The role of autolysins during vegetative growth of Bacillus subtilis 168

Steve A. Blackman, Thomas J. Smith and Simon J. Foster

INTRODUCTION

Seemingly, all bacteria possess a complement of autolysins, which are potentially lethal enzymes capable of hydrolysing peptidoglycan, the major structural component of bacterial cell walls (Ghysen et al., 1966). These enzymes have been postulated to have roles in motility, cell separation, competence, antibiotic-induced lysis, pathogenicity, cell wall growth and turnover, and differentiation (Rogers et al., 1983; Ward & Williamson, 1984). Bacillus subtilis has multiple autolysins and associated prophage lytic enzymes, the proposed functions of which were summarized by Smith et al. (1996).

The two major autolysins present during vegetative growth of B. subtilis are a 50 kDa N-acetylmuramoyl-L-alanine amidase (amidase) and a 90 kDa endo-β-N-acetylglucosaminidase (glucosaminidase), which have been purified and characterized (Herbold & Glaser, 1975; Rogers et al., 1984). Recently, their structural genes have been cloned and studied at the molecular level. The amidase is encoded by the lytC gene, which is part of a four-gene divergon, lytRABC (Kuroda & Sekiguchi, 1991; Lazarevic et al., 1992; Kuroda et al., 1992). The lytR gene is transcribed divergently from the lytABC operon and encodes a putative DNA-binding protein, which represses expression of itself and lytABC (Lazarevic et al., 1992). The lytA gene encodes a putative lipoprotein (Lazarevic et al., 1992; Kuroda et al., 1992) and lytB encodes a modifier protein which enhances the activity of the amidase (Herbold & Glaser, 1975; Lazarevic et al., 1992; Kuroda & Sekiguchi, 1993). Transcription of this operon proceeds from two promoters. One is controlled by σD, the sigma factor responsible for transcription of the genes involved in flagellar motility and chemotaxis (Helmann et al., 1988), which accounts for 70–90% of the transcription during vegetative growth. The other promoter upstream of lytABC is controlled by σA, the major house-keeping sigma factor of B. subtilis (Lazarevic et al., 1992; Kuroda & Sekiguchi, 1993). The glucosaminidase is encoded by the lytD gene (Margot et al., 1994; Rashid et al., 1995) and its active form is a dimer of about 190 kDa (Rogers et al., 1984). Transcription of lytD proceeds from a single promoter which is controlled by σD (Margot et al., 1994), although about 5% of transcription may be

A set of isogenic mutants of Bacillus subtilis 168, insertionally inactivated in the genes encoding a number of lytic enzymes and a sigma factor (σD, which controls the expression of a number of autolysins) was constructed. Phenotypic analysis of the mutants determined the individual and combined roles of the autolysins in vegetative growth. The major vegetative autolysins of B. subtilis, LytC (50 kDa amidase) and LytD (90 kDa glucosaminidase), were shown to have roles in cell separation, cell wall turnover, antibiotic-induced lysis and motility. LytC was also shown to have a role in general cell lysis induced by sodium azide. Renaturing SDS-PAGE of cell-wall-binding protein extracts of the mutant strains revealed the presence of a novel autolysin that was previously masked by LytC. This 49 kDa enzyme was shown to be σD-controlled and was identified as a candidate cell separation and cell wall turnover enzyme. A multiple mutant strain, lacking LytC, LytD and the 49 kDa enzyme, retained at least ten bands of autolytic activity. These may correspond to individual or proteolytically processed novel autolysins, the functions of which are unknown. The multiple mutant strains facilitate the study of these, and other lytic enzymes, to determine their cellular functions.

Keywords: Bacillus subtilis, autolysins, cell separation, cell wall turnover, motility

Abbreviation: CWBP(s), cell-wall-binding protein(s).

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controlled by another minor promoter activity (Rashid et al., 1995). The genes encoding LytC and LytD have been insertionally inactivated both singly (Kuroda & Sekiguchi, 1991; Margot & Karamata 1992) and together (Rashid et al., 1993; Margot et al., 1994). Despite this, the specific functions of these enzymes have not been fully elucidated. LytC has a role in cell wall turnover, motility and cell lysis (Margot & Karamata, 1992). It also has a mutually compensatory role with CwlC (a 30 kDa sporulation-specific amidase) in mother cell lysis at the end of sporulation (Smith & Foster, 1995). A lytC lytD double mutant has been shown to have reduced motility, however single mutants in lytC or lytD were unaffected (Rashid et al., 1993). It is surprising that the lytC lytD strain did not show abnormal filamentation under the conditions used as it has been previously postulated that one or both of the major autolysins has a role in cell separation (Fein & Rogers, 1976). This is exemplified by the fact that when the structural gene for SigD, which to a great extent controls the expression of both autolysins, is inactivated the mutant is filamentous (Helmann et al., 1988). A sigD mutant is still able to undergo septation but is filamentous owing to the lack of hydrolysis of the nascent septa, preventing daughter cell separation.

Most autolysins may perform more than one role in the cell and can also often compensate for the lack of each other (Foster, 1991). As a result of the possible functional redundancy of the autolysins of B. subtilis, it is important to identify and characterize the total complement of such enzymes to determine their individual and combined roles in any given cellular process. This study is ultimately concerned with identifying and characterizing novel autolysins which are expressed during, and so probably involved in, vegetative growth of B. subtilis. As part of this work multiple peptidoglycan hydrolase mutants of B. subtilis were constructed to facilitate the study of the role of the autolysins.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** Strains of B. subtilis and plasmids used in this study are shown in Table 1. Unless otherwise stated, growth of B. subtilis strains was carried out at 37°C in nutrient broth (Oxoid) with shaking (250 r.p.m.) or on nutrient agar plates at 37°C. Minimal medium was as described by Anagnostopoulos & Spizizen (1961), supplemented, where appropriate, with amino acids (50 µg L-tryptophan ml-1, 220 µg methionine ml-1). Chromosomal drug resistance markers in B. subtilis were selected with 1 µg erythromycin ml-1, 25 µg lincomycin ml-1, 5 µg chloramphenicol ml-1, 0.3 µg pleomycin ml-1, and 100 µg spectinomycin ml-1. Spores of B. subtilis were prepared by growing in CCY medium (Stewart et al., 1981) for 2 d at 37°C. Escherichia coli XLOLR (Stratagene) was grown in Luria-Bertani broth or on Luria-Bertani plates at 37°C. In E. coli, plasmids were selected with 50 µg ampicillin ml-1.

**Construction of mutants**

**lytC.** Plasmid p6302 (Lazarevic et al., 1992) was restricted with BglII, which cuts once, within the cloned B. subtilis lytC gene. The linearized plasmid was end-filled and dephosphorylated, and ligated with a gel-purified 1.5 kb phleomycin resistance cassette that had been excised from plasmid pUC22 (Steinmetz & Richter, 1994) by restriction with Smal and PvuII. Restriction analysis of the resultant plasmid, pTJS43, using BamHI showed that the phleomycin resistance cassette had been inserted into the lytC gene and was oriented in the same direction as the lytC coding sequence. The lytC-inactivated strain of B. subtilis (SH125) was made by transforming (Anagnostopoulos & Spizizen, 1961) B. subtilis 168 HR with pTJS43 (linearized by EcoRI digestion). Recombinants were selected by overlaying agar plates with top agar containing phleomycin (0.3 µg ml-1), as described by Cutting & Vander Horn (1990). Southern blot analysis showed that the phleomycin resistance cassette had been transferred from pTJS43 into the chromosomal copy of lytC by a double cross-over event to create strain SH125. The mutation was transferred into the parental background for this study (1A304) by transformation with SH125 genomic DNA.

**lytD.** The 1.1 kb PstI fragment, containing part of the lytD gene, from p5102 (Margot et al., 1994) was ligated into the PstI restriction site of pUBS1 (from G. Murphy, Institute of Plant Science Research, Cambridge, UK) to create plasmid pSAB100. The spectinomycin resistance cassette was excised from pC156 (Steinmetz & Richter, 1994) using Smal and ligated into the HpaI site of pSAB100 to create pSAB101. Restriction analysis of pSAB101, using BamHI, showed that the spectinomycin resistance cassette had been inserted into the lytD gene and was oriented in the opposite direction to the lytD coding sequence. Plasmid pSAB101 was linearized by cutting with EcoRI and transformed into B. subtilis 168 strain 1A304 to create strain SH119. Recombinants were selected on nutrient agar plates containing 100 µg spectinomycin ml-1 (Steinmetz & Richter, 1994). The construct was confirmed by Southern blot analysis.

Multiple mutant strains and other constructs were made by transformation with the appropriate chromosomal DNA (see Table 1 for crosses). All molecular biology methods were performed as described by Sambrook et al. (1989).

**Swarm plate assay.** Swarming motility of strains was measured using nutrient and minimal 0.5% agar (Difco) plates. Samples (1 µl) from overnight (30°C) liquid cultures were spotted onto swarm plates and incubated at 37°C (nutrient agar for 18–22 h, minimal agar for 44–48 h) or 25°C (nutrient agar for 44–48 h, minimal agar for 68–72 h). Extent of swarming motility was measured as percentage growth diameter relative to the parental control (1A304).

**Cell autolysis.** Cultures of parental and mutant strains of B. subtilis were grown to mid-exponential phase (OD600 0.25–0.3) in nutrient broth. Lysis of cells, after addition of 0.05 M sodium azide or 5 µg cloxacinil ml-1 (10 × MIC), was followed spectrophotometrically (10 mm path length, Pye Unicam SP6-400 spectrophotometer) while continuing incubation at 37°C and 250 r.p.m.

**Measurement of cell wall turnover.** This was determined in minimal medium using the method of Margot & Karamata (1992). N-Acetyl-d-[1-14C]glucosamine was obtained from Amersham and radioactivity of filters and filtrates was estimated in Safefluor-S (LUMAC) scintillation cocktail with a Beckman LS1801 liquid scintillation system.

**Preparation of cell-wall-binding protein (CWBP) extracts.** Two-litre cultures (OD600 1.0) of B. subtilis parental and mutant strains were harvested (11000 g, 4°C, 10 min), and the cells washed once with ice-cold 50 mM Tris/HCl (pH 7.5) and then resuspended in 40 ml of the same buffer. PMSF was
preparations were then stored at -20 °C. CWBs were passed twice through a French pressure cell (Aminco, glycerol] and stored at -20 °C.

Tris/HCl (pH

were then washed twice with ice-cold 100mM NaCl to extract (100 °C, 3 min) with SDS sample buffer

by centrifugation (27000 * g, 30 min) to break the cells. Cell walls were sedimented 15 min, 4 °C) and washed twice by

extracted (100 °C, 3 min) with SDS sample buffer

analysed by SDS-PAGE (Laemmli, 1970) using 11% (w/v) acrylamide gels and Coomassie blue staining to visualize

autolysins of Bacillus subtilis

Table 1. Bacterial strains and plasmids

<table>
<thead>
<tr>
<th>Strains</th>
<th>Genotype/phenotype</th>
<th>Source/reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis 168</td>
<td>trpC2</td>
<td>Laboratory stock</td>
</tr>
<tr>
<td>HR</td>
<td>trpC2 metB5 xin-1 SPβ(s)</td>
<td>BGSC†</td>
</tr>
<tr>
<td>1A304</td>
<td>trpC2 wapA::pSFG126 Eryr</td>
<td>Foster (1993)</td>
</tr>
<tr>
<td>SFG103</td>
<td>trpC2 sigD::pLM5 Cm′</td>
<td>Helmann et al. (1988)</td>
</tr>
<tr>
<td>DP-1</td>
<td>trpC2 sigD::pLM5 Cmr</td>
<td>This study</td>
</tr>
<tr>
<td>SH125</td>
<td>trpC2 lycC::ble</td>
<td>This study</td>
</tr>
<tr>
<td>SH119</td>
<td>trpC2 metB5 xin-1 SPβ(s) lycD::spc</td>
<td>SH125 → 1A304</td>
</tr>
<tr>
<td>SH115</td>
<td>trpC2 metB5 xin-1 SPβ(s) lycC::ble</td>
<td>DP-1 → 1A304</td>
</tr>
<tr>
<td>SH118</td>
<td>trpC2 metB5 xin-1 SPβ(s) sigD::pLM5 Cmr</td>
<td>SFG103 → 1A304</td>
</tr>
<tr>
<td>SH117</td>
<td>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Eyr</td>
<td>SH119 → SH115</td>
</tr>
<tr>
<td>SH128</td>
<td>trpC2 metB5 xin-1 SPβ(s) lycC::ble lycD::spc</td>
<td>SH118 → SH128</td>
</tr>
<tr>
<td>SH131</td>
<td>trpC2 metB5 xin-1 SPβ(s) lycC::ble lycD::spc</td>
<td>SH115 → SH117</td>
</tr>
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<td>SH120</td>
<td>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Eyr</td>
<td>SH119 → SH117</td>
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<td>SH121</td>
<td>trpC2 metB5 xin-1 SPβ(s) lycD::spc</td>
<td>SH118 → SH117</td>
</tr>
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<td>SH127</td>
<td>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Eyr</td>
<td>SH118 → SH117</td>
</tr>
<tr>
<td>SH123</td>
<td>trpC2 metB5 xin-1 SPβ(s) sigD::pLM5 Cmr</td>
<td>SH119 → SH120</td>
</tr>
<tr>
<td>SH124</td>
<td>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Eyr</td>
<td>SH118 → SH123</td>
</tr>
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<td>Plasmids</td>
<td>pJH101 with 2.3 kb BamHI–EcoRI insert carrying B. subtilis lycC gene</td>
<td>Lazarevic et al. (1992)</td>
</tr>
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<td>p5102</td>
<td>pMTL20EC with 4 kb BamHI insert carrying B. subtilis lycD gene</td>
<td>Margot et al. (1994)</td>
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<tr>
<td>pTJS43</td>
<td>p6302 with 1.5 kb phleomycin resistance cassette (ble) inserted into unique BglII site</td>
<td>This study</td>
</tr>
<tr>
<td>pSAB100</td>
<td>1.1 kb PstI fragment from p5102 cloned into unique PstI site of pUBS1</td>
<td>This study</td>
</tr>
<tr>
<td>pSAB101</td>
<td>pSAB100 with 1.2 kb spectinomycin resistance cassette (spc) inserted into unique HpaI site</td>
<td>This study</td>
</tr>
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</table>

* Arrows indicate transformation of recipient strain with donor chromosomal DNA.
† Bacillus Genetic Stock Center, Ohio State University, OH, USA.

added to a concentration of 0.5 mM and the suspension was passed twice through a French pressure cell (Amino, 180 MPa, 4 °C) to break the cells. Cell walls were sedimented by centrifugation (27000 g, 15 min, 4 °C) and washed twice by resuspension in ice-cold 50 mM Tris/HCl (pH 7.5). The walls were then washed twice with ice-cold 100 mM NaCl to remove any loosely associated proteins and washed twice again with ice-cold 50 mM Tris/HCl (pH 7.5). The cell wall preparations were then stored at -20 °C. CWBs were extracted (100 °C, 3 min) with SDS sample buffer [62.5 mM Tris/HCl (pH 6.8), 1 mM EDTA, 1% (w/v) SDS, 5% (v/v) β-mercaptoethanol, 0.0025% bromophenol blue, 10% (v/v) glycerol] and stored at -20 °C.

SDS-PAGE and renaturing SDS-PAGE. CWB samples were analysed by SDS-PAGE (Laemmli, 1970) using 11% (w/v) acrylamide gels and Coomassie blue staining to visualize proteins. Autolysin activity was detected by renaturing gel electrophoresis as described by Foster (1992) using purified B. subtilis vegetative cell walls as the substrate.

Cell separation. To study filamentation of parental and mutant strains using phase-contrast microscopy, cells were harvested at specific points during growth in nutrient broth and fixed in ethanol using the method of Hauser & Errington (1995). Cells were mounted on Poly-Prep poly-L-lysine-coated slides (Sigma) and stained with methylene blue. To study macrofilamentation (boli), cultures (10 ml) of B. subtilis strains were grown for 18 h at 45 r.p.m. and carefully poured into Petri dishes for photography. For electron microscopy, samples of late-exponential or early-stationary phase cultures of B. subtilis parental and mutant strains were taken.

N-terminal sequence determination. A CWB sample of the multiple mutant strain (SH124, wapA lycC lycD sigD) was
Fig. 1. Cell separation of stationary-phase cultures of *B. subtilis* strains illustrated by phase-contrast micrographs (bar, 10 μm) (a), liquid cultures (b) and electron micrographs (bar, 1 μm) (c). Phase-contrast samples were stained and mounted as described in Methods. Liquid cultures (10 ml nutrient broth) were grown for 18 h at 25 °C with gentle shaking (45 r.p.m.) and carefully poured into Petri dishes for photography. Electron micrograph samples were fixed in glutaraldehyde, embedded in araldite and sections stained in uranyl acetate followed by Reynolds lead citrate solution. Photographs are of representative samples from at least two independently grown cultures.
RESULTS

Construction of multiple lytic enzyme mutants of B. subtilis 168

B. subtilis strain 1A304 was used as the parental strain for mutant construction as it is unable to express three phage lytic enzymes usually associated with B. subtilis 168. The lytC gene was disrupted by insertionional inactivation using a phleomycin resistance cassette. The lytD gene was similarly inactivated using a spectinomycin resistance cassette. The correct insertion in both genes was confirmed by Southern blotting and renaturing gel analysis (results not shown). A set of isogenic strains was constructed (Table 1) to allow the individual and combined roles of the autolysins and associated components to be assessed.

Cell growth and division

All strains grew at equivalent rates in nutrient broth and reached the same final OD$_{600}$ value (results not shown). However, accurate OD$_{600}$ readings could not be obtained for strain SH131 (lytC lytD sigD) for reasons that are explained below. The effects of the single or multiple mutations on cell morphology during growth in batch culture were studied by phase-contrast microscopy. Strains HR and 1A304 showed identical morphology and division kinetics (results not shown). The chains of cells of the parental strain were shortest at the beginning of growth (lag phase), gradually becoming longer through exponential growth, reaching their maximum length (four to eight cells) as the cultures entered stationary phase. During stationary phase, the chains gradually shortened, eventually becoming unicellular and motile. All strains showed similar cellular dimensions and pattern of filamentation during growth (the degree of filamentation depending on the strain examined). Fig. 1 shows phase-contrast micrographs, liquid cultures and electron micrographs of representative samples from at least two cultures of selected strains. Strains SH115 (lytC) and SH119 (lytD) showed only very slight increases in chain length relative to the parental strain (Fig. 1a), four to ten cells per chain compared with two to six. A strain inactivated in sigD (SH118) was somewhat filamentous (12-18 cells per chain) (Fig. 1a). When lytC and lytD mutations were combined (SH128), extremely long chains of cells were formed that spanned many fields of view and often wrapped around each other forming large lumps when viewed by phase-contrast microscopy (Fig. 1a). These results suggested that both LytC and LytD have a role in cell separation but that they could mutually compensate for the lack of each other. The lytC lytD sigD triple mutant (SH131) had a similar phenotype to SH128 (lytC lytD) when viewed under the phase-contrast microscope (Fig. 1a), but in liquid culture large boli up to 10 mm across were formed (Fig. 1b). This suggested that another SigD-controlled enzyme (or enzymes) is involved in cell separation, as it was only in the multiple mutant strain that boli were formed. When the strains were viewed by electron microscopy it was noted that in the lytC-inactivated strain (SH115), the thickness of the cell walls (45-60 nm) and septa (60-95 nm) was greater than in wild-type (1A304) walls (25-40 nm) and septa (45-70 nm) (Fig. 1c). Also the walls were rougher when compared to the wild-type (Fig. 1c). The walls and septa of SH131 (lytC lytD sigD) showed even greater thickness at 60-70 nm and 100-120 nm, respectively (Fig. 1c).

Swarming motility

All strains bearing mutations in specific autolysin genes were motile in stationary phase when viewed by phase-contrast microscopy, apart from strain SH128 (lytC lytD), the immotility of which may be due to its very high degree of filamentation. The strain inactivated in sigD alone (SH118) was immotile as this sigma factor controls expression of genes essential for flagellar motility (Helmann et al., 1988). The strains were also analysed by growth on swarm plates since these are a measure not only of bacterial motility but also of chemotaxis. On swarm plates motile strains form a halo of diffuse growth around the tightly packed central colony. Swarming motility was measured on nutrient and minimal plates at 37 and 25 °C and the results were expressed as the percentage colony diameter relative to the parental strain (Table 2). Strain SH115 (lytC) showed a reduction in swarming motility, the halo diameter varying between 43 and 67% that of the parental strain, depending on the medium used and the temperature of incubation. Strain SH119 (lytD) also showed a reduction in motility. It swarmed to 40 and 34% the diameter of the parental strain on nutrient plates, and 77 and 60% the diameter of the parental strain on minimal plates at 37 and 25 °C, respectively. Strain SH118 (sigD) does not express the major flagellar protein, flagellin, and so it was predicted to be non-motile. However, it did apparently swarm to an extent, forming a small halo on nutrient plates (20 and 16% at 37 and 25 °C, respectively) and producing quite a large halo on minimal plates (48 and 52% at 37 and 25 °C, respectively). Strain SH128 (lytC lytD) does not swarm at all, forming no halo whatsoever under any conditions (11-16%). These results suggest that LytD and, to a lesser extent, LytC have a role in swarming motility and can partially compensate for each other.

Cell autolysis

Cellular lysis of B. subtilis was measured by adding either sodium azide or cloxacillin to exponentially growing cells and following lysis spectrophotometrically. Fig. 2(a) shows representative data from three independent experiments following lysis of cultures after addition of sodium azide. Lysis of 1A304 began immediately after the addition of sodium azide.
Table 2. Swarm plate assay of *B. subtilis* strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Swarm diameter (%)*</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NA, 37 °C</td>
</tr>
<tr>
<td>SH115</td>
<td>lytC*</td>
<td>65 ± 5.5</td>
</tr>
<tr>
<td>SH119</td>
<td>lytD*</td>
<td>40 ± 1.7</td>
</tr>
<tr>
<td>SH118</td>
<td>sigD</td>
<td>20 ± 1.1b</td>
</tr>
<tr>
<td>SH128</td>
<td>lytC lytD*</td>
<td>16 ± 1.5</td>
</tr>
</tbody>
</table>

* Swarm diameters were measured on nutrient agar (NA) or minimal agar (MA) plates at 37 or 25 °C as described in Methods. Results are expressed as a percentage of the parental strain (1A304) (70–85 mm) ± SEM. SEM equals *σ*/√*n* (Rashid et al., 1995), where *n* = 14 (a) or 5 (b).

and >95% lysis had occurred within 60 min. Strain SH115 (lytC) showed a marked reduction in rate of lysis, with only 17 and 49% reduction in OD600 occurring after 60 and 370 min, respectively. Conversely, strain SH119 (lytD) was only very slightly reduced in the rate of lysis, and this may not be significant. In fact strain SH128 (lytC lytD) lysed at the same rate as strain SH115 (lytC), suggesting that LytD is not involved in sodium-azide-induced cellular lysis. Strain SH118 (sigD) also showed a reduction in the rate of lysis compared to parental 1A304 with 36 and 95% lysis after 60 and 370 min, respectively. The decreased rate of lysis in SH118 was probably due to the reduced level of LytC or other SigD-controlled components. This phenomenon could not be investigated further as strain SH131 (lytC lytD sigD) was too filamentous to obtain accurate OD600 readings.

Lysis of *B. subtilis* after the addition of the cell-wall-synthesis-inhibiting antibiotic cloxacillin was studied with three independent experiments and representative data are shown in Fig. 2(b). The parental strain (1A304) lost >90% OD600 after 70 min. Both SH115 (lytC) and SH119 (lytD) had a reduced rate of lysis, losing 30 and 72%, respectively, of initial OD600 after 70 min. Strain SH118 (sigD) lysed at the same rate as SH115 (lytC), but in SH128 (lytC lytD) lysis was delayed by 40 min and then proceeded slowly. Thus both LytC and LytD have a role in antibiotic-induced lysis and are partially able to compensate for each other in this process.

Cell wall turnover

As *B. subtilis* cells grow and divide, old cell wall material is shed at the outer surface, presumably by the action of endogenous autolysins. This cell wall turnover was analysed in exponentially growing cells by two independent pulse-chase experiments using N-acetyl-D-[1-14C]glucosamine to label the cell wall peptidoglycan. Representative results are shown in Fig. 3. Strain 1A304 (parental) lost >90% of incorporated label after 90 min, which corresponds to about three generations. Strain SH115 (lytC) showed a marked reduction in the rate of wall turnover, with only 37% of incorporated label lost after 90 min. There was no delay in the initiation of release of labelled material. Strain SH119 (lytD) had almost identical wall turnover kinetics when compared with the parental strain. However, strain SH128 (lytC lytD) showed a greater reduction in wall turnover than SH115 (lytC). Thus both LytC and LytD were shown to have a role in wall turnover. The absence of LytD could, however, be totally compensated for by LytC, but LytD could only partially compensate for the lack of LytC. Strain SH118 (sigD) had a reduced rate of wall turnover when compared to the parental strain (56% release of label after 90 min) but the rate of
Analysis of minor vegetative cell autolysins

CWBP extracts from purified native cell walls give a fraction enriched for autolysins, as autolysins are primarily cell-wall-associated. The wapA gene encodes the proform of three major CWBPs in B. subtilis (Foster, 1993). Strains were constructed bearing the wapA mutation as this reduced total CWBP levels, allowing easier analysis and study of minor autolysins. Inactivation of wapA had no effect on the autolysin profile (Fig. 4, lane 2), cell morphology or pattern of cell division (results not shown). Renaturing SDS-PAGE analysis of CWBP preparations from the mutant strains confirmed the absence of LytC and LytD in the relevant strains (Fig. 4, lanes 3 and 4). Otherwise masked by the presence of LytC was another lytic enzyme of 49 kDa (lanes 3 and 6) (Smith et al., 1996), which may correspond to the CWBP49' of Margot & Karamata (1992). Expression of this enzyme is controlled by SigD as it was missing in the sigD background of the multiple mutant strain (SH124, lane 7). Despite the inactivation of both major vegetative autolysins and sigD, there were still at least ten bands of autolytic activity in the multiple mutant strain (lane 7). We have now obtained N-terminal sequences of seven of the corresponding proteins (results not shown). It is interesting to note that many of these are distinct from each other and so may correspond to different gene products or proteolytic products of larger preforms.

DISCUSSION

A set of isogenic strains has been created which has allowed in-depth physiological analysis of the roles of the major autolysins of B. subtilis. The parental strain used in these studies was 1A304, which is unable to express prophage lytic enzymes. This removes contaminating phage lytic activity. In all pertinent respects 1A304 is identical to HR (B. subtilis 168 trpC2) which can express phage enzymes. Mutants of B. subtilis 168 inactivated in the two major vegetative autolysin genes (lytC and lytD) have previously been constructed and studied phenotypically (Margot & Karamata, 1992; Margot et al., 1994; Rashid et al., 1993, 1995). However, our study has revealed a number of phenotypes that have not previously been recorded and has shown hitherto unknown roles for the autolysins during vegetative growth.

Margot et al. (1994) noted that a strain inactivated in lytC showed a 60 min delay in the release of radiolabelled cell-wall material, which subsequently occurred at a rate identical to that of the wild-type strain. Also, the lytD mutation had no effect on wall turnover either singly or in combination with lytC. However, our
experiments (Fig. 3) showed that SH115 (lytC) had a reduced rate of wall turnover but with no delay. Strain SH119 (lytD) was unaffected in wall turnover, but strain SH128 (lytC lytD) was reduced in the rate of wall turnover to a greater extent than SH115 (lytC). This shows that both LytC and LytD have a role in cell wall turnover. The absence of LytD could be totally compensated for by LytC, but LytD could only partially compensate for the lack of LytC. A strain inactivated in controlling motility and chemotaxis (Helmann et al., 1988), was slightly reduced in the rate of wall turnover.

The expression of both lytC and lytD is mostly under the control of SigD and so this reduced rate of turnover may be due to the lower levels of these enzymes in this strain. However, the greater reduction in the rate of wall turnover of strain SH131 (lytC lytD sigD) compared to SH128 (lytC lytD) suggests that a further SigD-controlled autolysin(s) may be involved.

It has long been suggested that one of the roles for autolysins in bacteria is to facilitate separation of cells after septation (Lominski et al., 1958; Forsberg & Rogers, 1971). Although filamentous mutants of B. subtilis have been isolated (Fein & Rogers, 1976), these mutations were subsequently found to be located in regulatory genes (Pooley & Karamata, 1984). These have been characterized as sigD (Helmann et al., 1988) and sin (Gaur et al., 1986), both of which show reduced autolysin levels. It was postulated that the reason for the filamentation of sigD and sin strains was the decreased amounts of LytC and LytD (Fein & Rogers, 1976). Surprisingly, when the structural genes encoding LytC and LytD were inactivated this had no effect on cell separation (Margot et al., 1994; Rashid et al., 1995). However, our mutants show notable filamentation changes compared to the parental strain (Fig. 1a). More physiological growth conditions, for example lower temperature, may affect the degree of filamentation. Also, to reduce the physical disruption effects on chain length, cultures were shaken at lower speeds. Aeration was maintained by use of a low culture-to-flask volume ratio (10 ml nutrient broth in a 250 ml flask). By growing our mutants under these conditions we observe substantial increases in chain lengths which highlights further differences between the strains (Fig. 1b). All strains showed similar kinetic patterns of filamentation to each other throughout growth. Single inactivations of lytC and lytD showed slight increases in cell chain lengths compared to the parental strain. The very obvious filamentous phenotype of SH128 (lytC lytD) suggests that both of the major vegetative enzymes have roles in cell separation. These enzymes can compensate for the lack of each other, as it is only when both genes are inactivated that there is gross filamentation. As described previously (Helmann et al., 1988), a sigD-inactivated strain is somewhat filamentous, suggesting that the autolysins involved in septal peptidoglycan hydrolysis are under the control of \( \sigma^D \). The lytC gene is 70–90% and lytD is 95% under the control of SigD (Lazarevic et al., 1992; Kuroda & Sekiguchi, 1993; Margot et al., 1994; Rashid et al., 1995). Therefore, the filamentation in a sigD strain may just be due to the reduced levels of LytC and LytD in this mutant. However, seemingly at least one other SigD-controlled enzyme is involved in cell separation, as only strain SH131 (lytC lytD sigD) forms large filamentation masses, or boli, in liquid culture (Fig. 1b). A candidate for the third cell separation enzyme under the control of SigD is the 49 kDa autolysin identified by renaturing gel electrophoresis in strain SH123 (Fig. 4, lane 6) (Smith et al., 1996). This is probably the protein described as CWBP49 by Margot & Karamata (1992), as this is also SigD-controlled. Therefore, at least three enzymes are involved in cell separation, all with mutually compensatory roles. This functional redundancy suggests that the enzymes are performing similar functions in cell separation.

The minor 49 kDa enzyme may also be involved in cell wall turnover as SH131 (lytC lytD sigD) shows reduced turnover compared to SH128 (lytC lytD). Thus LytC, LytD and the minor 49 kDa enzyme are also implicated in cell wall turnover, and so it is possible that cell separation is simply a function of cell wall turnover. Wall material is constantly being cleaved and released and when enough of this activity has occurred at a nascent septum the cells can separate. This hypothesis is supported by electron microscopy, as both SH115 (lytC) and SH131 (lytC lytD sigD) have thickened septa (Fig. 1c), concomitant with reduced wall turnover. However, strain SH118 (sigD) is more filamentous than strain SH115 (lytC) but is less affected in wall turnover. Furthermore, it has been shown that wall turnover is slower at the poles compared to the cylindrical regions of the cell (Archibald & Coapes, 1976), although the poles do still turn over (Clarke-Sturman et al., 1989). Although cell wall turnover may have a role to play in cell separation, there is seemingly a more specialized process occurring. What is special about the poles? It could be that autolysins are preferentially located here. This seems unlikely, however, as both LytC and LytD are involved in cell wall turnover which occurs primarily over the cylindrical surface. A change in the substrate peptidoglycan would allow autolysins to fulfil roles in cell wall turnover and cell separation. How the activity of autolysins is controlled at the post-translational level is unknown, but it has been postulated that it is due to membrane energization (Jolliffe et al., 1981), lipoteichoic acid inhibition (Fischer et al., 1981), substrate conformation (Koch et al., 1985), the wall ionic environment (Cheung & Freese, 1985) and extracellular protease activity (Jolliffe et al., 1980). Control may, therefore, be mediated by electrochemical interactions directly or indirectly by affecting the three-dimensional conformation of the substrate. Peptidoglycan conformation at the outer surface of the cell may be equivalent to that of the central divide of the nascent septum, allowing any one of a number of enzymes to act at either of these locations. In this model, when the cell is killed by sodium azide, destroying the proton motive force across the membrane, the conformation of the cell wall...
peptidoglycan is likely to change, allowing unrestricted autolysin activity and generalized cell lysis. There are, however, further levels of control, as LytD cannot compensate for the lack of LytC in this process. Antibiotic-induced death and lysis is a more complex process and its mechanism has yet to be fully explained (Rogers et al., 1983).

Fein (1979) noted that mutants FJ3, FJ6, FJ7 and NilS are 90% deficient in both the amidase (LytC) and the glucosaminidase (LytD) and are non-motile, suggesting that autolysins are required for flagellar morphogenesis. These mutations were found to be in regulatory genes (Pooley & Karamata, 1984) sigD and sin (Helmann et al., 1988; Gaur et al., 1986), which control the expression of flagellar proteins. Previous results are conflicting as to the role of the major autolysins in swarming. Margot et al. (1994) reported that a strain inactivated in \textit{lytC} alone showed a reduction in motility on swarm plates, but a \textit{lytD} single mutant was unaffected. Rashid et al. (1993) reported that a \textit{lytC} \textit{lytD} doubly inactivated strain showed a marked reduction in swarming motility, but the single mutants were unaffected. However, more recently published work has shown that both the \textit{lytC} and \textit{lytD} single mutants were reduced in swarming motility (Rashid et al., 1995). In our results, strains SH115 (\textit{lytC}) and SH119 (\textit{lytD}) were reduced in swarming motility, SH119 more so than SH115. Strain SH128 (\textit{lytC} \textit{lytD}) was non-motile on swarm plates (Table 2). When \textit{sigD}, the chemotaxis and motility sigma factor gene, is inactivated the strain is somewhat filamentous. Autolysins involved in hydrolysis of septal peptidoglycan, including LytC and LytD, are presumably co-regulated with the motility genes because it is advantageous for the bacteria to split into single cells when they become motile, to allow them to move more freely and disperse. CWBP49\textsuperscript{*}, which we postulate has a role in cell separation and cell wall turnover, is under the control of SigD and so it can be predicted that inactivation of the gene for this enzyme, either singly or in concert with other autolysin genes, would also impair motility. The lack of motility of SH128 (\textit{lytC} \textit{lytD}) is probably due to the fact that this strain is highly filamentous and so cannot move over the surface. The reduced swarming of strains SH115 (\textit{lytC}) and SH119 (\textit{lytD}) is probably due to the slightly more filamentous phenotype of these mutants. Both of these strains are motile when viewed microscopically. However, even two unseparated cells will be unable to chemotax effectively, as they will be transducing signals independently and will be attempting to roll and tumble in different directions from each other. Two unseparated cells will, therefore, form a type of pushmi-pullu (a llama with a head at both ends in 'The Story of Dr Dolittle' by Hugh Lofting). Therefore, the reduction in motility on swarm plates may be a result of their filamentous phenotypes. However, despite the obvious difference in motility between the \textit{lytC} and \textit{lytD} strains, the difference in cell separation of these strains is not great. This leaves open the possibility that autolysins are involved in other swarming-associated functions. How flagellum extrusion through the cell wall occurs is unknown but it may well involve the action of autolysins (Dijkstra & Keck, 1996). The apparent motility observed in the \textit{sigD} strain SH118 (Table 2) may be due to colony spreading as a function of growth. In support of this, the more filamentous strain (SH128) swarms less. Only two vegetatively expressed autolysins in \textit{B. subtilis} (\textit{lytC} and \textit{lytD}) have been studied at the molecular level. In this study we have identified roles for these enzymes in cell separation, motility, cell lysis and cell wall turnover. We have proposed that a minor 49 kDa autolysin, controlled by \textit{SigD}, has roles in cell separation and cell wall turnover. To determine the predicted roles of this enzyme it will be necessary to inactivate its structural gene. As this protein is produced at very low levels it has so far proved impracticable to purify enough protein for analysis. To circumvent this problem we have constructed a strain bearing an inducible \textit{sigD} gene in a \textit{flgM} (anti-sigma factor) (Fredrick & Helmann, 1996) background. Overexpression of the 49 kDa enzyme in this strain will allow further analysis.

There are many minor autolysins associated with vegetative cells of \textit{B. subtilis} (Fig. 4, lane 7). Also, the \textit{B. subtilis} genome sequencing project has revealed several autolysin homologues. Thus there are many more autolysins still to be analysed, whose function and regulation are completely unknown. We propose that the only rational way to investigate the complex interactive roles of these enzymes is by the study of isogenic multiple mutants using a combinatorial approach. We have set the foundations for this longer term strategy and have already revealed unexpected roles for the major enzymes. The central question still remains as to why this organism, and many others, have so many autolytic enzymes with seemingly overlapping functions.

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