The GTPase CpgA Is Implicated in the Deposition of the Peptidoglycan Sacculus in *Bacillus subtilis*[▽]

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Depletion of the *Bacillus subtilis* GTPase CpgA produces abnormal cell shapes, nonuniform deposition of cell wall, and five- to sixfold accumulation of peptidoglycan precursors. Nevertheless, the inherent structure of the cell wall appeared mostly unchanged. The results are consistent with CpgA being involved in coordinating normal peptidoglycan deposition.

The gene *cpgA*, encoding a multidomain GTPase, is located in a conserved gene cluster, downstream of and cotranscribed with two genes *prpC* and *prkC*, encoding, respectively, a Ser/Thr phosphatase and a sensor kinase (18, 27, 28, 31). PrkC contains an external domain with three PASTA repeats that are likely involved in binding peptidoglycan (34), while its *Mycobacterium tuberculosis* homologue, PknB, has been implicated in cell wall synthesis (20). We have recently shown that cells depleted for CpgA display marked morphological changes, including swollen cells and a variety of bizarre forms (8). Production of the peptidoglycan sacculus and rod shape morphology in *B. subtilis* is dependent on penicillin-binding proteins (PBPs) with transglycosylase and transpeptidase activities (17). However, recent evidence indicates that the actin-like proteins MreB, Mbl, and MreBH form a cytoskeletal structure under the membrane that is also required for the assembly of peptidoglycan (5, 6, 10, 14). We now suggest that, while *prkC* and *prpC* are involved in monitoring the status of peptidoglycan, CpgA (YloQ), based on its structure (23, 24), low abundance, and probable association with ribosomes (4, 8, 9, 16), is a translation factor affecting production of certain proteins involved in morphology determination. We examined in detail here the possible nature of the cell wall defect in cells depleted of CpgA.

We used a strain (BFS2823) with a copy of *cpgA* controlled by a *pspac* promoter in the chromosome (8, 15), allowing depletion of CpgA (confirmed by reverse transcription-PCR [data not shown]) in exponentially growing cells in E-medium, in the absence of IPTG (isopropyl-β-D-thiogalactopyranoside). Under these conditions, as shown in Fig. 1A, we observed a reduced growth rate (generation time of 56 min compared to 31 min for the nondepleted strain) and major morphological changes, with many swollen and irregular-shaped cells, as shown previously (8). The minimal E-medium (25) contains a low concentration of phosphate, but supplementation with increased phosphate had no effect on growth or cellular morphology. A significant increase in generation time (170 min compared to 110 min for the parental strain) and abnormal shapes were also observed (Fig. 1B) after depletion of CpgA in Spizizen medium (1). In contrast, in cultures of BFS2823 in medium grown with IPTG (Fig. 1) the cells appeared normal. However, as we observed previously (8), growth of depleted cultures in LB was not affected, although a small number of longer cells were observed (all data not shown). Therefore, the effect on growth rate and the greatly perturbed morphology, after depletion of CpgA, may be expressed in media supporting slow growth rates, indicating that CpgA is essential for normal growth under these conditions (see also reference 21).

To obtain more information on the nature of the abnormal morphology in CpgA depleted cells, we carried out a detailed study of electron microscopic (EM) sections of the normal and depleted cells in E-medium with sampling for EM sectioning and staining with uranyl acetate as described previously (26). In the absence of IPTG, up to 70% of cells displayed a range of marked abnormalities throughout the growth phase (Fig. 2 and 3), including swollen, coccoid-like cells and a variety of bizarre shapes. Importantly, in many cells, there appeared to be an irregular deposition of cell wall, producing various levels of thickness (see arrows in Fig. 3), compared to the wild type. This suggested impairment of the expansion of the cell wall, normally involving uniform deposition or distribution of the multilayered cell wall throughout the cell. Finally, up to 15% of the depleted cells showed multiple or otherwise abnormal septal cleavage planes (Fig. 3B). We confirmed that these shape changes were abolished in the presence of IPTG or when depleted cells of strain OMG501, derived from BFS2823 but ectopically expressing *cpgA* from a xylose dependent copy of *cpgA* (8), were used (data not shown).

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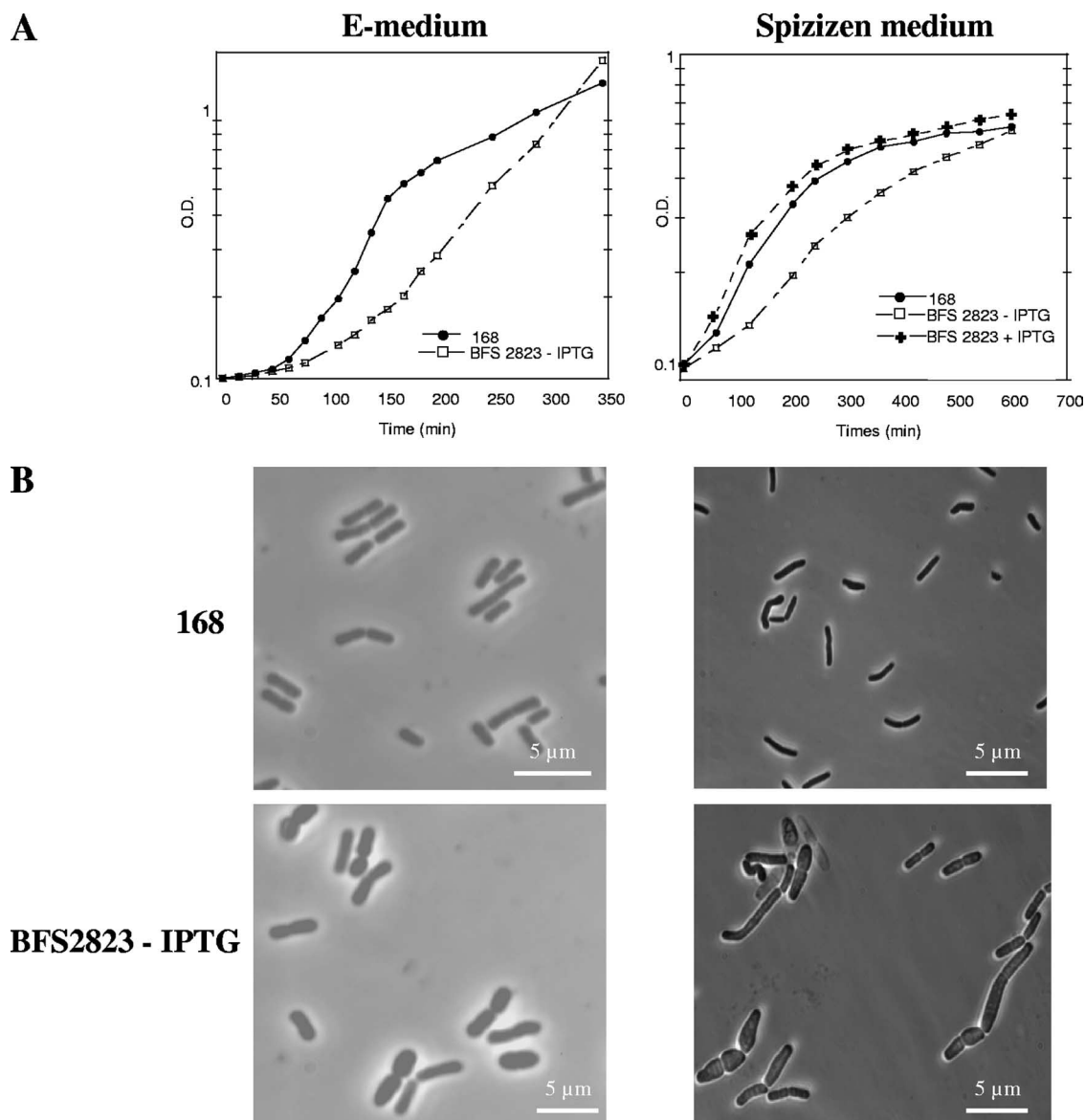▽ Published ahead of print on 14 March 2008.

FIG. 1. (A) Effect of depletion of CpgA on growth. Cultures were grown at 37°C for strain 168 *trpC* (●), its derivative BFS2823 without IPTG (□) or BFS2823 with 1 mM IPTG (✚) in E-medium or Spizizen medium. BFS2823 expresses *cpgA* from a *pspac* promoter. (B) Morphology defect of CpgA-depleted cells. Phase-contrast micrographs of strain 168 (wild type) and CpgA-depleted cells (BFS2823) grown either in E-medium or Spizizen medium, sampled at and optical density (OD) of 0.4, are shown.

These results indicated that in depleted cells, the nature of the cell envelope, probably the peptidoglycan, the major shape determinant, and/or its assembly, were perturbed. However, with depleted cells grown in E-medium, plus or minus IPTG (1 mM), in plate and liquid tests, we found no change in sensitivity (exponential- or stationary-phase cells) to ampicillin (PBP target), vancomycin (binds to late peptidoglycan precursors), or fosfomycin (inhibitor of MurA, first enzyme in the biosynthesis of peptidoglycan) (19). However, with cefuroxime or cefotaxime (cephalosporins also targeting PBPs) the depleted cells were more sensitive, with a reduced MIC in Spizizen medium, 0.25 μg/ml compared to 1 μg/ml for nondepleted cells. The depleted cells, in contrast, displayed a level of sensitivity to osmotic shock (4% NaCl), Triton X (0.01 to 0.5%),

or EGTA (25 mM) identical to that of the nondepleted cells or wild-type strain 168 (data not shown).

We also measured in depleted cells the level of teichoic acids (from cell wall phosphorus content, as described previously [11]), the major PBPs 1 to 5 (measured as described previously [12, 35]), the cytoskeleton protein Mbl and its association with the membrane (using Western blotting and microscopy analysis of an Mbl-GFP fusion as described in reference 18), peptidoglycan (diaminopimelic acid content [3]) and, as shown in Fig. 4, the muropeptide composition (determined by high-pressure liquid chromatography [HPLC] analysis). In every case, the depleted cells were essentially normal, compared to nondepleted cells or the wild type. Finally, in contrast to cells depleted of Mbl, MreB, MreBH (7, 14), or MreC/D, impli-
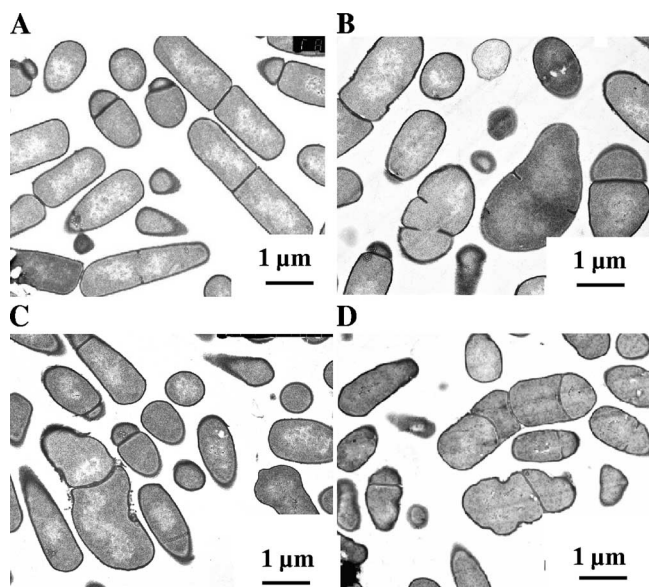
FIG. 2. EM sections of CpgA-depleted cells throughout the growth phase. Strain 168 (A) and strain BFS2823 (B to D), both grown in E-medium without IPTG, are shown. EM sections (samples were fixed with 2.5% glutaraldehyde in cacodylate buffer and stained with 1% uranyl acetate, and ultrathin sections were contrasted with lead acetate) were prepared as described previously (26) and are shown at different stages of the growth phase. (A) Mid-exponential phase, OD = 0.4; (B) OD = 0.4; (C) OD = 0.8; (D) stationary phase. In contrast to strain 168, a variety of misshapen cells with some abnormal division planes are visible throughout the growth phase with depleted cells. BFS2823 with IPTG or a derivative of BFS2823, ectopically expressing *cpgA* from a *xyl* promoter, produces largely normal cells (data not shown).

cated in morphogenesis through the coupling of Mbl cables to sites of extracellular cell wall synthesis (22), we found that the shape changes in CpgA-depleted cells were not reversed by 10 mM magnesium (data not shown), providing more evidence that morphogenetic factors, compromised in some way in CpgA-depleted cells, did not involve specific reductions in the major cytoskeleton components. Interestingly, however, EM
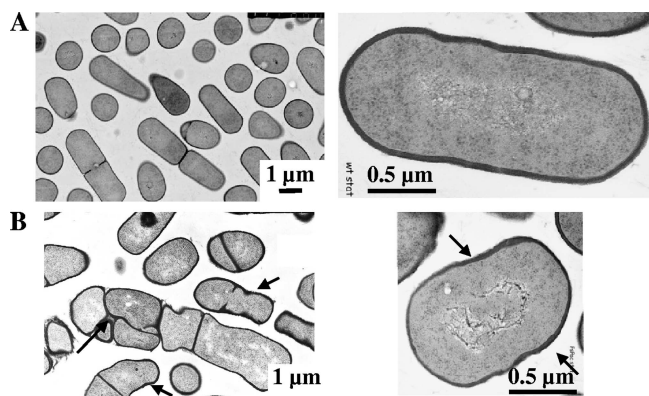

FIG. 3. EM sections of CpgA-depleted cells reveal nonuniform deposition of cell wall. (A) Wild-type strain 168; (B) BFS2823 grown without IPTG in E-medium at 37°C, with nonuniform deposition of cell wall (arrows) and multiple aberrant division planes in a minority of cells.
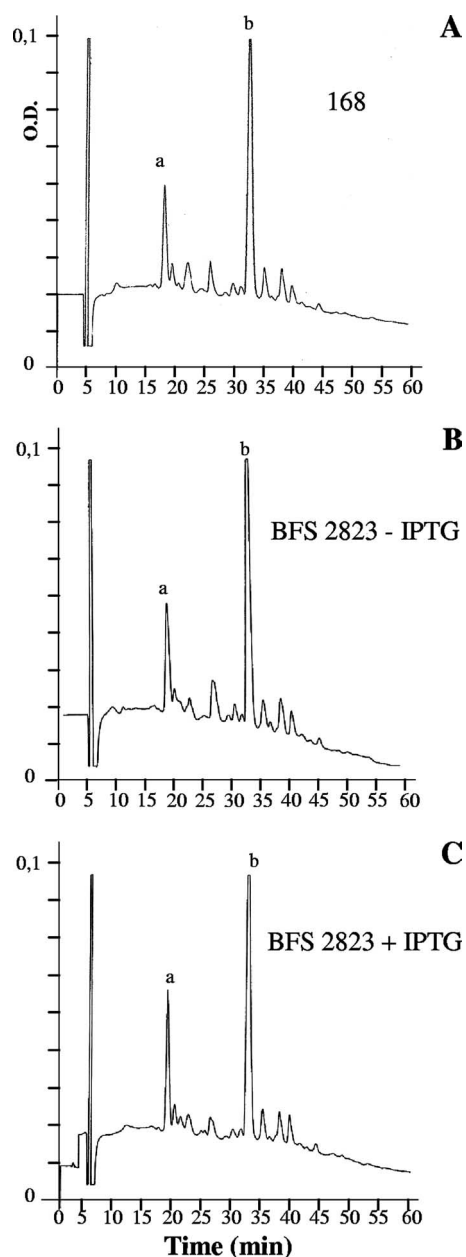

FIG. 4. HPLC analysis of the muropeptides in peptidoglycan from depleted and nondepleted strains. (A) Strain 168; (B) BFS2823 without IPTG; (C) BFS2823 with IPTG. Purified peptidoglycan (2) was digested with cellosyl *N*-acetylmuramidase, and the resulting muropeptides were reduced as described previously (3). The muropeptide mixtures were applied to a 5-μm Vydac C_{18} reversed-phase column and eluted as described previously (3) a, main monomer; b, main dimer.

sections of cells depleted of MreD, were also previously reported to show irregular deposition of cell wall material (22), indicating that MreD and CpgA may function in the same pathway.

From all of these above studies, the obvious defect in normal cell wall deposition (EM sections and precursor accumulation) could not be correlated, with the exception of some sensitivity to cephalosporins, with chemical defects in the assembled peptidoglycan. Finally, we therefore determined whether the nor-

TABLE 1. Peptidoglycan nucleotide precursor pools in cells from strain 168 and strain BFS2823 with or without IPTG

Strain	Pool level (pmol/mg of cell protein)[a]			
	UDP-MurNAc pentapeptide	UDP-MurNAc tripeptide	UDP-MurNAc dipeptide	UDP-GlcNAc
168	90	3	8	162
BFS2823 (without IPTG)	530	23	114	187
BFS2823 (with IPTG)	60	5	ND	196

[a] The peptidoglycan precursor fraction was obtained from a 2-liter exponential culture in E-medium as previously described (3). After a gel filtration step (Sephadex G-25); excluded fractions absorbing at 262 nm were pooled, lyophilized, and analyzed by HPLC as described previously (29, 30). Values are the average of two measurements. ND, not determined.

mal flow of peptidoglycan precursors was maintained in cells depleted of CpgA. As before, cells from strain 168 or strain BFS2823, in E-medium, with or without 1 mM IPTG, were harvested, and peptidoglycan precursors were extracted from the cytoplasm and quantified as described previously (3, 13, 29, 30). Assembly of *B. subtilis* peptidoglycan monomer units proceeds by a linear sequence of reactions via a series of UDP nucleotide precursors and lipid intermediates (33). The cytoplasmic steps culminate in the formation of the UDP-MurNAc pentapeptide precursor from UDP-*N*-acetylglucosamine (UDP-GlcNAc). In fact, as shown in Table 1, CpgA depletion resulted in a reproducible five- to sixfold increase in the level of UDP-MurNAc pentapeptide, with a similar increase in the immediate upstream precursors, the di- and tripeptides. In contrast, there was no significant change in the pool of the early precursor, UDP-GlcNAc. This indicated a bottleneck in the depleted cells, causing precursors to accumulate as the coordinated distribution of precursors into the expanding cell wall to ensure uniform construction of the multilayered sacculus was disrupted. Such a bottleneck would be consistent with the observed nonuniform deposition of cell wall material in the depleted strain, revealed by the EM analysis.

In conclusion, we suggest that CpgA acts as a translation factor to control the synthesis of certain factors required for a late step in biogenesis of peptidoglycan involving coordinated deposition of a uniform cell wall layer. When CpgA is depleted, we envisage that such factors (although not apparently Mbl or a major PBP) become limiting. Then, although peptidoglycan precursors are apparently largely inserted and cross-linked normally, a final step in the coordinated spatial deposition throughout the cell is perturbed, resulting in the accumulation of precursors, a cell wall of varying thickness and abnormal cell morphology. Importantly, we now have evidence that CpgA is a substrate, at least in vitro, for the kinase PrkC and the phosphatase PrpC (unpublished data), providing further support for functional linkage of both proteins in peptidoglycan biogenesis and homeostatic control.

REFERENCES

1. **Anagnostopoulos, C., and J. Spizizen.** 1961. Requirements for transformation in *Bacillus subtilis*. J. Bacteriol. **81:**741–746.
2. **Atrih, A., G. Bacher, G. Allmaier, M. P. Williamson, and S. J. Foster.** 1999. Analysis of peptidoglycan structure from vegetative cells of *Bacillus subtilis* 168 and role of PBP 5 in peptidoglycan maturation. J. Bacteriol. **181:**3956–3966.
3. **Billot-Klein, D., L. Gutmann, S. Sablé, E. Guittet, and J. van Heijenoort.** 1994. Modification of peptidoglycan precursors is a common feature of the low-level vancomycin resistant VANB-type *Enterococcus D366* and of the naturally glycopeptide-resistant species *Lactobacillus casei, Pediococcus pentosaceus, Leuconostoc mesenteroides,* and *Enterococcus gallinarum.* J. Bacteriol. **176:**2398–2405.
4. **Campbell, T. L., D. M. Daigle, and E. D. Brown.** 2005. Characterization of the *Bacillus subtilis* GTPase YloQ and its role in ribosome function. Biochem. J. **389:**843–852.
5. **Carballido-López, R.** 2006. Orchestrating bacterial cell morphogenesis. Mol. Microbiol. **60:**815–819.
6. **Carballido-López, R., and J. Errington.** 2003. The bacterial cytoskeleton: in vivo dynamics of the actin-like protein Mbl of *Bacillus subtilis*. Dev. Cell **4:**19–28.
7. **Carballido-López, R., A. Formstone, Y. Li, S. D. Ehrlich, P. Noirot, and J. Errington.** 2006. Actin homolog MreBH governs cell morphogenesis by localization of the cell wall hydrolase LytE. Dev. Cell **11:**399–409.
8. **Cladière, L., K. Hamze, E. Madec, V. M. Levdikov, A. J. Wilkinson, I. B. Holland, and S. J. Séror.** 2006. The GTPase, CpgA(YloQ), a putative translation factor, is implicated in morphogenesis in *Bacillus subtilis*. Mol. Genet. Genomics **275:**409–420.
9. **Daigle, D. M., and E. D. Brown.** 2004. Studies of the interaction of *Escherichia coli* YjeQ with the ribosome in vitro. J. Bacteriol. **186:**1381–1387.
10. **Daniel, R. A., and J. Errington.** 2003. Control of cell morphogenesis in bacteria: two distinct ways to make a rod-shaped cell. Cell **113:**767–776.
11. **De Siervo, A. J. D.** 1969. Alterations in the phospholipid composition of *Escherichia coli* B during growth at different temperatures. J. Bacteriol. **100:**1342–1349.
12. **Eymann, C., A. Dreisbach, D. Albrecht, J. Bernhardt, D. Becher, S. Gentner, L. T. Tam, K. Büttner, G. Buurman, C. Scharf, S. Venz, U. Völker, and M. Hecker.** 2004. A comprehensive proteome map of growing *Bacillus subtilis* cells. Proteomics **4:**2849–2876.
13. **Flouret, B., D. Mengin-Lecreulx, and J. van Heijenoort.** 1981. Reverse-phase high pressure liquid chromatography of uridine diphosphate *N*-acetylmuramyl peptide precursors of bacterial cell wall peptidoglycan. Anal. Biochem. **114:**59–63.
14. **Formstone, A., and J. Errington.** 2005. A magnesium-dependent *mreB* null mutant: implications for the role of *mreB* in *Bacillus subtilis*. Mol. Microbiol. **55:**1646–1657.
15. **Foulger, D., and J. Errington.** 1998. A 28-kbp segment from the *spoVM* region of the *Bacillus subtilis* 168 genome. Microbiology **144:**801–805.
16. **Himeno, H., K. Hanawa-Suetsugu, T. Kimura, K. Takagi, W. Sugiyama, S. Shirata, T. Mikami, F. Odagiri, Y. Osanai, D. Watanabe, S. Goto, L. Kalachnyuk, C. Ushida, and A. Muto.** 2004. A novel GTPase activated by the small subunit of ribosome. Nucleic Acids Res. **32:**5303–5309.
17. **Höltje, J. V.** 1998. Growth of the stress-bearing and shape-maintaining murein sacculus of *Escherichia coli*. Microbiol. Mol. Biol. Rev. **62:**181–203.
18. **Iwanicki, A., K. Hinc, S. Seror, G. Wegrzyn, and M. Obuchowski.** 2005. Transcription in the *prpC-yloQ* region in *Bacillus subtilis*. Arch. Microbiol. **183:**421–430.
19. **Kahan, F. M., J. S. Kahan, P. J. Cassidy, and H. Kropp.** 1974. The mechanism of action of fosfomycin (phosphonomycin). Ann. N. Y. Acad. Sci. **235:**364–386.
20. **Kang, C.-M., D. W. Abbott, S. T. Park, C. C. Dascher, L. C. Cantley, and R. N. Husson.** 2005. The *Mycobacterium tuberculosis* serine/threonine kinases PknA and PknB: substrate identification and regulation of cell shape. Genes Dev. **19:**1692–1704.
21. **Kobayashi, K., S. D. Ehrlich, A. Albertini, G. Amati, K. K. Andersen, M. Arnaud, K. Asai, S. Ashikaga, S. Aymerich, et al.** 2003. Essential *Bacillus subtilis* genes. Proc. Natl. Acad. Sci. USA **100:**4678–4683.
22. **Leaver, M., and J. Errington.** 2005. Roles for MreC and MreD proteins in helical growth of the cylindrical cell wall in *Bacillus subtilis*. Mol. Microbiol. **57:**1196–1209.
23. **Leipe, D. D., Y. I. Wolf, E. V. Koonin, and L. Aravind.** 2002. Classification and evolution of P-loop GTPases and related ATPases. J. Mol. Biol. **317:**41–72.
24. **Levdikov, V. M., E. V. Blagova, J. A. Brannigan, L. Cladière, A. A. Antson, M. N. Isupov, S. J. Séror, and A. J. Wilkinson.** 2004. The crystal structure of YloQ, a circularly permuted GTPase essential for *Bacillus subtilis* viability. J. Mol. Biol. **340:**767–782.
25. **Lévine, A., F. Vannier, C. Absalon, L. Kuhn, P. Jackson, E. Scrivener, J. V. Labas, J. Vinh, P. Courtney, J. Garin, and S. J. Séror.** 2006. Analysis of the dynamic *Bacillus subtilis* Ser/Thr/Tyr phosphoproteome implicated in a wide variety of cellular processes. Proteomics **6:**2157–2173.

26. **Londoño-Vallejo, J. A., C. Fréhel, and P. Stragier.** 1997. SpoIIQ, a forespore expressed gene required for engulfment in *Bacillus subtilis*. Mol. Microbiol. **24:**29–39.

27. **Madec, E., A. Laszkiewicz, A. Iwanicki, M. Obuchowski, and S. Séror.** 2002. Characterization of a membrane-linked Ser/Thr protein kinase in *Bacillus subtilis*, implicated in developmental processes. Mol. Microbiol. **46:**571–586.

28. **Madec, E., A. Stensballe, S. Kjellström, L. Cladière, M. Obuchowski, O. N. Jensen, and S. J. Séror.** 2003. Mass spectrometry and site-directed mutagenesis identify several autophosphorylated residues required for the activity of PrkC, a Ser/Thr kinase from *Bacillus subtilis*. J. Mol. Biol. **330:**459–472.

29. **Mengin-Lecreulx, D., B. Flouret, and J. van Heijenoort.** 1982. Cytoplasmic steps of peptidoglycan synthesis in *Escherichia coli*. J. Bacteriol. **151:**1109–1117.

30. **Mengin-Lecreulx, D., B. Flouret, and J. van Heijenoort.** 1983. Pool levels of UDP *N*-acetylglucosamine and UDP *N*-acetylglucosamine-enolpyruvate in *Escherichia coli* and correlation with peptidoglycan synthesis. J. Bacteriol. **154:**1284–1290.

31. **Obuchowski, M., E. Madec, G. D. Delattre, G. Boël, A. Iwanicki, D. Foulger, and S. J. Séror.** 2000. Characterization of PrpC from *Bacillus subtilis*, a member of the PPM phosphatase family. J. Bacteriol. **182:**5634–5638.

32. **Rosenthal, S., and N. Sharon.** 1964. The use of Sephadex G-25 for the isolation of nucleotide sugar derivatives from *Micrococcus lysodeikticus*. Biochim. Biophys. Acta **83:**378–380.

33. **van Heijenoort, J.** 2001. Recent advances in the formation of the bacterial peptidoglycan monomer unit. Nat. Prod. Rep. **18:**503–519.

34. **Yeats, C., R. D. Finn, and A. Bateman.** 2002. The PASTA domain: a beta-lactam binding domain. Trends Biochem. Sci. **27:**438.

35. **Zorzi, W., X. Y. Zhou, O. Dardenne, J. Lamotte, D. Raze, J. Pierre, L. Gutmann, and J. Coyette.** 1996. Structure of the low-affinity penicillin-binding protein 5 PBP5fm in wild-type and highly penicillin-resistant strains of *Enterococcus faecium*. J. Bacteriol. **178:**4948–4957.