A hallmark of bacterial endospore formation is engulfment, during which the membrane of one cell (the mother cell) migrates around the future spore, enclosing it in the mother cell cytoplasm. Bacteria lack proteins required for eukaryotic phagocytosis, and previously proteins required for membrane migration remained unidentified. Here we provide cell biological and genetic evidence that three membrane proteins synthesized in the mother cell are required for membrane migration as well as for earlier steps in engulfment. Biochemical studies demonstrate that one of these proteins, SpoIID, is a cell wall hydrolase, suggesting that membrane migration in bacteria can be driven by membrane-anchored cell wall hydrolases. We propose that the bacterial cell wall plays a role analogous to that of the actin and tubulin network of eukaryotic cells, providing a scaffold along which proteins can move.

[Keywords: Bacillus subtilis; sporulation; membrane movement; peptidoglycan hydrolysis; protein localization]

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The movement and localization of macromolecules within the cell is a conserved and essential feature of both prokaryotic and eukaryotic organisms. In bacteria, such events are essential for chromosome segregation, cell division, and DNA replication, as well as for pathogenesis, chemotaxis, and the development of specialized cell types (Shapiro and Losick 1997; Jensen and Shapiro 2000). However, in contrast to eukaryotic cells, little is known about how macromolecules are moved or localized in bacterial cells. Indeed, the mechanism for the rapid separation of bacterial chromosomes remains unclear. Thus far, neither the distant bacterial homologs of actin (FtsA and MreB) nor that of tubulin (FtsZ) have been implicated in this process, and the only bacterial motor proteins identified so far [Smc/MukA] are instead required to separately condense the segregated chromosomes [Gordon and Wright 2000; Hiraga 2000; Lemon and Grossman 2001]. Thus, the means by which bacteria move essential macromolecules such as DNA within their cell remains a mystery.

One dramatic example of the dynamic capabilities of bacterial cells is the phagocytosis-like process of engulfment [Fig. 1], a key step in the spore formation pathway of the endospore-forming bacteria, such as Bacillus subtilis, B. anthracis, and various Clostridia species (for reviews, see Stragier and Losick 1996; Piggot and Losick 2002). Shortly after an asymmetrically positioned cell division event generates the smaller forespore and larger mother cell, the mother cell membrane migrates around the forespore, until the leading edges of the engulfing membrane meet and fuse, releasing the forespore into the mother cell cytoplasm, where spore assembly is completed. Engulfment thereby mediates a striking reorganization of the sporangium, from two cells that lie side by side, to an endospore in which one cell lies within the cytoplasm of another. Despite the fact that engulfment is essential for sporulation in all endospore-forming bacteria, it remains unclear how bacterial cells are able to mediate this phagocytosis-like process.

Genetic studies of sporulation resulted in the identification of several proteins involved in engulfment, SpoIIβ [Margolis et al. 1993], SpoIIID [Lopez-Diaz et al. 1986], SpoIIIM [Smith et al. 1993], SpoIIIP [Frandsen and Stragier 1995], SpoIIQ [Londoño-Vallejo et al. 1997], and SpoIIIE [Sharp and Pogliano 1999]. It is now clear that mutants lacking either SpoIIβ [Perez et al. 2000] or SpoIIQ [Sun et al. 2000] are able to complete engulfment, albeit more slowly than wild type. Furthermore, although SpoIIIE is required for the membrane fusion event that is the final step of engulfment, it is not required for membrane migration [Sharp and Pogliano 1999]. Thus, the only known proteins essential for the early stages of engulfment are the mother cell-expressed proteins SpoIIID, SpoIIIM, and SpoIIIE.
SpoIID, in keeping with the observation that mother cell-specific, but not forespore-specific, gene expression is required for engulfment (Sun et al. 2000). These three proteins are found in all endospore-forming bacteria whose genomes have been sequenced (Stragier 2002), suggesting that they play conserved and essential roles in sporulation. In addition, SpoIID is homologous to another Bacillus protein, LytB, which is thought to regulate the activity of the major vegetative cell wall hydrolase, LytC (Lopez-Diaz et al. 1986; Kuroda et al. 1992), an N-acetyl-muramoyl-L-alanine amidase involved in cell separation (Blackman et al. 1998).

SpoIID, SpoIIM, and SpoIIP are essential for the first step of engulfment, septal thinning, during which the peptidoglycan within the sporulation septum becomes thinner when viewed by electron microscopy, starting in the middle of the septum and proceeding toward the edges (Piggot et al. 1994; Piggot and Losick 2002). They are also required to mediate the dissolution of partial septa formed when division initiates at the second potential division site within the mother cell (Fig. 1B; Pogliano et al. 1999; Eichenberger et al. 2001). Both activities likely require localized peptidoglycan hydrolysis, although no biochemical studies of these proteins have been performed, and none of the identified B. subtilis hydrolases are essential for engulfment (Foster and Popham 2002). Although dispensable for engulfment, SpoIIB also participates in septal thinning as, in its absence, the septal peptidoglycan appears ragged, as though incomplete septal thinning has occurred throughout the septum (Perez et al. 2000). Despite this defect, the spoIID mutant is able to slowly initiate and complete engulfment, with the engulfing membrane moving around the residual septal peptidoglycan. SpoIIB is therefore likely to be required for the complete dissolution of septal peptidoglycan, but not for membrane migration (Perez et al. 2000).

Thus far, despite the identification of mutants specifically defective in septal thinning or membrane fusion, and specific large-scale screens for engulfment-defective mutants, proteins essential for moving the engulfing membrane around the forespore remain unidentified. However, past studies of the essential mother cell-expressed engulfment proteins SpoIID, SpoIIM, and SpoIIP focused on the phenotypic analysis of null mutants, leaving open the possibility that these proteins are also required for later steps in engulfment. Here we report the isolation of spoIID and spoIIP mutants defective in both septal thinning and membrane migration, supporting a role for SpoIID and SpoIIP throughout engulfment. We also demonstrate that SpoIID, SpoIIM, and SpoIIP initially localize to the septal midpoint, then spread throughout the septum prior to becoming enriched at the leading edge of the engulfing mother cell membrane, where they remain until the completion of engulfment. These results suggest that SpoIID, SpoIIM, and SpoIIP are involved in moving the engulfing membrane around the forespore, as well as in septal thinning. Finally, our biochemical studies indicate that one of these proteins, SpoIID, is a peptidoglycan hydrolase, suggesting that membrane migration in bacteria may be driven by the activity of membrane-anchored cell wall hydrolases that drag the membrane with them as they hydrolyze peptidoglycan.

Results

A genetic screen for membrane migration-defective mutants

We performed a large-scale genetic screen to identify mutants blocked at various stages of engulfment (Sharp and Pogliano 1999). The mutants isolated were visually screened for engulfment defects using the fluorescent membrane stain FM 4-64, which allows clear visualization of the sporulation septum at various stages of engulfment (Fig. 2A,C), and readily identifies mutants blocked at different stages of engulfment (Pogliano et al. 1999, Sharp and Pogliano 1999, Perez et al. 2000; Sun et al. 2000). For example, mutants defective in septal thinning show a characteristic “bulge” phenotype caused when the forespore pushes through the center of the un-thinned septum and into the mother cell (arrows in Fig. 2E,G). The forespores of such sporangia have a central constriction imposed by the septal peptidoglycan (see cartoon, Fig. 2I), and, in strains with null mutations in either spoIID, spoIIM, or spoIIP, membrane migration is completely blocked. In contrast, mutants slow to complete membrane migration, such as the spoIIP mutant, show a smoothly curving septum (Sun et al. 2000; Fig. 2Q, arrow). The failure of such mutants to complete en-
Proteins required for *B. subtilis* engulfment

**Figure 2.** Engulfment phenotypes of spoIIP mutants. Sporangia were sampled 2 and 3 h after the onset of sporulation (first, second, third, and fourth columns, respectively), and the membranes stained with FM 4-64 (first and third columns) and the DNA stained with 6-diamidino-2-phenylindole (DAPI; second and fourth columns) and visualized with deconvolution microscopy. (A–D) Engulfment in the wild-type strain FY79. (A,B) The sporulation septum is initially flat, and then the mother cell membrane begins to migrate around the forespore (arrows). (C,D) After the completion of membrane migration but prior to membrane fusion, the forespore membranes stain with FM 4-64, which is membrane impermeable (arrows), whereas after membrane fusion (asterisks), the forespore membranes and chromosomes fail to stain with FM 4-64 (C) and DAPI (D). (E–H) Phenotype of a spoIP null strain (KP719), showing the constricted bulge phenotype caused when the forespore breaks through the center of an unthinned septum into the mother cell (arrows). (I) Model of a constricted bulge, based on electron microscopy studies [Frandsen and Stragier 1995; Pogliano et al. 1999; Perez et al. 2000]. The peptidoglycan (gray shading) remains within the sporulation septum, constricting the membrane was able to initiate but not complete membrane migration, and many sporangia have a partial constriction remaining at the original position of the septum (arrows). (J) Constricted bulges (arrowhead) are also seen. (N) Model of an open bulge, whose appearance suggests that the septal peptidoglycan no longer extends completely across the septum, and does not tightly constrict the forespore when a bulge forms. (O–R) Engulfment in the spoIIQ null strain [KP575], in which membrane migration is slow, but septal thinning is normal [Londoño-Vallejo et al. 1997], producing smoothly curving septa with no bulges (arrows). (S) Model of sporangium in which membrane migration but not septal thinning is defective. (R) Bar, 2 µm.

Engulfment can be unambiguously assayed using fluorescence microscopy because the fluorescent membrane stain FM 4-64 is membrane impermeable and so fails to stain the forespore membranes when added to sporangia that have completed membrane fusion [Sharp and Pogliano 1999; Fig. 2C, asterisk]. This screen resulted in the isolation of membrane fusion-defective mutants, as previously reported [spoIIIIE; Sharp and Pogliano 1999]. We also isolated mutants that appeared to be defective in membrane migration. These mutants included one with a point mutation in spoIIISA, a gene that is dispensable for engulfment and lacking from the genomes of most endospore-forming bacteria [Adler et al. 2001]. We also isolated an allele of spoIIIP with a phenotype distinct from that of the spoIIIP null mutant in several respects. First, spoIIIP95-2 sporangia have bulges that appear less constricted by septal peptidoglycan than those produced by spoIIIP null sporangia [Fig. 2J, arrow]. These “open bulges” suggest that the septal peptidoglycan no longer extends completely across the septum; indeed some spoIIIP95-2 sporangia appear to have completed septal thinning because no constriction is noted. Second, the mother cell membrane was able to initiate but not complete membrane migration [Fig. 2L, arrow], in about 26% of all sporangia [Table 1]. We observed sporangia in which the mother cell membrane had moved as far as the forespore pole, but we failed to observe sporangia that had completed the membrane fusion event that is the final step of engulfment. The production of sporangia with open bulges distinguishes the spoIIIP95-2 phenotype from that of a mutation conditionally defective in membrane migration but not in septal thinning [spoIIQ], which produces neither constricted nor open bulges [Fig. 2O,Q]. These observations suggest that the spoIIIP95-2 mutation decreases the rate of both septal thinning and membrane migration, and thus, that SpoIIIP is required for both processes.

Sequencing of spoIIIP95-2 identified a single mutation upstream of the spoIIIP coding region, changing the consensus ribosome binding site from GGAGG to GGAGA. No additional mutations were identified within or downstream of the spoIIIP coding region. Because the mutation changes the ribosome binding site away from the consensus sequence [Shine and Dalgarno 1974; Rocha et al. 1999; Ma et al. 2002], it is predicted to reduce spoIIIP translation, suggesting that the mutant phenotype is a consequence of reduced levels of SpoIIIP protein. Thus, the levels of SpoIIIP appear to be crucial for the rate of both septal thinning and membrane migration.
The engulfment phenotype was scored at $t_{30}$. Between 117 and 252 sporangia were scored for each strain.

**Table 1.** Percent sporangia showing the indicated engulfment phenotype

| Engagement stage | Bulge | Class | 37°C | 44°C |
|------------------|-------|-------|------|------|
| Early            | None  | 1     | 12   | 5    |
|                  | Closed| 2     | 1    | 12   |
|                  | Open  | 3     | 0    | 23   |
| Late             | None  | 4     | 16   | 3    |
|                  | Closed| 5     | 0    | 1    |
|                  | Open  | 6     | 0    | 12   |
| Complete         | None  | 7     | 70   | 10   |

The engulfment phenotype was scored at $t_{30}$. Between 117 and 252 sporangia were scored for each strain.

**Localised mutagenesis of spoIID**

We also performed localised mutagenesis of spoIID in an attempt to isolate mutations that block engulfment after septal thinning. A plasmid encoding spoIID was mutagenized by polymerase chain reaction (PCR), and introduced into a spoIID null strain of *B. subtilis*, where it integrated into the nonessential amyE locus by homologous recombination. Alleles that failed to fully complement the spoIID null mutation were isolated and characterized further. We isolated two mutants that were able to proceed past the stage of septal thinning and initiate membrane migration, but which were unable to complete membrane fusion. One of these mutations, spoIID39, is strongly temperature sensitive (Ts) for engulfment: engulfment and spore formation occurs at nearly wild-type levels at the permissive temperature, whereas at the nonpermissive temperature, the mutant appears similar to the spoIID null mutant in terms of both spor production (Table 2) and engulfment phenotype, producing constricted bulges and failing to initiate membrane migration (Supplementary Fig. 1). However, at the semipermissive temperature, the mutant produces an intermediate level of spores, and fluorescence microscopy demonstrated that it produces both open bulges (Fig. 3M, arrow) and closed bulges (Fig. 3O, arrowhead), and initiates but fails to complete membrane migration (Fig. 3Q, arrow). This suggests that under conditions in which SpolIID39 protein is partially active, the rate of both septal thinning and membrane migration are reduced.

The second spoIID mutation, spoIID38, reduced spore formation by twofold at all temperatures. The spoIID38 sporangia showed an engulfment defect similar to that of spoIIP95-2 and spoIID39 at 37°C, producing sporangia with slowed membrane migration (arrows in Fig. 3J,K). The spoIID38 sporangia were able to slowly complete engulfment, as by $t_{30}$, 10% of sporangia completed membrane fusion [Fig. 3K, asterisk], compared with 70% of wild-type sporangia [Table 1]. DNA sequence analysis revealed that the spoIID38 gene had three mutations, one of which changed an amino acid in the N-terminal hydrophobic segment of SpolIID from leucine to proline (L8 to P), another of which was upstream of the coding region (at nucleotide −50 from the ATG, C to T), and the third of which was a silent mutation changing an arginine codon from AGA to AGG (R111 to R). It seems probable that the L8-to-P mutation causes the mutant phenotype. Although this leucine is not conserved in any SpolIID homolog, the introduction of proline into the hy-
A drophobic core of a membrane-spanning segment or signal sequence is expected to dramatically affect its structure, perhaps inhibiting insertion into the membrane bilayer. The spoIID39 gene had two mutations, one changed a conserved threonine to an aspartate (T107D), and the other changed a nonconserved glutamate to glycine (E247G).

Localization of the mother cell-expressed engulfment proteins

If SpoIID, SpoIIM, and SpoIIP are involved in both membrane migration and septal thinning, then the proteins would be expected to localize to the septum and to the leading edge of the engulfment membrane. To test this prediction, we fused green fluorescent protein of Aequorea victoria (GFP) to the cytoplasmic N terminus of SpoIID, SpoIIM, and SpoIIP, because the C terminus of each is predicted to be extracellular and GFP is not fluorescent when exported from bacterial cells via the general secretory pathway (Feilmeier et al. 2000). The spoIID promoter was used to express the fusion genes, and each fully complemented the respective null mutations, producing wild-type levels of spores (Table 2).

We found that both GFP–SpoIIP and GFP–SpoIIM localized to the sporulation septum (Fig. 4A, arrow 1, B, arrow 4), and to the second division site within the mother cell (Fig. 4B, arrowhead), consistent with a role for these proteins in septal thinning and repressing division within the mother cell. Interestingly, both GFP–SpoIID [data not shown] and GFP–SpoIIM [Fig. 4B, arrow 3] initially localized to the septal midpoint, where septal thinning likely starts. Importantly, both GFP–SpoIIP and GFP–SpoIIM were most concentrated at the leading edge of the engulfing membrane (Fig. 4A, arrow 2, B, arrow 4), where they remained throughout membrane migration. This is in contrast to a protein required only for septal thinning (SpoIIB), which initially localizes to the septum, but delocalizes by the start of membrane migration (Perez et al. 2000). GFP–SpoIIM also localized to the forespore distal pole in later sporangia in which engulfment was more complete (Fig. 4B, arrow 3). This localization pattern might be caused by overexpression of GFP–SpoIIM from the spoIID promoter, which is about fourfold more active than the spoIIM promoter (A. Rubio, pers. comm.). Thus, the localization of SpoIIM and SpoIIP suggests that both proteins are required for membrane migration as well as for septal thinning.

GFP–SpoIID also localized to the sporulation septum [Fig. 4C, arrow 5], although a significant amount of GFP fluorescence was also observed throughout the mother cell cytoplasmic membrane. This diffuse fluorescence may be due to the presence of a potential leader peptidase recognition sequence within SpoIID, which if cleaved would release GFP fused to a signal sequence, which would likely freely diffuse within the mother cell membrane. In addition to this diffuse fluorescence, sporangia that had commenced membrane migration showed a high concentration of GFP–SpoIID at the leading edge of the engulfing membrane, similar to SpoIIP and SpoIIM [Fig. 4C, arrow 6]. Thus, similar to SpoIIP and SpoIIM, SpoIID localizes to the septum and to the mother cell-expressed engulfment proteins
leading edge of the engulfing membrane, consistent with our genetic data implicating SpoIID in membrane migration as well as septal thinning.

**SpoIID is a peptidoglycan hydrolase**

The previously described roles of SpoIID, SpoIIM, and SpoIIP in the thinning of septal peptidoglycan and in the retraction of partial septa within the mother cell suggested that they might be involved in peptidoglycan degradation (Pogliano et al. 1999). However, although all of these proteins are conserved in all endospore-forming bacteria, none are homologous to enzymes known to hydrolyze peptidoglycan [although SpoIID is homologous to a protein that regulates the activity of one such protein (Lopez-Diaz et al. 1986; Kuroda et al. 1992)]. We were therefore interested in determining if any of these proteins were able to degrade bacterial cell walls. For these biochemical studies, we focused on SpoIID and SpoIIP because each has a large extracellular domain, as do many known peptidoglycan hydrolases (Foster and Popham 2002), whereas SpoIIM is an integral membrane protein and lacks a substantial extracellular domain.

We overexpressed His-tagged SpoIID and SpoIIP in *Escherichia coli*, purified the proteins, and used renaturing polyacrylamide gel electrophoresis to test their ability to hydrolyze bacterial cell walls incorporated into a polyacrylamide gel (Foster 1992). This assay showed that SpoIID was able to degrade both *Micrococcus luteus* (Fig. 5B) and *B. subtilis* cell walls (Fig. 5C), clearing peptidoglycan from the gel at the position of full-length SpoIID and of a larger copurifying protein that might be a SpoIID multimer. Remarkably, less SpoIID than lysozyme was required to solubilize the peptidoglycan to the same extent. SpoIIP did not consistently demonstrate hydrolase activity in these gels (data not shown). Further tests of biochemical activities for SpoIIP, and the more precise description of the precise hydrolytic activity of SpoIID, will require the purification of both proteins in an active and soluble state. However, our biochemical studies to
The leading edge of the engulfing membrane advances adjacent to the cell wall

If movement of the mother cell membrane around the forespore is mediated by the peptidoglycan hydrolase activity of SpoIID, then one would expect that the engulfing membrane would advance most rapidly adjacent to the cell wall. We therefore used ultrathin section transmission electron microscopy to provide a high-resolution image of the leading edge of the engulfing membrane. We consistently observed that during membrane migration, the leading edge of the mother cell membrane is in close contact with the cell wall (Fig. 6, arrow), whereas the lagging portion of the engulfing membrane is away from the cell wall (see also Fig. 4 in Perez et al. 2000). Thus, electron microscopic analysis suggests that the leading edge of the engulfing membrane moves around the forespore immediately adjacent to the cell wall.

Discussion

Here we provide genetic and cell biological evidence that three mother cell-expressed proteins are required for the movement of the mother cell membrane around the forespore during the phagocytosis-like process of engulfment. First, we isolated mutations predicted to reduce the level of active SpoIID and SpoIIP protein, and found that these mutations reduce both the rate of septal thinning and membrane migration. Second, we localized SpoIID, SpoIIM, and SpoIIP, and found that each is enriched at the leading edge of the engulfing membrane, where they remain until the completion of membrane migration. Together, this cell biological and genetic evidence strongly suggests that these three proteins are involved in the movement of the mother cell membrane around the forespore, as well as in thinning of the septal peptidoglycan. SpoIID, SpoIIM, and SpoIIP are likely to be the only essential engulfment proteins that are dispensable for growth, because the genetic screen reported here, and a similar screen in another laboratory, failed to identify any new engulfment proteins [P. Eichenberger and R. Losick, pers. comm.]. Furthermore, we previously demonstrated that only mother cell-specific gene expression is essential for engulfment (Sun et al. 2000), and Eichenberger and Losick have inactivated all ntrB-transcribed genes identified by microarray analysis, yet failed to identify any new engulfment proteins [P. Eichenberger and R. Losick, pers. comm.]. Although it remains possible that other sporulation-specific proteins play subtle or redundant roles in engulfment, and that proteins essential for viability play a crucial role in engulfment, it
seems likely that, of the sporulation-specific proteins, SpoIID, SpoIIM, and SpoIIP comprise the essential engulfment machinery. We have demonstrated that one of these proteins, SpoIID, is a peptidoglycan hydrolase capable of solubilizing both B. subtilis and M. luteus cell peptidoglycan in a renaturing gel electrophoresis assay. This enzymatic activity is consistent with the requirement for SpoIID in septal thinning and is the first direct demonstration that septal thinning requires peptidoglycan hydrolysis. The observation that reducing the level of SpoIID activity also slows membrane migration suggests that peptidoglycan hydrolase activity is required for membrane migration. SpoIID is the founding member of a new class of peptidoglycan hydrolases that includes SpoIID homologs of other endospore-forming bacteria, proteins of unknown function in cyanobacterial genomes, and B. subtilis LytB (Kuroda et al. 1992), which has been previously reported to regulate the activity of a major peptidoglycan hydrolase (LytC). Our results demonstrate that at least certain members of this family of proteins are peptidoglycan hydrolases capable of mediating dynamic events in bacterial cells.

We can imagine two distinct mechanisms by which peptidoglycan hydrolase activity might contribute to membrane migration during engulfment. First, it is possible that peptidoglycan hydrolysis is necessary to remove bridges between the forespore membrane and the cell wall, such as those that might be formed by lipoteichoic acid. Such bonds might impede movement of the mother cell membrane around the forespore. If so, then membrane migration might be expected to occur more slowly immediately adjacent to the cell wall. In contrast, our electron micrographs show that engulfment proceeds most rapidly adjacent to the cell wall, with the cell wall distal portion of the leading edge lagging behind that adjacent to the cell wall. In addition, a previous study suggested that the cell wall was necessary for engulfment because spore formation was blocked if the wall was removed by enzymatic digestion in osmotically buffered medium before, but not after, engulfment (Fitz-James 1964). The second model proposes that peptidoglycan hydrolysis plays a more active role in engulfment because a membrane-anchored protein complex that includes a peptidoglycan hydrolase could drag the mother cell membrane around as it hydrolyzes peptidoglycan surrounding the forespore (Fig. 7). In this model, the energy for membrane movement could be provided by the hydrolysis of a large number of bonds in the peptidoglycan, which might be sufficient to power this relatively slow process (which requires ~45 min to complete). This model suggests that the bacterial cell wall provides an external scaffold along which motor proteins can move, similar to the eukaryotic cytoskeleton.

Previous observations support an analogy between the bacterial cell wall and the eukaryotic cytoskeleton. First, like the eukaryotic cytoskeleton, the bacterial cell wall plays a crucial role in determining and maintaining cell shape (Holtje 1998). Second, both the peptidoglycan biosynthesis machinery and several cell wall hydrolases are processive enzymes that likely move along the peptidoglycan strands as they synthesize or degrade cell wall polymers [Barrett et al. 1984; Holtje 1996, 1998]. Interestingly, biochemical studies of the SpoIID homolog LytB have suggested that it confers processivity on the enzyme with which it interacts [Herbold and Glaser 1975]. We therefore predict that the SpoIID peptidoglycan hydrolase [or a protein complex that includes SpoIID] is a processive enzyme that translocates along the glycan chains, thereby moving the mother cell membrane around the forespore.

**Materials and methods**

*Bacterial strains, genetic manipulations, and growth conditions*

*B. subtilis* strains (Table 3) used in this study are derivatives of wild-type strain PY79 [Youngman et al. 1984]. Mutations and the various plasmid constructs were introduced into PY79 by transformation [Dubnau and Davidoff-Abelson 1971]. *B. subtilis* was grown and sporulated at 37°C unless otherwise specified. Sporulation was induced by the resuspension method [Sterlini and Mandelstam 1969] or by nutrient exhaustion in Difco Sporulation Medium [DSM; Schaeffer et al. 1965]. Sporulation efficiency was determined after heating cultures at 80°C for 20 min at 48 h after induction of sporulation for 30°C cultures, 24 h for 37°C cultures, and 18 h for 44°C cultures. Standard PCR conditions [Qiagen Taq Polymerase Kit and Roche Expand Hi-
Fidelity PCR System] were used. Two E. coli strains were used to propagate the various plasmids used in this study, DH5α and KJ622 (TG1, pcnB uvc24-1). Sequencing of plasmid constructs or of PCR-amplified chromosomal DNA was conducted by the Shared Resource UCSD Cancer Center.

Localized mutagenesis of spoIID

An amyE integrational vector encoding wild-type spoIID was constructed by cloning a 2-kb EcoRI-to-BamHI fragment including the spoIID gene from p16-2 [a gift of A. Decatur and R. Losick] into EcoRI- and BamHI-digested pER82 (Driks et al. 1994). The resulting plasmid (pKP1) was PCR mutagenized with Taq DNA polymerase (Zhou et al. 1991), using the primers KJPO3 [5’-CTCCAGTTCATCAATAC-3’] and KJPO6 [5’-GCCCTCCTGATCCTAG-3’] to amplify an 8.1-kb fragment comprising most of the plasmid backbone and the amyE DNA that flanks both spoIID and the B. subtilis selectable marker kan. The PCR fragments were directly transformed into freshly prepared competent cells of the B. subtilis spoIID null mutant strain KP7. Transformants were selected by plating on DSM plates containing 3 µg/mL kanamycin, and incubated at either room temperature, 30°C, 37°C, or 44°C. The PCR mutagenesis was highly effective, as between 6% and 50% of all transformants failed to complement the spoIID null mutation. Transformants that were either partially or completely colony defective were colony purified and tested for temperature- or cold-sensitivity sporulation defects, and for mutations that only partially complemented the spoIID mutation. The mutations in two such strains, KP38 and KP39 were sequenced following PCR amplification of the spoIID coding region using primers AADO31 (5’-GAGGGATTTTTGACCTCGAAG-3’) and AADO32 (5’-CAAAAGCCTTTTCCCCTGG-3’), which hybridize to the amyE flanking DNA. In the course of these studies, we sequenced the spoIID298 mutation of KP7, and found that it changed codon 145 (normally encoding Gln) to an amber codon (CAG to TAG).

Isolation of spoIIP95-2

Possible peptidoglycan thinning or membrane migration mutants from a screen described previously (Sharp and Pogliano 1999) were introduced into a nonmutagenized background [strain KP555] by transformation as described (Sharp and Pogliano 1999), and microscopically screened to ensure that the original engulfment phenotype had been retained. To identify the mutant gene, we transformed into these strains a plasmid library consisting of B. subtilis genomic fragments cloned into the amyE integration vector pDG1730 [Guérout-Fleury et al. 1996], in which the cat gene was replaced with a spec gene. Transformants were screened for Spo+ colonies on DSM plates containing 80 µg/mL X-gal and 5 µg/mL chloramphenicol. Such transformants contained a DNA fragment that either complemented or marker rescued the original spo mutation. The cloned chromosomal DNA was identified by using ligation-mediated PCR using the Universal Genome Walker Kit (Clontech). In brief, chromosomal DNA was prepared and digested with either Dral or Ksal, and ligated to the GenomeWalker Adapters. Two specific primers were used in the PCR amplification, one hybridized to the amyE region of the plasmid (KXGSP1 5’-TGCGACTGTTAGTTATATGATAGT-3’), the other upstream of the cat gene KXGSP2 (5’-TATAATCAGTATTACGAAACGGGAAATACGC-3’), and two primers to the GenomeWalker Adapter (AP1, 5’-CTGGGCTGCCTATGGAACC-3’ and AP2 5’-ACTATAGGCGACCGCCGGTG-3’) were used to amplify the cloned B. subtilis DNA. The PCR product was sequenced using the second internal primer (KXGSP2) to identify the cloned genomic DNA.

Construction of GFP fusions

N-terminal GFP fusions to SpoIID, SpoIIM, and SpoIIP were constructed by PCR amplification of the respective wild-type genes from PY79 chromosomal DNA, using the following primers: spoIID [AAD03 5’-CTCCAGTTCATCAATAC-3’ and AADO4 5’-CTCCAGTTCATCAATAC-3’], spoIIM [AADO5 5’-GAACACGCGGCCAATCTGTTATATGATAGT-3’], spoIIP [AADO7 5’-CTCCAGTTCATCAATAC-3’], and pcnB (AAD05 5’-GAACACGCGGCCAATCTGTTATATGATAGT-3’), which hybridize to the amyE region of the plasmid (KXGSP1 5’-TGCGACTGTTAGTTATATGATAGT-3’), the other upstream of the cat gene KXGSP2 (5’-TATAATCAGTATTACGAAACGGGAAATACGC-3’), and two primers to the GenomeWalker Adapter (AP1, 5’-CTGGGCTGCCTATGGAACC-3’ and AP2 5’-ACTATAGGCGACCGCCGGTG-3’) were used to amplify the cloned B. subtilis DNA. The PCR product was sequenced using the second internal primer (KXGSP2) to identify the cloned genomic DNA.

Table 3. Bacterial strains used in this study

| Strain          | Genotype                  | Reference                  |
|-----------------|---------------------------|----------------------------|
| *Bacillus subtilis* |                           |                            |
| PY79            | Wild type                 | Youngman et al. 1984       |
| KP7             | spoIID298                 | Lopez-Diaz et al. 1986     |
| KP38            | amyE::spoIID38tkan, spoIID298 | This study               |
| KP39            | amyE::spoIID39tkan, spoIID298 | This study               |
| KP519           | spoIIM::Tn917             | Sandman et al. 1987       |
| KP555           | thr::cotD-lacZtkan, cotE-gfp::kan | This study               |
| KP621           | spoIIP95-2, thr::cotD-lacZtkan, cotE-gfp::kan | This study               |
| KP718           | amyE::spoIIDtkan, spoIID298 | This study               |
| KP719           | spoIIP::tet               | Frandsen and Stragier 1995 |
| KP720           | amyE::gfp-spoIIDtkan, spoIID298 | This study               |
| KP721           | amyE::gfp-spoIIMtkan, spoIIM::Tn917 | This study               |
| KP722           | amyE::gfp-spoIIPtkan, spoIIP::tet | This study               |
| *Escherichia coli* |                           |                            |
| KJ622           | Strain TG1, pcnB uvc24-1  | This study               |

Proteins required for *B. subtilis* engulfment

In brief, chromosomal DNA was prepared and digested with either DraI or KsaI, and ligated to the GenomeWalker Adapters. Two specific primers were used in the PCR amplification, one hybridized to the amyE region of the plasmid (KXGSP1 5’-TGCGACTGTTAGTTATATGATAGT-3’), the other upstream of the cat gene KXGSP2 (5’-TATAATCAGTATTACGAAACGGGAAATACGC-3’), and two primers to the GenomeWalker Adapter (AP1, 5’-CTGGGCTGCCTATGGAACC-3’ and AP2 5’-ACTATAGGCGACCGCCGGTG-3’) were used to amplify the cloned B. subtilis DNA. The PCR product was sequenced using the second internal primer (KXGSP2) to identify the cloned genomic DNA.

Construction of GFP fusions

N-terminal GFP fusions to SpoIID, SpoIIM, and SpoIIP were constructed by PCR amplification of the respective wild-type genes from PY79 chromosomal DNA, using the following primers: spoIID [AAD03 5’-CTCCAGTTCATCAATAC-3’ and AADO4 5’-CTCCAGTTCATCAATAC-3’], spoIIM [AADO5 5’-GAACACGCGGCCAATCTGTTATATGATAGT-3’], spoIIP [AADO7 5’-CTCCAGTTCATCAATAC-3’], and pcnB (AAD05 5’-GAACACGCGGCCAATCTGTTATATGATAGT-3’), which hybridize to the amyE region of the plasmid (KXGSP1 5’-TGCGACTGTTAGTTATATGATAGT-3’), the other upstream of the cat gene KXGSP2 (5’-TATAATCAGTATTACGAAACGGGAAATACGC-3’), and two primers to the GenomeWalker Adapter (AP1, 5’-CTGGGCTGCCTATGGAACC-3’ and AP2 5’-ACTATAGGCGACCGCCGGTG-3’) were used to amplify the cloned B. subtilis DNA. The PCR product was sequenced using the second internal primer (KXGSP2) to identify the cloned genomic DNA.

**Isolation of spoIIP95-2**

Possible peptidoglycan thinning or membrane migration mutants from a screen described previously (Sharp and Pogliano 1999) were introduced into a nonmutagenized background [strain KP555] by transformation as described (Sharp and Pogliano 1999), and microscopically screened to ensure that the original engulfment phenotype had been retained. To identify the mutant gene, we transformed into these strains a plasmid library consisting of B. subtilis genomic fragments cloned into the amyE integration vector pDG1730 [Guérout-Fleury et al. 1996], in which the cat gene was replaced with a spec gene. Transformants were screened for Spo+ colonies on DSM plates containing 80 µg/mL X-gal and 5 µg/mL chloramphenicol. Such transformants contained a DNA fragment that either complemented or marker rescued the original spo mutation. The cloned chromosomal DNA was identified by using ligation-mediated PCR using the Universal Genome Walker Kit (Clontech). In brief, chromosomal DNA was prepared and digested with either DraI or KsaI, and ligated to the GenomeWalker Adapters. Two specific primers were used in the PCR amplification, one hybridized to the amyE region of the plasmid (KXGSP1 5’-TGCGACTGTTAGTTATATGATAGT-3’), the other upstream of the cat gene KXGSP2 (5’-TATAATCAGTATTACGAAACGGGAAATACGC-3’), and two primers to the GenomeWalker Adapter (AP1, 5’-CTGGGCTGCCTATGGAACC-3’ and AP2 5’-ACTATAGGCGACCGCCGGTG-3’) were used to amplify the cloned B. subtilis DNA. The PCR product was sequenced using the second internal primer (KXGSP2) to identify the cloned genomic DNA.
The resulting plasmids were sequenced, and then transformed into the respective null mutant strains. Each fully complemented the null mutation and supported wild-type levels of spore production.

Microscopy and image analysis

To assess the completion of engulfment, we harvested samples of sporulating bacteria at the appropriate time, and stained them with a final concentration of 5 μg/mL FM 4-64, 0.2 μg/mL 4’, 6-diamidino-2-phenylindole (DAPI), and 30 μg/mL MitoTracker Green FM [MTG; Sharp and Pogliano 1999]. After the completion of the membrane fusion event that is the final step of engulfment, the membrane-impermeable stain FM 4-64 is excluded from the forespore membrane and DAPI is excluded from the forespore nucleoid. When visualizing GFP, live cells were stained with a final concentration of 0.1 μg/mL MitoTracker Red and 0.2 μg/mL DAPI (Sharp and Pogliano 2002). All fluorescent stains were obtained from Molecular Probes. An Applied Precision optical sectioning microscope and Delta Vision software were used to collect and deconvolve the images, as has been previously described (Pogliano et al. 1999). Following deconvolution, images from the medial focal plane were saved as TIF files and imported into Adobe Photoshop.

Overexpression and purification of His-SpoIID and His-SpoIIP

His-tagged SpoIID was constructed by cloning the promoters to EcoRI fragment of SpoIID from p16-2 [a gift of A. Decatur and R. Losick] into pRSETc, fusing the entire coding region of spoIID to the poly-His linker. The resulting plasmid (pKP4) was transformed into E. coli strain BL21. His-tagged SpoIIP was constructed by the PCR amplification of the entire spoIIP coding sequence using the primers 5’TIF files and imported into Adobe Photoshop.

Purification of B. subtilis

One liter of PY79 was grown at 37°C in liquid sporulation medium (DSM). Two hours after the initiation of sporulation, cells were harvested by centrifugation (7000g, 15 min, 4°C). The pellet was washed with 25 mM TrisCl at pH 8.0 for 30 min at 4°C. After spinning the cells at 7000g for 15 min at 4°C in microcentrifuge tubes, pellets were flash-frozen in liquid nitrogen and dried overnight in a speed-vac. One gram of the freeze-dried cells was resuspended in 80 mL of 4% (w/v) SDS. The suspension was shaken for 90 min at 150 rpm on a rotary shaker (RT) and sonicated for 5 min at 4°C (using ten 30-sec cycles of sonication followed by cooling on ice). After sonication, the suspension was incubated at 100°C for 15 min and centrifuged (12,000g, 15 min, RT). To remove the SDS and membranes, the pellet was resuspended in 80 mL of 0.1% (w/v) purified Triton X-100 and incubated for 30 min at RT with gentle shaking. The suspension was centrifuged and the pellet was washed 4× for 30 min in 13 mL of deionized water (dH2O). The final pellet was freeze-dried overnight and resuspended as a 2% (w/v) cell wall suspension in dH2O containing 0.02% (w/v) sodium azide. The suspension was stored at 4°C.

Renaturing gel electrophoresis for cell wall hydrolytic activity

Purified His-SpoIID and His-SpoIIP was subjected to SDS-PAGE, with gels containing 0.1% (w/v) M. luteus cells (Sigma) or 0.1% (w/v) purified B. subtilis cell wall as a substrate (Foster 1992). SDS-PAGE gels were run at 15 mA at room temperature. Following electrophoresis, gels were rinsed in deionized water, transferred to 300 mL of Renaturation solution [25 mM TrisCl at pH 7.2, 1% (w/v) Triton X-100], and incubated at 37°C for 16 h with gentle shaking. Gels were rinsed with deionized water, stained with 0.01% (w/v) methylene blue in 0.01% (w/v) KOH for 3 h, and destained with deionized water. Zones of clearing in the blue background indicated cell wall hydrolytic activity. Lysozyme and bovine serum albumin (BSA) were used as positive and negative controls, respectively, because we noted that overloaded proteins show a small amount of clearing in these assays, even when the protein completely lacks hydrolase activity. This small amount of clearing can be seen for BSA in Figure 5. However, such clearing is always partial and appears only after destaining, indicating that peptidoglycan remains in the region of the band and suggesting that the high concentration of protein somehow reduces the affinity of methylene blue for peptidoglycan. In contrast, peptidoglycan hydrolases such as SpoIID and lysozyme mediate the rapid and complete clearing of peptidoglycan even in the absence of overloading [such as Fig. 5, lanes 1,2, for SpoIID], and this clearing is apparent before destaining.

Electron microscopy

Electron microscopy was performed as described in Perez et al. [2000] using method IV.

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A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore

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