JOURNAL OF BACTERIOLOGY, July 1995, p. 3855–3862 Vol. 177, No. 13
0021-9193/95/$04.00+0

Characterization of the Involvement of Two Compensatory Autolysins in Mother Cell Lysis during Sporulation of *Bacillus subtilis* 168

THOMAS J. SMITH AND SIMON J. FOSTER*

Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2UH, United Kingdom

Received 7 February 1995/Accepted 26 April 1995

The 30-kDa sporulation-specific peptidoglycan hydrolase CwlC of *Bacillus subtilis* 168 was purified and characterized. It is an *N*-acetylmuramoyl-L-alanine amidase (amidase) that is associated with the mother cell wall of sporulating cells, and although it is secreted, it undergoes no N-terminal processing except removal of the initial methionine. It was found that mother cells of a strain insertionally inactivated in *cwlC* and *lytC* (the major vegetative amidase gene) did not lyse at the end of sporulation. Mutants with single mutations in *cwlC* or *lytC* lysed, and so the two autolysins must have mutually compensatory roles in mother cell lysis. Active CwlC and LytC are present at the time of mother cell lysis; however, reporter gene analysis revealed that *lytC* transcription ceases early in sporulation, and therefore the function that LytC has in mother cell lysis is performed by material remaining from presporulation expression. Autolytic enzymes similar in molecular mass to CwlC were detected in two other *Bacillus* species by their cross-reactivity with anti-CwlC antiserum.

Autolysins, enzymes that hydrolyze bacterial cell wall peptidoglycan, are ubiquitous among bacteria (11), although their precise roles remain uncertain. In the spore-forming gram-positive bacterium *Bacillus subtilis*, there may be 10 or more peptidoglycan hydrolases (8, 10). Such enzymes have been implicated in several functions during vegetative growth and sporulation (34, 41).

The differentiation process of sporulation involves a number of events of cell wall rearrangement, in which peptidoglycan-degradative enzymes evidently play a fulcral role (10). These include digestion of the asymmetric septum to permit prespore engulfment (5), cortex maturation (1, 42), mother cell lysis, and cortex autolysis during germination (5). Mother cell lysis to release the mature endospore is the final characteristic morphological event that occurs during sporulation. A 30-kDa mother cell-associated autolysin appears during sporulation and has been postulated to be responsible for mother cell lysis (8). A gene, *cwlC*, which appears to encode this protein has been cloned and sequenced; the deduced gene product is a 255-amino-acid, 27-kDa peptide. Its expression is controlled by σ^K (18), the mother cell-specific, late-sporulation sigma factor (6, 27). However, its role in sporulation was unknown, as the mother cells of a *cwlC* mutant still lyse (18).

LytC is the major vegetative cell amidase of *B. subtilis* 168 and is also present during sporulation (8). The *lytC* gene is part of a three-gene operon, *lytABC* (25). *lytR*, which is transcribed divergently from *lytABC*, encodes a putative DNA-binding protein, which represses expression of itself and *lytABC* (25). *lytA* encodes an acidic protein that is thought to be membrane associated (20, 25). *lytB* encodes a cell wall-associated modifier protein, which binds to the LytC amidase and enhances its activity (14). A study with a plasmid clone carrying a *lytA*::*lacZ* translational fusion suggested that, during vegetative growth, expression of *lytABC* peaks in late exponential phase and falls off in stationary phase (20). Two promoters for *lytABC* have been detected: one is recognized by the major housekeeping sigma factor, σ^A; the other, which directs 75 to 85% of the transcription during vegetative growth, is controlled by a minor sigma factor, σ^D (21, 22, 25). σ^D controls the expression of genes involved in motility and chemotaxis (13, 31). *sigD* mutants are immotile and form abnormally long filaments of cells (13).

In this study, CwlC was purified and characterized. Mutational and transcriptional analyses have revealed the compensatory roles of *lytC* and *cwlC* in mother cell lysis.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The plasmids and strains of *B. subtilis* 168 used in this study are shown in Table 1. *Bacillus megaterium* KM and QMB1551, *Bacillus licheniformis* ATCC 8480, *Bacillus circulans* ATCC 4513, *Bacillus cereus* T, and *Bacillus amyloliquefaciens* H were obtained from the *Bacillus* Genetic Stock Center. Vegetative cells of *Bacillus* species were grown in nutrient broth or on nutrient agar plates; sporulation was initiated in CCY medium (39). When appropriate, chromosomal drug resistance markers in *B. subtilis* were selected with chloramphenicol (3 μg/ml), erythromycin (1 μg/ml), and lincomycin (25 μg/ml). Synchronous sporulation was performed by the resuspension method of Sterlini and Mandelstam, in which the cells are grown to mid-log phase in a nutrient medium and then synchronous sporulation is initiated by transferring them to a starvation medium (38). *Escherichia coli* DH5α (Bethesda Research Laboratories) (12) and *Micrococcus luteus* ATCC 4698 were grown in Luria-Bertani (LB) broth or on LB agar plates; *Staphylococcus aureus* 8325-4 was grown in tryptic soy broth (TSB) or on TSB agar plates. In *E. coli*, plasmids were selected with ampicillin (50 μg/ml). All bacterial cultures were grown at 37°C.

Purification of bacterial cell wall substrates. Cell walls from vegetative cells of *B. subtilis*, *M. luteus*, and *S. aureus* and from spores of *B. megaterium* were prepared as described previously (8, 15).

Assay for autolysin activity. Various cell wall substrates were used in the spectrophotometric assay for autolysin activity (7). Each assay was carried out in duplicate. One unit of enzyme activity is defined as the amount of enzyme necessary to decrease the A_{450} of a wall suspension by 0.001 min^{-1} at 37°C, measuring the maximum initial rate (7).

Purification of the sporulation-specific autolysin CwlC and preparation of antisera. CCY broth (20 1-liter preparations) was inoculated with *B. subtilis* SH108 and shaken (250 rpm) at 37°C until the mother cells were beginning to lyse (stage VI to VII, reached after about 26 h). The cells were harvested by centrifugation (11,000 × *g*, 4°C, 5 min), washed with ice-cold 50 mM Tris-HCl (pH 7.5), and resuspended in 60 ml of the same buffer. Phenylmethylsulfonyl fluoride (PMSF; Sigma) was added to 0.5 mM, followed by 0.67 volumes of ice-cold 10 M LiCl–50 mM Tris-HCl (pH 7.5). The suspension was mixed by continual inversion at 4°C for 1 h. Particulate matter was removed by centrifugation (30,000 × *g*, 4°C, 15 min), and the supernatant, which contained salt-

* Corresponding author. Mailing address: Department of Molecular Biology and Biotechnology, University of Sheffield, P.O. Box 594, Firth Court, Western Bank, Sheffield S10 2UH, United Kingdom. Phone: 44 114 282 4411. Fax: 44 114 272 8697. Electronic mail address: s.foster@sheffield.ac.uk.

TABLE 1. *B. subtilis* 168 strains and plasmids

Strain or plasmid	Genotype or description[a]	Origin (reference)
B. subtilis 168 strains		
HR	*trpC2*	Laboratory stock
ANC1	*purB his-1 smo-1* Δ*cwlC*::*cat*	J. Sekiguchi (18)
DP1	*trpC2 sigD*::pLM112	M. J. Chamberlin (13)
L16332	*pheA1 purA16 hisA35 trpC2 metB5 lytC*::p6223	D. Karamata (28)
IA4	*trpC2 aroI906 dal-1 purE1*	*Bacillus* Genetic Stock Center
SH103	*trpC2* Δ*cwlC*::*cat*	ANC1→HR[b]
SH104	*trpC2* Δ*cwlC*::*cat lytC*::p6223	L16332→SH103
SH108	*trpC2 lytC*::p6223	L16332→HR
SH109	*trpC2 sigD*::pLM112	DP1→HR
SH110	*trpC2* Δ*cwlC*::*cat sigD*::pTJS42	Integration of pTJS42 into *sigD* locus of SH103
SH111	*trpC2 lytA*::pTJS40	Integration of pTJS40 into *lytA* locus of HR
SH112	*trpC2 sigD*::pLM112 *lytA*::pTJS40	DP1→SH111
SH113	*trpC2 lytC*::pTJS41	Integration of pTJS41 into *lytC* locus of HR
SH114	*trpC2 sigD*::pLM112 *lytC*::pTJS41	DP1→SH113
Plasmids		
p6302	pJH101 with 2.3-kb *Bam*HI-*Eco*RI insert carrying *B. subtilis lytC* gene; Ap^r Cm^r	D. Karamata (25, 28)
p6327	pMTL20EC with 1.1-kb *Eco*RV fragment carrying *B. subtilis lytRABC* promoter region cloned into *Sma*I site; Ap^r Cm^r Ery^r	D. Karamata (25, 28)
pJH1-1	pUC19 with 0.85-kb *Bam*HI-*Eco*RI insert carrying 3′ region of *B. subtilis sigD* gene; Ap^r	M. J. Chamberlin (13)
pTJS40	pAZ106 with end-filled 1.1-kb *Eco*RI-*Stu*I fragment of p6327 cloned into *Sma*I site; Ap^r Ery^r	This study
pTJS41	pAZ106 with end-filled 0.74-kb *Hin*dIII-*Bgl*II fragment of p6302 cloned into *Sma*I site; Ap^r Ery^r	This study
pTJS42	pAZ106 with end-filled 0.37-kb *Pvu*II-*Eco*RI fragment of pJH1-1 cloned into *Sma*I site; Ap^r Ery^r	This study

[a] Abbreviations: Ap^r, Cm^r, Ery^r, resistance to ampicillin, chloramphenicol, and erythromycin/lincomycin, respectively.
[b] Arrows indicate construction by transformation with chromosomal DNA.

extractable cell wall proteins, was dialyzed three times against 40 volumes of 10 mM Tris-HCl (pH 7.5) (buffer 1) at 4°C. The extract was centrifuged (30,000 × *g*, 4°C, 10 min) to remove any precipitate, brought to room temperature, and loaded onto a column (8 by 1 cm) of reactive red 120-agarose type 3000 (Sigma), previously equilibrated with buffer 1. The column was washed with buffer 1, and then the bound proteins were eluted with an 80-ml linear gradient of 0 to 400 mM NaCl in buffer 1 at a flow rate of 1 ml min^{-1} over 40 2-ml fractions. Fractions containing autolysin activity were pooled and dialyzed overnight at 4°C against 100 volumes of 20 mM Tris-HCl (pH 8.5) (buffer 2). The dialyzed material was centrifuged (34,000 × *g*, 4°C, 10 min) to remove any precipitate, brought to room temperature, and applied to a Bio-Rad 5-ml EconoPac-Q anion-exchange column equilibrated with buffer 2. The column was washed with buffer 2, and the autolysin was eluted with a 60-ml linear gradient of 0 to 30 mM NaCl in buffer 2 at a flow rate of 1 ml min^{-1} over 30 2-ml fractions. The fractions containing autolysin activity were pooled.

Anti-CwlC antiserum was prepared as follows. Purified CwlC protein was subjected to sodium dodecyl sulfate–11% polyacrylamide gel electrophoresis (SDS–11% PAGE). The gels were stained minimally with Coomassie blue; the barely visible band corresponding to CwlC was excised, and the protein was electroeluted (Electrophor, LKB) from it (32). The electroeluted protein solution was dialyzed exhaustively against distilled water and lyophilized; the resulting material was resuspended in a small volume of water and used for production of antisera in a dwarf lop rabbit. Three injections were made, each with 100 to 200 μg of CwlC.

Determination of hydrolytic bond specificity. The mode of action of purified CwlC was determined as previously described (7, 9). Purified 30-kDa lytic enzyme was dialyzed against 100 volumes of 1 mM Tris-HCl buffer (pH 7.5). Purified walls were digested at 5 mg ml^{-1} in 10 mM $MgCl_2$–1 mM Tris-HCl buffer (pH 7.5) containing 1,500 U of purified enzyme per ml at 37°C. Periodically, 0.5-ml samples were removed for A_{450} readings and determination of the appearance of new reducing and amino termini. The method of Margot et al. (29) as described by Foster (7) was used to determine to which isomer of alanine new amino termini could be attributed.

Preparation of autolysin-containing extracts for SDS-PAGE. SDS extracts were made from *B. subtilis* cultures as follows. The cells were harvested by centrifugation (14,000 × *g*, room temperature, 10 min), washed with 50 mM Tris-HCl (pH 7.5), resuspended in 1/100 of the original culture volume of SDS sample buffer (62.5 mM Tris-HCl [pH 6.8], 1 mM EDTA, 1% [wt/vol] SDS, 5% [vol/vol] β-mercaptoethanol, 0.0025% [wt/vol] bromophenol blue, 10% [vol/vol] glycerol), and heated at 100°C for 5 min. The suspension was centrifuged (14,000 × *g*, room temperature, 10 min), and the supernatant, containing the extracted proteins, was removed and stored at −20°C.

SDS-PAGE, renaturing SDS-PAGE, and Western immunoblotting. Protein samples were analyzed by SDS-PAGE (24). All gels contained 11% (wt/vol) acrylamide; protein was visualized with Coomassie blue. Autolysin activity was detected by renaturing gel electrophoresis exactly as described by Foster (8). The molecular masses of autolysins were estimated by comparison with standards of known sizes (Dalton Mark VII-L; Sigma), which were run on the same gel and stained with Coomassie blue.

Samples for Western blot analysis were separated by SDS–11% PAGE and then transferred to nitrocellulose and treated with anti-CwlC antiserum as described by Burnette (3). Antigen-antibody complexes were detected by reaction with goat anti-rabbit immunoglobulin G (IgG) conjugated to alkaline phosphatase (Sigma).

N-terminal sequence determination. A sample of purified CwlC was subjected to SDS–11% PAGE and then electrophoretically transferred onto a Problott membrane (Applied Biosystems) as described by the manufacturer. The 30-kDa band, corresponding to CwlC, was excised, and the peptide sequence was determined with an Applied Biosystems 476A protein sequencer.

Cell fractionation. A 4-liter CCY culture of *B. subtilis* 168 HR was grown until ≥70% of the mother cells contained phase-bright mature endospores (stage VI of sporulation, 20 h). The cells were harvested (11,000 × *g*, 4°C, 10 min), washed with ice-cold 50 mM Tris-HCl (pH 7.5), and resuspended in 40 ml of the same buffer. The suspension was passed through a French pressure cell (180 MPa, 4°C), which broke the mother cell walls but left the forespores intact. The lysate was then fractionated by a modification of the method of Todd and Ellar (40), as follows. The whole procedure was performed at 0 to 4°C to minimize protein and peptidoglycan degradation. The lysate was centrifuged (3,000 × *g*, twice for 2 min each) to remove forespores and debris before being centrifuged (27,000 × *g*, 20 min) to sediment mother cell wall fragments. The cell walls were washed twice by resuspension in 50 mM Tris-HCl (pH 7.5) and extracted (100°C, 5 min) with SDS sample buffer, to give fraction F1 (mother cell wall-associated proteins). The

supernatant fraction was centrifuged again (27,000 × *g*, 20 min) to remove remaining wall material, and then membrane fragments were sedimented by centrifugation (105,000 × *g*, 60 min). The pellet was washed in 50 mM Tris-HCl (pH 7.5), centrifuged again under the same conditions, resuspended in SDS sample buffer, and boiled (100°C, 5 min) to give fraction F2 (proteins associated with mother cell membranes). The supernatant from the first ultracentrifugation was centrifuged again under the same conditions to remove remaining membranous material. The protein from the final supernatant was precipitated with trichloroacetic acid (10% [wt/vol], 30 min, 4°C) and washed three times with 1 ml of ice-cold acetone by centrifugation (14,000 × *g*, 5 min, 4°C) and resuspension. The pellet was resuspended in SDS sample buffer and boiled (100°C, 5 min) before centrifugation (14,000 × *g*, 5 min, room temperature) to remove insoluble material. This gave fraction F3 (mother cell cytoplasm).

An independent fractionation based on protoplast formation was also performed. An 800-ml sporulating culture of *B. subtilis* 168 HR was grown and harvested as described above. The pellet was resuspended in 50 ml of SMM (0.5 M sucrose, 10 mM $MgCl_2$, 40 mM maleic acid [pH 6.5]), and lysozyme was added to 0.5 mg/ml. The suspension was incubated at 37°C for 30 min, by which time protoplasting of the sporangia was complete. The protoplasts were harvested (5,000 × *g*, room temperature, 10 min) and then washed by gentle resuspension in SMM at room temperature followed by centrifugation (5,000 × *g*, room temperature, 10 min). The washed protoplasts were extracted with SDS sample buffer to give fraction P1, comprising membranes and cytoplasm from the mother cells as well as SDS-extractable material from the forespores. The supernatant from the protoplasting reaction mix was centrifuged again (5,000 × *g*, room temperature, 10 min) to remove remaining protoplasts. PMSF (1 mM) was added, and the mixture was dialyzed overnight against 50 mM Tris-HCl, pH 7.5 (two times 2 liters, 4°C). Particulate matter was removed by centrifugation (30,000 × *g*, 4°C, 15 min); the protein from the supernatant was trichloroacetic acid precipitated, resuspended in SDS sample buffer, and boiled for 5 min. Insoluble material was removed by centrifugation (14,000 × *g*, room temperature, 5 min), leaving fraction P2, comprising proteins released by digestion of the mother cell walls.

Creation of insertionally inactivated mutants and β-galactosidase transcriptional fusions. (i) Construction of a *lytA*::*lacZ* transcriptional fusion. Plasmid pTJS40 was created by end-filling the 1.1-kb *Eco*RI-*Stu*I fragment of p6327, which contained the promoter region and the 5′ portions of *lytA* and *lytR* (25, 28), and ligating with pAZ106 (16) that had been cut with *Sma*I and dephosphorylated. The correct orientation of the insert, in which *lacZ* runs in the same direction as *lytA*, was verified by restriction analysis with *Hin*dIII and *Eco*RI. *B. subtilis* 168 HR was transformed (2) with pTJS40, and transformants were selected with erythromycin and lincomycin, to create strain SH111. *B. subtilis* SH111 has an intact copy of *lytRABC* as well as a *lytA*::*lacZ* transcriptional fusion.

(ii) Construction of a *lytC*::*lacZ* transcriptional fusion. Plasmid pTJS41 was made by end-filling the 0.74-kb *Hin*dIII-*Bgl*II fragment of p6302, which contained an internal fragment of the *lytC* gene (25, 28), and ligating with pAZ106 that had been cut with *Sma*I and dephosphorylated. The correct orientation of the insert, in which *lacZ* and the reading frame of *lytC* run in the same direction, was verified by restriction analysis with *Xba*I and *Sca*I. Transformation of *B. subtilis* 168 HR with pTJS41 and selection with erythromycin and lincomycin resulted in the creation of SH113. *B. subtilis* SH113 has a *lytC*::*lacZ* transcriptional fusion that results in insertional inactivation of *lytC*.

(iii) Creation of other insertionally inactivated mutants. The *sigD* derivatives of HR, SH111, and SH113 (SH109, SH112, and SH114, respectively) were made by transformation with chromosomal DNA from *B. subtilis* DP1; transformants were selected with chloramphenicol. The *cwlC* mutant SH103 was created by transforming HR with chromosomal DNA from *B. subtilis* ANC1 and selecting transformants with chloramphenicol. The *lytC* derivatives of SH103 and HR (SH104 and SH108, respectively) were made by transformation with chromosomal DNA from *B. subtilis* L16332; transformants were selected with erythromycin and lincomycin.

Plasmid pTJS42, which contains a fragment from within the coding region of the *B. subtilis sigD* gene, was made by end-filling the 0.37-kb *Pvu*II-*Eco*RI fragment of pJH1-1 (13) and ligating with pAZ106 that had been cut with *Sma*I and dephosphorylated. pTJS42 was transformed into the *cwlC* mutant SH103, and transformants were selected with erythromycin and lincomycin. The resulting strain was the *cwlC sigD* mutant SH110.

Chromosomal rearrangements made by transformation with plasmids were confirmed by Southern blot analysis (37); the appropriate plasmid insert was used as the probe. Probe labelling, hybridization, and detection were done with the Boehringer Mannheim nonradioactive DNA labelling and detection kit. The drug resistance markers of all strains were checked, as was the filamentous, immotile phenotype of *sigD* mutants.

β-Galactosidase assays. β-Galactosidase (LacZ) assays, with 4-methylumbelliferyl-β-D-galactopyranoside (MUG) as the substrate, were performed as described by Youngman (43).

RESULTS

Purification of sporulation-specific autolysin CwlC. A sporulation-specific autolysin was purified from 20 liters of a sporulating culture of *B. subtilis* SH108 by lithium chloride extraction and dye affinity chromatography (Table 2; Fig. 1). The *lytC* mutant SH108 was used in order to avoid contamination by the highly expressed amidase LytC. On the basis of SDS-PAGE, the purified CwlC protein appeared to be >98% homogeneous (Fig. 1A, lane 2). Renaturing gel electrophoresis (Fig. 1B, lane 2) revealed that a 30-kDa species is the only protein in the purified material that showed autolysin activity. Econopac-Q anion-exchange chromatography resulted in significant loss of activity of the protein, so this step was omitted from subsequent preparations.

TABLE 2. Purification of sporulation-specific autolysin CwlC

Purification step	Total protein (mg)	Activity (U)[a]	Sp act (U mg^{-1})	Yield (% initial activity)	Purification (fold)
LiCl extraction	64	11,000	170	100	1
Reactive red chromatography	1.3	4,000	3,100	37	18.2

[a] Peptidoglycan hydrolase activities were measured with *M. luteus* cell walls as the substrate.

The N-terminal amino acid sequence of the 30-kDa species was determined from material isolated from a band on an SDS-polyacrylamide gel. Ten residues of unambiguous sequence were obtained: VKIFIDPGHG. This sequence corresponds to the N terminus of the peptide sequence of the CwlC protein deduced from the gene sequence (18). It proves that the purified protein is indeed CwlC and shows that there is no posttranslational processing of the protein except removal of the initial methionine.

Enzymological properties of CwlC. The hydrolytic bond specificity of an autolysin can be deduced by analysis of the products of cell wall digestion. Enzymes that hydrolyze the glycosidic links between the amino sugars of the glycan chains (i.e., glucosaminidases and muramidases) cause an increase in the number of reducing termini; enzymes that hydrolyze the amides of the cross-linking peptides (i.e., endopeptidases and *N*-acetylmuramoyl-L-alanine amidases) increase the number of free amino groups. Hydrolysis of *B. subtilis* vegetative cell walls for 6 h at 37°C by purified CwlC (300 U/mg [dry weight] of walls), which resulted in >98% reduction in the A_{450}, caused an increase of 490 nmol of free amino groups per mg (dry

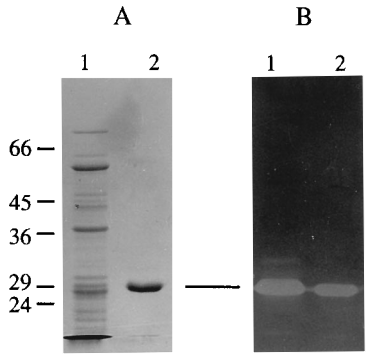

FIG. 1. Purification of CwlC. (A) Coomassie blue-stained SDS-PAGE gel of samples taken during the purification procedure. Lanes (each containing approximately 6 μg of total protein): 1, LiCl cell extract; 2, eluate from reactive red chromatography. (B) Renaturing SDS-PAGE, showing proteins with autolysin activity. Lanes: 1, LiCl cell extract (approximately 6 μg of total protein); 2, eluate from reactive red chromatography (approximately 1 μg of total protein). The molecular masses of standards (in kilodaltons) are indicated.

TABLE 3. Effects of divalent cations on the peptidoglycan hydrolase activity of CwlC

Addition[a]	Concn (mM)	Activity[b] (% of control)
$MgCl_2$ (control)	10	100 ± 5
$MgCl_2$	1.0	82 ± 9
None		25 ± 3
EDTA	1.0	20 ± 1
$CaCl_2$	10	120 ± 0
$CdCl_2$	1.0	36 ± 0
$ZnCl_2$	1.0	27 ± 1
$HgCl_2$ + $MgCl_2$	1.0 + 10	1.4 ± 0.3

[a] Each reaction mix contained CwlC (150 U/ml) and *B. subtilis* cell walls (140 μg/ml) in 20 mM Tris-HCl (pH 8.5) plus the addition(s) shown. Reaction mixes were incubated at 37°C.

[b] Activity is expressed as the percentage of the activity obtained in the presence of 10 mM $MgCl_2$. Each was determined in duplicate and is shown as mean ± difference. Control reactions with walls and additions but no enzyme gave activities of ≤2%.

weight) of walls. In contrast, the same reaction released only 6.1 nmol of new reducing equivalents per mg (dry weight) of walls. Acid hydrolysis of the 1-fluoro-2,4-dinitrobenzene-labelled samples followed by thin-layer chromatography analysis identified an increase in only *N*-2,4-dinitrophenyl-DL-alanine on hydrolysis of the walls with purified CwlC. The use of L-[^{14}C]alanine-labelled walls (29) identified this DNP-alanine increase to be accounted for solely by *N*-2,4-dinitrophenyl-alanine, and thus CwlC is an amidase.

The pH optimum for hydrolysis of *B. subtilis* vegetative cell walls by CwlC was found to be between pH 8.5 and 9.5. Lithium chloride had an inhibitory effect on enzyme activity, causing 52 and 91% inhibition at concentrations of 1 and 3 M, respectively. Table 3 shows the effects of various combinations of divalent ions and EDTA on autolytic activity. Activity was stimulated about fourfold by 10 mM Mg^{2+} or Ca^{2+}. Zn^{2+} and Cd^{2+} had little stimulatory effect, and the enzyme was almost completely inhibited by mercuric chloride. The activity of CwlC towards several bacterial cell wall substrates was investigated (7). In 10 mM $MgCl_2$–20 mM Tris-HCl (pH 8.5), the activities obtained with *M. luteus* cell walls, *S. aureus* cell walls, and *B. megaterium* spore cortex were, respectively, 560, 6, and 34% of the rate obtained with *B. subtilis* vegetative cell walls under the same conditions. These results suggested that *M. luteus* cell walls are a better substrate for CwlC than its presumed natural substrate, *B. subtilis* vegetative walls. This may be because *M. luteus* peptidoglycan has fewer cross-links than that of *B. subtilis* (35) and hence can be disrupted by hydrolysis of fewer bonds. The differences in activity may also be due to peptidoglycan modification (4) or secondary polymer effects (35).

Location of CwlC in sporulating cells. A sporulating culture of *B. subtilis* 168 HR was harvested when most of the sporangia were at stage VI (mature endospores, approaching mother cell lysis). Separate aliquots were fractionated by two methods. The first used a French pressure cell to break the mother cells but not the spores, followed by rounds of differential centrifugation to isolate mother cell walls, membranes, and cytoplasm. In the second fractionation method, the sporangia were protoplasted, producing the following two fractions: a soluble fraction of cell wall-associated material and a protoplast pellet that contained all the cytoplasmic and membrane material, as well as the whole forespores.

Renaturing gel and Western blot analyses of the various fractions suggested that CwlC was primarily associated with the mother cell wall (Fig. 2). The bands that reacted with anti-CwlC antiserum and the strongest bands of autolytic activity around 30 kDa were found primarily in the fractions containing mother cell wall-derived material (Fig. 2, lanes F1 and P2). The result from the protoplast fractionation was especially convincing, since almost all the CwlC protein was associated with the material that was released by the action of lysozyme on the sporangia; that is, CwlC was confined to the cell wall of the mother cell.

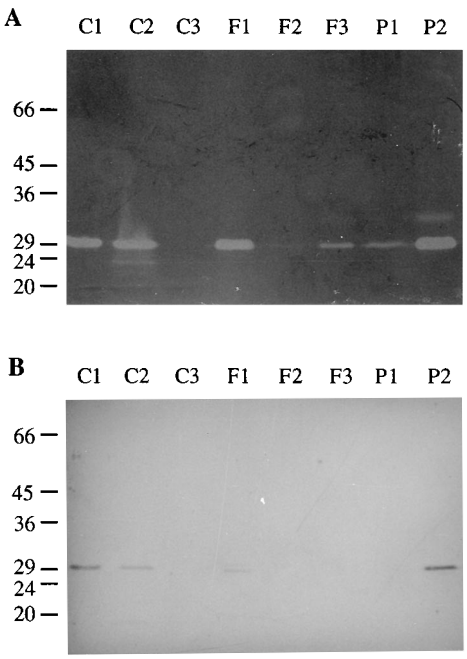

FIG. 2. Location of CwlC in sporulating cells. Sporulating cells of *B. subtilis* 168 HR were fractionated by the two procedures described in Materials and Methods. (A) Renaturing SDS-PAGE gel showing proteins with autolysin activity. (B) Western blot probed with anti-CwlC antiserum. Lanes: C1, purified CwlC; C2, SDS cell extract from whole sporulated *B. subtilis* 168 HR; C3, SDS cell extract from whole sporulated *B. subtilis* SH103 (*cwlC* mutant); F1, F2, and F3 (from differential centrifugation fractionation), mother cell wall, mother cell membrane, and mother cell cytoplasm fractions, respectively; P1 and P2 (from protoplasting fractionation), protoplasted mother cells and mother cell wall fractions, respectively. Lanes C2 and C3 each contained extract from 1 ml of original culture; all other lanes were loaded with material from 2 ml of original culture. The molecular masses of standards (in kilodaltons) are indicated.

Functional analysis of *cwlC* and the role of autolysins in mother cell lysis. Kuroda et al. (18) made a mutant that was deleted in *cwlC*, but they found no discernible phenotypic change during sporulation or germination or in the resistance of spores to heat and lysozyme. This suggested that there is some functional redundancy, so that the loss of *cwlC* can be compensated for, or that *cwlC* has no major role during differentiation. In order to identify a role for *cwlC* and to determine the influence of other autolysins in mother cell lysis, a set of isogenic mutants that included *cwlC* mutants that also lacked *lytC* or *sigD* was constructed (Table 1).

Cultures of each strain were grown in CCY medium and incubated at 37°C with shaking. Sporulation was monitored by phase-contrast microscopy. The single mutants SH103 (*cwlC*), SH113 (*lytC*), and SH109 (*sigD*) all sporulated and released on the same time scale as the parental strain HR. Release was complete after 48 h. SH114 (*lytC sigD*) also sporulated and released like the wild type. However, in SH104 (*cwlC lytC*), mother cell lysis had not happened even after 7 days (Fig. 3), and in SH110 (*cwlC sigD*), mother cell lysis was greatly re-

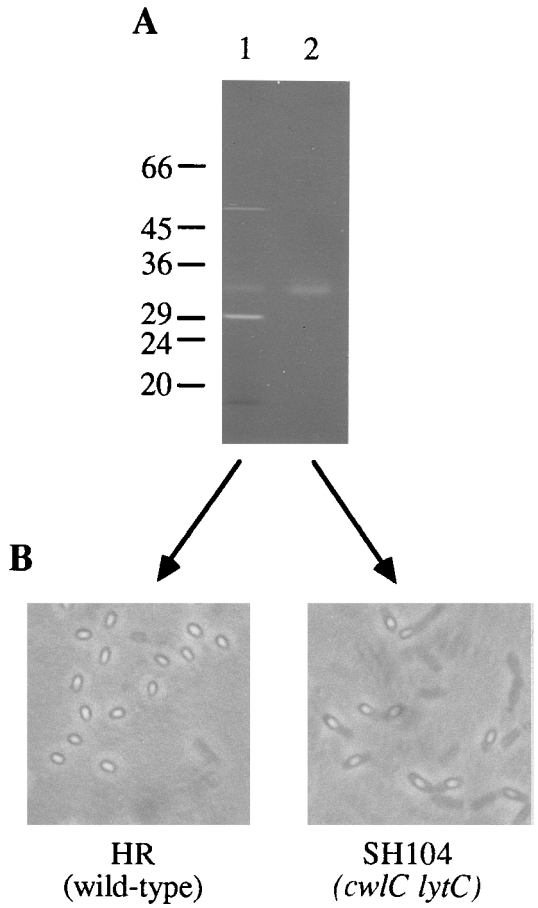

FIG. 3. Effect of simultaneous inactivation of *cwlC* and *lytC* on sporulation autolysin profile and mother cell lysis. (A) Renaturing SDS-PAGE of SDS cell extracts taken from resuspension cultures 8 h after resuspension. Lanes: 1, *B. subtilis* 168 HR; 2, *B. subtilis* SH104 (*cwlC lytC* mutant). Each lane contained material from 1 ml of original culture. The molecular masses of standards (in kilodaltons) are indicated. (B) Phase-contrast micrographs of CCY cultures of the same strains after shaking at 37°C for 48 h. The parental strain HR has sporulated and lysed completely, so only the phase-bright spores are visible. The *cwlC lytC* mutant SH104 has sporulated but not released, so the phase-bright spores remain within phase-dark mother cells. Similar results were obtained when sporulation was initiated by the resuspension method.

tarded and was not complete until 5 days after inoculation. It therefore appears that peptidoglycan hydrolysis, which is presumably essential for mother cell lysis, can be performed by LytC or CwlC independently. Hence, mother cell lysis is prevented only when both are inactivated. The retarded lysis of the *cwlC sigD* mutant may arise from the fact that the majority of *lytC* expression is controlled by σ^D, which is the flagellar motility and chemotaxis sigma factor, encoded by *sigD* (13, 25, 30).

Spores of the *cwlC* and *lytC* single mutants (SH103 and SH108, respectively) and the *cwlC lytC* double mutant (SH104) were tested for resistance to chloroform (15 min, room temperature) and heat (70°C, 30 min). The effect of the treatment on the viability of the spores was measured by taking viable-cell counts before and after chloroform and heat treatment. The proportion of spores of each of the mutant strains that survived chloroform and heat treatment was comparable to that for wild-type (HR) spores prepared and treated in the same way. Hence, neither CwlC nor LytC is necessary for the formation of viable lysozyme- and heat-resistant spores. This result suggests that the blockage of mother cell lysis in the *cwlC lytC* mutant arises directly from the absence of the lytic activities of CwlC and LytC and not as a secondary effect of some abnormality earlier in sporulation.

Expression of *cwlC* and *lytC* during sporulation. In view of the apparent involvement of *cwlC* and *lytC* in mother cell lysis, it was relevant to consider their control during sporulation. Expression of *cwlC* peaks late in sporulation, between stages V and VII, as was shown by using an in-frame translational fusion between *cwlC* and *lacZ* (18).

We directly investigated the amount of CwlC protein during sporulation by detecting the autolytic activity of CwlC and its reaction with anti-CwlC antiserum. A synchronous sporulating culture of *B. subtilis* HR was prepared by the resuspension method (38). Samples were taken at nine time points over 22 h after resuspension; proteins were extracted with SDS and analyzed by renaturing gel electrophoresis and Western blotting (results not shown). There was little CwlC in the early stages of sporulation, but CwlC levels increased rapidly between 6 and 8 h after resuspension, and a significant amount remained at 22 h after resuspension. The spores became phase bright (stage V) about 8 h after resuspension, and mother cell lysis (stage VII) had occurred by 11 h after resuspension. The CwlC protein, which has been shown previously to be active (8), accumulates associated with the mother cell wall before mother cell lysis, an event with which it is functionally linked.

Expression of *lytC* during sporulation was studied by means of the transcriptional fusion strains SH111, SH112, SH113, and SH114 (Table 1). SH111 and SH112 have a *lytA*::*lacZ* transcriptional fusion, followed by another, intact copy of *lytABC*. SH113 and SH114 have *lacZ* within *lytC* itself, although this insertion inactivates *lytC*. The fusions were designed to give complementary information. The SH112/SH113 fusion has a fully functional *lytABC* operon and so should be free of secondary effects caused by the disruption of any genes. However, between the 3′ end of the −10 box of the σ^D-dependent promoter and the first base of the *lytC* ribosome-binding site there are 2,494 bp of *lytAB*, which may contain unidentified promoters and/or terminators that could make the level of β-galactosidase an inaccurate measure of *lytC* transcription. The SH112/SH114 fusion measures transcription of *lytC* directly but may suffer from secondary effects arising from the inactivation of *lytC*. Each fusion was studied in *sigD*$^+$ and *sigD* backgrounds, in case a residue of the high level of vegetative σ^D-dependent expression of *lytC* masked any sporulation-specific expression.

Sporulation of the fusion strains was studied by the resuspension method, and β-galactosidase activity in each strain was measured as a function of time (Fig. 4). In all four strains, β-galactosidase activity fell off sharply as sporulation progressed. By the time that formation of phase-bright spores was complete (stage V, about 7 h after resuspension in these experiments), β-galactosidase activity had dropped almost to zero. Lysis (stage VII) did not occur until 11 h after resuspension. Inactivation of *sigD* reduced *lacZ* expression by about 1.7-fold in the *lytA*::*lacZ* fusion and by about 4-fold in the *lytC*::*lacZ* fusion. The *lacZ* gene in the *lytA*::*lacZ* fusion was expressed two to four times more strongly than that in the *lytC*::*lacZ* fusion. Similar results were obtained when the experiment was repeated.

In the light of the loss of *lytC* expression well before the mother cells lyse, it seems likely that any function that *lytC* has in mother cell lysis is performed by protein from expression before sporulation that remains undegraded. This hypothesis is consistent with the observation that the band due to LytC activity (i.e., the band at about 50 kDa) remains until after mother cell lysis (results not shown) (8).

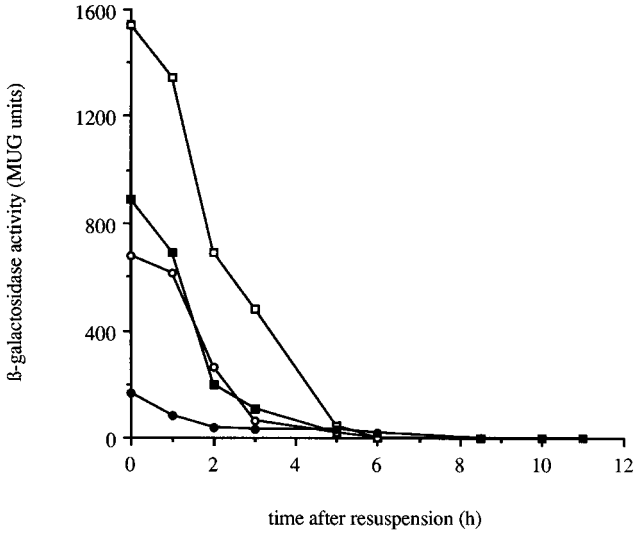

FIG. 4. β-Galactosidase expression during sporulation in *lacZ* transcriptional fusion strains. Sporulating cultures were prepared by the resuspension method. □, SH111 (*lytA*::*lacZ* fusion); ■, SH112 (*lytA*::*lacZ* fusion in *sigD* background); ○, SH113 (*lytC*::*lacZ* fusion); ●, SH114 (*lytC*::*lacZ* fusion in *sigD* background). One MUG unit (43) is defined as the activity that hydrolyzes 1 pmol of MUG per min per ml of culture, normalized to a postresuspension A_{600} of 1.0.

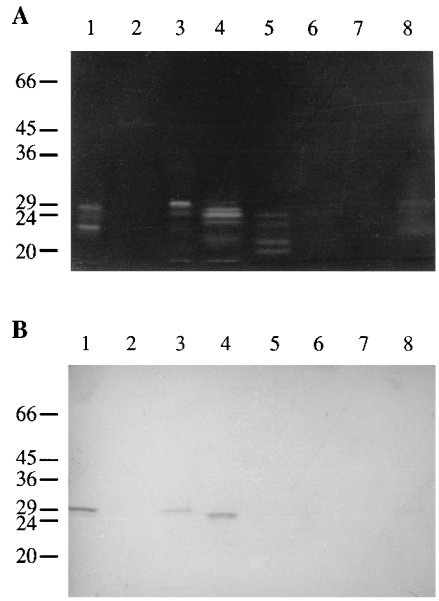

FIG. 5. Analysis of SDS cell extracts from various *Bacillus* species. (A) Renaturing SDS-PAGE gel, showing renaturable proteins that digest *B. subtilis* vegetative cell walls. Lanes: 1, *B. subtilis* HR; 2, *B. subtilis* HR, vegetative; 3, *B. megaterium* KM; 4, *B. megaterium* QMB1551; 5, *B. licheniformis* ATCC 8480; 6, *B. circulans* ATCC 4513; 7, *B. cereus* T; 8, *B. amyloliquefaciens* H. The vegetative extract of *B. subtilis* HR was prepared from a nutrient broth culture in stationary phase; all other samples shown were from sporulated extracts prepared by SDS extraction of fully sporulated and released cultures grown in CCY broth. Each lane contains 30 μg of protein. (B) Western blot with samples identical to those of panel A, probed with anti-CwlC antiserum. The molecular masses of standards (in kilodaltons) are indicated.

Active LytC was not detected in SDS cell extracts from strains that had been allowed to sporulate in CCY medium for 48 h (Fig. 2A, lanes C2 and C3). However, in CCY medium, the mother cells of the *cwlC* mutant SH103 lysed, but those of the *cwlC lytC* mutant SH104 did not (Fig. 3B). Hence, LytC can lyse the mother cell walls in the absence of CwlC in CCY cultures, so active LytC must remain into sporulation under these conditions. The extracts shown in Fig. 2A, lanes C2 and C3, were made from fully sporulated and released CCY cultures, by which time most of the remaining LytC was presumably degraded.

Screening of other *Bacillus* species for proteins that cross-react with anti-CwlC antiserum. SDS extracts were prepared from of a range of *Bacillus* species, using cultures grown in 30-ml aliquots of CCY medium until sporulation and release were complete. Samples were analyzed by renaturing gel electrophoresis and Western blotting (Fig. 5). Proteins that cross-reacted with anti-CwlC antiserum were found in the extracts from *B. amyloliquefaciens* H and two strains of *B. megaterium* (KM and QMB1551). The cross-reacting bands had a molecular mass of about 30 kDa, about the same size as CwlC. Each of the bands on the Western blot coincided with a band of enzyme activity on the renaturing gel, strongly suggesting that the cross-reacting proteins were peptidoglycan hydrolases. No cross-reactivity was seen in extracts made from stationary-phase vegetative cells of any of the strains (data not shown).

DISCUSSION

A method for purification of the sporulation-specific autolysin CwlC of *B. subtilis* 168 was developed. A single chromatographic separation, using reactive red-activated agarose, effected all of the purification (Table 2). The success of this step arose largely from the fact that CwlC is one of only a very few proteins that bind to reactive red under the conditions used.

CwlC was found to be primarily cell wall associated, so it must be secreted across the mother cell membrane. However, CwlC is not N-terminally processed except for removal of the initial methionine residue and does not have a hydrophobic signal sequence matching the conventional *sec*-dependent consensus (18). It must therefore be secreted by some other mechanism. CwlM, the *B. licheniformis* homolog of CwlC, also lacks a hydrophobic signal sequence. Moreover, judging from the apparent molecular mass of CwlM expressed in *B. subtilis* and analyzed by renaturing SDS-PAGE, little or none of the protein is cleaved posttranslationally (23). It is known that certain secreted proteins are exported across the cell membrane by an ATP-dependent system that recognizes signal sequences quite different from the highly hydrophobic ones recognized by the *sec* gene-encoded machinery (17).

The phenotype of the *cwlC lytC* double mutant gives evidence of the function of *cwlC*. A strain in which both genes were inactivated failed to undergo mother cell lysis, but strains with single mutations in *cwlC* or *lytC* grew, sporulated, and underwent mother cell lysis as well as the wild type did. This showed that there is some function in mother cell lysis, presumably hydrolysis of the mother cell wall peptidoglycan, which requires that either CwlC or LytC be present. In the single mutants, one gene product compensates for the loss of the other, and so a wild-type phenotype results. We suggest that mother cell lysis is the main function of CwlC, which is expressed almost solely in the later stages of sporulation. In contrast, LytC clearly has other roles, since it is produced primarily during vegetative growth (8, 25). Studies of chromosomally encoded transcriptional fusions between the *lytABC* operon and *lacZ* showed that *lytC* expression declines dramatically during sporulation and is effectively zero by the time that mother cell lysis occurs (Fig. 4). *lacZ* expression was two- to fourfold greater in the *lytA*::*lacZ* fusion than in the *lytC*::*lacZ* fusion. This suggested that some transcriptional termination

may occur between *lytA* and *lytC* or, alternatively, that inactivation of *lytC* in the *lytC*::*lacZ* fusion has a secondary effect on transcription of *lytABC*. However, the strong decline of *lytC* expression during sporulation was clearly shown by each of the fusion strains. It was suspected that there might be some evidence of increased or sustained *lytC* expression during sporulation, but none was found, even against the low background of a *sigD* mutant. However, renaturing gels (results not shown) (8) have shown that active LytC remains into sporulation and beyond the time of mother cell lysis, and so it appears that the role of *lytC* in mother cell lysis is performed by material that persists from earlier expression.

The retarded mother cell lysis of the *cwlC sigD* double mutant probably arises from the mutant's reduced level of *lytC* expression, since σ^D, the product of *sigD*, controls one of the two promoters identified upstream from *lytC* (25). As Fig. 4 shows, σ^D is responsible for 40 to 75% of the transcription through *lytC* at the start of sporulation. However, since *sigD* controls the expression of many genes (13, 31), other factors may be involved.

A previous report has suggested that CwlC is mostly located in the mother cell cytoplasm (18). In the present study, two separate fractionation methods indicated that CwlC is located primarily in association with the mother cell walls of sporulating cells. This is in agreement with our proposed role for CwlC, in hydrolysis of mother cell wall peptidoglycan.

Like the majority of low-molecular-mass (≤50 kDa) peptidoglycan hydrolases associated with gram-positive bacteria for which data are available (7, 9, 14, 19, 23, 26, 33, 36), CwlC is an *N*-acetylmuramoyl-L-alanine amidase. In particular, its substrate specificity is consistent with its amino acid sequence similarity to the CwlM amidase of *B. licheniformis* and LytC of *B. subtilis* (18, 23). We screened extracts of sporulated cells from several other *Bacillus* species for proteins that cross-reacted with anti-CwlC antiserum. Cross-reacting proteins similar in size to CwlC were found in *B. megaterium* and *B. amyloliquefaciens* (Fig. 5). These proteins are probably homologs of CwlC, and since they were not detected in extracts taken from stationary-phase vegetative cultures, they may well be expressed specifically during sporulation. Western blots failed to detect CwlM of *B. licheniformis.*

We have shown that two autolysin genes, *cwlC* and *lytC*, have mutually compensatory roles in mother cell lysis during sporulation of *B. subtilis* 168. The location and catalytic properties of the two enzymes are consistent with their being directly responsible for digesting the peptidoglycan of the mother cell wall. We have therefore defined the components involved in one of the most characteristic events during sporulation. Mother cell lysis is presumably advantageous as an aid to the dispersion of the mature endospores to a more favorable environment. The autolysins are expressed well in advance of mother cell lysis, and there is no evidence that the enzymes and substrate are held in different cell compartments. So, as is the case with many autolysin-catalyzed reactions, the question of how untimely cell lysis is prevented remains to be answered. Since our results underline the functional redundancy of bacterial autolysins, we anticipate that the genes involved in other autolytic events will only be elucidated by studying mutations at multiple loci. Furthermore, the existence of enzymes with compensatory functions poses the question of why natural selection has maintained a system of cell wall metabolism which, for laboratory growth at least, is unnecessarily complex.

ACKNOWLEDGMENTS

This work was supported by the BBSRC and the Royal Society.

We are grateful to Mike Chamberlin, Dimitri Karamata, and Junichi Sekiguchi for gifts of strains and plasmids.

REFERENCES

1. **Alderton, G., and N. S. Snell.** 1963. Base exchange and heat resistance in bacterial spores. Biochem. Biophys. Res. Commun. **10:**139–143.
2. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
3. **Burnette, W. M.** 1981. Western blotting: electrophoretic transfer of proteins from sodium dodecyl sulfate-polyacrylamide gels to unmodified nitrocellulose and radiographic detection with antibody and radioiodinated protein A. Anal. Biochem. **112:**195–203.
4. **Clarke, A. J.** 1993. Extent of peptidoglycan O acetylation in the tribe *Proteeae*. J. Bacteriol. **175:**4550–4553.
5. **Doi, R. H.** 1989. Sporulation and germination, p. 169–215. *In* C. R. Harwood (ed.), *Bacillus*. Plenum Press, London.
6. **Errington, J.** 1993. *Bacillus subtilis* sporulation: regulation of gene expression and control of morphogenesis. Microbiol. Rev. **57:**1–33.
7. **Foster, S. J.** 1991. Cloning, sequence analysis and biochemical characterisation of an autolytic amidase of *Bacillus subtilis* 168 *trpC2*. J. Gen. Microbiol. **137:**1987–1998.
8. **Foster, S. J.** 1992. Analysis of the autolysins of *Bacillus subtilis* 168 during vegetative growth and differentiation by using renaturing gel electrophoresis. J. Bacteriol. **174:**464–470.
9. **Foster, S. J.** 1993. Analysis of *Bacillus subtilis* 168 prophage-associated lytic enzymes: identification and characterisation of CWLA-related prophage proteins. J. Gen. Microbiol. **139:**3177–3184.
10. **Foster, S. J.** 1994. The role and regulation of cell wall structural dynamics during differentiation of endospore-forming bacteria. J. Appl. Bacteriol. **76:**25S–39S.
11. **Ghuysen, J.-M., D. J. Tipper, and J. L. Strominger.** 1966. Enzymes that degrade bacterial cell walls. Methods Enzymol. **8:**685–699.
12. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
13. **Helmann, J. D., L. M. Marquez, and M. J. Chamberlin.** 1988. Cloning, sequencing, and disruption of the *Bacillus subtilis* σ^{28} gene. J. Bacteriol. **170:**1568–1574.
14. **Herbold, D. R., and L. Glaser.** 1975. *Bacillus subtilis N*-acetylmuramic acid L-alanine amidase. J. Biol. Chem. **250:**1676–1682.
15. **Johnstone, K., and D. J. Ellar.** 1982. The role of cortex hydrolysis in the triggering of germination of *Bacillus megaterium* KM endospores. Biochim. Biophys. Acta **714:**185–191.
16. **Kemp, E. H., R. L. Sammons, A. Moir, D. Sun, and P. Setlow.** 1991. Analysis of transcriptional control of the *gerD* spore germination gene of *Bacillus subtilis* 168. J. Bacteriol. **173:**4646–4652.
17. **Kuchler, K.** 1993. Unusual routes of protein secretion: the easy way out. Trends Cell Biol. **3:**421–426.
18. **Kuroda, A., Y. Asami, and J. Sekiguchi.** 1993. Molecular cloning of a sporulation-specific cell wall hydrolase gene of *Bacillus subtilis*. J. Bacteriol. **175:** 6260–6268.
19. **Kuroda, A., M. Imazeki, and J. Sekiguchi.** 1991. Purification and characterisation of a cell-wall hydrolase encoded by the *cwlA* gene of *Bacillus subtilis*. FEMS Microbiol. Lett. **81:**9–14.
20. **Kuroda, A., M. H. Rashid, and J. Sekiguchi.** 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.
21. **Kuroda, A., and J. Sekiguchi.** 1991. Molecular cloning and sequencing of a major *Bacillus subtilis* autolysin gene. J. Bacteriol. **173:**7304–7312.
22. **Kuroda, A., and J. Sekiguchi.** 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.
23. **Kuroda, A., Y. Sugimoto, T. Funahashi, and J. Sekiguchi.** 1992. Genetic structure, isolation and characterisation of a *Bacillus licheniformis* cell wall hydrolase. Mol. Gen. Genet. **234:**129–137.
24. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
25. **Lazarevic, V., P. Margot, B. Soldo, and D. Karamata.** 1992. Sequencing and analysis of the *Bacillus subtilis lytRABC* divergon: a regulatory unit encompassing the structural genes of the *N*-acetyl muramoyl-L-alanine amidase and its modifier. J. Gen. Microbiol. **138:**1949–1961.
26. **Longchamp, P. F., C. Mauël, and D. Karamata.** 1994. Lytic enzymes associated with defective prophages of *Bacillus subtilis*: sequencing and characterisation of the region comprising the *N*-acetyl-muramoyl-L-alanine amidase gene of prophage PBSX. Microbiology **140:**1855–1867.
27. **Losick, R., and P. Stragier.** 1992. Crisscross regulation of cell-type-specific gene expression during development in *B. subtilis*. Nature (London) **355:** 601–604.
28. **Margot, P.** 1992. Ph.D. thesis. University of Lausanne, Lausanne, Switzerland.
29. **Margot, P., C.-A. H. Roten, and D. Karamata.** 1991. *N*-Acetylmuramoyl-L-alanine amidase assay based on specific radioactive labelling of muropeptide

L-alanine: quantitation of the enzyme activity in the autolysin-deficient *Bacillus subtilis* 168, *flaD* strain. Anal. Biochem. **198:**15–18.
30. **Marquez, L. M., J. D. Helmann, E. Ferrari, H. M. Parker, G. W. Ordal, and M. J. Chamberlin.** 1990. Studies of σ^D-dependent functions in *Bacillus subtilis*. J. Bacteriol. **172:**3435–3443.
31. **Marquez-Magaña, L. M., D. B. Mirel, and M. J. Chamberlin.** 1994. Regulation of σ^D expression and activity by *spo0*, *abrB*, and *sin* gene products in *Bacillus subtilis*. J. Bacteriol. **176:**2435–2438.
32. **McDonald, C., S. Fawell, D. Pappin, and S. Higgins.** 1986. Electroelution of proteins from SDS gels. Trends Genet. **2:**35.
33. **Oda, Y., R. Nakayama, A. Kuroda, and J. Sekiguchi.** 1993. Molecular cloning, sequence analysis and characterisation of a new cell wall hydrolase, CwlL, of *Bacillus licheniformis*. Mol. Gen. Genet. **241:**380–388.
34. **Pooley, H. J., and D. Karamata.** 1984. Flagellation and the control of autolysin activity in *Bacillus subtilis*, p. 13–19. *In* C. Nombela (ed.), Microbial cell wall synthesis and autolysis. Elsevier, Amsterdam.
35. **Rogers, H. J., H. R. Perkins, and J. B. Ward.** 1980. Microbial cell walls and membranes, p. 191–214. Chapman and Hall, London.
36. **Romero, A., R. Lopez, and P. Garcia.** 1990. Sequence of the *Streptococcus pneumoniae* bacteriophage HB-3 amidase reveals high homology with the major host autolysin. J. Bacteriol. **172:**5064–5070.
37. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed., p. 9.31–9.57. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
38. **Sterlini, J. M., and J. Mandelstam.** 1969. Commitment to sporulation in *Bacillus subtilis* and its relationship to development of actinomycin resistance. Biochem. J. **113:**29–37.
39. **Stewart, G. S. A. B., K. Johnstone, E. Hagelberg, and D. J. Ellar.** 1981. Commitment of bacterial spores to germinate. Biochem. J. **198:**101–106.
40. **Todd, J. A., and D. J. Ellar.** 1982. Alteration in the penicillin-binding profile of *Bacillus megaterium* during sporulation. Nature (London) **300:**640–643.
41. **Ward, J. B., and R. Williamson.** 1984. Bacterial autolysins: specificity and function, p. 159–166. *In* C. Nombela (ed.), Microbial cell wall synthesis and autolysis. Elsevier, Amsterdam.
42. **Warth, A. D.** 1978. Molecular structure of the bacterial spore. Adv. Microb. Physiol. **17:**1–47.
43. **Youngman, P.** 1990. Use of transposons and integrational vectors for mutagenesis and construction of gene fusions in *Bacillus* species, p. 221–266. *In* C. R. Harwood and S. M. Cutting (ed.), Molecular biology methods for *Bacillus*. John Wiley & Sons, New York.