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Cloning and sequencing the *degS-degU* operon from an alkalophilic *Bacillus brevis*

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Abstract The *sacU* region from an alkalophilic *Bacillus brevis* was cloned and sequenced. The two open reading frames of the *degS-degU* operon encode polypeptides that 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 cause hypersecretion of several extracellular enzymes in a *B. subtilis* rec⁻ strain as well as in a *B. subtilis sacU*(HY) strain.

Introduction

Bacillus subtilis can secrete a wide variety of extracellular enzymes. These include proteases, α-amylase, levansucrase and β-glucanase (Priest 1977). Many of these enzymes are of industrial importance. 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. 1988; Kunst et al. 1988; Tanaka and Kawata 1988).

The genes *degS* and *degU* are unlinked to any of the target genes and constitute an operon. 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. In this system one component accepts an environmental signal and transduces the information to the other component, resulting in activation of the target gene or cell machinery (Stock et al. 1989). Similarities were found between DegS and the histidine protein kinase family and between DegU and the response regulator proteins, suggesting that DegS might modify DegU through phosphorylation (Henner et al. 1988; Kunst et al. 1988; Msadek et al. 1990).

The *degS* and *degU* genes were initially defined by different classes of mutations leading either to deficiency of degradative enzyme syntheses (designated *degS* or *degU* mutations) or to overproduction of degradative enzymes [designated *degS*(HY) or *degU*(HY) mutations]. Several of these mutations have been characterized at the molecular level (Henner et al. 1988; Msadek et al. 1990). Both the *degS* and *degU* genes were found to be essential for the production of extracellular enzymes in *B. subtilis* and these gene products were found to exert control over degradative enzyme synthesis at a transcriptional level.

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; Tanaka et al. 1991). It was also found that mutations in *degS* or *degU* that lead to a deficiency of degradative enzyme synthesis promote the accumulation of the unphosphorylated form of DegU. It is in this form that DegU is necessary for competence gene expression (Dahl et al. 1992; Msadek et al. 1991). Thus DegU appears to be the first response regulator shown to have two active conformations controlling two distinct regulatory pathways: degradative enzyme production and expression of genetic competence (Msadek et al. 1993). The in-vivo equilibrium between the two forms of DegU is presumably regulated by DegS in response to an environmental signal that has not, as yet, been identified.

In this report we detail the cloning, nucleotide sequence and characterization of the *degS-degU* operon

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from *B. brevis* and evaluate its effect on extracellular enzyme production.

Materials and methods

Strains and plasmids

The *B. brevis* Alk 36 strain was isolated from soil (Louw et al. 1993). All the *B. subtilis* strains and plasmids were obtained from the *Bacillus* Genetic Stock Centre and the BGSC accession numbers are quoted: *B. subtilis* 1A46 *recE4 thr-5 trpC2*; *B. subtilis* 1A311 *amyE(+M) amyR2 metB5 pro(L) purF6 sacU9 str*; *B. subtilis* 1A201 *hisA1 sacA321 sacU42 trpC2*. The plasmid pPL703 is a promoter probe vector containing a promoter-less *cat* gene. The plasmid pPL708 is an expression vector derived from pPL703 containing the *Spo2* promoter (Mongkolsuk et al. 1983).

Media and qualitative tests

B. subtilis strains and *B. brevis* were grown in Luria broth (10 g tryptone, 5 g yeast extract and 10 g NaCl per litre) at pH 7.2 and pH 9.0, respectively. All strains were grown at 37° C.

DM3 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. 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 and neomycin were added at concentrations of 10 μg/ml when required, except for DM3 plates where chloramphenicol concentrations of 20 μg/ml were added.

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 β-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/l. 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%; $MgSO_4 \cdot H_2O$, 0.02%. The pH was adjusted to 7.2 with 1 M NaOH.

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

DNA manipulations and cloning procedures

Standard methods were used for chromosomal DNA isolation from *B. brevis* (Lovett and Keggins 1979) and plasmid DNA isolation (Gryczan et al. 1978), which was purified by caesium chloride-ethidium bromide equilibrium centrifugation according to the method of Sambrook et al. (1989). Partial *Sau*3A restriction of the *B. brevis* DNA was carried out and fragments in the size range of 3–6 kb were purified from agarose using the Geneclean kit (Bio101, Calif., USA.)

Restriction enzymes and T4 DNA ligase were from Boehringer Mannheim (Germany) and used according to the manufacturer's specifications.

Transformation

The polyethylene-glycol-induced protoplast transformation procedure of Chang and Cohen (1979) was used with the following modifications: stationary phase recipient cells were diluted 1:50 in Luria broth and grown with shaking to an optical density of 0.35 at 540 nm. Lysozyme treatment was continued for 45 min and 30% (w/v) polyethylene glycol (Merck approx. relative molecular mass 4000) was used for induction of DNA uptake.

Nucleotide sequencing

The *B. brevis* DNA insert could not be subcloned into Bluescript SK or KS as this proved lethal to the recipient *Escherichia coli* strain. A similar finding has been reported previously by Kunst et al. (1988). Sequencing templates were therefore generated by subcloning an internal *Hin*d III fragment of 0.4 kb and a series of *Sau*3A fragments from the *B. brevis* DNA insert into Bluescript-SK. Primers were then manufactured to complete the sequence in both directions.

The nucleotide sequences of the *degS-degU* genes were determined by the dideoxy-nucleotide-chain-termination method (Sanger et al. 1977) using the Sequenase II Kit (USB) according to the manufacturer's specifications. The nucleotide and deduced amino acid sequences were analysed using the Genetics Computer Group (GCG) software package (version 7.0). The GenBank/EMBL accession number is L15444.

Enzyme assays

Protease activity was determined using azocasein as substrate (Millet 1970). Alkaline protease activities were assayed at pH 9.5 in the presence of 20 mM ethylenediaminetetraacetic acid (EDTA) and total protease production was measured at pH 7.0. Activities were expressed in Anson units (Anson 1939) by using protease reference standards of known protease activity. α-Amylase was assayed according to the method of Bernfeld (1949). One unit was defined as the number of milligrams of maltose liberated in 3 min at 37° C by 1 ml of enzyme solution. β-Glucanase activities were defined as previously described (Louw et al. 1993) and levansucrase was determined according to the method of Kunst et al. (1988). One unit corresponds to 1 μmol glucose produced per minute.

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

In-vitro transcription and translation

Covalently closed circular plasmids were used as templates for the prokaryotic coupled transcription-translation system as recommended by the manufacturer (Amersham Corp., Arlington Heights, Ill., USA). Proteins were labelled with [^{35}S] methionine (specific activity, >37 TBq/mM) separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; 15% acrylamide to 0.4 bisacrylamide) and visualized by autoradiography.

Results

Cloning of the *degS-degU* genes from *B. brevis*

A genome library of *B. brevis* DNA was constructed by ligating approximately 0.5 μg of *B. brevis* size-fractionated DNA to 0.5 μg of *Bam*H1 linearized pPL703 and the ligation mix was transformed into *B. subtilis* 1A46. The transformants were plated onto the modif-

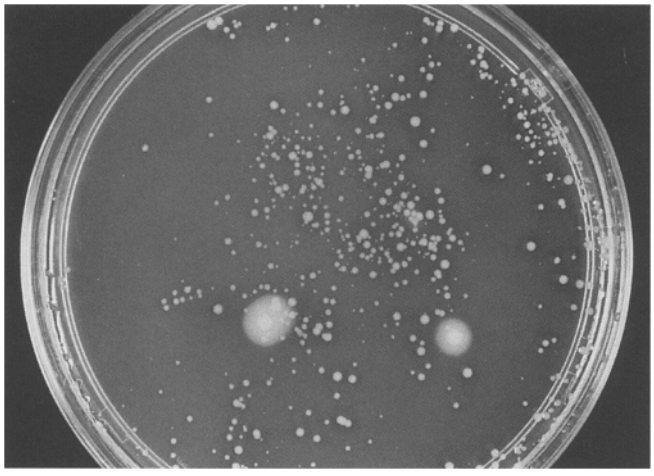

Fig. 1 Modified DM3 plate showing two colonies producing haloes, indicating increased protease production

ied 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 that gave rise to haloes on the DM3 plates, indicating increased protease production (Fig. 1). These three transformants, designated pML1, pML2 and pML3, as well as *B. subtilis* 1A46, were inoculated into protease screening medium and grown for 24 h. Protease production was determined at pH 9.5 (Table 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.

Nucleotide sequence determination

The 2.6 kb fragment of pML2 was sequenced in both directions using the dideoxy-chain termination method (Fig. 2). Two complete open-reading frames (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 *B. licheniformis* β-lactamase (Neugebauer et al. 1981), *B. 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 RBS, (5′-GGAGGG-3′), eight bases from the TTG start codon. The *degS* gene has no obvious RBS, 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 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. 1988; Kunst et al. 1988; Tanaka and Kawata 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. 3), whereas the *degU* genes have 71% identical amino acids (84% similarity) (Fig. 4).

Table 1 Extracellular protease activities of the three transformants and *Bacillus subtilis* 1A46 (*AU* Anson units, *EDTA* ethylenediaminetetraacetic acid)

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

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 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 that caused a Glu to Lys change at amino acid 107 in ORF2 (Henner et al. 1988). 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 ten- and 20-fold for total protease production and six- to 36-

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   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
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Fig. 2 Nucleotide sequence of part of the 2.6-kb *Eco*R1 fragment of pML2 containing the *Bacillus 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*.

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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
        ..* * . * .*   ** *.* .*..
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Fig. 3 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 *degS*200(HY) mutation in the *B. subtilis* gene is printed in *bold letters.*

fold for β-glucanase production by both *B. subtilis* strains 1A311 and 1A46 respectively. Levansucrase production increased two- and five-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 two-fold in 1A46 carrying the pML2 plasmid but were found to increase four-fold in the *B. subtilis* 1A311 strain, which carries the *sacU*(HY) mutation.

In-vitro expression of the *degS-degU* encoded polypeptides

The pML2 plasmid containing the *degS-degU* genes was incubated in-vitro with a coupled *E. coli* transcription-translation system (Materials and methods). The labelled polypeptides were separated by SDS-PAGE (Fig. 5). 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- and ORF2- encoded proteins (respectively 45 000 and 30 000 Da). 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 re-

Fig. 4 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.*

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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
        *...** *** ***.*.************************** .****** .*
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Table 2 Effect of the *degS-degU* operon on the production of extracellular enzymes in *B. subtilis* (*U* units)

Strains	Enzyme specific activity (U/mg of protein)				
	Protease (total)	β-Glucanase	α-Amylase	Levansucrase	
				Medium I[a]	Medium II[b]
B. subtilis					
1A46 (pML2)	1.20	18.3	0.85	0.78	2.68
1A46 (pPL708)	0.06	0.5	0.49	0.40	0.03
1A311 (pML2)	1.80	15.3	6.62	1.59	2.55
1A311 (pPL708)	0.17	2.6	1.60	0.30	1.95

[a] Cells grown in MMCH medium plus 2% sucrose (Kunst et al. 1988)
[b] Cells grown in Luria broth plus 1% sucrose

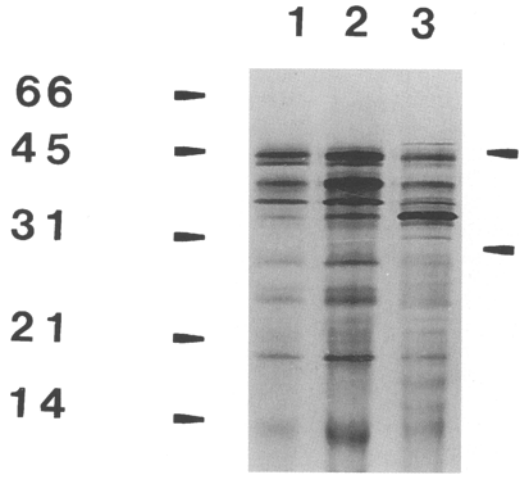

Fig. 5 Identification of DegS-DegU polypeptides. *Lanes*: *1*, vector pPL708; *2*, vector pPL703; *3*, recombinant plasmid pML2 containing both ORF1 and ORF2. Migration of standards and molecular mass in Kilodaltons is indicated in the *left margin*. *Arrowheads* in the *right margin* indicate bands of approximately 45 and 30 kDa

flect 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 that 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.

Discussion

The *degS-degU* genes were isolated from an alkalophilic *B. brevis* 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 giving rise to possible industrial application. 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, 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, from the published literature none 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* sp. 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 candidates for an autophosphorylation site were the Asp-152 and Asp-168 amino acids 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 may be 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; 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.

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