Characterization of an alkalophilic *Bacillus brevis* isolate with respect to its endo-(1,3-1,4)-ß-glucanase gene, protein hyperproduction and the *degS-degU* operon

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CERTIFICATION OF SUPERVISOR

In terms of paragraph **GP 8** of "General Rules for the degree of Doctor of Philosophy (PhD)" I, as supervisor of the candidate Maureen E. Louw, certify that I approve of the incorporation in this thesis of material that has already been published or submitted for publication.

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Abstract

Bacillus brevis Alk 36 was isolated from soil during a screening programme for the selection of extracellular enzyme producing strains. A gene coding for an endo(1,3-1,4)-ß-glucanase (or lichenase) was cloned from *B. brevis* Alk 36 and expressed in *Escherichia coli*. The nucleotide sequence of this gene was determined and found to encode a protein of 252 amino acid residues. The amino acid sequence of the *B. brevis* lichenase gene showed only a 50% similarity to previously published data for *Bacillus* endo-(1,3-1,4)-ß-glucanases. The enzyme exhibited some unique properties. The optimum temperature and pH for enzyme activity were 65-70°C and 8-10, respectively. When held at 75°C for 1 h, 75% residual activity was measured. The molecular mass was estimated to be 29 kDa and the enzyme was found to be resistant to sodium dodecyl sulphate (SDS).

B. brevis Alk 36 was evaluated as a potential host strain for the efficient production and secretion of foreign proteins and was found to grow optimally between pH 8.0 and pH 9.5 and between 42°C and 52°C. *B. brevis* was successfully transformed using vector DNA and was found to produce relatively low levels of protease. In addition, it was evaluated as a possible protein hyper-secreting strain. However, using PCR technology, the highly conserved cell wall protein genes could not be positively identified in *B. brevis* Alk 36.

The *sacU* region from *B. brevis* Alk 36 was cloned and sequenced. The *degS-degU* genes were selected for their ability to stimulate the production of extracellular proteases. The two open reading frames of the *degS-degU* operon encoded polypeptides which gave calculated molecular masses of 43.8 kDa and 27.0 kDa respectively. Sequence comparisons at the amino acid level to the *B. subtilis degS-degU* genes showed 74% and 84% similarity respectively. On a multicopy vector the *B. brevis degS-degU* genes were found to be relatively stable and to cause hypersecretion of several extracellular enzymes in a *B. subtilis* rec⁻ strain as well as in a *B. subtilis sacU*(HY) strain. These enzymes included proteases, α-amylase, endo-(1,3-1,4)-ß-glucanase and levansucrase.

Abbreviations

A_{540}	absorbance at 540 nm
aa(s)	amino acids
ATCC	American Type culture collection
ATP	adenosine 5'-triphosphate
BGSC	*Bacillus* Genetic Stock Culture Collection
bp	base pair
C-	carboxy terminal (end of a protein)
CsCl	caesium chloride
CMC	carboxymethylcellulose
Da	daltons
dNTP	deoxynucleotide triphosphate
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetra-acetic acid
g	standard gravitational acceleration
h	hour(s)
IPTG	isopropyl-ß-D-thiogalactopyranoside
kb	kilobase pairs
kDa	kilodalton(s)
K_m	Michaelis constant
LB	Luria-Bertani broth
MIC	Minimum inhibitory concentrations
min	minute(s)
M_r	relative molecular mass
MW	Middle Wall protein layer
N-	amino terminal (end of protein)
nt	nucleotides
OD	optical density
ORF	open reading frame
OW	Outer wall protein layer
p	plasmid
PAGE	polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PG	Peptidoglycan layer
PIs	Proteinaceous protease inhibitors

Abbreviations (cont.)

RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
sp(p)	species
TCA	trichloracetic acid
TEMED	*N,N,N',N'*-tetramethylethlenediamine
Tris	Tris(hydroxymethyl)aminomethane
U	units of enzyme activity
V_{max}	maximum reaction rate
v/v	volume/volume
w/v	weight/volume
μ_{max}	maximum obtainable growth rate per hour
α	alpha
ß	beta
μ	micro
ϕ	phi
δ	sigma

Chapter 1

General Introduction

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Chapter 1

General Introduction

1.1 *Bacillus brevis* as a model system for the production of heterologous proteins.

1.1.1 Introduction

Interest in *B. brevis* as an industrially important species initially grew in the early 1940's with the discovery that some *B. brevis* strains produce the antibiotic gramicidin. These gramicidin-producing strains were characterized and were found to be thermostable. (Gause and Brazhnikova 1944, cited in Nakamura 1991).

In the early seventies a study was initiated to isolate microorganisms that were able to secrete large amounts of proteins (Udaka 1976). It was thought that such organisms would be useful not only for practical applications, such as in the field of food production, but also for basic work in elucidating the mechanism underlying protein secretion. During the course of this work *in vitro* recombinant DNA technology emerged thereby providing an excellent vehicle for the applications of these protein-hyperproducing isolates, the majority of which were found to belong to the genus *B. brevis*. The development and successful application of these bacteria as a model system for the production of heterologous proteins will be discussed in this chapter.

1.1.2 Isolation and characterization of protein hyperproducers

Initially, five potential protein-producers were discovered by means of a novel screening method (Udaka 1976). Plates inoculated with many isolates from natural sources were incubated for a few days and then flooded with a protein-denaturing reagent, such as 5% trichloroacetic acid (TCA). Bacteria from colonies giving rise to an opaque area after TCA treatment were selected as potential protein-producing strains. A more efficient protein-producer gave a denser precipitate beneath the colony on addition of TCA. It was found that autolysis of bacteria usually does not

occur under these conditions so that the formation of an opaque area by acid is a measure of true protein secretion rather than the consequence of cellular lysis. Following primary screening on plates, the bacteria were further selected by assay of extracellular protein in liquid media after culture in shake flasks.

Of 1200 isolates tested, approximately 15% showed opaque areas after TCA treatment. In general, greater protein secretion was observed when bacteria were cultured in nutrient-rich media as opposed to chemically defined media containing glucose and an inorganic nitrogen source. Among these isolates, five strains exhibiting particularly marked responses to this test were selected and found to secrete large amounts of protein (Udaka 1976). During a more recent study (Takagi et al. 1989a), approximately 100 000 isolates were screened from which 32 were obtained producing more than 5 g/l of protein when cultured in optimized liquid media. On further characterization of these isolates, it was found that the majority of the protein hyperproducing strains belonged to the *Bacillus* genus, particularly *B. brevis*. Prominent features of these *B. brevis* strains were the accumulation of one or two high molecular mass proteins (around 100 kDa) in large amounts in the culture medium, resistance to kanamycin, and extracellular protease levels which ranged from nearly zero to a significant amount.

The most important consequence of this study was the isolation of protein-producers which showed no detectable extracellular protease activity. This was to prove particularly important for the successful expression of high levels of eukaryotic proteins within this system (Takagi et al. 1989b).

Cultural conditions for protein production. *B. brevis* 47 isolated by Udaka (1976) was used for detailed studies on cultural conditions for protein production. It was found that a large amount of protein could be produced relatively easily in nutrient rich media. However, the efficiency of protein production varied greatly, depending on the medium composition (Shaku et al. 1980). *B. brevis* was found to grow well in a medium containing yeast extract or meat extract, but significant amounts of protein were not accumulated. "Polypeptone" which is an enzymatic hydrolysate of casein was found to stimulate protein secretion. Optimal cultural conditions depended on the *B.*

brevis strains used, and in addition, temperature, pH and aeration were found to be important factors. Glycine and L-isoleucine were found to be prominent among various amino acids tested to enhance protein production by *B. brevis* in nutrient rich media (Miyashiro et al. 1980). Wright et al. (1989) and Wight et al. (1992) found that when grown in a semi-defined medium containing fructose and polypeptone, *B. brevis* 47 was able to reach protein yields almost similar to those obtained in complex media.

Studies on extracellular protein production by *B. brevis* 47 in a chemically defined medium were carried out by Tsuchida et al. (1980). Protein production was found to be significantly lower in this medium when compared to complex medium. The effect of phosphate in a chemically defined medium on protein production was evaluated (Tsukagoshi et al. 1981) and it was found not only to affect the quantity of protein produced, but in addition, to influence the composition of the proteins secreted. At lower phosphate concentrations (below 1%) fewer proteins with lower molecular weights were secreted, while increased phosphate levels favoured higher concentrations of proteins with greater molecular masses.

1.1.3 Mechanism of extracellular protein production

Cell wall structure. The cell wall structure of the protein producing *B. brevis* is quite different from that of most Gram-positive bacteria: These strains can be classified into two groups:
Group I with a double-layered cell wall consisting of a middle wall protein layer (MW) and a peptidoglycan layer (PG), as in the case of strains HPD31 and HPD52.
Group II with a three-layered cell wall consisting of two protein layers [outer wall (OW) and middle wall (MW)], as well as a peptidoglycan layer, as in strain 47 (Yamada et al. 1981; Gruber et al. 1988).

B. brevis 47, was found to belong to the second group described above. The outer two protein layers, are composed of proteins with approximate molecular masses of 130 kDa (OWP, outer wall protein) and 150 kDa (MWP, middle wall protein), respectively as judged by SDS-polyacrylamide gel electrophoresis (Yamada et al. 1981). The OWP and MWP are serologically different (Tsuboi et al. 1982; Ohmizu et al. 1983). The

antibody to MWP of strain 47 cross-reacted with cell wall proteins of strains HPD31 and HPD52, while the antibody to OWP of strain 47 did not cross-react with any of the cell wall proteins of strains examined to date. This indicates that the protein of the middle wall layer, the protein layer adjacent to the peptidoglycan layer, appears to be well conserved among protein-producing *B. brevis* strains but the OWP may be lost or replaced (Gruber et al. 1988).

In *B. brevis* 47, it was found that during the stationary phase of growth the cell wall proteins (MWP and OWP) are shed from the bacterial surface and accumulate extensively in the culture medium (Yamada et al. 1981). Under optimal conditions this can reach between 14 and 30 g/l depending on the *B. brevis* strain used. Both the 130 kDa and 150 kDa proteins isolated from the culture broth had almost identical amino-acid compositions and, upon limited proteolysis, generated the same peptide fragments as the respective proteins isolated from the cell wall. Furthermore, antisera prepared against the 130 kDa and 150 kDa proteins purified from the culture broth, cross-reacted with the respective cell wall proteins. Antisera to the 130 kDa and 150 kDa proteins purified from the cell wall also cross-reacted with the respective extracellular proteins. However, antisera to the 130 kDa protein isolated from both extracellular and cell wall proteins formed no reaction with the 150 kDa protein isolated from the same sources and *vice versa* (Tsuboi et al. 1982; Ohmizu et al. 1983), proving that the two major extracellular proteins were derived from the respective cell wall proteins.

Cell wall protein genes. Molecular cloning and characterization of the genes coding for the *B. brevis* 47 cell wall proteins (MWP and OWP) was carried out in order to determine the precise properties of these proteins and the mechanisms involved in their biosynthesis, translation across the membranes and assembly at the external cell surface.

Immunological procedures were used to clone the genes coding for the cell wall proteins, since no easily measurable biological activities were detected for either protein. Regions of the structural genes for the MWP and OWP were cloned in *E.*

coli (Tsukagoshi et al. 1984). With the cloned DNA fragment as a hybridization probe, the restriction map of the region surrounding the cell wall protein genes on the *B. brevis* 47 genome was constructed. This work showed that these genes form a gene cluster, while Northern blot analysis of *B. brevis* 47 RNA indicated that these genes constitute a co-transcriptional unit and that they are transcribed from a promoter(s) located upstream of the MWP gene (Tsuboi et al. 1986). Therefore, the cell wall protein genes of *B. brevis* 47 seem to constitute an operon (*cwp* operon).

The 5' region of the operon, containing transcription and translation initiation sites was cloned in *B. subtilis*, after many unsuccessful attempts using *E. coli* as the cloning host (Yamagata et al. 1987). The entire nucleotide sequence of both cell wall protein genes was determined (Tsuboi et al. 1986; Yamagata et al. 1987 and Tsuboi et al. 1988). MWP and OWP genes were interrupted by an untranslated sequence of 130 base pairs (bp) and consisted of 3090 bp (1030 amino acid residues with a molecular mass of 114 830 Da) and 2940 bp (980 amino acid residues with a molecular mass of 103 740 Da), respectively. Both MWP and OWP were synthesized in precusor forms with signal peptides of 23 and 24 amino acids, respectively. The nucleotide sequence of the MWP gene, was found to be highly conserved among protein-producing *B. brevis* strains such as HPD31 and HPD52. This is consistent with the results of the immunological studies.

The 5' region of the *cwp* operon. Primer extension assay of *cwp* operon transcripts showed the existence of six different 5' ends. This confirmed the results of the S1 nuclease protection assay and suggested the existence of six tandemly arranged promoters within 300 bp upstream from the MWP coding sequence (Yamagata et al. 1987; Adachi et al. 1989). The putative promoters were designated P1, P2, P2*, P3, P4 and P5 according to the positions of the 5' ends of the corresponding transcripts (Fig1.1). Deletion analysis of the promoter region with the aid of promoter-probe vectors carrying the promoter-less *Bacillus licheniformis* α-amylase (EC 3.2.1.1) gene showed that P1, P2 and P3 promoters are located within three distinct fragments derived from this region. P2 and P3 promoted efficient synthesis of α-amylase when they were placed upstream from the α-amylase gene, whereas P1 promoted synthesis

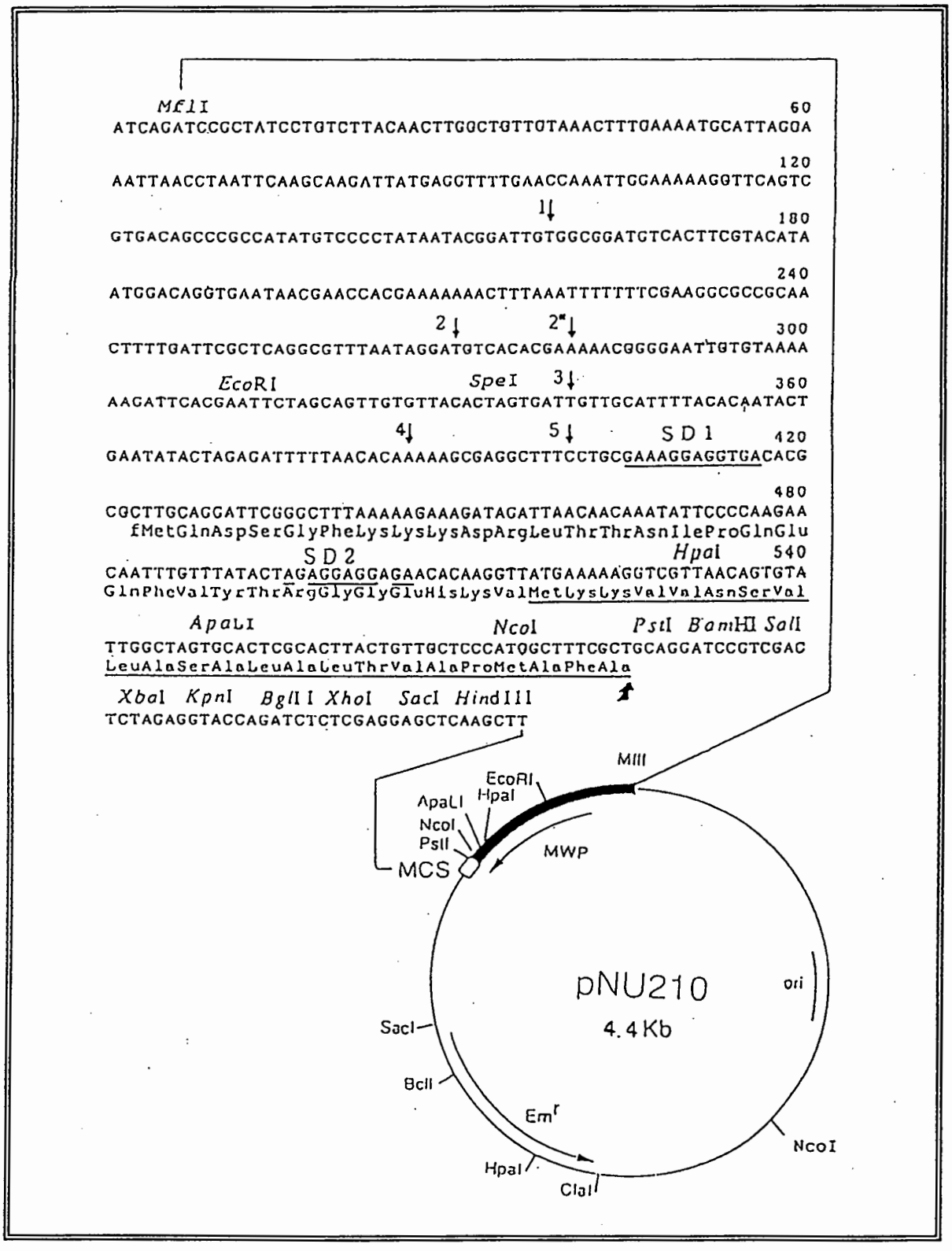


Fig. 1.1 (After Udaka and Yamagata, 1993). Structure of the expression-secretion vector pNU210. The closed bar indicates the 5' region of the *mwp* gene containing multiple promoters and the signal peptide-coding sequence. The open bar indicates a multicloning site (MCS). The DNA and amino acid sequences of these regions are shown in upper part of the figure. Vertical arrows along the top of the DNA sequence indicate transcription start sites, 1 to 5. SD1 and SD2 are the ribosome-binding sites located upstream of the dual translation initiation sites (TTG at nucleotides 424 and 426 and ATG at nucleotides 517 and 519). The signal peptide-coding sequence is underlined

only at a very low level. The use of P2*, P4 and P5 remains uncertain at present (Adachi et al. 1989).

The P1, P2 and P3 promoters have similar sequences around the transcription initiation site (TACGGATTGT, TAGGATGT and TAGTGATTGT, respectively). The -35 and -10 regions of P1 and P3 resemble the consensus sequence of the *B. subtilis* vegetative promoters recognized by the sigma-43 RNA polymerase (TTGACA in the -35 and TATAAT in the -10 regions), and the spacer between the -35 and -10 regions corresponds to the preferred internal length (16-18 bp) for *B. subtilis* promoters (Moran et al. 1982). The P2 promoter resembles only the consensus sequence in the -10 region. The P1 and P3 promoters were used to the same extent in *B. subtilis* and *B. brevis*, whereas the P2 promoter was used more frequently in *B. brevis* than in *B. subtilis*. The P2 promoter is used constitutively in *B. brevis* 47 at all stages of growth whereas P3 is used only at the exponential phase of growth. P2 could be a promoter of an unknown type that is preferentially used in *B. brevis* and might be responsible for the constitutive synthesis and secretion of the cell wall proteins into the medium during the stationary phase of growth (Adachi et al. 1989). Tandem or overlapping promoters have been found in several genes of *B. subtilis* (Johnson et al. 1983, Wang and Doi 1987). Several genes expressed over relatively long periods are transcribed by more than one form of RNA polymerase (EC 2.7.7.6) which initiate transcription from either tandem or overlapping promoters (Johnson et al. 1983). The promoters in cell wall protein genes of *B. brevis* HPD31, HPD52 were found to be almost identical to that of strain 47 (Ebisu et al. 1990).

Translation and secretion signals. The 5' region of the *cwp* operon contained two possible translation start sites located tandemly in the same reading frame. The first site (the one located upstream) contained a possible ribosome-binding site designated SDI (Fig 1.1) and an initiation codon TTG. This sequence is used as an initiation codon in *Bacillus* species (Neugebauer et al. 1981). The second site also contains a ribosomal-binding site, designated SD2 and an initiation codon ATG. SDI has 12 bases and SD2 has 9 bases complementary to the 3'ends of *B. brevis* and *B. subtilis* 16S rRNA (McLaughlin et al. 1981; Kop et al. 1984). The amino-acid sequence from Met at -23, the NH_2-terminus of the translational products from the second initiation site,

to Ala at -1 shows characteristics of signal peptides of secretary precursors and is highly homologous with the putative signal peptide of the OWP of *B. brevis* 47 (Tsuboi et al. 1988). The amino-acid sequence from Ala at +1 to Ala at +9 is in agreement with the NH_2-terminal amino-acid sequence of MWP determined chemically (Tsuboi et al. 1988). Although both of the two possible translation start sites are utilized in *B. brevis*, translation appears to start at the second site far more frequently than at the first site.

1.1.4 Production of heterologous proteins

In order to study the expression of cloned genes in *B. brevis* it was essential to establish a method of transformation, especially by plasmid DNA. Various transformation techniques were attempted. Protoplast transformation in the presence of polyethylene glycol (PEG) was found to be successful in many bacteria especially *B. subtilis* (Chang and Cohen 1979), *B. stearothermophilus* (Imanaka et al. 1982) and *B. thuringiensis* (Martin et al. 1981). However, this method could not be applied to *B. brevis* 47 due to the extreme difficulty in regenerating its protoplasts. A new method for transforming *B. brevis* 47 was developed (Takahashi et al. 1983) whereby the two protein layers (OWP and MWP) which constitute part of the cell wall were removed by incubation with Tris-hydrochloride buffer of alkaline pH. The resultant cells were surrounded by a thin peptidoglycan layer and a cytoplasmic membrane. As the peptidoglycan layer is very thin, DNA-uptake can be induced with polyethylene glycol as in the case of protoplasts. This method was successful for *B. brevis* 47 but was less efficient when applied to *B. brevis* HDP31. A method utilizing electroporation was then optimized for transforming this strain with some success (Takagi et al. 1989c). This method was, however, not applicable to a number of *B. brevis* strains including *B. brevis* 47, possibly due to the presence of two S-layers in the cell wall. Thus for each *B. brevis* isolate a different transformation technique had to be optimized.

Vector development. Plasmids pHW1 (Horinouchi and Weisblum 1982) and pUB110 (Gryczan et al. 1978) of *Staphylococcus aureus* origin can be used to transform *B. brevis* to erythromycin and neomycin resistance, respectively. pHW1 is a low-copy-number plasmid in *B. brevis* and was found to be useful as a cloning vector, especially when products of the cloned genes were deleterious to host cells. pUB110 is a high-copy-number plasmid in *B. brevis*, and found to be useful for overproduction of polypeptides from cloned genes.

Another series of vectors were constructed from a low-copy-number cryptic plasmid, pWT481, found in *B. brevis* 481 (Yamagata et al. 1984). pHY481 was constructed by inserting a fragment containing the erythromycin-resistance gene derived from pHW1 into a *Hin*dIII site of pWT481. pHY481 was found to be stably maintained in *B. brevis* 47 even in the absence of the selective drug.

As the cell wall proteins of *B. brevis* 47 are synthesized and secreted into the medium very efficiently, even during the stationary phase of growth, it was postulated that the 5' region of the *cwp* operon would greatly facilitate the expression of downstream heterologous genes and secretion of the gene products (Adachi et al. 1989). Thus a 600 bp *Alu*1-*Alu*1 fragment containing all the tandem promoters, dual translation initiation sites and the MWP signal peptide-coding region was isolated and used to construct expression and secretion vectors. The structure of one such expression - secretion vector, pNU210, is shown in Fig1.1. pNU210 is a multicopy plasmid with the replication origin of pUB110 and the erythromycin resistance gene of pHW1. The multicloning site on the plasmid is convenient for the insertion of foreign genes to construct transcriptional fusion with the *cwp* operon, or translational fusion with the 5' terminal portion of the *mwp* gene. In the latter case, the gene product will be fused to the *mwp* signal peptide. Provided that the fusion protein does not have any structures that interfere with its translocation across the cytoplasmic membrane, the gene products should be efficiently secreted into the medium after cleavage of the MWP signal peptide (Udaka and Yamagata 1993).

An *Apa*L1 site located within the MWP signal peptide coding region is useful for constructing transcriptional or translational fusion of the MWP gene with foreign

genes. By inserting the appropriate synthetic DNA encoding the COOH-terminal portion of the MWP signal peptide between the *Apa*L1 and the foreign gene, the foreign proteins directly fused with the MWP signal peptide can be synthesized and processed efficiently, resulting in accumulation in the medium of the foreign proteins with no additional amino-acid residues at their NH_2-termini. The majority of foreign proteins thus secreted into the medium retain their natural conformation and biological activities.

Production of heterologous bacterial proteins. *B. licheniformis* α-amylase gene was cloned in *E. coli* and sequenced (Yuuki et al. 1985). The gene comprised the coding sequences of a signal peptide of 29 amino acids and the mature enzyme of 483 amino acids. The gene was initially cloned into pHY481 giving rise to the recombinant plasmid pHY483. A plasmid pHY4831 was then constructed by subcloning the entire gene into pHY481, in addition to the multiple promoter region, signal sequence and the coding sequence for the nine NH_2-terminal amino acids of MWP, joined through a linker DNA to the coding sequence for the α-amylase. A substantial increase in extracellular α-amylase was obtained (50-fold), with *B. brevis* 47 (pHY4831) as opposed to *B. brevis* 47 (pHY483) under the same culture conditions (Yamagata et al. 1987). Further increase in enzyme production was obtained by introducing pHY4831 into *B. brevis* 47 cop11, a mutant where the copy number of the plasmid increases about tenfold (Yamagata et al. 1985). Similar attempts with an expression-secretion vector such as pNU210 have been made with other enzyme genes, such as those encoding cyclomaltodextrin glucanotransferase (CGTase, EC 2.4.1.19) of *B. macerans* (Takano et al. 1992); thermophilic ß-amylase (EC 3.2.1.2) of *Clostridium thermosulfurogenes* (Mizukami et al. 1992); a thermostable carboxyl esterase (EC 3.1.1.1) from *B. stearothermophilus* (Tulin et al. 1991; Amakai et al. 1992 and Tulin et al. 1992) and a thermostable D-xylose isomerase (EC 5.3.1.5) originally cloned from the thermophile *Thermus thermophilus* (Dekker et al. 1992). In all cases a large amount of each enzyme (0.3 to 1 g/l) was produced under the appropriate culture conditions when *B. brevis* 47 or HPD31 was used as the host. The amount of enzyme produced by *B. brevis* carrying the genes under the control of the *cwp* promoter was at least ten times higher than that produced by *B. brevis* carrying the genes under the control of their own promoters.

Production of heterologous mammalian proteins. Human epidermal growth factor (hEGF) has important pharmaceutical applications, and as its production in other host-vector systems has been low, Yamagata et al. (1989) decided to investigate its production in the *B. brevis* system. A synthetic hEGF gene was inserted into the cleavage site of the MWP signal sequence on pNU210, so that the fused gene encodes exactly the same amino acid sequence as that of mature hEGF directly following the MWP signal peptide. Under optimal conditions the hEGF synthesized by *B. brevis* was one of the major extracellular proteins with the same apparent molecular weight as that of authentic hEGF. The amount of hEGF in the culture medium remained constant even after 3 days, suggesting that hEGF did not undergo proteolytic degradation in the medium. The amount of hEGF produced by *B. brevis* was increased a further 4-fold by subcloning the hEGF gene into the more stable pWT481 vector with a lower copy number but which still contained the 5' region of the cell wall protein gene and the signal sequence region of the cell wall protein gene (Ebisu et al. 1992).

Other mammalian proteins successfully secreted into the culture medium in a soluble biologically active form by the *B. brevis* host-vector system include swine pepsinogen (Takao et al. 1989), cholera toxin B subunit (Ichikawa et al. 1993) and human α-amylase (Konishi et al. 1990).

It was, however, noticed that in *B. brevis* the levels of production of eukaryotic proteins are generally much lower than those of bacterial proteins, except for human epidermal growth factor. One of the possible reasons is that the eukaryotic proteins synthesized are toxic to the bacterial cells and so might inhibit growth. It was therefore felt that the transcriptional regulation of a gene by using a controllable promoter system (such as the *E. coli lac* system) would be important to eukaryotic protein production. By this means, the cyto-toxic effects can be minimized in the host bacterium. Takagi et al. (1992) were able to construct a functional *lac* repressor-operator system in *B. brevis* using the *B. licheniformis* α-amylase (BLA) and the human salivary α-amylase (HAMY) as model genes. The production of α-amylase could be induced by isopropyl-ß-D-thio-galactopyranoside (IPTG). The promoters in the 5' region of the MWP gene of *B. brevis* were used for the induction of the *lac* operator and repressor genes. Takagi et al. (1992) found that the expression of BLA was

increased up to 100-fold by induction with IPTG but HAMY was only induced 6-fold by the addition of IPTG. However, the production levels of BLA and HAMY by the inducible plasmids are more than 100-fold lower than those by the constitutive plasmids. Therefore, before this inducible system can be put to practical use, further optimization studies would have to be carried out.

The efficient production of eukaryotic proteins in *B. brevis* was thus only achieved after some of the following strategies were adopted:

(i) isolation of hyper-secreting mutants of the host bacterium;
(ii) use of a very stable plasmid found in *B. brevis* as a vector;
(iii) engineering of the signal peptide used in expression-secretion vectors and
(iv) extensive survey and improvement of cultivation conditions.

Additional factors influencing successful heterologous protein production have been identified. These include proteinaceous protease inhibitors (PIs). PIs have been isolated from a large number of animals and plants, and have been well characterized with regard to their protein nature and gene structures. In contrast, bacterial PIs have not been extensively characterized.

B. brevis HPD31 was found to secrete vast amounts of proteins with very little protease activity (Takagi et al. 1989b). The heterologous proteins produced by this strain remained stable in the medium showing no sign of protease degradation. For these reasons Shiga et al (1992) screened the culture supernatant of this strain for strong inhibitor activity towards trypsin. A novel proteinaceous protease inhibitor was isolated from the culture supernatant and designated (BbrPI). It was found to be produced extracelluarly in multiple forms having at least three different molecular masses. The gene coding for BbrPI was cloned and sequenced. The amino acid sequence of the protein was found to contain the amino termini of the three inhibitors a, b and c. The molecular masses were deduced to be 22 kDa, 23.5 kDa and 24 kDa respectively. It was hypothesized that the secreted precusor protein, which is probably inactive, is cleaved by protease into several active protease inhibitor molecules. Although no protease activity was detected in the culture supernatant of *B. brevis* HPD31 it was hypothesized that the role of BbrPI could involve protecting the protein layers on the outermost cell surface from proteolytic attack.

In a further contribution towards successful heterologous protein production by the *B. brevis* system, Ito et al. (1992) cloned and characterized a gene from *B. brevis* HPD31 analogous to the *E. coli lon* gene. This is the structural gene for the ATP-dependent serine protease La, which is thought to catalyze rate-limiting steps in the degradation of many short-lived or abnormal proteins in *E. coli*. *E. coli* mutants deficient in protease La show mucoidy, a filamentous form, increased sensitivity to UV light, and an inability to lysogenize some bacteriophages. These properties result from a failure to rapidly degrade proteins that regulate the corresponding cellular processes. The mutants also show a diminished rate of degradation of abnormal proteins such as puromycyl polypeptides, amino acid analog-containing polypeptides and products of heterologous genes. Since *E. coli* protease La plays such important roles in protein metabolism and the regulation of various cellular processes, it was thought to be of interest to determine whether a mutant of *B. brevis* HPD31 containing an insertion in the *lon* gene also showed a diminished rate of degradation of puromycyl peptides. This was found to be the case, indicating that the *B. brevis lon* gene plays a role similar to that of the *E. coli lon* gene in the degradation of abnormal and probably short-lived regulatory proteins. However, the other phenotypes shown by *E. coli lon* mutants were not observed in *B. brevis*. Thus together with the other factors mentioned here, the use of the *B. brevis lon* mutant is expected to further improve the efficiency of heterologous protein production in this system.

1.2 *Bacillus* endo-(1,3-1,4) ß-glucanases

1.2.1 Introduction

Of all the industrially important enzymes produced by *Bacillus* spp. more than 80% were found to be hydrolytic in action and used for the depolymerization of natural substances. Almost 60% of these were proteolytic and used in the detergent, dairy and leather industries. The carbohydrases used in baking, brewing, distilling, starch and textile industries were found to represent almost 30% of the total enzyme usage leaving the lipases and highly specialized enzymes with the remainder (Godfrey 1983). Among the extracellular carbohydrases the amylases are probably the most ubiquitous and widely employed in industry. Other extracellular carbohydrases from *Bacillus* include cellulases, hemicellulases, xylanases and a variety of ß-glucanases. In this

chapter the focus will be on the ß-glucanases, specifically the endo-(1,3-1,4)-ß-glucanases.

1.2.2 Endo-glucanases

Cellulose and related ß-glucans are the most abundant carbohydrates in plant biomass. Although *Bacillus* are not truly cellulolytic micro-organisms, several strains secrete endo-glucanases. These include ß-1,3 glucanases (EC 3.2.1.39); ß-1,4 glucanases (EC 3.2.1.4); ß-(1,3-1,4) glucanases (EC 3.2.1.73) and ß-1,6 glucanases (EC 3.2.1.75). The distinction between these enzymes is based on substrate specificity. The ß-1,3 glucanases hydrolyse polysaccharides consisting of anhydroglucose units connected by ß(1 → 3) linkages (Nogi and Horikoshi, 1990). They are also known as laminarinases. The ß-1,4 glucanases are capable of degrading non crystalline cellulosic substrates such as carboxymethyl cellulose (CMC) and trinitro-phenyl-carboxymethyl cellulose (TNP-CMC) but are unable to degrade more crystalline forms such as filter paper, cotton or Avicel. They are also known as CM-cellulases. The lichenases or endo-(1,3-1,4)-ß-glucanases degrade the mixed linkage barley ß-(1,3-1,4) glucan specifically hydrolysing the (1 → 4)-ß-glucosyl linkages in cereal ß-D-glucans and lichenan only where the glucosyl residue is substituted at the C(0) 3 position producing two major products: 3-0-ß-D-cellobiosyl-D-glucose and 3-0-ß-D-cellotriosyl-D-glucose (Cantwell et al. 1988). The unique action of lichenases precludes its ability to hydrolyse repeating sequences of ß 1,3 - or ß 1,4-linked glucans as in laminarin or CMC respectively. In this respect the *B. subtilis* enzyme behaves like that isolated from malted barley (Hinchliffe 1984). The 1,6 glucanases are able to hydrolyse fungal cell walls composed of pustulan or 1,6-ß-linked glucan.

The most important commercial application of *Bacillus* ß-glucanases is the use of lichenase in the brewing industry. It is able to hydrolyse the main component of the barley endosperm cell wall which is endo(1,3-1,4)-ß-glucan. Thermostable ß-glucanases are particularly suited for this, as they are not inactivated as rapidly during the kilning and mashing processes. A high proportion of ß-1,3-1,4-glucan in barley will increase the viscosities of wort, slow filtration rates and even cause undesirable hazes and precipitates during beer fermentations. Although *Saccharomyces cerevisiae* can

produce several kinds of ß-glucanases, none of them is able to degrade the ß-1,3-1,4-mixed linkage glucan. Lichenase is also used in modifying agricultural wastes for animal feed production and in the enzymic stabilization of wines (Cantwell et al. 1988).

The CM-cellulases have potential application in the hydrolysis of cellulosic materials and wastes to fermentable sugars for subsequent conversion to liquid fuels, chemical feedstocks and single cell protein (Cantwell et al. 1988). In addition to their many commercial uses, *Bacillus* ß-glucanases provide a model system for the study of mechanisms involved in the regulation, expression and export of enzymes involved in polysaccharide (especially cellulose) degradation (Klier and Rapoport 1988).

1.2.3 Molecular cloning of endo-(1,3-1,4) ß-glucanase-encoding genes.

Information on *Bacillus* ß-glucanases has largely been provided by the cloning and overexpression of the corresponding genes. This greatly facilitates studies of the physicochemical properties of these enzymes and their mode of action, for when overexpressed in *E. coli* they are not contaminated by other ß-glucanases, cellobiohydrolases or ß-glucosidases.

The Congo red agar plate assay which is based on complex formation between Congo red dye and ß-D-glucans is the most frequently used detection method and was first used for cloning the lichenase gene from *B. subtilis* by Cantwell and McConnell (1983).

At present, in total nine endo-(1,3-1,4) ß-glucanase genes from six different *Bacillus* species have been cloned and most have been expressed in heterologous hosts such as *E. coli*, *B. subtilis* and yeasts (Table 1.1). The molecular masses of several of these lichenases have been estimated using SDS-polyacrylamide gel electrophoresis and were found clustered within the range of 24 kDa to 27 kDa with the exception of the *B. circulans* lichenase which was reported to have a molecular mass of 40.5 kDa. The validity of this enzyme being classified as a true lichenase will be discussed in section 1.2.4.

Table 1.1 A review of cloned lichenases from *Bacillus* spp.

Donor Organism	Expression in heterologous hosts	References
B. subtilis	*E. coli; S. cerevisiae; B. subtilis*	Cantwell and Mc-Connell (1983) Cantwell et al. (1986)
B. subtilis	*E. coli; S. cerevisiae*	Hinchliffe (1984) Hinchliffe and Box (1984)
B. amyloliquefaciens	*E. coli; B. subtilis*	Borriss et al. (1985); Hofemeister et al. (1986)
B. macerans	*E. coli*	Borriss et al. (1988)
B. subtilis	*E. coli; B. subtilis*	Tezuka et al. (1989)
B. circulans	*E. coli*	Bueno et al. (1990a)
B. polymyxa	*E. coli*	Gosalbes et al. (1991)
B. licheniformis	*E. coli*	Lloberas et al. (1988) Lloberas et al. (1991)
B. subtilis	*E. coli; S. cerevisiae*	Chen et al. (1992)

Bacillus lichenases were found to have pH optima which varied from pH 6.0 to pH 8.0 and thermal sensitivity above 60°C (Godfrey 1983). The enzyme from *B. macerans* was, however, found to have a half-life of 40 minutes at 65°C indicating an increased thermostability when compared to mesophilic *Bacillus* endo-(1,3-1,4) ß-glucanases (Borriss et al. 1988).

1.2.4 Sequence analysis of *Bacillus* endo-(1,3-1,4) ß-glucanase genes.

Many of the ß-glucanase-encoding genes have been sequenced: from *B. subtilis*, (Murphy et al. 1984, Tezuka et al. 1989) *B. amyloliquefaciens*, (Hofemeister et al. 1986) *B. macerans*, (Borriss et al. 1990) and *B. licheniformis* (Lloberas et al. 1991).

Detailed sequence analysis among the mesophilic *Bacillus* spp. revealed that the minor differences between them are typical of the divergence of homologous genes in related species. Moreover, the sequences can be aligned with few gaps, and the amino acid sequences of the encoded proteins are not dramatically changed by the nucleotide differences. When the sequences of the two *B. subtilis* lichenases (Murphy et al. 1984, Tezuka et al. 1989) were compared, differences were found in only 26 nucleotides. This discrepancy resulted in four amino acid substitutions giving rise to homologies of 99%. The sequences of the *B. licheniformis* and *B. amyloliquefaciens* lichenases were compared to that of the *B. subtilis* gene, and they were found to give rise to homologies of 78% and 88% respectively.

A comparison of the amino acid sequences of the mesophilic ß-glucanases specifically that of *B. amyloliquefaciens* and the thermophilic *B. macerans* ß-glucanase indicated striking homologies. The extent of similarity was found to be 70% (Borriss et al. 1990). It was found that 157 amino acid residues present in both mature ß-glucanases were identical and that a further 55 residues could be matched if four gaps were introduced in both sequences. Areas of 100% homology were detected in the central and C-terminal parts of the proteins. In contrast, only relatively weak similarity was found in the N-terminal end of the mature proteins of both the mesophilic and thermophilic *Bacillus* spp.

Borriss et al. (1990) identified a short sequence between amino acids 126 and 161 which was found to be highly conserved in all *Bacillus* ß-glucanase enzymes and contained the putative active residues Glu (128) and Asp (137). It is also present in barley lichenase, which does not show significant overall homology to bacterial lichenases. Within this region, Gosalbes et al. (1991) found that the motif W-X_3-BIZ-X_3-K (where X is any amino acid and B and Z represent Asn and Gln respectively) is conserved in all bacterial lichenases excluding the *B. circulans* enzyme. Bueno et al. (1990b) found that there was, in fact, no significant amino acid sequence homology between the ß-glucanase of *B. circulans* and those from the mesophilic *Bacillus* spp. This data together with the results reported by Bueno et al. (1990a) on the carboxymethyl cellulase activity shown by the *B. circulans* enzyme suggest that this enzyme may not be a true lichenase but rather an endo-ß-glucanase with broad substrate specificity.

Comparison of *Bacillus* endo-(1,3-1,4)-ß-glucanase sequences with those of the bacterial ß-1,4 glucanases revealed no discernable homology. In addition, no significant homology was found with plant endo-(1,3-1,4)-ß-glucanases. It was concluded that *Bacillus* lichenases evolved independently of their plant counterparts and of other microbial endo-ß-glucanases (Borriss et al. 1990).

1.2.5 Biochemical properties of hybrid *Bacillus* endo-(1,3-1,4)-ß-glucanases.

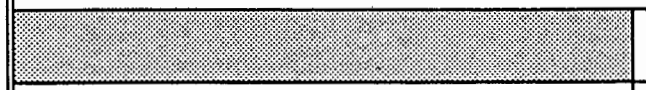
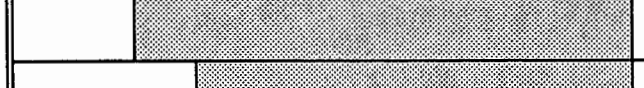
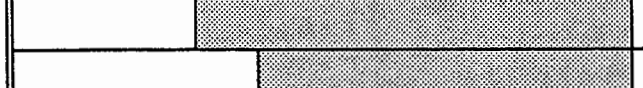
During the brewing process a large proportion of the malt ß-glucanase is irreversibly heat inactivated during kilning and the remaining activity is rapidly destroyed during mashing. Therefore, thermostable endo-(1,3-1,4)-ß-glucanases of fungal or bacterial origin are often added during mashing. Many *Bacillus* lichenases have been characterized and the enzyme from *B. macerans* (MAC) was found to be the most thermostable. This enzyme was, however, found to be rather labile in an acidic environment, and to have an activity maximum around pH 7.0, while the endo-(1,3-1,4) ß-glucanase from *B. amyloliquefaciens* (AMY) was found to be far less affected by acidic environments and to have an activity maximum around pH 6.0 (Olsen et al. 1991).

Borriss et al. (1989) and Olsen et al. (1991) undertook the construction of hybrid endo-(1,3-1,4)-ß-glucanases in an attempt to combine the thermostability of the MAC enzyme with the tolerance towards acidic conditions of the AMY enzyme.

Borriss et al. (1989) constructed these hybrid genes by reciprocal exchanges of the amino-terminal and carboxy-terminal halves of the ß-glucanase-encoding genes from *B. amyloliquefaciens* and *B. macerans*, via a common *Eco*RV restriction endonuclease site located in a homologous region in the central part of the two genes. The hybrid ß-glucanase genes were expressed in *E. coli* cells and the biochemical properties of the hybrid enzymes were analysed. The level of ß-glucanase expression from hybrid genes in *E. coli* differed dramatically. Cells harbouring the H1 gene (containing 5' half of the *B. amyloliquefaciens* gene) had ~1000 fold more ß-glucanase activity than cells carrying the H2 construction. As the enzyme kinetic parameters K_m and relative V_{max} for two substrates of the enzymes are similar, the difference was attributed to either degradation of the H2 protein or variation in the rate of initiation of transcription.

Compared to the parental enzymes, the hybrid proteins exhibited novel biochemical properties such as different pH optima, thermostability and differences in pH tolerance. The H1 protein is of special interest for the brewing industry since, in this protein, the tolerance to lower pH and a low pH optimum of enzymatic activity has been combined with a thermostability comparable to that of the *B. macerans* ß-glucanase at high pH. (Table 1.2)

Table 1.2 Comparison of biochemical characteristics of parental and hybrid lichenases from *B. amyloliquefaciens* and *B. macerans*

Diagramatic representation of hybrid enzyme constructs N-terminal C-terminal		**Temperature for optimal enzyme activity**	**Temperature range for >80% of optimal activity**	**pH for optimal activity at 50°C**	**pH range for >80% activity at 50°C**
	MAC	65°C	55 - 75°C	7.6	5.9 - 8.5
	AMY	55°C	37 - 65°C	6.5	5.5 - 7.0
	H(A16-M)	65°C	55 - 80°C	7.0	5.9 - 6.5
	H(A36-M)	65°C	50 - 80°C	6.0 - 7.0	5.9 - 6.5
	H(A78-M)	65°C	50 - 70°C	6.0 - 7.0	5.9 - 8.0
	H(A107-M)/H1	55°C	37 - 65°C	5.9	5.0 - 8.0
	H(A152-M)	55°C	50 - 65°C	6.5	5.5 - 7.0

Olsen et al. (1991) constructed hybrid endo-(1,3-1,4)-ß-glucanase genes using an approach based on the previous results of Borriss et al. (1989). Overlapping segments of the endo-(1,3-1,4)-ß-glucanase genes from *B. amyloliquefaciens* and *B. macerans* were generated by the polymerase chain reaction (PCR). The mature hybrid enzymes contained a 16, 36, 78 or 152 amino acid N-terminal sequence derived from *B. amyloliquefaciens* endo-(1,3-1,4)-ß-glucanase followed by a C-terminal sequence of 198, 178, 136 and 62 residues, respectively, derived from *B. macerans* endo-(1,3-1,4)-ß-glucanase. Biochemical characterization of parental and hybrid enzymes showed a significant increase in thermostability of three of the hybrid enzymes (Table 1.2) when exposed to an acidic environment thus combining two important enzyme characteristics within the same molecule. At pH 4.1, 85% to 95% of the initial activity was retained after 1 hour at 65°C by the hybrid enzymes H(A16-M), H(A36-M) and H(A78-M). In contrast, the parental enzymes from *B. amyloliquefaciens* and *B. macerans* retained only 5% and 0% of initial activity. After 60 min. incubation at 70°C, pH 6.0, the parental enzymes retained 5% or less of the initial activity whilst one of the hybrids, H(A-16M) still exhibited 90% of the initial activity.

Comparison of the N-terminal amino acid sequences of the AMY and MAC enzymes as well as prediction of hybrid enzyme secondary structures and hydrophobic/hydrophilic regions did not reveal the reasons for the dramatic thermostabilization of the hybrid enzymes. Possibly the hybrid molecules follow different folding pathways leading to more stable conformations with residue changes in the exterior parts of the molecules (Olsen et al. 1991).

Oslen and Thomsen (1991) investigated the effect of glycosylation on the thermostability of two different lichenases (from *B. macerans* and the hybrid enzyme H1). The corresponding genes were expressed in *E. coli* and *Saccharomyces cerevisiae*. Both the enzymes secreted from yeast cells were glycosylated with differences in the type and extent of glycosylation. Thermostability analysis of the glycosylated enzymes and their unglycosylated counterparts synthesized by *E. coli* disclosed a substantially higher thermotolerance of the glycosylated enzymes, particularly of the *B. amyloliquefaciens* - *B. macerans* hybrid. At 70°C the half life of the glycosylated H1-hybrid enzyme was increased by 20-fold.

1.2.6 Expression and regulation of endo-(1,3-1,4)-ß-glucanases.

The transfer of genes encoding *Bacillus* lichenases to heterologous hosts provided an ideal opportunity for studying the regulatory regions of these genes. The cloned lichenases also offered a useful system for the study of protein export both in *Bacillus* species and in *E. coli*. Secreted proteins from *Bacillus* are synthesized with N-terminal leader peptides which are proteolytically cleaved during translocation across the cell membrane. However, in contrast, very few proteins are secreted into the medium by *E. coli*. Those that are, usually require accessory proteins to alter the permeability of the outer membrane. It was, therefore, somewhat surprising to find that in most cases when *Bacillus* lichenases have been cloned and expressed in *E. coli* a significant fraction of the active enzyme was located in the extracellular medium (Borriss et al. 1985; Hinchliffe 1984; Bueno et al. 1990a; Lloberas et al. 1991). Cantwell et al. (1988) carried out a detailed analysis of the location of the *B. subtilis* lichenase in *E. coli* in order to clarify this further. They found that the enzyme was distributed between the extracellular, periplasmic and intracellular spaces in the ratio 24:30:46. This was not due to cell lysis as confirmed by the normal localisation of ß-galactosidase (an intracellular protein in *E. coli*). Secretion of the cloned lichenase was found to be accompanied by leakage of periplasmic space proteins into the medium suggesting that the permeability of the outer membrane of *E. coli* was altered by the presence of the ß-glucanase protein (Cantwell et al. 1988).

The feasibility of expressing endo-(1,3-1,4)-ß-glucanase in yeasts in order to overcome the problems caused by excess barley ß-glucans in the brewing process was investigated. Hinchliffe and Box (1984) were able to demonstrate a very low level of enzyme activity in the cell extract of *S. cerevisiae* containing a *B. subtilis* lichenase gene operating under its own natural promoter and secretory signal sequences. Cantwell et al. (1986) were initially unable to obtain any activity when a *B. subtilis* lichenase gene was cloned into *S. cerevisiae* on an *E. coli*-yeast shuttle vector. Expression was only achieved when the 5' non-translated region of the lichenase gene was removed and the gene was placed under the control of a yeast promoter. Enzyme expression levels were further increased by placing a yeast gene terminator at the 3' end of the ß-glucanase gene. Cantwell et al. (1986) were able to induce high levels of lichenase expression in two commercially used

brewing yeasts by placing the gene under the control of the yeast alcohol dehydrogenase (ADH1) promoter on a high copy number vector. Lancashire and Wilde (1987) used both a yeast promoter and secretion signal sequence to replace the bacterial signal sequences and have demonstrated effective secretion of the enzyme from yeast which effectively degraded the ß-glucans in wort during brewing fermentation conditions. Recently Chen et al. (1992), using an *E. coli*-yeast shuttle vector were able to detect low levels of lichenase activity extra-cellularly as well as in the cell extract. They found that the expression levels were affected by the orientations of the insert.

Thus by using yeast promoter and secretion signal sequences it appears feasible to utilize recombinant yeasts expressing bacterial endo-(1,3-1,4)-ß-glucanase genes to optimize the brewing process.

1.3 Positive regulation in *B. subtilis*

1.3.1 Introduction

Bacillus subtilis, as a soil bacterium, has developed many regulatory mechanisms in order to adapt to environmental changes, as growth in the environment is very often limited by the availability of basic nutrients. Requirements for bacterial survival include a continuous monitoring of these changes and the flexibility to respond rapidly to a wide variety of stimuli. A common pattern in responding to environmental changes is through positive control. This can involve the sensing of a signal and its transduction to the transcription machinery in order to stimulate the transcription of the target genes.

In order to optimize nutrient use, many different classes of positive regulators have evolved in *B. subtilis* and these can be categorised according to their mechanisms of action. These include accessory regulatory polypeptides, classical positive regulators that bind to target sites located just upstream from the promoter, ambiactive regulators that can act both positively and negatively, antiterminators, two-component signal transduction systems, and positive regulators associated with specific secondary σ factors (Klier et al. 1992). In this chapter only two of these will be discussed in detail, namely accessory regulatory polypeptides and two-component signal transduction systems. Both of these play an important, though not exclusive role in the regulation of extracellular

enzyme expression and have a direct bearing on this study.

1.3.2 Positive regulation by accessory polypeptides.

B. subtilis can secrete a wide variety of extracellular enzymes. These include proteases, α-amylase, levansucrase and ß-glucanases (Priest 1977). Expression of genes encoding extracellular enzymes fall under the control of many transcriptional regulatory factors. Among these are a group of regulatory genes which have been cloned from a number of different *Bacillus* spp., and which give rise to gene products of varying size. They do, however, share a number of characteristics: they all act as positive regulators when on multicopy plasmids, and stimulate the production of extracellular enzymes at the transcriptional level, although they show no sequence homology with each other. Deletion of the corresponding genes from the *B. subtilis* chromosome does not lead to any detectable phenotype, and they appear to be specific to Gram-positive bacteria (Klier et al. 1992). The target sites of these regulatory polypeptides are often located upstream from the promoters of genes encoding degradative enzymes.

degR. Nagami and Tanaka (1986) cloned a DNA fragment from *B. natto* which, when on a multicopy plasmid, was found to enhance the production of both alkaline and neutral extracellular proteases as well as levansucrase, but showed no detectable effects on the production of other extracellular enzymes such as α-amylase. The gene *prtR* (or *degR*) coded for a 60-amino-acid polypeptide. Yang et al (1987) cloned the same gene from *B. subtilis*, and showed that deletion of the gene indicated that it was nonessential for growth or expression of proteases and levansucrase. Tanaka et al. (1987) investigated the mode of action of the *degR* gene and found that it activated target genes at the transcriptional level and was shown to increase mRNA levels of the protease genes. This enhancement was, however, not due to the stabilization of the mRNA.

degQ. A small 46-amino-acid polypeptide identified as the *degQ* (or *sacQ*) gene was cloned and characterized from *B. subtilis* (Yang et al. 1986) as well as *B. licheniformis* (Amory et al. 1987). It was found not to have sequence homology with any of the other regulators acting on extracellular enzyme production. When overexpressed this polypeptide was found to activate the expression of a number of target genes in *B.*

subtilis all encoding extracellular enzymes such as alkaline protease, levansucrase, ß-glucanase(s), xylanase and α-amylase. A similar phenotype was obtained with the *B. subtilis* strain harbouring the *degQ*36 mutation. The *degQ* genes from *B. subtilis* and *B. licheniformis* were cloned in the same multicopy plasmid and compared under similar conditions. It was found that the *degQ* gene from *B. licheniformis* was more efficient than the *degQ* gene from *B. subtilis* in producing the hypersecretion phenotype (Amory et al. 1987). A deletion of the *degQ* gene showed no apparent phenotype indicating that it was not an essential gene. A detailed study of one of the target genes levansucrase, indicated that the target site for *degQ* regulation was upstream from the ribosome-binding site of this gene, suggesting transcriptional control.

***senS* and *senN*.** Wong et al. (1988) cloned a DNA fragment from *B. natto* able to stimulate the expression of neutral and alkaline protease, α-amylase, and alkaline phosphatase at a moderate but significant level of two- to four-fold. Although this gene, *senN,* coded for a polypeptide of 60 amino acids in length (similar to *degR*), no sequence homology was found with the other accessory polypeptides. A similar gene was cloned from *B. subtilis* by Wang and Doi (1990). The *senS* gene was found to code for a highly charged basic protein containing 65 amino acid residues with a similar phenotype to *senN* and a complex gene structure. The gene is characterized by the presence of a transcription terminator (attenuator) located between the promoter and open reading frame, a strong ribosome-binding site, and a strong transcription terminator at the 3' end of this monocistronic gene. The deduced amino acid sequence of *senS* showed a typical helix-turn-helix motif of DNA binding proteins and has significant homology with several *B. subtilis* RNA polymerase sigma factors. The *senS* gene organisation of a promoter followed by a stem-and-loop region indicates that *senS* expression is highly regulated and seems to indicate control by a cascade type of regulatory mechanism.

***degT*.** Another trans-acting regulatory gene was cloned from *B. stearothermophilus* (Takagi et al. 1990). When on a multicopy vector it was found to enhance the production of extracellular enzymes such as alkaline protease, levansucrase, xylanase and cellulase as well as alter sporulation control, decrease transformation efficiency for *B. subtilis* and cause flagella loss and abnormal cell division. The phenotypes of *degT* were

found to be very similar to those of *degQ* and *degR*, although the *degT* gene was far larger, coding for a polypeptide of 372 amino acid residues. No significant homology was, however, found at the amino acid level. The primary structure of the DegT protein was divided into two regions: Region I which was found to be highly hydrophobic and thought to function as a membrane sensor for environmental stimuli, such as starvation of a nutrient source. Region II which has a region analogous to DNA binding proteins and was postulated to bind to DNA. In the C-terminal part of this molecule a α-helix structure was located similar to that identified in several transcriptional activators, and it was thought to play a role in activating and/or repressing transcription of several target genes.

***tenA* and *tenI*.** Two novel *B. subtilis* genes that regulate the production of several extracellular enzymes were cloned and characterized (Pang et al. 1991). These two genes were found to be organized as part of an operon. When cloned on a multicopy plasmid the first gene ,*tenA* was found to stimulate alkaline protease production at the transcriptional level, while the second gene *tenI* was found to exert the opposite effect and to reduce alkaline protease production. The stimulatory effect of *tenA* on various extracellular enzymes such as neutral/alkaline proteases or levansucrase was found to be between 11- and 55-fold. The stimulatory effect of *tenA* was found to require functional *degS-degU* genes. This is in agreement with the stimulatory effects exerted by *degQ* and *degR*. Chromosomal inactivation of either *tenA* or *tenI* was found to have no effect on the production of neutral or alkaline proteases but did cause a delay in sporulation. However, as *B. subtilis* has several genes similarily affecting extracellular enzyme production it is possible that they could compensate for *tenA* in regulating the expression of target genes.

Although functionally *tenA* is similar to *degQ*, *degR*, *senS* and *senN* and can stimulate the production of extracellular enzymes at a transcriptional level, structurally neither *tenA* nor *tenI* share any significant homology with any of the above mentioned genes and were found to code for proteins of 236 and 205 amino acid residues respectively. Sequence analysis also revealed a terminator-like structure between the promoter and the ribosome-binding site of *tenA*. Deletion of the structure was found to double the expression of *tenA*. This structure is similar to that reported for the *senS* gene.

degM. The regulatory gene, *degM*, was cloned from a *Bacillus* sp. and was found to enhance the production of a minor serine protease when expressed in a *B. subtilis* strain with a Npr⁻ Apr⁻ phenotype (Masui et al. 1992). Nucleotide sequence analysis showed that the *degM* gene was composed of 373 amino acid residues and that there were regions within the gene with homology to conserved regions of sensor proteins of two component regulatory systems in *Bacillus* spp.

The pleiotropic effects of many of these genes suggest that they are part of a global control mechanism affecting the synthesis of degradative enzymes.

1.3.3 Two-Component regulatory systems.

As the requirements for bacterial survival include both the continuous monitoring of external conditions and the ability to respond rapidly to extracellular and cytoplasmic signals reflecting changes in the environment, a dual process was developed, termed signal transduction (Msadek et al. 1993). Since two regulatory proteins play a central role in this process they have been referred to as "two-component systems", although in many cases more than two regulatory proteins are involved (Kunst et al. 1993).

Interaction of these two regulatory proteins typically involves the first component, a histidine protein kinase, receiving an extracellular signal directly or indirectly, possibly through its amino-terminal domain, which often includes transmembrane sequences. This signal is then transduced via the carboxy-terminal domain of the kinase to the second component the response regulator, which is generally a transcriptional activator. The common mechanism underlying this signal transduction process involves a now-classic phosphotransfer reaction between the two proteins. The protein kinase is first autophosphorylated at a conserved histidine residue in an ATP-dependent reaction. In a second step, the phosphoryl group is transferred to an aspartate residue in the amino-terminal domain of the response regulator. Several members of the histidine protein kinase family also act as phosphatases, catalyzing dephosphorylation of the associated response regulator. Regulation may therefore take place by modulating either the kinase activity or the phosphatase activity of the protein (Msadek et al. 1993).

Signal transduction proteins in *B. subtilis*. As *B. subtilis* is normally found in environments such as soil, where nutrients are limited and conditions tend to fluctuate widely, growth in this environment is generally limited by the availability of essential nutrients. It is therefore critical for cell survival that strict control mechanisms play a role.

Molecular genetic analysis has allowed the identification of a network of interacting signal-transduction proteins. To date five known histidine-kinase proteins in *B. subtilis* (CheA, ComP, SpoIIJ, DegS and PhoR) are thought to interact with six cognate response regulators (CheY, ComA, Spo0A, Spo0F, DegU and PhoP). These will be briefly reviewed in this section.

In *B. subtilis*, two component systems have been identified affecting phosphate assimilation (PhoR/PhoP). PhoR is thought to be the sensor histidine kinase for the PhoP response regulator. (Seki et al. 1987). In chemotaxis, sequence similarity suggests that (CheA/CheY) form a histidine kinase-response regulator pair, however no biochemical evidence yet exists for transfer of a phosphoryl group between the two proteins, although strong homology with the corresponding *E. coli* proteins suggests that such a transfer is more than likely (Bischoff and Ordal 1991; Fuhrer and Ordal 1991).

Sporulation involves a number of genes in this multicomponent regulatory system: *spo0A; spo0F; spo0B; spoII* and *kinB* (Burbulys et al. 1991). The *spo0A* and *spo0F* genes have been cloned and sequenced; *spo0F* was found to encode a protein containing the characteristic N-terminal domain of the phosphorylated response regulators (reviewed in Msadek et al. 1993), while SpoOA is a response regulator with a unique carboxy-terminal domain. In addition, SpoOA was known to be a DNA-binding protein that regulates *abrB* gene expression (Strauch et al. 1990, *in* Klier et al. 1992). The phosphorylation of SpoOA results from a series of reactions that have been termed a phosphorelay. The initial event in the cascade is the phosphorylation of SpoOF by the SpoII kinase as well as by KinB (Burbulys et al. 1991). The key enzyme in the phosphorelay is SpoOB, a phosphotransferase that catalyses the concurrent dephosphorylation of SpoOF and the phosphorylation of SpoOA (Burbulys et al. 1991). This is the only known example of such a phosphotransfer between two response

regulators. The end product of the phosphorelay is the phosphorylated form of SpoOA, whose accumulation is essential for triggering subsequent steps in the sporulation process (Fig.1.2). When phosphorylated it is an effective repressor of the AbrB gene (Strauch et al. 1990 *in* Klier et al. 1992, Strauch and Hoch 1993) which in turn controls many genes whose functions are not required during conditions of nutrient excess.

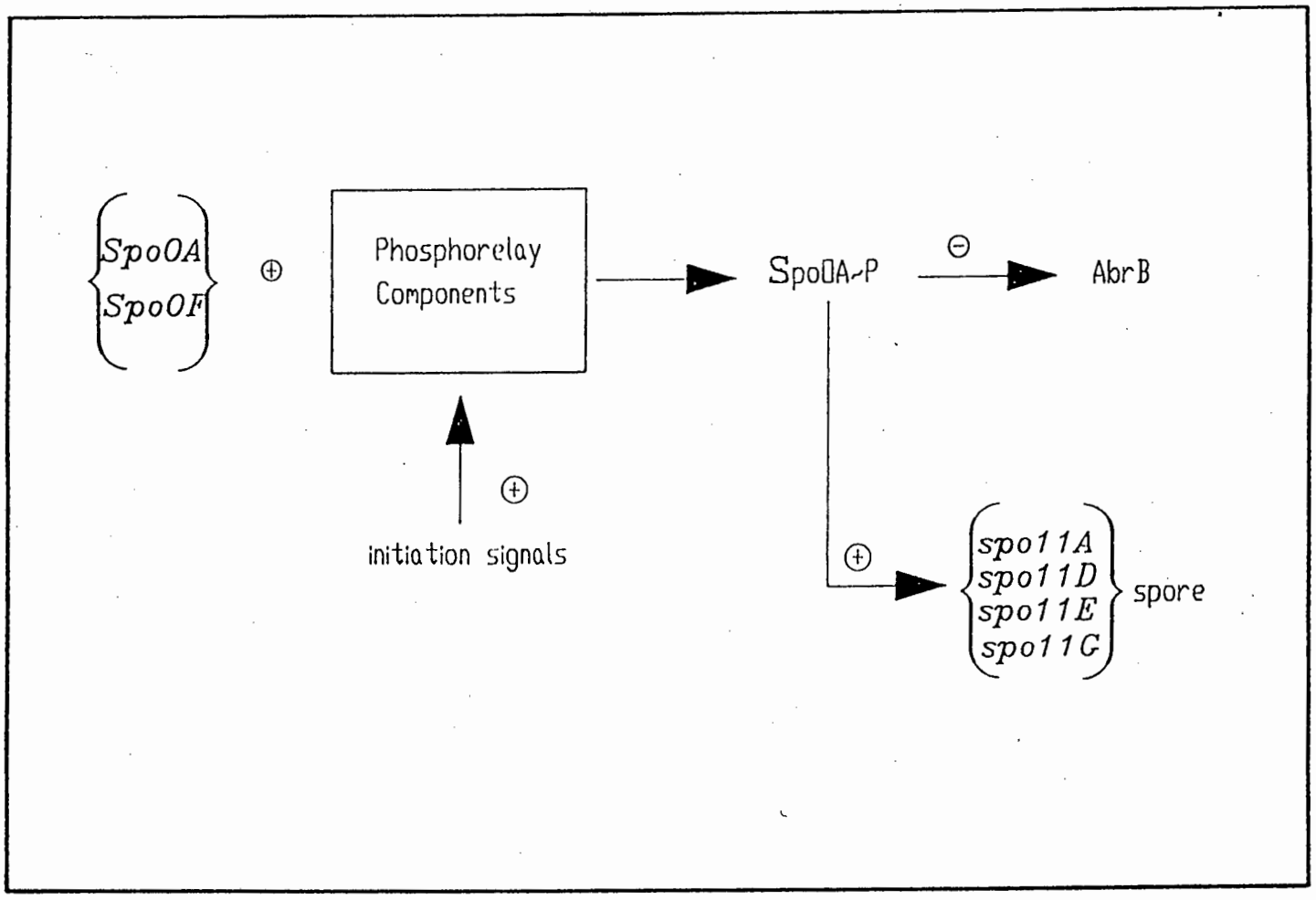


Fig. 1.2 Involvement of Spo0A in early sporulation-specific gene expression: +, positive effects; -, negative effects

Competence in *B. subtilis* is a natural physiological state allowing the uptake of exogenous DNA, and involves an environmentally induced and temporally regulated series of events whose control overlaps with the control of sporulation and the regulation of certain genes that are expressed only during stationary phase. Specific regulatory proteins controlling the expression of late competence genes include the products of the *comP, comA, comQ, srfA* (*comL*) and *comK* genes. Other proteins controlling the expression of late competence genes include DegS, DegU, Spo0A, Sin and AbrB (Dubnau 1991). The regulatory pathway controlling competence gene expression is shown in Fig. 1.3. The *comP* and *comA* genes encode histidine kinase and response regulator proteins, respectively. Thus in response to an as-yet-unidentified signal, ComA would be phosphorylated by ComP and act in conjunction with ComQ to allow expression of *srfA*, an operon required for biosynthesis of the lipopeptide antibiotic surfactin, and expression of *comK* and late competence genes. This appears to be the major role of the ComP, ComA and ComQ proteins in the development of competence (reviewed in Kunst et al. 1993 and Msadek et al. 1993).

Mutants capable of expressing late competence genes in complex medium have been isolated (Dubnau and Roggiani 1990; Roggiani et al. 1990), and were called *mec* mutants (for *m*edium-independent *e*xpression of *c*ompetence). The *mecA* gene appeared to act as a negative regulator of *comK* since disruption of the gene bypasses the requirement of the early regulatory genes *comP, comA, degS* and *degU* for the expression of late competence genes.

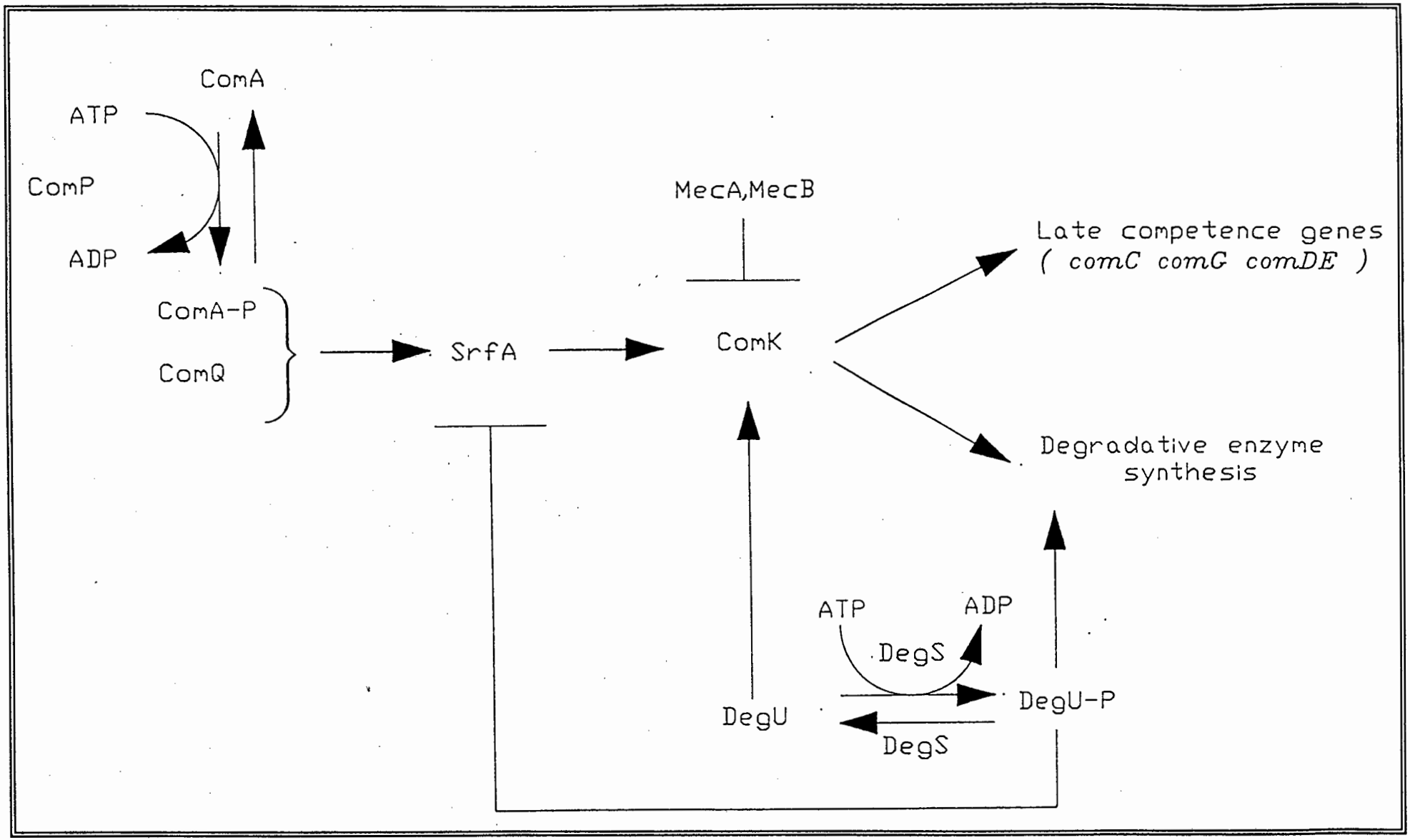


Fig. 1.3 Regulation of competence gene expression in *B. subtilis* (after Msadek et al. 1993). Arrows indicate positive regulation, and perpendicular bars indicate negative regulation.

degS-degU Operon. In *B. subtilis,* the expression of genes encoding extracellular enzymes was found to be under control of the *sacU* region which consists of two genes *degS* and *degU* (Henner et al. 1988a; Kunst et al. 1988 and Tanaka and Kawata 1988). These two genes are unlinked to any of the target genes and constitute an operon (Msadek et al. 1990). The operon encodes the DegS and DegU proteins both of which display amino acid sequence homology with the sensor and effector proteins respectively of the bacterial two-component regulatory system (Henner et al. 1988a; Kunst et al. 1988 and Msadek et al. 1990). The DegS modulator was hypothesized to control the DegU effector, which was thought to be a transcriptional activator. In agreement with other modulator/effector pairs, the DegS and DegU proteins contain several conserved domains. Several modulators have been identified as protein kinases which become autophosphorylated in the presence of ATP at a histidine residue located within the conserved "transmitter" domain at the C-terminal end of the modulator. Autophosphorylation of DegS modulator using a purified preparation of the protein has been demonstrated (Mukai et al. 1990 and Dahl et al. 1991). The DegU effector contains a conserved "receiver" domain at its N-terminal end consisting of two conserved aspartate residues corresponding to positions 11 and 56 in the DegU amino acid sequence (Msadek et al. 1990).

The model for DegS/DegU mediated regulation was proposed based on the general model for such regulatory pairs. (Fig.1.4). It was proposed that the cytoplasmic DegS protein received an as yet unidentified signal from the environment which led to phosphorylation in the presence of ATP at a histidine residue within the "transmitter" domain and subsequent transfer of the phosphate group to an aspartate residue in the "receiver" part of the effector. The phosphorylated effector then activates the expression of target genes encoding degradative enzymes by binding to DNA sequences upstream from target gene promoters. However, no biochemical evidence for specific binding of phosphorylated DegU to these target sites has been found. Other intermediates may be involved in the regulation of degradative enzyme synthesis. The *degU*(HY) and *degQ*(HY) mutations leading to hyperproduction of degradative enzymes have been used to locate cis-acting target sequences upstream from the *sacB* (coding for levansucrase) and *aprE* (coding for alkaline protease) promoters (Shimotsu and Henner 1986; Henner et al. 1988b).

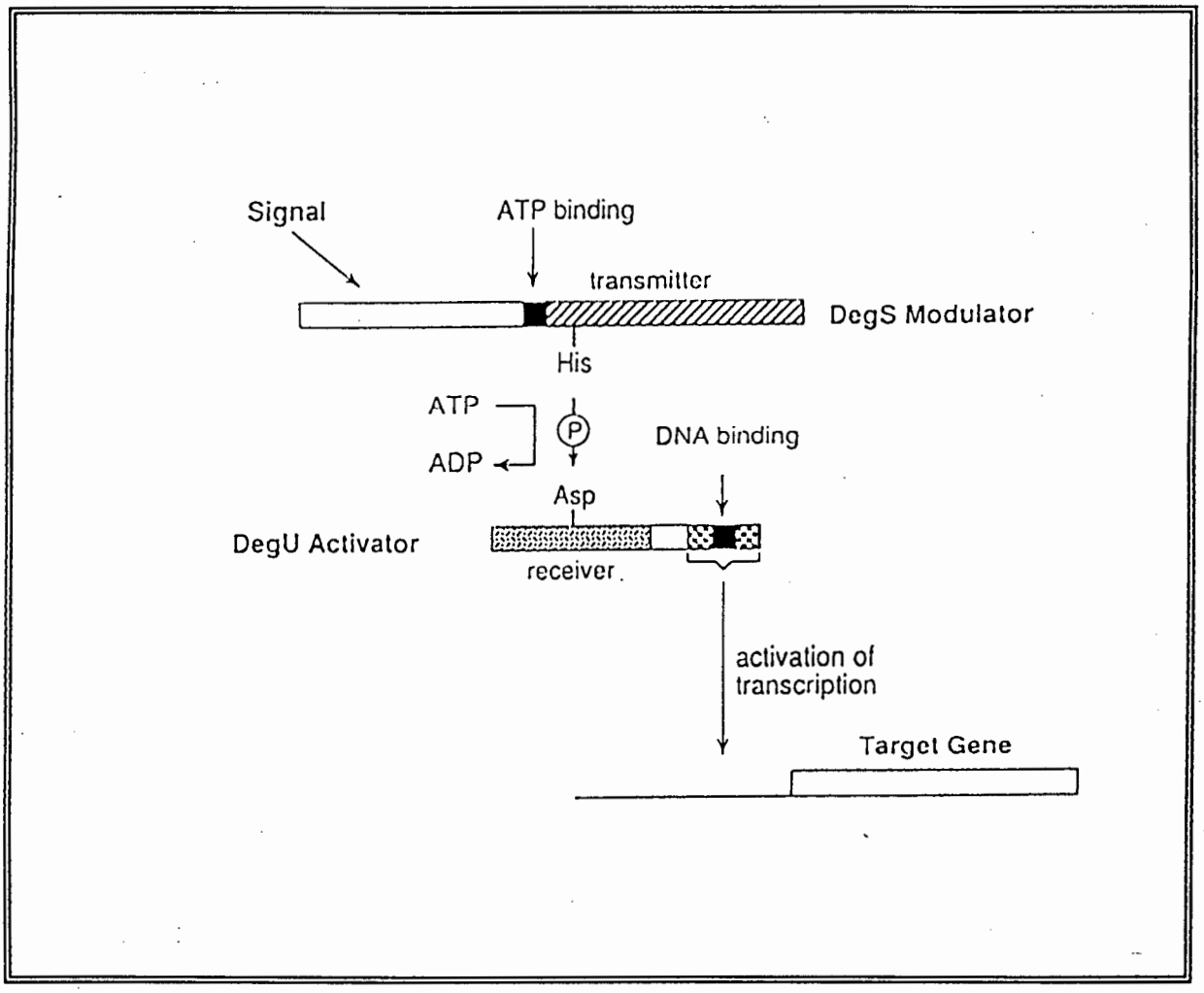


Fig 1.4 Model for DegS/DegU mediated regulation (after Kunst et al. 1990)

The *degS* and *degU* genes were initially defined by different classes of mutations leading either to deficiency of degradative enzyme synthesis (designated *degS* or *degU* mutations) or to overproduction of degradative enzymes [designated *degS*(HY) or *degU*(HY) mutations]. The pleitropic HY phenotype not only caused hyper-production of degradative enzymes but also decreased genetic competence, caused flagella loss and the ability to sporulate in the presence of glucose.

Several of these mutations have been characterized at a molecular level and the amino acid modifications in DegS or DegU and associated phenotypes are illustrated in Table 1.3. Missense mutations in *degS* and *degU* that abolish degradative-enzyme production do not affect the competence pathway, while those that lead to hyperproduction of degradative enzymes result in lowered transformation frequency.

Table 1.3 Mutations in *degS* and *degU* genes and associated phenotypes (after Msadek et al. 1993)

Mutation	Phenotype			References
	In vitro	In vivo		
		Transformation frequency	Degradative enzyme production	
Wild type	Normal DegU phosphatase activity	high	low	Henner et al. (1988a) Tanaka et al. (1991)
degS100(HY)	Reduced DegU phosphatase activity	deficient	high	Henner et al. (1988a) Tanaka et al. (1991)
degS200(HY)	Reduced DegU phosphatase activity	deficient	high	Henner et al. (1988a) Tanaka et al. (1991) Dahl et al. (1992)
degS42	Loss of autophosphorylation activity	high	deficient	Msadek et al. (1990) Tanaka et al. (1991)
degS220	Deficiency in phospho-transfer to DegU	high	deficient	Msadek et al. (1990) Dahl et al. (1991)
degU24(HY)	Increased DegU phosphorylation	deficient	high	Msadek et al. (1990) Dahl et al. (1991)
degU31(HY)	Increased DegU phos-phorylation	deficient	high	Msadek et al. (1990) Dahl et al. (1991)
degU32(HY)	Decreased DegU dephos-phorylation	deficient	high	Henner et al. (1988a) Dahl et al. (1992)
degU146	Deficiency in DegU phos-phorylation	high	deficient	Msadek et al. (1990) Dahl et al. (1991) Dahl et al. (1992)

In vitro phosphorylation experiments using modified DegS and DegU proteins support the hypothesis that *degS*(HY) and *degU*(HY) mutations favour accumulation of the phosphorylated form of DegU by increasing the phosphorylation rate of the response regulator or by enhancing the stability of the phosphorylated protein by decreasing its dephosphorylation rate (Dahl et al. 1991 and Tanaka et al. 1991). In addition, Mukai et al. (1992) purified the DegR protein and studied its *in vitro* effect on the phosphorylation reaction involving DegS and DegU. They found that the enhancing effect of DegR on the formation of DegU-phosphate was due to its protection of the DegU-phosphate from dephosphorylation. Dahl et al. (1992) showed that DegS also has phosphatase activity and is consequently capable of inactivating DegU by dephosphorylation. It was also found that mutations in *degS* or *degU* that lead to a deficiency of degradative enzyme synthesis also promote the accumulation of the unphosphorylated form of DegU. The *in vitro* equilibrium between the two forms of DegU is presumably regulated by DegS in response to an environmental signal which has not, as yet, been identified.

1.3.4 Interaction of the signal transduction network in controlling degradative enzyme synthesis, sporulation and competence in *B. subtilis*.

In *B. subtilis*, the different two-component systems controlling postexponential-phase responses appear to interconnect to a large extent to form a sensory transduction network. This network appears to be involved in a hierarchy of environmental signal responses, involving a choice between competence gene expression, degradative-enzyme production, and finally, sporulation (reviewed in Kunst et al. 1993 and Msadek et al. 1993).

The *degS* and *degU* genes are both required for the synthesis of degradative enzymes in *B. subtilis* as well as for the functioning of *degQ* and *degR* on high-copy-number plasmids leading to the increased production of degradative enzymes. In addition, expression of the *degQ* gene was shown to be controlled by both the ComP/ComA and DegS/DegU two component systems. Separate regulatory targets for DegS-DegU and ComP-ComA were localized by deletion analysis upstream from the *degQ* gene. (Msadek et al. 1991). The ComP/ComA regulatory pair has also been shown to be required for expression of

a degradative enzyme (levansucrase)in certain mutants such as *mecA* and *mecB* (Kunst et al. 1993).

Both the ComP-ComA and DegS-DegU two component systems control the expression of late competence genes. They appear to act through two different branches in the competence regulatory pathway (Fig.1.3). The ComP-ComA branch involves at least one intermediate regulatory locus *srfA* for which phosphorylated ComA is required. The DegS-DegU regulatory pair is, however, not required for the expression of *srfA*. (Dubnau 1991). It was shown by Roggiani et al. (1990) that the presence of a *degS*(HY) or a *degU*(HY) mutation or a disruption of the *degU* gene results in decreased expression of late competence genes, whereas disruption of the *degS* gene had little or no effect on competence gene expression.

Thus the effect of HY mutations on competence appears to be two-fold. These mutations favour accumulation of the phosphorylated form of DegU thereby causing a lack of the unphosphorylated form of the response regulator which is required for the expression of late competence genes. In addition, the phosphorylated form of DegU appears to act as a repressor of essential components of the competence development pathway encoded by the *srfA* operon. DegU appears to be the first response regulator described as having two active conformations; a phosphorylated form that is necessary for degradative enzyme production and a non-phosphorylated form that is required for the expression of genetic competence. (Dahl et al. 1992).

Another intermediate regulatory gene which is common to both DegS-DegU and ComP-ComA pathway is the *comK* gene whose expression requires both ComA and DegU. In addition, this gene is repressed by the *mecA/mecB* genes, and *mec* mutations allow a bypass of both ComP-ComA and DegS-DegU for competence gene expression, as well as a bypass of DegS-DegU for degradative enzyme synthesis. The *mec* gene products therefore play a central role affecting both competence gene expression and degradative-enzyme synthesis (reviewed in Kunst et al. 1993).

Both ComP and DegS have been proposed to affect sporulation to a minor extent especially in the absence of the SpoIIJ (KinA) kinase, possibly through "cross talk" with the Spo0A phosphorelay. (Smith et al. 1992). Both the DegS-DegU two component

system and the SpoOA phosphorelay control the expression of the *aprE* and *nprE* genes encoding the major *B. subtilis* proteases (Msadek et al. 1993). The Spo0A and AbrB regulatory proteins are also required for the expression of late competence genes (Dubnau, 1991). *Spo0A* regulates *abrB* whose gene product plays both positive and negative roles in competence.

At least three regulatory systems involving histidine kinase-response regulator proteins appear to form a signal transduction network in *B. subtilis*. At the onset of stationary phase, when growth is limited by the depletion of nitrogen sources such as amino acids but glucose is still present as a carbon source, the ComP-ComA system would allow competence gene expression, and the DegS-DegU regulatory pair could be involved in the choice between producing degradative enzymes or expressing competence genes. Finally, when glucose is no longer present and no alternative nutrient sources are accessible as substrates for degradative enzymes, the Spo0A phosphorelay would trigger sporulation initiation.

1.4 Characterization of *Bacillus brevis* Alk 36 isolate

B. brevis Alk 36 was isolated from soil during a screening programme for the isolation of *Bacillus* strains producing extracellular enzymes. This strain was found to produce an endo-(1,3-1,4)-ß-glucanase enzyme which exhibited some unique properties. These will be discussed in Chapter 3.

With the discovery of *B. brevis* strains able to hypersecrete proteins without detectable extracellular protease activity and the subsequent development of these strains for the production of heterologous proteins, there was renewed interest in the isolation of new *B. brevis* strains. Some of these isolates varied significantly from the standard strains of the species. The wide discrepancies in maximum growth temperatures, unusual characteristics in selected strains and a wide range of G+C values suggest that *B. brevis* is a phenotypically heterogeneous species. With this in mind it was decided to characterize *B. brevis* Alk 36 with a view to possibly utilizing it as a potential host strain for secreting recombinant proteins.

During the course of this study, the *degS-degU* operon was cloned from *B. brevis* Alk 36 and it was found to have homology with the sensor and effector proteins respectively of the bacterial two-component regulatory system of *B. subtilis* as reviewed in this chapter. The phenotype of the *degS-degU* operon on a multicopy vector and its effect on extracellular enzyme production will be described in Chapter 4.

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Chapter 2

Isolation and characterization of *Bacillus brevis* Alk 36

2.0 Summary

A *Bacillus brevis* strain was isolated from soil and designated *B. brevis* Alk 36. It was evaluated as a potential host strain for the efficient production and secretion of foreign proteins, and was found to grow optimally between pH 8.0 and pH 9.5, and between 42°C and 52°C. *B. brevis* Alk 36 was successfully transformed using vector DNA and was found to produce relatively low levels of protease. It was evaluated as a possible protein hyper-secreting strain. The levels of extracellular proteins produced were, however, found to be relatively low, and when analysed on SDS-PAGE, the high molecular mass cell wall proteins characteristic of these strains were not over-expressed. In addition, by using PCR technology the highly conserved cell wall protein genes could not be positively identified in *B. brevis* Alk 36.

2.1 Introduction

The development of *B. brevis* as a model system for the production of heterologous proteins has proved extremely successful (Udaka and Yamagata 1993). This was primarily due to a number of unique characteristics found only among this group of microorganisms. These characteristics have been reviewed in detail in the General Introduction.

While screening and characterizing protein hyperproducing isolates, Takagi et al. (1989a) observed that most protein hyperproducing *Bacillus* isolates appeared to be strains of *B. brevis* and were found to share many characteristics. They occurred rarely in nature and many of them accumulated one or two major high molecular weight protein species which were secreted in large quantities into the culture fluid. These varied in size from 75 kDa to 150 kDa depending on the *B. brevis* strain (Ebisu et al. 1990). They produced little cell bound protein and were resistant to kanamycin in addition to a number of other antibiotics, the resistance profiles of which varied from strain to strain (Takagi et al. 1989a). There were, however, also considerable differences in other characteristics, such as extracellular protease activities which ranged from nearly zero to a significant level.

The genes for both bacterial and mammalian secretary proteins have been efficiently expressed in the *B. brevis* host-vector system, and large amounts of structurally correct and biologically active proteins were secreted into the medium, where they remained stable for a number of days. Mammalian proteins were found to be produced in active form 10-100 times more efficiently in *B. brevis* than has been reported for other systems, such as *B. subtilis* where the recombinant proteins are degraded by extracellular proteases, or *E. coli* where they are generally not secreted into the medium, or yeast where often overall yields were found to be low.

Very efficient secretion of heterologous proteins in active form by *B. brevis* suggests that the bacterium has extracellular enzymes for protein-folding after secretion. Recently peptidyl-prolyl-cis-trans isomerase (PPI) and disulphide bond-forming activities were detected in the culture medium of *B. brevis*, and in addition, PPI was also detected in the

cytoplasm (Yamagata and Udaka 1993). The structural gene for disulphide bond forming enzyme (Dsb) of *B. brevis* was cloned in *E. coli* and is at present being characterized (Yamagata and Udaka 1993).

As such *B. brevis* protein-hyperproducing strains were found to be excellent hosts for the production of many heterologous proteins, it was decided to characterize the *B. brevis* Alk 36 isolate with a view to possibly utilizing it as a potential host strain for secreting recombinant proteins.

2.2 Materials and Methods

2.2.1 Isolation of *B. brevis* Alk 36. Approximately 3 g soil was pasteurized (Norris et al. 1981) before being used to inoculate 100 ml of alkalophilic enrichment broth, pH 10.0 (0.5% yeast extract, 0.5% polypeptone, 0.1% di-potassium hydrogen phosphate, 0.2% magnesium sulphate and 1% sodium carbonate) as modified from Takeuchi et al (1989) and incubated for 4-5 days at 37°C. Aliquots from the turbid broth cultures were then inoculated onto the same medium solidified with agar (15 g/l) and incubated at 37°C for 24-48 h. The cultures were purified on nutrient agar plates pH 9.0 at 37°C. They were then classified as *Bacillus* spp. after a Gram stain, a spore stain and a catalase test gave positive results. Further strain identification was done by utilizing the API 50CH and 20E strips (Bio Merieux, France).

2.2.2 Bacterial strains and plasmids. *Bacillus subtilis* strains 1A46, 1A654 and *Bacillus* sp. ATCC 31408 (Appendix C) were used as control strains in selected experiments. The multicopy plasmid pPL708 is an expression vector containing the *Spo2* promoter (Duvall et al. 1983). A restriction map of the vector is given in Appendix E. Plasmid pML2 contains the *degS-degU* genes cloned from *B. brevis* Alk 36 (Chapter 4). Plasmid pVC102 contains an α-amylase gene cloned from a *Bacillus* sp. (Corfield et al. 1984).

2.2.3 Media and growth conditions. Media, buffers and solutions not described in the text are listed in Appendix B.

2.2.4 Determination of the pH optimum of *B. brevis* Alk 36. Aliquots (50ml) of Luria-Bertani broth (Sambrook et al. 1989) were dispensed into 250 ml Erlenmeyer flasks and autoclaved. Measured volumes of sterile 10% ($^{w}/_{v}$) Na_2CO_3 solution was used aseptically to adjust the pH value of each flask, giving rise to a range of pH values from pH 7.5 to pH 10.5. An inoculum was prepared by growing *B. brevis* Alk 36 in Luria broth pH 8.0 for 16 h at 37°C. This was used to inoculate the pre-warmed broth samples to give a starting OD of 0.05 at A_{540nm}. Growth rates were measured at 37°C. Aliquots were taken at 30 minute intervals up to the end of log phase, and the OD measured at A_{540nm}. From these results the natural log was determined. The natural log value was plotted

against time for each pH value. From each graph the maximum growth rate (μ_{max}) over a given time period was calculated. The μ_{max} value was then plotted against pH to give the optimum pH growth range.

2.2.5 Determination of thermostability of *B. brevis* Alk 36. Luria broth, pH 8.0 was prepared in 50 ml aliquots, pre-warmed and inoculated as described in section 2.2.4. These flasks were incubated at different temperatures ranging from 37°C to 57°C. Samples were taken at half hourly or 15 minute intervals depending on the growth temperature and the OD measured at A_{540nm} up to the end of log phase. The μ_{max} was then calculated for each temperature as described in section 2.2.4 and the μ_{max} value was then plotted against temperature to give a thermostability range.

2.2.6 Isolation of chromosomal DNA from *Bacillus* spp. The method of Lovett and Keggins (1979) was followed with some modifications (Appendix A).

2.2.7 Isolation of plasmid DNA from *Bacillus* spp. *B. subtilis* plasmids were isolated by the sodium dodecyl sulphate-NaCl (SDS-NaCl) method as modified by Gryczan et al. (1978) (Appendix A).

2.2.8 Genetic transformation of *B. brevis* Alk 36. The polyethylene glycol-induced (PEG) protoplast transformation procedure of Chang and Cohen (1979) was followed with a number of modifications. An overnight culture of *B. brevis* Alk 36 was diluted 1:50 in pre-warmed Luria broth pH 8.0 and grown with shaking at 37°C until an OD of 0.25-0.30 at A_{540} was reached. The cells were harvested by centrifugation at 8 000 rpm for 10 minutes and resuspended in 0.1 volume of SMMP (Appendix B). Lysozyme was added to 2 mg/ml and the cells were shaken very gently in a 37°C waterbath for 90-120 minutes. The protoplasts were harvested by centrifugation at 4 000 rpm for 15 minutes using a Beckman model TJ-6 bench centrifuge. The protoplasts were washed with 5 ml SMMP and finally brought to 0.1 of the original volume with SMMP. Transforming plasmid DNA was added to a sterile corex glass tube. After the addition of an equal volume of 2x SMM buffer (Appendix B), 0.5 ml protoplast suspension was pipetted into

the tube. The contents were immediately treated with 1.5 ml 30% ($^{w}/_{v}$) PEG (Merck approximate molecular weight 4000) in 1x SMM buffer for exactly 2 minutes, followed by dilution with 5 ml SMMP medium. The protoplasts were centrifuged at 4 000 rpm for 15 minutes and resuspended in 1 ml SMMP and shaken gently at 37°C for 90 minutes to allow phenotypic expression. Transformed protoplasts were then plated onto modified DM3 regeneration plates (Appendix A) containing 10 μg/ml chloramphenicol.

2.2.9 Evaluation of extracellular protease production by *B. brevis* Alk 36. The medium for protease production was developed from that of Horikoshi and Ikeda (1977) and contained Bactotryptone 1%; yeast extract 0.5%; NaCl 1%; soluble starch (Merck) 2%; K_2HPO_4 0.1%; $MgSO_4.H_2O$ 0.02%. The pH was adjusted to 7.2 with 1N NaOH. Aliquots (50 ml) of protease production medium in 250 ml Erlenmeyer flasks were inoculated (1%) with overnight cultures of *B. brevis* Alk 36 and *Bacillus* sp. ATCC 31408 respectively. The inoculated flasks were incubated in a New Brunswick controlled environment orbital shaker at 37°C for 3 days at 170 rpm. At each 24 hour time period samples were taken, centrifuged and the supernatant assayed for protease activity while the pellets were used for protein determination.

2.2.10 Protein and protease determinations. Protease was assayed using azocasein as substrate (Millet 1970). Total protease activities were assayed at pH 9.5. Activities were expressed in Anson units (Anson 1939) by using protease reference standards of known protease activity. For calculating specific activities the total amount of protein was estimated from the bacterial culture pellets using the biuret method (Herbert et al. 1971).

2.2.11 Sensitivities toward antibiotics. The minimum inhibitory concentrations (MIC) of various antibiotics were measured by inoculating *B. brevis* Alk 36 on Luria agar plates pH 8.0 containing various antibiotics at a range of concentrations. These were incubated at 42°C for one to two days and scored for growth.

2.2.12 Plate screen for protein producing bacteria. Screening for protein producing bacteria was performed essentially as described by Udaka (1976). Plates of T2 medium (Appendix B) were inoculated with *B. brevis* Alk 36 and *B. subtilis* 1A46. The plates were incubated at 42°C for 2 days, then flooded with 5% perchloric acid. After standing

for more than 10 minutes, the bacterial colonies on the plates were scraped off gently with a glass rod. The plates were washed with tap water to determine whether an opaque area was present under each colony. Bacteria forming turbid zones after acid treatment were selected as protein producers.

2.2.13 Determination of extracellular protein in the culture broth. *B. brevis* Alk 36 was inoculated into 50 ml of T2 broth in 250 ml Erlenmeyer flasks and incubated on an orbital shaker at 42°C for two days. So as to maintain an alkaline pH the T2 broth was modified (Appendix B). In order to measure cell bound and extracellular protein an equal volume of 0.2 N NaOH was added to the culture broth. After 30 minutes the mixture was centrifuged to remove the cells and an equal volume of 10% ($^{w}/_{v}$) TCA was added to the clear supernatant. This was stirred for 1 hour before centrifugation at 10 000 rpm for 20 minutes. The protein content of the pellet was measured as set out in section 2.2.10.

2.2.14 Characterization of extracellular proteins. The extracellular protein produced by *B. brevis* Alk 36 was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After precipitation with TCA (section 2.2.13) the protein was solubilized with SDS-PAGE splitting buffer (Appendix B), heated at 100°C for 3 min and then separated by 10% SDS-PAGE electrophoresis according to the method of Laemmli (1970) (Appendix A). Protein bands were visualized by staining in Coomassie blue and destaining in 20% Acetic acid.

2.2.15 Screening for the presence of cell wall protein genes using PCR technology. Primers were designed with the aid of PRIMER, (version 0.5, Whitehead Institute for Biomedical Research, Cambridge, Massachusetts) software programme, using the sequence of the homologous 5' region of the *B. brevis* cell wall protein genes (Ebisu et al. 1990). The nucleotide primers were synthesized on a Autogen 6500 synthesizer.

Foreward primer:

5'TGTCACACGAAAAACGG (17-mer)

Reverse primer:

5'GCCAATACACTGTTAACGAC (20-mer).

Chromosomal DNA was used in the PCR reaction at a concentration of 100 ng and primers at a concentration of 1.0 μM. The PCR reaction mix was as described in the Boehringer Mannheim PCR core kit. The samples were overlaid with 50 μl light mineral oil (Sigma). Amplification of the DNA was accomplished with the Omnigene temperature cycler (Hybaid) by using the step-cycle programme set to denature at 94°C for 45 seconds, anneal at 45°C for 45 seconds and extend at 72°C for 1 minute for a total of 30 cycles (Carozzi et al. 1991). Following amplification, the PCR reaction mix was analysed by agarose gel electrophoresis as described by Sambrook et al. (1989). A total of 20 μl of each PCR reaction mix was electrophoresed on a 1.6% agarose gel in Tris-acetate buffer, stained with ethidium bromide and photographed.

2.3 Results

2.3.1 Isolation of *B. brevis* Alk 36. Approximately 100 *Bacillus* spp. were evaluated for their extracellular enzyme production capabilities. One of the strains, designated Alk 36, was found to produce a thermostable endo-(1,3-1,4)-ß-glucanase. This strain was identified as belonging to the species *B. brevis* using the API 50CH and 20E strips, with an 85% positive identification.

According to the API results the next best identification of the isolate was for *B. sphaericus* at 15%. The differences between the two strains are illustrated in Table 2.1.

Table 2.1 Summary of the characters used in the simplified key for *Bacillus* species (after Norris et al. 1981).

Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14
B. brevis	+	-	-	+	-	-	0	-	-	-	-	+	+	-
B. sphaericus	+	-	-	-	0	-	-	-	-	0	-	-	0	-

\+ Greater than 85% of strains examined positive;
\- Greater than 85% of strains negative
0 Variable character

1 Catalase
2 V-P reaction
3 Growth in anaerobic agar
4 Growth at 50°C
5 Growth in 7% NaCl
6 Acid and gas in glucose
7 NO_3 reduced to NO_2
8 Starch hydrolyzed
9 Growth at 65°C
10 Rods 1.0 µm wide or wider
11 pH in V-P medium <6.0
12 Acid from glucose
13 Hydrolysis of casein
14 Parasporal bodies

2.3.2 Growth characteristics of *B. brevis* Alk 36. *B. brevis* was found to be alkalotolerant with an optimum growth range between pH 8.0 and pH 9.5 (Fig 2.1). It was also found to be moderately thermophilic with an optimum growth range between 42°C and 52°C (Fig 2.2).

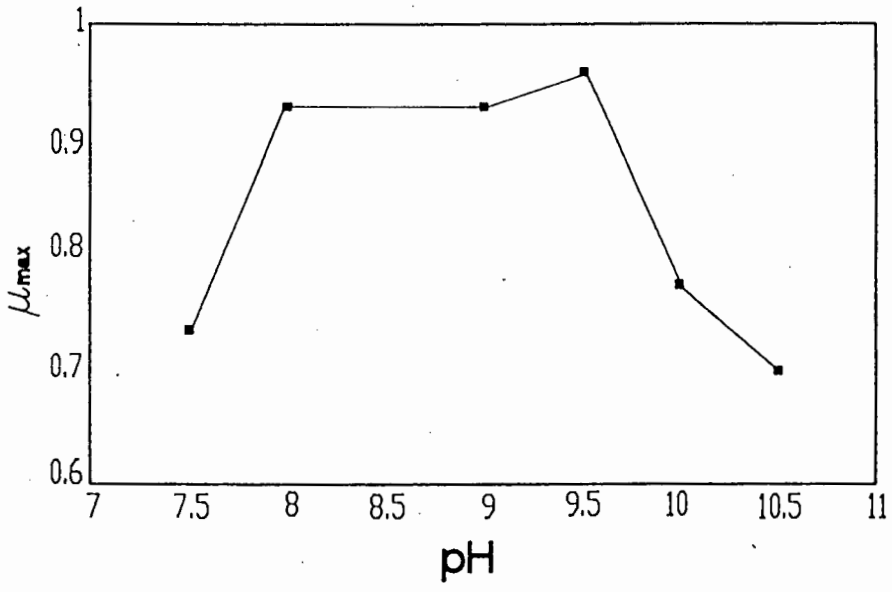

Fig. 2.1 Effect of pH on the growth rate of *B. brevis* Alk 36

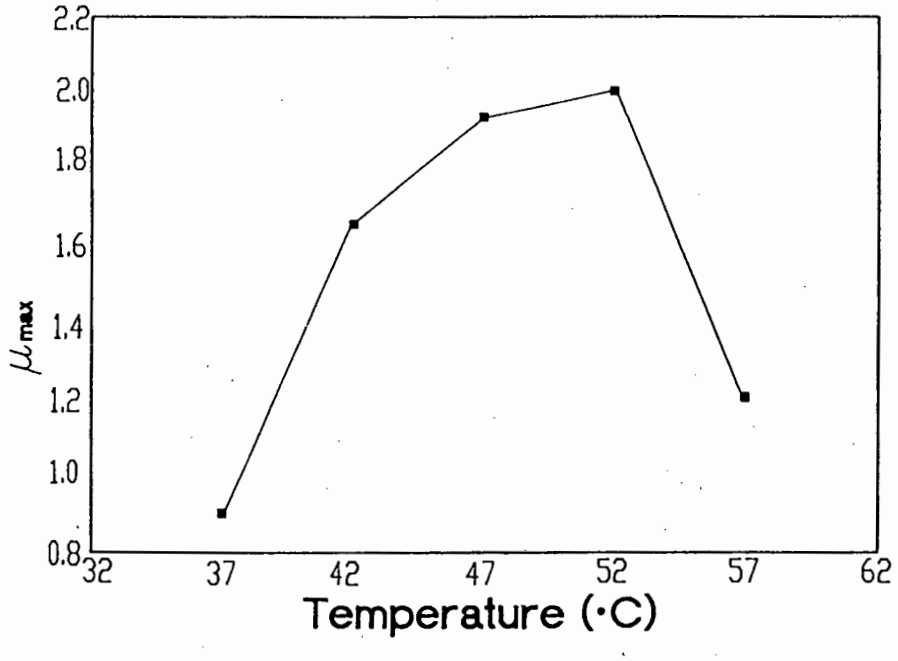

Fig 2.2 Effect of temperature on the growth rate of *B. brevis* Alk 36

2.3.3 Genetic manipulation of *B. brevis* Alk 36. It was found that *B. brevis* Alk 36 could be transformed using the protoplasting technique of Chang and Cohen (1979) with some modifications. The multicopy vector pPL708 was used as a control plasmid. This plasmid was stably maintained within the strain and could be easily re-isolated. The number of transformants were, however, variable and despite optimization of the procedure only between 5 and $7x10^2$ transformants were obtained per μg vector DNA.

Other transformation techniques were attempted such as electroporation (Takagi et al. 1989c; Bron 1990); competent cell (Anagostopoulos and Spizizen 1961); and the alkaline Tris/PEG method (Takahashi et al. 1983). All of these methods proved unsuccessful when applied to *B. brevis* Alk 36 and no transformants were obtained. When the recombinant plasmid pML2 containing the *degS-degU* genes cloned from *B. brevis* Alk 36 (Chapter 4) were transformed back into this strain it was found that only the vector with approximately 500 base pairs of insert was recovered (Fig 2.3). The plasmid pVC102 containing an α-amylase gene cloned from a *Bacillus* sp. was transformed into *B. brevis* Alk 36 but no transformants were obtained.

2.3.4 Evaluation of extracellular protease production by *B. brevis* Alk 36. Protease production was quantified by growing *B. brevis* Alk 36 and an alkalophilic Bacillus sp. (ATCC 31408), Takeuchi et al. (1989) in medium under alkaline conditions and assaying for total protease produced (Table 2.2). The amount of protease produced by *B. brevis* Alk 36 was found to be between 5- and 10-fold lower than that produced by the *Bacillus* sp. depending on time of analysis.

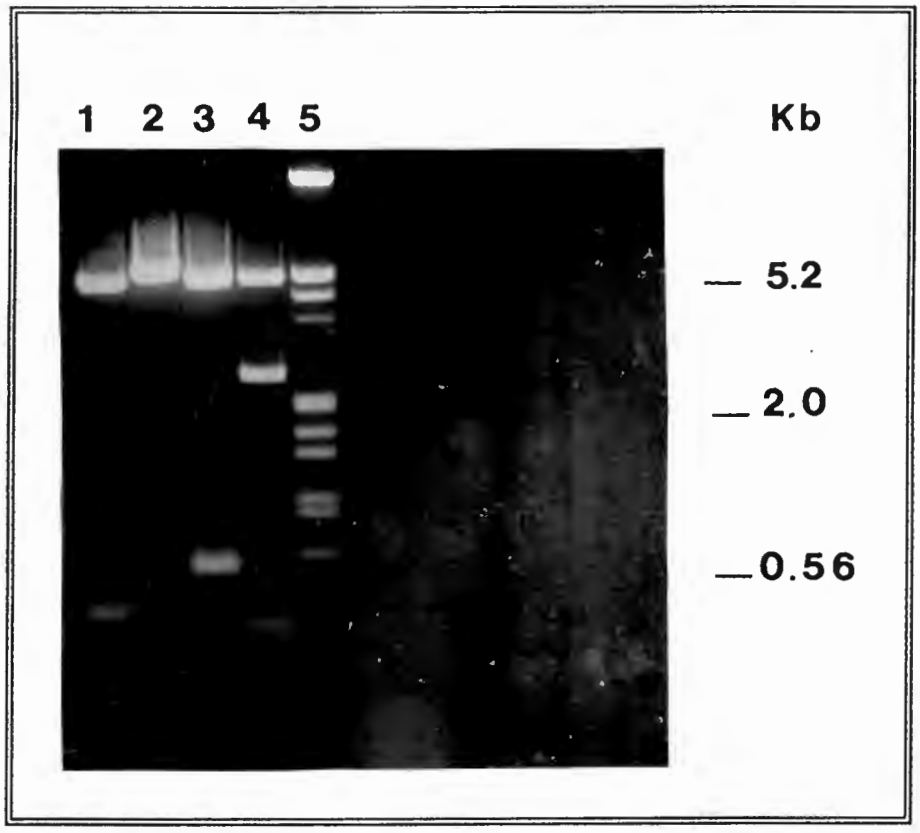

Fig. 2.3 Agarose gel showing restriction pattern of pML2 after transformation into *B. brevis* Alk 36. Lanes 1: pPL708 digested with *Eco*R1; 2: pML2 isolated from *B. brevis* Alk 36 digested with *Eco*R1; 3: As for lane 2 except pML2 was digested with *Pst*1 in addition to *Eco*R1; 4: pML2 isolated from *B. subtilis* 1A46 and digested with *Pst*I/*Eco*R1; 5: λIII.

Table 2.2 Evaluation of extracellular protease production by *B. brevis* Alk 36 and an alkalophilic *Bacillus* sp.

Sample Time (h)	Total protease activity (μAu/mg protein)	
	Bacillus sp. ATCC 31408	*Bacillus* brevis Alk 36
24	72	7
48	77	18
72	94	19

2.3.5 Sensitivity of *B. brevis* Alk 36 towards antibiotics. The sensitivity of *B. brevis* Alk 36 towards a spectrum of antibiotics was investigated. For kanamycin the MIC was found to be 30 μg/ml and erythromycin >200 μg/ml. *B. brevis* Alk 36 was found to be sensitive to ampicillin; neomycin and chloramphenicol. Most of the *B. brevis* isolates

that accumulated one or two major high molecular weight protein species had an MIC of >200 μg/ml to kanamycin, except for HPD52 which had an MIC of 30 μg/ml. (Takagi et al 1989a).

2.3.6 Determination of extracellular protein production by *B. brevis* Alk 36. Initially *B. brevis* Alk 36 was screened for extracellular protein production using the agar plate screen as described in 2.2.12. After two days incubation at 42°C the plates were processed and *B. brevis* Alk 36 was found to have formed an opaque area after acid treatment which indicated protein secretion (Fig. 2.4).

An attempt was made to quantify the amount of protein secreted by growing *B. brevis* Alk 36 in T2 broth at pH 8.0. After 24 hours growth, the cells were found to have lysed and the pH to have dropped to below pH 7.0. The T2 medium was then buffered to maintain a pH of 8.0 over the two day period and the optical density and pH was monitored. After two days the cells had reached an OD of 8.40 at A_{540nm} and the amount of extracellular protein produced was found to be 0.45 mg/ml.

This falls into the middle to lower end of the range of protein producing bacteria as defined by Udaka (1976), but falls far short of the yields obtained by Takagi et al. (1989a) using optimized media.

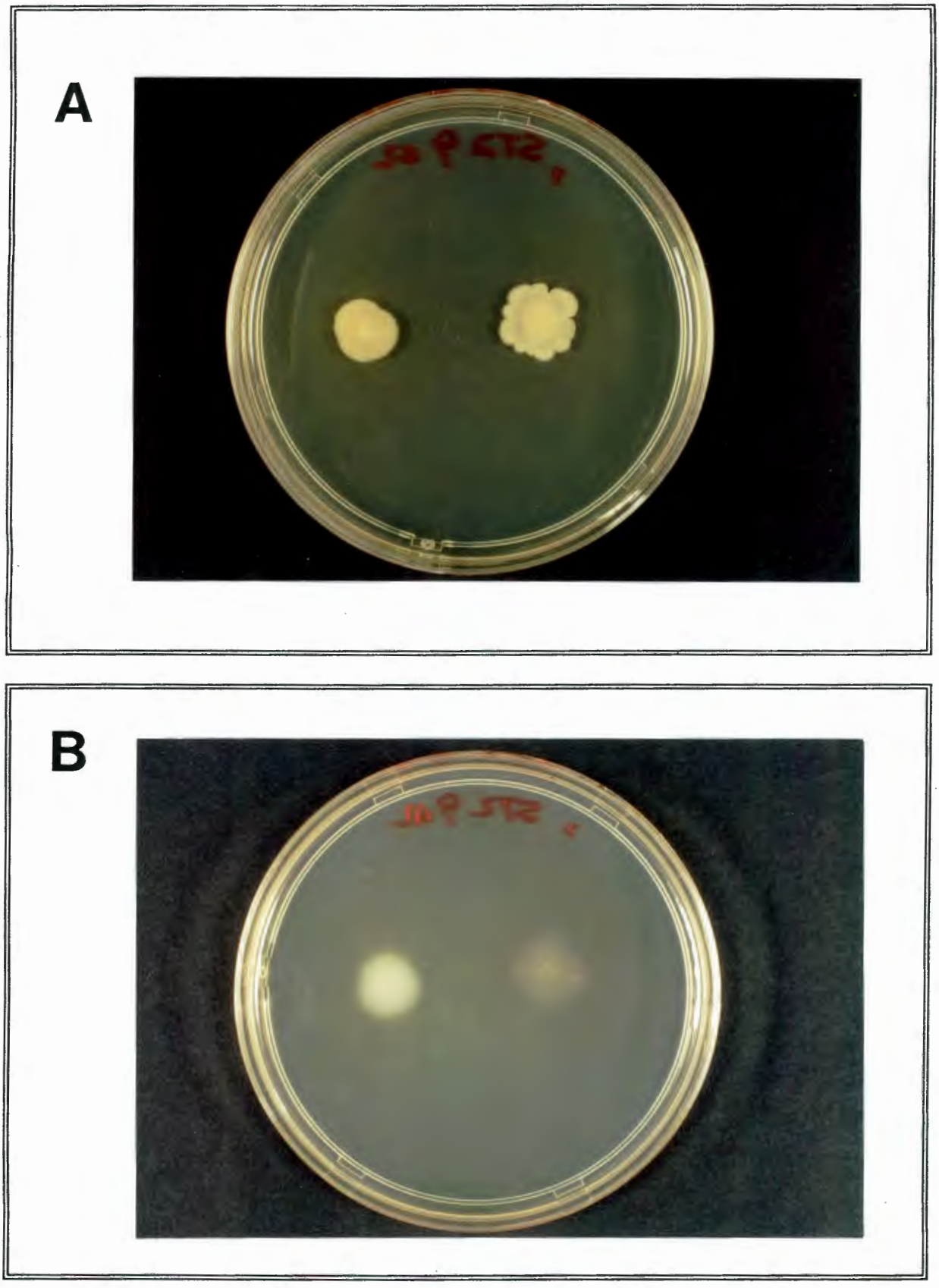

Fig. 2.4 Detection of protein secretion on T2 agar medium. (A) colonies of *B. brevis* Alk 36 and *B. subtilis* 1A46 before treatment. (B) Illustrates the opaque areas beneath the colonies after acid treatment. The colony on the left hand side of the plates is *B. brevis* Alk 36 and the one on the right hand side is *B. subtilis* 1A46.

2.3.7 Characterization of extracellular proteins. When the extracellular proteins produced by *B. brevis* Alk 36 were analysed by SDS-PAGE electrophoresis, two high molecular weight proteins were identified at ~89 kDa and ~178 kDa (Fig. 2.5). These were, however, not overproduced.

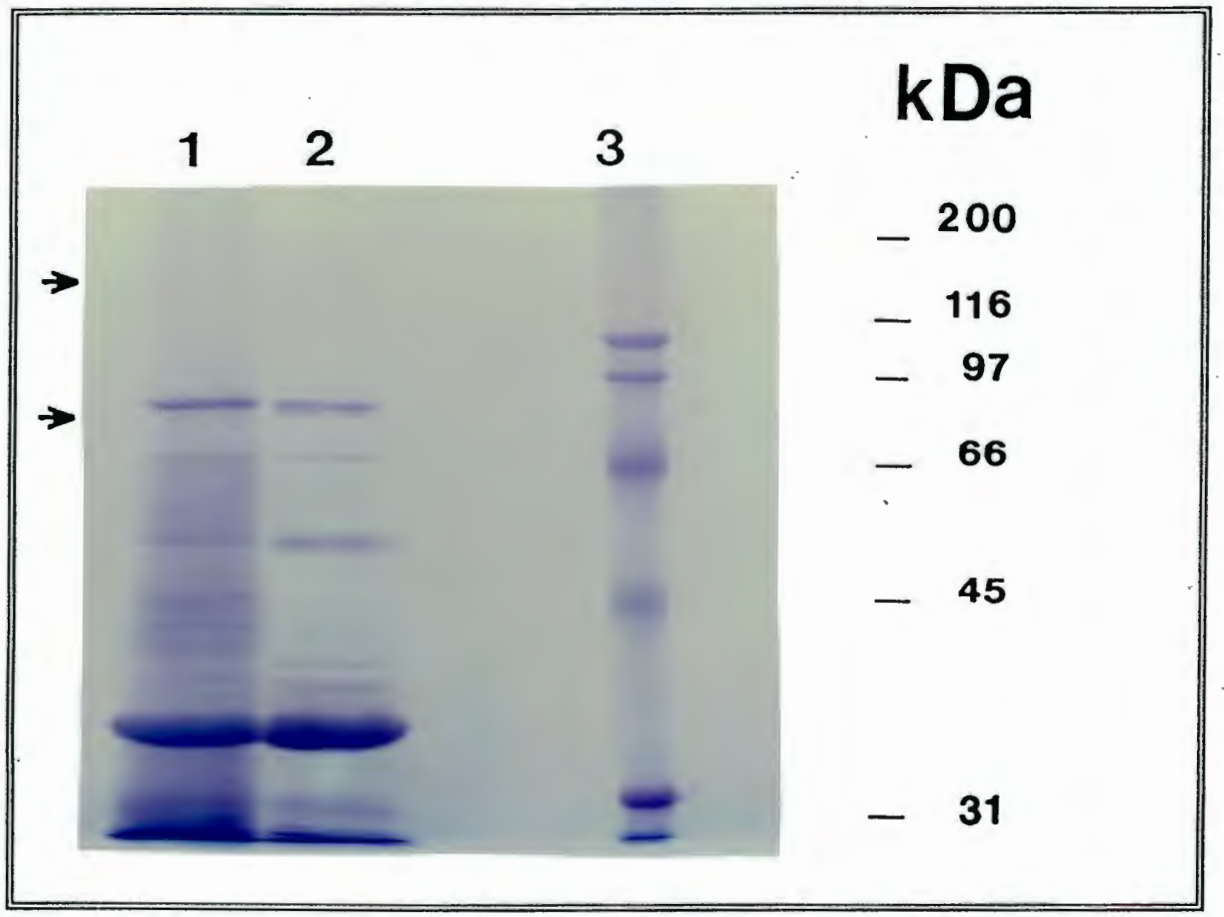

Fig 2.5 Evaluation of extracellular high molecular weight proteins produced by Alk 36 on SDS-PAGE. Lanes: 1, 2 Alk 36 samples grown at 42°C; 3, migration of high molecular weight markers. Sizes are indicated in the right hand margin. Arrowheads in the left hand margin indicate bands of approximately 89 kDa and 178 kDa.

2.3.8 Screening for the presence of cell wall protein genes using PCR technology. When the cell wall protein genes of four different protein producing *B. brevis* strains were evaluated by Ebisu et al. (1990), it was found that all sequences in the regulatory regions of the four cell wall protein genes were highly conserved. On the basis of this, an attempt was made to characterize the high molecular weight proteins of *B. brevis* Alk 36 using PCR technology. Primers were designed to amplify the highly homologous 5' region of the cell wall protein genes giving rise to a product of 276 base pairs in length.

Due to the inability in obtaining any of the four *B. brevis* protein producing strains for use as a positive control, it was decided to use a *B. subtilis* strain IA654 as a negative control. When the PCR samples were analysed on an agarose gel, it was found that *B. brevis* Alk 36 DNA gave rise to three PCR products of 2.2 kb, 1.3 kb and 600 base pairs in length. The *B. subtilis* control also gave rise to three DNA products of 1.8 kb, 1.6 kb and 380 base pairs. No product of 276 base paris was obtained. (Fig.2.6).

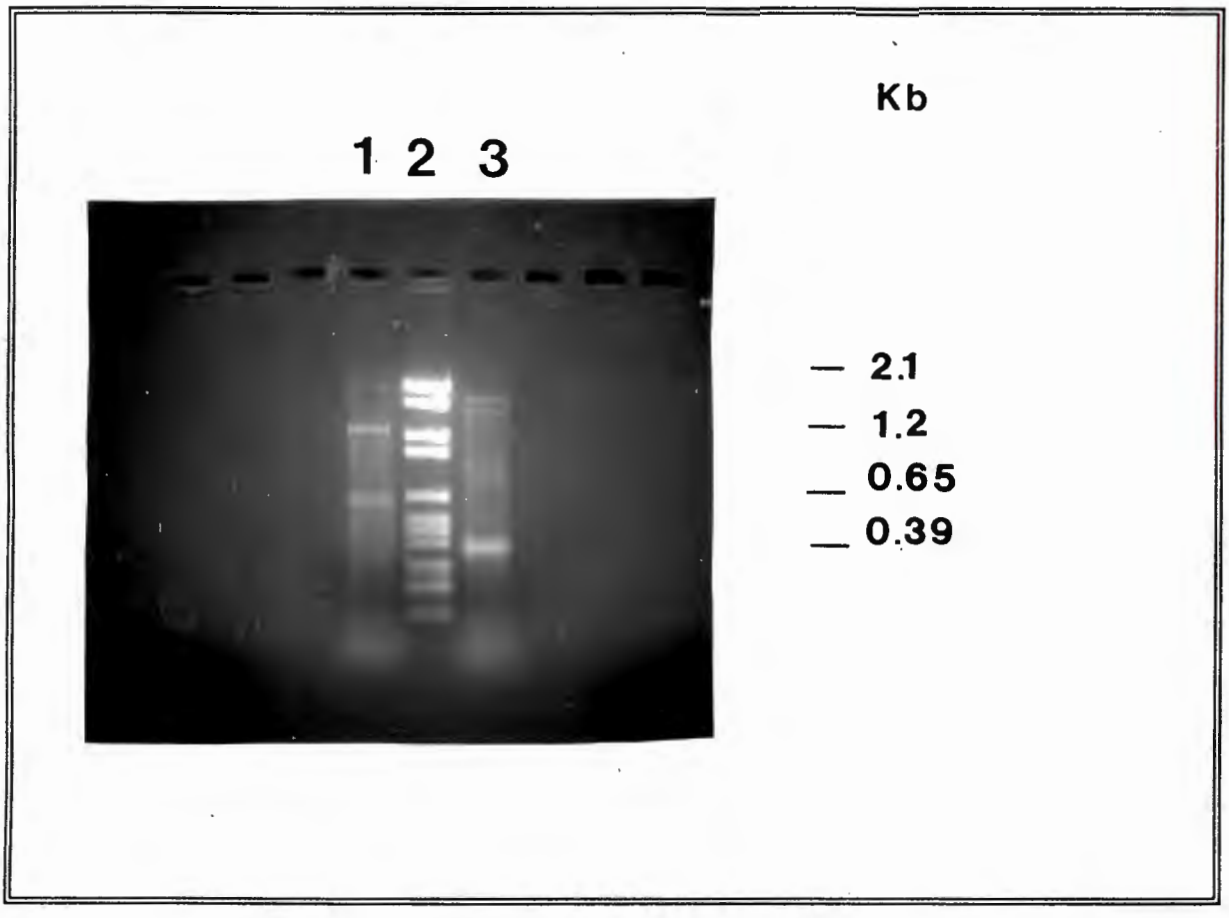

Fig 2.6 Agarose gel showing PCR products. Lane 1: *B. brevis* Alk 36; 2: λ marker VI; 3: *B. subtilis* 1A654

2.4 Discussion

In evaluating *B. brevis* Alk 36 as a potential host strain for the efficient production and secretion of foreign gene products, it was found that its ability to grow optimally over a wide pH and temperature range could be a decided advantage for use in a number of different fermentation processes, particularly if an alkaline environment would facilitate the expression of certain recombinant proteins.

B. brevis Alk 36 was transformed with plasmid DNA which was found to be stably maintained within the strain. However, when a chimeric plasmid was constructed containing a functional gene, either no transformants were obtained (if the gene was foreign) or transformants containing only part of the insert were re-isolated. This could be due to a number of factors such as an intramolecular recombination event or to site-specific restriction endonucleases which are very common in *Bacillus* species. Some of these have been demonstrated to play a role in the "restriction" of foreign DNA which invades the cell (Maru and Yoshikawa 1989). Thus further studies would have to be undertaken to determine the precise reasons for the failure in obtaining the expression of foreign genes by *B. brevis* Alk 36.

In evaluating the protease production by *B. brevis* Alk 36 an alkalophilic *Bacillus* sp. ATCC31408 was used for comparative purposes. It could be seen that *B. brevis* Alk 36 produces significantly less protease than the *Bacillus* sp., although these levels are not as low as those obtained by Takagi et al. (1989b) for some of their isolates, such as HPD31. It is, however, difficult to compare these results directly as the alkalophilic strains were grown in different growth media and the analytical methods also differed.

The results of the evaluation of *B. brevis* Alk 36 as a possible protein producing strain were found to be rather ambiguous, for although the initial plate screen gave positive results, this could have been due to cellular lysis. However, the colony sizes are comparable, and according to Udaka (1976), autolysis of bacteria does not usually occur in an agar medium. In this, *B. brevis* Alk 36 could differ as in liquid culture containing glucose, autolysis was found to occur when the pH dropped below pH 7.0. After buffering the medium, and carefully monitoring the pH, the amount of extracellular

protein produced was found to be relatively low, and when analysed on SDS-PAGE the high molecular mass proteins were found not to be over-expressed.

The 276 base pair product of the highly conserved 5' region of the *B. brevis* cell wall protein genes could not be isolated by PCR technology. As the DNA of *B. brevis* Alk 36 and *B. subtilis* 1A654 both gave rise to three PCR products, each of which was roughly in the same size range, it could be speculated that the primers were not specific enough for the 5' region of the *B. brevis* cell wall protein genes. The region in which the reverse primer was located was found to be highly homologous with the putative signal peptide of the outer cell wall protein (OWP) of *B. brevis* 47 (Tsuboi et al. 1988) and shows characteristics of signal peptides of secretory precursors (Inouye and Halegoua 1980). However, in the absence of a positive control, and without further analysis by sequencing the PCR products obtained from *B. brevis* Alk 36, no further conclusions can be drawn from this experiment.

From the above results it would seem that *B. brevis* Alk 36 was not a member of the protein hyper-producing *B. brevis* group, unless the optimum cultural conditions needed for protein production by this strain differed greatly from those reported by Udaka (1976) and Takagi et al. (1989a). However, together with its relatively low extracellular protease production and its ability to grow over a wide pH and temperature range *B. brevis* Alk 36 could make a promising candidate for the expression of recombinant heterologous proteins if its genetic transformation systems can be optimized to include the successful expression of foreign genes.

Chapter 3

Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4)-ß-glucanase-encoding gene from *B. brevis* Alk 36

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Chapter 3

Characterization, cloning and sequencing of a thermostable endo-(1,3-1,4)-ß-glucanase-encoding gene from *B. brevis* Alk 36

3.0 Summary

A *Bacillus brevis* gene coding for an endo-(1,3-1,4)-ß-glucanase was cloned in *Escherichia coli* and sequenced. The open reading frame contains a sequence of 759 nucleotides encoding a polypeptide of 252 amino acid residues. The amino acid sequence of the ß-glucanase gene showed only a 50% similarity to previously published data for *Bacillus* endo-(1,3-1,4)-ß-glucanases. The optimum temperature and pH for enzyme activity were 65-70°C and 8-10, respectively. When held at 75°C for 1h, 75% residual activity was measured. The molecular mass was estimated to be about 29 kDa on sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis and the enzyme was found to be resistant to SDS.

3.1 Introduction

Few reports have been published on the isolation and characterization of extracellular enzymes from *B. brevis* strains. Much work has, however, been done on the cloning and characterization of intracellular multifunctional enzymes from *B. brevis* strains involved in the biosynthesis of the cyclic antibiotics gramicidin S and tyrocidine. Mittenhuber et al. (1989) reported the cloning of the structural genes for tyrocidine synthetases 1 and 2 from a *B. brevis* strain and proposed that they form an operon. These genes code for two of the three multifunctional enzymes involved in the non-ribosomal biosynthesis of the cyclic decapeptide antibiotic tyrocidine. Krätzschmar et al (1989) determined the nucleotide sequence of a major part of the gramicidin S biosynthesis operon (*grs*). They identified three ORFs two of which, *grsA* and *grsB*, code for the gramicidin S synthetases 1 and 2 respectively, which were found to show high homology to the tyrocidine synthetases.

Diderichsen et al. (1990) cloned an α-acetolactate decarboxylase from *B. brevis*. This extracellular enzyme was found to be suitable for the conversion of α-acetolactate, produced during the brewing process, to acetoin thereby preventing the formation of diacetyl. To date, no reports have been published on the characterization and cloning of extracellular carbohydrases from *B. brevis* such as endo-glucanases. In addition, prior to this study, no bacterial endo-(1,3-1,4)-ß-glucanases with an alkaline pH optimum have been reported. A number of reports have been published dealing with the cloning and characterization of endo-(1,3-1,4)-ß-glucanases from various *Bacillus* spp. and these have been reviewed in the General Introduction.

In this chapter the biochemical properties of the *B. brevis* lichenase have been characterized and the gene cloned and sequenced. The secretion and location of the cloned lichenase in *E. coli* was examined. The sequence of the encoded polypeptide was compared with the sequences from lichenases cloned from other *Bacillus* spp. It was thought that as this enzyme exhibited certain novel biochemical properties, the differences in the amino acid sequence could give insight into key functional domains among conserved regions.

3.2 Materials and Methods

3.2.1 Bacterial strains and vectors. *B. brevis* Alk 36 was isolated from soil as described in section 2.2.1. The *E. coli* host strain for the original cloning experiment was JM101 (Messing 1979) while for sequencing, the *E. coli* rec⁻ strain LK112 was used (Zabeau and Stanley 1982). These strains are described in Appendix C. The cloning vector was the positive selection vector p*Eco*R251, (Zabeau and Stanley 1982). Subcloning for sequencing was done using the M13-derived Bluescript SK plasmid (Stratagene, San Diego). Restriction maps of the vectors are given in Appendix E.

3.2.2 Media, buffers and enzymes. Lichenan from *Cetraria islandica*, carboxymethyl cellulose (CMC), xylan (from birchwood) and laminarin were all purchased from Sigma Chemical Company (St Louis, Mo). Avicel from Fluka Chemie AG (Switzerland). Restriction endonucleases, T4 ligase, Mungbean nuclease and Exonuclease III were all obtained from Boehringer Mannheim Biochemicals. DNase-free agarase was obtained from CalBiochem. All media and buffers not described in the text are given in Appendix B.

3.2.3 Growth conditions. *E. coli* strains were grown in Luria broth pH 7.2 and *B. brevis* Alk 36 was grown in Luria broth pH 9.0. All strains were grown at 37°C.

3.2.4 Isolation of *B. brevis* chromosomal DNA. Chromosomal DNA isolation was as described in section 2.2.6.

3.2.5 Preparation of DNA

3.2.5.1 Partial digestion and isolation of size fractionated fragments. The chromosomal DNA was partially digested with *Sau*3A endonuclease in order to produce fragments ranging from 1.0 - 10.0 kb in size (Sambrook et al. 1989). Approximately 15 μg of DNA was digested and loaded onto a 0.8% low-melting point agarose gel (Sambrook et al. 1989) and electrophoresed at 80 volts for 5 hours. Slices of agarose gel containing DNA fragments ranging from 1.5 - 4 kb in size were cut out and placed in Eppendorf tubes together with a equal volume of TES buffer. The agarose gels were

melted at 65°C and equilibrated at 37°C before agarase was added at a concentration of 2 units per 100 μg of gel. The tubes were then incubated at 37°C for 16 hours during which time the agarose was digested to oligosaccharides. The DNA was recovered by extracting twice with phenol. After the second extraction, the aqueous phase was transferred to a fresh tube and 2 volumes of TE pH 7.6 (Appendix B) was added. To this 0.05 volume of 5 M NaCl was added followed by 2 volumes of ethanol. The tube was then incubated at 0°C for 15 min followed by centrifugation at 12 000 g for 15 min at 4°C. The ethanol was removed and the DNA pellet dried at room temperature.

3.2.5.2 Preparation of vector DNA. The vector p*Eco*R251 was prepared by digesting 2 μg of the plasmid with 10 units of *Bgl*II endonuclease for 1 hour at 37°C. A sample of the DNA was analysed by agarose gel electrophoresis to confirm complete digestion.

3.2.6 Construction of a *B. brevis* genomic library in *E. coli*.

3.2.6.1 Ligation of vector and insert DNA. p*Eco*R251 is a positive selection vector in *E. coli* and the *Eco*R1 gene is lethal unless insertionally inactivated. DNA concentrations of 1 μg vector to 2 μg insert were used. The ligation reaction contained DNA, 5μl ligation buffer (10x), T4 DNA ligase (1 unit) and water to 50 μl. The reaction was incubated at 16°C for 16 hours.

3.2.6.2 Transformation of *E. coli* and screening for transformants. *E. coli* JM101 cells were made competent according to the $CaCl_2$ method of Sambrook et al. (1989). *E. coli* LK112 cells were made competent according to the method as described in Armitage et al. (1988), (Appendix A). The competent cells were aliquoted and stored at -70°C until needed. The entire 50 μl ligation mix was transformed into competent *E. coli* cells and left to express for 1 hour at 37°C in Luria broth before plating onto Luria agar plates containing 0.1% lichenan and ampicillin (100 μg/ml). Positive transformants were selected for their ability to form clear haloes on Luria agar plates after flooding with 0.1% Congo red.

3.2.7 Restriction endonuclease mapping and Southern blot analysis. Recombinant plasmid DNA was prepared by the large-scale (maxiprep) alkali lysis method (Sambrook et al. 1989). Restriction endonuclease mapping was carried out using standard

procedures (Sambrook et al. 1989). Southern hybridization using the cloned DNA fragment as a probe was used to confirm that the insert DNA originated from *B. brevis* Alk 36. Chromosomal and recombinant plasmid DNA were digested with restriction endonuclease and the DNA fragments separated by electrophoresis in 0.8% agarose gels. The DNA was transferred to Hybond N+ membranes (Amersham) by the DNA/RNA alkali blotting procedure (Appendix A). The non-radioactive digoxigenin (DIG) DNA labelling and detection kit (Boehringer Mannheim) was used for probe-DNA labelling, hybridization and detection. The manufacturer's procedures were followed exactly. The probe was isolated from agarose gels using the Geneclean ™ kit (Bio101, Calif. USA).

3.2.8 Preparation of supernatant and cell lysate fractions. The recombinant *E. coli* strain was grown overnight in Luria broth plus 0.1% lichenan and ampicillin (100 μg/ml). The supernatant as well as the periplasmic and sonicated fractions were assayed separately. The recovery of extracellular, periplasmic and cytoplasmic enzymes was done as described by Cornelis et al. (1982).

3.2.9 Enzyme assays. The determination of endo-(1,3-1,4)-ß-glucanase activity was carried out using lichenan as a substrate (Tezuka et al. 1989). One unit was defined as the amount of enzyme that produced 1 μmol reducing sugar calculated as glucose per minute under the conditions of assay. ß-galactosidase activity was assayed as detailed by Clowes and Hayes (1968) and ß-lactamase according to Sykes and Nordstrom (1972).

3.2.10 Substrate specificity of the recombinant endo-(1.3-1.4)-ß-glucanase. In order to determine enzyme specificity, the activity of the sonicated fraction of the recombinant *E. coli* strain JM101 containing the cloned ß-glucanase gene was assayed as described in section 3.2.9 at pH 9.0 using, in addition to lichenan, other high molecular mass substrates such as carboxymethylcellulose (CMC), avicel, laminarin and xylan at a final concentration of 0.5% ($^{w}/_{v}$). For calculating specific activities, protein concentration of the sonicated fraction was measured using the biuret method (Herbert et al. 1971).

3.2.11 Influence of pH and temperature on ß-glucanase activity. The activity of the crude enzyme extract was determined at various pH values ranging from pH 3 to pH 11. The pH was adjusted using Sørensen phosphate buffer from acid to neutral pH values

and Tris buffer at alkaline values. Enzyme activity was measured at 40°C. Other conditions were as for the standard assay method (3.2.9). Influence of temperature on enzyme activity was measured by incubating the enzyme extract at various temperatures ranging from 40°C to 80°C under standard assay conditions at pH 7.0. Thermal stability of the enzyme was measured at pH 7.0 by incubating the enzyme at different temperatures for the indicated time period. Samples were withdrawn, immediately cooled on ice and the residual activity was then measured.

3.2.12 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) protein assay. Proteins were analysed on 15% SDS-PAGE gels (Appendix A). To visualize enzyme activity, samples were pre-treated by incubating for 40 minutes at 60°C in sample buffer and run on SDS-PAGE activity gels containing 0.1% lichenan (Zverlov and Velikodvorskaya 1990). To remove the SDS the gels were washed in phosphate buffer, pH 6.3 (Béguin 1983). The bands of enzyme activity were detected by staining the lichenan/PAGE gel with 0.1% Congo red.

3.2.13 Sequencing strategy. The endo-(1,3-1,4)-ß-glucanase gene was located on a 1.75 kb restriction fragment of pNA3 (Fig.3.1). As there were no suitable restriction endonuclease sites flanking the insert, the *Hin*dIII - *Pst*I sites found in the vector, p*Eco*R251 were used in order to subclone the insert into *Hin*dIII - *Pst*I endonuclease digested pBluescript.SK giving rise to SKpNA3. This recombinant plasmid was then transformed into *E. coli* LK112 as it was found to be more stable in this rec$^-$ strain. SKpNA3 was then used to generate overlapping deletions of the gene using the exonuclease III shortening technique (Henikoff 1984).

3.2.14 Exonuclease III digestion. Exonuclease III deletions of SKpNA3 were generated by an adaptation of the method of Henikoff (1984). Progressive deletions from the 5' end of the insert were obtained by unidirectionally digesting *Kpn*I-*Cla*I restriction endonuclease digested SKpNA3. *Sac*I-*Bam*H1 restriction endonuclease digested SKpNA3 was used to generate progressive deletions from the 3' end of the insert. After digestion the linearized plasmid DNA was ethanol precipitated and 10 μg resuspended in 100 μl exobuffer. Exonuclease III digestion was then carried out as described in Appendix A. Mung Bean nuclease was then used to digest the single

stranded DNA and create blunt ends. The re-ligated deletion plasmids were transformed into competent *E. coli* LK112 and the transformants were selected on Luria agar plates containing 100 μg/ml Ampicillin. The shortened recombinant plasmids were selected by restriction endonuclease mapping before being purified by cesium chloride-ethidium bromide density gradient ultracentrifugation. (Sambrook et al. 1989).

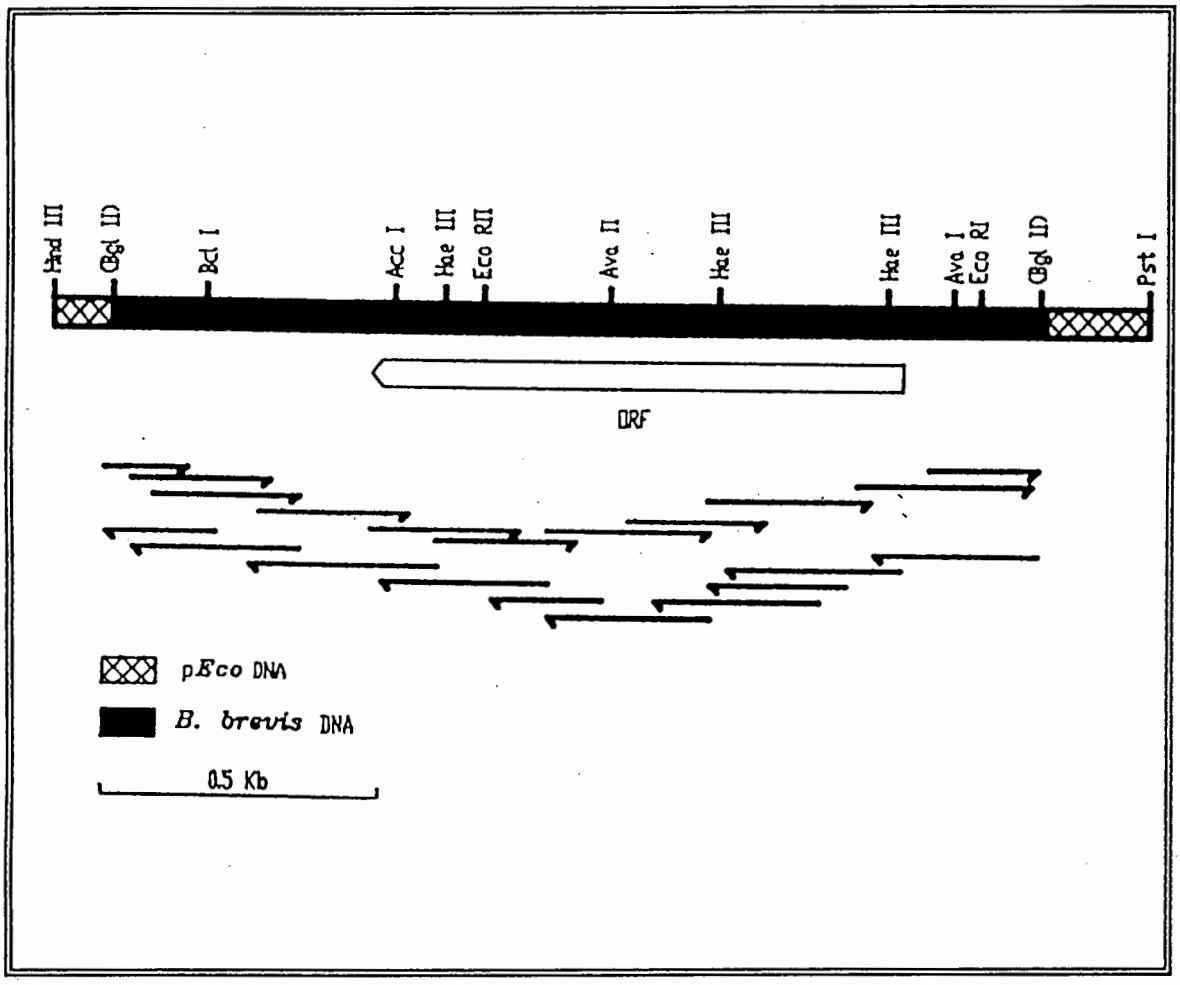

Fig 3.1 Restiction endonuclease map of insert DNA on pNA3 encoding the *B. brevis* Alk 36 endo-(1,3-1,4)-ß-glucanase gene. The ORF and direction of transcription are indicated by an open arrow. The thin arrows represent the extent and direction of sequencing templates generated by exonuclease III digestion.

3.2.15 Nucleotide sequence determination and protein sequence comparison. Sequencing was carried out by the dideoxynucleotide triphosphate chain termination method using the overlapping DNA fragments generated by exonuclease III digestion (Appendix A). The sequence of the entire insert was determined from both strands.

Sequence data was analysed using the Genetics Computer Group Inc. (CGC) software package (version 7.0). The TFASTA subroutine was used to screen the databases for sequences having similarity to the amino acid sequence of the *B. brevis* endo-(1,3-1,4) ß-glucanase gene.

3.2.16 Nucleotide sequence accession number. The nucleotide sequence reported has been assigned the GenBank accession number M84339.

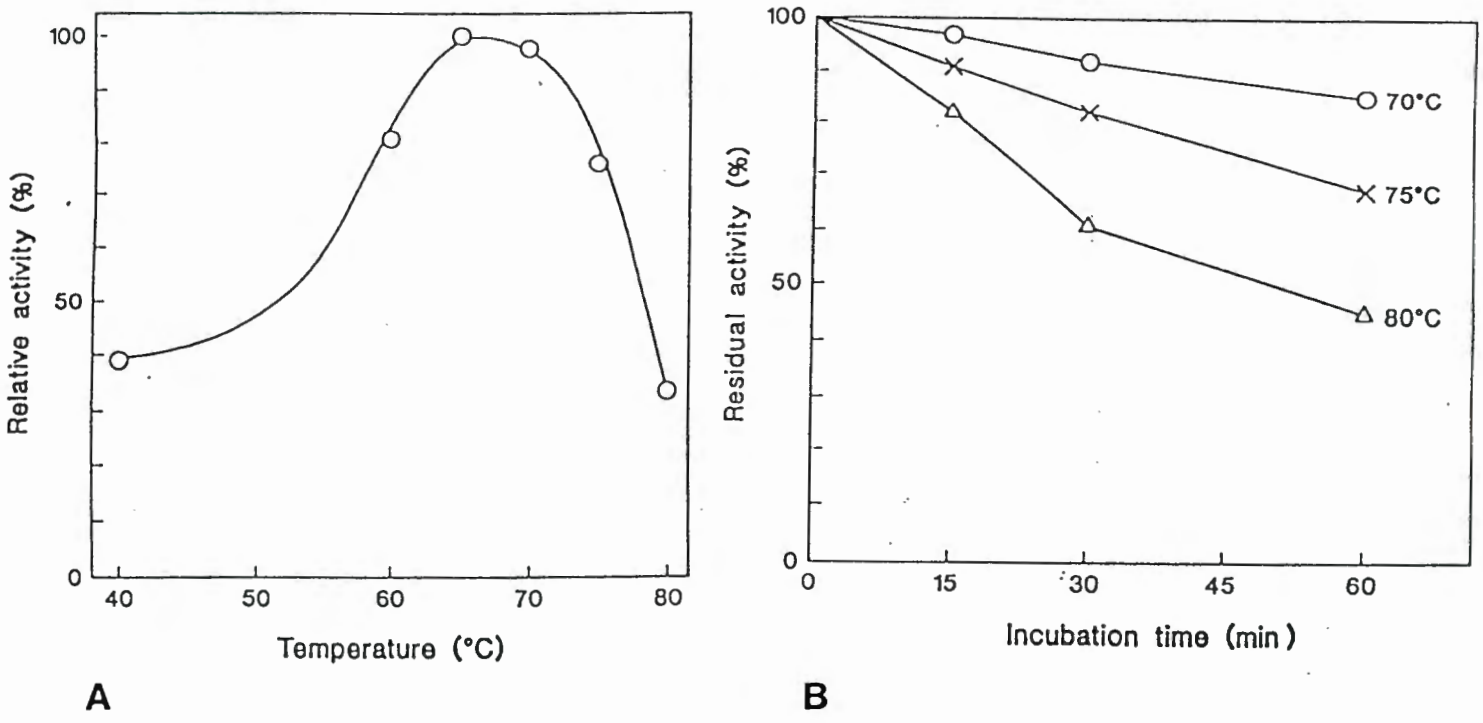

Fig 3.3 (A) Influence of temperature on endo-(1,3-1,4)-ß-glucanase acitvity. The enzyme activities at various temperatures were measured by the standard assay at pH 7.0 (B) Influence of temperature on the stability of endo(1,3-1,4)-ß-glucanase. The enzyme was assayed at pH 7.0.

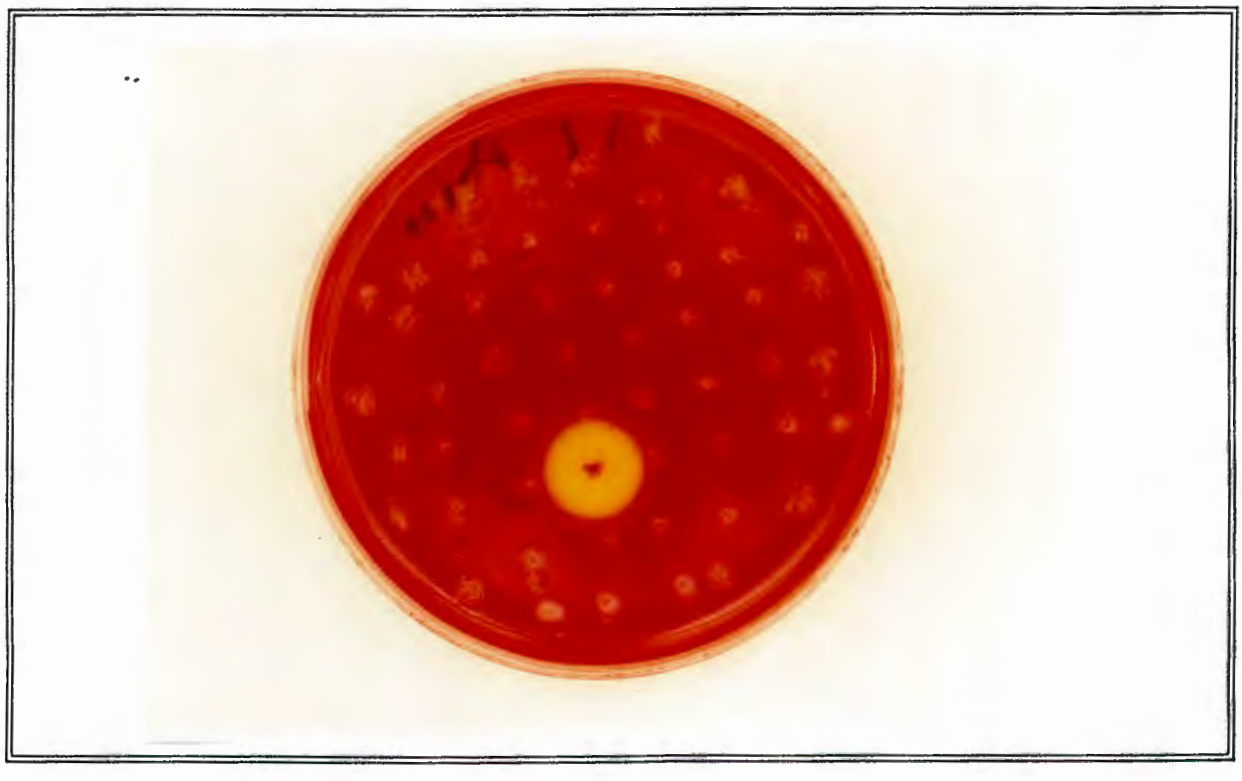

Fig. 3.4 *E. coli* transformant showing hydrolysis of lichenan using the Congo red plate assay.

3.3.3 Southern hybridization. The origin of the 1.75 kb insert on pNA3 was determined by Southern blotting and DNA hybridization. (Fig. 3.5). An internal 1.1 kb *Acc*I-*Ava*I fragment was labelled using dioxigenin d-UTP and was used as a probe. *B. brevis* Alk 36 DNA was digested with *Acc*I to completion and in a separate experiment partially with *Sau*3A. The *Acc*I-*Ava*I probe hybridized to a 3.2 kb *Acc*I fragment as well as to *Sau*3A digested chromosomal DNA. No hybridization could be detected to partially digested *Sau*3A chromosomal DNA from *B. subtilis* 1A654.

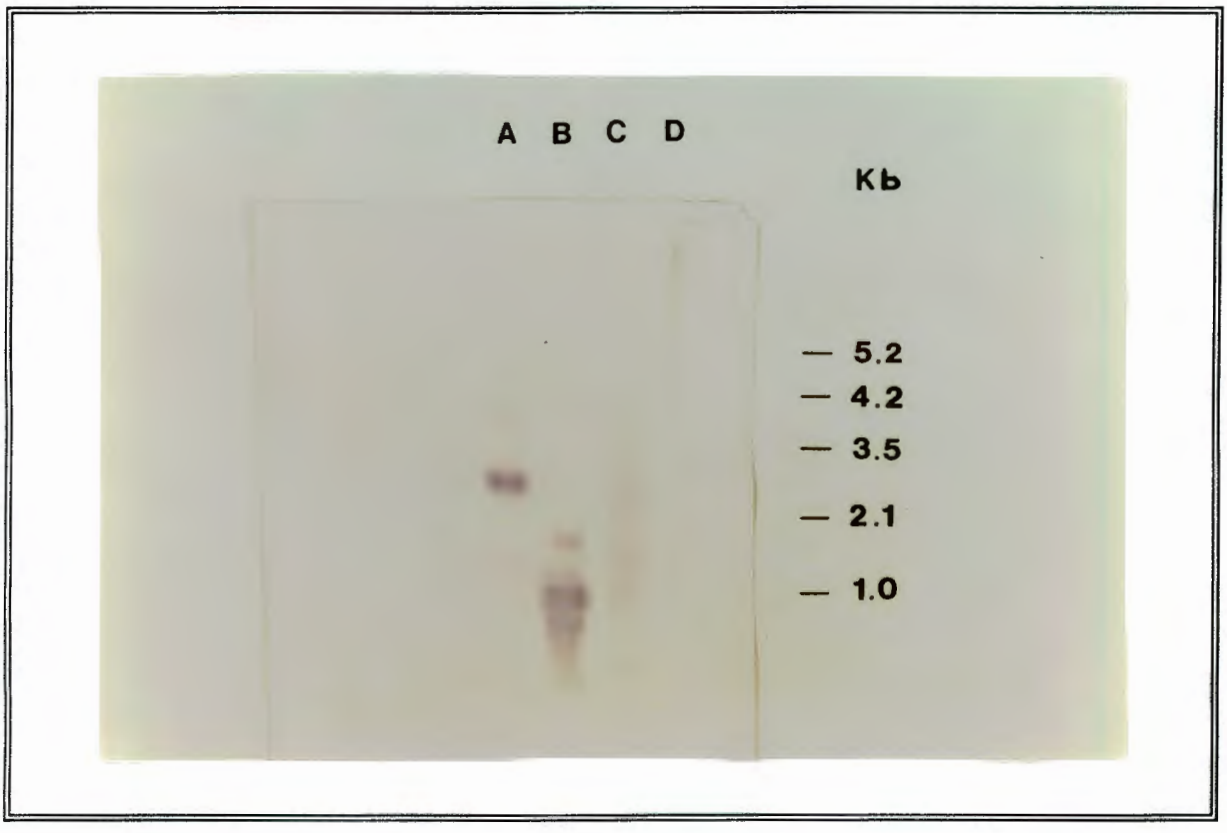

Fig 3.5 Southern blot hybridization using nonradioactively labelled *Acc*1-*Ava*1 fragment. *B. brevis* Alk 36 DNA digested with *Acc*1 (lane A), internal *Acc*1-*Ava*1 fragment (lane B), *B. brevis* Alk 36 DNA digested with *Sau*3A (lane C) and *B. subtilis* 1A654 DNA digested with *Sau*3A (Lane D).

3.3.4 Substrate specificity of the enzyme. The cloned *B. brevis* Alk 36 enzyme was characterized as an endo-(1,3-1,4)-ß-glucanase or lichenase on the basis of its substrate specificity (Table.3.1).

Table 3.1 Substrate specificity of the ß-glucanase enzyme produced by *E. coli* JM101 (pNA3)

Substrates	U/ml	U/mg protein
Lichenan	279.90	39.78
Laminarin	0.89	0.13
Carboxymethylcellulose	0.29	0.04
Xylan (birchwood)	0.43	0.06
Avicel	0.24	0.03

3.3.5 Cellular localization of ß-glucanase activity in *E. coli* JM101. In order to study the distribution of the ß-glucanase synthesized in *E. coli*(pNA3), extracellular, periplasmic and cytoplasmic fractions were isolated and enzyme activities assayed (Table. 3.2). Most of the ß-glucanase enzyme activity was located in the cytoplasmic fraction and periplasmic region with very little enzyme being secreted into the extracellular medium. The location of the ß-galactosidase (a cytoplasmic enzyme) and ß-lactamase (a periplasmic enzyme) activity confirmed the correct fractionation of the cell components.

Table 3.2 Localization of the ß-glucanase enzyme produced by *E. coli* JM101 (pNA3).

Cell fraction	Distribution of total activity (%)		
	ß-glucanase	ß-galactosidase	ß-lactamase
Extracellular	18	5	8
Periplasmic	36	4	90.5
Cytoplasmic	46	97	1.5

3.3.6 SDS-PAGE protein analysis. Zymograms that detected ß-glucanase activity of the renatured proteins separated by SDS-PAGE, gave a molecular mass of approximately 29 kDa. When the acrylamide gels, containing lichenan were stained, a zone of activity was detected running the length of the PAGE gel (Fig.3.6). This indicated SDS-resistance of the enzyme, and it was found that SDS-PAGE gels could be stained directly with Congo red without the removal of SDS. On further de-staining, bands in lane 2 can be seen to correspond to those in lane 3. Enzyme activity was assayed in the presence of different concentrations of SDS (ranging from 0.1% to 10%). It was found that no inhibition of enzyme activity occurred up to a concentration of 10% SDS.

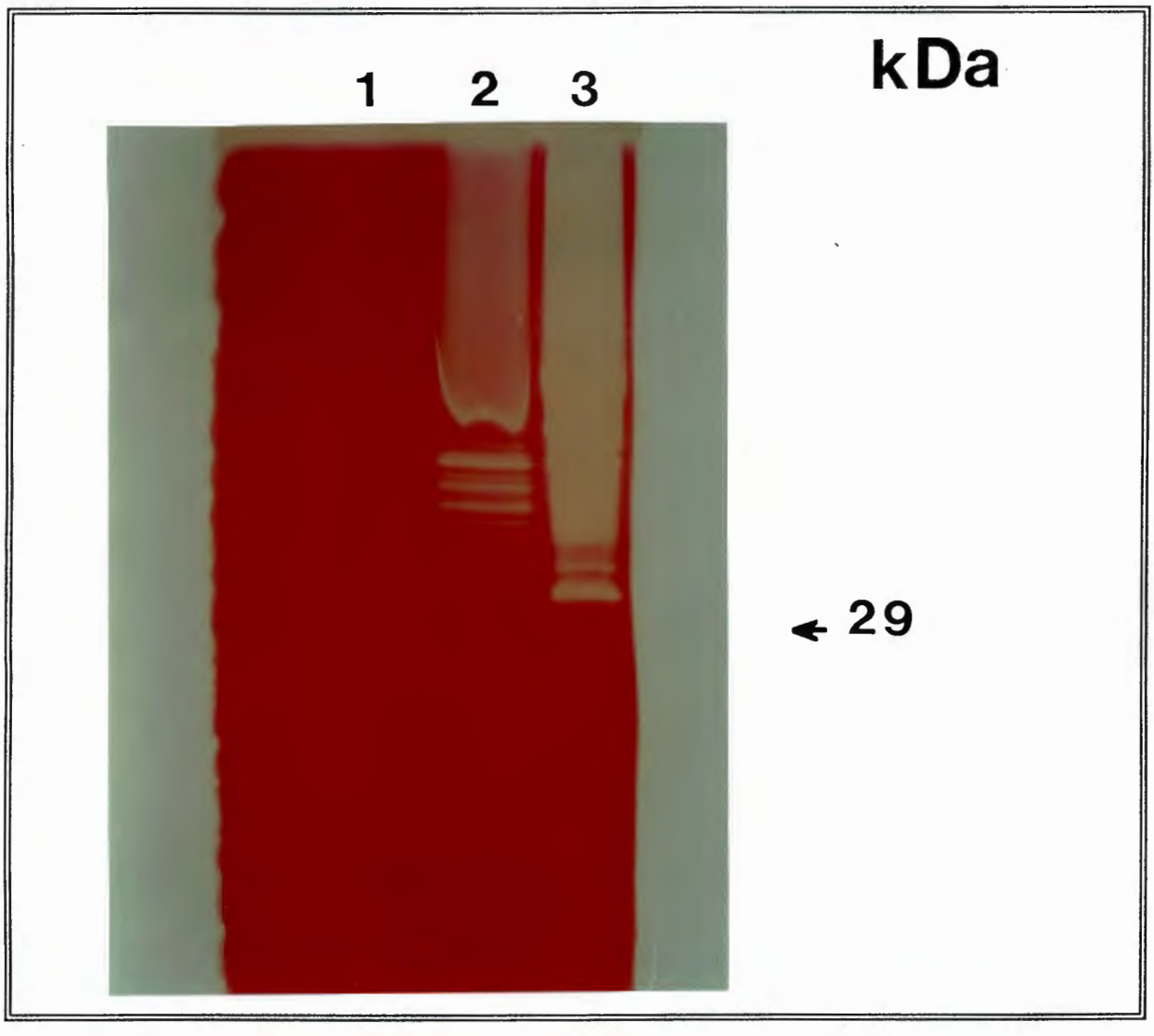

Fig 3.6 SDS-PAGE activity gel stained with Congo red. Lane 1, sonicated extract of *E. coli* (p*Eco*R251); Lane 2, Supernatant from *B. brevis* Alk 36 grown for 24h in Luria broth plus 0.1% lichenan; Lane 3, sonicated extract of *E. coli* (pNA3).

3.3.7 Nucleotide sequence of the *B. brevis bgl* (Bb) gene. The nucleotide sequence of the insert in plasmid pNA3 was determined (Fig.3.7) and found to contain a single open reading frame (ORF) of 759 nt, which began with an ATG at position 407 and ended with a TAG at position 1165. The predicted size of the polypeptide encoded by this ORF was 252 amino acids, which has a calculated relative molecular mass (M_r) of 29 050. A classical ribosome binding site was located 6-14 nt upstream of the ATG initiation codon (5'-AAGGAGGA-3'). A putative promoter sequence, (5'-GATTGAAT-N_{16}-TATAAT-3') was located upstream from the ATG start codon from position 241 to 270 (Fig.3.7).

```
TATGGAACCAATCCTGTAGGGGCTTTATTTTAGCATTTAATCAAATGTTGAGGTAGTGAATGAGGCAAAATTGTTTATTGAGAAAAGAATCGTTTACAAA   100

AAGTTAGCATAAATCGAATTCTCCGATATTTAGCTATTTCAGTTGATTTTGGGGGATTTTACCCTCGGGAAACAATATTTTTTTCTACGCGCTGTATGAT   200

AACCTGACTTTTTGCAGTTAATAGAAAAAGTAGTGAAAAAGATTGAATTTATTTAAGATTCATTTATAATAAGATTGAAAGCGCTTTCGAAAATCCCGAA   300
                                          -35                    -10
TTTTTAAAGTTGTTACCGAAAAAGTAACAACAAATTTTTTCGCTGTTTACTAACGCGCGTTAGTAAACGGATAGTAACAAAATGATCCCTATAAGGAGGA   400
                                                                                                  SD
TGGAAGATGGTAAAAAGTAAATATTTAGTTTTCATTTCTGTTTTTTCTTTGTTGTTTGGAGTATTTGTTGTTGGGTTTAGTCATCAAGGGGTAAAAGCTG   500
      M  V  K  S  K  Y  L  V  F  I  S  V  F  S  L  L  F  G  V  F  V  V  G  F  S  H  Q  G  V  K  A  E

AAGAAGAGAGGCCAATGGGAACAGCGTTTTATGAGTCGTTTGATGCTTTTGATGACGAACGTTGGTCTAAAGCAGGCGTCTGGACAAATGGCCAGATGTT   600
  E  E  R  P  M  G  T  A  F  Y  E  S  F  D  A  F  D  D  E  R  W  S  K  A  G  V  W  T  N  G  Q  M  F

CAATGCAACATGGTATCCAGAACAGGTGACGGCTGACGGTCTAATGAGACTTACTATTGCAAAGAAGACAACAAGTGCTAGAAACTATAAAGCAGGAGAG   700
 N  A  T  W  Y  P  E  Q  V  T  A  D  G  L  M  R  L  T  I  A  K  K  T  T  S  A  R  N  Y  K  A  G  E

CTTCGTACCAATGATTTCTATCATTATGGACTCTTTGAAGTGAGTATGAAGCCTGCGAAGGTAGAAGGGACCGTGTCATCCTTTTTTACCTACACAGGGG   800
L  R  T  N  D  F  Y  H  Y  G  L  F  E  V  S  M  K  P  A  K  V  E  G  T  V  S  S  F  F  T  Y  T  G  E

AATGGGATTGGGATGGAGATCCTTGGGATGAAATTGATATTGAGTTCTTAGGAAAGGACACGACGAGAATACAATTTAATTACTTTACAAATGGAGTAGG   900
  W  D  W  D  G  D  P  W  D  E  I  D  I  E  F  L  G  K  D  T  T  R  I  Q  F  N  Y  F  T  N  G  V  G

AGGAAATGAATTTTACTATGATTTAGGGTTTGATGCATCAGAGTCATTTAATACGTATGCCTTTGAATGGAGAGAGGATTCCATTACCTGGTATGTTAAT  1000
 G  N  E  F  Y  Y  D  L  G  F  D  A  S  E  S  F  N  T  Y  A  F  E  W  R  E  D  S  I  T  W  Y  V  N

GGAGAAGCGGTTCATACAGCGACAGAAAACATTCCACAGACCCCGCAGAAGATCATGATGAATTTATGGCCGGGCGTGGGAGTAGATGGATGGACAGGTG  1100
G  E  A  V  H  T  A  T  E  N  I  P  Q  T  P  Q  K  I  M  M  N  L  W  P  G  V  G  V  D  G  W  T  G  V

TATTCGATGGCGATAATACGCCTGTATATTCATACTATGATTGGGTGAGGTATACACCACTTTAGAATTATCAGATCCACCAGTAACACCAGAACCACCA  1200
  F  D  G  D  N  T  P  V  Y  S  Y  Y  D  W  V  R  Y  T  P  L

ATTGAAGAGCCGACAGAAGAACCAATTGAAGAACCAATCGAAAAACCAATTGAAGAGCCTATCGAGAAAATCGAAATAGATGAGAACGACGAAACGAAAG  1300

AAAACGTAAACGACTTTAAAGGAATCTGGAGGTGAAAGATTTACCTTAATACAGGCGACCTTCAATGTATTCATTTATTTTACTATTTTGGACTTCTAGC  1400

TTTTGCAAATAGGAGTAATGTTACTTAAGGGCTAGAGTAACAGAAAAGTAGGCTTTCAAGTATTAAGAAATTACTGATTATCGTAAAATACATTTTTTGT  1500
```

Fig 3.7 Nucleotide sequence of the insert in pNA3, containing the *B. brevis bgl*Bb gene. The nucleotide sequence is numbered throughout. The deduced amino acid sequence of the ß-glucanase is shown in single-letter code below the coding sequence. Putative promoter sequences (-35 and -10 regions) and ribosome binding site (SD) are underlined

3.3.8 Sequence comparisons with other *Bacillus* endo-(1,3-1,4)-ß-glucanase genes. The TFASTA subroutine was used to screen nucleotide sequence databases for other bacterial endo-(1,3-1,4)-ß-glucanases specifically those cloned from *Bacillus* spp. The similarity of the deduced amino acid sequence of *B. brevis* ß-glucanase to the other reported ß-glucanases was determined on the bases of identical amino acid sequence comparisons (Table. 3.3).

Table 3.3 Similarity of nucleotides in different endo-(1,3-1,4)-ß-glucanase genes (%)

	Bm	Bs	Bs2	Bl	Ba	Bp	Ct
Bb	53	51	50	51	49	54	46
Bm	-	69	69	68	70	88	49
Bs	-	-	99	87	91	72	48
Bs2	-	-	-	86	90	72	47
Bl	-	-	-	-	84	73	54
Ba	-	-	-	-	-	71	46
Bp	-	-	-	-	-	-	46

Sequence data of the proteins were taken from Tezuka et al (1989) for *B. subtilis* (Bs) and Murphy et al. (1984) for a second *B. subtilis* strain (Bs2), Lloberas et al. (1991) for *B. licheniformis* (Bl), Hofemeister et al. (1986) for *B. amyloliquefaciens* (Ba), Borriss et al. (1990) for *B. macerans* (Bm), Gosalbes et al. (1991) for *B. polymyxa* (Bp), Zverlov and Velikodvorskaya (1990) for *C. thermocellum* (Ct). and *B. brevis* (Bb) (this chapter).

The greatest similarity was found to the polypeptide sequence from *B. macerans* (53%) and to that of *B. polymyxa* (54%), but all sequences showed significant sequence similarities. It is interesting to note that these two sequences from *B. macerans* and *B. polymyxa* are very closely related (88% identity), whereas the mesophilic ß-glucanases form a separate cluster based on homology as previously reported (Hofemeister et al. 1986). The *B. brevis* ß-glucanase gene and the *Clostridium thermocellum* laminarinase show a greater divergence in sequence similarity. Alignment of the predicted polypeptides from the different *Bacillus* ß-glucanases and the *C. thermocellum* laminarinase (Fig.3.8) revealed considerable homology in a number of highly conserved blocks, particularly in the central and the C-terminal parts of the proteins. The N-terminal region has the most pronounced variation between all the different sequences.

```
                                                                      60
Bl    MSYRVKRMLMLLVTGLFLSLSTFAASASAQT-------GGSFYEPF-NNYNTGLWQKADG
Bs    MPY-LKRVLLLLVTGLFMSLFAVTSTASAQT-------GGSFFDPF-NGYNSGFWQKADG
Ba    M----KRVLLILVTGLFMSLCGITSSVSAQT-------GGSFFEPF-NSYNSGLWQKADG
Bs2   MPY-LKRVLLLLVTGLFMSLFAVTATASAQT-------GGSFFDPF-NGYNSGFWQKADG
Bm    M--KKKSC-FTLVTTFAFSLI---FSVSALA-------GSVFWEPL-SYFNRSTWEKADG
Bp    M--MKKKSWFTLMITGVISLF---FSVSAFA-------GNVFWEPL-SYFNSSTWQKADG
Bb    MV-KSKYLVFISVFSLLFGVFVVGFSHQGVKAEEERPMGTAFYESF-DAFDDERWSKAGV
Ct    M---KNRVISLLMASLLLVLSVIVAPF--YKAEAATVVNTPFVAVFRSNFDSVQWKK---
      *    .      .      .                          *   .   ..   * *
                                                                     120
Bl    YSNGNMFNCTWRANNVSMTSLGEMRLSLTSP--SYNKFDCGENRSVQTYGYGLYEVNMKP
Bs    YSNGNMFNCTWRANNVSMTSLGEMRLALTSP--SYNKFDCGENRSVQTYGYGLYEVRMKP
Ba    YSNGDMFNCTWRANNVSMTSLGEMRLALTSP--SYNKFDCGENRSVQTYGYGLYEVRMKP
Bs2   YSNGNMFNCTWRANNVSMTSLGEMRLALTSP--AYNKFDCGENRSVQTYGYGLYEVRMKP
Bm    YSNGGVFNCTWRANNVNFTNDGKLKLGLTSS--AYNKFDCAEYRSTNIYGYGLYEVSMKP
Bp    YSNGQMFNCTWRANNVNFTNDGKLKMSLTSP--ANNKFDCGEYRSTNNYGYGLYEVSMKP
Bb    WTNGQMFNATWYPEQVTADGLMRLTIAKKTT--SARNYKAGELRTNDFYHYGLFEVSMKP
Ct    --RWAKFVSTVLEAFTGDISNGKMILTLDREYGGSYPYKSGEYRTKSFFGYGYYEVRMKA
            *  *                . .       .   .  .* *.   . ** .** **.
                                                                     180
Bl    AKNVGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGVGNHEKIVNLGFD
Bs    AKNTGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKIVDLGFD
Ba    AKNTGIVSSFFTYTGPTE--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKFADLGFD
Bs2   AKNTGIVSSFFTYTGPTD--GTPWDEIDIEFLGKDTTKVQFNYYTNGAGNHEKIVDLGFD
Bm    AKNTGIVSSFFTYTGPAH--GTQWDEIDIEFLGKDQTKVQFNYYTNGVGGHEKVISVGFD
Bp    AKNTGIVSSFFTYTGPSH--GTQWDEIDIEFLGKDTTKVQFNYYTNGVGGHEKIINLGFD
Bb    AKVEGTVSSFFTYTGEWDWDGDPWDEIDIEFLGKDTTRIQFNYFTNGVGGNEFYYDLGFD
Ct    AKNVGIVSSFFTYTGPS--DNNPWDEIDIEFLGKDTTKVQFNWYKNGVGGNEYLHNLGFD
      **   * *********        ************ *..*** . ** * .*    .***
                                                                     240
Bl    AANSYHTYAFDWQPNSIKWYVDGQLKHTATTQIPQTPGKIMMNLWNGAGVDEWLGSYNGV
Bs    AANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIPTTPGKIMMNLWNGTGVDEWLGSYNGV
Ba    AANAYHTYAFDWQPNSIKWYVDGQLKHTATTQIPAAPGKIMMNLWNGTGVDDWLGSYNGV
Bs2   AANAYHTYAFDWQPNSIKWYVDGQLKHTATNQIPTTLGKIMMNLWNGTGVDEWLGSYNGV
Bm    ASKGFHTYAFDWQPGYIKWYVDGVLKHTATANIPSTPGKIMMNLWNGTGVDDWLGSYNGA
Bp    ASTSFHTYAFDWQPGYIKWYVDGVLKHTATTNIPSTPGKIMMNLWNGTGVDSWLGSYNGA
Bb    ASESFNTYAFEWREDSITWYVNGEAVHTATENIPQTPQKIMMNLWPGVGVDGWTGVFDGD
Ct    ASQDFHTYGFEWRPDYIDFYVDGKKVYRGTRNIPVTPGKIMMNLWPGIGVDEWLGRYDG-
      *.   ..**.*.*.   *  **.*     .* .** .  ******* * *** * * ..*
                  256
Bl    T-PLSRSLHWVRYTKR
Bs    N-PLYAHYDWVRYTKK
Ba    N-PIYAHYDWMRYRKK
Bs2   N-PLYAHYDWVRYTKK
Bm    N-PLYAEYDWVKYTSN
Bp    N-PLYAEYDWVKYTSN
Bb    NTPVYSYYDWVRYTPL
Ct    RTPLQAEYGICKILS-
        *.       .
```

Fig 3.8 Aligment of the amino acid sequence of the *B. brevis* (Bb) beta-glucanase with enzymes from different *Bacillus* strains. Identical and conserved amino acids among the eight proteins are marked with asterisks and dots respectively.

3.4 Discussion

The endo-(1,3-1,4)-ß-glucanase isolated from *B. brevis* Alk 36 has some unique properties. It has a higher temperature optimum then previously reported for *Bacillus* lichenases, with the exception of *B. macerans*. The temperature profiles of the *B. macerans* and *B. brevis* lichenases are similar, however, the *B. brevis* lichenase exhibits a greater residual activity at a higher temperature than that of the *B. macerans* enzyme *viz* 75% activity after one hour at 75°C for *B. brevis* lichenase as opposed to approximately 10% activity after one hour at 70°C for *B. macerans* enzyme.

The pH optimum of the enzyme is unusual as the pH optima for bacterial lichenases tend to fall between pH 6.0 and pH 8.0 while that of the *B. brevis* lichenase exhibits activities of greater then 80% from pH 7.0 to pH 10.0. This pH profile is, however, similar to that of the ß-1,3-glucanase isolated from an alkalophilic *Bacillus* sp. by Nogi and Horikoshi (1990) and to the ß-1,3-glucanase or laminarinase isolated from *Clostridium thermocellum* by Zverlov and Velikodvorskaya (1990). These alkalophilic laminarinase enzymes both have similar thermo-stability profiles to that of the *B. brevis* lichenase. The distinction between ß-1,3-glucanases and (1,3-1,4)-ß-glucanases can be rather tenuous as it is based on substrate specificity for laminarin as opposed to lichenan, respectively. Since the substrate specificity of the *B. brevis* enzyme was found to be 300 fold more for lichenan than laminarin it was classified as a (1,3-1,4)-ß-glucanase. The laminarinase from *C. thermocellum* was, however, classified as such despite a hundred fold higher activity on lichenan (Zverlov and Velikodvorskaya 1990).

Another novel characteristic of the *B. brevis* (1,3-1,4)-ß-glucanase enzyme is its resistance to SDS; this is the first report of an SDS-resistant ß-glucanase. A molecular weight of approximately 29 kDa was derived by SDS-PAGE. This agrees with the molecular weight calculated from the sequence of 29,050 Da and is similar to the previously published endo-(1,3-1,4)-ß-glucanases which are estimated to be between 25 and 30 kDa. An exception is the endo-(1,3-1,4)-ß-glucanase cloned from *B. circulans* which was reported to have a molecular weight of 40.5 kDa (Bueno et al. 1990a).

The N-terminal portion of the ß-glucanase polypeptide deduced from the nucleotide

Chapter 4

Cloning and sequencing the *degS-degU* operon from *Bacillus brevis* Alk 36.

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Chapter 4

Cloning and sequencing the *degS-degU* operon from *Bacillus brevis* Alk 36.

4.0 Summary

The *sacU* region from *Bacillus brevis* Alk 36 was cloned and sequenced. The *degS-degU* genes were selected for their ability to stimulate the production of extracellular proteases. The two open reading frames of the *degS-degU* operon encoded polypeptides which gave calculated molecular masses of 43.8 kDa and 27.0 kDa respectively. Sequence comparisons at the amino acid level to the *B. subtilis degS-degU* genes showed 74% and 84% similarity respectively. Within the DegS protein it was hypothesized that Asp-152 and Asp-168 amino acids formed a domain potentially involved in ATP binding through Mg^{2+} salt bridges. The DegU protein was found to contain three aspartate residues which were clustered to form an acidic binding pocket, as determined from the three dimensional structure of other effectors. These were found at positions: Asp-10; Asp-11 and Asp-56 and were conserved in both *B. subtilis* and *B. brevis* DegU proteins. On a multicopy vector the *B. brevis degS-degU* genes were found to be relatively stable and to cause hypersecretion of several extracellular enzymes in a *B. subtilis* rec^- strain as well as in a *B. subtilis sacU*(HY) strain. These enzymes included proteases, α-amylase, endo-(1,3-1,4)-ß-glucanase and levansucrase.

4.1 Introduction

The *degS* and *degU* genes, which together make up the *Bacillus subtilis sacU* locus, and belong to the family of two-component systems, were found to positively regulate the synthesis of several extracellular enzymes. A number of reports describing various signal transduction proteins isolated from *B. subtilis*, as well as their interaction in controlling degradative enzyme synthesis, sporulation and competence have been reviewed in the General Introduction.

To date the only studies published on the *degS-degU* genes at a molecular level have been on those cloned from *B. subtilis*. As direct screening for the *sacU* phenotype proved difficult, the *degS-degU* genes from *B. subtilis* were cloned by two groups utilizing different strategies. Kunst et al. (1988) utilized chromosome "walking", in addition to screening for the restoration of levansucrase synthesis in a *sacU* mutant strain. Henner et al. (1988) utilized chromosome "walking" and screened for transformants containing DNA able to convert the *sacU*(HY) phenotype to *sacU*. The *sacU*(HY) phenotype was determined by clearing zones obtained from colonies on milk plates. The *degU* gene was inadvertently cloned by Tanaka and Kawata (1988) as it was found to significantly decrease extracellular protease production when on a multicopy plasmid. It was initially designated *iep* until sequence comparisons confirmed that it was in fact a *degU* gene. In order to obtain the intact operon the downstream region containing the *degS* gene was cloned. When this construct was transformed into a *B. subtilis* strain the transformants showed an increase in exoprotease production of between two to five fold. The transformants were, however, found to be unstable and in addition, cells carrying the *degU* gene on a multicopy vector tended to lyse after two days incubation at 37°C (Tanaka, personal communication).

On the basis of sequence similarities, several histidine kinase-response regulator pairs have been proposed to exist in other gram-positive bacteria. Examples are the regulation of exoprotein synthesis in *Staphylococcus aureus*; the regulation of copper metabolism and melanin pigmentation in *Streptomyces lividans*; the regulation of cell wall precursor synthesis conferring vancomycin resistance on *Enterococcus faecium* as well as a putative response regulator of unknown function in *B. megaterium*. These were all mentioned in a review by Msadek et al (1993). In addition, Turgay and Marahiel (1993) identified an

ORF at the 3' end of the cloned gramicidin S operon which showed homology to a conserved motif in the histidine protein kinase, the sensor protein of the two component systems. They cloned and sequenced the *grs* associated two-component genes (*gtcO* and *gtcE*) and found similarity to the *envZ-ompR* genes involved in osmoregulation in *E. coli*.

In this Chapter the cloning, selection, nucleotide sequence and characterization of the *degS-degU* two component system from *B. brevis* Alk 36 is described, and its effect, when on a multicopy plasmid, on extracellular enzyme production is evaluated.

4.2 Materials and Methods

4.2.1 Bacterial strains. All the *B. subtilis* strains and plasmids were obtained from the *Bacillus* Genetic Stock Centre and the BGSC accession numbers are quoted. The genotypes of *B. subtilis* strains 1A46, 1A311, 1A201 are detailed in Appendix C. The plasmid pPL703 is a promoter probe vector containing the promoter-less *cat* gene (Mongkolsuk et al. 1983). The plasmid pPL708 was described in Chapter 2. The *E. coli* strains and vectors utilized in sequencing were described in Chapter 3.

4.2.2 Media, buffers and enzymes. All media and buffers not described in the text are given in Appendix B. Azocasein was obtained from Sigma Chemical Co. Polyethylene glycol (approximate molecular weight 4000) and soluble starch was purchased from Merck (Darmstat, Germany). Soluble starch was also obtained from Saarchem (South Africa). [^{35}S] methionine was purchased from Amersham laboratories (England). Restriction endonucleases and T4 DNA ligase were from Boehringer Mannheim (FRG).

4.2.3 Growth conditions. *B. subtilis* strains and *B. brevis* were grown in Luria broth as described in section 3.2.3. Chloramphenicol and neomycin were added to finale concentrations of 10 μg/ml when required.

Extracellular enzyme production was determined using the following media: Luria broth plus 1% soluble starch (Merck) for α-amylase determination; Luria broth plus 0.1% lichenan from *Cetraria islandica* (Sigma) for ß-glucanase determination. For levansucrase production two types of media were used, Luria broth plus 1% sucrose (Amory et al. 1987) and MMCH medium containing 2% sucrose (Kunst et al. 1988). Auxotrophic requirements were added at a concentration of 100 mg per litre. The medium for protease production was described in section 2.2.9.

All enzyme activities were measured after 24h growth of the strains in the respective media except for levansucrase production where the culture supernatants of exponentially growing cells were used for analysis.

4.2.4 DNA manipulations. The isolation of chromosomal DNA and plasmid DNA from *Bacillus* spp. have been described in section 2.2.6 and 2.2.7 respectively. Partial *Sau*3A restriction of the *B. brevis* DNA was carried out as described in section 3.2.5.1. Size fractionated fragments in the range of 3 to 6 kb were purified from agarose gel slices using the Geneclean™ kit (Bio 101, Calif., USA).

The vector pPL703 was restricted with *Bam*H1 as described in section 3.2.5.2. The ligation reaction contained DNA (0.5 μg vector and 0.5 μg size fractionated fragments), 10 μl ligation buffer (10x), T4 DNA ligase (1 unit) and water to 100 μl. The reaction was incubated at 16°C for 16 hours.

4.2.5 Transformation of *B. subtilis* and screening of transformants. The polyethylene glycol-induced protoplast transformation procedure was followed essentially as described in section 2.2.8 but with the following modifications: stationary phase recipient cells were diluted 1:50 in Luria broth and grown with shaking to an OD of 0.35 at 540nm, and Lysozyme treatment was continued for 45 minutes. Protoplasts were resuspended in SMMP broth and stored frozen at -70°C until needed.

DM3 selection plates were made up as described by Corfield et al. (1984) and further modified so as to include both 1% soluble starch (Saarchem) and 1% casein (Saarchem) in the plates (Appendix B). Saarchem soluble starch was used in preference to Merck as it was found to form a more opaque background when incorporated into the DM3 plates together with casein. The volumes were adjusted accordingly to accommodate both the casein and starch. Chloramphenicol was added to a finale concentration of 20 μg/ml.

4.2.6 Restriction endonuclease mapping and Southern blot analysis. This was carried out as described in section 3.2.7.

4.2.7 Sequencing strategy. The 2.8 kb *B. brevis* DNA cloned into pPL703 could not be subcloned into pBluescript.SK or KS as this proved lethal to the recipient *E. coli* strain. A similar finding has been reported previously by Kunst et al. (1988). Sequencing templates were therefore generated by subcloning an internal *Hin*dIII fragment of 0.4 kb and a series of *Sau*3A fragments from the *B. brevis* DNA insert into

pBluescript-SK (Fig.4.1). Primers were then manufactured to complete the sequence in both directions.

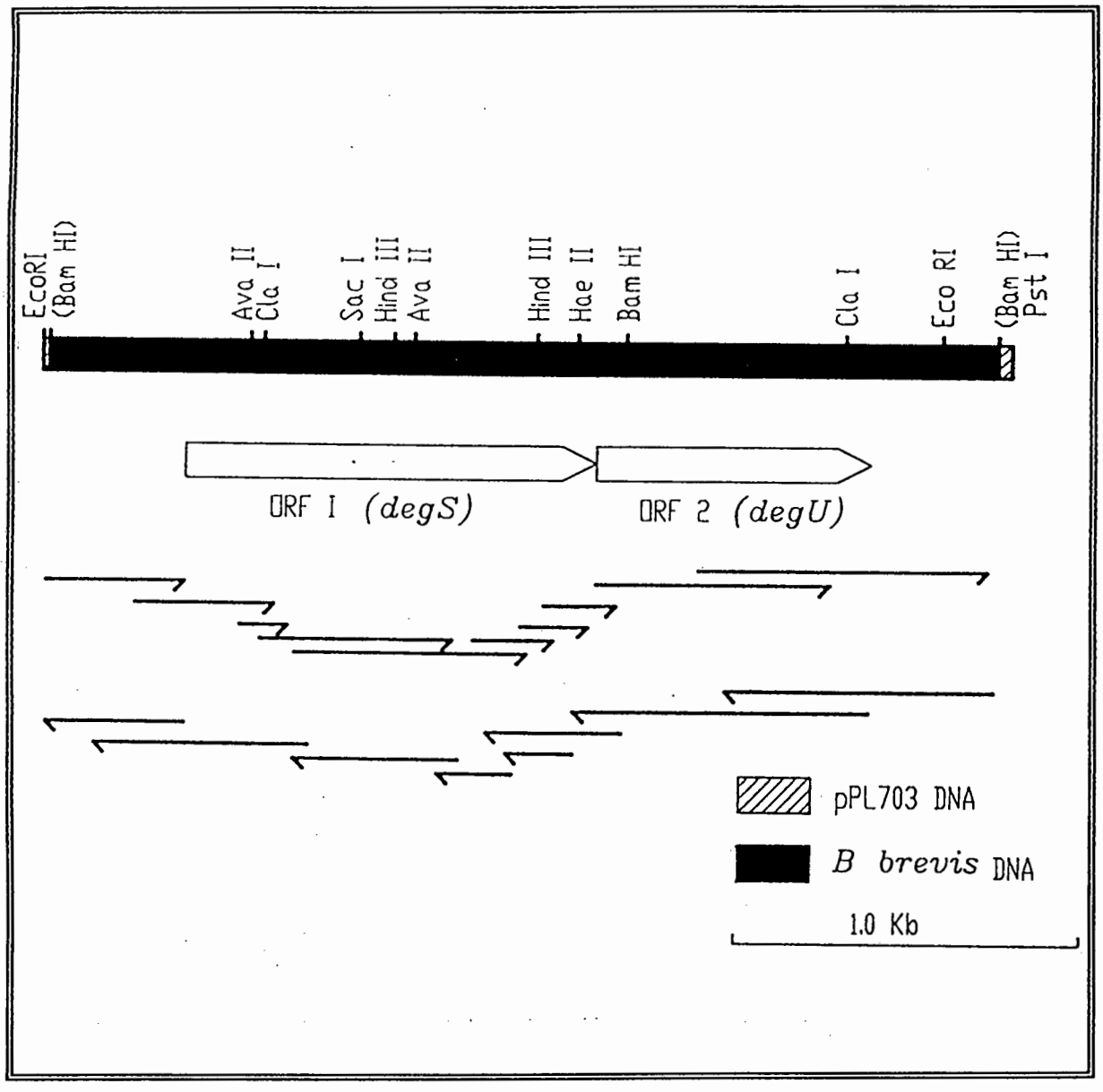

Fig 4.1 Restriction endonuclease map of insert DNA on pML2 encoding *B. brevis* Alk 36 *degS-degU* genes. The ORF's and direction of transcription are indicated by open arrows. Thin arrows show the direction and extent of the sequencing reactions.

4.2.8 Nucleotide sequencing. The nucleotide sequences of both strands of DNA coding for the *degS-degU* genes were determined using the overlapping DNA fragments generated as described in section 4.2.7. DNA sequencing was carried out by the dideoxynucleotide-chain-termination method of Sanger et al. (1977). This method is described in Appendix A. The nucleotide and deduced amino acid sequences were

analysed as described in section 3.2.15.

4.2.9 Qualitative tests for extracellular enzyme production. The hydrolysis of different substrates was evaluated on agar plates containing the following media: Luria agar containing either 1% soluble starch (Saarchem); 0.1% lichenan (Sigma) or 1% skim milk powder (Oxoid). In order to adjust the pH of the skim milk plates to pH 8.0, 14 ml/l of sterile 10% ($^{w}/_{v}$) Na_2CO_3 was added after autoclaving. ST plates (Kunst et al. 1988) contained sucrose 20g, Bactotryptone 1 g, agar 17 g, 13 mM KCl; 1 mM $MgSO_4$ and auxotrophic requirements (100 mg/l). Neomycin and chloramphenicol were added at concentrations of 10 μg/ml. Agar plates containing the different media were inoculated with colonies of *B. subtilis* 1A46 (pPL708) and a transformant *B. subtilis* (pML2) containing the *degS-degU* operon and incubated at 37°C for 24 hours.

4.2.10 Enzyme Assays. Protease activity was determined as described in section 2.2.10. Alkaline protease activities were assayed at pH 9.5 in the presence of 20 mM EDTA and total protease production was measured at pH 7.0. ß-Glucanase activity was determined as described in section 3.2.9. α-Amylase was assayed according to the method of Bernfeld (1949). One unit was defined as the number of mg of maltose liberated in 3 min at 37°C by 1 ml of enzyme solution. Levansucrase was determined according to the method of Kunst et al. (1988). One unit corresponds to 1 μmol of glucose produced per min.

For calculating specific activities the total amount of protein was estimated from the bacterial culture pellets using the biuret method (Herbert et al. 1971).

4.2.11 SDS-PAGE protease activity gel. Extracellular proteases produced by *B. subtilis* 1A46 and three transformants carrying the *degS-degU* genes were characterized by 12% PAGE gels containing SDS and gelatin (0.1%) as a copolymerized substrate (Heussen and Dowdle 1980). After 24 h growth in protease production medium (section 2.2.9) cultures were sedimented by centrifugation and supernatant samples (100 μl) were added to an equal volume of SDS-PAGE splitting buffer (Appendix B). The samples were incubated at 37°C for 30 min before loading approximately 50 μl of sample per well. Electrophoresis was carried out at a constant voltage of 40 mA for approximately

six hours. After electrophoresis gels were incubated in Triton X-100 (2.5% $^{w}/_{v}$) for 16 h at 4°C to remove SDS and restore enzyme activity. After incubation in 0.1 M glycine buffer pH 8.5 at 37°C for 3.5 h, bands of proteolytic activity were detected by staining with amido black (1%) and destaining with methanol:acetic acid:water in a ratio of 30:10:60.

4.2.12 *In vitro* transcription and translation. A prokaryotic DNA-directed cell -free transcription-translation system (Amersham Corp., Arlington Heights, Ill.) was used to investigate the synthesis of proteins by the recombinant plasmid containing the *degS-degU* genes. The protocol as specified by the manufacturers was followed throughout. The resulting polypeptides were labelled with L-[^{35}S] methionine (specific activity >37 TBq/mM) and resolved by 15% SDS-PAGE by the method of Laemmli (1970). (Appendix A). Translated polypeptides were visualized by autoradiography. Bio-rad molecular-mass standards were used as molecular markers.

4.2.13 Nucleotide sequence accession number. The nucleotide sequence reported has been assigned the GenBank accession number L15444.

4.3 Results

4.3.1 Cloning of the *degS-degU* genes from *B. brevis* Alk 36. A genome library of *B. brevis* DNA was constructed by ligatin size fractionated *B. brevis* DNA into pPL703 and transforming the ligation mix into *B. subtilis* 1A46. The transformants were plated onto the modified DM3 plates. The inclusion of both starch and casein in the DM3 medium was found to facilitate the screening for hyperproducing protease transformants. From approximately 500 colonies, three were identified which gave rise to haloes on the DM3 plates, indicating increased protease production (Fig.4.2). These three transformants, designated pML1, pML2 and pML3, as well as *B. subtilis* 1A46, were inoculated into protease production medium and grown for 24 h. Protease production was determined at pH 9.5 (Table 4.1). When compared to the protease produced by the recipient strain it was found that the production of both neutral and alkaline proteases by the recombinants was substantially increased. A *Pst*1 and *Eco*R1 double digestion of pML1, pML2 and pML3 indicated that all three transformants contained the same 2.6 kb insert. Thus, the recombinant plasmid with the smallest insert, pML2, was chosen for further study.

Table 4.1 Extracellular protease activities of the three transformants and *B. subtilis* 1A46

Strain	Protease activity (μAU/ml)	
	With EDTA	Without EDTA
pML1	625	1025
pML2	725	1475
pML3	400	675
B. subtilis 1A46	35	47

4.3.2 Southern hybridization. A preliminary restriction endonuclease map of pML2 was constructed (Fig.4.1). The origin of the 2.6 kb insert on pPL703 was determined by Southern blotting and DNA hybridization. An internal 1.6 kb *Cla*1-*Cla*1 fragment was used as a probe. *B. brevis* Alk 36 DNA was digested with *Cla*1 to completion. The 1.6 kb *Cla*1-*Cla*1 probe was found to hybridize to a *B. brevis* chromosomal fragment of the same size confirming the origin of the cloned fragment. (Fig 4.3)

4.3 Results

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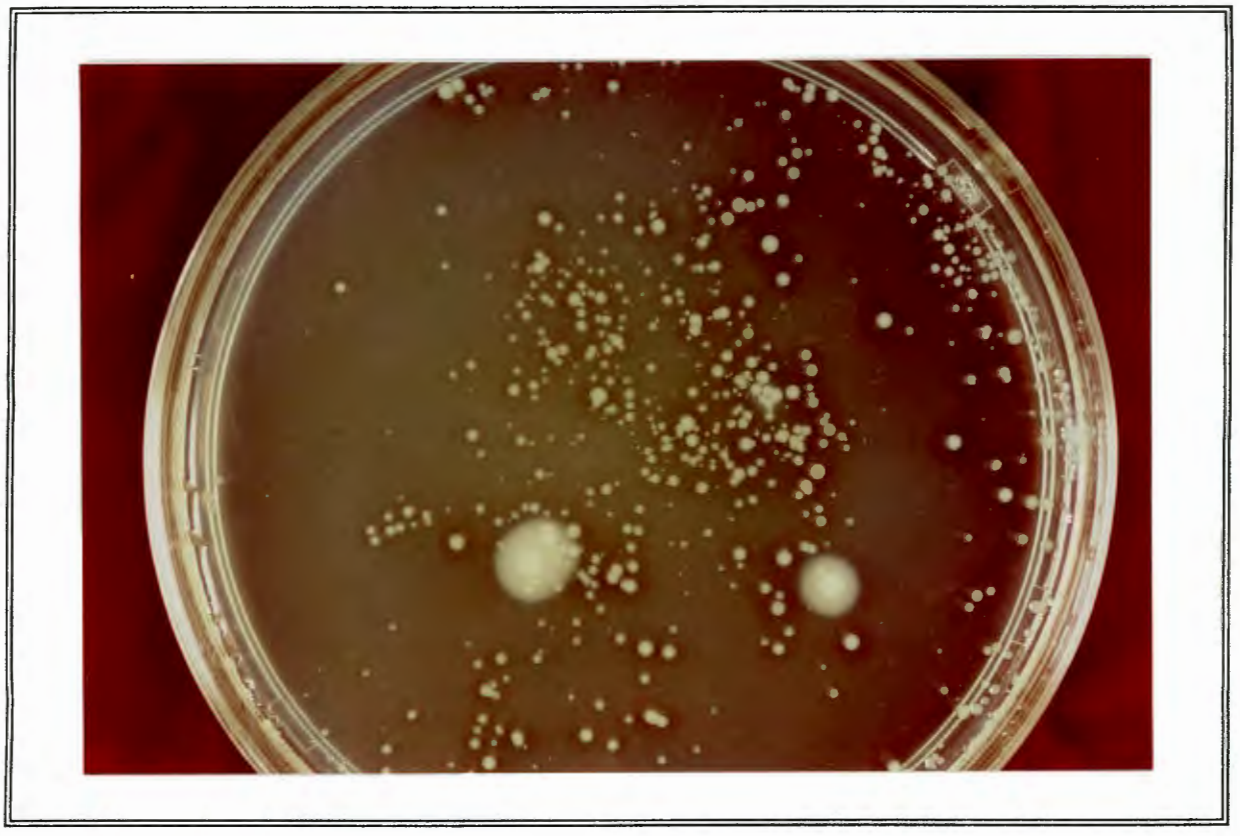

Fig 4.2 Modified DM3 plate showing two colonies producing haloes indicating increased protease production

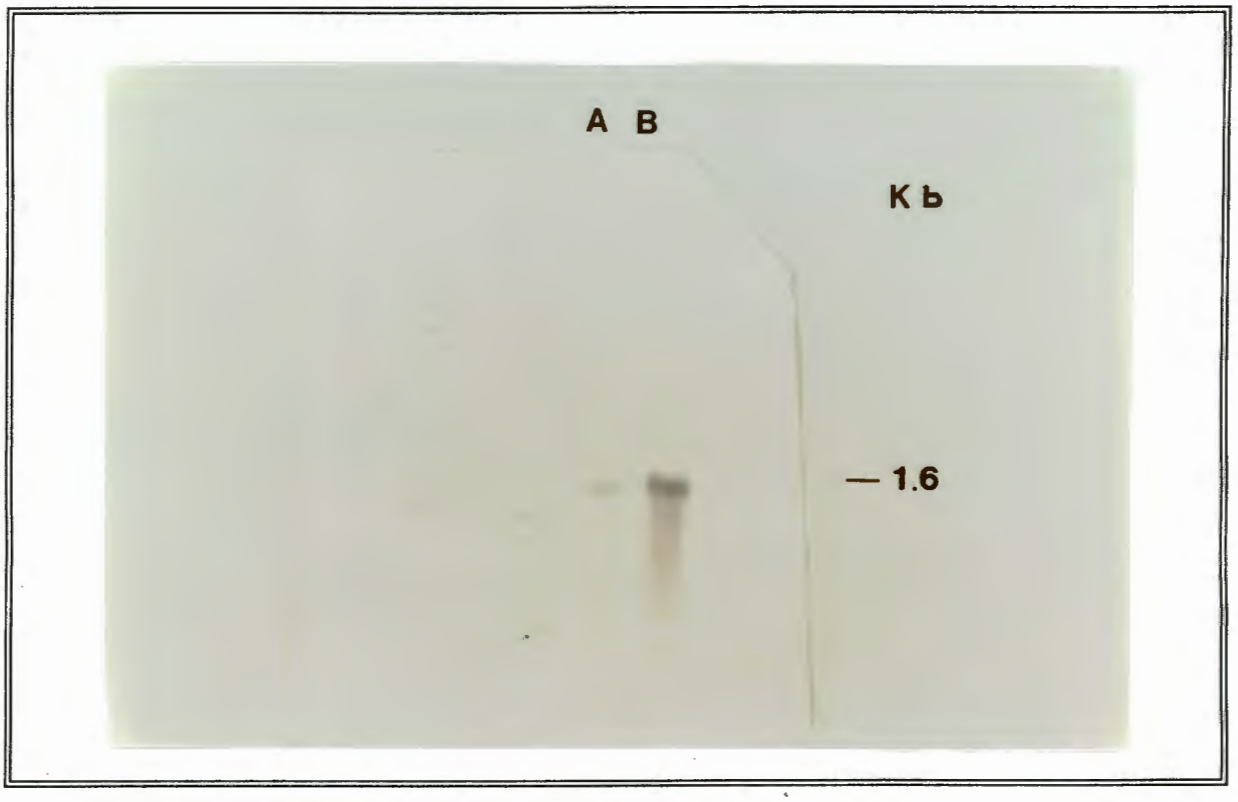

Fig 4.3 Southern blot hybridization using non-radioactively labelled *Cla*I-*Cla*I fragment. *B. brevis* Alk 36 DNA digested with *Cla*I (lane A), internal *Cla*I-*Cla*I fragment (lane B)

4.3.3 Nucleotide sequence determination. The 2.6 kb fragment of pML2 was sequenced in both directions (Fig.4.4). Two complete ORFs were identified in the sequence; the *degS* gene (1133 nt) which has a presumptive GTG start codon at position 126 and ends with an TAG at position 1259, and the *degU* gene (710 nt), which has a presumptive TTG start codon at position 1294 and ends with a TAA at 2004. These codons initiate translation in a number of *Bacillus* genes: the *Bacillus licheniformis* ß-lactamase (Neugebauer et al. 1981), *Bacillus pumilus cat*-86 (Harwood et al. 1983), and *B. subtilis* alkaline phosphatase genes (Bookstein et al. 1990). The *degU* gene is preceded by a strong ribosome binding site, (5'GGAGGG-3') 8 bases from the TTG start codon. The *degS* gene has no obvious ribosome binding site, but the GTG is followed by GCT, which has been shown to increase initiation at GTG initiation codons (Ringquist et al. 1992). No termination or promoter sequences are located in the 34 bases separating the two ORFs, and no transcription terminator sequences could be identified downstream of the *degU* gene. The predicted sizes of the polypeptides encoded by the *degS* and the *degU* genes are 378 and 237 amino acids giving calculated molecular masses of 43.8 kDa and 27.0 kDa, respectively.

Sequence comparisons at the nucleotide and amino acid levels indicated that these ORFs show significant similarity to the *degS* and *degU* genes of *B. subtilis* (Henner et al 1988a; Kunst et al. 1988 and Tanaka et al. 1988). The similarity of the deduced amino acid sequences of the *B. brevis* genes to those of *B. subtilis* was determined on the basis of identical amino acid sequence comparisons. The alignments revealed that 54% of the amino acids were identical (73% similarity) in the *degS* genes (Fig.4.5) while the *degU* genes have 71% identical amino acids (84% similarity) (Fig.4.6).

```
   1  ATGTCGAAGGATTCATTTATAAAAATGAGAAAAAAAGAAGGGAATTTTTTTCTTGCTCGCGAAAGATAACATGGGAAAAC   80
  81  GAATGCCTTTTTCATCTCCATGCCTTTTAAAGGTTGGTGGTTCAAGTGGCTGATCCTCAAACATTAGATAAGATTATTGA  160
                                                  M  A  D  P  Q  T  L  D  K  I  I  D
 161  TAAAACGCTAGATACTGTAGGGAAAAGTAGAGAACAGATCTTTGAAATTAGTGAGCAATCTAGAAATGAGTACGTCTCTC  240
       K  T  L  D  T  V  G  K  S  R  E  Q  I  F  E  I  S  E  Q  S  R  N  E  Y  V  S  L
 241  TAGAACAAGAACTGCAAGAAGTCCGCATGAAAGTTGCAGAGATTATAGACCAATCGGACCGGGCGGAAGTACACGCTCGA  320
       E  Q  E  L  Q  E  V  R  M  K  V  A  E  I  I  D  Q  S  D  R  A  E  V  H  A  R
 321  TTTGCACGCAATCGATTGGCTGAAGTGAGTAAGCAATTTCATAGATATTCAAATGAAGAGATACGTAAAGTGTACGAGCA  400
       F  A  R  N  R  L  A  E  V  S  K  Q  F  H  R  Y  S  N  E  E  I  R  K  V  Y  E  Q
 401  AGCCAATGAGCTCCAAGTGAAGCTAGCATTGCTCCAGCAAGAAGAACAGCAGCTTCGGGATCGAAGAGATGCAATTGAAC  480
       A  N  E  L  Q  V  K  L  A  L  L  Q  Q  E  E  Q  Q  L  R  D  R  R  D  A  I  E  R
 481  GCCGATTGTTGAATCTAAAAGATACCATCGAGCGTGCGGAAGAATTAGTTGGGCAGATGACCGTTGTCTACAACTTTCTT  560
       R  L  L  N  L  K  D  T  I  E  R  A  E  E  L  V  G  Q  M  T  V  V  Y  N  F  L
 561  ACAGGAGATCTTCGTCAAGTTGGAGAAGCACTTGAGGATGCAAGGGAAAAGCAGGCGTTTGGTCTACAAATTATTCAAGC  640
       T  G  D  L  R  Q  V  G  E  A  L  E  D  A  R  E  K  Q  A  F  G  L  Q  I  I  Q  A
 641  TCAAGAGGAAGAACGTCGAAAGCTTTCCCGGGAAATTCATGACGGACCTGCTCAAATGATGGCGAATGTTCTATTGCGCT  720
       Q  E  E  E  R  R  K  L  S  R  E  I  H  D  G  P  A  Q  M  M  A  N  V  L  L  R  S
 721  CAGAACTGGTTGAGCGCATTTATCACGACAAAGGCATTGATGAAGCGTTAAAGGAAATTCGTGATTTGCGCAAGATGGTG  800
       E  L  V  E  R  I  Y  H  D  K  G  I  D  E  A  L  K  E  I  R  D  L  R  K  M  V
 801  AAGTCTTCTCTTGCTGAAGTCCGGCGAATTATCTATGATTTACGCCGGATGGCCCTTGATGACCTTGGACTCATTCCAAC  880
       K  S  S  L  A  E  V  R  R  I  I  Y  D  L  R  R  M  A  L  D  D  L  G  L  I  P  T
 881  ATTGAAGAAGTATGTAAAAACCTTTGAAGAACATACGGGAATATTTGTTGATTTTAAACACATAGGAAAAGGAGAGCGCT  960
       L  K  K  Y  V  K  T  F  E  E  H  T  G  I  F  V  D  F  K  H  I  G  K  G  E  R  F
 961  TCCCAGAGCATGTGGAGATCGCACTATTTCGCCTTGTTCAGGAAGCGTTACAAAACACACGAAAGCATGCAAAAGCTTCA 1040
       P  E  H  V  E  I  A  L  F  R  L  V  Q  E  A  L  Q  N  T  R  K  H  A  K  A  S
1041  CATGTCCATGTGAAAATCGAAGAGCAGAAAACGAAATTCACTGTCGTGATAAAAGATAATGGAAAAGGATTCGATCAAAC 1120
       H  V  H  V  K  I  E  E  Q  K  T  K  F  T  V  V  I  K  D  N  G  K  G  F  D  Q  T
1121  CGAGAAAAAGGAAGGGTCATTTGGTTTAGTTGGAATGAAAGAGAGGGTCAACATGTTGAAAGGCCAGCTTGTCATTCGAA 1200
       E  K  K  E  G  S  F  G  L  V  G  M  K  E  R  V  N  M  L  K  G  Q  L  V  I  R  T
1201  CGAAGCCAAACGATGGAACAACGATCATCATTTCCATTCCTATCACAACCGAAGAATAGACTGCGTAGGTGCAAAAATTG 1280
       K  P  N  D  G  T  T  I  I  I  S  I  P  I  T  T  E  E  *
1281  GAGGGTGATTTAATTGAACGAGCAAGTAAACGAAAACAAAATTCAAATTGTCATCATTGATGACCATCAACTATTCCGTG 1360
                     M  N  E  Q  V  N  E  N  K  I  Q  I  V  I  I  D  D  H  Q  L  F  R  E
1361  AAGGGGTTAAACGGATCCTAGCCATGGAGCCAGAATTTGAGGTTGTGGCTGATGGAGAAGACGGCGAAAACGCTGTTGAA 1440
       G  V  K  R  I  L  A  M  E  P  E  F  E  V  V  A  D  G  E  D  G  E  N  A  V  E
1441  CTAGTAGAAAAATATAATCCAGATGTCATCCTTATGGATATTAACATGCCAAAGGTGAATGGTGTTGAAGCGACAAGAGA 1520
       L  V  E  K  Y  N  P  D  V  I  L  M  D  I  N  M  P  K  V  N  G  V  E  A  T  R  D
1521  TTTAATTCAAAGGTACCCTGACGTAAAGGTATTGGTTCTCTCGATTCACGACGATGAGTCCTATGTCACTCATGTACTAA 1600
       L  I  Q  R  Y  P  D  V  K  V  L  V  L  S  I  H  D  D  E  S  Y  V  T  H  V  L  K
1601  AAACGGGAGCTTCCGGTTATCTATTAAAAGAAATGGACGCCGACGCTCTAATTGAAGCAGTAAAAGTCGTAGCGCAAGGC 1680
       T  G  A  S  G  Y  L  L  K  E  M  D  A  D  A  L  I  E  A  V  K  V  V  A  Q  G
1681  GGGGCGTACATTCATCCGAAGGTGACACATAATCTCATCAAGGAATACCGTCGTCTAGTAAATGAAGATGAACAAGAAAG 1760
       G  A  Y  I  H  P  K  V  T  H  N  L  I  K  E  Y  R  R  L  V  N  E  D  E  Q  E  S
1761  CTCTGAAATCGGCTTTAAAGAAGTCGAATATCGCAAGCCGTTGCACATTTTAACTCGTCGTGAATGTGAAGTGCTACAGC 1840
       S  E  I  G  F  K  E  V  E  Y  R  K  P  L  H  I  L  T  R  R  E  C  E  V  L  Q  L
1841  TCATGACTGACGGTTATAGCAACCGAATGATTGGTGAAGCTCTATACATTAGTGAGAAAACAGTCAAAAATCATGTTTCA 1920
       M  T  D  G  Y  S  N  R  M  I  G  E  A  L  Y  I  S  E  K  T  V  K  N  H  V  S
1921  AATATTTTGCAAAAGATGAATGTAAACGATCGAACACAAGCGGTAGTAGAATCGATCAAAAACGGTTGGGTAAAAGTACG 2000
       N  I  L  Q  K  M  N  V  N  D  R  T  Q  A  V  V  E  S  I  K  N  G  W  V  K  V  R
2001  TTAATCCAAAAAGCGATCGCTTCATTACGCCCTCTTGAAAGGTCTTTCAATTAAAGGTACAGTAAAAAAGACAAGATCCG 2080
       *
2081  GGGAATTCACATCAATGCGA  2100
```

Fig 4.4 Nucleotide sequence of the 2.6 kb *Eco*R1 fragment of pML2 containing the *B. brevis degS* and *degU* genes. The nucleotide sequence is numbered throughout. The deduced amino acid sequence is shown in single-letter code below the coding sequence. The ribosome binding site of the *degU* gene is underlined and the GTG and TTG start codons are indicated in bold letters. The TAG ending the *degS* gene and TAA at the end of the *degU* gene are indicated by asterisks.

```
BB        MA----DPQTLDKIIDKTLDTVGKSREQIFEISEQSRNEYVSLEQELQEVRMKVAEIIDQ
BS        MNKTKMDSKVLDSILMKMLKTVDGSKDEVFQIGEQSRQQYEQLVEELKQIKQQVYEVIEL
          *     *.. ** *. * * **. *....*.*.****..*  * .**.... .* *.*.

BB        SDRAEVHARFARNRLAEVSKQFHRYSNEEIRKVYEQANELQVKLALLQQEEQQLRDRRDA
BS        GDKLEVQTRHARNRLSEVSRNFHRFSEEEIRNAYEKAHKLQVELTMIQQREKQLRERRDD
          .*. **..* *****.***..***.*.****. **.*. *** *...** *.***.***

BB        IERRLLNLKDTIERAEELVGQMTVVYNFLTGDLRQVGEALEDAREKQAFGLQIIQAQEEE
BS        LERRLLGLQEIIERSESLVSQITVVLNYLNQDLREVGLLLADAQAKQDFGLRIIEAQEEE
          .***** *.. ***.* **.*.*** *.*  ***.**  * **. ** ***.**.*****

BB        RRKLSREI|H|DGPAQMMANVLLRSELVERIYHDKGIDEALKEIRDLRKMVKSSLAEVRR
BS        RKRVSREI|H|DGPAQMLANVMMRSELIERIFRDRGAEDGFQEIKNLRQNVRNALYEVRR
          *...****|*|******.***..****.***..*.* .....**..**. *...* ****

BB        IIYDLRRMALDDLGLIPTLKKYVKTFEEHTG-IFVDFKHIGKGE--RFPEHVEIALFRLV
BS        IIYDLRPMALDDLGLIPTLRKYLYTTEEYNGKVKIHFQCIGETEDQRLAPQFEVALFRLA
          ****** ************.**. * **  * . ..*. **  *  *.. . *.*****

BB        QEALQNTRKHAKASHVHVKIEEQKTKFTVVIKDNGKGFD---QTEKKEGSFGLVGMKERV
BS        QEAVSNALKHSESEEITVKVEITKDFVILMIKDNGKGFDLKEAKEKKNKSFGLLGMKERV
          ***. *. **. . .. **.*  *    ..*********     ***. ****.******

BB        NMLKGQLVIRTKPNDGTTIIISIPITTEE
BS        DLLEGTMTIDSKIGLGTFIMIKVPLSL
          ..* * . * .*   ** *.* .*..
```

Fig 4.5 Alignment of the deduced amino acid sequence of the *B. brevis* (BB) and *B. subtilis* (BS) *degS* genes. Identical and conserved amino acids are marked with asterisks and dots respectively. The conserved histidine-189 residue is boxed and the site of the *degS200*(HY) mutation in the *B. subtilis* gene is printed in bold letters.

```
BB      MNEQVNENKIQIVII DD HQLFREGVKRILAMEPEFEVVADGEDGENAVELVEKYNPDVIL
BS      M------TKVNIVII DD HQLFREGVKRILDFEPTFEVVAEGDDGDEAARIVEHYHPDVVI
        *       *..**** ** ************  ** *****.*.**..*  .** *.***..

BB      M D INMPKVNGVEATRDLIQRYPDVKVLVLSIHDDESYVTHVLKTGASGYLLKEMDADALI
BS      M D INMPNVNGVEATKQLVELYPESKVIILSIHDDENYVTHALKTGARGYLLKEMDADTLI
        * * ****.*******..*.. **. **..*******.**** ***** **********.**

BB      EAVKVVAQGGAYIHPKVTHNLIKEYRRLVNED-EQESSEIGFKEVEYRKPLHILTRRECEVL
BS      EAVKVVAEGGSYLHPKVTHNLVNEFRRLATSGVSAHPQHEVYPEI--RRPLHILTRRECEVL
        *******.**.*.********..*.***   .   .. .  . *.  *.************

BB      QLMTDGYSNRMIGEALYISEKTVKNHVSNILQKMNVNDRTQAVVESIKNGWVKVR
BS      QMLADGKSNRGIGESLFISEKTVKNHVSNILQKMNVNDRTQAVVVAIKNGWVEMR
        *...** *** ***.*.**************************** .****** .*
```

Fig 4.6 Alignment of the deduced amino acid sequence of the *B. brevis* (BB) and *B. subtilis* (BS) *degU* genes. Identical and conserved amino acids are marked with asterisks and dots respectively. The three conserved aspartate residues are boxed.

4.3.4 Characterization of proteases by gelatin-SDS-PAGE. The production of extracellular proteases by *B.subtilis* 1A46 and the three transformants: pML1, pML2 and pML3 carrying the *degS-degU* genes, as well as *B. brevis* Alk 36 was determined using the gelatin-SDS-PAGE technique (Fig.4.7). It can be seen that the three transformants overproduce protease in comparison to the recipient strain *B. subtilis* 1A46, thereby confirming the quantitative results set out in Table.4.1. In addition, it can be seen that *B. brevis* Alk 36 produces significantly fewer protease activity bands when compared to *B. subtilis* 1A46 under the same conditions.

4.3.5 Qualitative effect of the *degS-degU* operon on the production of extracellular enzymes. In order to determine the hydrolysis of starch by α-amylase and lichenan by endo-(1,3-1,4)-ß-glucanase, the plates were flooded with 1% iodine and 1% Congo red respectively. Clearing zones indicating enzyme activity could be clearly seen (Fig.4.8). Protease activity was indicated by zones of activity on Luria agar milk plates and the mucoid aspect of levans surrounding colonies on ST plates was clearly visible (Fig.4.9). In all cases the transformant pML2 containing the *degS-degU* operon exhibited greater hydrolytic activity indicating increased extracellular enzyme secretion.

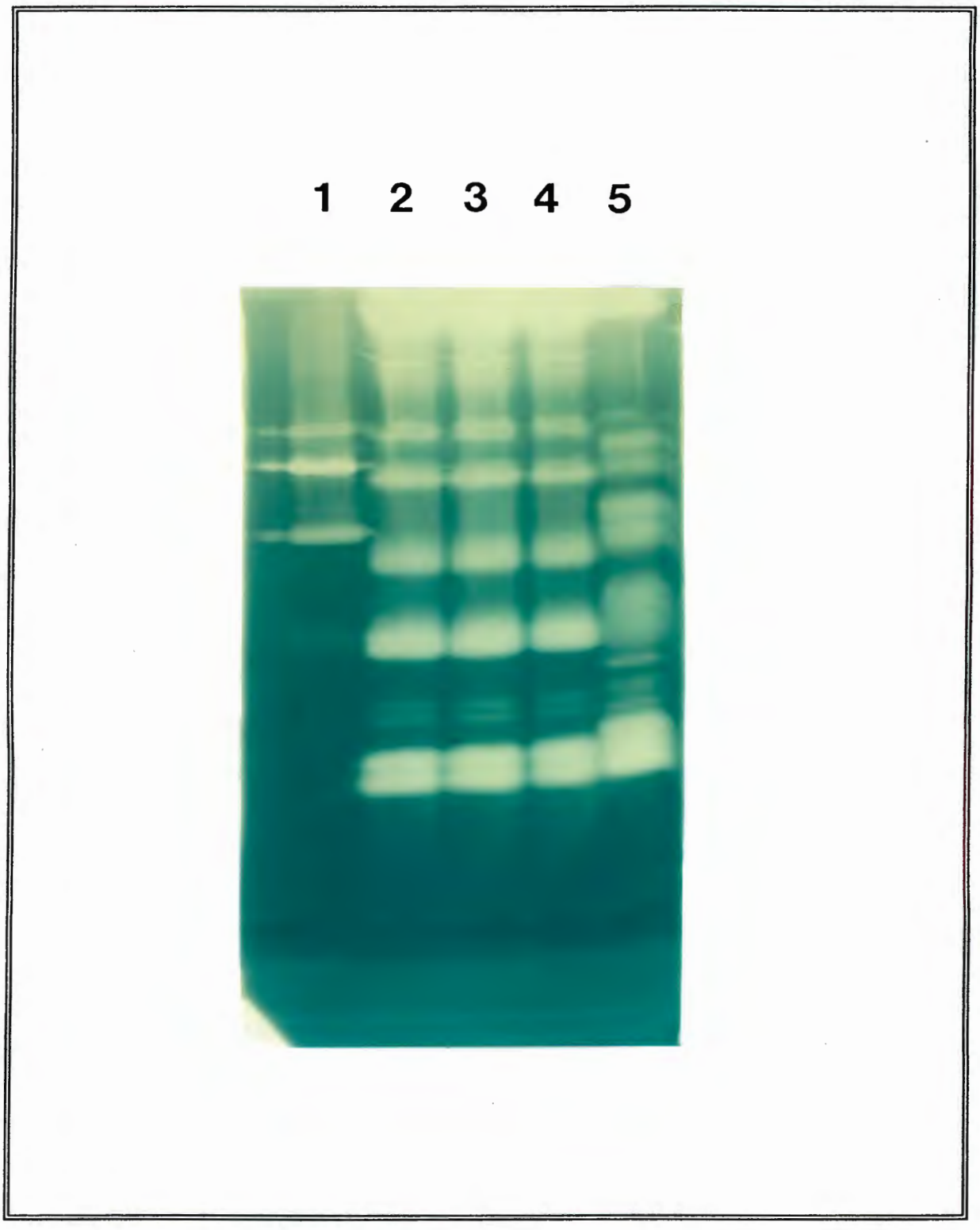

Fig 4.7 SDS-PAGE protease activity gel. Lanes: 1, *B. brevis* Alk 36; 2-4, *B. subtilis* 1A46 transformants containing the *degS-degU* operon on a multicopy plasmid; 5, *B. subtilis* 1A46.

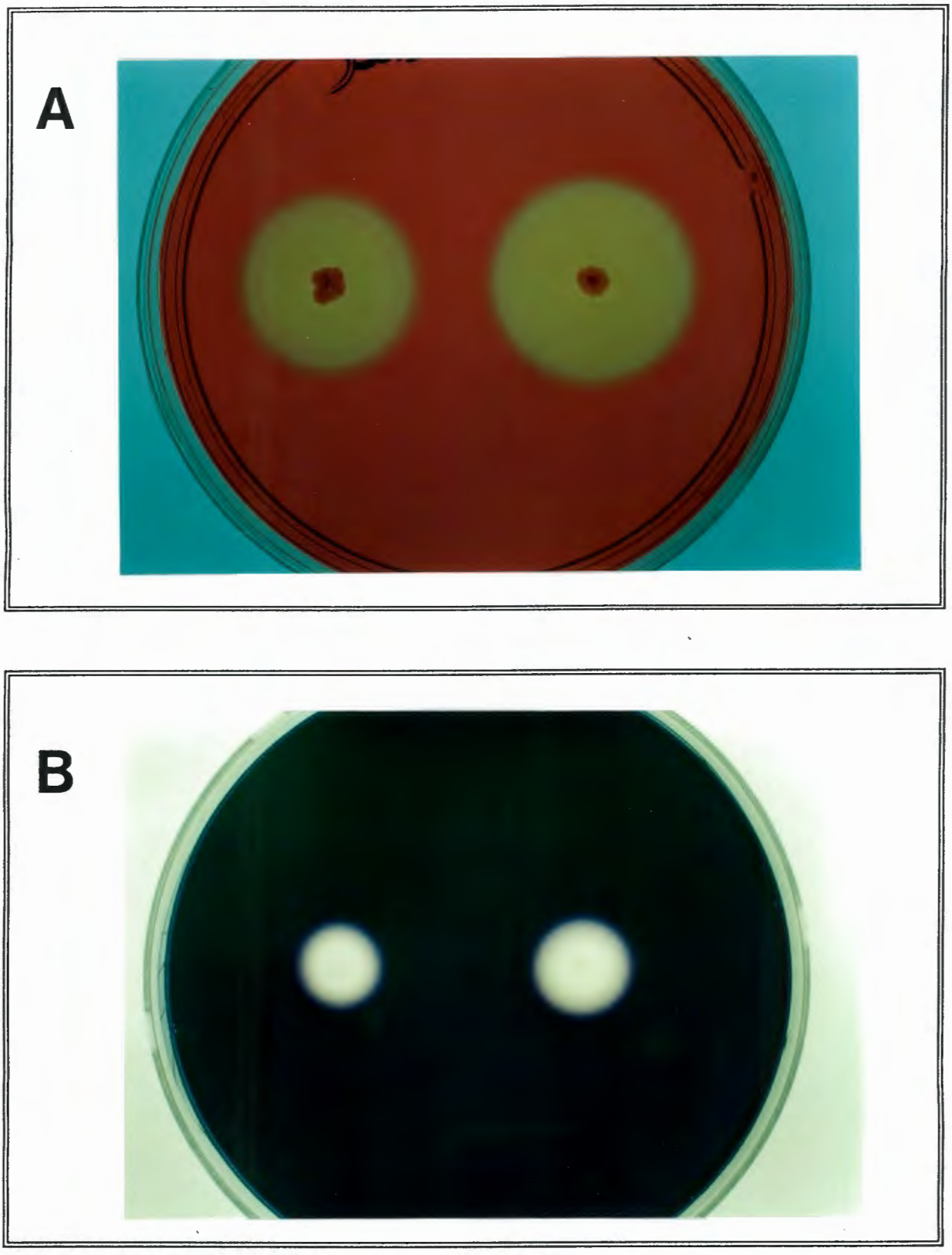

Fig 4.8 Qualitative effect of the *degS-degU* operon on the production of lichenase and α-amylase. (A) Lichenan plate flooded with Congo red (B) Starch plate flooded with iodine. The colony on the right hand side of the plates is *B. subtilis* 1A46 (pML2) and the one on the left hand side is *B. subtilis* 1A46 (pPL708)

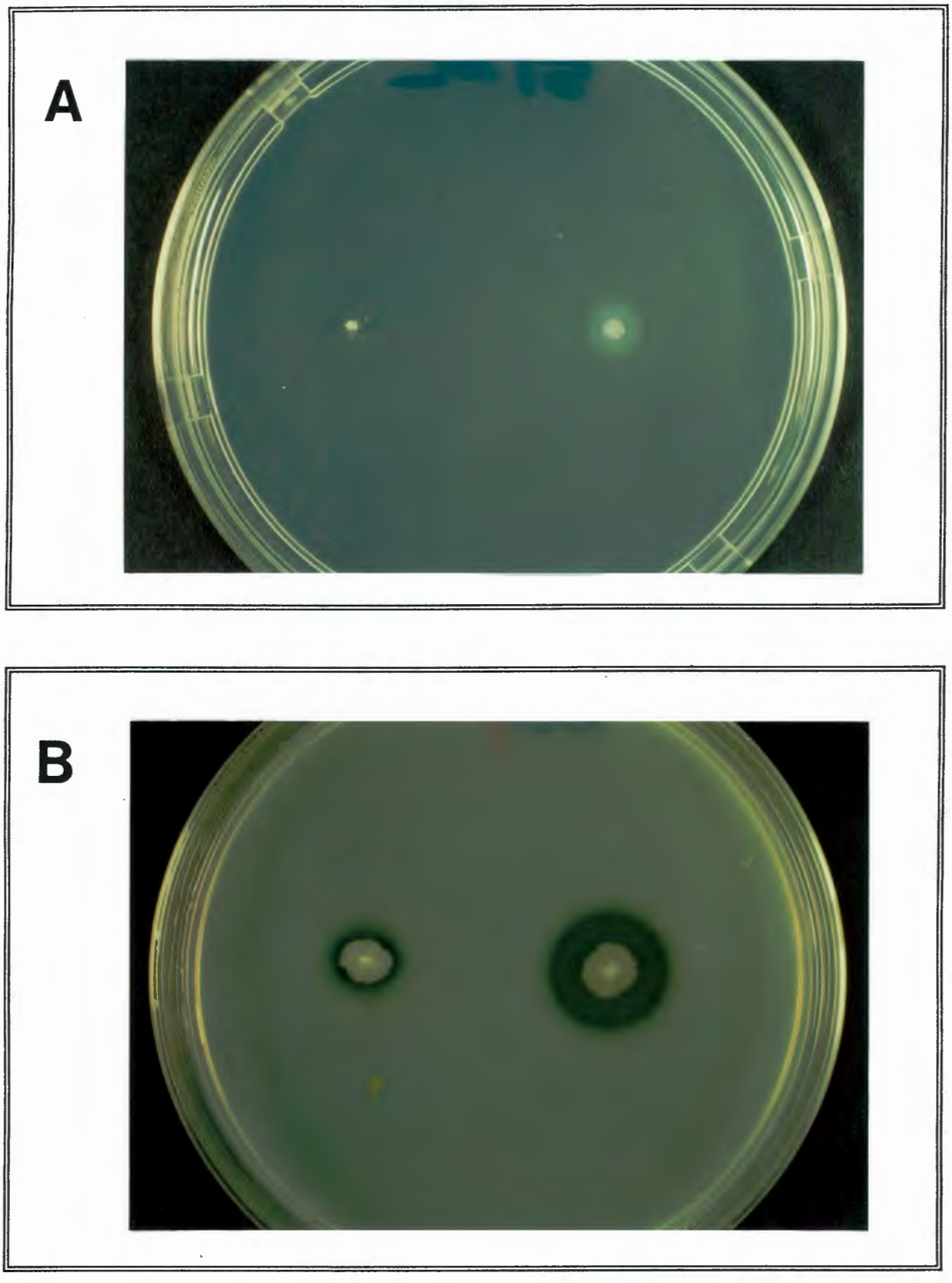

Fig 4.9 Qualitative effect of the *degS-degU* operon on the production of levansucrase and protease. (A) ST plate showing the mucoid aspect of levans. (B) Milk plate indicating protease activity. The colony on the right hand side of the plates in *B. subtilis* 1A46 (pML2) and the one on the left hand side is *B. subtilis* 1A46 (pPL708)

4.3.6 Quantitative effect on the *degS-degU* operon on the production of extracellular enzymes. The plasmid pML2 containing the *degS-degU* genes was transformed into the *sacU* mutant *B. subtilis* 1A201 and was found to restore levansucrase synthesis in this strain. The *degS-degU* genes were also transformed into a *sacU*(HY) mutant strain, *B. subtilis* 1A311. By using *B. subtilis* 1A311 (pPL708) and *B. subtilis* 1A46 (pPL708) as control strains, the effect of the *degS-degU* operon on extracellular enzyme production could be assessed (Table 4.2.). In all cases the plasmid pML2 carrying the *degS-degU* genes was found to enhance extracellular enzyme production. It was particularly interesting that pML2 was able to enhance the *sacU9*(HY) mutation found in strain 1A311. This mutation was due to a single nucleotide change which caused a Glu to Lys change at amino acid 107 in ORF2 (Henner et al. 1988a). Despite the fact that *B. subtilis* 1A46 and *B. subtilis* 1A311 are not a set of isogenic strains the effects of the *degS-degU* operon are sufficiently clear cut for this not to be a significant factor.

The increases in enzyme levels varied between 10- and 20-fold for total protease production and 6- to 36-fold for ß-glucanase production by both *B. subtilis* strains 1A311 and 1A46 respectively. Levansucrase production increased 2- and 5-fold when strains 1A46 and 1A311 were grown in minimal medium. It was, however, found that when 1A311 and 1A46 were grown in Luria broth plus sucrose the levansucrase production levels for 1A311 were only increased by 1.3-fold but for 1A46 these levels were increased by 90-fold. When grown in this medium however, 1A46 took twice as long as 1A311 to reach log phase growth. The α-amylase levels were increased 2-fold in 1A46 carrying the pML2 plasmid but were found to increase 4-fold in the *B. subtilis* 1A311 strain which carries the *sacU*(HY) mutation.

4.3.7 *In vitro* expression of the *degS-degU* encoded polypeptides. An *in vitro E. coli* cell free transcription-translation system was used to determine the M_r of the proteins expressed by the plasmid pML2 containing the *degS-degU* genes. The labelled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.4.10). Comparison of the polypeptides synthesized by the recombinant plasmid with those encoded by the vector plasmid pPL703, and pPL708 (containing the *cat* gene with a functional promoter) indicated several additional bands, two of which had apparent molecular masses in agreement with those deduced for the ORFI- an ORF2- encoded proteins (respectively 45 000 and 30 000 daltons).

4.3.6 Effect of the *degS-degU* operon on the production of extracellular enzymes. The plasmid pML2 containing the *degS-degU* genes was transformed into the *sacU* mutant *B. subtilis* 1A201 and was found to restore levansucrase synthesis in this strain. The *degS-degU* genes were also transformed into a *sacU*(HY) mutant strain, *B. subtilis* 1A311. By using *B. subtilis* 1A311 (pPL708) and *B. subtilis* 1A46 (pPL708) as control strains, the effect of the *degS-degU* operon on extracellular enzyme production could be assessed (Table 4.2.). In all cases the plasmid pML2 carrying the *degS-degU* genes was found to enhance extracellular enzyme production. It was particularly interesting that pML2 was able to enhance the *sacU9*(HY) mutation found in strain 1A311. This mutation was due to a single nucleotide change which caused a Glu to Lys change at amino acid 107 in ORF2 (Henner et al. 1988a). Despite the fact that *B. subtilis* 1A46 and *B. subtilis* 1A311 are not a set of isogenic strains the effects of the *degS-degU* operon are sufficiently clear cut for this not to be a significant factor.

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4.3.7 *In vitro* expression of the *degS-degU* encoded polypeptides. An *in vitro E. coli* cell free transcription-translation system was used to determine the M_r of the proteins expressed by the plasmid pML2 containing the *degS-degU* genes. The labelled polypeptides were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig.4.10). Comparison of the polypeptides synthesized by the recombinant plasmid with those encoded by the vector plasmid pPL703, and pPL708 (containing the *cat* gene with a functional promoter) indicated several additional bands, two of which had apparent molecular masses in agreement with those deduced for the ORFI- an ORF2- encoded proteins (respectively 45 000 and 30 000 daltons).

Table 4.2 **Effect of the *degS-degU* operon on the production of extracellular enzymes in *B. subtilis*.**

Strains	Enzyme specific activity (U/mg of protein)				
				Levansucrase	
	Protease (total)	ß-glucanase	α-amylase	Medium I*	Medium II**
B. subtilis					
1A46 (pML2)	1.2	18.3	0.85	0.78	2.68
1A46 (pPL708)	0.06	0.5	0.49	0.40	0.03
1A311 (pML2)	1.8	15.3	6.62	1.59	2.55
1A311 (pPL708)	0.17	2.6	1.60	0.30	1.95

* Cells grown in MMCH medium plus 2% sucrose
** Cells grown in Lura broth plus 1% sucrose

A discrepancy was noticed in the case of the ORF2 polypeptide between the experimental value and the one deduced from the DNA sequence. A similar disparity was previously recorded for the molecular mass of the *degU* gene (Kunst et al. 1988), and may reflect the transcription-translation of the gene in a heterologous *in vitro* system derived from *E. coli* rather than *B. subtilis*.

The 32.0 kDa protein which was considerably overproduced in pML2 but not in pPL703 or pPL708, was attributed to a fusion protein formed from read-through of an ORF downstream of the *degU* gene into the *cat* gene.

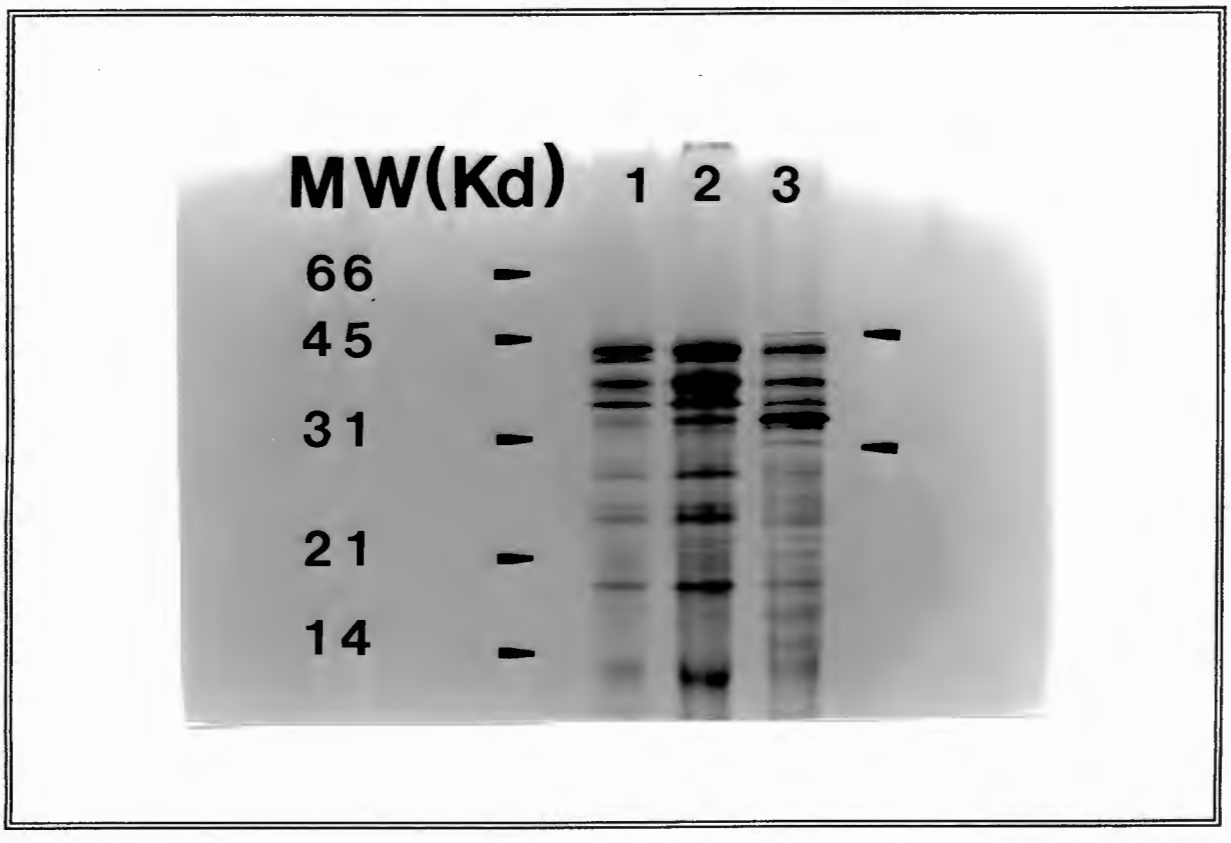


Fig 4.10 Identification of DegS-DegU polypeptides. Lanes: 1, vector pPL708; 2, vector pPL703; 3, recombinant plasmid pML2 containing both ORF1 and ORF2. Migration of standards is indicated in the left margin. Arrowheads in the right margin indicate bands of approximately 45 000 and 30 000 daltons.

4.4 DISCUSSION

The *degS-degU* genes were isolated from *B. brevis* Alk 36 and were selected for their ability to stimulate the production of extracellular proteases. When on a high copy number plasmid, the *B. brevis degS-degU* operon was found to be relatively stable and to cause the hypersecretion of several extracellular enzymes. This phenotype is similar to that obtained from *B. subtilis* strains carrying the *sacU*(HY) mutation as well as to *B. subtilis* strains overexpressing various accessory polypeptides such as *degQ* or *degR* on multicopy plasmids (Amory et al. 1987; Nagami and Tanaka 1986; Tanaka et al. 1987; Yang et al. 1986; Yang et al. 1987). Comparison of the increase in extracellular enzyme production in the presence of these genes has been difficult to quantify, as in each case parameters such as growth media, times of analysis and analytical methods have differed. However, none of the published literature has shown that a recombinant plasmid carrying the *degS-degU* genes increases extracellular enzyme production in a *B. subtilis sacU*(HY) strain. On the contrary, Podvin and Steinmetz (1992) found that when a recombinant phage carrying the wild type *degU* allele was introduced into a *degU32*(HY) strain, a partial suppression of the hyperproduction phenotype associated with the *degU32* mutation occurred.

To our knowledge this is the first published sequence of a *degS-degU* operon to have been cloned from a *Bacillus* spp. other than *B. subtilis*. Sequence comparisons between the *B. subtilis* and *B. brevis degS-degU* regions show considerable homology throughout the protein in a number of conserved blocks in both genes. Within the DegS protein it was hypothesized that Asp-152 and Asp-168 amino acids formed a domain which may interact with the phosphate group of ATP through Mg^{2+} salt bridges. This domain is located in the vicinity of the histidine residue (His-189) which is conserved in seven modulators (Msadek et al. 1990) and maybe the site of autophosphorylation of DegS. Both the Asp-152 and the His-189 are present in the *B. brevis degS* gene.

The DegU protein contains three aspartate residues which are clustered to form an acidic binding pocket, as determined from the three dimensional structure of other effectors. These are found at positions: Asp-10; Asp-11 and Asp-56 and were found

to be conserved in both the *B. subtilis* and *B. brevis* DegU proteins. In the *B. subtilis* DegU protein the replacement of Asp-56 by Asn in the *degU* mutant abolishes the capacity of the DegU protein to activate degradative enzyme synthesis, (Msadek et al 1990) and consequently, Asp-56 is proposed as the potential site of phosphorylation.

Primary structure modifications causing the mutational changes giving rise to the *degS*(HY) and *degU*(HY) phenotypes were compared to that of the sequence of the *B. brevis degS* and *degU* genes. No corresponding changes could be observed except in the region of the *degS200* mutation where in *B. subtilis* Gly was replaced by Glu which gave rise to the (HY) phenotype. This phenotype was found to be due to the mutated DegS protein being able to stabilize the DegU - phosphate (Dahl et al. 1992 and Tanaka et al. 1991). In the *B. brevis* DegS protein the Gly was replaced by Ala and the two amino acids either side of this mutation were also found to differ. Whether these amino acid changes could play a role in the (HY) phenotype of the *B. brevis degS-degU* operon by stabilizing the DegU-phosphate in a similar manner would have to be verified by further studies involving an *in vitro* system utilizing purified *B. brevis* DegS and DegU proteins.

Chapter 5

General Conclusions

Chapter 5

General Conclusions

This study focused on characterizing a *Bacillus brevis* Alk 36 strain which was isolated from soil during a screening programme for the selection of extracellular enzyme producing strains. In addition to characterizing the *B. brevis* Alk 36 endo-(1,3-1,4)-ß-glucanase enzyme, a positive regulator of extracellular enzyme production was cloned and sequenced and found to belong to the family of two-component signal transduction systems. This strain was also evaluated as a potential member of the protein-hypersecreting *B. brevis* group, with a view to its use as a host for the production of heterologous proteins.

The endo-(1,3-1,4)-ß-glucanase (or lichenase) was found to exhibit some unique properties: It had a pH optimum in the alkaline range, it was found to have a greater residual activity at a higher temperature than previously reported for *Bacillus* lichenases and, in addition, it was resistant to SDS. The gene was successfully cloned and expressed in *E. coli* with the aid of a positive selection vector and the Congo red plate screen.

Comparison of the amino acid sequences of the different lichenases indicated that the *B. brevis* polypeptide sequence shows the greatest homology to two sequences from *B. macerans* (53%) and *B. polymyxa* (54%) which are closely related, whereas the mesophilic lichenases form a separate cluster based on homology as previously reported (Hofemeister et al. 1986). It was proposed that comparisons of the sequences of different endo-(1,3-1,4)-ß-glucanase proteins exhibiting novel characteristics should facilitate the identification of the roles played by particular amino acid residues, leading ultimately to the modification of these proteins to suit specific industrial processes. With this in view, work is at present being carried out by Borriss (personal communication), who is utilizing the *B. brevis* endo-(1,3-1,4)-ß-glucanase gene to construct hybrid ß-glucanases with altered pH behaviour, to determine which sequences of the gene are responsible for ß-glucanase pH optima.

A completely different approach was used for cloning the *sacU* locus containing the *degS-degU* genes from *B. brevis* Alk 36. A *Bacillus* promoter probe vector was used and the genes were transformed and expressed in a rec⁻ *Bacillus* strain and selected for their ability to increase extracellular protease production. This was achieved by developing a sensitive plate screen whereby the hydrolysis of casein in the medium was intensified by the presence of starch, causing a definate halo around positive colonies. This proved sensitive enough so as to discriminate between the normal levels of protease secreted by cells and the increased levels secreted by hyperproducing strains.

When cloned on a high copy number plasmid, the *degS-degU* operon was found to be relatively stable and to cause the hypersecretion of several extracellular enzymes. In addition, this phenotype was found to occur even when the gene was expressed in a *degU*(HY) stain, which is not the case with previously reported *deg* operons. From the literature it would seem that the only other *degS-degU* sequence to have been published was from *B. subtilis* (Kunst et al. 1988, Henner et al. 1988a and Tanaka and Kawata 1988).

It is tempting to speculate as to why the *B. brevis degS-degU* operon is more efficient at producing the hypersecretion phenotype. This could be due to a number of reasons, such as higher expression levels of the *degS-degU* genes, or more probably, the polypeptides exhibiting a greater efficiency as effectors or receptors within the two-component system. When the *B. subtilis* DegS and DegU proteins responsible for the (HY) phenotype were investigated in an *in vitro* system, it was found that the (HY) phenotype was associated with increasing the phosphorylation rate of the DegS response regulator or by enhancing the stability of the phosphorylated protein by decreasing its dephosphorylation rate (Tanaka et al. 1991, Dahl et al. 1991, Dahl et al. 1992). Whether the *B. brevis degS-degU* operon could cause the (HY) phenotype by stabilizing the DegU-phosphate in a similar manner would have to be determined by further work involving *in vitro* studies of the purified proteins.

The fact that the *B. brevis deg* operon can be stably expressed in a variety of *Bacillus* genetic backgrounds, may throw more light on the nature of the environmental signals to which the *degS* gene responds and facilitate the identification of the DNA targets of the DegU protein. In addition, it might prove interesting to investigate the possible

presence of the *deg* operon in a number of different Gram positive strains and, if present, to clone and compare the sequences and phenotypes of the different genes.

Very little has been published to date on possible industrial applications of the *deg* operon in increasing extracellular enzyme expression. This was possibly due to the unstable nature of the *B. subtilis deg* operon. A report was, however, published by Vehmaanperä and Korhola (1986) investigating the stability of a recombinant plasmid containing the *B. amyloliquefaciens* α-amylase gene when expressed in a *B. subtilis* strain carrying the *degU*(HY) mutation. They found that against this genetic background the recombinant strain proved to be unstable. It would be of interest to determine whether a similar instability would occur if the *B. brevis deg* operon and an extracellular enzyme gene were both expressed from multicopy vectors in the same *Bacillus* strain.

The *B. brevis* Alk 36 isolate was evaluated as a potential host for the efficient production and secretion of foreign gene products. It was found not to be a member of the protein secreting *B. brevis* group. However, as it produced very little extracellular protease and it was able to grow over a wide pH and temperature range it was felt to be a promising candidate for the expression of recombinant heterologous proteins providing its genetic transformation systems could be optimized. Further work would, however, have to be carried out to ensure the stable expression of recombinant genes. This could involve the use of integrative vectors and possibly insertionally inactivating the *recA* gene. This method has been previously reported by Quivey and Faustoferri (1992) and used to inactivate the *Streptococcus mutants recA* gene. An integrative vector containing a *Sau*3A fragment of the *recA* allele is at present available from the *Bacillus* Genetic Stock Culture Collection, but unfortunately, it carries the erythromycin resistance gene as a marker and so would have to be suitably adapted for use in *B. brevis* Alk 36.

The characterization of *B. brevis* Alk 36 has so far highlighted a number of interesting findings and clearly illustrates the versatility of using both cloning approaches *viz E. coli*, which was the route of choice for cloning the endo-(1,3-1,4)-ß-glucanase gene, and *B. subtilis* which proved vital for the successful cloning the *degS-degU* genes as they were found to be lethal in *E. coli*.

APPENDIX A

General Techniques

A.1 **Isolation of chromosomal DNA**

A.2 **Isolation of plasmid DNA from *Bacillus* spp. (maxiprep)**

A.3 **SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)**

A.3.1 Resolving gel

A.3.2 Stacking gel

A.3.3 Staining/de-staining protocols

A.3.3.1 Protein SDS-PAGE

A.3.3.2 Lichenase activity gel

A.3.3.3 Protease activity gel

A.3.4 SDS-PAGE solutions

A.4 **Preparation and transformation of competent *E. coli* cells**

A.5 **DNA hybridization**

A.6 **Exonuclease III digestion**

A.7 **Nucleotide sequencing**

A.7.1 Primer annealing reaction

A.7.2 Sequencing reactions

A.7.3 Gel electrophoresis and autoradiography

A.1 Isolation of chromosomal DNA from *Bacillus* spp.

The method of Lovett and Keggins (1979) was followed with some modifications (Appendix A). An overnight culture in 200 ml Luria broth was washed once with TES pH 7.5 (30 mM Tris; 5mM EDTA; 50 mM NaA) and the pellet resuspended in 50 ml TES pH 7.5. Lysozyme was added to a final concentration of 500 μg/ml and heat-treated RNase to 100 μg/ml. The resulting solution was incubated at 37°C for 20-25 minutes. It was then diluted with an equal volume of TES buffer followed by the addition of proteinase K to 100 μg/ml and sarkosyl to 0.8%. The solution was incubated for 30 minutes until it had cleared and the viscosity increased. An equal volume of TE-saturated phenol was added and the tube rotated gently for 10 minutes. The layers were separated by centrifugation (10 000g for 10 minutes), and the phenol extraction repeated twice on the top aqueous phase. An equal volume of cold chloroform:isoamyl alcohol (24:1) was added, the solution mixed gently and centrifuged (10 000g for 10 minutes). The upper aqueous phase was transferred to a fresh polypropylene tube.

This was repeated twice. To this upper phase an equal volume of water-saturated ether was added, mixed gently and the phases were allowed to separate by standing 2-5 minutes. The upper layer was removed and discarded and the extraction repeated on the lower layer. Traces of ether were removed by heating the DNA solution to 60°C with gentle mixing. To the final aqueous phase two volumes of ice-cold 96% ethanol was added and the DNA precipitated overnight at -20°C. The precipitate was spooled out on a hooked pasteur pipette, air-dried and resuspended in 5 ml TE pH 8.0 (10 mM Tris; 1 mM EDTA). Finally the DNA was dialysed in 3 litres of TE buffer pH 8.0 with 3 buffer changes for 24 hours.

A.2 Isolation of plasmid DNA from *Bacillus* spp.

B. subtilis plasmids were isolated by the sodium dodecyl sulphate-NaCl (SDS-NaCl) method as modified by Gryczan et al. (1987) (Appendix A). An overnight culture grown in 200 ml Luria broth with selective antibiotics was centrifuged

washed with 0.05 M Tris pH 7.5 and resuspended in 20 ml of 25% ($^{w}/_{v}$) sucrose; 0.1 M NaCl; 0.05 M Tris pH 7.5. Lysozyme was added to a final concentration of 0.5 mg/ml and the cells incubated at 37°C for 15 minutes. The reagents listed were then added in the following amounts and order: 4.8 ml of 5 M NaCl; 1.2 ml of 0.5 M EDTA pH 8.5 and 26 ml of 2% sodium dodecyl sulphate in 0.7 M NaCl. The suspension was then inverted once and placed on ice for 18 hours. The lysate was centrifuged at 15 000 g for 45 minutes, the supernatant collected and made 0.3 M in sodium acetate. Two values of ice-cold 96% ethanol were added and the solution held at -20°C for 2 hours. The precipitate was collected by centrifugation at 15 000 g for 30 minutes and dissolved in 4 ml TE buffer. Heat treated RNase was added to 50 μg/ml and the sample incubated at 37°C for 30 minutes. After the addition of 100 μg/ml proteinase K, incubation was continued for a further 30-60 minutes until the solution had cleared. Purification of the plasmid DNA was performed by cesium chloride-ethidium bromide equilibrium centrifugation (Sambrook et al. 1989).

A.3 SDS-Polyacrylamide gel electrophoresis (SDS-PAGE)

Discontinuous SDS-PAGE was done according to the method of Laemmli (1970), using an LKB 2001 vertical slab electrophoresis unit (Hoefer Scientific Instruments, for LKB, San Francisco, CA, USA). The 3.0 mm thick gel spacers were used. the resolving gel was prepared and degassed before pouring. Distilled water was layered on the gel to promote a sharp interface. After the gel had polymerized (~60 min), the water was poured off and the stacking gel was cast and a Teflon comb inserted for well formation.

To each sample an equal volume of splitting buffer was added (Section A.3.4) and the samples were pre-treated as set out in the Materials and Methods section of the relevant chapters. Electrophoresis was continued at 35 mA (constant current) until the dye front migrated to the end of the gel (four to five hours). After electrophoresis the gels were stained overnight in Coomassie blue staining solution, then destained (Section A.3.4).

The acrylamide gels were prepared as follows:

A.3.1 Resolving Gel

Stock Solutions (ml)	**Final acrylamide concentration in the separating gel (%)**		
	10	**12**	**15**
30% acrylamide/ 0.8% bisacrylamide	10	12	15
4 x Tris/SDS solution pH 8.8	7.5	7.5	7.5
distilled deionized H_2O*#	12.5	10.5	7.5
10% ammonium persulfate	0.1	0.1	0.1
TEMED	0.02	0.02	0.02

* For lichenase activity gels 0.1% lichenan (0.03 g) was added to the distilled water aliquot and microwaved until dissolved. After cooling the gel preparation was continued as described above.

\# For protease activity gels the ratio of acrylamide:bisacrylamide was altered to

30%:1%. A 1% gelatin stock solution was autoclaved, and a 3.0 ml aliquot taken and made up to the required volume of water as indicated.

A.3.2 Stacking Gel

The stacking gel contained the following:

Solution (ml)	Stacking gel
Acrylamide solution	1.3
4 x Tris/SDS solution pH 6.8	2.5
distilled H_2O	6.1
10% Ammonium persulfate	0.05
TEMED	0.01

A.3.3 Staining/destaining protocols

A.3.3.1 **Protein SDS-PAGE gel.** The gels were stained overnight in Coomassie blue and de-stained in 20% acetic acid.

A.3.3.2 **Lichenase activity gel.** The gels were initially washed in Na-citrate buffer pH 6.3 (Béguin 1983) and incubated in Tris buffer pH 8.0 at 37°C for 2 h prior to staining with 0.1% Congo red. As the enzyme was found to be SDS-resistant the citrate buffer washing step was omitted and the gels were incubated directly in Tris buffer before staining.

A.3.3.3 **Protease activity gel.** The gels were incubated overnight in 2.5% Triton X-100 at 4°C to remove the SDS and then in 0.1 M glycine buffer pH 8.5 at 37°C for 3.5 h. The gels were then stained for activity with 1% amido black for at least 1h and then destained in methanol:acetic acid:water in the ratio of 30:10:60.

A.3.4 SDS-PAGE solutions

Acrylamide solution

Acrylamide	30.0 g
Bis-acrylamide	0.8 g
Distilled water	to 100 ml

Filter through Whatman's paper (no.1) and store in dark bottle at 4°C.

4 x Tris/SDS	**(pH 8.8)**
Tris (1.5M)	30 g
SDS	0.66 g
Distilled water	to 100 ml

Adjust pH to 8.8 with HCl

4 x Tris/SDS	**(pH 6.8)**
Tris (0.5M)	6.05 g
SDS	0.4 g
Distilled water	to 100 ml

Adjust pH to 6.8 with HCl

Ammonium persulphate (10% $^{w}/_{v}$)

A fresh solution was made immediately before use and stored at 4°C for not longer than 4 days.

PAGE electrophoresis buffer (x5)

Tris	15.1 g
Glycine	72.0 g
SDS	5.0 g
distilled water	1000 ml

Splitting buffer

Stacking gel buffer	2.5 ml
SDS (10%)	4 ml
Glycerol	2 ml
2-mercaptoethanol	1 ml
Bromophenol blue	0.1 mg
Distilled water	0.5 ml

The solution was stored in aliquots at -20°C

Coomassie blue staining solution

Coomassie blue R250 (0.25% w/v)	2.5 g
Destaining solution	1000 ml

The solution was stirred vigorously to dissolve the dye and then filtered through Whatman's paper (No.1)

Destain solution

Acetic acid	100 ml
Methanol	300 ml
Distilled water	600 ml

Citrate buffer pH 6.3

$Na_2H\ PO_4$	7.09 g
Citric acid	2.62 g
Distilled water	1000 ml

The pH was adjusted to 6.3

The first two washes contained 25% isopropanol

Congo Red

Congo Red	1 g
Distilled water	to 1000 ml

Adjust pH to approximately 12.0 with NaOH

Triton X-100 (2.5%)

Triton X-100		50 ml
Distilled water	to	2000 ml

Gelatin solution (1%)

Gelatin	1 g
Distilled water	80 ml

Boil solution until the gelatin has dissolved, then make volume up to 100 ml with water and autoclave.

Amido Black stain

"Amidoswartz" (for electrophoresis)		1 g
Distilled water	to	50 ml

Mix for 10 min. Made up to 500 ml with destain. Stir for 30 min. Filter through Whatman's paper (No.3). Stain can be re-used several times.

Glycine buffer pH 8.0

Glycine aminoacetic acid		7.51 g
Distilled water	to	1000 ml

The pH was adjusted to 8.0 with NaOH.

A.4 Preparation and transformation of competent *E. coli* cells

E. coli cells were made competent for DNA uptake according to the method as described by Armitage et al. (1988). A single colony from a freshly inoculated bacterial plate was resuspended in 5 ml ψ-broth (Appendix B) in a sterile 50 ml Erlenmeyer flask and shaken at 37°C for 2-3 h. This starter culture was then used to inoculate 100 ml prewarmed ψ-broth in a 1 litre Erlenmeyer flask and shaken at 37°C until OD_{550} reaches 0.35. This generally takes about 2-3 h. The culture was then transferred to 2 x 35 ml capped centrifuge tubes and chilled on ice for 15 min, the tubes were then centrifuged at 2500 r.p.m for 5 min at 4°C and the supernatant was decented. The cells were then resuspended by gentle vortexing in 10.5 ml ice-cold TFB1 (Appendix B), incubated on ice for 90 min and again centrifuged at 2500 r.p.m for 5 min at 4°C and the supernatant decanted. The cells were resuspended gently in 2.8 ml ice-cold TFB2 (Appendix B) and 200 μl aliquoted into 1.5 ml Eppendorf tubes. These cells were now competent and

could be used immediately for transformation or stored for many months at -70°C with little reduction in transformation efficiency.

In order to transform the competent cells, they were incubated on ice for 10 min and then DNA added, mixed gently and incubated on ice for a further 20 min. The cells were then heat shocked by placing the tubes in a 37°C waterbath for 60 sec and returned immediately to ice for a further 2 min. To the cells, 800 µl ψ-broth was added, and they were then shaken at 37°C for 60 min after which they were plated.

A.5 **DNA hybridization**

DNA fragments resolved by agarose gel electrophoresis were transferred to a Hybond-N hybridization membrane (Amersham) as follows:

After electrophoresis the gel was left under UV light for 10 min to depurinate the DNA. The gel was shaken gently in 0.25 N HCl for 15 min and washed twice with distilled water. The gel was then shaken gently in 1 M NaCl-0.5 M NaOH for 30 min and washed with distilled water. In order to neutralize the gel it was shaken gently in 3 M NaCl-0.5 M Tris pH 7.0 for 2x 30 min and washed with distilled water in between and after. The gel was then soaked in 10xSSC (Appendix B) for 10 min and placed on a glass plate. A sheet of Hybond-N was immersed in distilled water followed by 10xSSC and layered carefully over the gel. The edges of the gel were sealed with strips of parafilm and two pieces of Whatman 3mm filter paper soaked in 10xSSC were placed on top of the membrane, followed by a 4 cm thick layer of absorbent paper. A light weight was placed on top of this and the transfer left to continue overnight. The DNA labelling, hybridization and detection was as set out in the Boehringer Mannheim nonradioactive DNA labelling and detection manual.

A.6 **Exonuclease III digestion**

The DNA was prepared for sequencing by the construction of ordered deletions of the endo-(1,3-1,4)-ß-glucanase gene in both directions in the "Bluescript" plasmid SKpNA3, using exonuclease III (Henikoff 1984). Approximately 10 µg of SKpNA3 previously digested with the appropriate restriction endonucleases (section 3.2.14) and resuspended in 100 µl exobuffer (Appendix B) was equilibrated at 37°C for 5 min. A sample (9 µl) was first removed to a tube containing 20 µl 10 x Mungbean nuclease buffer and 170 µl distilled water and placed on ice. Exonuclease III (350 units) was added to the

equilibrated DNA, the mixture briefly vortexed, and after a lag period of 30 sec nine samples (9μl) were removed at regular time intervals. Each sample was immediately transferred to a tube as for time 0. When all the samples had been collected the Exonuclease III was heat inactivated at 70°C for 10 min. To each tube 15 units of Mungbean nuclease in 1 x Mungbean nuclease dilution buffer (Appendix B) was added, and the tubes were incubated at 30°C for 30 min to remove the single stranded DNA remaining after exonuclease III digestion. To each tube: 4 μl of 20% SDS; 10 μl of 1M Tris pH 8; 20 μl of 8 M LiCl and 250 μl phenol/chloroform was added. The mixture was briefly vortexed and the upper layer extracted with chloroform and precipitated with ethanol (Sambrook et al. 1989). Each DNA precipitate was resuspended in 15 μl TE buffer (Appendix A), blunt-end ligated in a final volume of 20 μl (Section 3.2.6.1) and transformed into competent *E. coli* Lk112 cells (section A4). The transformants were selected on Luria agar plates containing 100 μl/ml Ampicillin.

A.7 **Nucleotide sequencing**

A.7.1 **Primer annealing reaction**

The supercoiled DNA (6-10 μg, in TE buffer) was diluted to a final volume of 20 μl in distilled water. Alkaline denaturation in 0.2 N NaOH (5 min at room temperature) was followed by the addition of 5 μl of 3M sodium acetate (pH 5.2), 25 μl of distilled water and 150 μl of ethanol. This mixture was chilled to -70°C, centrifuged at 4°C for 20 min in a microfuge and washed with 200 μl of ethanol (70%). The DNA pellet was dried and resuspended in a final volume of 10 μl of sequencing buffer (40 mM Tris-HCl, pH 7.5; 20mM $MgCl_2$; 50 mM NaCl) and 12 ng of primer. This mixture was annealed for 15 min at 40°C immediately prior to sequencing. The primers used were the forward sequencing primer as supplied in the Sequenase DNA sequencing kit (US Biochemical Corp., Cleveland, Ohio) and the M13 reverse sequencing primer (Amersham).

A.7.2 **Sequencing reactions**

DNA sequencing was done by the dideoxy chain termination method of Sanger et al. (1977) according to the protocol of Tabor and Richardson (1987), using T7 DNA polymerase and a "Sequanase" sequencing kit supplied by the US Biochemical Corporation, Cleveland, Ohio. The DNA chain was radiolabelled with [α-^{35}S]dATP (1200 Ci/mmol; Amersham).

A.7.3 **Gel electrophoresis and autoradiography**

The sequencing reactions were analyzed on standard 6% denaturing acrylamide urea sequencing gels. The composition and running conditions of the gels were as described in the Amersham M13 Sequencing Handbook. After electrophoresis the gels (0.2mm thick) were dried onto Whatman No.3 filter paper using a Dual Temperature Slab Gel Dryer (Model 1125B; Hoefer Scientific Instruments, San Francisco). Gels containing ^{35}S-labelled DNA were placed under XAR-5 autoradiographic film and exposed for 1-2 days. The autoradiographs were developed using Kodak GBX X-ray developer and fixer.

APPENDIX B

Media, buffers, and solutions

All media, buffers, and solutions were sterilized by autoclaving at 121°C for 20 min unless otherwise indicated. Heat labile substances were sterilized by filtration through 0.22 μm membrane filters (Millipore). Polyacrylamide gel solutions and buffers are given in Appendix A.

B.1 Media

Penassay broth (x4)

Biolab beef extract	6 g
Biolab yeast extract	6 g
Biolab peptone	20 g
Glucose	4 g
NaCl	14 g
K_2HPO_4	14.72 g
KH_2PO_4	5.28 g
Distilled water	1000 ml

The pH was adjusted to 7.0.

Modified DM3 medium

To make 500 ml the following solutions were prepared separately in water:

a)	Biolab agar	4 g
	Starch	5 g
	Distilled water	100 ml

solution a) was mixed well and microwaved before autoclaving.

b)	Casein	5 g

To b), 50 ml of 0.1N NaOH was added, the solution microwaved, cooled and 0.1N HCl added until pH 7.6. The solution was then made up to 100 ml with distilled water.

c)	Sodium succinate	67.5 g

To c), 100 ml of distilled water was added, the pH adjusted to 7.3 and the solution made up to 150 ml.

The following solutions were made up separately with the volumes of water as indicated.

d)	Casamino acids	5.0 g	50 ml
	Biolab yeast extract	2.5 g	25 ml
	K_2HPO_4	1.75g}	50 ml
	KH_2PO_4	0.75g}	
	Glucose	2.5 g	12,5 ml
	$MgCl_2.6H_2O$	4.38 g	10 ml

All components were autoclaved separately, cooled to approximately 50°C and mixed. To this, 2.5 ml of 2% filter sterilized Bovine serum albumen (fraction five, Boehringer

Manheim) was added as well as chloramphenicol at a concentration of 20μg/ml.

Modified T2 medium

Glucose*	10 g
Polypeptone	10 g
Meat extract	10 g
Yeast extract	4 g
0.2 M phosphate buffer pH 8.0	500 ml
Distilled water	to 1000 ml

The pH was adjusted to 8.5.
*Glucose and phosphate buffer were autoclaved separately. The pH was maintained after inoculation of *B.brevis* Alk36 by the addition of 2 ml of 10% ($^{w}/_{v}$) Na_2CO_3 at the end of log phase growth.

MMCH medium

Sucrose*	20 g
K_2HPO_4	10.45g
KH_2PO_4	5.99 g
MgSO4	0.49 g
$(NH_4)_2SO_4$	1.98 g
10 mM $MnCl_2$	1.0 ml
Ferric Ammonium citrate	22 mg
Casein hydrolysate	0.5 g
Distilled water	1000 ml

*Sucrose was sterilized separately.
Filter sterilized amino acids were added at a concentration of 100 mg/l after autoclaving.

ST medium

Sucrose*	20 g
Tryptone	1 g
Agar	17 g
KCl	0.97 g
$MgSO_4$	0.25 g
Distilled water	1000 ml

*Sucrose sterilized separately.
Amino acids were added after autoclaving as for MMCH medium.

ψ-broth

Bacto tryptone	20.00g
Yeast extract	5.00 g
$MgSO_4.7H_2O$	4.00 g
KCl	0.75 g
Distilled water	1000 ml

Antibiotics

Antibiotic stock solutions were as follows:

Ampicillin	100 mg/ml water
Chloramphenicol	20 mg/ml ethanol (50%)
Erythromycin	20 mg/ml water

Kanamycin	62.5 mg/ml water
Neomycin	20 mg/ml water

B.2 Buffers and Solutions

2X SMM buffer

Sucrose	342.3 g
Maleic acid	4.64 g
$MgCl_2$	8.13 g
Distilled water	1000 ml

The pH was adjusted to 6.5 prior to autoclaving.

PEG solution (30%)

PEG (Merck approximate molecular weight 4000)		30 g
2xSMM buffer		50 ml
Distilled water	to	1000 ml

SMMP buffer

Consists of equal volumes of 4x Pennassay broth plus 2x SMM buffer autoclaved separately then mixed.

Tris buffer (1 M)

Dissolve 121 g Tris base in 800 ml distilled water. Adjust to desired pH with concentrated HCl. Mix and add distilled water to 1000 ml.

TE buffer pH 7.5 or 8.0 (100X)

Tris.Cl (pH 7.5 or 8.0)		121 g
EDTA (O.5 M, pH 8.0)		200 ml
Distilled water	to	1000 ml

Autoclave and dilute with sterile water before use.

TFBI

1 M RbCl		5.00 ml
$MnCl_2.4H_2O$		0.495 g
KOAc		0.147 g
750 mM $CaCl_2.2H_2O$		0.67 ml
50% glycerol		15.00 ml
Distilled water	to	50 ml

The pH was adjusted to 5.8 with glacial acetic acid and the solution filter sterilized.

TFB2

100 mM MOPS (Sigma) pH 7.0 (with NaOH)		5.0 ml
1 M RbCl		0.5 ml
750 mM $CaCl_2$		5.0 ml
50% glycerol		15.0 ml
Distilled water	to	50 ml

The solution was filter sterilized.

Sørensen buffer	(pH 6)	(pH 7)	(pH 8)

Na_2HPO_4 (0.2M)	6.15 ml	30.5 ml	47.35 ml
NaH_2PO_4 (0.2M)	43.85 ml	19.5 ml	2.65 ml
Distilled water up to	1000 ml		

	(pH 3)	(pH 4)	(pH 5)
Citric acid (0.1 M)	79.45 ml	61.45 ml	48.5 ml
Na_2HPO_4 (0.2 M)	20.55 ml	38.55 ml	51.5ml

20X SSC buffer

NaCl		175.3 g
Sodium citrate		88.2 g
Distilled water	to	1000 ml

pH was adjusted to 7.0 with NaOH (10 N) and the solution autoclaved. The 20x SSC buffer was diluted ½ to give 10x strength.

Exonuclease III shortening solutions

Exonuclease III buffer

Tris Cl (0.05 M, pH 8)		0.606 g
$MgCl_2$ (5 mM)		0.102 g
Distilled water	to	100 ml

Mungbean nuclease buffer (x5)

Sodium acetate (pH 5, 150 mM)		1.23 g
NaCl (250 mM)		1.46 g
$ZnCl_2$		0.068 g
Glycerol 25%		25 ml
Distilled water	to	100 ml

Mungbean nuclease dilution buffer (X1)

Sodium acetate (pH 5, 100 mM)		10 ml
Zinc acetate (1 mM)		10 ml
Cysteine (10 mM)		1 ml
Triton X-100 (0.1%)		0.1 ml
Glycerol (50%)		50 ml
Distilled water	to	100 ml

APPENDIX C

Bacterial strains, genotypes and references

Strain	Genotype/description	Reference/origin
E. coli:		
JM101	*supE thi* Δ *(lac-proAB)/*F'[*traD36 proA*$^+$ *proB*$^+$ *lacI*^{q} *lacZ*Δ*M15]*	Messing (1979)
LK112	*thr1 leu6 thi1 supE44 tonA2* r^-_k M^-_k laqI^q *lacZ δM15 lacY1*	Zabeau and Stanley (1982)
B. subtilis		
1A46	*recE4 thr-5 trpC2*	Dubnau et al. (1973)
1A311	*amyE(+M) amyR2 metB5 pro(L) purF6 sacU9 str*	Yoneda et al. (1973)
1A201	*hisA1 sacA321 sacU42 trpC2*	Lepesant et al. (1972)
1A654	*aroD120 bgl-33 trpC2*	Borriss et al. (1986)
ATCC 31408	soil isolate producing alkaline protease	te Nijenhuis (1977)

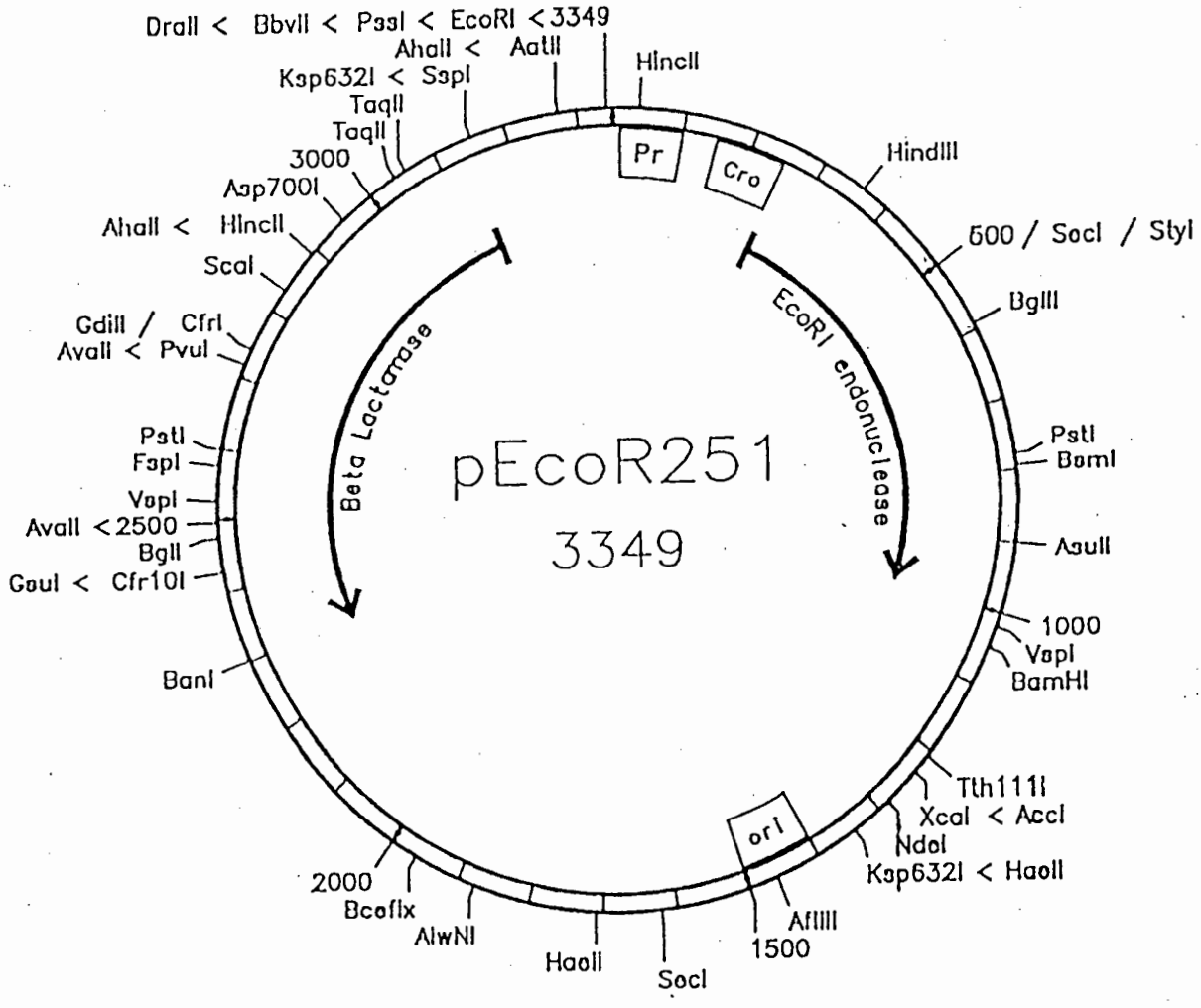
APPENDIX D

One- and three-letter codes used for amino acids

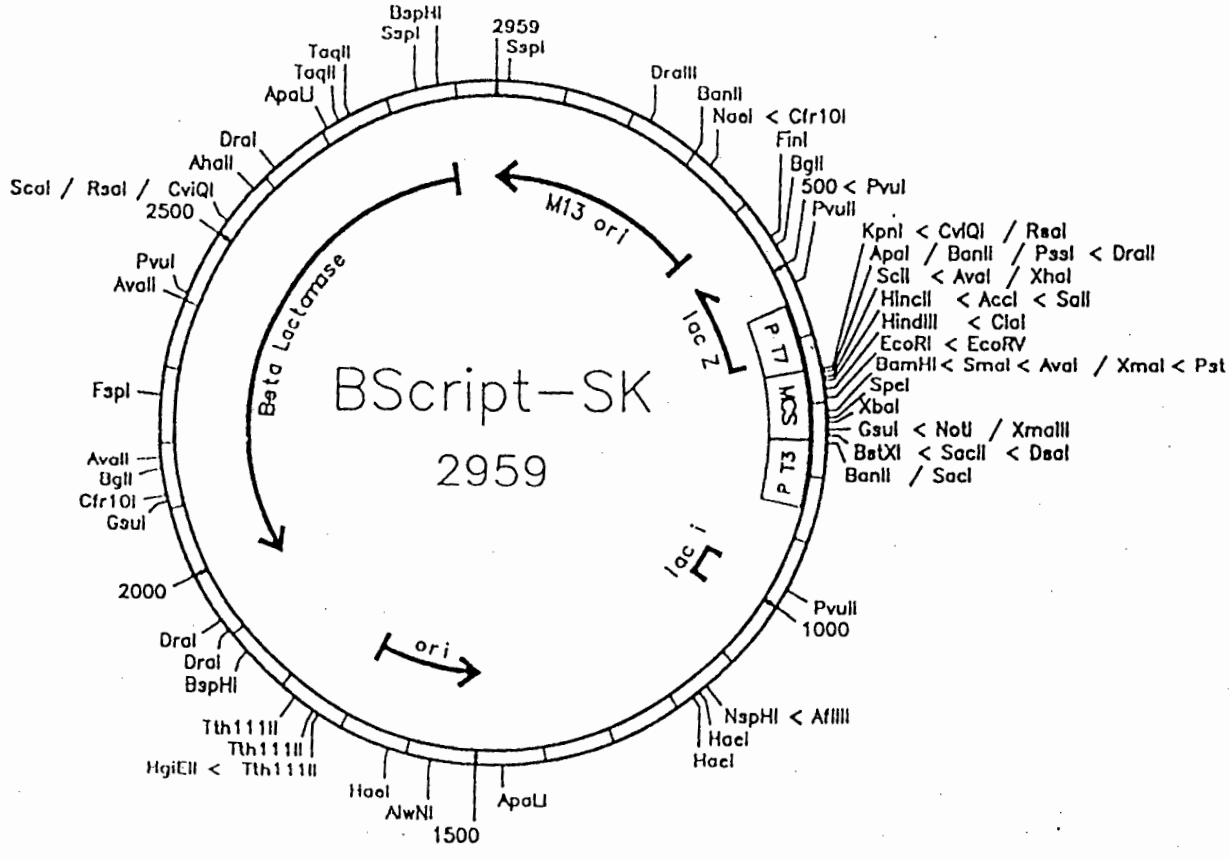
Amino acid	Codes		Code	Amino acid
Alanine	Ala	A	A	Alanine
Arginine	Arg	R	C	Cysteine
Asparagine	Asn	N	D	Aspartic acid
Aspartic acid	Asp	D	E	Glutamic acid
Cysteine	Cys	C	F	Phenylalanine
Glutamine	Gln	Q	G	Glycine
Glutamic acid	Glu	E	H	Histidine
Glycine	Gly	G	I	Isoleucine
Histidine	His	H	K	Lysine
Isoleucine	Ile	I	L	Leucine
Leucine	Leu	L	M	Methionine
Lysine	Lys	K	N	Asparagine
Methionine	Met	M	P	Proline
Phenylalanine	Phe	F	Q	Glutamine
Proline	Pro	P	R	Arginine
Serine	Ser	S	S	Serine
Threonine	Thr	T	T	Threonine
Tryptophan	Trp	W	V	Valine
Tyrosine	Tyr	Y	W	Tryptophan
Valine	Val	V	Y	Tyrosine

APPENDIX E

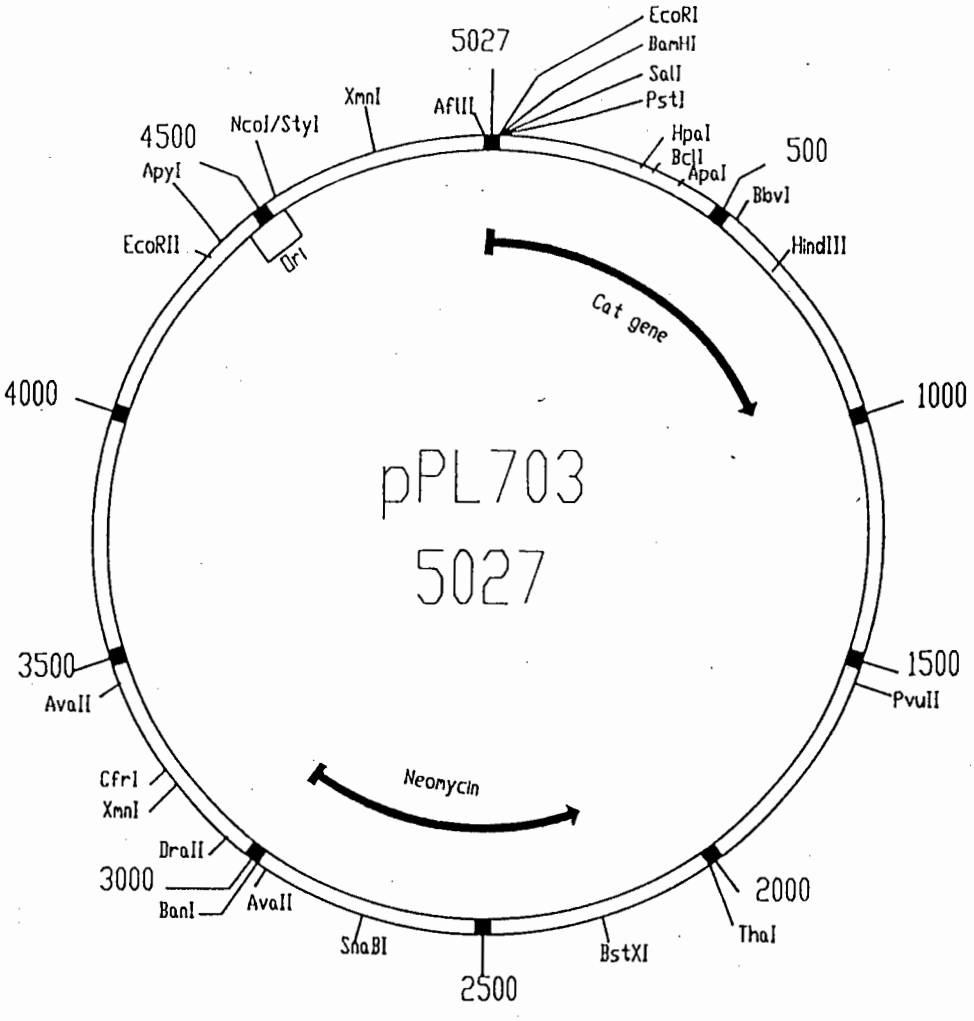
Plasmid vector maps



APPENDIX E


BscpHI
SspI
2959
SspI
TaqII
TaqII
ApaLI
DraIII
BanII
NaeI < Cfr10I
FinI
BglII
500 < PvuI
PvuII
DraI
AhaII
ScaI / RsaI / CviQI
2500
PvuI
AvaII
FspI
AvaII
BglII
Cfr10I
GsuI
2000
DraI
DraI
BspHI
Tth111II
Tth111II
HgiEII < Tth111II
HaeI
AlwNI
1500
ApaLI
NspHI < AflIII
HaeI
HaeI
PvuII
1000
KpnI < CviQI / RsaI
ApaI / BanII / PssI < DraII
ScII < AvaI / XhoI
HincII < AccI < SalI
HindIII < ClaI
EcoRI < EcoRV
BamHI < SmaI < AvaI / XmaI < PstI
SpeI
XbaI
GsuI < NotI / XmaIII
BstXI < SacII < DsaI
BanII / SacI
P T7
MCS
P T3
M13 ori
lac Z
lac i
ori
Beta Lactamase
BScript–SK
2959

APPENDIX E


pPL703
5027
5027
EcoRI
BamHI
SalI
PstI
AflII
XmnI
4500
NcoI/StyI
ApyI
EcoRII
Ori
HpaI
BclI
ApaI
500
BbvI
HindIII
Cat gene
1000
1500
PvuII
2000
ThaI
BstXI
2500
ShaBI
AvaII
BanI
3000
DraII
XmnI
CfrI
Neomycin
3500
AvaII
4000

Literature Cited

Adachi T, Yamagata H, Tsukagoshi N and Udaka S (1989) Multiple and tandemly arranged promoters of the cell wall protein gene operon in *Bacillus brevis* 47. J Bacteriol **171:**1010-1016

Amaki Y, Tulin EE, Ueda S, Ohmiya K and Yamane T (1992) Purification and properties of a thermostable esterase of *Bacillus stearothermophilus* produced by recombinant *Bacillus brevis*. Biosci Biotech Biochem **56:**238-241

Amory A, Kunst F, Aubert E, Klier A and Rapoport G (1987) Characterization of the *sacQ* genes from *Bacillus licheniformis* and *Bacillus subtilis*. J Bacteriol **169**:324-333

Anagostopoulos C and Spizizen J (1961) Requirements for transformation in *Bacillus subtilis*. J Bacteriol **81:**74-76

Anson ML (1939) The estimation of pepsin, trypsin, papain and cathepsin with hemoglobin. J Gen Physiol **22:**79-89

Armitage P, Walden R and Draper J (1988) Vectors for the transformation of plant cells using *Agrobacterium*. **In:** Draper J et al. (eds) Plant genetic transformation and gene expression. Blackwell scientific publications, Oxford, pp 46-49.

Béguin P (1983) Detection of cellulase activity in polyacrylamide gels using Congo red-stained agar replicas. Anal Biochem **131:**333-336

Bernfeld P (1949) Amylases, α and ß. Meth Enzymol **1:**149-150

Bischoff DS and Ordal GW (1991) Sequence and characterization of *Bacillus subtilis* CheB, a homolog of *Escherichia coli* CheY, and its role in a different mechanism of chemotaxis. J Biol Chem **266:**12301-12305

Bookstein C, Edwards CW, Kapp NV and Hulett FM (1990) The *Bacillus subtilis* 168 alkaline phosphatase III gene: impact of a *phoAIII* mutation on total alkaline phosphatase synthesis. J Bacteriol **172:**3730-3737

Borriss R, Bäumlein H and Hofemeister J (1985) Expression in *Escherichia coli* of a cloned ß-glucanase gene from *Bacillus amyloliquefaciens*. Appl Microbiol Biotechnol **22:**63-71

Borriss R, Süss K-H, Süss M, Manteuffel R and Hofemeister J (1986) Mapping and properties of *bgl* (ß-glucanase) mutants of *Bacillus subtilis*. J Gen Microbiol **132:**431-442

Borriss R, Manteuffel R and Hofemeister J (1988) Molecular cloning of a gene coding for thermostable beta-glucanase from *Bacillus macerans*. J Basic Microbiol **28:**3-10

Borriss R, Olsen O, Thomsen KK and von Wettstein D (1989) Hybrid *Bacillus* endo-(1,3-1,4)-ß-glucanases: construction of recombinant genes and molecular properties of the gene products. Carlsberg Res Commun **54:**41-54

Borriss R, Buettner K and Maentsaelae P (1990) Structure of the beta-1,3-1,4 -glucanase gene of *Bacillus macerans:* homologies to other beta-glucanases. Molec Gen Genet **222:**278-283

Bron S (1990) Plasmids **In:** Harwood CR and Cutting SM (eds) Molecular biological methods for *Bacillus*. John Wiley and Sons, England, pp 156-157

Bueno A, Vazquez de Aldana CR, Correa J, Villa TG and del Rey F (1990a) Synthesis and secretion of a *Bacillus circulans* WL-12 1,3-1,4-ß-D-glucanase in *Escherichia coli*. J Bacteriol **172:**2160-2167

Bueno A, Vazquez de Aldana, Correa J and del Rey F (1990b) Nucleotide sequence of a 1,3-1,4-ß-glucanase-encoding gene in *Bacillus circulans* WL-12. Nucleic Acid Res **18:**4248

Burbulys D, Trach KA and Hoch JA (1991) Initiation of sporulation in *B. subtilis* is controlled by a multicomponent phosphorelay. Cell **64:**545-552

Cantwell BA and McConnell DJ (1983) Molecular cloning and expression of a *Bacillus subtilis* ß-glucanase gene in *Escherichia coli* Gene **23:**211-219

Cantwell BA, Brazil G, Murphy N and McConnell DJ (1986) Comparison of expression of the endo-ß-1,3-1,4-glucanase gene from *Bacillus subtilis* in *Saccharomyces cerevisiae* from the CYC1 and ADH1 promoters. Curr Genet **11:**65-70

Cantwell BA, Sharp PM, Gormley E and McConnell DJ (1988) Molecular cloning of *Bacillus* ß-glucanases. **In:** J-P Aubert et al. (eds) Biochemistry and genetics of cellulose degradation. Academic Press, London, FEMS Symp **43:**181-201

Carozzi NB, Kramer VC, Warren GW, Evola S and Koziel MG (1991) Prediction of Insecticidal activity of *Bacillus thuringiensis* strains by polymerase chain reaction product profiles. Appl Environ Microbiol **57:**3057-3061

Chang S and Cohen S (1979) High frequency transformation of *Bacillus subtilis* protoplasts by plasmid DNA. Molec Gen Genet **168:**111-115

Chen Y, Huang X, Song D, Yang F and Zheng W (1992) Molecular cloning and expression of a *Bacillus subtilis bglS* gene in *Saccharomyces* cerevisiae. Curr Microbiol **25:**279-282

Clowes RC and Hayes W (1968) The control and expression of related genes in the *lac* operon. In: Clowes RC and Hayes W (eds) Experiments in microbial genetics. Blackwell scientific publications, Oxford, pp 57-59.

Corfield VA, Reid SJ, Bodmer J and Thomson JA (1984) A modified protoplast-regeneration protocol facilitating the detection of cloned exoenzyme genes in *Bacillus subtilis*. Gene **30:**17-22

Cornelis P, Digneffe G and Willemot K (1982) Cloning and expression of a *Bacillus coagulans* gene in *Escherichia coli*. Mol Gen Genet **186:**507-511

Dahl MK, Msadek T, Kunst F and Rapoport G (1991) Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. J Bacteriol **173:**2539-2547

Dahl MK, Msadek T, Kunst F and Rapoport G (1992) The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. J Biol Chem **267:**14509-14514

Dekker K, Sugiura A, Yamagata H, Sakaguchi K and Udaka S (1992) Efficient production of thermostable *Thermus thermophilus* xylose isomerase in *Escherichia coli* and *Bacillus brevis*. Appl Microbiol Biotechnol **36:**727-732

Diderichsen B, Wedsted U, Hedegaard L, Jensen BR and Sjoholm C (1990) Cloning of *aldB*, which encodes α-acetolactate decarboxylase, an exoenzyme from *Bacillus brevis*. J Bacteriol **172:**4315-4321

Dubnau D, Davidoff-Abelson R, Scher B and Cirigliano C (1973) Fate of transforming Deoxyribonucleic acid after uptake by competent *Bacillus subtilis*: phenotypic characterization of radiation-sensitive recombination-deficient mutatns. J Bacteriol **114:**273-286.

Dubnau D and Roggiani M (1990) Growth medium-independent genetic competence mutants of *Bacillus subtilis*. J Bacteriol **172:**4048-4055

Dubnau D (1991) Genetic competence in *Bacillus subtilis*. Microbiol Rev **55:**395-424

Duvall EJ, Williams DM, Lovett PS, Rudolph C, Vasantha N and Guyer M (1983) Chloramphenicol-inducible gene expression in *Bacillus subtilis*. Gene **24:**171-177

Ebisu S, Tsuboi A, Takagi H, Naruse Y, Yamagata H, Tsukagoshi N and Udaka S (1990) Conserved structures of cell wall protein genes among protein - producing *Bacillus brevis* strains. J Bacterial **172:**1312-1320

Ebisu S, Takagi H, Kadowaki K, Yamagata H and Udaka S (1992) Production of human epidermal growth factor by *Bacillus brevis* increased with use of a stable plasmid from *B. brevis* 481. Biosci Biotech Biochem **56:**812-813

Fuhrer DK and Ordal GW (1991) *Bacillus subtilis* CheN, a homolog of CheA, the central regulator of chemotaxis in *Escherichia coli*. J Bacteriol **173:**7443-7448

Godfrey T (1983) Comparison of key characteristics of industrial enzymes by type and source. **In:** Godfrey T and Reichelt J (eds) Industrial enzymology: the application of enzymes in industry. Macmillan, United Kingdom, pp 466-502

Gosalbes MJ, Pérez-González JA, González R and Navarro A (1991) Two ß-glycanase genes are clustered in *Bacillus polymyxa*: molecular cloning, expression, and sequence analysis of genes encoding a xylanase and an endo-ß-(1,3)-(1,4)-glucanase. J Bacteriol **173:**7705-7710

Gruber K, Tanahashi H, Tsuboi A, Tsukagoshi N and Udaka S (1988) Comparative study on the cell wall structure of protein-producing *Bacillus brevis*. FEMS Microbiol Lett **56:**113-118

Gryczan TJ, Contente S and Dubnau D (1978) Characterization of *Staphylococcus aureus* plasmids introduced by transformation into *Bacillus subtilis*. J Bacteriol **134:**318-329

Harwood CR, Williams DM and Lovett PS (1983) Nucleotide sequence of a *Bacillus pumilus* gene specifying chloramphenicol acetyl transferase. Gene **24:**163-169

Heijne G von (1988) Transcending the impenetrable: how proteins come to terms with membranes. Biochim Biophys Acta **947:**307-333

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

Henner DJ, Yang M, and Ferrari E (1988a) Localization of *Bacillus subtilis sacU*(HY) mutations to two linked genes with similarities to the conserved procaryotic family of two-component signalling systems. J Bacteriol **170:**5102-5109

Henner DJ, Ferrari E, Perego M and Hoch JA (1988b) Location of the targets of the *hpr-97, sacU32*(HY), and *sacQ36*(HY) mutations in upstream regions of the subtilisin promoter. J Bacteriol **170:**296-300

Herbert D, Phipps PJ and Strange RE (1971) Chemical analysis of microbial cells. Methods Microbiol **5B:**209-344

Heussen C and Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. Anal Biochem **102**: 196-202.

Hinchliffe E (1984) Cloning and expression of a *Bacillus subtilis* endo-1,3-1,4-ß-D-glucanase gene in *Escherichia coli* K12. J Gen Microbiol **130:**1285-1291

Hinchliffe E and Box WG (1984) Expression of the cloned endo-1,3-1,4-ß-glucanase gene of *Bacillus subtilis* in *Saccharomyces cerevisiae*. Curr Genet **8:**471-475

Hofemeister J, Kurtz A, Borriss R and Knowles J (1986) The ß-glucanase gene from *Bacillus amyloliquefaciens* shows extensive homology with that of *Bacillus subtilis*. Gene **49:**177-187

Horikoshi K and Ikeda Y (1977) Preparation of an alkaline protease. US patent No. 4 052 262

Horinouchi S and Weisblum B (1982) Nucleotide sequence and functional map of pE194, a plasmid that specifies inducible resistance to macrolide, lincosamide and streptogramin type B antibiotics. J Bacteriol **150:**804-814

Ichikawa Y, Yamagata H, Tochikubo K and Udaka S (1993) Very efficient extracellular production of cholera toxin B subunit using *Bacillus brevis*. FEMS Microbiol Lett **111:**219-224

Imanaka T, Fujii M, Aramori I and Aiba S (1982) Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J Bacteriol **149:**824-830

Inouye M and Halegoua S (1980) Secretion and membrane localization of proteins in *Escherichia coli*. CRC Crit Rev Biochem **7:**339-371

Ito K, Udaka S and Yamagata H (1992) Cloning, characterization and inactivation of the *Bacillus brevis lon* gene. J Bacteriol **174:**2281-2287

Johnson WC, Moran CP and Losick R (1983) Two RNA polymerase sigma factors from *Bacillus subtilis* discriminate between overlapping promoters for a developmentally regulated gene. Nature **302:**800-804

Klier AF and Rapoport G (1988) Genetics and regulation of carbohydrate catabolism in *Bacillus*. Ann Rev Microbiol **42:**65-95

Klier A, Msadek T and Rapoport G (1992) Positive regulation in the gram-positive bacterium: *Bacillus subtilis*. Annu Rev Microbiol **46:**428-459

Konishi H, Sato T, Yamagata H and Udaka S (1990) Efficient production of human α-amylase by a *Bacillus brevis* mutant. Appl Microbiol Biotechnol **34:**297-302

Kop J, Kopylov AM, Magrum L, Siegel R, Gupta R, Woese CR and Noller, HF (1984) Probing the structure of 16S ribosomal RNA from *Bacillus brevis*. J Biol Chem **259:**15287-15293

Krätzschmar J, Krause M and Marahiel MA (1989) Gramicidin S biosynthesis operon containing the structural genes *grsA* and *grsB* has an open reading frame encoding a protein homologous to fatty acid thioesterases. J Bacteriol **171:**5422-5429

Kunst F, Debarbouille M, Msadek T, Young M, Mauel C, Karamata D, Klier A, Rapoport G and Dedonder R (1988) Deduced polypeptides encoded by the *Bacillus subtilis sacU* locus share homology with two-component sensor-regulator systems. J Bacteriol **170**:5093-5101

Kunst F, Msadek T, Martin-Verstraete I, Debarbouille M, Arnaud M, Klier A and Rapoport G (1990) The sucrose system of *Bacillus subtilis*. **In:** Heslot H et al. (eds) Proc 6th International Symposium on genetics of industrial microorganisms, Strasbourg, pp 703-713

Kunst F, Msadek T and Rapoport G (1993) Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis*. **In:** Piggot PJ et al. (eds). Regulation of bacterial differentiation (pp 1-20)

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

Lancashire WE and Wilde RJ (1987) Secretion of foreign proteins by brewing yeasts. Proc Eur Brewery Convention, 21st Congress pp 513-520

Lepesant J-A, Kunst F, Lepesant-Kejzlarova J and Dedonder R (1972) Chromosomal location of mutations affecting sucrose metabolism in *Bacillus subtilis* Marburg. Mol Gen Genet **118**(2):135-160

Lloberas J, Querol E and Bernués J (1988) Purification and characterization of endo-ß-1,3-1,4-D-glucanase activity from *Bacillus licheniformis*. Appl Microbiol Biotechnol **29:**32-38

Lloberas J, Perez-Pons JA and Querol E (1991) Molecular cloning expression and nucleotide sequence of the endo-ß-1,3-1,4-D-glucanase gene from *Bacillus* licheniformis. Eur J Biochem **197:**337-343

Lovett PS and Keggins KM (1979) *B. subtilis* as a host for molecular cloning. Meth Enzymol **68:**342-357

Martin PAW, Lahr JR and Dean DH (1981) Transformation of *Bacillus thuringiensis* protoplasts by plasmid deoxyribonucleic acid. J Bacteriol **145:**980-983

Maru B and Yoshikawa H (1989) DNases involved in restriction and recombination in *B. subtilis* and its relatives. **In:** Maru B and Yoshikawa H (eds) *Bacillus subtilis*: Molecular biology and Industrial application. Elsevier publishers, Amsterdam, pp 123-139

Masui A, Fujiwara N, Takagi M and Imanaka T (1992) Cloning and nucleotide sequence of the regulatory gene, *degM*, for minor serine protease in *Bacillus subtilis*. J Ferm Bioeng **74:**230-233

McLaughlin JR, Murray CL and Rabinowitz JC (1981) Unique features in the ribosome binding site sequence of the gram-positive *Staphylococcus aureus* ß-lactamase gene. J Biol Chem **256:**11283-11291

Messing J (1979) A multipurpose cloning system based on single stranded DNA bacteriophage M13. Recomb DNA Tech Bull **2:**43

Millet J (1970) Characterization of proteinases excreted by *Bacillus subtilis* Marburg strain during sporulation. J Appl Bacteriol **33:**207-219

Mittenhuber G, Weckermann R and Marahiel MA (1989) Gene cluster containing the genes for tyrocidine synthetases 1 and 2 from *Bacillus brevis*: evidence for an operon. J Bacteriol **171**:4881-4887

Miyashiro S, Enei H, Hirose Y and Udaka S (1980) Effect of glycine and L-isoleucine on protein production by *Bacillus brevis* No. 47. Agric Biol Chem **44:**105-112

Mizukami M, Yamagata H, Sakaguchi K and Udaka S (1992) Efficient production of thermostable *Clostridium thermosulfurogenes* ß-Amylase by *Bacillus brevis.* J Ferm Bioeng **73:**112-115

Mongkolsuk S, Chang Y, Reynolds RB and Lovett PS (1983) Restriction fragments that exert promoter activity during post exponential growth of *Bacillus subtilis*. J Bacteriol **155:**1399-1406

Moran CP, Lang N, Legrice SFJ, Lee G, Stephens M, Sonenshein AL, Pero J and Losick R (1982) Nucleotide sequences that signal the initiation of transcription and translation in *Bacillus subtilis*. Molec Gen Genet **186:**339-346

Msadek T, Kunst F, Henner D, Klier A, Rapoport G and Dedonder R (1990) Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: Expression of the regulatory genes and analysis of mutations in *degS* and *degU*. J Bacteriol **172:**824-834

Msadek, T, Kunst F, Klier A and Rapoport G (1991) DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleitropic regulatory gene *degQ*. J Bacteriol **173:**2366-2377

Msadek T, Kunst F and Rapoport G (1993) Two-component regulatory systems. **In**: Sonenshein AL et al. (eds) *Bacillus subtilis* and other gram-positive Bacteria: Biochemistry physiology and molecular genetics. American society for microbiology, Washington, D.C. pp 729-745

Mukai K, Kawata M and Tanaka T (1990) Isolation and phosphorylation of the *Bacillus subtilis degS* and *degU* gene products. J Biol Chem **265:**20000-20006

Mukai K, Kawata M, Mukai M and Tanaka T (1992) Stabilization of phosphorylated *Bacillus subtilis* DegU by DegR. J Bacteriol **174:**7954-7962

Murphy N, McConnell DJ and Cantwell BA (1984) The DNA sequence of the gene and genetic control sites for the excreted *B. subtilis* enzyme ß-glucanase. Nucleic Acid Res **12:**5355-5367

Nagami Y and Tanaka T (1986) Molecular cloning and nucleotide sequence of a DNA fragment from *Bacillus natto* that enhances production of extracellular proteases and levansucrase in *Bacillus subtilis*. J Bacteriol **166:**20-28

Nakamura LK (1991) *Bacillus brevis* Migula 1900 taxonomy: reassociation and base composition of DNA. Int J Syst Bacteriol **41:**510-515

Neugebauer K, Sprengel R and Schaller H (1981) Penicillinase from *B. licheniformis:* a nucleotide sequence of the gene and implications for the biosynthesis of a secretory protein in a gram-positive bacterium. Nucleic Acids Res **9:**2577-2588

Nijenhuis te B (1977) Alkaline protease produced by a *Bacillus.* US Patent No. 4002 572.

Nogi Y and Horikoshi K (1990) A thermostable alkaline ß-1,3-glucanase produced by alkalophilic *Bacillus* sp. AG-430. Appl Microbiol Biotechnol **32:**704-707

Norris JR, Berkeley RC, Logan NA and O'Donnell AG (1981) The genera *Bacillus* and *Sporolactobacillus.* **In:** Starr MP et al. (eds) The prokaryotes, Vol II, Springer Berlin, pp 1724-1731

Ohmizu H, Sasaki T, Tsukagoshi N, Udaka S, Kaneda N and Yagi K (1983) Major proteins released by a protein-producing bacterium, *Bacillus brevis* 47, are derived from cell wall protein. J Biochem **94:**1077-1084

Olsen O and Thomsen KK (1991) Improvement of bacterial ß-glucanase thermostability by glycosylation. J Gen Microbiol **137:**579-585

Olsen O, Borriss R, Simon O and Thomsen KK (1991) Hybrid *Bacillus* (1,3-1,4)-ß-glucanases: engineering thermostable enzymes by construction of hybrid genes. Mol Gen Genet **225:**177-185

Pang AS-H, Nathoo SA and Wong S-L (1991) Cloning and characterization of a pair of novel genes that regulate production of extracellular enzymes in *Bacillus subtilis.* J Bacteriol **173:**46-54

Podvin L and Steinmetz M (1992) A *degU*-containing SPß prophage complements superactivator mutations affecting the *Bacillus subtilis degSU* operon. Res Microbiol **143:**559-567

Priest FG (1977) Extracellular enzyme synthesis in the genus *Bacillus*. Bacteriol Rev **41:**711-753

Quivey RG and Faustoferri RC (1992) *In vivo* inactivation of the *Streptococcus mutans recA* gene mediated by PCR amplification and cloning of a *recA* DNA fragment. Gene **116:**35-42

Ringquist S, Shinedling S, Barrick D, Green L, Binkley G, Stormo GD and Gold L (1992) Translation initiation in *Escherichia coli*: sequences within the ribosome-binding site. Mol Microbiol **6:**1219-1229

Roggiani M, Hahn J and Dubnau D (1990) Suppression of early competence mutations in *Bacillus subtilis* by *mec* mutations. J Bacteriol **172:**4056-4063

Sambrook J, Fritsch EF, Maniatis DF (1989) Molecular cloning: a laboratory manual. Cold Spring Harbour laboratory, Cold Spring Harbour, N.Y.

Sanger F, Nicklen S and Coulson AR (1977) DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA **74:**5463-5467

Seki T, Yoshikawa H, Takahashi H and Saito H (1987) Cloning and nucleotide sequence of *phoP*, the regulatory gene for alkaline phosphatase and phosphodiesterase in *Bacillus subtilis*. J Bacteriol **169:**2913-2916

Shaku M, Koike S and Udaka S (1980) Cultural conditions for protein production by *Bacillus brevis* No.47. Agric Biol Chem **44:**99-103

Shiga Y, Hasegawa K, Tsuboi A, Yamagata H and Udaka S (1992) Characterization of an extracellular protease inhibitor of *Bacillus brevis* HPD31 and nucleotide sequence of the corresponding gene. Appl Environ Microbiol **58:**525-531

Shimotsu H and Henner D (1986) Modulation of *Bacillus subtilis* levansucrase gene expression by sucrose and regulation of the steady-state mRNA level by *sacU* and *sacQ* genes. J Bacteriol **168:**380-388

Smith I, Dubnau E, Predich M and Rudner R (1992) Early *spo* gene expression in *Bacillus subtilis*: the role of interrelated signal transduction systems. Biochimie **74:**669-678

Strauch MA and Hoch JA (1993) Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Molec Microbiol **7:**337-342

Sykes RB and Nordstrom K (1972) Microiodometric determination of ß-lactamase activity. Antimicrob Agents Chemother **1:**94-99.

Tabor S and Richardson CC (1987) DNA sequence analysis with a modified bacteriophage T7 DNA polymerase. Proc Natl Acad Sci USA **84:**4767-4771

Takagi H, Kadowaki K and Udaka S (1989a) Screening and characterization of protein-hyperproducing bacteria without detectable exoprotease activity. Agric Biol Chem **53:**691-699

Takagi H, Miyauchi A, Kadowaki K and Udaka S (1989b) Potential use of *Bacillus brevis* HPD31 for the production of foreign proteins. Agric Biol Chem **53**:2279-2280

Takagi H, Kagiyama S, Kadowaki K, Tsukagoshi N and Udaka S (1989c) Genetic transformation of *Bacillus brevis* with plasmid DNA by electroporation. Agric Biol Chem **53:**3099-3100

Takagi M, Takada H and Imanaka T (1990) Nucleotide sequence and cloning in *Bacillus subtilis* of the *Bacillus stearothermophilus* pleiotropic regulatory gene *degT*. J Bacteriol **172:**411-418

Takagi H, Yamagata H and Udaka S (1992) Gene expression in *Bacillus brevis* under control of the *Lac* repressor-operator system from *Escherichia coli*. J Ferm Bioeng **74:**1-6

Takahashi W, Yamagata H, Yamaguchi K, Tsukagoshi N and Udaka S (1983) Genetic transformation of *Bacillus brevis* 47, a protein-secreting bacterium by plasmid DNA. J Bacteriol **156:**1130-1134

Takano T, Miyauchi A, Takagi H, Kadowaki K, Yamane K and Kobayashi S (1992) Expression of the Cyclodextrin Glucanotransferase gene of *Bacillus macerans* in *Bacillus brevis*. Biosci Biotech Biochem **56**:808-809

Takao M, Morioka T, Yamagata H, Tsukagoshi N and Udaka S (1989) Production of swine pepsinogen by protein-producing *Bacillus brevis* carrying swine pepsinogen cDNA. Appl Microbiol Biotechnol **30:**75-80

Takeuchi K, Nishino T, Oders M, Shiiuogaki H (1989) Alkaline proteases and microorganisms producing same. US Patent no. 4797 362

Tanaka T, Kawata M, Nagami Y and Uchiyama H (1987) *prtR* Enhances the mRNA level of the *Bacillus subtilis* extracellular proteases. J Bacteriol **169:**3044-3050

Tanaka T and Kawata M (1988) Cloning and characterization of *Bacillus subtilis iep*, which has positive and negative effects on production of extracellular proteases. J Bacteriol **170**: 3593-3600

Tanaka T, Kawata M and Mukai K (1991) Altered phosphorylation of *Bacillus subtilis* DegU caused by single amino acid changes in DegS. J Bacteriol **173:**5507-5515

Tezuka H, Yuuki T and Yabuuchi S (1989) Construction of a ß-glucanase hyperproducing *Bacillus subtilis* using the cloned ß-glucanase gene and a multi-copy plasmid. Agric Biol Chem **53:**2335-2339

Tsuboi A, Tsukagoshi N and Udaka S (1982) Reassembly *in vitro* of hexagonal surface arrays in a protein-producing bacterium *Bacillus brevis* 47. J Bacteriol **151:**1485-1497

Tsuboi A, Uchihi R, Tabata R, Takahashi Y, Hashiba H, Sasaki T, Yamagata H, Tsukagoshi N and Udaka S (1986) Characterization of the genes coding for two major cell wall proteins from protein-producing *Bacillus brevis* 47: Complete nucleotide sequence of the outer wall protein gene. J Bacteriol **168**:365-373

Tsuboi A, Uchihi R, Adachi T, Sasaki T, Hayakawa S, Yamagata H, Tsukagoshi N and Udaka S (1988) Characterization of the genes for the hexagonally arranged surface layer proteins in protein-producing *Bacillus brevis* 47: Complete nucleotide sequence of the middle wall protein gene. J Bacteriol **170**:935-945

Tsuchida T, Miyashiro S, Enei H and Udaka S (1980) Protein production in chemically defined medium by *Bacillus brevis* No.47. Agric Biol Chem **44:**2291-2295

Tsukagoshi N, Yamada H, Tsuboi A and Udaka S (1981) Effects of phosphate in medium on protein secretion in a protein-producing bacterium, *Bacillus brevis* 47. Appl Environ Microbiol **42:**370-374

Tsukagoshi N, Tabata R, Takemura T, Yamagata H and Udaka S (1984) Molecular cloning of a major cell wall protein gene from protein-producing *Bacillus brevis* 47 and its expression in *Escherichia coli* and *Bacillus subtilis*. J Bacteriol **158:**1054-1060

Tulin EE, Takagi H, Ueda S, Yamagata H, Yamane T and Udaka S (1991) Characteristics of batch culture of a recombinant *Bacillus brevis* excreting a foreign esterase. Biotechnol Bioeng **38:**1247-1252

Tulin EE, Ueda S, Yamagata H, Udaka S and Yamane T (1992) Effective extracellular production of *Bacillus stearothermophilus* esterase by pH-stat model fed-batch culture of recombinant *Bacillus brevis*. Biotechnol Bioeng **40:**844-850

Turgay K and Marahiel MA (1993) Two genes *gtcO* and *gtcE* encoding a response regulator and a sensor protein similar to those in "two component" regultory systems are localized at the 3' end of *grs* operon in *Bacillus brevis*. Abstract P32, Seventh International Conference on *Bacillus*, Paris

Udaka S (1976) Screening for protein-producing bacteria. Agric Biol Chem **40:**523-528

Udaka S and Yamagata H (1993) High-level secretion of heterologous proteins by *Bacillus brevis*. **In:** R Wu (ed) Methods in Enzymology. Part H. Academic Press, New York, pp 23-33

Vehmaanperä JO and Korhola MP (1986) Stability of the recombinant plasmid carrying the *Bacillus amyloliquefaciens* α-amylase gene in *B.subtilis*. Appl Microbiol Biotechnol **23:**456-461

Wang, LF and Doi RH (1987) Promoter switching during development and the termination site of the sigma-43 operon of *Bacillus subtilis*. Molec Gen Gent **207:**114-119

Wang L-F and Doi RH (1990) Complex character of *senS*, a Novel gene regulating expression of extracellular-protein genes of *Bacillus subtilis*. J Bacteriol **172:**1939-1947

Wong S-L, Wang L-F and Doi RH (1988) Cloning and nucleotide sequence of *senN*, a novel "*Bacillus natto*" (*B. subtilis*) gene that regulates expression of extracellular protein genes. J Gen Microbiol **134:**3269-3276

Wight CP, Daugulis AJ, Lau RH and White BN (1989) Stimulation of extracellular protein production in *Bacillus brevis* 47. Appl Microbiol Biotechnol **31:**338-341

Wright CP, Whitney GK, Daugulis AJ and White BN (1992) Enhancement and regulation of extracellular prtoein production by *Bacillus brevis* 47 through manipulation of cell culture conditions. Biotechnol Bioeng **40:**46-52

Yamada H, Tsukagoshi N and Udaka S (1981) Morphological alterations of cell wall concomitant with protein release in a protein-producing bacterium, *Bacillus brevis* 47. J Bacteriol **148:**322-332

Yamagata H, Takahashi W, Yamaguchi K, Tsukagoshi N and Udaka S (1984) Detection of plasmids in *Bacillus brevis* species and introduction of a plasmid into *B. brevis* 47, a protein-producing bacterium by cotransformation. Agric Biol Chem **48:**1069-1071

Yamagata H, Nakagawa K, Tsukagoshi N and Udaka S (1985) A stable plasmid vector and control of its copy number in *Bacillus brevis* 47 a protein-producing bacterium. Appl Environ Microbiol **49**:1076-1079

Yamagata H, Adachi T, Tsuboi A, Takao M, Sasaki T, Tsukagoshi N and Udaka S (1987) Cloning and characterization of the 5' region of the cell wall protein gene operon in *Bacillus brevis* 47. J Bacteriol **169:**1239-1245

Yamagata H, Nakahama K, Suzuki Y, Kakinuma A, Tsukagoshi N and Udaka S (1989) Use of *Bacillus brevis* for efficient synthesis and secretion of human epidermal growth factor. Proc Natl Acad Sci USA **86:**3589-3593

Yamagata H and Udaka S (1993) Very efficient secretion of human proteins by *Bacillus brevis* and enzymes for protein folding after secretion. Abstract L23, Seventh International Conference on *Bacillus*

Yamaguchi K, Ueda M and Udaka S (1993) Efficient production of *Bacillus stearothermophilus* α-amylase in *Bacillus brevis* by altering its signal peptide. Biosci Biotech Biochem **57:**1384-1386

Yang M, Ferrari E, Chen E and Henner DJ (1986) Identification of the pleiotropic *sacQ* gene of *Bacillus subtilis*. J Bacteriol **166:**113-119

Yang M, Shimotsu H, Ferrari E and Henner DJ (1987) Characterization and mapping of the *Bacillus subtilis prtR* gene. J Bacteriol **169:**434-437

Yoneda Y, Yamane K and Maru B (1973) Membrane mutation related to the production of extracellular α-amylase and protease in *Bacillus subtilis*. Biochim Biophys Res Commun **50:**765-770.

Yuuki T, Nomura T, Tezuka H, Tsuboi A, Yamagata H, Tsukagoshi N and Udaka S (1985) Complete nucleotide sequence of a gene coding for heat and pH-stable α-amylase of *Bacillus licheniformis:* Comparison of the amino acid sequences of three bacterial liquefying α-amylases deduced from the DNA sequences. J Biochem **98**:1147-1156

Zabeau M and Stanley KK (1982) Enhanced expression of cro-ß-galactosidase fusion proteins studied under the control of the P_R promoter of bacteriophage λ. EMBO J **1**:1217-1224

Zverlov VV and Velikodvorskaya GA (1990) Cloning the *Clostridium thermocellum* thermostable laminarinase gene in *Escherichia coli*; the properties of the enzyme thus produced. Biotechnol Lett **12:**811-816