 4.7).

Figure 4.10 (A) and (B) show the analysis of these strains by SDS P A G E. Figure 4.10 (A) shows the Coomassie stained gel and figure 4.10 (B) the ^{35}S methionine labelled plasmid coded proteins. In tracks 2-5 (figure 4.10 (A)), the pullulanase protein can be clearly seen, however when one looks at the ^{35}S methionine labelled proteins (figure 4.10 (B)) there is not a labelled band corresponding to the stained pullulanase band. There is a band of $\approx M_r$ 94,000 in track 2 (pPT14) which is absent from tracks 3-5 (pullulanase sub-clones). The 94,000 band is also present in track 6 (Hind III deleted, pullulanase negative pPT15. This band is also absent from track 7 (pPT73). From this it can be concluded that this protein must be expressed from a gene anticlockwise of the Kpn I site in pPT14 (figure 4.3). The reason why the pullulanase can not be seen as a labelled product is unclear. However attempts to label both the K.pneumoniae amylase and the B.licheniformis α-amylase in maxi-cells also ended in failure (results not shown). This is interesting because strains carrying tyrB and aspC plasmids (Fotheringham et al., 1986) which were processed at the same time, gave labelled products of the correct M_r. However, these proteins are cytoplasmic, therefore as the amylolytic proteins will be exported into the periplasm, the absence of a labelled band may be because the proteins have 'leaked' out of the periplasm during this procedure. This possibility could be easily checked by running some of the culture fluid on an SDS-P A G E gel.

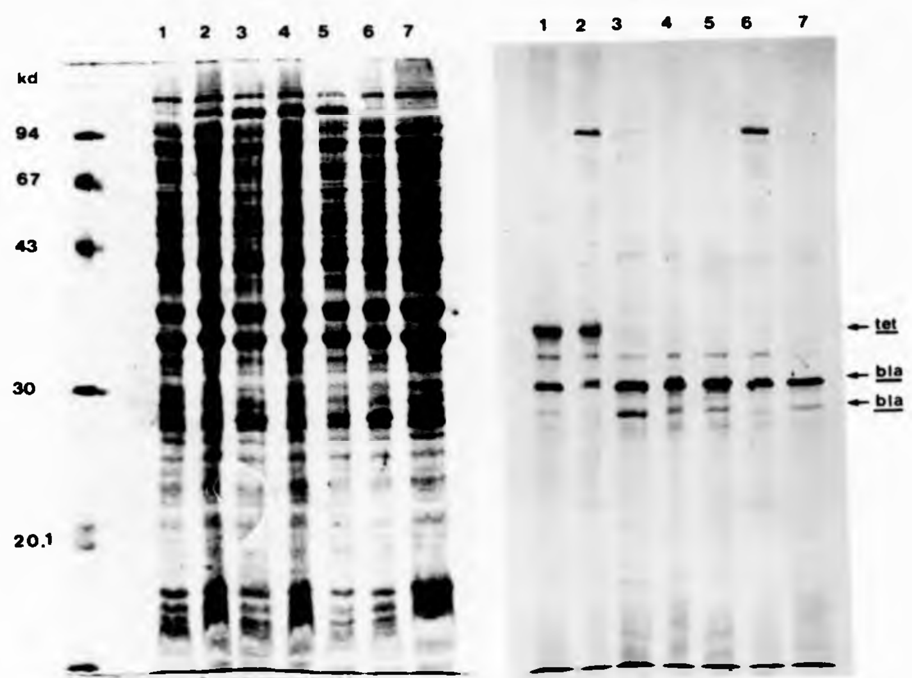


Figure 4.10

Maxi-cell Expression of pPT14 Derived Plasmids.

All strains were cultured under inducing conditions (maltose) and treated as described in Chapter 2. A) is the Coomassie stained gel and B) the autoradiograph of the stained gel. The tracks are: 1) pAT153 control; 2) pPT14; 3) pPT76; 4) pPT77; 5)pPT78; 6) pPT15; 7) pPT73; M - molecular weight markers. The pullulanase band can be clearly seen in A) tracks 2-5 (arrow), but a band corresponding to this protein can not be seen on the autoradiograph. bla: β-lactamase gene products ≈M_r 30,000 and 28,000. tet: tetracyclin gene product ≈M_r 34,000.

4.2.4 Two-Dimensional Electrophoresis of Proteins from E.coli HW87/pPT14 and HW87/pPT99.

HW87/pPT14 (pullulanase clone) and HW87/pPT99 (pullulanase/amylase double clone; see section 4.3.3) were subjected to two-dimensional electrophoresis as descibed in Chapter 2. As neither the amylase nor pullulanase was labelled in maxi-cells, it was of interest to find out if they could be labelled in whole cells. Figure 4.11 (A) shows HW87 plasmid free control; figure 4.11 (B) is HW87/pPT14 and the putative pullulanase spot, which is absent in the control, can be clearly seen. Figure 4.11 (C) shows the proteins from HW87/pPT99 and again the putative pullulanase spot is evident as is the putative amylase spot (see Chapter 3 for gel of amylase clone alone). From this it can be concluded that both genes are functioning normally when in the same host. However, it is difficult to assign a spot to the M_r 94,000 protein which was expressed in maxi-cells. However, as was noted in chapter 3, there are many diffences between the gels and unless the protein is highly expressed it is difficult to determine if a particular protein spot is plasmid encoded.

4.2.5 Action Pattern of the K.pneumoniae Pullulanase on Pullulan.

To prove that pPT14 did in fact code for the K.pneumoniae pullulanase gene its action pattern on pullulan was determined. Digestion of pullulan by

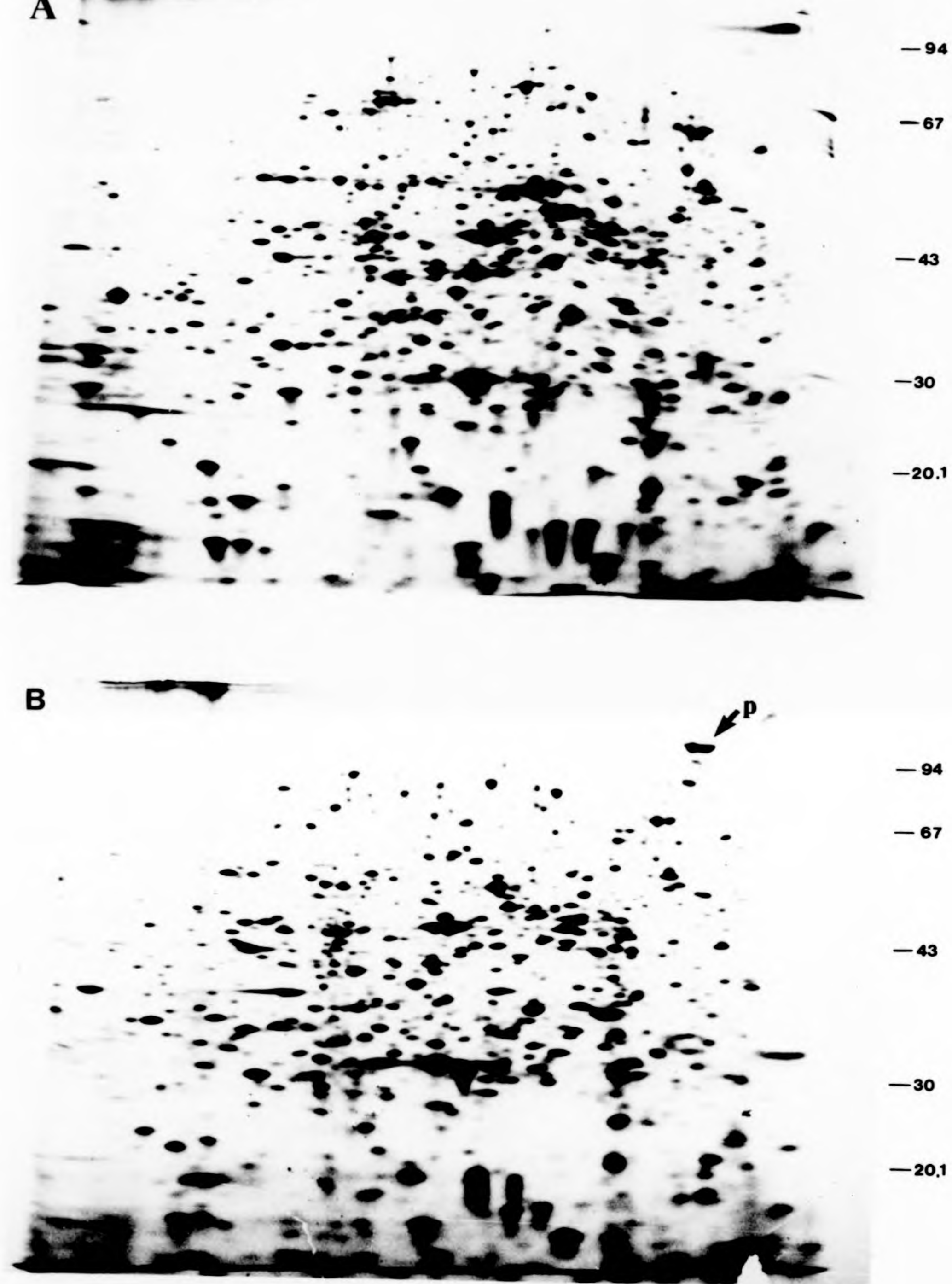


Figure 4.11

Figure 4.11

Two-Dimensional Electrophoresis of Proteins from HW87 carrying pPT14 and pPT99.

Cells were labelled with ^{35}S-methionine and processed as descibed in Chapter 2. The first dimension isolectric focusing to equilibrium (7500 V hr) was in a 1.6% pH 5-7 and 0.4% pH 3-10 ampholine mixture and the second dimension was 10% SDS-P A G E A) HW87; B) HW87/pPT14 (pullulanase); C) HW87/pPT99 (pullulanase/amylase double clone). a - putative amylase; p - putative pullulanase.

pullulanase should quantitatively yield maltotriose as the end product. An initial attempt to show this using extracts made from HW87/pPT14 proved inconclusive. No build up of maltotriose could be seen, only a range of maltodextrins. One explanation for this, was that intracellular enzymes involved in maltodextrin catabolism were breaking down any end products formed by the pullulanase, mainly by the amylomaltase. To lessen this effect a crude extract was prepared from MH70/pPT14 (malQ) and the digestion products were analysed by T.L.C. It is clear from (Figure 4.12) that the gene cloned is in fact pullulanase for, from 5-30mins, maltotriose is the only product. It is interesting that the maltotriose is broken down further by other enymes present in the crude extract. The appearance of maltotetraose (G4) shows that the pullulan must have a significant amount of α-1,6 linked maltotetraose units in its structure.

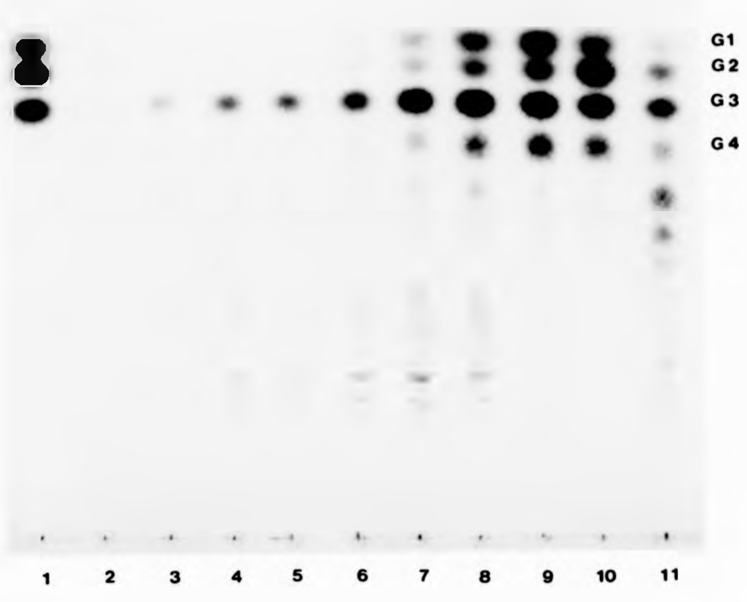


Figure 4.12

Action Pattern of the Cloned Pullulanase on Pullulan.

Crude extracts were prepared from MH70/pPT14 (malQ strain), incubated with pullulan (5%) then subjected to T.L.C. as described in Chapter 2. Samples are 2 ul of; 1) glucose (G1), maltose (G2), maltotriose (G3) standard mixture (0.2%); 2) 0 time digest; 3) 5 min; 4) 10 min; 5) 15 min; 6) 30 min; 7) 1 hr; 8) 2 hrs; 9) 4 hrs; 10) 24 hrs; 11) starch hydrolysate.

4.3 Pullulan Utilization using the K.pneumoniae Pullulanase Gene.

4.3.1 Growth on Glucose, Maltose and Pullulan of E.coli HW87 carrying pPT14, pPT76, pPT77 and pPT78.

The pullulanase clones were grown in L-broth + cb, washed twice in saline then streaked onto M9 + leu medium containing either glucose, maltose or pullulan and incubated at 37°C. Table 4.4 summarizes the result of this experiment. The full sized clone (pPT14) grew quite well on pullulan, however the three sub-clones (pPT76, pPT77, pPT78) all exhibit growth defects on pullulan. No appreciable growth could be detected until 72hrs (result not shown) and it was not clear until 96hrs that they were definitely growing. This growth was not just a few isolated colonies that could have been explained by mutations allowing better utilization of pullulan. As the subclones also showed no difference from pPT14, when grown on maltose, it is also unlikely that the slow growth rate was due to a toxic effect of the protein itself.

To rule out the possibity that the slow growth rate was a toxic effect due to overexpression of the protein two approaches were taken; 1) WT264 (chromosomal low copy number strain) was transformed with pPT14, pPT76, pPT77, pPT78 and their growth characteristics on solid media were re-examined; 2) the growth rates of

HW87, HW87/pPT14, and HW87/pPT76 were determined in M9 casamino acids in the presence of glucose and maltose (a clone carrying pPT12, the K.pneumoniae amylase, was also used at the same time). Table 4.5 shows that none of the pullulanase subclones can grow on pullulan in a low copy

Table 4.4

Comparative Growth of HW87 carrying pPT14, pPT76, pPT77, pPT78 on Glucose, Maltose and Pullulan.

Strain	Carbon Source	24hrs	48hrs	96hrs
HW87/pPT14	glucose	++	+++	+++
HW87/pPT14	maltose	+	+++	+++
HW87/pPT14	pullulan	+	++	+++
HW87/pPT76	glucose	++	+++	+++
HW87/pPT76	maltose	+	+++	+++
HW87/pPT76	pullulan	-	-	+
HW87/pPT77	glucose	++	+++	+++
HW87/pPT77	maltose	+	+++	+++
HW87/pPT77	pullulan	-	-	+
HW87/pPT78	glucose	++	+++	+++
HW87/pPT78	maltose	+	+++	+++
HW87/pPT78	pullulan	-	-	+

The medium was M9 + leu plates containing either glucose, maltose or pullulan. - (no significant growth) to +++ (full growth).

number background, whereas the full size clone (pPT14) can (albeit at a slower growth rate). This is extremely interesting as it would appear that pPT14 has some activity which allows growth on pullulan even at low copy number. Loss of this 'activity' might also explain why the subclones grow very slowly on pullulan.

Table 4.5

Comparative Growth of WT264 carrying pPT14, pPT76, pPT77, pPT78 on Glucose and Pullulan.

Strain	Carbon Source	48hrs	144hrs
WT264/pPT14	glucose	+++	+++
WT264/pPT14	pullulan	+	+++
WT264/pPT76	glucose	+++	+++
WT264/pPT76	pullulan	-	-
WT264/pPT77	glucose	+++	+++
WT264/pPT77	pullulan	-	-
WT264/pPT78	glucose	+++	+++
WT264/pPT78	pullulan	-	-

The medium was M9 + leu plates containing glucose or pullulan. - (no significant growth) to +++ (full growth).

The growth rate of HW87 carrying the various clones was affected by maltose (figure 4.13), but the subclone (pPT76) did not show a more pronounced detrimental effect than the parental plasmid (pPT14). In

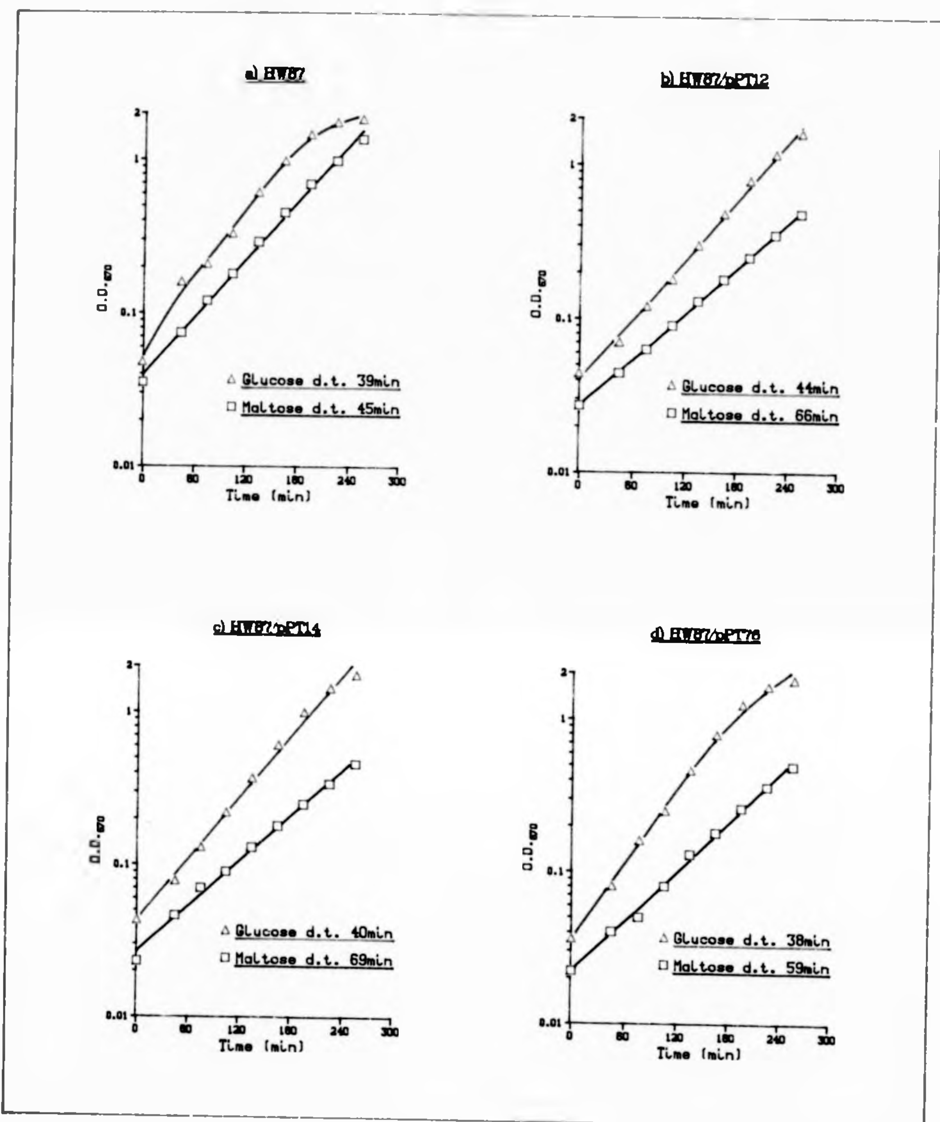


Figure 4.13

Effect of Maltose Induction on the Growth Rate of HW87 Carrying pPT12, pPT14 and pPT76.

0.5 ml of an LB overnight culture of each strain was inoculated into 50 ml of M9 + casamino acids containing either glucose or maltose in a 500 ml baffle flask and the OD was followed. a) HW87 control; b) HW87/pPT12 (amylase); c) HW87/pPT14 (pullulanase); d) HW87/pPT76 (pullulanase subclone). Both the pullulanase clones and the amylase clone show a reduced growth rate when compared to the HW87 maltose control. Under repressing conditions (glucose) the growth rate is unaffected.

fact pPT14 has a longer doubling time (69 min) than pPT76 (59 min) when induced. Also this effect is only in the range of ≈20 min per generation and would not account for the extremely slow growth rate seen in all of the subclones when utilizing pullulan.

4.3.2 Effect of mal Mutations on Pullulan Utilization.

A variety of malA and malB strains were transformed with pPT14 and pPT76 and their abilty to confer growth on maltose and pullulan was tested. The results are shown in table 4.6. All of the strains gave the expected phenotype with respect to maltose, but when grown on pullulan there is a very interesting differance between the two clones. Both clones have a Pul$^-$ phenotype when expressed in malE, F, G and K mutants, but in a lamB background pPT14 can utilise pullulan whereas pPT76 grows very slowly (no appreciable growth until after 6 days). The fact that pPT14 can utilize pullulan in a lamB backround is also different to the result found with both the K.pneumoniae amylase (Chapter 3) and the B.licheniformis α-amylase (Chapter 5), where the LamB protein is essential for growth on starch. The implications of this will be discussed further in the discussion below and in the overall discussion.

Both plasmids conferred pullulan utilization in a malQ background, although growth was slow. This reflects the fact that maltose phosphorylase can use maltotriose

Table 4.6

Effect of *mal* Mutations on Pullulan Utilization by Various *K.pneumoniae* Pullulanase Clones.

Strain	Growth: Maltose	Growth: Pullulan
lamB^{+}/pPT14(MC4100)	+++	+++
lamB/pPT14(pop3208)	+++	+++
malE/pPT14(TST1)	-	-
malF/pPT14(TST6)	-	-
malG/pPT14(TST2)	-	-
malK/pPT14(pop3295)	-	-
malQ/pPT14(MH70)	-	(+)
lamB^{+}/pPT76	+++	++
lamB/pPT76	+++	(+)
malE/pPT76	-	-
malF/pPT76	-	-
malG/pPT76	-	-
malK/pPT76	-	-
malQ/pPT76	-	(+)

The medium was M9 containing maltose or pullulan and incubated at 37 ^{o}C. + to +++, comparative growth after 48 hrs. (+), little growth after 48 hrs, but was definitely growing after 6 days. - , no significant growth after 6 days. This experiment was repeated with pPT77 and the same growth characteristics as pPT76 were observed (results not shown).

as a substrate. However, it does so at a 100 fold lower rate than with larger maltodextrins (Schwartz and Hofnung, 1967), thus explaining the inefficient growth on pullulan in this background.

4.3.3 Construction of Amylase/Pullulanase Double Clone and its Effect on Starch Utilization.

The 3.9 kb *Eco*R I fragment from pPT12 (*K.pneumoniae* amylase) was ligated into the *Eco*R I site of pPT77 in both orientations, thus making amylase/pullulanase double clones pPT99 and pPT100 (Figure 4.14). The plasmids were then tested for their ability to utilize starch and pullulan (Table 4.7) on solid medium. From the results it can be concluded that both activities function normally and that the orientation of the amylase gene is irrelevant. It is of note that HW87/pPT12 grows on amylose better than on starch presumably because it has no α-1,6 linkages to contend with. Also it is interesting that the double clones do not show this difference, presumably because the α-1,6 can be hydrolysed by pullulanase. To determine if the double clone utilizes starch more efficiently than the amylase clone alone, the doubling times of HW87/pPT12 and HW87/pPT99 grown on starch minimal medium were determined. Figure 4.15 shows the comparison of these two clones and it can be concluded that the pullulanase gene does not make any significant difference to the growth rate on starch, in fact the double clone has a slightly longer doubling time.

Table 4.7

Growth Characteristics of HW87/pPT99 and HW87/pPT100 and Controls.

Strain	Carbon Source	48hrs	72hrs	120hrs
HW87/pPT12	amylose	+	++	+++
HW87/pPT12	starch	-	+	+++
HW87/pPT12	pullulan	-	-	-
HW87/pPT14	amylose	-	-	-
HW87/pPT14	starch	-	-	-
HW87/pPT14	pullulan	++	+++	+++
HW87/pPT99	amylose	+	++	+++
HW87/pPT99	starch	+	++	+++
HW87/pPT99	pullulan	-	+	+++

The medium was M9 + leu containing either amylose, starch or pullulan. - (no significant growth) to +++ (good growth). HW87/pPT100 gave the same response as HW87/pPT99.

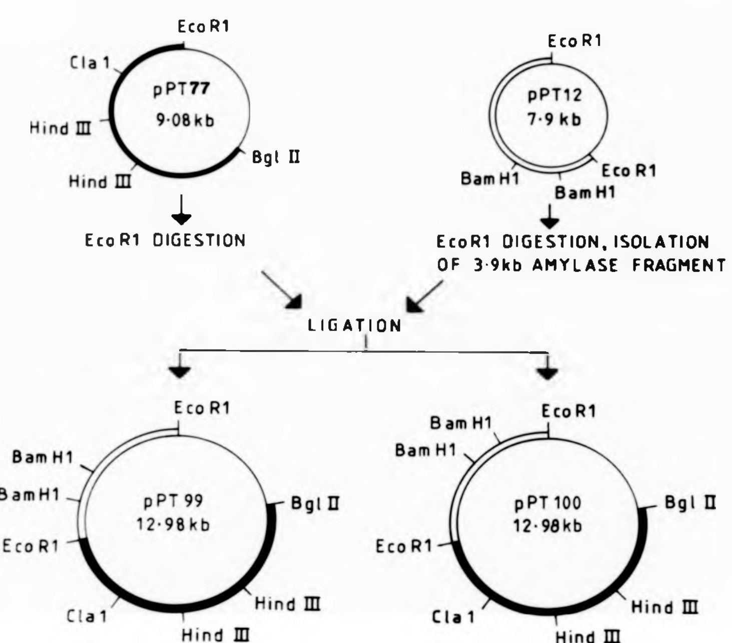


Figure 4.14

Contruction of pPT99 and pPT100; K.pneumoniae Pullulanase/ Amylase Double Clone.

All manipulations were carried out as described in Chapter 2. Essentially, the 3.9 kb amylase fragment from pPT12 (open box) was cloned into EcoR I cut pPT77 (subcloned pullulanase fragment, filled box) in both orientations.

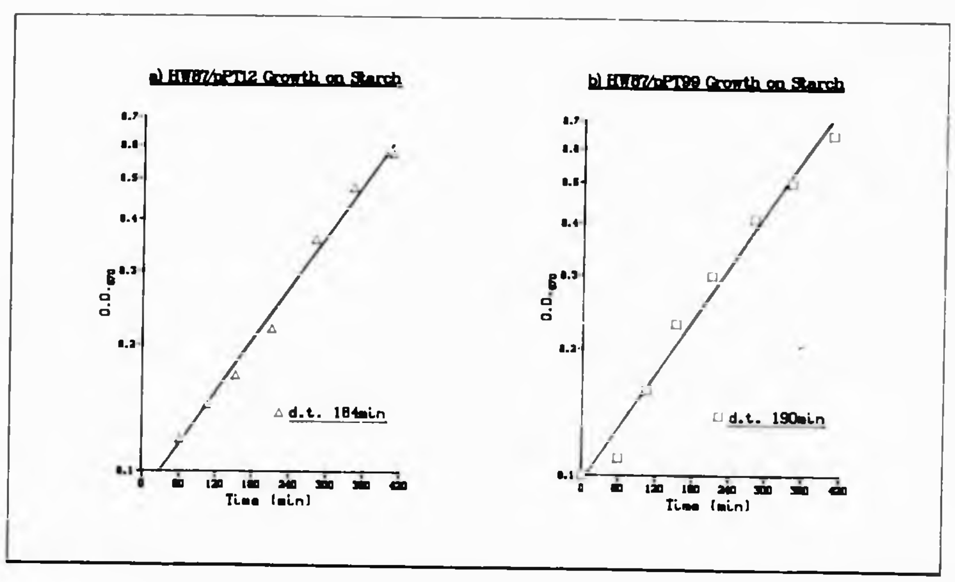


Figure 4.15

Comparative Growth Rates of HW87/pPT12 and HW87/pPT99.

0.5 ml of an overnight culture grown in M9 + starch + leu + Cb was inoculated into 50 ml of the same medium in a 500 ml baffle flask and the OD was followed. Each culture was set up in duplicate.

Discussion.

The *K.pneumoniae* pullulanase gene has been cloned and expressed in *E.coli*. Strains carrying pPT14 produce a protein of the same molecular weight as purified pullulanase, can degrade pullulan to maltotriose and can utilize pullulan as sole carbon source. Extracts made from *malQ* strains still retain the ability to degrade maltotriose to maltose and glucose, similar to the result obtained with the *K.pneumoniae* amylase (Chapter 2).

Strains containing the pullulanase gene show some very interesting growth characteristics:

a) Insertions over a large area of the insert have an effect on pullulan utilization in that they either lead to a Pul^- phenotype, a slow growth phenotype or have no effect on growth on pullulan.

b) Subcloning of the pullulanase structural gene similarly affect pullulan utilization, in that all the subclones exhibit a slow growth phenotype on pullulan.

c) In a *lamB* background the parental plasmid can still grow well on pullulan, whereas the subclones grow poorly. This contradicts the result found with both the *B.licheniformis*

α-amylase and K.pneumoniae maltohexaose producing amylase where the lamB gene product was essential for growth on starch (Chapters 3 and Chapter 5).

d) In a wild type background strains carrying the subcloned pullulanase plasmids (pPT76, pPT78 and pPT79) grow better than when in a lamB background, but still not as well as the parental plasmid (pPT14).

e) In a low copy number background the parental plasmid confers pullulan utilization ability, but the subclones do not.

These observations suggest that pullulan is utilized in a different way to starch. Clearly the growth is lamB-independent, suggesting that the pullulan is broken down extra-cellularly, the maltotriose produced would then be transported via the outer membrane porin proteins. It also seems likely that the parental plasmid expresses another gene product or products which function in the utilization of pullulan. It is tempting to speculate that this may be in the form of a pullulan permease. However, as the pullulanase is exported into the medium in its K.pneumoniae host (Wallenfels et al., 1966) this is unlikely. One could also envisage a mechanism similar to that of hemolysin, where other gene products are thought to function in its export across the outer membrane (Goebel and Hedgpeth, 1983), however

pullulanase was not detected in the culture medium in significant amounts with any of the pullulanase clones. However, it has been shown that pullulanase can be either membrane bound or extra-cellular depending on the growth conditions (Wallenfels et al., 1966; Hope and Dean, 1974, 1975). As specific culture effects have not been assessed, the possibility that the parental plasmid contains information which targets the pullulanase in the outer membrane in such a way that it can act on the substrate can not be ruled out. The fact that pullulanase is a lipoprotein (see section 4.1 and below) and is processed and modified correctly in E.coli (Pugsley et al., 1986), but not correctly localised, suggests that at least one other gene product which is absent in E.coli is required for correct localization and secretion. The large area (≈8 kb) in which Bgl II insertions affect growth on pullulan of strains carrying these plasmids suggests that more than one gene product may be involved. A candidate for at least one gene product which may be involved is the the M_r 94,000 protein which was expressed in maxicells (section 4.2.3).

The pullulanase gene from K.pneumoniae has been cloned by two other groups (Takizawa and Murooka, 1985; Michaelis et al., 1985: see section 4.1). It is interesting that these two clones show strikingly different restriction maps. When comparing the restriction map of pPT14 to these clones it shows greatest homology with the clone isolated by Takizawa and Murooka, but is not identical. Both of the previously

cloned pullulanase fragments have been cloned on relatively small fragments and could not contain all of the region which affects growth in pPT14. Michaelis et al., 1985 have shown that their pullulanase clone does not allow growth on pullulan in a malT+ background even when preinduced on maltose, but in a malTC background it can grow, albeit at a slow rate. This suggests that there is a problem with induction of the pullulanase and the maltose maltodextrin transport system, presumably because the pullulanase can not reach the substrate and thus produce maltotriose which would induce the system. This is similar to the findings in this study when comparing the effect on growth on pullulan of the parental plasmid and the subclones or the insertions. Michaelis et al., 1985 has also shown that pullulanase expressed in E.coli is loosely associated with the outer membrane and that at least a small fraction of the fully induced pullulanase expressed in E.coli is accessible to the subtrate.

Taking all this information into consideration it is possible to put forward an explanation for the pullulan utilization data from this study. On the assumption that pullulan can not gain access to the periplasm through the lamB porin (this is unknown, but the results above suggest this) then the pullulanase can not get access to its substrate without being either correctly localised in the outer membrane or secreted into the medium. That the strain carrying the parental plasmid can grow on pullulan suggests that it can come

into contact with the substrate, possibly because the pullulanase is correctly orientated in the outer membrane. In the case of the strains carrying the subclones and if they are analogous to the clone used by Michaelis et al., 1985, then the enzyme will be incorrectly orientated in the inner and outer membranes as well as the periplasm. The slow growth phenotype could be explained by the over expression of the subclones causing correct localization of a small number of molecules or by causing the membrane to become leaky (thus explaining the Pul^- phenotype in a low copy number background). It is also possible that the lamB porin may transport pullulan, but ineffectually. Obviously a lot more work is needed to determine the molecular events which occur within the cell during growth on pullulan. There are a number of interesting questions which remain unanswered. Can pullulan gain access to the periplasm in E.coli? Is the M_r 94,000 protein involved in export/ localisation of pullulanase? Is it possible to improve the growth rate of strains carrying the pullulanase subclones or insertions by "complimenting" them with genes of the putative export apparatus? Is the pullulanase protein, expressed in strains carrying pPT14, found in the outer membrane, and is the active site of the enzyme exposed to the extracellular medium?

CHAPTER 5

CLONING AND CHARACTERISATION OF THE *B.licheniformis* α-AMYLASE GENE.

5.1 Introduction.

The α-amylases of *B.licheniformis* have been purified and characterised. There are two types reported in the literature, one with a M_r 62,000 (Saito, 1973; Chiang *et al*., 1979; Morgan and Priest, 1981), and one with an M_r 28,000 (Krishnan and Chandra, 1983). The high molecular weight α-amylases produced by *B.licheniformis* NCIB 6346 (Morgan and Preist, 1981) and *B.licheniformis* BLM 1777 (Chiang *et al*., 1979) have similar characteristics with maximal activity at 70-90°C; a pH range of 7-10 (maximal activity pH 7); they are thermostable at 65-70°C; and they yield mainly maltopentaose from starch.

The low molecular weight α-amylase from *B.licheniformis* CUMC305 is thermostable up to 80°C. It shows maximal activity at 90°C and retains 91% of its optimal activity at 100°C. In the presence of substrate it is fully stable at 100°C for four hours and has a pH maximum of 10. The action pattern has not been determined. It is of note that strain CUM305 is an industrial enzyme production strain and has been subjected to mutagenesis. Therefore the amylase may not be characteristic of the wild type enzyme.

Recently, the structural genes for two α-amylases from *B.licheniformis* have been cloned (Ortlepp *et al*., 1983; Guerineau and Heslot, 1984; Piggot *et al*., 1984; Sibakov and Palva, 1984) and the 5' regions of these two genes have been sequenced (Stephens, *et al*.,1984; Sibakov and Palva, 1984). The coding sequence obtained from these genes and the *B.amyloliquefaciens* (Takkinen *et al*., 1983) α-amylase are very homologous. This will be discussed in more detail in the discussion of section 5.2.

I decided to clone the α-amylase gene from *B.licheniformis* NCIB 6346 because it was a "natural" isolate and the enzyme had been well characterised from this strain (Morgan and Priest, 1981).

5.1.2 Cloning Strategy.

Initially, the cloning strategy, was a direct "shotgun cloning" into pAT153. However, this approach proved unsuccessful. There are a number of reasons why this approach sometimes fails:

a) when using total restriction digests to create gene banks the target gene may have an internal restriction site. Thus only a non-functional piece of the gene is cloned.

b) the gene may be carried on a very large restriction fragment and as the restriction digests were not fractionated, small fragments would be cloned preferentially.

c) the high copy number of the vector will cause high expression of the cloned gene and this can be detrimental to the host, especially when cloning amylases (Yang et al., 1983; Willemot and Cornelis, 1983).

A different approach was needed, so it was decided to clone the amylase using a λ cloning vehicle.

5.1.3 Cloning of the B.licheniformis α-Amylase in λ Charon 28.

λ Charon 28 (Rimm et al., 1980) was used as the cloning vehicle. When restricted with BamH I, thus removing the internal stuffer fragment, 15-20 kb fragments can be cloned. The restriction enzyme Sau3A I has compatible ends with BamH1. As this enzyme cuts frequently within DNA, partially digested fragments in the 15-20 kb range can be isolated and cloned into the λ Charon 28 vector to make a fairly representative library of chromosomal DNA fragments. This method also has the advantage in that many thousands of clones can be easily screened.

A partial *BamH* I *B.licheniformis* λ library was constructed and *in vitro* packaged (Collins and Bruning, 1978). This preparation was then used to infect HW14 then was plated on NZCYM medium + starch. Plaques were screened by exposure to iodine vapors. A positive plaque was identified as a clear zone around the plaque where the starch had been broken down by the amylase. Out of approximately 10^4 plaques screened, 11 positive clones were obtained. DNA from four of these was isolated by the rapid plate lysate procedure (Davis *et al*., 1980) and was restricted with *Eco*R I and *Bgl* II. Figure 5.1 shows the result of these digests. Although the *Bgl* II digest is ambiguous, the *Eco*R I digest results show that all of the inserts are related. Clone 3 was chosen for further analysis.

5.1.4 Subcloning of the α-Amylase Gene from λ Charon 28 Clone 3.

CsCl purified DNA was isolated from λ Charon 28, clone 3. The DNA was cleaved with *Eco*R I, run on an LGT-agarose/TBE slab gel, and the three *Eco*R I fragments (6.0, 3.4 and 1.5 kb) were isolated. The fragments were ligated into three plasmid vectors:-

a) pBR322 - a Col EI derived cloning vehicle which has a copy number of 50 per chromosome (Bolivar, 1977).

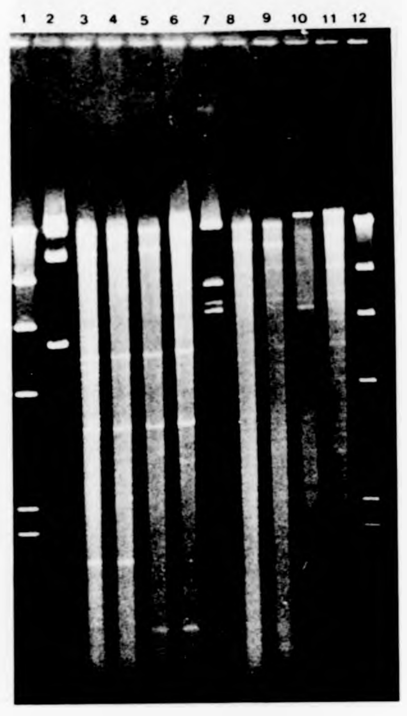


Figure 5.1

EcoR I and Bgl II Digestion of Putative B.licheniformis α-amylase λ Clones (quick prep. DNA).

DNA was prepared, restricted and run on a 1% agarose/ T.B.E. slab gel as descibed in Chapter 2. The tracks are: 1) & 12) λ Hind III markers; 2) Charon 28/EcoR I; 3) Clone 1/EcoR I; 4) Clone 2/EcoR I; 5) Clone 3/EcoR I; 6) Clone 4/EcoR I; 7) Charon 28/Bgl II; 8) Clone 1/Bgl II; 9) Clone 2/Bgl II; 10) Clone 3/Bgl II; 11) Clone 4/Bgl II. The bands were visualised against a background of E.coli chromosomal DNA.

b) pAT153 - a high copy-number vector (150 per chromosome) derived from pBR322 (Twigg and Sherratt, 1980).

c) pFF2 (Flock *et al*., 1984) - a hybrid plasmid of pAT153 and pUB110 (Jalanko *et al*., 1981). This is an *E.coli*/*B.subtilis* shuttle vector and thus allows the expression of the amylase in *B.subtilis* as well as *E.coli*.

d) pLG338 (Stoker *et al* 1982) - a low copy number plasmid derived from pSC101 (Cohen *et al*., 1973). This plasmid was used in case over-expression of the amylase was detrimental to the host.

HW87 was transformed with the ligation products and was plated on L-agar + starch + Cb for the pBR322, pAT153 and pFF2 ligations and L-agar + starch + Tc for the pLG338 ligations. After overnight growth at 37°C the colonies were screened by exposure to iodine vapours. Clear halos, due to starch degradation, could be seen around colonies derived from the 3.4 kb fragment ligation showing that the amylase activity was associated with this fragment. It should be noted that the pAT153 derivative proved difficult to obtain and it took several attempts before it was cloned. Initially it was thought that this might be because the amylase gene was detrimental to the cell when cloned on a high copy number plasmid. However, this seems unlikely as pFF2

derivative (pPT80) was obtained quite easily and has the same relative copy number as pAT153 (see below).
The plasmids were named:-

1) pPT80 - pFF2 derivative.

2) pPT81 - pAT153 derivative.

3) pPT83 - pBR322 derivative.

4) pPX2 - pLG338 derivative.

5.1.5 Copy Number of Plasmids pPT80, pPT81, pPT83 and pPX2 in E.coli HW87.

The copy number was determined by comparison with pBR322 and assuming it had a copy number of 50 per chromosome (see Chapter 2). The results are shown in table 5.1. All of the ColE1-derived plasmids have a copy number withun the expected range. However the pLG338 derivative had a copy number which was much higher than the expected six per chromosome (Stoker et al., 1982).

5.1.6 Restriction Analaysis of pPT81.

A restriction map for pPT81 was constructed (Fig 5.2) and the approximate position of the gene was determined by deletion analysis (figure 5.3). From this analysis it was concluded that the structural gene for

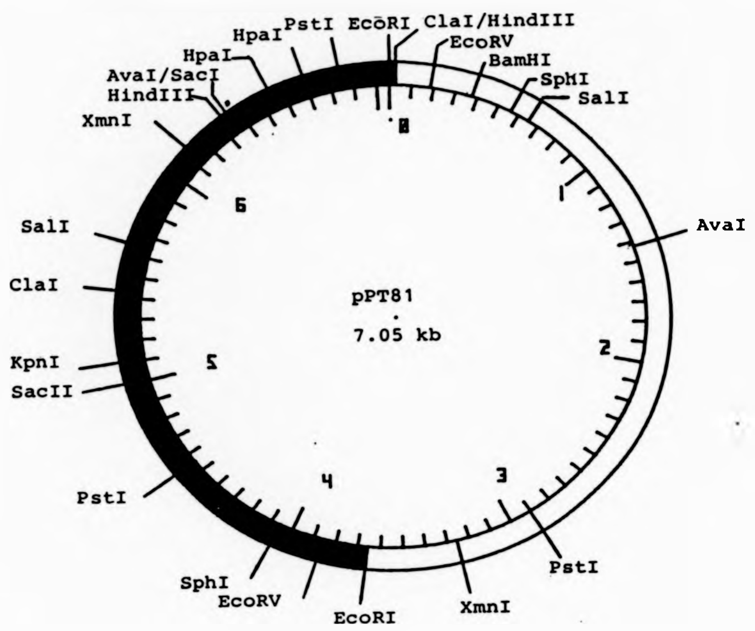


Figure 5.2

Restriction Map of pPT81 (α-amylase/pAT153).

Restriction mapping was carried out as described in Chapter 2. The plasmid vector is pAT153 and the 3.4 kb EcoR I α-amylase insert is shown in black.

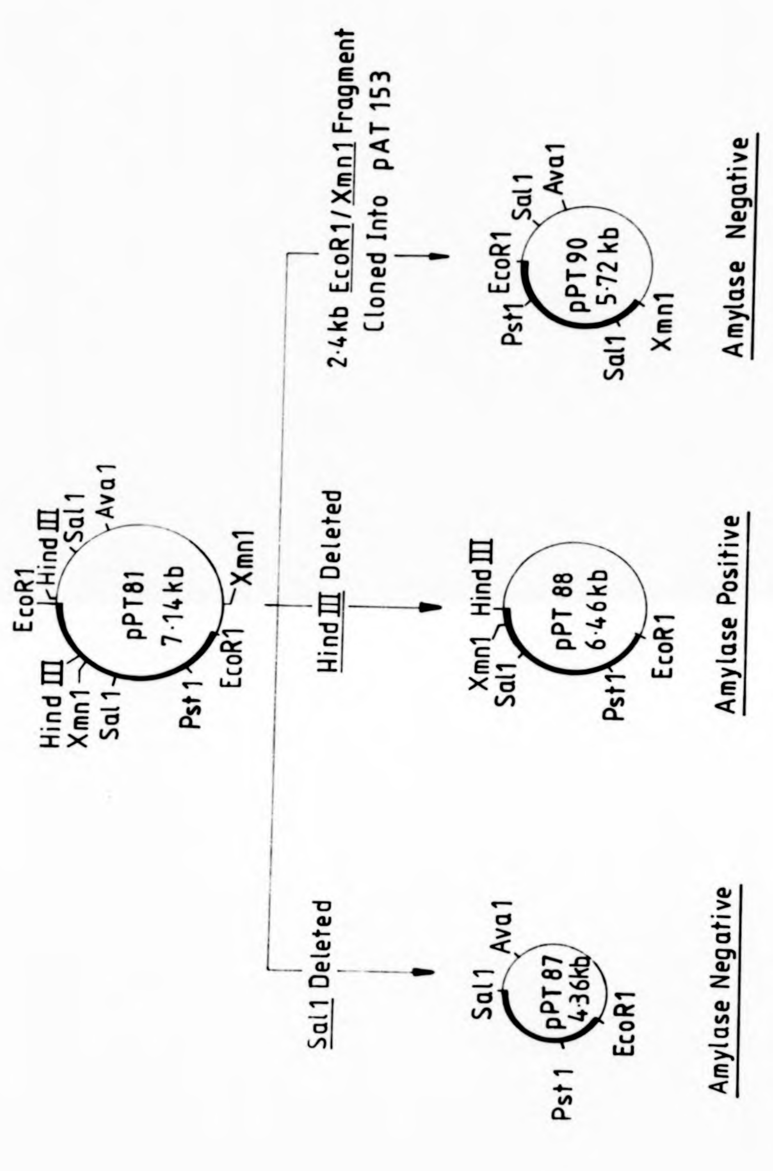


Figure 5.3

Figure 5.3

Deletion Analysis of pPT81.

All manipulations were carried out as described in Chapter 2. The Sal I and EcoR I/Xmn I deletions interrupt the structural integrity of the α-amylase gene, but the Hind III deletion does not showing that the α-amylase gene is situated on an EcoR I/Hind III fragment.

the *B.licheniformis* α-amylase was situated on the *Hind* III/*Eco*R I fragment.

Table 5.1

Relative Copy Number of pPT80, pPT81, pPT83 and pPX2.

Plasmid	Rel. Copy No.
pPT80	87
pPT81	89
pPT83	42
pPX2	27

Copy number was determined as described in Chapter 2. Figures were calculated relative to pBR322 assuming it had a copy number of 50.

5.2 Sequence Analaysis of the α-Amylase Insert.

5.2.1 Sequencing Strategy.

As the approximate location of the gene was known (see above), the initial strategy was to clone specific fragments into M13mp8 and mp9 and sequence them by the dideoxy chain termination method (Sanger *et al*., 1977). This approach took two forms:

1) Specific fragments were generated using restriction endonucleases with hexanucleotide recognition sites (which cut near, or within, the amylase gene) and were cloned into M13mp8 or mp9 and sequenced.

2) The 3.4 kb fragment was recircularised at its EcoR I ends, cut with Sau3A I and the fragments were cloned into BamH1 cut M13 mp8 then sequenced.

This approach was initially quite productive (Figure 5.4). Four of the Sau3A I fragments were completely sequenced on both strands. Of the remaining three, two were partially sequenced. The 682 bp fragment containing the 5' end of the gene was not isolated. Combined with the data from the specific hexanucleotide restriction fragments, the whole α-amylase gene had in fact been sequenced, but not completely on both strands.

At this point it became obvious that in order to obtain the complete sequence on both strands of the DNA unambiguously by this method would be difficult and time consuming. Therefore, a random "shotgun" method was used to complete the sequence (Bankier and Barrell, 1983).

5.2.2 Shotgun Sequencing of the 3.4 Kb Amylase Insert.

Figure 5.5 shows a schematic representation of this method. It has a number of advantages over the procedure outlined above:-

a) Large numbers of M13 clones can be produced from one cloning experiment and it is restriction enzyme independent. This avoids time consuming subcloning experiments.

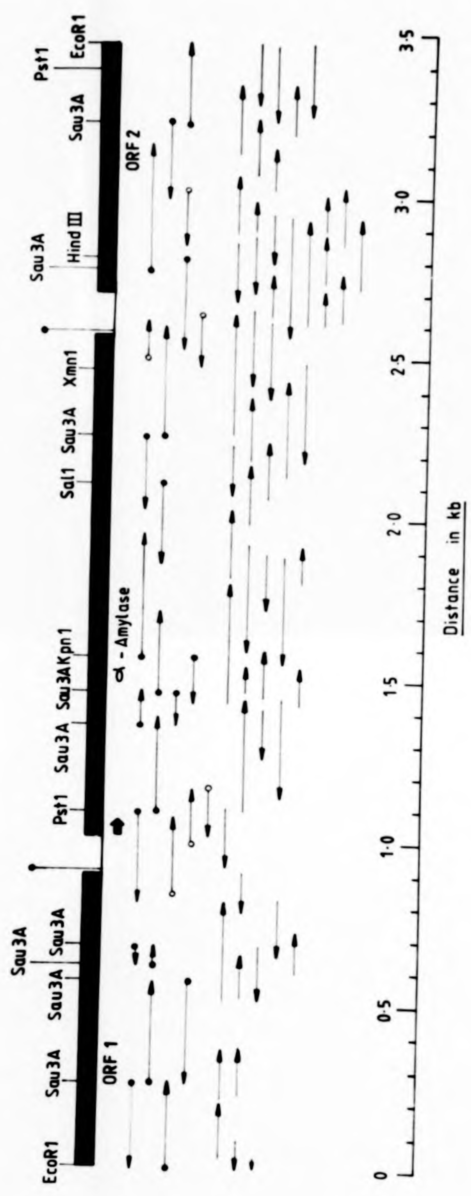


Figure 5.4

Figure 5.4

Sequencing Strategy for the B.licheniformis α-amylase.

Key

- sequence obtained from specifically cloned fragments.

- sequence obtained by primer extension.

- sequence obtained from randomly cloned fragments.

- direction of transcription of α-amylase.

ORF 1 - open reading frame 1.

ORF 2 - open reading frame 2.

- putative transcription terminators.

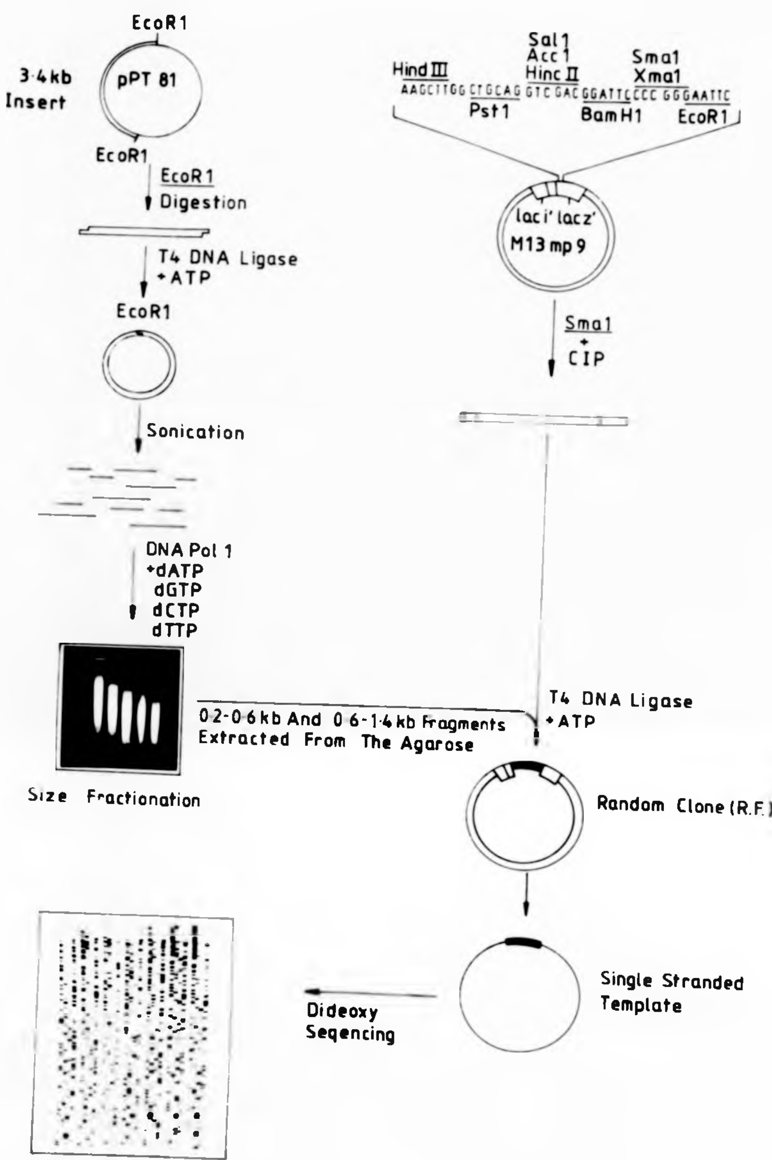


Figure 5.5

Figure 5.5

Schematic Representation of the Random Sequencing Protocol.

All manipulations were carried out as described in Chapter 2. Briefly, the 3.4 kb EcoR I fragment was isolated, recircularised, sonicated and the ends were made blunt with DNA polymerase I. After size fractionation (LGT agarose/T.B.E. slab gel) the blunt fragments were ligated into Sma I cut M13 mp9 and sequenced.

b) As the clones are essentially random, the sequence will develop from multiple overlapping sequences, thus giving data of higher fidelity. This is extremely important where there is secondary structure in the DNA.

c) This method automatically produces sequence from both strands as the DNA fragments are randomly cloned in both orientations.

All of the sequencing data was processed using the computer programs developed by Staden (Staden, 1982; Staden, 1984 a, 1984b & 1984c). By this approach the whole insert was sequenced on both strands with the exception of two small areas. These remaining areas were completed by primer extension (see Chapter 2). The complete strategy, using all of these methods is shown in figure 5.4.

5.2.3 <u>Sequence Analysis.</u>

The sequence was subjected to computer analysis and has three major open reading frames (Fig 5.6). The central frame is the only one which forms a complete gene and corresponds to the α-amylase gene. Translation of this sequence reveals a protein of 512 amino acids with a M_r 58492 (figure 5.7 and table 5.2). As has been previously found with <u>B.subtilis</u> chromosomal genes (Ogasawara, 1985) the <u>B.licheniformis</u> α-amylase codon usage (table 5.2) is unbiased when compared to <u>E.coli</u>

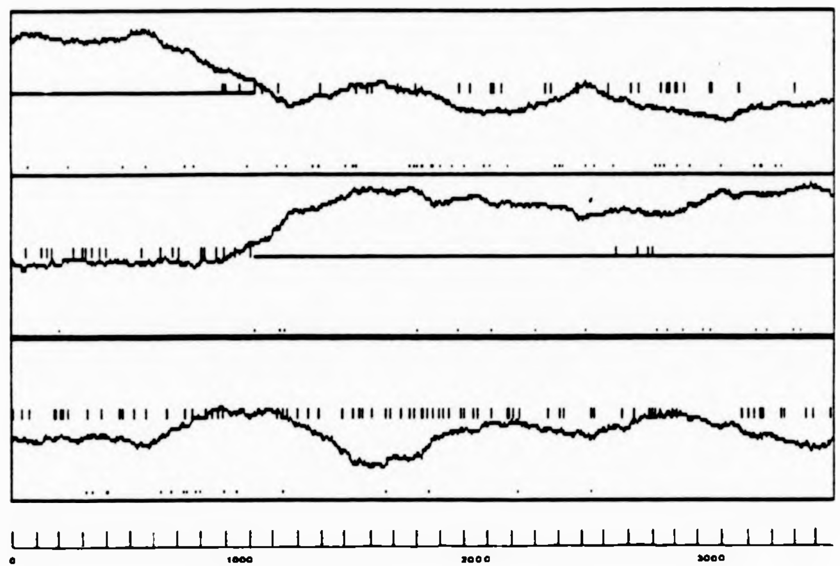


Figure 5.6

Computer Analysis of the Open Reading Frames of the 3.466 kb α-amylase Fragment: The Position Base Preference Method.

The DNA sequence was analysed by the positional base preference method (Staden, 1984c). The dots depict the start codons (met) and the upright bars depict the stop codons. The numbering refers to the nucleotides as shown in figure 5.7. All three reading frames are shown. The output shows that there is a high probabilty of three coding regions. The central coding region (1000-2600) belongs to the B.licheniformis α-amylase.

Figure 5.7

The Nucleotide Sequence of the 3.4 kb EcoR I B.licheniformis α-amylase Fragment.

The nucleotide sequence and translation of ORF1, α-amylase and ORF2 is shown. The predicted amino acid sequence is presented above the nucleotide sequence by the single letter amino acid code. The nucleotide positions are assigned relative to the 5′ terminal base of the EcoR I site. The regions of dyad symetry are shown by arrows above the nucleotide sequence. ORF1 codes for the last 296 amino acids of an unknown protein. α-amylase codes for a preprotein of 512 amino acids with a 29 amino acid signal sequence. The -35 and -10 regions

are underlined and the mRNA start is at position 992 as determined by reverse run-off priming. The ribsome binding site is also underlined. The translation of ORF2 is from the first methionine after the inframe TAA stop codon at position 2705 (249 amino acids). Regions complimentary to the 3' end of the B.subtilis 16S rRNA are shown by the filled circles.

(Ikemura, 1981; Konisberg and Godson, 1983). Only three codons are not used, pro (CCA) and the two cys codons (UGU, UGC).

Table 5.2

<u>B.licheniformis</u> α-amylase Codon Usage.

F	TTT	17.	S	TCT	4.	Y	TAT	18.	C	TGT	0.
F	TTC	5.	S	TCC	5.	Y	TAC	13.	C	TGC	0.
L	TTA	4.	S	TCA	4.	*	TAA	0.	*	TGA	0.
L	TTG	13.	S	TCG	9.	*	TAG	1.	W	TGG	17.
L	CTT	8.	P	CCT	4.	H	CAT	18.	R	CGT	3.
L	CTC	2.	P	CCC	4.	H	CAC	7.	R	CGC	6.
L	CTA	1.	P	CCA	0.	Q	CAA	13.	R	CGA	3.
L	CTG	9.	P	CCG	8.	Q	CAG	9.	R	CGG	4.
I	ATT	13.	T	ACT	3.	N	AAT	11.	S	AGT	1.
I	ATC	7.	T	ACC	4.	N	AAC	14.	S	AGC	4.
I	ATA	1.	T	ACA	13.	K	AAA	19.	R	AGA	3.
M	ATG	8.	T	ACG	8.	K	AAG	11.	R	AGG	4.
V	GTT	12.	A	GCT	10.	D	GAT	19.	G	GGT	7.
V	GTC	12.	A	GCC	7.	D	GAC	18.	G	GGC	16.
V	GTA	4.	A	GCA	12.	E	GAA	18.	G	GGA	12.
V	GTG	4.	A	GCG	11.	E	GAG	7.	G	GGG	11.

Total codons= 513, total amino acids= 512, M_r 58492. Amino acids are shown in the single letter code (Appendix 3).

The complete sequence of the whole α-amylase insert is shown in figure 5.7, which also shows the translation of open reading frame 1 (ORF1) and open reading frame 2 (ORF2). The 5′ end of the α-amylase gene is identical to that published by Stephens <u>et al</u>., (1984), except for one base, a T to C transition in the

putative -10 of the promoter at position 984 (figure 5.7). It also has a putative signal sequence of 29 amino acids (figure 5.7) which is typical of Gram positive signal sequences (Lofdahl et al., 1983; Kroyer and Chang. 1981; Palva et al., 1981). The putative N-terminus of the mature protein is identical to the published protein sequence for this enzyme (Kuhn et al., 1982). Preceding the AUG start codon is a typical Bacillus ribosome binding site (McLaughlin et al., 1981) showing a high degree of complementarity to the 3' end of the B.subtilis 16S rRNA (10 out of the first 15 bases (figure 5.8)).

The two open reading frames, ORF1 and ORF2 are partial sequences of unknown proteins. No homology with DNA sequences from the NBRF, EMBL and GENBANK DNA databases could be found. Likewise, no protein homology with protein sequences from the NBRF protein database could be found either. The putative protein derived from the DNA sequence of ORF2, has two possible start points (position 2720 and 2765; Figure 5.7), both are preceded by a region which is homologous to the 3' end of the B.subtilis 16S rRNA. The second possible putative start site also has a putative σ55 promoter sequence (-35 position 2691, -10 position 2716; Figure 5.7). If this is the correct start point it is interesting that the ribosome binding site is homologous to the first 6 bases of the 3' B.subtilis 16S mRNA and does not contain the GGAGG sequence found in most of the Bacillus genes

Figure 5.8

Complementarity Between the Putative Ribosome Binding Site of the B.licheniformis α-amylase gene and the 16S rRNA of B.subtilis.

```
                                                  M   K   Q   Q
5' ATGTTTCACATTGAAAGGGGAGGAGAATC ATG AAA CAA CAA....3'
                 ******* *   **
              3' UCUUUCCUCCACUAG.....5'
```

The upper sequence shows the B.licheniformis α-amylase DNA sequence in the vicinity of the ATG start codon. The lower sequence refers to the 16S B.subtilis 3' rRNA. * defines the matches.

sequenced to date. The only exception is the ribosome binding site of the phage ϕ29 early genes (Murray and Rabinowitz, 1982).

5.2.4 Determination of the Transcription Start Site of the B.licheniformis α-amylase in E.coli.

The 5' flanking region of the α-amylase gene was screened for Bacillus promoter sequences (Johnson et al., 1983). The only promoter like sequence was of the σ^{55} type with a good -35 region (TTGTTA at 956) but an ill defined -10 region (TATACAAC at 977, figure 5.7). A computer search of this region (Staden, 1984b) also shows this as the main possibility (figure 5.9.1), with an mRNA start at position 992. To determine if this was the actual promoter, the mRNA start was mapped by reverse run-off priming (RRP; Hudson and Davidson, 1984).

mRNA from HW1023 (HW2/pPT81) was prepared (see Chapter 2). Two primers were used which primed at different distances from the putative promoter and were complementary to the mRNA transcript. The first RRP1 was an 18mer starting at position 1159 on the DNA sequence. The second RRP2 was a 23mer starting at position 1097 (figure 5.9.2). The primers were annealed to the mRNA and reverse transcribed using AMV reverse transcriptase and labelled with ^{35}S ATP. The transciption products were then run against a sequencing ladder produced using the same primers on a suitable DNA template, thus the transcript is a direct comparison and therefore very

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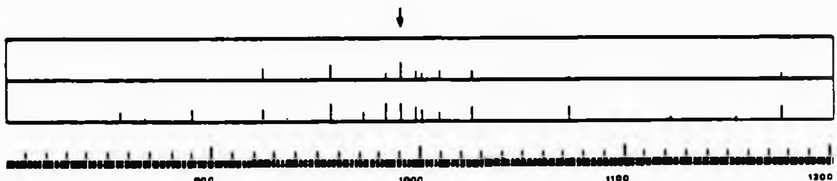


Figure 5.9.1

Computer Search for E.coli Promoter Sequences.

Computer analysis of the DNA sequence between 800 and 1200 nucleotides is shown (Staden, 1984b). Upright bars depict the most likely mRNA start points, the lower box is without gap penalties and the upper box with gap penalties. The major start point at position 992 is arrowed.

```
         -10              ┌►mRNA Start
                                                                 M  K  Q  Q  K  R  L  Y  A  R  L  L  T  L  L  F  A  L  I  F  L
AAAAATTCGGAATATTTATACAACATCATATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACGGCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTT
                                                                                                                      ||||||
                                                                                                                   3' GAAGAA
    970       980       990      1000      1010      1020      1030      1040      1050      1060      1070      1080

 L  P  H  S  A  A  A  A  A  N  L  N  G  T  L  M  Q  Y  F  E  W  Y  M  P  N  D  G  Q  H  W  K  R  L  Q  N  D  S  A  Y  L
GCTGCCTCATTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAATGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTT
||||||||||||||||                                              ||||||||||||||||||
CGACGGACTAAGACGTC 5'  RRP2 (23 mer)                        3' TTACCATGTACGGGTTAC 5'  RRP1 (18 mer)
    1090      1100      1110      1120      1130      1140      1150      1160      1170      1180      1190      1200
```

Figure 5.9.2

Figure 5.9.2

Sequence and Position on the DNA Sequence of Reverse Runoff Primers RRP1 and RRP2.

The position of RRP1 and RRP2 on the DNA sequence is shown. The mRNA start point as determined by reverse transcribing the α-amylase mRNA is a G at position 992 on the DNA sequence (see figure 5.9.3). The putative -10 region is underlined, the -35 region is not shown.

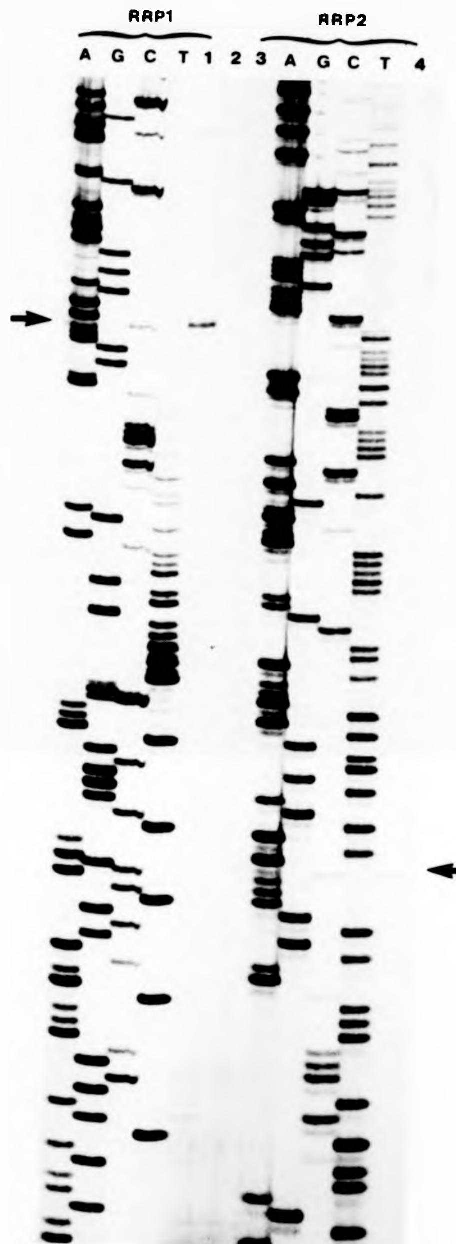


Figure 5.9.3

Figure 5.9.3

Reverse Transcription of the B.licheniformis α-amylase mRNA.

Reverse transcripts were prepared and run on a 6% acrylamide/urea/T.B.E. gel against a sequencing ladder using RRP1 and RRP2 as sequencing primers as described in Chapter 2. The template used for sequencing was the EcoR I/Sal I fragment of the α-amylase cloned into M13mp8 (antisense strand). The tracks are; 1) mRNA reverse transcript using RRP1; 2) No primer control; 3) mRNA reverse transcript using RRP2; 4) As 3, but x2 loading. The major transcripts are arrowed, both run alongside the C residue (which will be G on the sense strand) at position 992 on the DNA sequence (figure 5.9.2).

accurate. Figure 5.9.3 shows the result of this experiment. In both instances the transcript starts at postion 992 at a guanine residue 27 bases upstream from the translation start site. With this information and the fact that there is normally 7 bases between the purine start point (Aoyama and Takanami, 1985) and the end of the -10 region, the -10 is most likely TACAAC. This would make the spacing between the -35 and -10 17 bases (16-18 bases being the conserved distance; Russell and Bennett, 1982). Although this is good evidence that this is the B.licheniformis α-amylase promoter, it still needs confirmation by mapping α-amylase transcripts produced in B.subtilis.

5.2.5 Determination of the 5' Sequence of pPX2.

As the -10 region differed from the published sequences (Stephens et al., 1984; Sibakov and Palva, 1984) of the 5' end of the gene, the sequence of the 5' end of the low copy number derivative (pPX2) was determined. This would rule out the possibility that a promoter "down" mutation had been selected due to cloning the gene on a high copy number vector. The 5' sequence of pPX2 was found to be identical to that of pPT81 (result not shown).

5.2.6 Transcription Termination.

The α-amylase gene is flanked at either end by two G/C rich palindromic sequences, followed by poly T

```
                 A
               T   T
               A   C
                C-G
                C-G
                G-C
                C-G
                G-C
                A-T
                A-T
                A-T
                G-C^A
                A-T
5'.....TGAATAAATGAGT   TTTGGAAGAAAA.....
        890                   940
```

```
                  G A
                 T   A
                  C-G
                  C-G
                  T-A
                  T-A
                  T-A
                  A-T
                  G-C
                  G-C
                  C-G
                  A-T
......TAGAAGAGCAGAGAGG   TTTTTTATTTTGCC.....3'
   2555                        2608
```

<u>Figure 5.10</u>

Hypothetical secondary structures of the putative terminators flanking the α-amylase coding sequence. The numbers refer to the nucleotides in figure 5.7. The calculated free energies of these structures are -18.9 and -17.2 kcal/mol respectively (Tinoco *et al*., 1973).

where there is 74% identity. When looking at the choice of codons of the conserved amino acids there is no codon bias when choosing synonomous codons. From this it can be concluded that, both structurally and functionally, these enzymes are almost identical. It is of interest that these two enzymes show slightly different end product specificity. The major initial breakdown products of the B.amyloliquefaciens α-amylase are maltose and maltotriose, followed by significant amounts of maltohexaose (G6) and maltoheptaose (G7) (Welker and Campbell, 1967), whereas the B.licheniformis α-amylase forms mainly maltopentaose (G5) (Morgan and Priest, 1981; this work). It follows that these enzymes would be ideal candidates for "protein engineering" as small changes in the protein may lead to changes in end product specificity. The B.licheniformis α-amylase also shows a 59% homology at the protein level with the B.stearothermophilus α-amylase (Ihara et al., 1985). As neither of these enzymes are homologous with the B.subtilis saccharifying α-amylase (Yang et al., 1983). It therefore seems likely that the liquefying α-amylases exist as a homologous class distinct from the saccharifying α-amylases.

A comparison of the 5′ DNA sequence of the α-amylase gene from B.licheniformis NCIB 6346 (this work) and the 5′ sequence of the α-amylase gene from B.licheniformis NCIB 14580 shows a number of differences (Figure 5.13). It is interesting that the putative -10 region of the NCIB 14580 α-amylase gene (TACAAT) is

closer to the consensus (only one base different from the consensus sequence, TATAAT) than the -10 region of the NCIB 6346 α-amylase gene (TACAAC). The plasmid carrying the α-amylase from B.licheniformis NCIB 14580 is very unstable in E.coli (Sibakov and Palva, 1984). As the cloned α-amylase from B.licheniformis NCIB 6346 is stable in E.coli (section 5.4.5) this instabilty is presumably due to growth defects caused by the overexpression of the α-amylase from B.licheniformis NCIB 14580 because it has a stronger promoter. Evidence that the B.licheniformis α-amylase is lethal when overexpressed in E.coli is presented in section 5.3.3.

```
-29                                            +1                                 20
M K Q Q K R - L Y A R L L T L L F A L I F L L P H S A A A A A N L N G T L M Q Y F E W Y M P N D G Q H W K R L Q N D S A Y
M I Q K R K R T V S F R L V L M C T L L F V S L P I T K T S A V N G T L M Q Y F E W Y T P N D G Q H W K R L Q N D A E H
-31                                                +1                                 20

            40                           60                           80
L A E H G I T A V W I P P A Y K G T S Q A D V G Y G A Y D L Y D L G E F H Q K G T V R T K Y G T K G E L Q S A I K S L H
L S D I G I T A V W I P P A Y K G L S Q S D N G Y G P Y D L Y D L G E F Q Q K G T V R T K Y G T K S E L Q D A I G S L H
              40                           60                           80

           100                          120                          140
S R D I N V Y G D V V I N H K G G A D A T E D V T A V E V D P A D R N R V I S G E H L I K A W T H F H F P G R G S T Y S
S R N V Q V Y G D V V L N H K A G A D A T E D V T A V E V N P A N R N Q E T S E E Y Q I K A W T D F R F P G R G N T Y S
              100                          120                          140

           160                          180                          200
D F K W H W Y H F D G T D W D E S R K L N R I Y K F Q - - G K A W D W E V S N E N G N Y D Y L M Y A D I D Y D H P D V A
D F K W H W Y H F D G A D W D E S R K I S R I F K F R G E G K A W D W E V S S E N G N Y D Y L M Y A D V D Y D H P D V V
              160                          180                          200

              220                          240                          260
A E I K R W G T W Y A N E L Q L D G F R L D A V K H I K F S F L R D W V N H V R E K T G K E M F T V A E Y W Q N D L G A
A E T K K W G I W Y A N E L S L D G F R I D A A K H I K F S F L R D W V Q A V R Q A T G K E M F T V A E Y W Q N N A G K
              220                          240                          260

               280                          300                          320
L E N Y L N K T N F N H S V F D V P L H Y Q F H A A S T Q G G G Y D M R K L L N G T V V S K H P L K S V T F V D N H D T
L E N Y L N K T S F N Q S V F D V P L H F N L Q A A S S Q G G G Y D M R R L L D G T V V S R H P E K A V T F V E N H D T
               280                          300                          320

               340                          360                          380
Q P G Q S L E S T V Q T W F K P L A Y A F I L T R E S G Y P Q V F Y G D M Y G T K G D S Q R E I P A L K H K I E P I L K
Q P G Q S L E S T V Q T W F K P L A Y A F I L T R E S G Y P Q V F Y G D M Y G T K G T S P K E I P S L K D N I E P I L K
               340                          360                          380

               400                          420                          440
A R K Q Y A Y G A Q H D Y F D H H D I V G W T R E G D S S V A N S G L A A L I T D G P G G A K R M Y V G R Q N A G E T W
A R K E Y A Y G P Q H D Y I D H P D V I G W T R E G D S S A A K S G L A A L I T D G P G G S K R M Y A G L K N A G E T W
               400                          420                          440

               460                          480
H D I T G N R S E P V V I N S E G W G E F H V N G G S V S I Y V Q R   B.licheniformis
Y D I T G N R S D T V K I G S D G W G E F H V N D G S V S I Y V Q K   B.amyloliquifaciens
               460                          480
```

<u>Figure 5.11</u>

<u>Comparison of the Amino Acid Sequences of the B.licheniformis and B.amyloliquefaciens α-amylases.</u>

Homology is denoted with an asterisk (*), upright bars denote similar amino acids. The mature proteins start at +1, the signal sequences are shown in minus figures. The <u>B.amyloliquefaciens</u> sequence is from Takkinen <u>et al</u>., 1983.

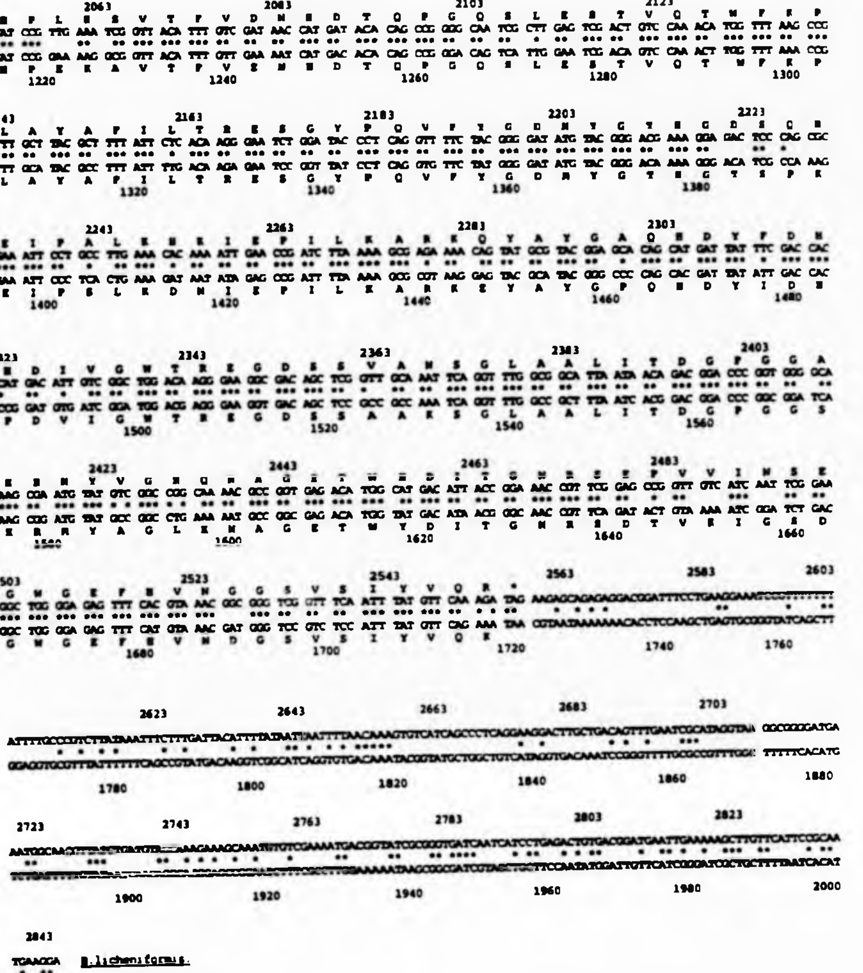


Figure 5.12

Comparison of the B.licheniformis and the B.amyloliquefaciens α-amylases.

The best alignment is achieved by assuming a deletion of six bases at residue 796 on the B.licheniformis α-amylase sequence (upper sequence). The numbers on the B.licheniformis sequence refer to the nucleotides in figure 5.7. The B.amyloliquefaciens sequence is as numbered by Takkinen et al., (1983). Asterisks denote base identity.

```
910                930                950                970
GCCATATCGGCGCTTTTCTTTTGGAAGAAAATATAGGGAAAATGGTACTTGTTAAAAATTCGGAATATTTATACAACATC
                   *   *                  *    *             **             * *
GCCATATCGGCGCTTTTCTGTTGCAAGAAAATATAGGGAAAACGATATTTGTTAAAAATTCAAAATATTTATACAATAGC
                  20                40                60                80


990           1010                            1030                        1050
                                   M   K   Q   q   K   R   L   Y   A   R   L   L
ATATGTTTCACATTGAAAGGGGAGGAGAA—TC  ATG AAA CAA CAA AAA CGG CTT TAC GCC CGA TTG CTG
  *        *            **   **                *        *       *
ATGTGTTTCACTTTGAAAGGGGAGAGGAAAATC ATG AAA CAA CAC AAA CGC CTT TAT GCC CGA TTG CTG
                                   M   K   Q   h   K   R   L   Y   A   R   L   L
            100                           120                     140


                1070                        1090                    1110
 t   L   L   F   A   L   I   F   L   L   P   H   S   A   A   A   A   A   N   L   N
ACG CTG TTA TTT GCG CTC ATC TTC TTG CTG CCT CAT TCT GCA GCA GCG GCG GCA AAT CTT AAT
 *                                           *           *
CCG CTG TTA TTT GCG CTC ATC TTC TTG CTG CCT CAC TCT GCA GCT GCG GCG GCA AAT CTT AAT
 p   L   L   F   A   L   I   F   L   L   P   H   S   A   A   A   A   A   N   L   N
        160                         180                         200


            1130                        1150                        1170
 G   T   L   M   Q   Y   F   E   W   Y   M   P   N   D   g   Q   H   W   K   R   L
GGG ACG CTG ATG CAG TAT TTT GAA TGG TAC ATG CCC AAT GAC GGC CAA CAT TGG AAG CGT TTG
                 *           *               *           *                   *   *
GGG ACG CTG ATG CAA TAT TTT GAG TGG TAC ATG CCA AAT GAC CGT CAA CAT TGG AAA CGC TTA
 G   T   L   M   Q   Y   F   E   W   Y   M   P   N   D   r   Q   H   W   K   R   L
        220                     240                         260


        1190                        1210                    1230
 Q   N   D   S   A   Y   L   a   E   H   G   I   T   a   v   W   I   P   P   A   Y
CAA AAC GAC TCG GCA TAT TTG GCT GAA CAC GGT ATT ACT GCC GTC TGG ATT CCC CCG GCA TAT
     *                       *       ?               *   *           *
CAA AAT GAC TCG GCA TAT TTG GTT GAA GAC GGT ATT ACT GTC GCC TGG ATT TCC CCG GCA TAT
 Q   N   D   S   A   Y   L   v   E   H   G   I   T   v   a   W   I   S   P   A   Y
  280                       300                     320


    1250                    1270                    1290
 K   G   T   S   Q   a   D   V   G   Y   G   A   Y   D   L   Y   D   L   G   E   F
AAG GGA ACG AGC CAA GCG GAT GTG GGC TAC GGT GCT TAC GAC CTT TAT GAT TTA GGG GAG TTT
             *       **      *           *           *   *
AAG GGA ACG AGT CAA GAC GAT GTA GGC TAC GGC GCT TAC GAT CTG TAT GAT TTA GGG GAG TTT
 K   G   T   S   Q   d   D   V   G   Y   G   A   Y   D   L   Y   D   L   G   E   F
340                     360                         380                         400


1310                        1330
 H   Q   K   G   T   V   R   T   k   y   g   t
CAT CAA AAA GGG ACG GTT CGG ACA AAG TAC GGC ACA     B.licheniformis NCIB 6346
                                 ** *** *** * *
CAT CAA AAA GGG ACG GTT CGG ACA AGT CTT CAT TCC     B.licheniformis NCIB 14580
 H   Q   K   G   T   V   R   T   s   l   h   s
                    420
```

Figure 5.13

Figure 5.13

Comparison of the N-Terminal α-amylase Sequences of *B.licheniformis* NCIB 6346 and NCIB 14580.

The differences in the DNA sequence are denoted as asterisks (*). Amino acid changes are shown in lower case. The question mark indicates what is presumably an error in the manuscript (Sibakov and Palva, 1984), GAC codes for an aspartate residue.

5.3 Expression of the B.licheniformis α-amylase in E.coli.

For a gene cloned into a multicopy plasmid, the expression of the B.licheniformis α-amylase gene is very poor. For example a protein band corresponding to the α-amylase can not be visualised on SDS-PAGE gels, whereas for the K.pneumoniae amylase and pullulanase, a protein band is observed (Chapter 3: Figure 3.10; Chapter 4: Figure 4.9). However, the B.licheniformis α-amylase does exhibit a much higher specific activity than the K.pneumoniae amylase (see section 5.4.1).

5.3.1 Over Expression of the B.licheniformis α-amylase in E.coli.

There are a number of reasons why it would be advantageous to overexpress the α-amylase:-

a) Eventually it would be desirable to construct a strain which could grow on starch using one copy of the gene integrated into the chromosome. This would relieve the cell of the added burden of making multicopy plasmids which is a significant energy drain during a fermentation. Single copy chromosomal insertion also removes any potential problems of plasmid stability (although not a problem in this case;see section 5.4.5). Unfortunately the natural

promoter of the α-amylase gene does not give high enough expression for at least 50 copies are needed for efficient growth (see section 5.5.1).

b) It would make large scale production and purification of the protein much easier so that the purified enzyme could be studied. However, overexpression of secreted proteins has been shown to be deleterious to the growth of E.coli (Yang et al., 1983; Willemot et al., 1983). Thus it was of interest to determine if this was the case with the α-amylase from B.licheniformis.

The strategy for overexpression was to alter the promoter. In order to do this it was necessary to put a restriction site in the ribosome binding or Shine Dalgarno (SD; Shine and Dalgarno, 1975) site of the natural promoter such that the GGA of the BamH I site would act as the ribosome binding site. Once the restriction site was created the α-amylase could then be put under control of the tac promoter (or any promoter containing a 3' BamH I site. This strategy has been used successfully on the cloned structural genes (aspC, tyrB and aroF) of the aromatic amino acid biosynthetic pathway (M.Edwards pers. comm.).

5.3.2 Site Directed Mutagenisis and Cloning of the tac Promoter onto the B.licheniformis α-amylase Gene.

A BamH I site was inserted into the SD box by changing GGA at position 1011 to TCC, using a 3 base mismatch primer (figure 5.14). Figure 5.15 shows how this was acheived to produce pPT109. HW87 carrying pPT109 grew normally on starch thereby showing that the SD box was still functional. The sequence of the 5′ end of pPT109 was checked by dideoxy sequencing and was found to be correct (figure 5.16). This construct then allowed the α-amylase gene to be put under tac promoter control. The tac promoter is a hybrid promoter consisting of the trp -35 and lacUV5 -10 region (trp::lac; Russell and Bennett, 1982). The new construct was called pPT172 (figure 5.17) and its sequence was confirmed by dideoxy sequencing (figure 5.18). The tac promoter was chosen because it can be controlled (lac repressor) and is a very strong promoter when induced (lactose or IPTG). HW1111 ($lacI^q$ tyrA) was transformed with pPT172. This $lacI^q$ strain (over produces 10x the lac repressor; Calos, 1978) was used to improve the lac repressor control as the promoter was present on a multicopy plasmid and would titrate out normal levels of the repressor.

5.3.3 Expression of B.licheniformis α-amylase Under tac Control.

HW1111/pPT172 was streaked onto L-agar + Cb plates plus and minus IPTG and incubated at 37 ^{o}C. In the

```
                              (TCC)      M   K   Q   Q
5'....ATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAA....3'
                 |||||||||    ||||||||
           3'   CTTTCCCCTAGGCTTAGTAC   5' 20 mer

   990       1000       1010       1020       1030
```

Figure 5.14

Mutagenesis of the Ribsome Binding Site of the B.licheniformis α-amylase Gene.

The 3 base mismatch primer is shown as it would anneal to the template (wild type sequence). Matches are shown as upright bars. The (TCC) depicts the sequence after mutagenesis which creates a BamH I site (GGATCC).

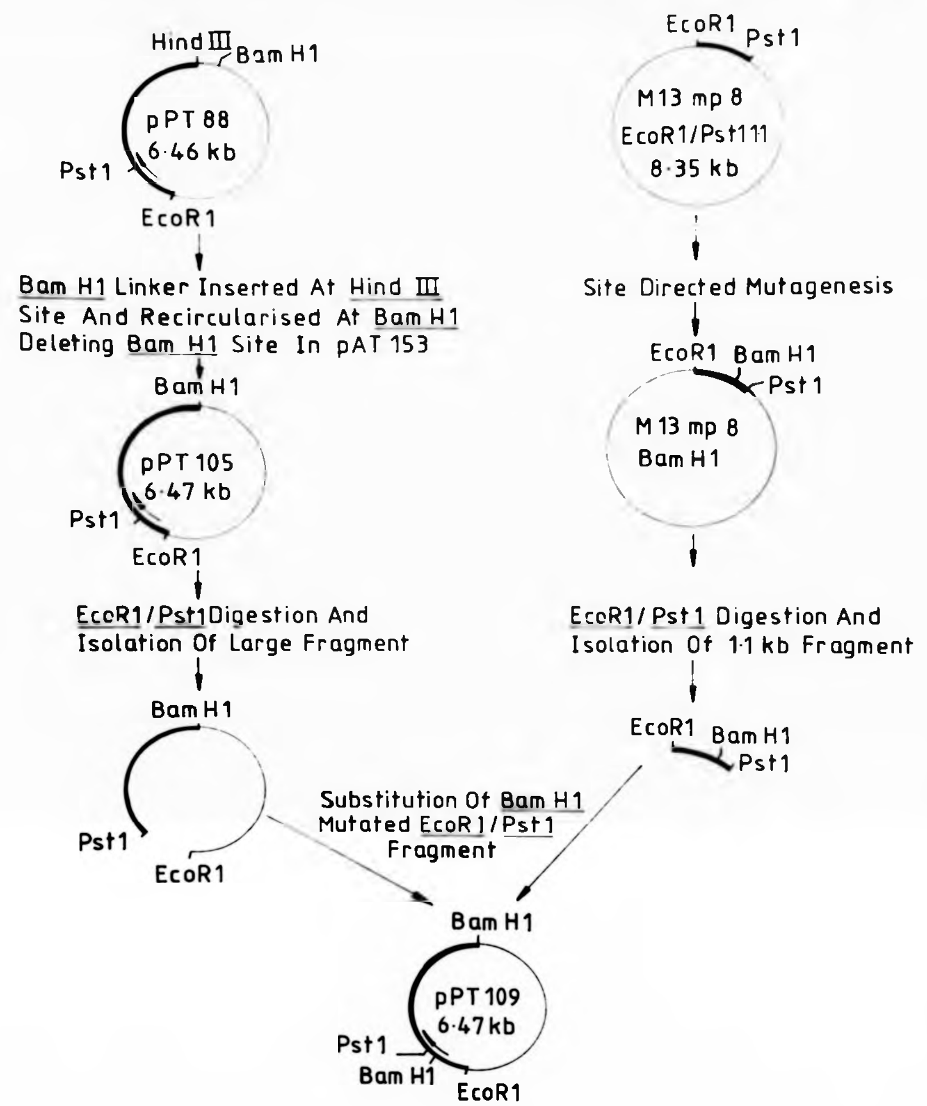
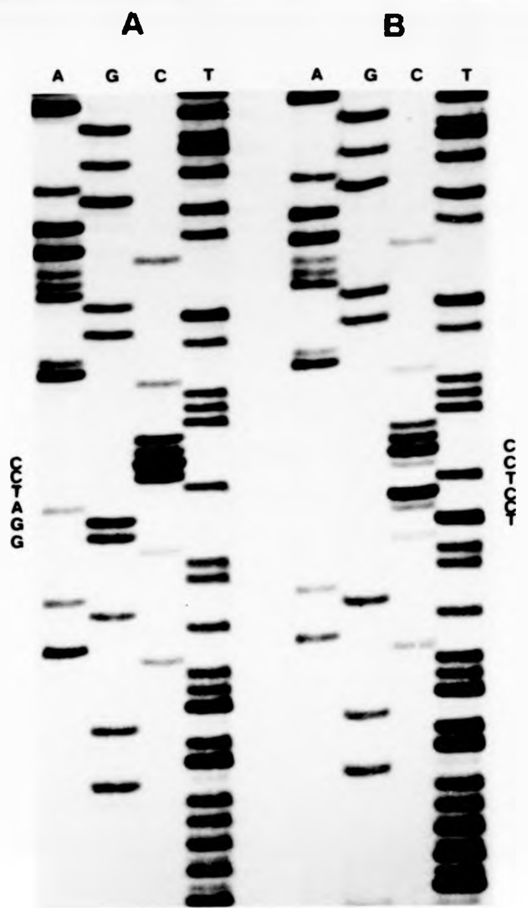


Figure 5.15

Construction of pPT109; α-amylase with a BamH I site in the Ribosome Binding Site.

All manipulations were carried out as described in Chapter 2. Construction of pPT88 is shown in figure 5.3. M13mp8 EcoR I/Pst I 1.1 carries the EcoR I/Pst I, 1.1 kb fragment from pPT81 (figure 5.2).



<u>Figure 5.16</u>

<u>Sequence Confirmation of the BamH I Mutated Ribosome Binding Site.</u>

The 1.1 kb <u>Eco</u>R I/<u>Pst</u> I fragment from pPT109 (figure 5.15) was cloned into M13mp8 and single stranded DNA was prepared as described in Chapter 2. A) The dideoxy sequence of this fragment showing the <u>Bam</u>H I site GGATCC B) The dideoxy sequence of the 1.1 kb <u>Eco</u>R I/<u>Pst</u> I fragment from pPT81 (wild type gene; figure 5.2) cloned in M13mp8. The sequence reads 5′ top to 3′ bottom.

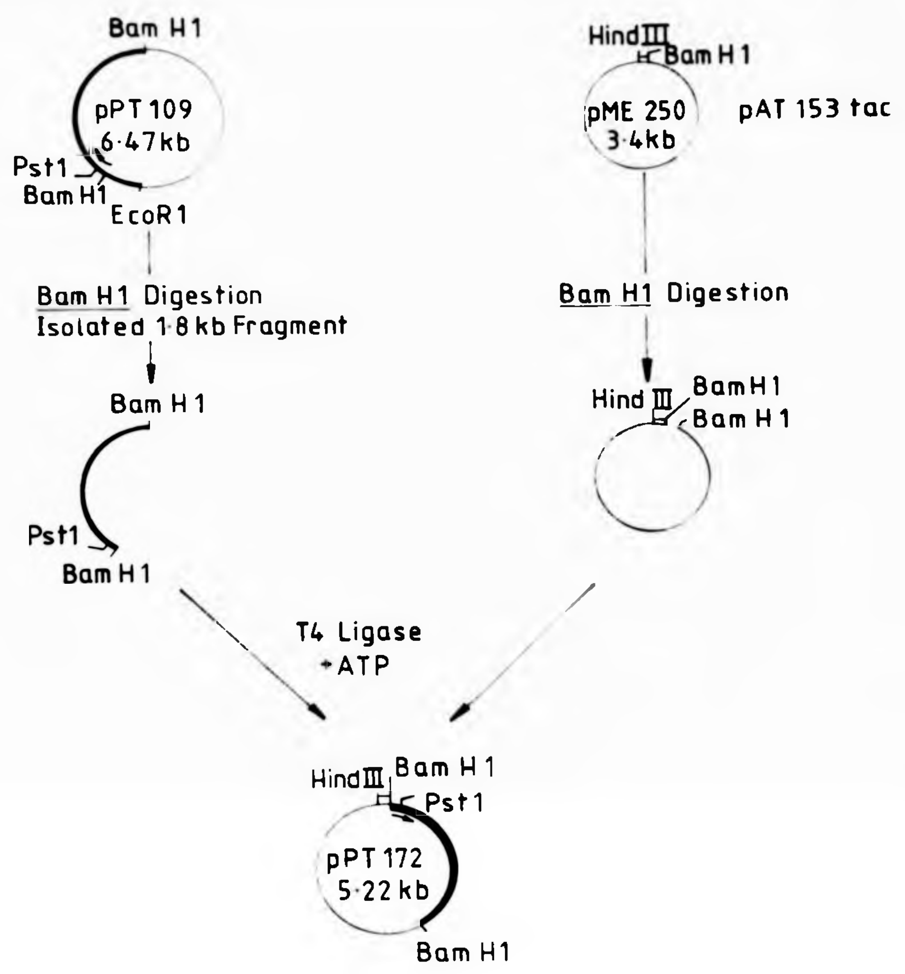


Figure 5.17

Construction of pPT172 (tac/amylase).

All manipulations were carried out as described in Chapter 2. Construction of pPT109 is shown in figure 5.15. pME250 is pAT153 with the tac promoter fragment (Russell and Bennett, 1982) cloned between the *Hind* III and *BamH* I sites.

Figure 5.18

Sequence of the tac/amylase Promoter.

The EcoR I/Pst I fragment from pPT172 was cloned into M13mp8 and sequenced as described in Chapter 2. The complementary strand of this sequence is shown below the gel and the trp -35 (TTGACA) and lacUV5 -10 (TATAAT) are underlined. The sequence is numbered with respect to the mRNA start point at +1.

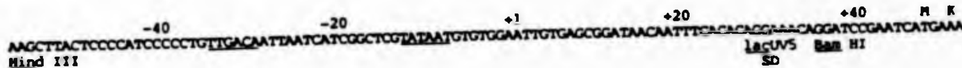


Figure 5.18

presence of IPTG the cells did not grow on minimal or complex medium (Table 5.3). However, even when uninduced the plasmid still allowed growth on starch showing that *lac* control is not 100% efficient. This is very interesting because it shows that relatively low levels of expression of the α-amylase allow efficient growth on starch.

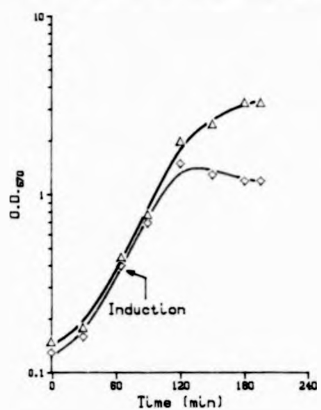
Table 5.3

Effect of IPTG Induction of HW1111/pPT172 on Growth.

	L-agar	M9 + glucose	M9 + starch
Uninduced	+	+	+
Induced	-	-	-

All plates were supplemented with Cb. Minimal plates contained tyrosine (HW1111 is a *tyr* auxotroph). Incubation temperature was 37°C for up to 144 hr.

The lethal effect was studied in more detail by following the growth curve in shake flasks containing L-broth. Figure 5.19, a and b show the effect of inducing with IPTG at OD_{670} 0.2 and 0.4. The effect is the same in both cases. After induction the culture goes through approximately two generations then stops growing (in fact cell lysis occurs because the OD_{670} drops). After a lag of 90 min growth recommences, but at a much reduced rate (figure 5.19, b). This may be due to cells which have either lost the plasmid or mutants which are resistant to the lethal effect. SDS-PAGE of induced samples failed to show a protein band corresponding to



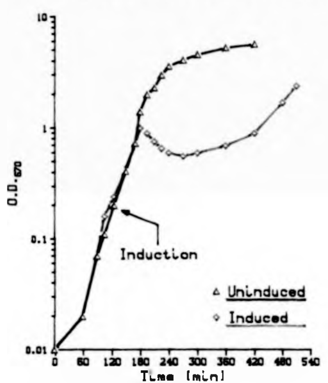


Figure 5.19

Effect of Induction of pPT172 (tac/α-amylase) in Complex Media on Growth.

50 ml cultures in 500 ml baffle flasks were inoculated with an overnight culture of HW1111/pPT172. The medium in both cases was L-broth + Cb. The OD was followed before and after induction with IPTG (0.2mM final concentration).

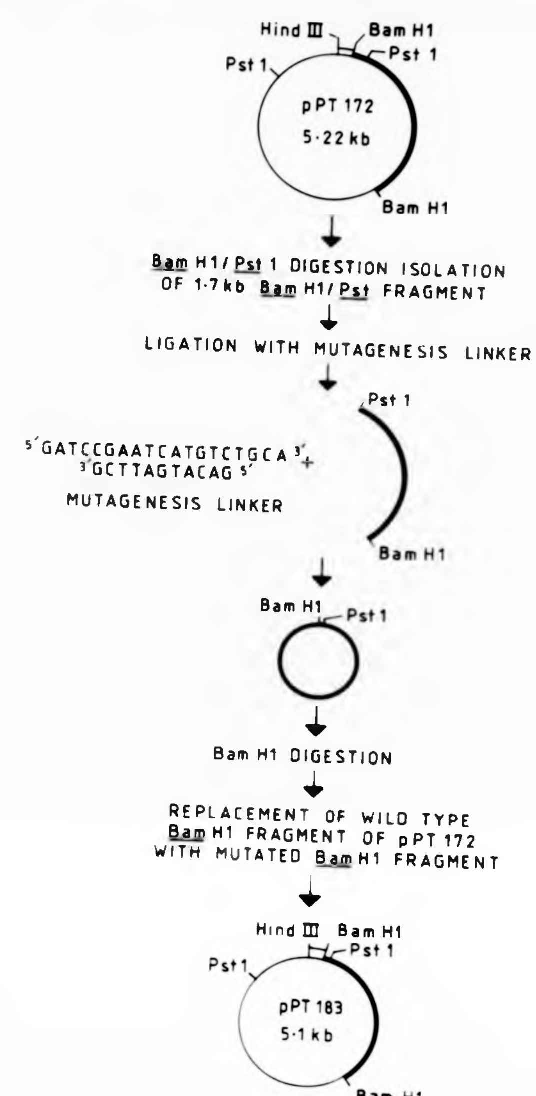
the α-amylase (results not shown). This is evidence that only low expression of the α-amylase in E.coli is needed to allow starch utilization and that over-expression is detrimental to growth.

5.3.4 Mutagenesis of the B.licheniformis α-amylase Signal Sequence.

To determine if the α-amylase was directly toxic to the cell or had the effect of blocking export sites in the cell membrane, the DNA sequence was mutated so that the major part of the signal sequence was removed. Essentially the 84 bp BamH I/Pst I fragment from pPT172 (tac/α-amylase) was replaced with a BamH I/Pst I linker which deletes 23 amino acids from the signal sequence to give pPT183 (figure 5.20). The construct identity was confirmed by restriction analysis and sequencing (figure 5.21).

5.3.5 Expression of HW1111/pPT183

HW1111/pPT183 was grown on L-agar + Cb plates plus and minus IPTG. As can be seen from table 5.4 the IPTG had no effect on growth on complex media. In liquid medium induction with IPTG had no effect (figure 5.22). pPT183 was also tested for its ability to confer starch utilization, no growth was observed on M9 + starch medium plus and minus IPTG after 48 hrs. However, on incubation for a further 24-48 hrs a background of amy^+ revertants were observed, especially on the plates + IPTG. This was



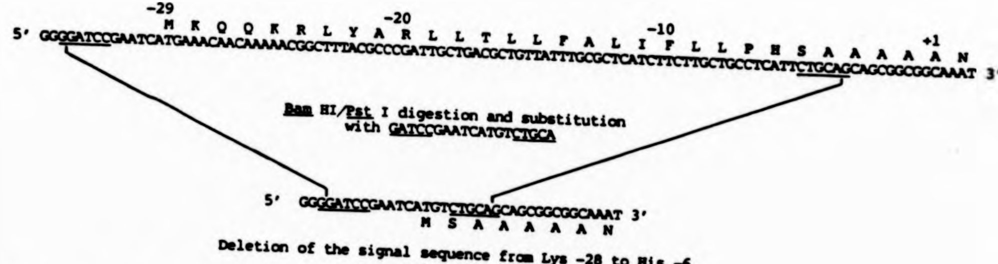
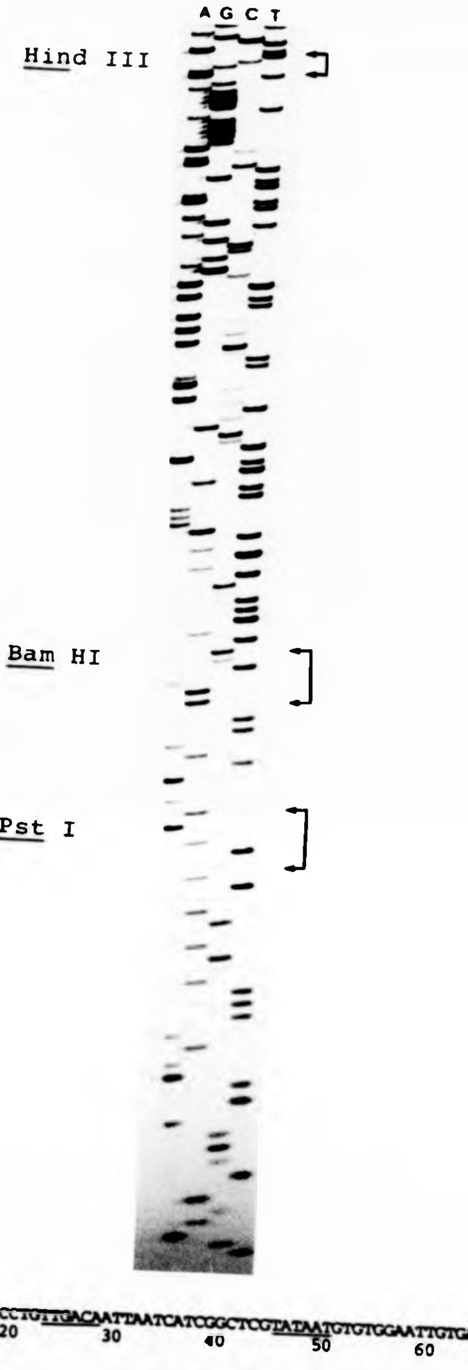


Figure 5.20

Figure 5.20

Construction of pPT183 - tac/α-amylase with Deleted Signal Sequence.

All manipulations were carried out as described in Chapter 2. pPT172 is described in figure 5.17. The predicted change at the DNA level is shown below the construction diagram. The mutagenesis primer effectively deletes 23 amino acids from lysine -28 to histidine -6 inclusive. The numbers refer to the start of the mature protein at +1.



5' AAGCTTACTCCCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGAT
10 20 30 40 50 60 70

M S A A A A A N L N G T L
AACAATTTCACACAGGAAACAGGATCCGAATCATGTCTGCAGCAGCGGCGGCAAATCTTAATGGGACGCTG...3'
80 90 100 110 120 130 140

Figure 5.21

Figure 5.21

Confirmation of the 5' Sequence of tac/α-amylase N-terminal Deletion (pPT183).

The EcoR I/Sal I fragment from pPT183 (figure 5.20) containing the 5' end of the deleted α-amylase gene was cloned into M13mp8 and dideoxy sequenced using sequencing primer RRP1 (figure 5.9.2). The complementary strand of this sequence is shown below the gel. The trp -35 (TTGACA) and lacUV5 -10 (TATAAT) are underlined. The sequence is numbered with respect to the 5' end of the Hind III site (AAGCTT). The sequence is identical to that which was predicted (figure 5.20).

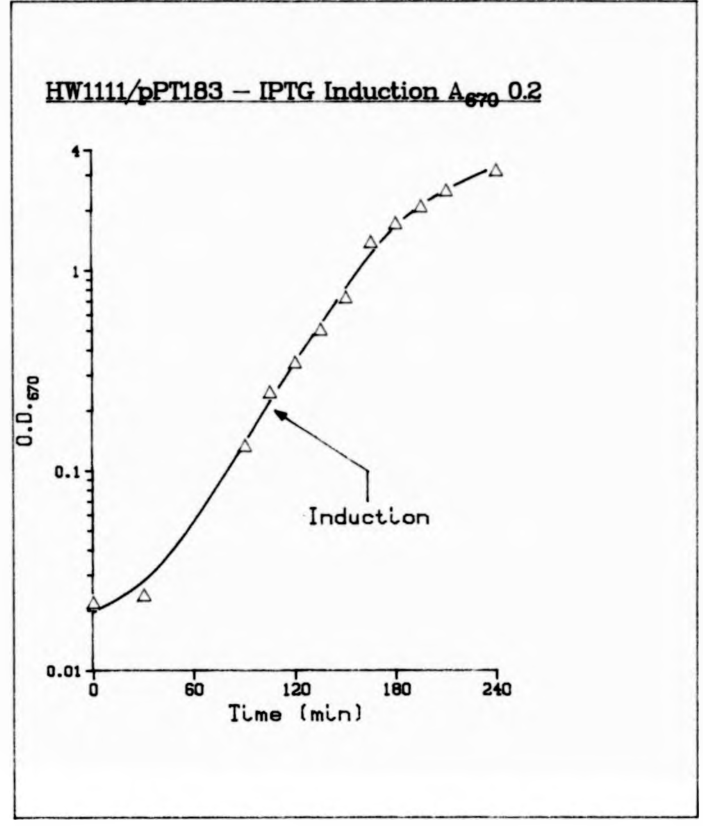


Figure 5.22

Induction of pPT183 - tac/N-termΔ α-amylase in Complex Media.

50 ml cultures in 500 ml baffle flasks were inoculated with an overnight culture of HW1111/pPT183. The medium was L-broth + Cb. The OD was followed before and after induction. A duplicate culture which was not induced was also monitored. The growth curve was identical to the induced curve and so is not shown.

initially taken as evidence that the α-amylase had to be exported to the periplasm in order to utilize starch. However, when extracts from HW1111/pPT183 were assayed they contained no significant α-amylase activity. Under inducing conditions the truncated α-amylase could not be visualised by SDS-PAGE either (result not shown). It is clear from this that the expression of this construct is very low. As the 5′ sequence is correct the transcription/expression should be high. However, it may be that another mutation downstream of the area sequenced has occured during the cloning or possibly the mRNA is extremely unstable. Nevertheless, some expression of the truncated α-amylase does occur because, upon iodine staining, a weak halo was observed when HW1111/pPT183 was grown on L-agar + starch plates, but only under inducing conditions and incubation for 48 hr. Further analysis is required to determine the explanation for these conflicting results.

Table 5.4

Effect of IPTG Induction on Growth of HW1111/pPT183.

	L-agar	M9 + glucose	M9 + starch
Uninduced	+	+	- [a]
Induced	+	+	- [a]

All plates were supplemented with Cb. Minimal plates contained tyrosine (HW1111 is a *tyr* auxotroph). Incubation temperature was 37°C for up to 144 hr. a, no initial growth, but slow growing putative revertants appeared after 72 hrs (see text).

5.3.6 Determination of the Transcription Start Point of pPT172 (tac/amy).

The main reasons for determining the start site were as follows. Firstly it was necessary to make sure the mRNA start was not altered and secondly, as the mRNA start site of this promoter is known, it was a good test of the method (especially as the same primers could be used as in the determination of the natural promoter). As can be seen from figure 5.23 there are two major bands at position -1 and +1 corresponding to two A residues. It is interesting that the published start point for the lacUV5 promoter is from either the G and A at positions -2 and -1 and the start point for the *trp* promoter is at the A at +1 (Russell and Benett, 1982). It would appear from this that the hybrid promoter compromises between these two different mRNA start points.

Discussion

The main conclusion from this data is that the *B.licheniformis* α-amylase is only required in relatively low abundance in order to confer starch utilization on *E.coli*. As *tac* promoter constructs expressed in a *lac*I^q background have been shown to have a 90% reduction in expression to that of the induced expression level (Russell and Benett, 1982), this should allow the the *tac*/α-amylase to be integrated into the chromosome and still allow starch utilization when the promoter is induced. This is an important consideration in the

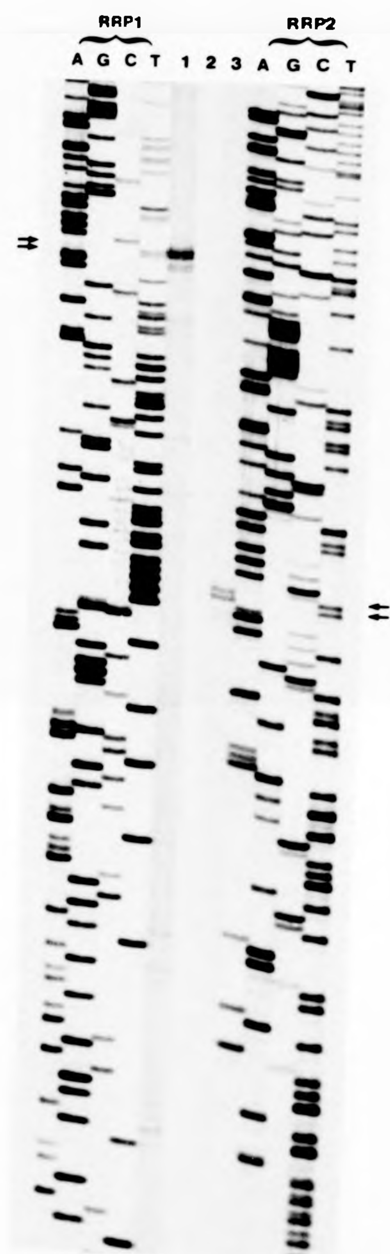


Figure 5.23

Figure 5.23

Reverse Transcription of the B.licheniformis tac/α-amylase (pPT172) mRNA.

Reverse transcripts were prepared and run on a 6% acrlamide/urea/T.B.E. gel against a sequencing ladder using RRP1 and RRP2 as sequencing primers as described in Chapter 2. The template for the sequencing reaction was the EcoR I/Sal I fragment of tac/α-amylase gene (pPT172) cloned into M13mp8 (antisense DNA strand). The template for the reverse transcript was mRNA prepared from HW1111/pPT172 which had been induced for 30 min with IPTG. The reverse transcription was carried out as described in Chapter 2. The numbered tracks are; 1) mRNA reverse transcipt using RRP1; 2) mRNA minus primer (control); 3) mRNA reverse transcript using RRP2. There are two major bands of equal abundance corresponding to two T residues on the gel (arrows). In the mRNA these will be two A residues at positions -1 and +1 (see DNA sequence in figure 5.18), +1 being the expected mRNA start point (Russell and Bennett, 1982).

industrial sense, because there is a general reluctance in using so called "recombinant organisms" and in this sense if the gene is integrated into the chromosomal DNA it is no longer considered a "recombinant". Obviously the _tac_ promoter would not be the final promoter of choice as induction by IPTG would not be feasible on an industrial scale. The obvious choice would be to put the gene under a _malT_ controlled promoter, so the α-amylase would be self regulating when grown on starch.

The reason for the apparent lack of expression from the truncated α-amylase (pPT183) is unclear. Obviously with the major part of the signal sequence removed the α-amylase will not be transported and will be produced cytoplasmically. In this "foreign" enviroment it may be very unstable (subject to proteolysis) or may even be toxic to the cell leading to selection of deletion mutants. If structural instabilty is the problem it was not immediately evident as no major deletions or rearrangments were seen when plasmid preparations were subjected to agarose gel electrophoresis. However, this does not preclude minor structural alterations. Alternatively, it could be that the truncated α-amylase is inactive with its mutated N-terminus. As protein corresponding to the α-amylase could not be observed by SDS-PAGE even under inducing conditions, lack of amylase detection is most likely due to a transciptional or translational problem. There is also the possibility a mutant which is analogous to the class III signal sequence mutants of the _lamB_ gene (see

Chapter 1; 1.4.6) has inadvertently been constructed. Mutants of this class have a block in expression caused by a mutation in the signal sequence of the LamB protein. Further analysis of the Amy^+ "revertants" should help determine the molecular events responsible for these results.

5.4 Properties of the B.licheniformis α-Amylase Expressed in E.coli.

5.4.1 Specific Activity of the Enzyme in Crude Extracts made from HW87/pPT80; The Effect of Glucose, Maltose and Starch.

Extracts were prepared from overnight cultures grown in M9 + leu medium containing glucose, maltose or starch and were assayed as described in Chapter 2.

Table 5.5

Specific Activity of Crude Extracts made from HW87/pPT80 grown on Glucose, Maltose and Starch Minimal Medium.

Carbon Source.	umoles maltose/min/mg protein
Glucose	1.125
Maltose	6.900
Starch	3.870

The medium was M9 + leu + Cb containing either glucose maltose or starch as sole carbon source. Extracts were prepared as described in Chapter 2 and assayed by the DNSA reducing end method.

From the information in table 5.5 it is clear that α-amylase activity is stimulated by both maltose and starch. However, it is difficult to determine if the gene is subject to glucose repression as a high α-amylase activity is found in the presence of glucose (extracts

made from HW87 grown on glucose, which did not contain the plasmid had 0 activity).

5.4.2 Temperature Profile of the Enzyme.

An S10 crude extract was prepared from HW87/pPT81 and the reducing activity was measured at temperatures ranging from 37-100°C. As can be seen from figure 5.24, the enzyme has a temperature optimum around 70°C. Activity is quickly lost above 80°C and is totally absent at 100°C.

5.4.3 Temperature Stability of the Enzyme.

The enzyme is completely stable at temperatures up to 65°C under the conditions used (figure 5.24). Above this temperature activity is quickly lost.

5.4.4 Action Pattern of the Amylase on Starch.

Figure 5.25 shows the action pattern of S10 α-amylase extracts on soluble starch. The major end products after prolonged digestion are maltopentaose (G5), maltotetraose (G4), maltotriose (G3), maltose (G2) and glucose (G1). The reported end products after digestion of starch with the purified enzyme (Morgan and Priest, 1981) are mainly maltopentaose with smaller amounts of glucose, maltose and maltotriose.
The high levels of G1-G4 are probably due to the intracellular degradative enzymes of E.coli.

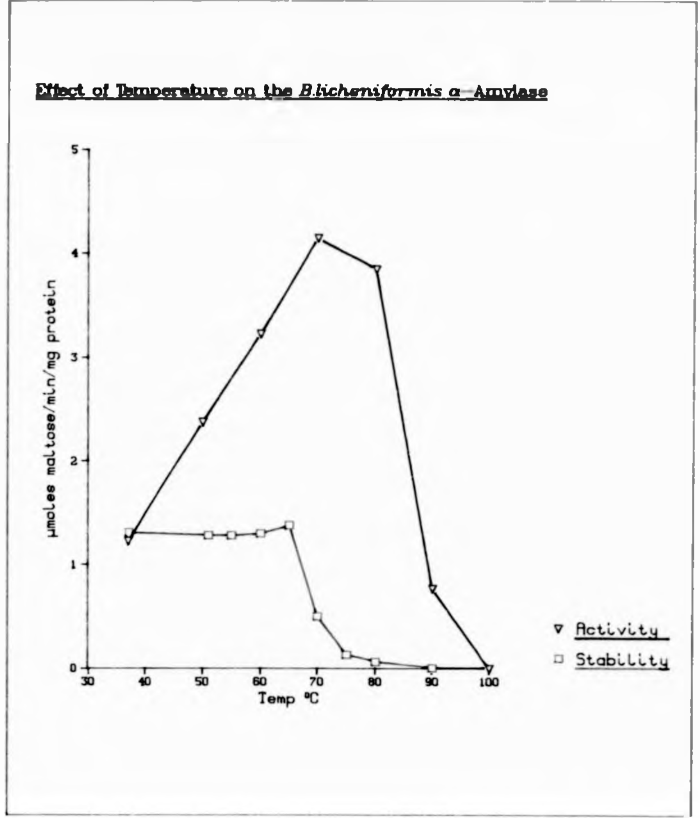


Figure 5.24

Effect of Temperature on the B.licheniformis α-amylase.

Extracts were prepared as described in Chapter 2. The temperature profile of activity (triangles) was determined by assaying the reducing activity at the temperatures indicated using the DNSA method at pH 7 (umoles maltose/min/mg protein). The temperature optimum for activity is 70-80 ^{O}C. The stability was determined by heating the extract for 15 min at temperatures ranging from 37-100°C. After cooling on ice the reducing activity was determined by the DNSA method at 37°C, pH 7. The enzyme is stable up to 65°C.

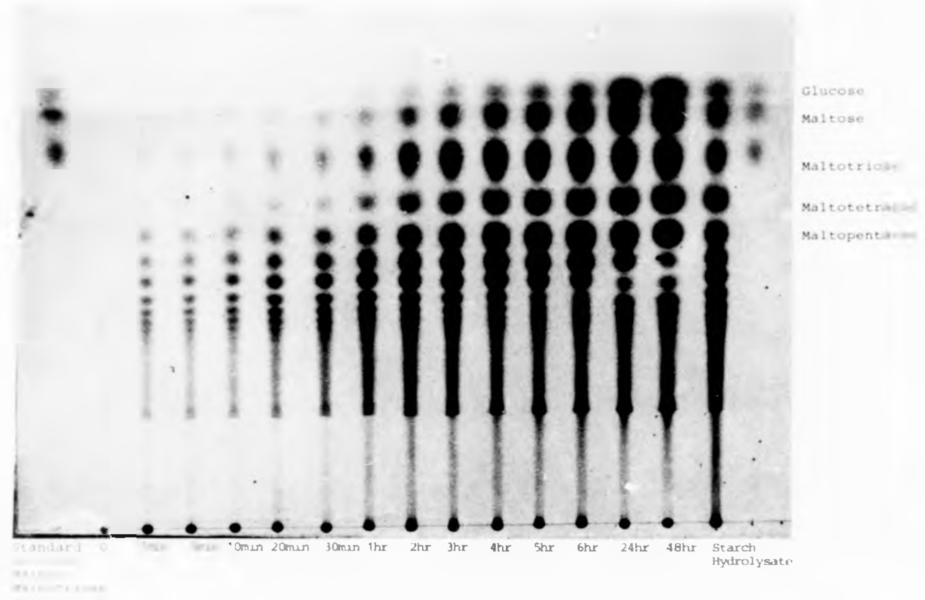


Figure 5.25

The Action Pattern of the B.licheniformis α-amylase on Starch.

Crude extracts were prepared from HW87/pPT81, incubated with 2% starch for the times shown and subjected to T.L.C. as described in Chapter 2.

5.4.5 Stability of Amylase Plasmids in HW87.

HW87 carrying pPT80 (high copy number) and pPX2 (low copy number) was subcultured without antibiotic selection in M9 + glucose + leu six times in succession (50-60 generations). The cells were then plated on L-agar and 100 individual colonies were replicated to M9 + starch + leu and L-agar with either Cb or Tc as appropriate. The results in table 5.6 show that both of these plasmids are structurally and segregationally stable in *E.coli* HW87. The *B.licheniformis* α-amylase cloned from a different strains has been shown to be unstable in *E.coli* (Sibakov and Palva, 1984; Joyet *et al.*, 1984; and Ortlepp *et al.*, 1983).

Table 5.6

Stability of pPT80 and pPX2 in *E.coli* HW87 Grown on Starch.

Strain	M9 + starch	LA + Cb	LA + Tc
HW87/pPT80	100/100	100/100	N/A
HW87/pPX2	100/100	N/A	100/100

M9 was supplemented with Leu. The ratios refer to the number of colonies picked to the number exhibiting either starch utilization or antibiotic resistance after six successive subcultures in M9 + glucose + leu in the absence of antibiotic selection.

5.4.6 Sub-cellular Location of the Amylase in E.coli.

In Gram negative bacteria, proteins can be located in five different compartments - the cytoplasm; inner membrane, periplasmic space, outer membrane or the culture medium - depending on the function of the protein. As the *B.licheniformis* α-amylase is exported into the medium in its Gram positive host (Morgan and Priest, 1981), its location in *E.coli* was of interest.

Compartmentalisation of the proteins was carried out using a cold osmotic shock procedure (Willis *et al.*, 1974). To ascertain that the osmotic shock procedure is working correctly, it is necessary to assay for a cytoplasmic marker(β-galactosidase) and a periplasmic marker (alkaline phosphatase) along side the protein under investigation. Ideally, a strain in which both of these activities are constitutively expressed, should be used. However, in the absence of such a strain both, of these enzymes can be induced. For this experiment HB101 was transformed with the various α-amylase constructs and alkaline phosphatase and β-galactosidase were induced.

5.4.7 Induction of Alkaline Phosphatase and β-galactosidase.

Alkaline phosphatase is regulated by the amount of phosphate (Pi) in the medium. When there is no available Pi, it is induced. β-galactosidase can be induced using IPTG, a gratuitous inducer of the *lac* operon. Initially

both of the enzymes were induced together, by growing the cells on NoPi minimal medium + maltose + IPTG. However, induction of both of these enzymes together was found to be detrimental and the cultures grew very slowly. Therefore it was necessary to induce and assay them separately.

β-galactosidase was induced by growing the cells to OD 0.4 in M9 + maltose medium, then IPTG was added and the culture grown to OD_{670} 0.6 before being "shocked". The alkaline phosphatase was induced by growth on NoPi medium + maltose to OD_{670} 0.6. After osmotic shock the cytoplasmic, periplasmic and culture medium fractions were assayed for α-amylase (Bernfeld, 1955), β-galactosidase (Miller, 1972) and alkaline phosphatase (Glenn and Mandelstam, 1971). Activity was expressed as units/ml of the original culture for each enzyme and the percentage activity in the cytoplasm, periplasm and culture medium was determined. Table 5.7 summarises the results of these experiments. The β-galactosidase and alkaline phosphatase were found predominantly in their correct locations (cytoplasm and periplasm) while the amylase was found almost exclusively in the periplasmic space. The same osmotic shock procedure was applied to cells grown to stationary phase (16 hrs) in M9 + maltose + Cb. Again, the location of the α-amylase was mainly in the periplasm (Table 5.8), but there was also a significant amount found in the culture medium. This extracellular α-amylase is probably due to cell lysis and not specific secretion.

Table 5.7

	CULTURE MEDIUM %			PERIPLASM%			CYTOPLASM%		
	β-Gal	Alk.Phos.	Amy	β-Gal	Alk.Phos.	Amy	β-Gal	Alk.Phos.	Amy
HW23[1]	0	11.7	0	0.35	76.0	0	99.65	12.3	0
HW23/pPT80[2]	0	9.3	0	0.38	82.4	96.9	99.62	8.3	4.0
HW23/pPT81[2]	ND	16.7	0	ND	76.6	97.0	ND	6.7	3.0
HW23/pPT83[2]	ND	11.0	0	ND	78.0	93.0	ND	11.0	7.0
HW23/pPX2[3]	ND	10.8	0	ND	81.4	99.0	ND	7.8	1.0

All osmotic shocks were carried out on mid-exponential cells (OD_{670} = 0.6)

ND = Not Determined

1 - No antibiotic selection

2 - Carbenicillin selection (200μg ml^{-1})

3 - Tetracyclin selection (10μg ml^{-1})

β-Gal - β-Galactosidase

Alk.Phos. - Alkaline Phosphatase

Amy - Amylase

Table 5.8

Subcellular Location of the B.licheniformis α-amylase in HW87/pPT80 Grown to Stationary Phase.

Location	% Activity
Culture medium	14
Periplasm	86
Cytoplasm	0

HW87/pPT80 was grown on M9 + maltose + leu + Cb for 16 hrs then osmotically shocked and fractionated as described previously.

Discussion.

The results obtained are consistent with the fact that the maltopentaose (G5) α-amylase has been cloned in E.coli. The cloned enzyme has similar properties to the purified enzyme from B.licheniformis (Saito, 1973; Chiang et al., 1979; Morgan and Priest, 1981). When the α-amylase is expressed in E.coli there are only significant amounts of α-amylase in the culture medium when the cells are approaching stationary phase. It therefore follows that the α-amylase does not have to be exported into the culture medium in order to utilize starch as a growth substrate.

Unlike other α-amylase genes which have been cloned in E.coli (Cornelis et al., 1982; Willemot and Cornelis, 1983; Yang et al., 1983; Ortlepp et al., 1983; Joyet et al., 1984; Sibakov and Palva, 1984) the

α-amylase gene from <u>B.licheniformis</u> NCIB 6346 does not show structural and segregational instability in <u>E.coli</u>. This is probably due to the fact that this α-amylase is poorly expressed. Therefore toxic effects caused by overexpression of this enzyme are not seen.

5.5 Growth of E.coli on Starch using the Cloned B.licheniformis α-amylase Gene.

All of the B.licheniformis amylase plasmids confer starch utilization on E.coli K12 in both a recA (HW87) and $recA^+$ (HW2) background (Table 5.9). As expected neither strain was able to utilize starch without the amylase plasmid.

Table 5.9

Growth Characteristics of HW87 and HW2 on Starch Minimal Medium.

Strain	M9 + starch	M9 + glucose
HW87[1]	-	+
HW87/pPT80	+	+
HW87/pPT81	+	+
HW87/pPT83	+	+
HW87/pPX2	+	+
HW2[2]	-	+
HW2/pPT80	+	+
HW2/pPT81	+	+
HW2/pPT83	+	+
HW2/pPX2	+	+

Plates were incubated at 37°C. 1 - media supplemented with leu (HW87 is a leu auxotroph). 2 - media supplemented with met (HW2 is a met auxotroph)

5.5.1 <u>Relative Growth Rates of HW87/pPT80 on Glucose, Maltose and Starch Minimal Medium.</u>

Initially the growth rate of HW87/pPT80 was determined on glucose, maltose and starch. The result of this experiment (figure 5.26) was that *E.coli* was able to grow almost as efficiently as it did on glucose and better than when grown on maltose. In order to determine if any cell free amylase (which might have been present in the inoculum due to cell lysis) helped in the initial growth on starch a culture was inoculated with cells that had been washed in saline. Although there is an initial lag the growth rate is only slightly affected. This rules out the possiblity that the growth was due to the amylase being released by lysis during growth.

Because HW87 has a fairly slow growth rate even when grown on glucose (d.t. 81 min) the question would the growth rate on starch be as good in a *rec*A$^+$ background was asked, so the growth rates of all of the HW2 α-amylase derivatives were determined. In liquid medium the high copy number derivatives have a doubling time which is comparable to growth on glucose whereas pPX2 has a much reduced growth rate. The growth curves are shown in figures 5.27.1 to 5.27.4 and the information is summarised in table 5.10. From this data it can be concluded that the gene must be present in a copy number of pBR322 level (≈50 per chromosome) for efficient growth on starch. It is interesting, that strains carrying plasmids containing the Km^r gene grow more slowly when

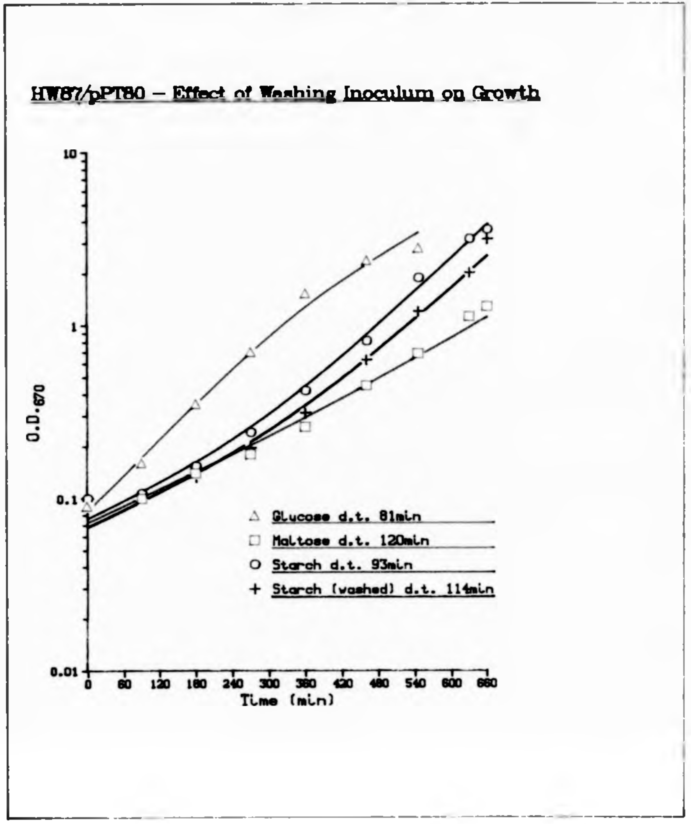


Figure 5.26

Growth Rates of HW87/pPT80 on Glucose Maltose and Starch.

0.5 ml of an overnight culture of HW87/pPT80, grown on M9 + starch + leu + Cb, was inoculated into 50 ml of the same medium in a 500 ml baffle flask and the OD was followed. Each culture was set up in duplicate. Starch (washed) refers to the inoculum being washed with saline before inoculation.

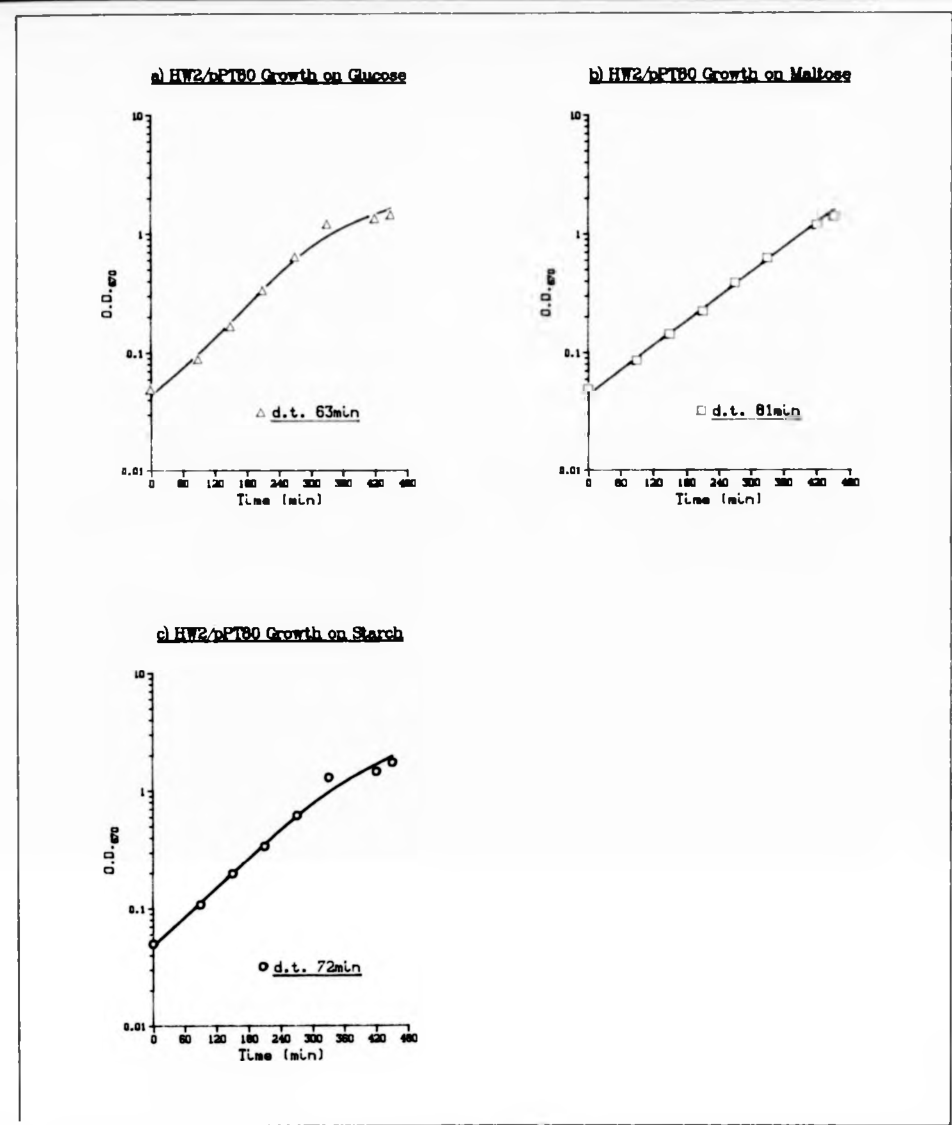


Figure 5.27.1

The medium was M9 + leu + Cb containing either glucose, maltose or starch, 50 ml in a 500 ml baffle flask. OD was followed at regular time intervals. pPT80 is a high copy number (87 per genome; table 5.1) plasmid derivative of the α-amylase gene (pAT153/ pUB110 B.subtilis shuttle vector).

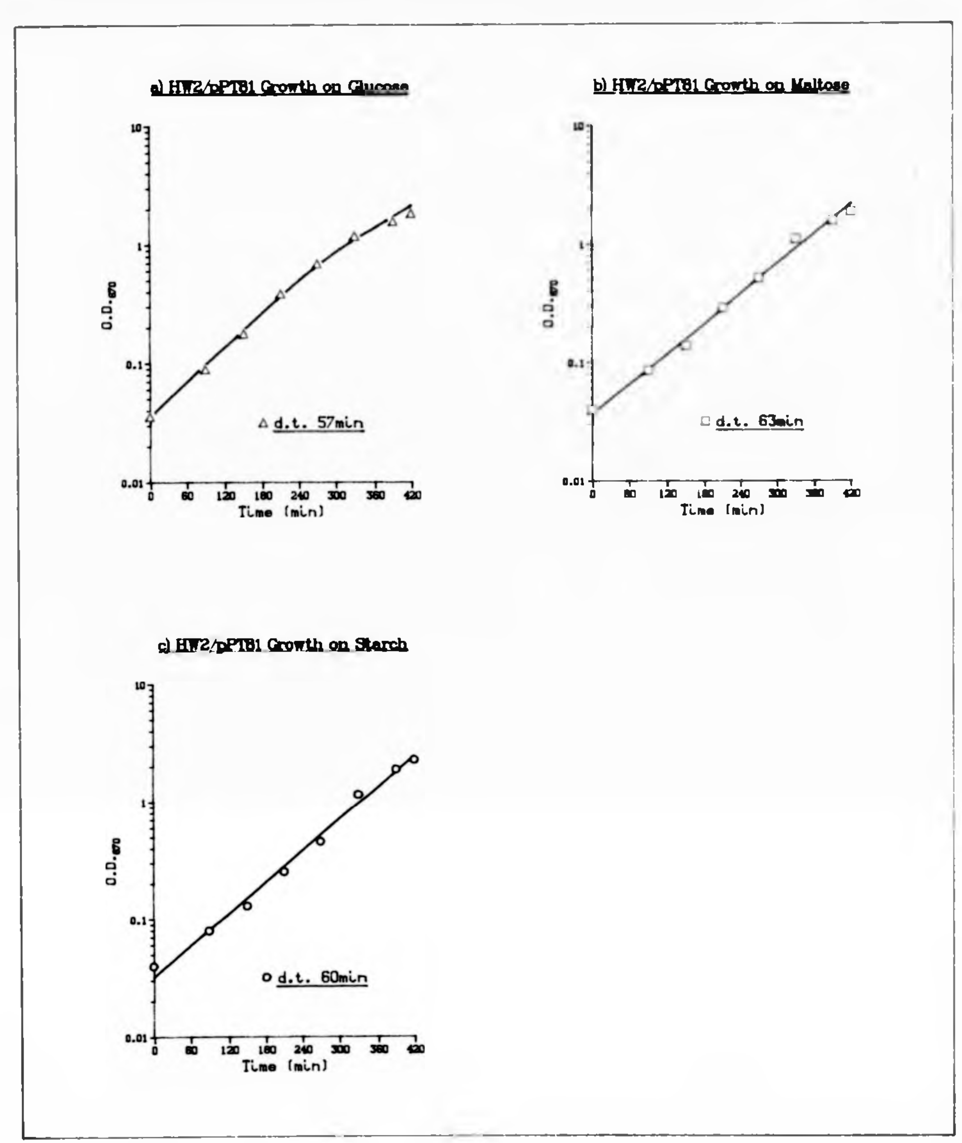


Figure 5.27.2

Growth conditions were as described previously (figure 5.27.1). pPT81 is a high copy number (89 per genome; table 5.1), pAT153 derivative carrying the α-amylase gene.

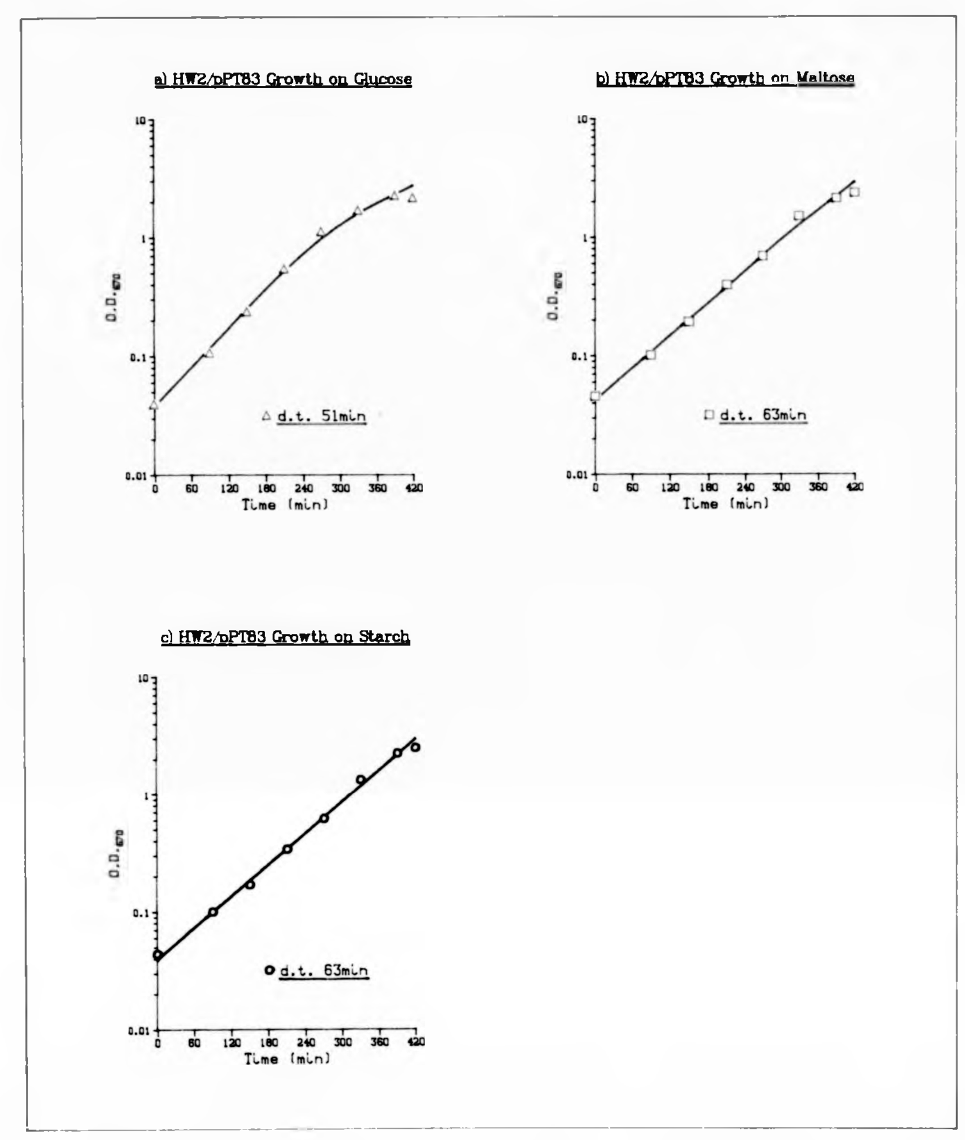


Figure 5.27.3

Growth conditions were as described previously (figure 5.27.1). pPT83 is the medium copy number (42 per genome), pBR322 derivative carrying the α-amylase gene.

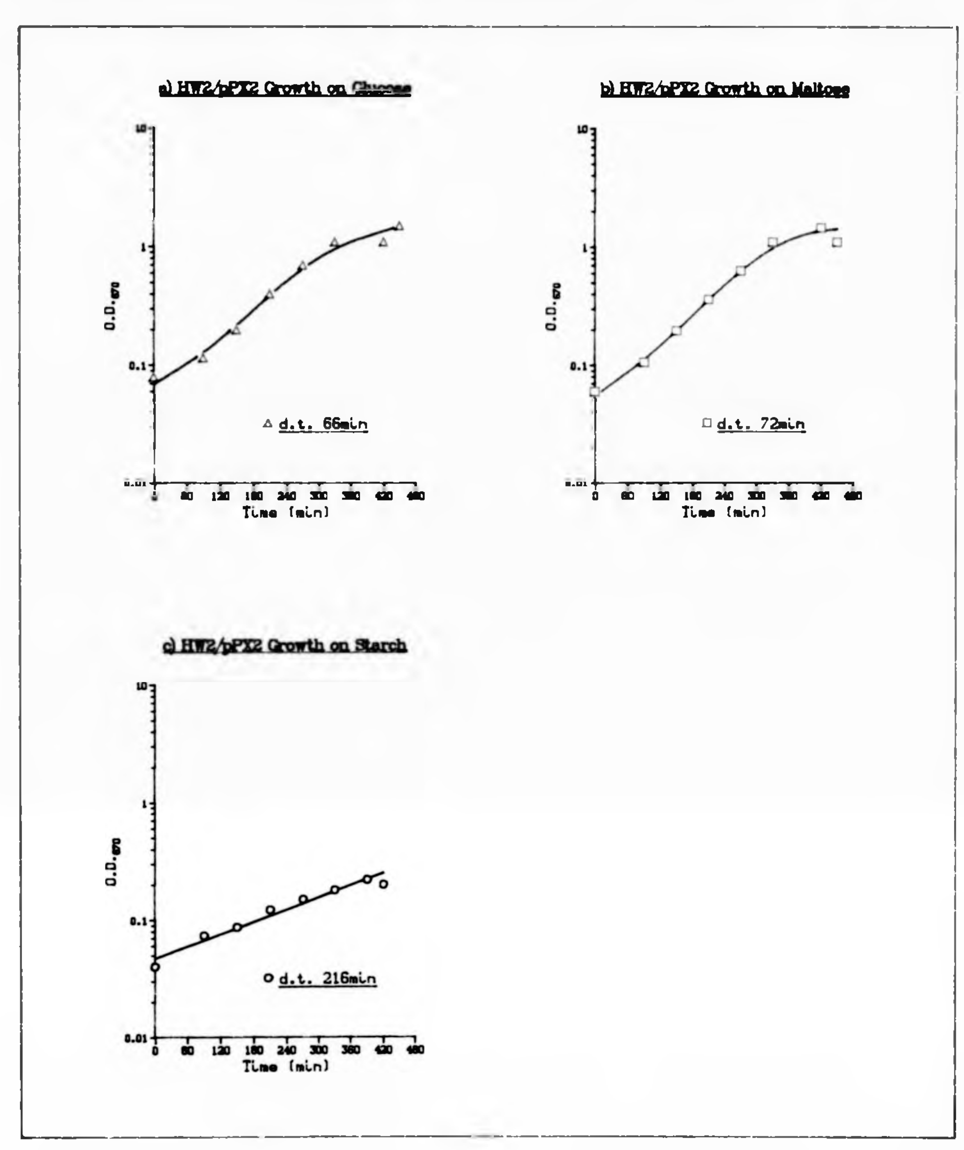


Figure 5.27.4

Growth conditions were as described previously (figure 5.27.1). pPX2 is the low copy number (27 per genome), pLG338 derivative carrying the α-amylase gene.

using maltose as a carbon source, but not when growing on glucose or starch.

Table 5.10

Growth in Liquid Minimal of HW2 carrying pPT80, pPT81, pPT83 and pPX2.

Strain	Copy No.	Glucose	Maltose	Starch
HW2/pPT80[a]	87	63 min	81 min	72 min
HW2/pPT81[a]	89	57 min	63 min	60 min
HW2/pPT83[a]	42	51 min	63 min	63 min
HW2/pPX2[b]	27	66 min	72 min	216 min

The medium was M9 + met + sugar as shown above. a: supplemented with Cb. b: supplemented with Tc. Copy number figures are from table 5.1.

5.5.2 The Effect of Maltose Mutations on Starch Utilization.

A variety of mal mutants were transformed with pPT81 and their growth characteristics were examined on M9 + starch and maltose. The results in table 5.11 show that the lamB gene product is an absolute requirement for growth on starch as is the malF gene product. It is suprising that both the malE, malG and malK mutants do not prevent growth on starch. This is unexpected because lesions in these genes should lead to a Mal$^-$ phenotype and a Dex$^-$ phenotype as was found previously in the case of the K.pneumoniae amylase (Chapter 3; section 3.3.2). This observation, and the general mechanism for starch utilization in E.coli will be discussed in more detail below.

Table 5.11

Effect of mal Mutations on Starch Utilization.

Strain		Growth 48 hr		Growth 144 hr	
		Maltose	Starch	Maltose	Starch
lamB+	(MC4100)	+++	+++	+++	+++
lamB	(pop3208)	+++	-	+++	-
malE	(TST1)	-	-	-	++
malF	(TST6)	-	-	-	-
malG	(TST2)	-	-	-	+
malK	(pop3295)	-	-	-	++
malQ	(MH70)	-	++	-	+++

The medium was M9 containing maltose or starch. Plates were incubated at 37°C. - (no significant growth) to +++ (full growth as compared to growth on maltose). All strains carried the amylase plasmid pPT81.

Discussion

It can be concluded from the results above that the B.licheniformis α-amylase expressed in E.coli allows this organism to use starch as sole carbon and energy source. A plasmid copy number of ≈50 is required for efficient growth on starch and a copy number of 90-100 is not detrimental to growth. This suggests that an α-1,6 hydrolysing enzyme is not essential for efficient utilization of starch (in contrast to what was thought at the start of this project). The lamB gene product is essential for starch utilization by the B.licheniformis

α-amylase (as was also found with the K.pneumoniae amylase; Chapter 3). This may be the key to how E.coli carrying an amylase gene can utilize starch. It has been shown conclusively that starch can access the periplasm via the lamB maltoporin (see Chapter 1). As the B.licheniformis α-amylase is found almost exclusively in the periplasmic space it can clearly come into contact with its substrate. Once the starch is cleaved, molecules smaller than maltoheptaose (G7) can be efficiently transported across the cytoplasmic membrane by the rest of the maltose/maltodextrin transport system into the cytoplasm where they can be degraded to glucose. Why the malE, malG and malK mutants carrying the amylase gene can grow on starch (albeit at a much slower rate) is unclear. The simplest explanation is that the mutations are leaky, however equivalent strains grown on maltose all show a Mal^- phenotype. Also, these same strains carrying the K.pneumoniae maltohexaose-producing amylase show a Dex^- phenotype. A possible explanation for these observations comes from the fact that the B.licheniformis α-amylase produces significant amounts of glucose on prolonged digestion. This glucose could be utilized. It has been shown that purified α-amylase from B.licheniformis does, in fact, produce significant amounts of glucose on prolonged digestion (Medda and Chandra, 1980; Morgan and Priest, 1981). Therefore in contrast to the B.licheniformis α-amylase this could explain why the K.pneumoniae amylase does not confer starch utilization in these strains, i.e. because it is an exoamylase and produces only maltohexaose (G6) as its

end product (Kainuma *et al*., 1975). If this is the explanation then it is unclear why the *malF* mutant is not also able to grow on starch. However, a more likely reason comes from the recent discovery that *E.coli* has a periplasmic α-amylase (Freundlieb and Boos, 1986). That *E.coli* contains an α-amylase (*amyS*) comes as somewhat of a surprise, especially as it was cloned from MC4100 (Casadaban, 1979) which was used in this study. The *E.coli* α-amylase has an M_r 66,000; is a periplasmic enzyme and can hydrolyse maltodextrins longer than maltose. The major end products after hydrolysis of maltodextrins are glucose, maltose and maltotriose. It is obviously an α-amylase but why it does not allow growth on starch is unclear. From the data (Freundlieb and Boos, 1986) it would appear that this enzyme is mainly active against maltodextrins in the G3-G15 size class and because of this, strains carrying the *amyS* gene do have a growth advantage over *amyS* mutants when grown on maltodextrins in the G7-G15 size range. Mutations in the *amyS* gene lead to a slow growth phenotype on maltodextrins larger than maltoheptaose (G7). It is interesting that *malE* containing strains carrying the *amyS* gene on a multicopy plasmid could grow on maltoheptaose (G7). This is presumably similar to what was found above when strains carrying the *B.licheniformis* α-amylase were able to grow even with a *malE*, *malF* or *malG* background, presumably due to feeding on the glucose produced by both the *B.licheniformis* α-amylase and the *amyS* gene product. It would also explain the difficulty experienced in determining the end products of the

K.pneumoniae maltohexaose-producing amylase and the K.pneumoniae pullulanase, as the end prodcuts would be quickly degraded by the amyS gene product. However, unlike what was found with the B.licheniformis α-amylase, malB strains carrying the K.pneumoniae maltohexaose-producing amylase do not grow on starch. This is presumably because maltohexaose is not produced quickly enough or in sufficient quantity for the amyS gene product to produce enough glucose to support growth. Why amyS mutants were not observed in previously performed Dex^- selections is unclear, especially as it has been reported previously that E.coli grows very poorly on maltodextrins larger than G7 (Ferenci, 1980). However it is clear that the enzyme is not very active on high molecular weight starch, as MC4100 or any of the various strains which were used throughout this project are unable to use starch as sole carbon and energy source without adding another amylolytic activity.

Overall Discussion and Conclusions.

From the results obtained in Chapter 3 and Chapter 5 it is clear that E.coli can be engineered to grow on starch by introduction of an amylase gene. This should hold true for any amylase gene cloned into E.coli as long as, a) it is not toxic to the cell, b) it is exported to the periplasm and c) the recipient strain has a fully functioning maltose/maltodextrin transport and utilization system. As was originally thought (Chapter 1) an α-amylase (endo-acting) is more efficient at starch

utilization and breakdown than an exo-acting amylase. The actual efficiency of carbon conversion by _E.coli_ carrying the _B.licheniformis_ α-amylase has not been determined, and there may be a resistant starch fraction (α-1,6,α-1,4 linked maltodextrins) which can not be utilized. It is interesting that overexpression of the _K.pneumoniae_ maltohexaose-producing amylase (or the _K.pneumoniae_ pullulanase) does not appear to be detrimental to _E.coli_, whereas only a low level expression of the _B.licheniformis_ α-amylase is lethal to the _E.coli_. This may be a reflection of the source of the proteins for the _K.pneumoniae_ amylase comes from a very closely related organism and the _B.licheniformis_ α-amylase from a distantly related organism. It would be of great interest to find out if the _B.licheniformis_ α-amylase is directly toxic or if it is blocking export sites, as this may show subtle differences in the interaction of Gram positive extracellular proteins with the _E.coli_ export machinery.

The preliminary results from the pullulanase cloning experiments suggest that a gene or number of genes involved in the export of pullulanase enzyme may have been cloned. This was probably fortuitous because of the direct screen used when cloning the pullulanase (i.e. for growth on pullulan). Gene banks which did not contain the full determinant would either not have grown or would have been missed due to reduced growth. However, at the moment much of this is speculation and a lot more experimental work is required to sort out the

molecular events which lead to the different phenotypes of the various pullulanase clones.

Appendix 1.

After the completion of this manuscript a paper was published with direct relevance to this project namely "Structural Genes Encoding the Thermophilic α-amylases of B.stearothermophilus and B.licheniformis" (Gray et al., 1986). This paper described the sequencing of the α-amylase gene from B.licheniformis NCIB 8061. The nucleotide sequence of this gene is identical to the nucleotide sequence of the α-amylase from B.licheniformis NCIB 6346 described in Chapter 5. The α-amylase gene from B.licheniformis NCIB 8061 is stable in E.coli and its product is exported to the periplasm confirming the results of this study. It is interesting that the α-amylase gene from B.stearothermophilus described by Gray et al., (1986) is essentially the same as the previously sequenced B.stearothermophilus α-amylase (Ihara et al., 1985), except it is 32 amino acids smaller.

Appendix 2.

DNA M_r Markers.

λ *Hind* III digest

1) 23,130 bp
2) 9,419 bp
3) 6,557 bp
4) 4,371 bp
5) 2,322 bp
6) 2,028 bp
7) 564 bp
8) 125 bp

φX174 *Hae* III digest

1) 1,353 bp
2) 1,078 bp
3) 872 bp
4) 603 bp
5) 310 bp
6) 281 bp
7) 271 bp
8) 234 bp
9) 194 bp
10) 118 bp
11) 72 bp

Appendix 3.

Amino Acid Codon Table.

F	TTT	Phe.	S	TCT	Ser.	Y	TAT	Tyr.	C	TGT	Cys.
F	TTC	Phe.	S	TCC	Ser.	Y	TAC	Tyr.	C	TGC	Cys.
L	TTA	Leu.	S	TCA	Ser.	*	TAA	Stop.	*	TGA	Stop.
L	TTG	Leu.	S	TCG	Ser.	*	TAG	Stop.	W	TGG	Trp.
L	CTT	Leu.	P	CCT	Pro.	H	CAT	His.	R	CGT	Arg.
L	CTC	Leu.	P	CCC	Pro.	H	CAC	His.	R	CGC	Arg.
L	CTA	Leu.	P	CCA	Pro.	Q	CAA	Gln.	R	CGA	Arg.
L	CTG	Leu.	P	CCG	Pro.	Q	CAG	Gln.	R	CGG	Arg.
I	ATT	Ileu.	T	ACT	Thr.	N	AAT	Asn.	S	AGT	Ser.
I	ATC	Ileu.	T	ACC	Thr.	N	AAC	Asn.	S	AGC	Ser.
I	ATA	Ileu	T	ACA	Thr.	K	AAA	Lys.	R	AGA	Arg.
M	ATG	Met.	T	ACG	Thr.	K	AAG	Lys.	R	AGG	Arg.
V	GTT	Val.	A	GCT	Ala.	D	GAT	Asp.	G	GGT	Gly.
V	GTC	Val.	A	GCC	Ala.	D	GAC	Asp.	G	GGC	Gly.
V	GTA	Val.	A	GCA	Ala.	E	GAA	Glu.	G	GGA	Gly.
V	GTG	Val.	A	GCG	Ala.	E	GAG	Glu.	G	GGG	Gly.

The single letter code is shown before each codon and the three letter equivalent code is shown after each codon. Codons are shown in the form of DNA.

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