Glucosaminidase of *Bacillus subtilis*: cloning, regulation, primary structure and biochemical characterization

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The 90 kDa glucosaminidase protein was purified to apparent homogeneity from vegetative cells of *Bacillus subtilis* AC327, and then the corresponding gene was cloned into *Escherichia coli* in two inactive forms by standard procedures. Nucleotide sequencing of the glucosaminidase region revealed a monocistronic operon, (designated *lytD* = *cwlg*) encoding a 95.6 kDa protein, comprising 880 amino acid residues, which has a typical signal peptide. Moreover, another monocistronic operon (designated *pmi* = *orfX*), encoding a 35.4 kDa protein, was found upstream of the glucosaminidase gene. Expression of a *lytD*–*lacZ* fusion gene, driven by *lytD* regulatory sequences, was observed during the exponential growth phase. The introduction of a *sigD* null mutation greatly reduced (by about 95%) the expression of the fusion. Amino acid sequence analysis of the glucosaminidase showed two types of direct repeats, each type being present twice, in the N-terminal-to-central region of the glucosaminidase: these repeats probably represent the cell-wall-binding domain. Zymographic analysis revealed that the 90 kDa glucosaminidase is partly processed to several smaller proteins (35–39 kDa), retaining lytic activity. Processing of these proteins occurred between the N-terminal cell-wall-binding and C-terminal catalytic domains of the glucosaminidase, the site being located between the 569th and 606th codons of the glucosaminidase. Serial deletions from the N-terminus of the glucosaminidase revealed that the loss of more than one repeating unit drastically reduces its lytic activity toward cell walls. The *lytD* gene product, in either an intact or a truncated form, was found to be lethal for *E. coli*, and the N-terminally truncated glucosaminidase proteins, produced in *E. coli*, were very unstable. The partially purified glucosaminidase from *B. subtilis* was found to be very unstable at low ionic strength at 37 °C, but this instability was overcome by the addition of either SDS-purified cell wall or protease inhibitor (PMSF) to the enzyme or after purification of the glucosaminidase to apparent homogeneity.

Keywords: *Bacillus subtilis*, autolysin, endo- β -N-acetylglucosaminidase, σ^P , protein domain

INTRODUCTION

The rod-shaped, Gram-positive soil bacterium, *Bacillus subtilis*, produces a complement of enzymes capable of dissolving the shape-maintaining and stress-bearing

Abbreviations: CBB, Coomassie Brilliant Blue; S/FMP, slow/fast-migrating protein.

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peptidoglycan layer of its own cell wall (Rogers *et al.*, 1980; Doyle & Koch, 1987; Shockman & Hölte, 1994). Some of these peptidoglycan hydrolases can trigger cell lysis: therefore, they can truly be called 'autolysins' or 'suicide enzymes' (Rogers *et al.*, 1980). Autolysins have been implicated in several important cellular processes, such as cell wall turnover, cell separation, competence and flagellation (motility), in addition to cell lysis, and they act as pacemaker and spacemaker enzymes for cell wall growth (Rogers *et al.*, 1980; Fein & Rogers, 1976; Fein,

1979; Ayusawa *et al.*, 1975; Pooley & Karamata, 1984). Therefore, fine tuning of autolysin activity through efficient and strict regulation is a must for bacterial survival (Höltje & Tuomanen, 1991).

Two major vegetative-phase autolysins [a 50 kDa *N*-acetylmuramoyl-L-alanine amidase (amidase) and a 90 kDa endo- β -*N*-acetylglucosaminidase (glucosaminidase)] were initially purified and characterized from *B. subtilis* (Herbold & Glaser, 1975; Rogers *et al.*, 1984). Recently, the gene encoding the 50 kDa amidase (CwlB = LytC) was cloned and studied at the molecular level (Kuroda & Sekiguchi, 1991; Lazarevic *et al.*, 1992). The *cwlB*(*lytC*) gene is part of an operon encoding a putative lipoprotein (*lytA* = *lppX*), a modifier protein (*lytB* = *cwlA*), and the *cwlB* gene, in that order (Kuroda *et al.*, 1992a; Kuroda & Sekiguchi, 1993). Transcription of this operon proceeds from a distal σ^A -type and a proximal σ^D -type promoter, the latter transcripts being predominant in the exponential growth phase (Kuroda & Sekiguchi, 1993). At least two pleiotropic mutations, *flaD*(= *sin*) and *degS*-*degU*, affect the transcription of the *cwlB* operon (Kuroda & Sekiguchi, 1993; Tokunaga *et al.*, 1994). Previously, we reported the cloning of a part of the glucosaminidase gene, *lytD*(= *cwlG*), its inactivation on the chromosome, and the construction of a CwlB- and LytD-deficient strain (Rashid *et al.*, 1993). A search for the physiological functions of CwlB and LytD revealed that CwlB is responsible for cell lysis at the stationary phase (Kuroda & Sekiguchi, 1991) and that both proteins, but only in concert, are required for the motility function (Rashid *et al.*, 1993).

Several other amidase genes have been cloned from the genus *Bacillus*. From *B. subtilis*, two prophage-encoded amidase genes (*cwlA* and *xlyA*) (Kuroda & Sekiguchi, 1990; Foster, 1991; Longchamp *et al.*, 1994), and a sporulation-specific amidase gene (*cwlC*) (Kuroda *et al.*, 1993) have been cloned, in addition to *cwlB*. From *B. licheniformis*, two amidase genes (*cwlM* and *cwlL*), and from *Bacillus* sp., a cell-wall hydrolase (probably an amidase) gene have also been cloned and studied (Kuroda *et al.*, 1992b; Oda *et al.*, 1993; Potvin *et al.*, 1988). Evidence has accumulated that these amidases are composed of a cell-wall-binding domain and a catalytic domain (Kuroda *et al.*, 1992b). On the basis of the amino acid sequence similarity in their catalytic domains, these amidases can be classified into two groups. Class I includes CwlA, CwlL, XlyA and *Bacillus* sp. amidase, and class II includes CwlB, CwlC and CwlM. The cell-wall-binding domains of these amidases contain several (usually two or three) tandemly repeated sequences. Interestingly, three tandemly repeated sequences have also been observed in the non-catalytic cell-wall-binding proteins, LytB and WapA (Kuroda *et al.*, 1992a; Foster, 1992). However, until now, the primary structure of the glucosaminidase has remained unexplored. To further our understanding of the regulation and primary structure of the glucosaminidase, we report here the nucleotide sequence, transcriptional regulation, protein structure and biochemical characterization of the major glucosaminidase of

B. subtilis. In addition, we present the nucleotide sequence of the adjacent upstream monocistronic operon.

Recently, Margot *et al.* (1994) independently reported the cloning and nucleotide sequence of the glucosaminidase gene (glucosaminidase and adjacent upstream genes designated as *lytD* and *pml*, respectively), using an entirely different approach. For consistency and ease of comparison with these data, we use the nomenclature *lytD*, and not *cwlG* (Rashid *et al.*, 1993) in this paper.

METHODS

Bacterial strains, plasmids and phage. The strains, plasmids and phage used in this study are listed in Table 1. Plasmid pMW118 is a low-copy vector (five copies per cell) for *E. coli*, and plasmid pHY300PLK is a shuttle vector for *E. coli* and *B. subtilis* (Takara). Plasmid pDEB1 is a transcriptional *lacZ* fusion vector (Schreier & Sonenshein, 1986).

Media and culture conditions. *B. subtilis* strains were grown in either bacto-peptone (BP) medium (Rashid *et al.*, 1993) or Schaeffer's sporulation medium (Schaeffer *et al.*, 1965) at 28 °C with shaking. *E. coli* was grown in LB medium at 37 °C. If necessary, ampicillin (Ap) and/or chloramphenicol (Cm) were added to final concentrations of 100 and 5 $\mu\text{g ml}^{-1}$, respectively. Tetracycline (Tc) was added to a final concentration of 20 $\mu\text{g ml}^{-1}$ for the selection of multicopy plasmids and 8 $\mu\text{g ml}^{-1}$ for the selection of a single chromosomal copy.

Purification of the glucosaminidase. *B. subtilis* AC327 was cultured in 110 l BP medium (Rashid *et al.*, 1993), in a 200 l fermenter, at 28 °C to OD₆₆₀ 2.5 (UV-1200, Shimadzu). The culture was then rapidly cooled to 4 °C and the cells, after centrifugation at 10000 *g* for 5 min, were stored at -80 °C. About 300 g wet weight (equivalent to 50 l culture) frozen cells was melted on ice and then washed with 500 ml buffer T solution (25 mM Tris/HCl, pH 7.2). The cells were suspended in 500 ml 5 M LiCl/buffer T solution and then incubated at 0 °C for 60 min. The supernatant (500 ml), obtained by centrifugation at 27000 *g* for 10 min, was used as a crude extract (extract A). Extract A was then diluted 10-fold with buffer T, followed by the addition of *M. luteus* cell wall (2.0 g) treated with SDS, and then the mixture was incubated with slow stirring at 0 °C for 30 min. After washing with a 0.5 M LiCl/buffer T solution, the cell wall pellet was extracted with 100 ml 5 M LiCl/buffer T solution at 0 °C for 30 min. The supernatant (100 ml) was used as a cell wall extract (extract B). Extract B was then concentrated by ultrafiltration to approximately 7.0 ml. The concentrated extract (extract C) was applied to a column (2.0 $\times$ 150 cm) of Toyopearl HW-55 Superfine (Tosoh) equilibrated with 2 M LiCl/buffer T. The column was eluted with the same buffer. Fractions containing cell-wall-lytic activity were pooled and used as the purified enzyme sample (HW-55 fraction).

Preparation of cell walls and assaying cell-wall-lytic activity. Cell walls of *B. subtilis* 168S and *M. luteus* ATCC4698 were prepared essentially as described previously (Fein & Rogers, 1976; Kuroda & Sekiguchi, 1990). Lytic activity was measured as described by Rogers *et al.* (1984). The reaction mixture (5 ml), consisting of *M. luteus* cell wall (to a final OD₅₄₀ 0.3) in a 50 mM sodium succinate/HCl (pH 5.5) buffer and the enzyme extract, was incubated with slow shaking at 37 °C. The final salt concentration of the reaction mixture was maintained at 0.25 M LiCl. One unit (U) of enzyme activity was defined as the amount of the enzyme necessary to decrease OD₅₄₀ by 0.001 in 1 min.

Determination of reducing sugar. The release of free reducing groups during the enzymic reaction was assayed by the

Table 1. Bacterial strains, phage and plasmids

	Genotype/phenotype	Source/reference
B. subtilis		
168	<i>trpC2</i>	This laboratory
168S	<i>trpC2 strA smo-1</i>	Sekiguchi <i>et al.</i> (1988)
AC327	<i>purB his-1 smo-1</i>	Sekiguchi <i>et al.</i> (1988)
AG1	<i>purB his-1 smo-1</i> <i>lytD::cat</i>	Rashid <i>et al.</i> (1993)
AN8	<i>purB his-1 smo-1</i> <i>cw/B::cat</i>	Kuroda & Sekiguchi (1991)
AGB1	<i>purB his-1 smo-1</i> <i>lytD::cat cw/B::tet</i>	Rashid <i>et al.</i> (1993)
168G1	<i>trpC2 lytD::cat</i>	This study
168N8	<i>trpC2 cw/B::cat</i>	This study
168GB1	<i>trpC2 lytD::cat</i> <i>cw/B::tet</i>	This study
1A680	<i>trpC2 lacA17 lacR1</i>	Errington & Vogt (1990); BGSC*
680GL20	<i>trpC2 lacA17 lacR1</i> <i>lytD-lacZ Cm^r</i>	This study
680GL22	<i>trpC2 lacA17 lacR1</i> <i>lytD-lacZ Cm^r</i> <i>sigD::tet</i>	This study
M. luteus		
ATCC4698		Sigma
E. coli		
JM109	<i>recA1 Δ(lac-proAB)</i> <i>endA1 gyrA96 thi1</i> <i>hsdR17 relA1 supE44</i> [F': <i>traD36 proAB</i> <i>lacI^qΔM15</i>]	Takara
JM103	<i>supE sbcB15 hsdR4 rpsL</i> <i>thi Δ(lac-proAB)</i> [F': <i>traD36 proAB</i> <i>lacI^qΔM15</i>]	Takara
Phage		
M13mp18		Takara
Plasmid		
pUC118/119	Ap ^r Lac ⁺	Takara
pMW118	Ap ^r Lac ⁺	Takara
pHY300PLK	Ap ^r Tc ^r	Takara
pEH4	Ap ^r 3' <i>lytD</i> †	Rashid <i>et al.</i> (1993)
pWCEV4	Ap ^r 5' <i>lytD</i> ‡	This study
pHYG	Ap ^r Tc ^r <i>lytD</i>	This study
pHYEB	Ap ^r Tc ^r <i>tytD</i> §	This study
pDEB1	Ap ^r Cm ^r	Schreier & Sonenshein (1986); BGSC
pDEB1G	Ap ^r Cm ^r <i>lytD-lacZ</i>	This study
pUCTC	Ap ^r Tc ^r	Rashid <i>et al.</i> (1993)
pSD	Ap ^r <i>sigD</i>	This study
pSDTC	Ap ^r <i>sigD::tet</i>	This study

* *Bacillus* Genetic Stock Center, Ohio State University.† 5'-Terminal 440 bp of the *lytD* gene are deleted.‡ 3'-Terminal 424 bp of the *lytD* gene are deleted.

§ C-Terminal 187 amino acids of LytD are replaced by 19 alien amino acids.

Thompson & Shockman (1968) modification of the method of Park & Johnson using *N*-acetylglucosamine as the standard.

Cloning of the 5'-terminal-to-upstream region of the glucosaminidase gene. Southern hybridization analysis (Sambrook *et al.*, 1989) of *B. subtilis* AC327 chromosomal DNA with the α -³²P-labelled 0.4 kb *Pst*I segment as a probe, which is internal to the glucosaminidase gene (Rashid *et al.*, 1993), indicated that a 4.0 kb *Cl*aI-*Eco*RV DNA segment contains the 5'-terminal-to-upstream region of the glucosaminidase gene. Thus, *Cl*aI/*Eco*RV-digested fragments of *B. subtilis* AC327 chromosomal DNA were separated by agarose gel electrophoresis, and 3.5 to 4.5 kb fragments were purified with a Gene-Clean kit II (Bio 101). The DNA fragments were then ligated into the *Acc*I-*Sma*I site of pMW118. After transformation of *E. coli* JM109 with the ligated DNA, Ap^r colonies were selected and colony hybridized (Sambrook *et al.*, 1989) with the α -³²P-labelled 0.4 kb *Pst*I fragment of pEH4 (Rashid *et al.*, 1993) as a probe. Nine positive clones were isolated from 300 Ap^r colonies harbouring an identical plasmid, pWCEV4 (plasmid pMW118 containing a 4.1 kb insert).

Construction of pEH4- and pWCEV4-derivatives for sequencing. pEH4-derivatives pEVH, pBP, pHHII, pBB, pHIIP, pPP, pPH and pHI were constructed by subcloning 2.4 kb *Eco*RV-*Hind*III, 1.8 kb *Bam*HI-*Pst*I, 1.2 kb *Hind*III-*Hinc*II, 0.8 kb *Bam*HI, 0.54 kb *Hinc*II-*Pst*I, 0.4 kb *Pst*I, 0.23 kb *Pst*I-*Hind*III and 0.33 kb *Hinc*II fragments from pEH4 (Rashid *et al.*, 1993) into the respective sites of pUC118 (Fig. 2). pWCEV4 derivatives pCCS and pWSEV were obtained by subcloning the 2.3 kb *Sph*I fragment of pWCEV4 into the *Sph*I site of pUC118, and by self-circularization of the vector fragment upon *Sph*I digestion, respectively. pCPS was obtained by self-circularization of the pCCS vector fragment upon *Pst*I digestion, and pCSH was a pUC118-derivative containing the 1.2 kb *Hind*III fragment of pWSEV. To construct plasmid derivatives for sequencing of the 1.2 kb *Hind*III-*Sph*I fragment of pWCEV4 (Fig. 2), the random subcloning approach was taken. The 1.2 kb *Hind*III fragment of pWSEV was digested with *Sau*3AI or *Msp*I, followed by ligation with *Bam*HI- or *Acc*I-digested and de-phosphorylated pUC118, respectively. After transformation of *E. coli*, colonies were randomly chosen as sequencing templates.

DNA sequencing. Nucleotide sequencing was performed with double-stranded plasmid DNA as templates, using M13 universal (M4) and reverse (RV) primers (Takara). Sequencing was performed with an Applied Biosystems 373A DNA Sequencer using a Taq Dye-Deoxy Terminator Cycle Sequencing kit. To fill in the gap or to determine the opposite strand sequence (where needed), oligonucleotide primers were synthesized with an Applied Biosystems 391S DNA synthesizer. The sequences of both strands were determined for the 4.2 kb *Hind*III-*Eco*RV fragment, and partially for the *tagABC* and *gerB* regions.

Construction of a *lytD-lacZ* fusion strain and introduction of a *sigD* null mutation in the *lytD-lacZ* background. pWCEV4 was digested with *Hinc*II and *Hind*III and the 1.7 kb *Hind*III fragment was subcloned into pUC118, resulting in plasmid pUC1.7 (Fig. 2). The 1.7 kb *Hind*III fragment of pUC1.7, containing the 5'-terminus and upstream regulatory sequences of the glucosaminidase gene, was then inserted into the unique *Hind*III site of plasmid pDEB1 (Schreier & Sonenshein, 1986). The orientation of the insert in one of the resultant plasmids, pDEB1G, was determined from the distance between the unique *Pst*I site of the vector and the unique *Sph*I site of the insert. Competent cells of *B. subtilis* 1A680 (Errington & Vogt, 1990)

were transformed with the integrative plasmid, pDEB1G, and Cm^r transformants were selected. One of the Cm^r transformants was designated *B. subtilis* 680GL20.

Plasmid pSD is a pUC119-derivative containing the entire *sigD* gene and was constructed as follows. A 0.59 kb *SalI*–*EcoRI* fragment containing the 5'-terminus of *sigD* from pJH6-2 (Helmann *et al.*, 1988) and a 0.91 kb *EcoRI*–*PstI* fragment containing the 3'-terminus of *sigD* from pLM3 (Màrquez *et al.*, 1990) were inserted into the *PstI*–*SalI* site of pUC119 by triple ligation. A 1.5 kb *EcoRI*–*HindIII* DNA fragment containing a Tc^r gene, *tet*, from pUCTC (Rashid *et al.*, 1993) was blunt-ended with the Klenow fragment and then inserted into the Klenow-treated unique *EcoT14I* site of pSD, resulting in plasmid pSDTC in which the *sigD* gene is disrupted at the 59th codon. Plasmid pSDTC was digested with *AvaI* and *AccI* and the 3.0 kb *AccI* fragment containing the *sigD::tet* gene cluster was purified with Gene-Clean. *B. subtilis* 680GL20 was transformed with the purified DNA, followed by selection for Tc^r. One Tc^r transformant was designated *B. subtilis* 680GL22. The integration was confirmed by the filamentation and non-motility of the growing cells of strain 680GL22 (Màrquez *et al.*, 1990).

β -Galactosidase assay. The β -galactosidase assay was performed as described by Shimotsu & Henner (1986). *B. subtilis* cells were centrifuged for 5 min in a microcentrifuge and then stored at –80 °C. The frozen cells were suspended in 1 ml Z-buffer (Miller, 1972) containing 300 μ g lysozyme ml^{–1} and 0.01% (v/v) Triton X-100, and then incubated at 37 °C for 5 min after brief (5 s) vortexing. Samples were assayed for β -galactosidase activity by the method of Miller (1972).

Expression of the glucosaminidase and a truncated form of the glucosaminidase (tLytD/G10) in *B. subtilis* AG1. The 3.9 kb *EcoRI* (multicloning site, mcs)–*BamHI* fragment of pWCEV4 (Fig. 2), containing the *pml* (*orfX*) and *tlytD* (C-terminally deleted for 187 codons) genes was cloned into the respective sites of pHY300PLK, resulting in plasmid pHYEB. After amplification in *E. coli*, pYEB was introduced into *B. subtilis* AG1 by the conventional method (Anagnostopoulos & Spizizen, 1961). To construct a plasmid, pHYG, which expresses the intact glucosaminidase, the 0.8 kb *BamHI* fragment of pEH4 (Fig. 2) was ligated with *BamHI*-cut and dephosphorylated pYEB. *B. subtilis* AG1 was transformed with the ligated solution directly by the protoplast transformation method (Akamatsu & Sekiguchi, 1987a, b). Proper orientation of the insert in pYHG was confirmed by the sizes of the *AccI* and *AccIII* fragments.

B. subtilis AG1 harbouring pY300PLK, pYEB or pYHG was cultured in 200 ml BP medium with 20 μ g tetracycline ml^{–1} at 28 °C for 12 h. After harvesting the cells by centrifugation, protein samples (extracts A and B) were prepared as previously described (Rashid *et al.*, 1993).

Construction of plasmids producing N-terminally truncated glucosaminidases in *E. coli*. The basic strategy used to construct truncated glucosaminidase-producing plasmids was to fuse in-frame restricted and modified (if necessary) DNA fragments containing truncated glucosaminidase genes into the multicloning sites of pUC vectors. Thus, synthesis of the modified glucosaminidases uses the promoter, RBS and initiation codon of the *E. coli lacZ* gene, with the addition of several alien amino acids to their N-termini, and are inducible with IPTG.

A DNA fragment containing the 5'-terminus of the glucosaminidase gene without the codon of the signal peptide was obtained by PCR with forward primer, 5' **GAGCAAGCTTTGCCGCGTATACCGACTACTCA** 3', and reverse primer, 5' **CTGAGCTGCTCCGTAAGCTT** 3'; the

boldface type indicates the inserted sequence and the underlining indicates a *HindIII* site. pG1, expressing protein G1, was then constructed by subcloning a 360 bp *HindIII* fragment from the PCR product which contains codons 28–148 of the glucosaminidase gene into the *HindIII* site of pEH4 (Fig. 2). This resulted in the addition of seven alien amino acids instead of the 27 amino acid residues for the signal peptide of the glucosaminidase.

pG2, expressing protein G2, was constructed by subcloning the Klenow-treated 2.4 kb *HindIII*–*EcoRV* fragment of pEH4 into the *HincII* site of pUC118. A plasmid, pG3, expressing protein G3 was obtained by inserting a 2.2 kb *XcmI*–*EcoRV* fragment of pEH4, which was blunt-ended with T4 polymerase, into the *SmaI* site of pUC119. The 1.8 kb *PstI*–*EcoRV* fragment of pEH4, after being blunt-ended with T4 polymerase, was inserted into the *SmaI* site of pUC118 to obtain plasmid pG4. pG5 and pG6 were obtained by subcloning the Klenow-treated 1.5 kb *BsrFI*–*EcoRV* and 1.24 kb *AvaI*–*EcoRV* fragments of pEH4 into the *SmaI* sites of pUC119 and pUC118, respectively. The 1.0 kb *AvaII*–*EcoRV* fragment, which was end-filled by Klenow treatment, was inserted into the *SmaI* site of pUC118 to obtain pG8. The 1.17 kb *PvuII*–*EcoRV* and 0.8 kb *BamHI* fragments were used to obtain plasmids pG7 and pG9 by subcloning them into the *SmaI* and *BamHI* sites of pUC119 and pUC118, respectively.

Controlled expression of modified glucosaminidases in *E. coli*. *E. coli* JM109 harbouring different plasmids was cultured in LB medium plus 50 μ g ampicillin ml^{–1} with shaking at 37 °C. IPTG (final concentration, 0.1 mM) was added when the cell density reached OD₆₆₀ 0.6. After further incubation for 1 h, the cells were quickly harvested by centrifugation. If incubation was continued for more than 1 h after the addition of IPTG, *E. coli* cells were found to lyse rapidly in the case of all plasmids except pG8 and pG9. Thus, even in the absence of signal sequence, modified glucosaminidases were very toxic for *E. coli*. Different samples were prepared as follows. For preparing an SDS-extract sample (extract S), 1 ml culture (OD₆₆₀ 1) was pelleted by centrifugation, and the cell pellet was then suspended in a mixture of distilled water (100 μ l) and SDS-PAGE sample buffer (100 μ l). After boiling at 100 °C for 5 min, cell debris was pelleted by centrifugation and the supernatant was used as extract S. For LiCl-extracts, cell pellets from 50 ml cultures were suspended in 5 ml 5 M LiCl/buffer T solution, followed by ultrasonication (30 s, 10 $\times$). After separation of cell debris, the supernatant (0.5 ml) was used as a crude extract (extract A). The rest of the crude extract (4.5 ml) was used for cell-wall-binding assays (20 mg *M. luteus* cell wall). Cell-wall-binding proteins were re-extracted with 1 ml 5 M LiCl/buffer T solution and the supernatant was used as a cell-wall extract (extract B).

SDS-PAGE and zymography. SDS-PAGE of proteins was performed in 12% (w/v) polyacrylamide gels as described by Laemmli (1970). Zymography was performed essentially as described by Leclerc & Asselin (1989), using an SDS-polyacrylamide gel containing 0.1% (w/v) *M. luteus* cell wall as a substrate.

Assays for the stability and stimulation of the glucosaminidase and effect of inhibitors on the enzyme activity.

The stability of the glucosaminidase was assayed by incubating the enzyme (extract B, 100 μ l) in a 0.25 M LiCl/buffer T (25 mM Tris/HCl, pH 7.2) solution at 37 °C with slow shaking for various times. The purification of LytB and the assay for stimulation toward the glucosaminidase were performed according to Kuroda & Sekiguchi (1992) and Kuroda *et al.* (1992a), respectively. The effect of the inhibitors EDTA, 2-mercaptoethanol, PMSF, diisopropyl fluorophosphate, diethyl

pyrocarbonate and *N*-ethylmaleimide on the enzyme activity was determined using *M. luteus* cell wall as a substrate.

Swarm plate assay. Swarm plate assay was performed on soft agar plates (0.3% agar). Tryptone and minimal medium swarm plates containing mannitol as an attractant were prepared as described by Rosario *et al.* (1994) and Márquez *et al.* (1990), respectively. Cells from overnight cultures (1 µl) were spotted onto swarm plates, and swarm formation was allowed to proceed for 8–12 h at 37 °C.

Other methods. Transformation of *E. coli* was performed as described by Sambrook *et al.* (1989). *B. subtilis* transformation was performed by the competent cell method (Anagnostopoulos & Spizizen, 1961), and the protoplast transformation method (Akamatsu & Sekiguchi, 1987a, b). Cell lysis in buffer was measured according to the method of Svarachorn *et al.* (1989). Southern and colony hybridizations were performed as described by Sambrook *et al.* (1989). Protein was determined using BCA protein assay reagent (Pierce), with BSA as a standard. Assays for competence, sporulation and germination of autolysin-deficient strains were performed according to the methods of Kuroda & Sekiguchi (1991).

RESULTS

Purification of the glucosaminidase to apparent homogeneity

The major glucosaminidase of *B. subtilis* AC327 was extracted from intact cells with a 5 M LiCl/buffer T solution, and the resultant crude enzyme (extract A) was bound to and eluted from *M. luteus* cell walls to obtain a partially purified enzyme (extract B). Then extract B was concentrated by ultrafiltration and subjected to gel filtration on a Toyopearl HW-55 column. On gel filtration, the specific activity was 26000 U mg⁻¹, having increased about 2200-fold, and the enzyme recovery was 11% (Table 2). The homogeneity of the glucosaminidase was determined by SDS-PAGE, which gave a single band of 90 kDa (Fig. 1a, lane 4). The glucosaminidase was present in a much lower amount compared to the major amidase in vegetative cells (as judged by SDS-PAGE of LiCl-extractable proteins), which is consistent with the previous study (Fan & Beckman, 1972). The purified glucosaminidase was added to the *M. luteus* cell walls and the mixture was incubated at 37 °C for various times. The products resulting from the enzymic hydrolysis were investigated by the modified Park & Johnson method

Fig. 1. SDS-PAGE of proteins at different steps of purification of the glucosaminidase (a), and zymography of the purified glucosaminidase (LytD) (b). Lane M, protein standards [1.5 µg of each protein for panel (a) and 1.0 µg of each protein for panel (b), BioRad], the molecular masses of which are shown at the left. Lane 1, 5 M LiCl crude extract (extract A, 120 µg protein); lane 2, *M. luteus* cell wall extract (extract B, 37.5 µg); lane 3, concentrated extract (extract C, 9 µg); lanes 4 and 5, LytD fractions obtained by Toyopearl HW-55 column chromatography (HW-55 fractions, 3 and 2 µg, respectively). Electrophoresis was in 12% SDS-polyacrylamide gels and proteins were stained with CBB. The zymographic gel (b) contained 0.1% (w/v) *M. luteus* cell wall as the enzyme substrate. After SDS-PAGE, the glucosaminidase protein in the zymographic gel (b, lane 5) was renatured by treatment with buffer T (25 mM Tris/HCl, pH 7.2) and 1% (v/v) Triton X-100. Before being photographed, the zymographic gel (b, lane 5) was stained with methylene blue (0.1%, w/v) in 0.01% (w/v) KOH.

(Thompson & Shockman, 1968). Free reducing groups were liberated during the enzyme reaction (data not shown). Therefore, this protein is a glycosidase. Surprisingly, zymographic analysis of the purified glucosaminidase revealed another lytic activity at about 35 kDa in addition to the 90 kDa activity (Fig. 1b, lane 5), but no protein band corresponding to this 35 kDa activity on Coomassie Brilliant Blue (CBB)-staining of the SDS-PAGE gel was detected (Fig. 1a, lane 4).

Cloning of the glucosaminidase gene in *E. coli*

Previously, we reported the cloning of a 3.8 kb *Hind*III–*Eco*RI DNA fragment containing, at least, a part of the glucosaminidase gene (Rashid *et al.*, 1993). A 0.4 kb *Pst*I

Table 2. Purification of the glucosaminidase

Fraction	Volume (ml)	Activity (U ml ⁻¹)	Total activity (U)	Specific activity (U mg ⁻¹)	Yield (%)
5 M LiCl extract	500	14	7000	11.7	100
<i>M. luteus</i> cell wall extract	100	30	3000	400	43
Concentrated extract	7	128	900	2140	13
Gel filtration on Toyopearl HW-55 column	10	78	780	26000	11

Fig. 2. Physical map of the chromosomal region encompassing *lytD*, the structural gene of the major endo- β -N-acetylglucosaminidase of *B. subtilis* AC327. pEH4 is a pUC119-derivative containing a 3.8 kb *HindIII*–*EcoRI* insert of *B. subtilis* chromosomal DNA (Rashid, 1993; Rashid *et al.*, 1993). pWCEV4 contains another 4.1 kb *EcoRV*–*ClaI* insert of *B. subtilis* chromosomal DNA in pMW118 (this study). Large open arrows indicate the coding regions of the respective genes with their transcriptional directions. Stem-loop structures indicate putative rho-independent terminators. The *tagABC* and *gerB* operons are depicted on the basis of the nucleotide sequence data obtained by Mauel *et al.* (1991) and Corfe *et al.* (1994), respectively. The 0.4 kb *PstI* fragment (filled bar) was used for insertional mutagenesis of the *lytD* gene (Rashid, 1993; Rashid *et al.*, 1993). Open bars with plasmid names above the chromosomal map indicate the segments of pEH4 and pWCEV4 inserted into the corresponding plasmids. Restriction sites: B, *BamHI*; C, *ClaI*; E, *EcoRI*; H, *HindIII*; Hi, *HincII*; P, *PstI*; S, *SphI*; V, *EcoRV*.

	701		765
CWLG. AWI	KYFFQFLKLSQTAGLSVTEVQKVLGKGLTGRKAFIDAANQVSI NELYLISHALLETCNGTS		
ATL. AWI	KY--QFLRLDQPNISIDKINQF-LKCKGYLENQGAAPNKAQMGVINEVYLI SHALLETCNGTS		
	1073		1134
	766		822
CWLG. AWI	ALANGLTLY-NGKTVYNN-----YGI GAYDSNPYYGAKYAYEQWFTPEAAI IGGAKFICSSY		
ATL. AWI	QLAKGADVNNKYVTSNKTNYHVFGLAAYDNDPLREGIKYAKQAGWDTVSKAIVGGAKFICNSY		
	1135		1199
	823		880
CWLG. AWI	IHTAYNQDTLYKWRWS--ATATHQYATD IGWAYKQVNRWYSLYSLDCYTLFYDFVPEYR		
ATL. AWI	VKAG---QNTLYKWRWNPAPGTHQYATD VDWANAKI I KGYDYDK IGEVGKYPDI PQYK		
	1200		1256

Fig. 3. Alignment of the deduced C-terminal amino acid sequences of the *B. subtilis* LytD (880 residues) and the glucosaminidase (GL) domain of *S. aureus* Atl (Oshida *et al.*, 1995; 1256 residues). Colons indicate identical amino acid residues and hyphens represent breaks introduced to maximize similarity. Amino acids are numbered from the N-termini of the proteins.

fragment, which is internal to the glucosaminidase gene (Rashid *et al.*, 1993), was located 0.23 kb away from the *HindIII* site in pEH4 (Fig. 2) and hybridized with probe B (Rashid *et al.*, 1993). Probe B was designed according to the amino acid sequence of an internal 34 kDa CNBr-peptide of the glucosaminidase (Rashid *et al.*, 1993). These observations together suggested that the whole region of the glucosaminidase gene cannot be located in the 3.8 kb *HindIII*–*EcoRI* cloned fragment of pEH4 (Rashid, 1993). Nucleotide sequencing of the region encompassing the *HindIII* and *EcoRV* sites in pEH4 indicated that the glucosaminidase gene is truncated at its 5'-terminus (Fig. 2 and below).

From Southern hybridization analysis of *B. subtilis* AC327 chromosomal DNA, using the 0.4 kb α - 32 P-labelled *PstI* fragment as a probe, it was concluded that a 4.5 kb *EcoRV* DNA fragment contains the intact glucosaminidase gene (data not shown). But an attempt to clone this DNA fragment in *E. coli* was unsuccessful. This raised the question of the possible toxicity of the glucosaminidase gene for *E. coli*. Therefore, we cloned a 4.1 kb *EcoRV*–*ClaI* DNA fragment containing the 5'-to-central region of the glucosaminidase gene by colony hybridization into the *SmaI*–*AccI* site of pMW118 (a low copy vector), the resultant plasmid being designated pWCEV4. Thus, the glucosaminidase gene was cloned in *E. coli* as a 5'-terminally truncated form in pEH4 and as a 3'-terminally truncated form in pWCEV4, and, therefore, was not expressed in *E. coli* in an active form (Fig. 2).

Nucleotide sequencing and structure of the glucosaminidase region

The basic approach used to construct plasmid derivatives for sequencing was to exploit the restriction enzyme sites present in the insert for subcloning into pUC118. This forced subcloning strategy, in combination with random subcloning for a 1.2 kb *HindIII*–*SphI* fragment, enabled complete sequence determination of the 4.2 kb *HindIII*–*EcoRV* fragment, and partially from the *EcoRI* site of pEH4 and from the *EcoRV* site of pWCEV4. The restriction enzyme map and location of potential ORFs are shown in Fig. 2.

Computer analysis of the 4162 bp sequence encompassing the right *HindIII* and left *EcoRV* sites revealed two ORFs, designated *pmi* and *lytD*, transcribed in the same direction as two monocistronic operons (Fig. 2). The *lytD* ORF encodes a highly basic (pI 10.1) polypeptide of 880 amino acid residues with a molecular mass of 95.6 kDa. The N-terminal amino acid sequences of the mature glucosaminidase protein and a CNBr-peptide derived from it, which had previously been determined (Rashid *et al.*, 1993), were found in the deduced amino acid sequence of the *lytD* gene. The N-terminal amino acid sequence of the mature protein corresponds to the derived polypeptide sequence beginning at amino acid 28. Thus, the pre-protein may be cleaved, during or after translocation across the cell membrane, by a signal peptidase after Ala₂₇ in the sequence, Ala₂₅–Gln₂₆–Ala₂₇–Ala₂₈. Typical features of a signal peptide were conserved in the putative leader peptide of the glucosaminidase (Nagarajan, 1993). There are two types of direct repeats, each type being present twice, in the N-terminal-to-central region of the glucosaminidase, and separated by an insertion of 226 amino acid residues (Fig. 6). A computer search for sequence similarity with the glucosaminidase revealed significant similarity with the glucosaminidase (GL) domain of *Staphylococcus aureus* Atl autolysin (Fig. 3) (Oshida *et al.*, 1995), but no similarity was found with the glucosaminidases of *Streptomyces plicatus* (Robbins *et al.*, 1984) and *Flavobacterium* sp. (Takegawa *et al.*, 1991). The degree of identity is 51% for 177 amino acids between the C-terminal catalytic domain of LytD and the C-terminal part of Atl glucosaminidase (GL) domain (Fig. 3). *pmi* (*orfX*)

Fig. 4. Expression of a *lytD-lacZ* transcriptional fusion protein and effect of a *sigD* null mutation on its expression. Cells were cultured in Schaeffer's sporulation medium at 28 °C. *lytD* promoter-driven β -galactosidase activity was measured at the indicated times relative to the start of sporulation (t_0) in *B. subtilis* 680GL20 (*lytD-lacZ*) (●), 680GL22 (*lytD-lacZ sigD::tet*) (▲), and parent strain, 1A680 (■). t_0 was defined as the point at which cell growth was no longer exponential. These data are representative of two independent experiments.

located upstream of *lytD*, encodes an acidic (pI 4.94) polypeptide of 316 amino acid residues with a molecular mass of 35.4 kDa. A computer search revealed significant amino acid sequence similarity between the Pmi, and the *E. coli manA* (Miles & Guest, 1984) and *Salmonella typhimurium pmi* (Collins & Hackett, 1991) gene products (not presented). Each gene product is the phosphomannose isomerase of the respective organism, and interconverts mannose 6-phosphate and fructose-6-phosphate. Thus, the *pmi* gene product may be the phosphomannose isomerase of *B. subtilis*.

Expression of a *lytD-lacZ* fusion gene in the wild-type and the *sigD*-deficient strains

To study the expression of the glucosaminidase gene *in vivo*, a *lytD-lacZ* transcriptional fusion gene was constructed and integrated (see Methods) into the chromosome of *B. subtilis* 1A680, which has a reduced level of endogenous β -galactosidase activity (Errington & Vogt, 1990). To confirm the proper integration event, chromosomal DNA from *B. subtilis* 680GL20 and a control, 1A680, were extracted and Southern hybridized with pDEB1G as a probe. Upon *Pst*I digestion, one hybridizing band at 5.5 kb for *B. subtilis* 1A680, and two hybridizing bands at 2.7 and 17 kb for *B. subtilis* 680GL20 confirmed the Campbell-type integration of pDEB1G for *B. subtilis* 680GL20 (data not shown). Upon *Pst*I and *Hind*III digestion, one hybridizing band at 1.7 kb for *B. subtilis* 1A680, and three hybridizing bands at 0.8, 1.7 and 11.7 kb for *B. subtilis* 680GL20 also supported the integration event (data not shown). Márquez *et al.* (1990)

reported the reduction of glucosaminidase activity in a *sigD* null mutant strain and our preliminary observation of the upstream nucleotide sequence of the glucosaminidase gene revealed a σ^D -type promoter sequence (Arnosti & Chamberlin, 1989; Helmann, 1991) upstream of the glucosaminidase gene. Therefore, we decided to introduce a *sigD* null mutation into the chromosome of *B. subtilis* 680GL20, as follows. A 1.5 kb *Eco*RI-*Hind*III DNA fragment containing a *Tc^r* gene (*tet*) from pUCTC (Rashid *et al.*, 1993) was inserted into the unique *Eco*T14I site of the *sigD* gene, resulting in plasmid pSDTC. The 3.0 kb *sigD::tet* gene cluster was purified and inserted into the chromosome of *B. subtilis* 680GL20 by the conventional method. A double-crossover recombination event disrupted the chromosomal copy of the *sigD* gene resulting in the *Tc^r* *B. subtilis* 680GL22 strain. Filamentous growth and non-motility of strain 680GL22 served as confirmation of *sigD* disruption (Márquez *et al.*, 1990).

Fig. 4 shows the time course of expression of the *lytD-lacZ* transcriptional fusion gene. In the parent strain, *B. subtilis* 680GL20, the fusion gene expression started from the early exponential growth phase, reaching a maximum at the onset of sporulation (t_0) and then sharply decreased. This type of temporal expression is characteristic of SigD-dependent genes, including those of flagellin (*hag*) and major amidase (*lytC*) (Mirel & Chamberlin, 1989; Kuroda & Sekiguchi, 1993). A low level of β -galactosidase activity (about 5% of that of strain 680GL20 at t_0) was observed for SigD-deficient strain 680GL22, which was almost growth-phase independent and certainly higher than that of the control strain, 1A680 (Errington & Vogt, 1990). Thus, the glucosaminidase gene probably has another minor promoter activity in addition to a major σ^D -like promoter activity.

Physiological roles of the glucosaminidase and/or the amidase in *B. subtilis*

A search for the physiological functions of the glucosaminidase and/or the amidase revealed that these two abundant activities are not required, either singly or in concert, for competence, sporulation and germination, as measured by conventional methods. Cellular autolysis was studied by (i) suspending exponentially growing cells (OD_{660} 0.3) in a buffer optimized for the measurement of the glucosaminidase or the amidase activity, (ii) continuing the culture for a prolonged time to measure stationary phase lysis, and (iii) exposing exponentially growing cells (OD_{660} 0.3) to cold-shock treatment (unpublished results). With all the methods tested, cellular autolysis was found to depend mainly on the amidase (unpublished results). The absence of both endolysins did not significantly alter the lytic action of penicillin toward exponentially growing cells (not presented). Inactivation of either the amidase or the glucosaminidase led to a modest reduction in swarm diameter (Table 3). In contrast, the double mutants, strains AGB1 and 168GB1, had greatly reduced swarm diameter (30–34% of the wild-type diameter). However, the synthesis of flagellin protein in the mutant strains was not affected, as judged

Table 3. Swarm plate assay of the autolysin-deficient strains

Strain	Characteristic(s)	Swarm diameter (% wt $\pm$ SEM)*	
		Mannitol	Tryptone
AC327	Wild-type	100 $\pm$ 2.3	100 $\pm$ 2.8
AG1	LytD ⁻	56 $\pm$ 0.9	65 $\pm$ 1.7
AN8	CwlB ⁻	67 $\pm$ 1.9	51 $\pm$ 1.4
AGB1	LytD ⁻ CwlB ⁻	34 $\pm$ 0.2	33 $\pm$ 0.9
168	Wild-type	100 $\pm$ 1.8	100 $\pm$ 3.6
168G1	LytD ⁻	54 $\pm$ 1.9	72 $\pm$ 1.5
168N8	CwlB ⁻	63 $\pm$ 0.9	64 $\pm$ 2.1
168GB1	LytD ⁻ CwlB ⁻	32 $\pm$ 0.5	30 $\pm$ 0.8

* Swarm diameter was measured after 8–12 h on 0.3% agar plates containing either tryptone or minimal medium with mannitol. SEM equals $\sigma_{n-1}/\sqrt{n}$ (Rosario *et al.*, 1994), where $n = 10$.

by SDS-PAGE analysis (not presented). Thus, the precise roles of these endolysins in flagellation (motility) remain obscure.

Recombinant glucosaminidase in *B. subtilis* AG1 (*lytD*)

Plasmid pHYG contains the intact glucosaminidase gene, and plasmid pHYEB contains a truncated form of the glucosaminidase gene in which the 3'-terminal 187 codons of the native glucosaminidase gene have been replaced by 19 alien codons of the vector (Fig. 6b). Plasmid pHYG could not be constructed in *E. coli*. We also did not succeed in constructing an intact glucosaminidase-producing plasmid in a low copy vector (pMW118). These observations confirmed our suspicion that the intact glucosaminidase is lethal for *E. coli*, even at a low copy number (five copies per cell). *B. subtilis* AG1 harbouring plasmid pHYG, pHYEB or, as a control, pHY300PLK was cultured in BP medium for 12 h at 28 °C and samples (extracts A and B) were then prepared as previously described (Rashid *et al.*, 1993). SDS-PAGE analysis of extracts A and B demonstrated the production of the intact glucosaminidase protein, and its amplification upon binding and elution from *M. luteus* cell wall (Fig. 5a, lanes 2 and 5). The truncated form of the glucosaminidase [tLytD(G10)] also remained tightly bound to the cell wall (Fig. 5a, lane 3), and the estimated molecular mass was less than the predicted size by about 14 kDa. However, in contrast to the intact glucosaminidase, this truncated glucosaminidase [tLytD(G10)] exhibits no catalytic activity toward cell wall, as seen by zymographic analysis (Fig. 5b, lanes 3 and 6).

Previously, we reported the disappearance of the lytic activities of several fast-migrating proteins (FMPs) of about 35–39 kDa in addition to one of 90 kDa for strain AG1 on zymography (Rashid *et al.*, 1993). As Fig. 5(b) shows, lytic activities of FMPs were found in addition to

Fig. 5. SDS-PAGE (a) and zymography (b) of the recombinant glucosaminidase protein produced in *B. subtilis* AG1 (*lytD*). The electrophoretic conditions and renaturation of proteins after electrophoresis (b) were as in the legend to Fig. 1. LiCl (5 M) crude extract (extract A) and cell-wall-extract (extract B) samples were prepared as previously described (Rashid *et al.*, 1993b). Lanes: M, protein standards (BioRad); 1–3, extract B samples; 4–6, extract A samples; 1 and 4, *B. subtilis* AG1(pHY300PLK); 2 and 5, *B. subtilis* AG1(pHYG); 3 and 6, *B. subtilis* AG1(pHYEB). The wild-type glucosaminidase protein (90 kDa) and activity (90 and 35 kDa) bands, and the truncated glucosaminidase (tLytD/G10) protein (63 kDa) band are indicated by arrowheads on the right.

that of the 90 kDa one for both extracts A and B (lanes 2 and 5). Protein bands corresponding to these activities could not be detected upon CBB staining (Fig. 5a) of the SDS-polyacrylamide gel. It is presumed that a certain amount of the intact glucosaminidase protein is processed *in vivo* to the FMPs by proteolytic cleavage. The activities of the FMPs were not amplified upon cell-wall-binding (Fig. 5b, compare lanes 2 and 5), whereas the 90 kDa activity was amplified.

Characterization of the truncated glucosaminidase proteins produced in *E. coli*

Enzymic characterization of the truncated glucosaminidase proteins (Fig. 6b) by zymographic analysis is presented in Fig. 7. Crude SDS-extract (extract S) samples prepared from equal amounts of *E. coli* cells harbouring various plasmids were loaded together with a control. For

Fig. 6. Intramolecular repeated sequences in the N-terminal-to-central region of the glucosaminidase (a), and the primary structure and serial deletions of the glucosaminidase (b). A1 and A2, and B1 and B2 are two distinct types of tandemly repeated sequences, and are separated by an insertion of 226 amino acids. The numbers at the left or right of the direct repeat sequences (a) are the positions with respect to the N-terminal amino acid of the glucosaminidase. Identical, similar (conservative substitutions) and unrelated amino acids are indicated by stars, colons and spaces, respectively. Conservative substitutions are: I, L, V and M; K and R; S and T; D and E; F and Y; N and Q; G and A. The numbers at the left or right of the truncated proteins (b) indicate alien amino acids. Hatched and rectangular regions indicate the signal peptide and catalytic domain of the glucosaminidase, respectively. The square region between *PvuII* and *Avall* sites indicates a putative 38 amino acid processing region for the activities of FMPs on zymography (Figs 1b, 5b and 7). Abbreviations: A, *Aval*; Av, *Avall*; B, *BamHI*; Bs, *BsrFI*; H, *HindIII*; P, *PstI*; Pv, *PvuII*; X, *XcmI*.

Fig. 7. Zymographic analysis of the truncated glucosaminidase proteins produced in *E. coli* JM109 harbouring different plasmids. Crude SDS-extract samples (extract S) were prepared as described in Methods and loaded on a 12% SDS-polyacrylamide gel containing 0.1% (w/v) *M. luteus* cell wall as a substrate. After electrophoresis, the proteins were renatured as previously described (Rashid *et al.*, 1993). The gel was stained with 0.01% methylene blue in 0.01% KOH before being photographed. Lanes: 1–8, proteins G1–G8, respectively; C, control strain, *E. coli* JM109(pUC118). The molecular masses of the protein standards (BioRad) are indicated on the left.

all proteins except G8, a certain amount was processed to several FMPs that retained lytic activity (lanes 1–7). Proteins G1–G6 exhibited two activities, the activities for the slow-migrating proteins (SMPs) corresponding to those of the proteins of the predicted molecular masses and the activities for the FMPs corresponding to those of the processed ones. The intensities of the activities of FMPs were almost identical for proteins G1–G5. For the

activities of SMPs, the intensities for proteins G1 and G2 were almost equal, but those for proteins G3, G4 and G5 gradually decreased. Thus, the loss of more than one repeating unit seemed to drastically reduce the activities of SMPs of the truncated glucosaminidases. However, the activities for SMPs of G6 and G7 and for FMPs of G1–G6, which are devoid of a substrate-binding domain, did not decrease, as judged by zymography (lanes 1–7). This discrepancy may be due to the more efficient renaturation on zymography of the smaller proteins, which completely lack a substrate-binding domain. Protein G8 (lane 8) exhibited no activity on zymographic analysis. We also attempted to characterize these truncated glucosaminidases in terms of their binding affinity to the substrate cell wall. For protein G1, we did not find any apparent change in binding affinity (data not shown). For proteins G2–G7, SMPs and activity bands could not be detected on the respective gels in the case of LiCl extracts (extracts A and B) (data not shown), although for SDS-extract samples (extract S) they were easily detected (Fig. 7). This may suggest rapid degradation of the truncated glucosaminidases by *E. coli* proteases during the preparation of samples. Previous investigators (Rogers *et al.*, 1984; Brown, 1977; Brown & Young, 1970) reported that the native glucosaminidase is very sensitive to proteases. Thus, deletions may render the modified glucosaminidases more sensitive to *E. coli* proteases.

In vitro processing of the partially purified glucosaminidase and the activity of processed forms of the glucosaminidase

Partially purified wild-type glucosaminidase (extract B) from *B. subtilis* AG1 (pHYG) was used to study the time-course of processing *in vitro*. On incubation at 37 °C for

Fig. 8. SDS-PAGE analysis of the stability of the glucosaminidase. (a) The partially purified 90 kDa glucosaminidase (extract B in 5 M LiCl/buffer T) is degraded to smaller proteins when incubated at 37 °C after dilution to 0.25 M LiCl (lanes 2–4). This degradation is inhibited when SDS-purified *B. subtilis* cell wall (5 mg ml⁻¹) was added to the enzyme (for 30 min on ice) before the start of incubation at 37 °C (lanes 5–7). (b) When 0.1 mM PMSF was added to the extract B sample, the degradation was also stopped. The electrophoretic conditions were as in the legend to Fig. 1. All incubations were performed in 0.25 M LiCl/buffer T (25 mM Tris/HCl, pH 7.2) at 37 °C for various times, and samples were prepared for loading by 2% TCA precipitation after incubation. Lane M, protein standards (BioRad). (a) and (b) 100 µl extract B sample was loaded in each lane. (a) Lane 1, 0 h incubation (control); lanes 2, 3 and 4, incubation without cell wall for 1, 2 and 3 h, respectively; lanes 5, 6 and 7, incubation with cell wall for 1, 2 and 3 h, respectively. (b) Lane 1, 0 h incubation (control); lanes 2 and 3, incubation without PMSF for 0.5 and 1.5 h, respectively; lanes 4 and 5, incubation with 0.1 mM PMSF for 0.5 and 1.5 h, respectively. (c) Lanes 1, 2 and 3, incubation with 150 µl HW-55 fraction for 1, 2 and 3 h, respectively.

1.5 h in 0.25 M LiCl/buffer T solution, the 90 kDa glucosaminidase protein and activity band disappeared with concomitant increases in several FMPs and their activity bands (Fig. 8a, lanes 2–4; 8b, lanes 2 and 3; zymography data not shown). It seems that only a small amount of contaminating protease was sufficient for its degradation. Our *in vitro* study supports our speculation that the truncated glucosaminidases, produced in *E. coli*, were very unstable, and hence the cell-wall-binding assay was unsuccessful.

Since the specific activities of the processed proteins (FMPs) seemed to increase in zymographic gels (Figs 1b, 5b and 7), the partially purified glucosaminidase (extract B) was processed completely *in vitro*. The cell-wall-lytic activities of these processed proteins were then measured in an aqueous buffer optimized for the glucosaminidase activity (Rogers *et al.*, 1984). The specific activities of these processed proteins did not increase in the aqueous buffer assay system (data not shown). Therefore, possible reasons for the strong activities of the processed proteins in zymographic gels may be efficient renaturation and activation of the processed proteins, and/or the existence of several contiguous proteins (35–39 kDa) which together exhibit a broad, seemingly stronger, activity band.

Stability of the purified glucosaminidase against proteolytic enzymes

The glucosaminidase was purified to apparent homogeneity from *B. subtilis* AG1 (pHYG) as described in Methods with the omission of the concentration step by ultrafiltration. This purified enzyme was found to be resistant to degradation compared to the partially purified

enzyme (extract B) as shown in Fig. 8(c). However, the partially purified enzyme also became resistant to proteolysis when SDS-purified *B. subtilis* (Fig. 8a) or *M. luteus* (not shown) cell walls were added (lanes 5–7). Moreover, the stability of the partially purified enzyme (extract B) was found to increase when PMSF (final concentration, 0.1 mM) was added to the enzyme (Fig. 8b, lanes 4 and 5).

Biochemical characterization of the glucosaminidase

B. subtilis cell-wall-binding protein, LytB, was purified and then added to the glucosaminidase in an equimolar amount to test for the stimulatory effect. It did not stimulate the lytic activity of the glucosaminidase (data not shown). Thus, the stimulatory effect of LytB seems to be only specific to the amidases.

The effect of various enzyme inhibitors on the glucosaminidase activity was also tested. No inhibition was observed for PMSF (5 mM), diisopropyl fluorophosphate (1 mM), 2-mercaptoethanol (25 mM) and *N*-ethylmaleimide (5 mM). However, the enzyme was inhibited strongly by diethyl pyrocarbonate which is known to react with active histidine (33% at 1 mM and 86% at 5 mM) and only slightly by EDTA (7% at 5 mM and 23% at 25 mM). Thus, it seems likely that the His residue(s) of the catalytic domain is important for the catalytic activity of the glucosaminidase.

DISCUSSION

Rogers *et al.* (1984) initially reported the purification of the high-molecular-mass autolytic glucosaminidase of *B. subtilis* 168. They found it to be a dimer of 190 kDa in

aqueous buffer consisting of identical subunits of 90 kDa, and very sensitive to proteolytic enzymes. We simplified the procedure of Rogers *et al.* (1984), as follows. (i) The tedious step of isolation of the autolysin-containing native cell walls was eliminated by directly extracting autolysins from the intact cells with a high concentration (5 M instead of 2 M) of LiCl, which is a more efficient method (Brown, 1973); (ii) the two-step column chromatography (affinity followed by gel filtration) was replaced by a single-step gel filtration chromatography. During the purification of the glucosaminidase, the growth medium significantly affected the yield, which is consistent with the study of Brown (1977). However, transcriptional studies with a *lytD-lacZ* fusion gene did not reveal significant dependence on growth medium (not presented). Thus, the difference in yield may be due to translational and/or post-translational phenomena.

Exploiting the amino acid sequences of the purified glucosaminidase, the corresponding gene, *lytD*, was cloned. Nucleotide sequencing revealed a monocistronic operon encoding a polypeptide of 880 amino acids which is endowed with a typical signal peptide. The existence of the signal peptide was confirmed by N-terminal sequencing of the mature protein (Rashid, 1993; Rashid *et al.*, 1993). There is a 12 bp nucleotide sequence discrepancy in the upstream non-coding region of *pmi* between our sequence and that of Margot *et al.* (1994), which is complementary to each other (nucleotides 48–59 in our accession number D45048). Transcriptional analysis with a *lytD-lacZ* fusion gene revealed that the glucosaminidase gene is transcribed during the early- to late-exponential growth phase. Introduction of a *sigD* null mutation greatly reduced (by about 95 %) this expression, suggesting that the glucosaminidase gene is mainly transcribed by an E- σ^D holoenzyme. The *flaD* and *degS-degU* pleiotropic mutations may affect transcription of the glucosaminidase gene through the level of the SigD protein, as is the case for the major amidase operon (Kuroda & Sekiguchi, 1993; Tokunaga *et al.*, 1994). Recently, Mirel *et al.* (1994) reported identification of the *flgM* gene product as an anti- σ^D factor which represses σ^D -dependent transcription in response to an unlinked mutation in a gene directing early flagellar synthesis. Thus, the glucosaminidase gene transcription may be affected in the *flaA4*, *flaA15* and *ifm* mutants by the action of the *flgM* gene product (Pooley & Karamata, 1984; Barilla *et al.*, 1994; Hauser *et al.*, 1991; Mirel *et al.*, 1994).

Studies with the strains inactivated in the glucosaminidase and/or amidase gene(s) revealed that the glucosaminidase and the amidase are dispensable, either singly or in concert, with respect to growth, morphology, competence, sporulation and germination. The contribution of the amidase to cellular autolysis was several times higher than that of the glucosaminidase, but, with respect to swarming, the contribution of each protein was indistinguishable from the other. The double mutant had greatly reduced swarming compared to the single mutants, which supports our previous result (Rashid *et al.*, 1993). This result is, however, in contradiction with the reports of Margot & Karamata (1992) and Margot *et*

al. (1994) suggesting that only the amidase-deficient strain has greatly impaired motility on semi-solid medium. Our amidase-deficient strains in the genetic backgrounds of 168 and AC327 were not as impaired as the double mutant in motility on minimal swarm plates (Márquez *et al.*, 1990) as well as on tryptone plates containing 0.3 % agar (Table 3). The reason for this discrepancy regarding the role of the amidase and glucosaminidase in flagellar motility remains obscure. Since these endolysins are not required for the synthesis of flagellin protein, the earlier hypothesis of Fein (1979) that these activities are required for the morphogenesis of the flagellum basal body does not remain plausible in the light of recent findings (Barilla *et al.*, 1994; Mirel *et al.*, 1994).

Amidases of the genus *Bacillus*, independent of phage or host origin, are generally composed of two domains – a catalytic domain and a cell-wall-binding domain containing several (usually two or three) tandemly repeated sequences (Kuroda *et al.*, 1992b). Other cell-wall-binding proteins [LytB, a modifier of major amidase, CwlB (Kuroda *et al.*, 1992a); WapA, a major wall-associated protein (Foster, 1992)], which apparently have no catalytic function also contain three tandemly repeated sequences. Interestingly, a stage II sporulation gene, *spoIIB*, also contains two tandemly repeated sequences homologous to *cwlM* and *cwlC* (Margolis *et al.*, 1993; Kuroda *et al.*, 1993). However, the organization of two domains in amidases, i.e. whether the N-terminal domain is catalytic or the C-terminal domain is substrate-binding, is not conserved. In the case of the glucosaminidase, a truncated form (tLytD/G10) lacking the C-terminal 187 amino acids still remained tightly bound to cell walls but lacked catalytic activity (Fig. 5). However, another truncated form of the glucosaminidase, protein G7, containing only the C-terminal 312 amino acids, had catalytic activity (Fig. 7, lane 7). Further truncation (protein G8, 274 amino acids) caused the complete loss of catalytic activity (Fig. 7, lane 8). Thus, it seems that the glucosaminidase, like the amidases, is also composed of two domains, the N-terminal two-thirds for substrate binding and the C-terminal one-third for the catalytic function, and that these domains are functionally independent.

We have demonstrated that FMPs were derived from the intact glucosaminidase upon putative proteolytic processing and included its C-terminal catalytic domain (Figs 1b, 5b and 7). A region comprising 38 amino acid residues in between the N- and C-terminal domains has been identified as a putative processing one (Figs 6b and 7). The molecular mass of tLytD(G10) determined on CBB staining, which was produced in *B. subtilis*, was about 14 kDa smaller than the predicted one (Fig. 5a). Assuming proteolytic cleavage of tLytD(G10) at the above-mentioned region, the discrepancy in molecular mass can be explained. Two *B. subtilis* proteins (CwlA, an amidase, and WapA) and a *B. megaterium* autolysin (29 kDa, cortex-lytic) are proteolytically processed to smaller forms (Kuroda *et al.*, 1991; Foster, 1991; Foster & Johnstone, 1988). Two autolytic muramidases (M-1 and M-2) of *Enterococcus hirae* ATCC9790 and one amidase (LytA) of *Staphylococcus aureus* NCTC8325 have also been reported to

be proteolytically processed (Kariyama & Shockman, 1992; Wang *et al.*, 1992). Taking these observations together, the processing of the glucosaminidase is not at all surprising. However, the physiological necessity of this processing remains unclear.

The instability of the partially purified glucosaminidase was due to the residual proteases present in the enzyme mixture (Fig. 8b). Our *in vitro* study indicates that the cell-wall-bound glucosaminidase probably achieves a conformational state which is relatively resistant to proteolytic attacks compared to the glucosaminidase alone (Fig. 8a). A similar phenomenon was also observed for the pneumococcal cell-wall hydrolases (Sanz *et al.*, 1992). In contrast to the amidase genes cloned in this laboratory, the *lytD* gene product was found to be very toxic to *E. coli*. It is unclear why the glucosaminidase hydrolyses *M. luteus* cell walls more efficiently than *B. subtilis* cell walls *in vitro* (Fan & Beckman, 1973; Rashid *et al.*, 1993), though there is no significant difference between the peptidoglycans of these two organisms (L-lysine instead of mesodiamino pimelic acid in the peptide subunit of *M. luteus*; teichuronic acid instead of teichoic acid in *M. luteus*) (Hase & Matsushima, 1977; Karl & Kandler, 1972; Nasir-ud-Din *et al.*, 1985). The absence of cysteine residues in the deduced polypeptide of the glucosaminidase gene, the non-inhibition of the glucosaminidase activity by thiol-directed reagents (*N*-ethylmaleimide and 2-mercaptoethanol), and the retention of lytic activity by the modified glucosaminidases on zymographic analysis (Fig. 7) raised ambiguity about the dimeric nature of this protein reported by Rogers *et al.* (1984). The role of the intervening 226 amino acids between the repeated sequences is also obscure. Experiments will be designed to address these questions in the future.

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