The role of autolysins during vegetative growth of *Bacillus subtilis* 168

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

Author for correspondence: Simon J. Foster. Tel: +44 114 222 4411. Fax: +44 114 272 8697.
e-mail: s.foster@sheffield.ac.uk

Department of Molecular Biology and Biotechnology, University of Sheffield, Firth Court, Western Bank, Sheffield S10 2TN, UK

A set of isogenic mutants of *Bacillus subtilis* 168, insertionally inactivated in the genes encoding a number of lytic enzymes and a sigma factor (σ^D , 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 σ^D -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

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 (Ghuysen *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 σ^D , 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 σ^A , 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 σ^D (Margot *et al.*, 1994), although about 5% of transcription may be

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

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 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 phleomycin 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* XL0LR (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 *Bgl*II, 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 pIC22 (Steinmetz & Richter, 1994) by restriction with *Sma*I and *Pvu*II. Restriction analysis of the resultant plasmid, pTJS43, using *Bam*HI 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 *Eco*RI 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 *Pst*I fragment, containing part of the *lytD* gene, from p5102 (Margot *et al.*, 1994) was ligated into the *Pst*I restriction site of pUBS1 (from G. Murphy, Institute of Plant Science Research, Cambridge, UK) to create plasmid pSAB-100. The spectinomycin resistance cassette was excised from pIC156 (Steinmetz & Richter, 1994) using *Sma*I and ligated into the *Hpa*I site of pSAB100 to create pSAB101. Restriction analysis of pSAB101, using *Bam*HI, 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 *Eco*RI 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 0.3% 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 cloxacillin 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 (11 000 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

Table 1. Bacterial strains and plasmids

Strain or plasmid	Genotype/phenotype	Source/reference*
Strains		
<i>B. subtilis</i> 168		
HR	<i>trpC2</i>	Laboratory stock
1A304	<i>trpC2 metB5 xin-1 SPβ(s)</i>	BGSC†
SFG103	<i>trpC2 wapA::pSFG126 Ery^r</i>	Foster (1993)
DP-1	<i>trpC2 sigD::pLM5 Cm^r</i>	Helmann <i>et al.</i> (1988)
SH125	<i>trpC2 lytC::ble</i>	This study
SH119	<i>trpC2 metB5 xin-1 SPβ(s) lytD::spc</i>	This study
SH115	<i>trpC2 metB5 xin-1 SPβ(s) lytC::ble</i>	SH125 → 1A304
SH118	<i>trpC2 metB5 xin-1 SPβ(s) sigD::pLM5 Cm^r</i>	DP-1 → 1A304
SH117	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r</i>	SFG103 → 1A304
SH128	<i>trpC2 metB5 xin-1 SPβ(s) lytC::ble lytD::spc</i>	SH119 → SH115
SH131	<i>trpC2 metB5 xin-1 SPβ(s) lytC::ble lytD::spc sigD::pLM5 Cm^r</i>	SH118 → SH128
SH120	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r lytC::ble</i>	SH115 → SH117
SH121	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r lytD::spc</i>	SH119 → SH117
SH127	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r sigD::pLM5 Cm^r</i>	SH118 → SH117
SH123	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r lytC::ble lytD::spc</i>	SH119 → SH120
SH124	<i>trpC2 metB5 xin-1 SPβ(s) wapA::pSFG126 Ery^r lytC::ble lytD::spc sigD::pLM5 Cm^r</i>	SH118 → SH123
Plasmids		
p6302	pJH101 with 2·3 kb <i>Bam</i> HI– <i>Eco</i> RI insert carrying <i>B. subtilis</i> <i>lytC</i> gene	Lazarevic <i>et al.</i> (1992)
p5102	pMTL20EC with 4 kb <i>Bam</i> HI insert carrying <i>B. subtilis</i> <i>lytD</i> gene	Margot <i>et al.</i> (1994)
pTJS43	p6302 with 1·5 kb phleomycin resistance cassette (<i>ble</i>) inserted into unique <i>Bgl</i> II site	This study
pSAB100	1·1 kb <i>Pst</i> I fragment from p5102 cloned into unique <i>Pst</i> I site of pUBS1	This study
pSAB101	pSAB100 with 1·2 kb spectinomycin resistance cassette (<i>spc</i>) inserted into unique <i>Hpa</i> I site	This study

* 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 (Aminco, 180 MPa, 4 °C) to break the cells. Cell walls were sedimented by centrifugation (27 000 *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. CWBPs 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. CWBP 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 25 °C with gentle shaking (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 CWBP sample of the multiple mutant strain (SH124, *wapA lytC lytD sigD*) was

Fig. 1. Cell separation of stationary-phase cultures of *B. subtilis* strains illustrated by phase-contrast micrographs (bar, 10 μ m) (a), liquid cultures (b) and electron micrographs (bar, 1 μ m) (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 $^{\circ}$ 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.

subjected to 11% SDS-PAGE and then electrophoretically transferred onto ProBlott membrane (Applied Biosystems) as described by the manufacturer. A number of bands, corresponding to lytic bands on the renaturing gel, were excised and the peptide sequence was determined with an Applied Biosystems 476A protein sequencer.

RESULTS

Construction of multiple lytic enzyme mutants of *B. subtilis* 168

B. subtilis 168 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 insertional 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 OD₆₀₀ value (results not shown). However, accurate OD₆₀₀ 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* had 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

Strain	Genotype	Swarm diameter (%)*			
		NA, 37 °C	NA, 25 °C	MA, 37 °C	MA, 25 °C
SH115	<i>lytC</i> ^a	65 ± 5.5	43 ± 2.3	48 ± 2.9	67 ± 1.4
SH119	<i>lytD</i> ^a	40 ± 1.7	34 ± 3.3	77 ± 6.5	60 ± 2.3
SH118	<i>sigD</i>	20 ± 1.1 ^b	16 ± 1.8 ^b	48 ± 9.4 ^a	52 ± 3.3 ^a
SH128	<i>lytC lytD</i> ^b	16 ± 1.5	15 ± 1.0	13 ± 0.3	11 ± 0.7

* 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 $\sigma_{n-1}/\sqrt{n}$ (Rashid *et al.*, 1995), where $n = 14$ (a) or 5 (b).

Fig. 2. Cell lysis of exponential-phase cultures of *B. subtilis* wild-type and mutant strains after the addition of sodium azide (a) or cloxacillin (b). Sodium azide (0.05 M) or cloxacillin (5 µg ml⁻¹, 10×MIC) was added to exponentially growing cultures (OD₆₀₀ 0.25–0.3) and lysis followed spectrophotometrically at 600 nm. □, 1A304 (parent); ■, SH115 (*lytC*); ○, SH119 (*lytD*); ●, SH118 (*sigD*); △, SH128 (*lytC lytD*). Data are representative of three independent experiments.

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 labelled material 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

Fig. 3. Cell wall turnover of exponential-phase cultures of *B. subtilis* wild-type and mutant strains. Cells were labelled for five generations with *N*-acetyl-D-[1-¹⁴C]glucosamine and transferred to chase medium (Margot & Karamata, 1992). Samples, withdrawn at regular intervals, were precipitated with ice-cold 5% trichloroacetic acid, filtered and washed. Radioactivity of filters and filtrates was determined and the proportion of label released through wall turnover was calculated as: $100 \times \text{d.p.m. on filters} / (\text{d.p.m. on filters} + \text{d.p.m. in filtrates})$. □, 1A304 (parent); ■, SH115 (*lytC*); ○, SH119 (*lytD*); ●, SH118 (*sigD*); △, SH128 (*lytC lytD*); ▲, SH131 (*lytC lytD sigD*). Data are representative of two independent experiments.

wall turnover was still greater than that of strain SH115 (*lytC*). This could be explained by the small proportion of *lytC* expression remaining in SH118 (*sigD*). Strain SH131 (*lytC lytD sigD*) exhibited only 5% wall turnover after 90 min, even less than SH128 (*lytC lytD*), which exhibited 15% wall turnover after 90 min. This suggested that a further, as yet unknown, SigD-controlled autolysin has a role in cell wall turnover.

Differentiation

No observable, significant differences were seen in sporulation frequency and germination rate between any of the strains studied in this work.

Analysis of minor vegetative cell autolysins

CWBP extracts from purified native cell walls give a fraction enriched for autolysins, as autolysins are primarily 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). Ex-

Fig. 4. Renaturing SDS-PAGE of CWBP extracts of *B. subtilis* 168 wild-type and mutant strains. Samples were prepared as described in Methods and renaturing SDS-PAGE was carried out as previously described (Foster, 1992) using purified *B. subtilis* cell walls as the substrate. Lanes: 1, 1A304 (parent); 2, SH117 (*wapA*); 3, SH120 (*wapA lytC*); 4, SH121 (*wapA lytD*); 5, SH118 (*wapA sigD*); 6, SH123 (*wapA lytC lytD*); 7, SH124 (*wapA lytC lytD sigD*).

pression 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 proforms.

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 radio-labelled 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 *sigD*, the gene that encodes σ^D , the sigma factor 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 σ^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 Nil5 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* (Helmman *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 *lytC* alone showed a reduction in motility on swarm plates, but a *lytD* single mutant was unaffected. Rashid *et al.* (1993) reported that a *lytC 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 *lytC* and *lytD* single mutants were reduced in swarming motility (Rashid *et al.*, 1995). In our results, strains SH115 (*lytC*) and SH119 (*lytD*) were reduced in swarming motility, SH119 more so than SH115. Strain SH128 (*lytC lytD*) was non-motile on swarm plates (Table 2). When *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', 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 (*lytC 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 (*lytC*) and SH119 (*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-pullyu (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 *lytC* and *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 *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 *B. subtilis* (*LytC* and *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 *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 *sigD* gene in a *flgM* (anti-sigma factor) (Fredrick & Helmman, 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 *B. subtilis* (Fig. 4, lane 7). Also, the *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.

ACKNOWLEDGEMENTS

This work was supported by the BBSRC and the Royal Society. We are grateful to M. Chamberlin, G. Murphy and D. Karamata for gifts of strains and plasmids.

REFERENCES

- Anagnostopoulos, C. & Spizizen, J. (1961). Requirements for transformation in *Bacillus subtilis*. *J Bacteriol* 81, 741–746.
- Archibald, A. R. & Coapes, H. E. (1976). Bacteriophage SP50 as a marker for cell wall growth in *Bacillus subtilis*. *J Bacteriol* 125, 1195–1206.
- Cheung, H. Y. & Freese, E. (1985). Mono-valent cations enable cell-wall turnover of the turnover-deficient *lyt-15* mutant of *Bacillus subtilis*. *J Bacteriol* 161, 1222–1225.
- Clarke-Sturman, A. J., Archibald, A. R., Hancock, I. C., Harwood, C. R., Merad, T. & Hobot, J. A. (1989). Cell wall assembly in *Bacillus subtilis*: partial conservation of polar wall material and the effect of growth conditions on the pattern of incorporation of new material at the polar caps. *J Gen Microbiol* 135, 657–665.
- Cutting, S. M. & Vander Horn, P. B. (1990). Genetic analysis. In *Molecular Biology Methods for Bacillus*, pp. 27–74. Edited by C. R. Harwood & S. M. Cutting. Chichester: John Wiley.

- Dijkstra, A. J. & Keck, W. (1996). Peptidoglycan as a barrier to transenvelope transport. *J Bacteriol* 178, 5555–5562.
- Fein, J. E. (1979). Possible involvement of bacterial autolysin enzymes in flagellar morphogenesis. *J Bacteriol* 137, 933–946.
- Fein, J. E. & Rogers, H. J. (1976). Autolytic enzyme deficient mutants of *Bacillus subtilis* 168. *J Bacteriol* 127, 1427–1442.
- Fischer, W., Rosel, P. & Koch, H. U. (1981). Effect of alanine ester substitution and other structural features of lipoteichoic acids on their inhibitory activity against autolysins of *Staphylococcus aureus*. *J Bacteriol* 146, 467–475.
- Forsberg, C. & Rogers, H. J. (1971). Autolytic enzymes in growth of bacteria. *Nature* 229, 272–273.
- Foster, S. J. (1991). Cloning, expression, sequence analysis and biochemical characterization of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. *J Gen Microbiol* 137, 1987–1998.
- Foster, S. J. (1992). Analysis of the autolysins of *Bacillus subtilis* 168 during vegetative growth and differentiation by using renaturing polyacrylamide gel electrophoresis. *J Bacteriol* 174, 464–470.
- Foster, S. J. (1993). Molecular analysis of three major wall-associated proteins of *Bacillus subtilis* 168: evidence for processing of the product of a gene encoding a 258 kDa precursor two-domain ligand-binding protein. *Mol Microbiol* 8, 299–310.
- Fredrick, K. & Helmann, J. D. (1996). FlgM is a primary regulator of σ^D activity, and its absence restores motility to a *sinR* mutant. *J Bacteriol* 178, 7010–7013.
- Gaur, N. K., Dubnau, E. & Smith, I. (1986). Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. *J Bacteriol* 168, 860–869.
- Ghuysen, J.-M., Tipper, D. J. & Strominger, J. L. (1966). Enzymes that degrade bacterial cell walls. *Methods Enzymol* 8, 685–699.
- Hauser, P. M. & Errington, J. (1995). Characterization of cell cycle events during the onset of sporulation in *Bacillus subtilis*. *J Bacteriol* 177, 3923–3931.
- Helmann, J. D., Márquez, L. M. & Chamberlin, M. J. (1988). Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. *J Bacteriol* 170, 1568–1574.
- Herbold, D. R. & Glaser, L. (1975). *Bacillus subtilis* N-acetylmuramic acid L-alanine amidase. *J Biol Chem* 250, 1676–1682.
- Jolliffe, L. K., Doyle, R. J. & Streips, U. N. (1980). Extracellular proteases modify cell wall turnover in *Bacillus subtilis*. *J Bacteriol* 141, 1199–1208.
- Jolliffe, L. K., Doyle, R. J. & Streips, U. N. (1981). The energised membrane and cellular autolysis in *Bacillus subtilis*. *Cell* 25, 753–763.
- Koch, A. L., Kirchner, G., Doyle, R. J. & Streips, U. N. (1995). How does a *Bacillus* split its septum right down the middle? *Ann Inst Pasteur Microbiol* 136, 91–98.
- Kuroda, A. & Sekiguchi, J. (1991). Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. *J Bacteriol* 173, 7304–7312.
- Kuroda, A. & Sekiguchi, J. (1993). High-level transcription of the major *Bacillus subtilis* autolysin operon depends on expression of the sigma D gene and is affected by a *sin(flaD)* mutation. *J Bacteriol* 175, 795–801.
- Kuroda, A., Rashid, M. H. & Sekiguchi, J. (1992). Molecular cloning and sequencing of the upstream region of the major *Bacillus subtilis* autolysin gene: a modifier protein exhibiting sequence homology to the major autolysin and the *spoIID* product. *J Gen Microbiol* 138, 1067–1076.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227, 680–685.
- Lazarevic, V., Margot, P., Soldo, B. & Karamata, D. (1992). Sequencing and analysis of the *Bacillus subtilis* *lytRABC* divergon: a regulatory unit encompassing the structural genes of the N-acetylmuramoyl-L-alanine amidase and its modifier. *J Gen Microbiol* 138, 1949–1961.
- Lominski, I., Cameron, J. & Wyllie, G. (1958). Chaining and unchaining of *Streptococcus faecalis*: a hypothesis of the mechanism of bacterial separation. *Nature* 181, 1477.
- Margot, P. & Karamata, D. (1992). Identification of the structural genes for N-acetylmuramoyl-L-alanine amidase and its modifier in *Bacillus subtilis* 168: inactivation of these genes by insertional mutagenesis has no effect on growth or cell separation. *Mol Gen Genet* 232, 358–366.
- Margot, P., Mauël, C. & Karamata, D. (1994). The gene of the N-acetylglucosaminidase, a *Bacillus subtilis* cell wall hydrolase not involved in vegetative cell autolysis. *Mol Microbiol* 12, 535–545.
- Pooley, H. & Karamata, D. (1984). Genetic analysis of autolysin-deficient and flagellaless mutants of *Bacillus subtilis*. *J Bacteriol* 160, 1123–1129.
- Rashid, M. H., Kuroda, A. & Sekiguchi, J. (1993). *Bacillus subtilis* mutant deficient in the major autolytic amidase and glucosaminidase is impaired in motility. *FEMS Microbiol Lett* 112, 135–140.
- Rashid, M. H., Mori, H. & Sekiguchi, J. (1995). Glucosaminidase of *Bacillus subtilis*: cloning, regulation, primary structure and biochemical characterization. *Microbiology* 141, 2391–2404.
- Rogers, H. J., Thurman, P. F. & Burdett, I. D. J. (1983). The bactericidal action of β -lactam antibiotics on an autolysin-deficient strain of *Bacillus subtilis*. *J Gen Microbiol* 129, 465–478.
- Rogers, H. J., Taylor, C., Rayter, S. & Ward, J. B. (1984). Purification and properties of autolytic endo- β -N-acetylglucosaminidase and the N-acetylmuramoyl-L-alanine amidase from *Bacillus subtilis* strain 168. *J Gen Microbiol* 130, 2395–2402.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Smith, T. J. & Foster, S. J. (1995). Characterisation of the involvement of two compensatory autolysins in mother cell lysis during sporulation of *Bacillus subtilis* 168. *J Bacteriol* 177, 3855–3862.
- Smith, T. J., Blackman, S. A. & Foster, S. J. (1996). Peptidoglycan hydrolases of *Bacillus subtilis* 168. *Microb Drug Resist* 2, 113–118.
- Steinmetz, M. & Richter, R. (1994). Plasmids designed to alter the antibiotic resistance expressed by insertion mutations in *Bacillus subtilis*, through *in vivo* recombination. *Gene* 142, 79–83.
- Stewart, G. S. A. B., Johnstone, K., Hagelberg, E. & Ellar, D. J. (1981). Commitment of bacterial spores to germinate. *Biochem J* 198, 101–106.
- Ward, J. B. & Williamson, R. (1984). Bacterial autolysins: specificity and function. In *Microbial Wall Synthesis and Function*, pp. 159–166. Edited by C. Nombela. Amsterdam: Elsevier.

Received 24 June 1997; revised 1 September 1997; accepted 12 September 1997.