Peptidoglycan hydrolysis is required for assembly and activity of the transenvelope secretion complex during sporulation in *Bacillus subtilis*

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Summary

Sporulating *Bacillus subtilis* cells assemble a transenvelope secretion complex that connects the mother cell and developing spore. The forespore protein SpoIIQ and the mother-cell protein SpoIIIAH interact across the double membrane septum and are thought to assemble into a channel that serves as the basement layer of this specialized secretion system. SpoIIQ is absolutely required to recruit SpoIIIAH to the sporulation septum on the mother-cell side, however the mechanism by which SpoIIQ is localized has been unclear. Here, we show that SpoIIQ localization requires its partner protein SpoIIIAH and degradation of the septal peptidoglycan (PG) by the two cell wall hydrolases SpoIID and SpoIIP. Our data suggest that PG degradation enables a second mother-cell-produced protein to interact with SpoIIQ. Cells in which both mother-cell anchoring mechanisms have been disabled have a synergistic sporulation defect suggesting that both localization factors function in the secretion complex. Finally, we show that septal PG degradation is critical for the assembly of an active complex. Altogether, these results suggest that the specialized secretion system that links the mother cell and forespore has a complexity approaching those found in Gram-negative bacteria and reveal that the sporulating cell must overcome similar challenges in assembling a transenvelope complex.

Keywords

LytM; protein localization; sporulation; cell wall hydrolase; specialized secretion

Introduction

During spore formation in *Bacillus subtilis*, the mother cell and forespore follow distinct programs of developmental gene expression that are linked to each other through cell-cell signaling pathways (Errington, 2003, Hilbert & Piggot, 2004, Higgins & Dworkin, 2012). In addition to these traditional modes of communication, the mother cell and forespore assemble a transenvelope secretion complex that spans the double membrane that separates them (Meisner *et al.*, 2008, Camp & Losick, 2008, Doan *et al.*, 2009, Camp & Losick, 2009, Meisner *et al.*, 2012, Levdikov *et al.*, 2012). This complex is required to maintain forespore physiology and for forespore gene expression at late stages in development (Doan et al., 2009, Camp & Losick, 2009). Little is known about this complex that appears to be assembled from components normally found in distinct Gram-negative secretion systems (Camp & Losick, 2008, Doan et al., 2009, Meisner et al., 2008). It is not known what this machine transports; what it looks like and how it is assembled; nor is it known whether a complete parts list for the complex has been identified. Here, we provide evidence for the

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involvement of an additional mother-cell protein in the assembly of the complex and show that, like Gram-negative secretion systems, degradation of peptidoglycan is important for the assembly of an active machine.

Upon the initiation of sporulation, the developing cell divides asymmetrically, generating two cells of unequal size and dissimilar fate (Higgins & Dworkin, 2012, Hilbert & Piggot, 2004, Errington, 2003). The smaller cell differentiates into the dormant spore and is called the forespore. The larger cell nurtures the developing spore and is referred to as the mother cell. The two cells follow distinct programs of gene expression that are linked to each other by cell-cell signaling pathways. At early stages of sporulation, forespore transcription under the control of ^{F} is required to activate the mother-cell transcription factor ^{E}. At late stages, the forespore transcription factor ^{G} is required to activate mother-cell gene expression under ^{K} control. Initially, the forespore and mother cell lie side by side separated by a double membrane septum. However, shortly after polar division, the first mother-cell transcription factor ^{E} directs the synthesis of cell wall hydrolases that degrade the septal peptidoglycan and facilitate the migration of the mother-cell membranes around the forespore in a process called engulfment. Upon completion of engulfment, a membrane fission event releases the forespore into the mother-cell cytoplasm as a free protoplast surrounded by two membranes. At this stage, the mother packages the forespore in protective layers in preparation for dormancy. When the spore is fully mature, the mother cell lyses releasing it into the environment, where it stays dormant until nutrients trigger germination and outgrowth.

During the morphological process of engulfment, the mother cell and forespore assemble a transenvelope complex that spans the double membrane that separates them. Several lines of evidence suggest that the basement layer of this complex is composed of the mother-cell membrane protein SpoIIIAH and the forespore membrane protein SpoIIQ. First, the extracytoplasmic domains of these two proteins interact in the space between the two membranes (Blaylock *et al.*, 2004, Doan *et al.*, 2005). Second, SpoIIIAH shares weak sequence similarity with inner membrane ring proteins, YcsJ/EscJ and FliF, found in type III secretion systems and the flagellar basal body (Camp & Losick, 2008, Meisner et al., 2008); and recent co-crystal structures of the extracytoplasmic domains of SpoIIIAH and SpoIIQ revealed that SpoIIIAH is indeed a structural homolog of EscJ (Meisner et al., 2012, Levdikov et al., 2012). Finally, forespore-expressed biotin ligase can access and biotinylate the extracytoplasmic domain of SpoIIIAH in a SpoIIQ-dependent manner (Meisner et al., 2008). Collectively, these findings suggest that SpoIIIAH and SpoIIQ form a channel that connects the mother cell and forespore. Seven additional mother-cell proteins encoded in the *spoIIIA* operon (along with SpoIIIAH) are required to assemble an active secretion complex (Doan et al., 2009, Camp & Losick, 2009). Several of these proteins share sequence similarity to components of Type II/IV or Type III secretion systems found in Gram-negative bacteria (Camp & Losick, 2008, Meisner et al., 2008, Doan et al., 2009). In the absence of any of the SpoIIIA proteins or SpoIIQ, the forespore appears to collapse and lose its integrity (Doan et al., 2009). Moreover, consistent with a loss of integrity, gene expression in the forespore compartment ceases, suggesting that the forespore lose its metabolic potential (Camp & Losick, 2009). It is not known what this transenvelope complex transports, however, the presence of the secretion ATPase on the mother-cell side and the phenotypes of the forespore in the absence of the components of the machine suggest that transport occurs from mother cell to forespore and that the secreted factor(s) are necessary to maintain forespore metabolic potential and forespore gene expression (Camp & Losick, 2009, Doan et al., 2009).

Assembly of this apparatus appears to begin with the interaction of SpoIIIAH and SpoIIQ across the polar septum. SpoIIIAH localization to the septal membrane requires SpoIIQ in

the forespore (Blaylock et al., 2004, Doan et al., 2005). Interestingly, SpoIIQ localizes to the septum on the forespore side in the absence of SpoIIIAH (Blaylock et al., 2004, Doan et al., 2005). The underlying mechanism by which SpoIIQ is anchored in the septal membrane is not known. However, Pogliano and co-workers have shown that in cells lacking the mother-cell transcription factor σ^E, SpoIIQ becomes mislocalized and is found in all membranes of the forespore (Blaylock et al., 2004, Rubio & Pogliano, 2004) (Fig. 1). This observation indicates that one (or several) mother-cell proteins under σ^E control are required to localize SpoIIQ and suggests that these localization factors could function in the assembly of the secretion complex. To gain a deeper understanding of this transenvelope machine, we set out to identify the localization determinants of SpoIIQ.

Here, we report that septal localization of SpoIIQ requires the σ^E-controlled proteins SpoIIIAH, SpoIID, and SpoIIP. Unlike SpoIIIAH that helps anchor SpoIIQ through direct protein-protein interaction, we show that SpoIID and SpoIIP act indirectly. Our data indicate that the catalytic activities of SpoIID and SpoIIP are necessary for SpoIIQ localization and suggest that septal PG degradation by these cell wall hydrolases allows an additional mother-cell protein under σ^E control to interact with and participate in anchoring SpoIIQ in the septal membrane. Mutational analysis supports the idea that this interaction occurs in the LytM groove of SpoIIQ. Cells in which both anchoring mechanisms have been disabled are severely impaired in sporulation, suggesting that both localization factors function in the secretion complex that maintains forespore integrity and late forespore gene expression. Finally, we show that peptidoglycan degradation in the intermembrane space is critical for the assembly of an active secretion complex. These results suggest that the secretion apparatus assembled during sporulation may have a complexity akin to those found in Gram-negative bacteria and that *B. subtilis* has come up with similar solutions to the challenges of assembling a transenvelope machine.

Results

SpoIID, SpoIIP and SpoIIIAH are required for proper localization of SpoIIQ

In the course of our analysis of the cell wall hydrolases SpoIID and SpoIIP (Morlot *et al.*, 2010), we noticed that the forespore membrane protein SpoIIQ frequently localized within the septal bulge that was generated in strains harboring point mutants in SpoIID (Fig. 1). Under these conditions, SpoIID and SpoIIP are also present at or near these bulges on the mother cell side and fail to migrate around the forespore (Gutierrez *et al.*, 2010). Accordingly, we wondered whether SpoIID and/or SpoIIP participate in localizing SpoIIQ in the septal membrane. To investigate this, we monitored the localization of a fully functional GFP-SpoIIQ fusion during sporulation in wild-type and in cells lacking SpoIID, SpoIIP, or both. In the absence of either SpoIID or SpoIIP the septal membrane bulges into the mother cell cytoplasm and GFP-SpoIIQ was frequently localized within the bulge (Rubio & Pogliano, 2004) (Fig. 1 and data not shown). In the absence of both proteins, degradation of the septal peptidoglycan is blocked and the asymmetric septum remains flat (Pogliano *et al.*, 1999, Abanes-De Mello *et al.*, 2002, Eichenberger *et al.*, 2001) (Fig. 1). Under these conditions, GFP-SpoIIQ principally localized to the septal membrane with a small amount of protein present throughout the peripheral forespore membranes (Fig. 1). This pattern of SpoIIQ localization was qualitatively similar to what has been observed previously in cells lacking SpoIIIAH (Blaylock et al., 2004, Rubio & Pogliano, 2004) (Fig. 1). Interestingly, in a subset of sporulating cells lacking both SpoIID and SpoIIP (8%, n>600), GFP-SpoIIQ was completely mislocalized and was uniformly distributed throughout the forespore membranes (Fig. S1).

It has been reported previously that SpoIIIAH is less enriched at the sporulation septum in mutants blocked in septal PG thinning (Blaylock et al., 2004). Accordingly, the partial

mislocalization of GFP-SpoIIQ in cells lacking SpoIID and SpoIIP could be due to reduced SpoIIIAH at the septum. Alternatively, the three [E]-controlled genes (*spoIIIAH, spoIID, spoIIP*) could be responsible for localizing SpoIIQ in the septal membrane. To test this, we examined GFP-SpoIIQ localization during spore formation in a *spoIIP, spoIID, spoIIIAH* triple mutant. Strikingly, in most cells, GFP-SpoIIQ was localized uniformly throughout the forespore membrane, closely mimicking the localization observed in a *sigE* null mutant (Fig. 1). In a subset of the triple mutant cells (16%, n>600), there was a small degree of enrichment of GFP-SpoIIQ in the septal membrane (Fig. S1), suggesting that additional [E]-controlled genes participate in SpoIIQ localization. Nonetheless, our data indicate that SpoIIIAH, SpoIID, and SpoIIP play critical roles in localizing SpoIIQ. The requirement for these proteins in SpoIIQ localization was independently discovered by K. Pogliano and co-workers (Fredlund *et al.*, 2013).

SpoIIQ localizes in the wake of SpoIID and SpoIIP during the morphological process of engulfment

SpoIID and SpoIIP have been reported previously to localize at the leading edge of the engulfing septal membrane where they degrade the cell wall peptidoglycan and are thought to pull or guide the mother-cell membranes around the forespore (Abanes-De Mello et al., 2002, Chastanet & Losick, 2007, Aung *et al.*, 2007, Morlot et al., 2010, Gutierrez et al., 2010). By contrast, during engulfment, SpoIIQ localizes more uniformly along the septal membrane on the forespore side (Rubio & Pogliano, 2004) (Fig. 1). Accordingly, it seemed unlikely that SpoIID and SpoIIP anchor SpoIIQ through direct protein-protein interactions. However, to compare their localization in the same cells, we performed double-labeling experiments using a mCherry-SpoIIQ fusion and either GFP-SpoIID or GFP-SpoIIP. Strains harboring the two fluorescent fusions as the sole source of these proteins sporulated at levels similar to wild-type and had no significant defects in engulfment (data not shown). At hour 2 of sporulation when the mother-cell membranes were migrating around the forespore, mCherry-SpoIIQ localized along the septal membrane as a curved band, or in sporulating cells that had just initiated the engulfment process, as a focus at the center of the forespore septal membrane (Fig. 2A and SD1). By contrast, GFP-SpoIIP (and separately GFP-SpoIID) localized weakly along the septal membrane, presumably on the mother-cell side, and as distinct and bright foci at the leading edge of the engulfing septal membranes (Fig. 2 and S2B). In merged images, mCherry-SpoIIQ appeared to localize behind the foci of GFP-SpoIIP suggesting that SpoIIQ localizes in the wake of the cell wall degrading activities of SpoIID and SpoIIP. Similar results were obtained using a GFP-SpoIID fusion (Fig. S2A and S2B). These data favor the idea that SpoIID and SpoIIP do not physically interact with SpoIIQ and therefore only indirectly participate in localizing it. However, since some GFP-SpoIIP and GFP-SpoIID were present in the septal membrane behind the leading edge of the engulfing membrane, these experiments cannot rule out a direct protein-protein interaction in the intermembrane space.

SpoIID and SpoIIP catalytic activities are required for SpoIIQ localization

The double-labeling experiment described above suggests that SpoIIQ localizes to the septal membrane in places where SpoIID and SpoIIP have thinned the septal PG. If this is true, then catalytic mutants of SpoIID and SpoIIP should be defective in localizing SpoIIQ. To test this, we generated a strain in which the only copy of SpoIID was the catalytic mutant SpoIID[E88A] and the only copy of SpoIIP was SpoIIP[E359A] (Morlot et al., 2010, Shida *et al.*, 2001). Sporulating cells harboring these two catalytic point mutants phenocopied a *spoIID, spoIIP* double mutant: engulfment was completely blocked and GFP-SpoIIQ localized to the septal membrane with a small amount of protein present throughout the peripheral forespore membranes (data not shown).

Next, we introduced the *spoIIIAH* deletion into the strain harboring the catalytic point mutants and monitored GFP-SpoIIQ localization during sporulation. In most cells, GFP-SpoIIQ localized uniformly throughout the forespore membranes, with no obvious enrichment at the forespore septal membrane, mimicking the localization observed in *spoIID, spoIIP, spoIIIAH* triple mutant and the *sigE* null (Fig. 3A and Fig. 1). Importantly, both catalytic mutants were expressed at levels comparable to their wild-type counterparts (Fig. 3B). Moreover, YFP fusions to both mutants localized to the asymmetric septum in match-controlled genetic backgrounds (Fig. 3C). Collectively, these data suggest that the catalytic activities of SpoIID and SpoIIP are required for the proper localization of SpoIIQ. Although we cannot exclude the possibility that the mutated residues in SpoIID and SpoIIP are required for interaction with SpoIIQ, in light of the double-labeling experiments described above, we favor the idea that the role of SpoIID and SpoIIP in SpoIIQ localization is in degrading the cell wall peptidoglycan that resides between the double membrane separating the mother cell and forespore.

SpoIID and SpoIIP activities allow another σ^E-controlled protein to anchor SpoIIQ

How might the degradation of septal peptidoglycan by SpoIID and SpoIIP promote specific localization of SpoIIQ to the septal membrane? Since SpoIIQ contains a degenerate LytM domain that in some proteins confers PG binding activity (Uehara *et al.*, 2009, Sabala *et al.*, 2012), one possibility that we considered is that thinning of the septal PG by SpoIID and SpoIIP generates modifications to the cell wall to which SpoIIQ binds. However, extensive attempts by us, and others (Meisner & Moran, 2011), to uncover an interaction between SpoIIQ and PG (with or without treatment by SpoIID and SpoIIP) were unsuccessful (Fig. S3). Accordingly, we favor a second model in which an additional mother-cell anchoring protein interacts with SpoIIQ across the septal membrane. However, unlike SpoIIIAH, which can localize SpoIIQ in the presence of septal PG (Fig. 1; *spoIIP spoIID*), this additional protein requires thinning of the PG to interact with SpoIIQ across the intermembrane space.

If such a protein exists, it is either produced under the control of ^{E} or it is synthesized prior to ^{E} activation. To distinguish between these two possibilities, we generated a *sigE* null mutant strain that expresses SpoIID, SpoIIP and their partner protein SpoIIM under IPTG-inducible control (Fig. 4A). SpoIIM is a polytopic membrane protein produced under ^{E} control that is thought to form a complex with SpoIID and SpoIIP and help localize this cell wall degrading machine to the septal membrane (Eichenberger et al., 2001, Chastanet & Losick, 2007, Aung et al., 2007). We reasoned that if the second anchoring protein is produced independently of ^{E}, then septal thinning by SpoIID and SpoIIP should be sufficient to localize GFP-SpoIIQ to the forespore membrane. If, on the other hand, this second protein is under ^{E} control, then GFP-SpoIIQ should fail to localize even if the septal PG had been degraded (Fig. 4A).

We induced sporulation in the *sigE* null mutant engineered to produce SpoIID, SpoIIP, and SpoIIM and analyzed GFP-SpoIIQ localization in the presence and absence of IPTG (Fig. 4A). In the absence of IPTG, the septal membranes remained flat in the vast majority of cells and, as expected, GFP-SpoIIQ localized uniformly in the forespore membranes (Fig. 4C). In a parallel culture, IPTG (1mM final) was added at hour 1.5 of sporulation at a time when most sporulating cells were undergoing asymmetric division. Thirty minutes later, when both SpoIID and SpoIIP were produced to levels similar to wild-type, as assayed by immunoblot (Fig. 4B), the polar septa began to curve (Fig. 4C, yellow carets) indicative of active thinning of the septal PG and the onset of engulfment. Importantly, in the sporulating cells that contained curved septa, GFP-SpoIIQ localized uniformly throughout the forespore membranes, with no specific localization at the septum (Fig. 4C, white carets). Collectively,

these data support the idea that SpoIIIAH and a second protein under ^{E} control interact with and localize SpoIIQ in the septal membrane.

Mutations in the LytM groove of SpoIIQ define a SpoIIIAH-independent interaction surface

We reasoned that if a second protein interacts with SpoIIQ that it would bind SpoIIQ on a different surface from the one to which SpoIIIAH binds. Accordingly, we examined the recently published crystal structures of the SpoIIQ-SpoIIIAH complex (Meisner et al., 2012, Levdikov et al., 2012). One region of the SpoIIQ protein that is not occluded by SpoIIIAH includes the groove in the degenerate LytM domain (Fig. 5A). In active PG hydrolases that contain a LytM domain, the residues within the groove are required to interact with peptidoglycan (Sabala et al., 2012). Interestingly, these enzymes are produced in a latent form that is held inactive by their amino-terminal segments, which occlude the catalytic residues within the LytM groove (Odintsov *et al.*, 2004). Thus, this groove can also engage in protein-protein interactions. Accordingly, we identified surface exposed residues in the LytM groove and mutated them individually to Alanine in the context of the GFP-SpoIIQ fusion (Fig. 5A and Fig. S4). We then tested these mutants for their ability to localize in a wild-type background and in cells lacking SpoIIIAH. If these mutants disrupt the interaction between SpoIIQ and a second ^{E}-controlled protein then the mutant proteins should still localize to the septal membrane on their own but fail to localize in the absence of SpoIIIAH. The localization phenotypes of the various mutants could be broadly categorized into four classes: 1) mutants that had no impact on GFP-SpoIIQ localization (S164A, V166A and E156A); 2) mutants that were uniformly localized in the forespore membranes at early stages of sporulation but became cytoplasmic at later stages, presumably due to degradation (H202A, E206A and R208A); 3) mutants that were almost uniformly localized in the forespore membranes throughout engulfment (D123A and Q168A), and 4) mutants that produced a mixture of class 1 and 2 (S119A) (Fig. 5 and S4). Class 3 mutants had the expected phenotype and we present the data for one of these (SpoIIQ^{Q168A}) in Figure 5.

In the presence of wild-type SpoIIIAH, the GFP-SpoIIQ^{Q168A} mutant (and the other class 3 mutant) retained its specific localization at the forespore septum (Fig. 5B and data not shown). However, in cells lacking SpoIIIAH, the GFP-SpoIIQ^{Q168A} mutant (and GFP-SpoIIQ^{D123A}) was almost completely mislocalized (Fig. 5B and S4). For comparison, we examined conservative and non-conservative point mutants (S164A, V166A and E156A) that also reside in the LytM groove (Fig 5A and Fig. S4). These mutants had no impact on localization of SpoIIQ in the presence or absence of SpoIIIAH. Altogether, these results identify residues within the LytM groove of SpoIIQ that are important for its localization and likely make up an interaction surface for a second mother-cell anchoring protein.

The second SpoIIQ anchoring protein helps maintain forespore integrity and σ^G activity

Our data are consistent with a model in which SpoIID and SpoIIP degrade septal PG to allow SpoIIQ to interact with a second ^{E}-controlled protein. To investigate whether the second anchoring protein also functions in the secretion apparatus that maintains forespore physiology, we took advantage of the class 3 mutant (SpoIIQ^{Q168A}) that fails to localize in the absence of SpoIIIAH. Specifically, we examined the affect of the SpoIIQ^{Q168A} mutant on the production of heat-resistant spores and on its ability to activate the forespore transcription factor ^{G} that requires an intact transenvelope complex (Doan et al., 2009, Camp & Losick, 2009). ^{G} activity was assessed in single cells by fluorescence microscopy using a ^{G}-responsive promoter (P$_{sspB}$) fused to *cfp* (Doan et al., 2009).

Alone, the SpoIIQ^{Q168A} mutant had no discernable impact on the production of heat-resistant spores (Fig. 6A; Table S1). Moreover, it did not affect the activation of ^{G}. At 3.5 hours after the onset of sporulation, 69% of the SpoIIQ^{Q168A} sporulating cells (n>600)

contained strong fluorescent signal in the forespore (Fig. 6B, yellow caret). A similar percentage of sporulating cells (75%) had σ^G activity in wild-type (Fig. 6B). Since the SpoIIQ^{Q168A} mutant resulted in SpoIIQ mislocalization only in the absence of SpoIIIAH, we reasoned that its effect on sporulation would be more pronounced in *a spoIIIAH* null background. To this end, we examined the production of heat-resistant spores and σ^G activity of a *spoIIIAH, spoIIQ*^{Q168A} double mutant. We observed a 60-fold reduction in the number of heat-resistant spores in the *spoIIIAH, spoIIQ*^{Q168A} mutant compared to the *spoIIIAH* mutant alone (Fig. 6A; Table S1). Importantly, the double mutant produced almost the same number of heat-resistant spores as cells lacking SpoIIQ (Fig. 6A; Table S1). This synthetic sporulation defect was specific to the *spoIIIAH* null, since *spoIIQ*^{Q168A} had no impact on the sporulation efficiency of a strain lacking a sporulation protein (SpoIIB) that is not involved in the secretion apparatus (Perez *et al.*, 2000) (Fig. 6A). Importantly, we also observed a reduction in the number of cells with σ^G activity in the *spoIIIAH, spoIIQ*^{Q168A} mutant relative to the *spoIIIAH* null (Fig 6B and S6). At hour 3.5 of sporulation, only 5% (n>600) of the *spoIIIAH, spoIIQ*^{Q168A} mutant cells contained strong σ^G-dependent CFP fluorescence in the forespore, compared to 18% (n>600) in the *spoIIIAH* null mutant (Fig. 6B, yellow carets). Finally, as reported previously (Doan et al., 2009), we observed that the forespores containing little or no CFP signal (i.e. reduced or no σ^G activity) were also smaller in appearance (Fig 6 and S6, white carets). This reduced size is indicative of a collapsed forespore (Doan et al., 2009), and was particularly pronounced in cells lacking SpoIIQ and in the *spoIIIAH, spoIIQ*^{Q168A} double mutant. Altogether, these results suggest that the second anchoring protein also functions in the secretion apparatus and can partially compensate for the absence of SpoIIIAH (see Discussion).

Septal peptidoglycan thinning is required for σ^G activity

We have shown that in sporulating cells lacking SpoIID and SpoIIP, GFP-SpoIIQ localizes to the septal membrane in a SpoIIIAH-dependent manner (Fig. 1). This suggests that SpoIIIAH and SpoIIQ are able to interact across the polar septum in the absence of PG thinning. We therefore wondered whether this interaction is sufficient to assemble an active secretion complex. We have previously shown that in cells lacking SpoIID, 11% of the sporulating cells maintain σ^G-dependent gene expression and therefore have active secretion complexes (Doan et al., 2009) (Fig. 7). However, sporulating cells lacking SpoIID have septal bulges that are thought to contain partially degraded PG raising the possibility that thinning of septal PG may be important for proper assembly and/or activity of the complex. To more directly test this idea, we analyzed σ^G activity in sporulating cells lacking *both* SpoIID and SpoIIP. In the absence of both cell wall hydrolases, the septal PG remains largely intact.

As described above, σ^G activity was assessed in single cells by fluorescence microscopy using a σ^G-responsive promoter (P$_{sspE}$) fused to *cfp* (Doan et al., 2009). At hour 3 of sporulation, 44% of wild-type cells contained strong σ^G-dependent CFP fluorescence (Fig. 7A and S7). Similar to and in extension of what was reported previously, at the same timepoint, 14% and 16% of the sporulating cells lacking SpoIID or SpoIIP, respectively, contained strong σ^G-dependent forespore fluorescence. Furthermore, those forespores with σ^G activity also contained septal bulges (Fig. 7A and S7). Consistent with the idea that septal PG degradation is necessary to assemble active secretion complexes, in the *spoIID, spoIIP* double mutant, septal bulging was rarely observed and only a small fraction of the sporulating cells (2%) contained σ^G activity. The few cells that had σ^G-dependent fluorescence appeared to exhibit some bulging and/or to have deformed septal membranes (data not shown). Moreover, the fluorescence signal observed in these cells was weaker than in wild-type and in the single mutants, indicating a lower level of σ^G activity.

If, as our data suggest, the secretion complex is inactive in the absence of PG thinning, then the forespores should collapse in the *spoIID, spoIIP* double mutant. Although it is unclear what a collapsed forespore would look like in the absence of engulfment, we note that at hour 3 of sporulation (when forespores have begun to collapse in cells lacking SpoIIQ or SpoIIIA proteins), the septal membranes in the *spoIID, spoIIP* mutant bulge inward toward the forespore cell pole (Fig. 7B). Cells lacking σ[E] (and do not produce SpoIID, SpoIIP and the SpoIIIA proteins) have a similar forespore phenotype (Fig. 7B). Interestingly, some of the early electron micrographs of mutants that lack σ[E] activity reveal a phenotype similar to the one described here (Illing & Errington, 1991). We hypothesize that this is indeed "forespore collapse" and is a manifestation of the loss of metabolic potential. Altogether, these data suggest that SpoIID- and SpoIIP-mediated septal thinning is critical to assemble an active secretion complex.

Discussion

We have shown that the localization of SpoIIQ to the septal membrane on the forespore side depends on the mother cell protein SpoIIIAH and the thinning of the septal PG during engulfment. Our data further suggest that PG degradation allows an additional mother-cell protein to interact with and participate in anchoring SpoIIQ in the apposing membrane. Finally, our results support a model in which PG degradation is critical to assemble an active transenvelope secretion complex and that this second SpoIIQ-interacting protein functions with SpoIIIAH in its assembly (Figure 8). Type III secretion systems found in Gram-negative bacteria typically possess 20-25 proteins, of which 9-10 are highly conserved core components (Abby & Rocha, 2012, Buttner, 2012, Diepold *et al.*, 2010, Ghosh, 2004). Type II and Type IV secretion systems are generally composed of 12-16 proteins (Filloux, 2004, Johnson *et al.*, 2006, Korotkov *et al.*, 2012, McLaughlin *et al.*, 2012, Douzi *et al.*, 2012, Christie *et al.*, 2005, Fronzes *et al.*, 2009, Chandran, 2013), of which a subset are highly conserved and likely to serve as the core machinery. Our findings raise the possibility that there are many unidentified proteins that similarly participate in the assembly and activity of this Gram-positive specialized secretion machine. Finally, our data highlight similar challenges and solutions that these macromolecular complexes must attend to in traversing the cell wall.

Although our data cannot formally rule out the possibility that the second anchor for SpoIIQ is modified PG, we favor a model in which this anchor is a mother-cell-produced protein that interacts directly with SpoIIQ. This is based on our inability to detect an interaction between the extracellular domain of SpoIIQ and PG in a collection of different assays using purified cell wall from vegetative and sporulating *B. subtilis* cells, in addition to *E. coli* sacculi. Furthermore, our data showing that induction of SpoIID, SpoIIP and SpoIIM in cells lacking σ[E] argue that if there is a modification to septal PG that SpoIIQ recognizes, it is not generated by SpoIID and SpoIIP but yet another cell wall modifying enzyme activated by σ[E]. Alternatively, cell wall synthesis under σ[E] control could generate new PG that SpoIIQ binds. We do not favor this model because GFP-SpoIIQ remained properly localized in strains lacking SpoIIIAH and the cell wall synthesis machinery (SpoVD and SpoVE) (Daniel *et al.*, 1994, Henriques *et al.*, 1992, Fay *et al.*, 2010, Meyer *et al.*, 2010) under σ[E] control (data not shown). Accordingly, we favor a simpler model that the second anchor for SpoIIQ is a protein synthesized in the mother, and that this protein unlike SpoIIIAH, requires the removal of septal PG to stably interact with SpoIIQ (Fig. 8). Importantly, this unidentified protein is not encoded in the *spoIIIA* operon, as GFP-SpoIIQ localization in a strain deleted for the entire operon was indistinguishable from the *spoIIIAH* null (Fig. 1).

As described in the Introduction, experiments from several groups suggest that SpoIIQ and SpoIIIAH form the basement layer (or scaffold) of the transenvelope secretion apparatus

(Camp & Losick, 2008, Camp & Losick, 2009, Meisner et al., 2008, Doan et al., 2009). One observation that appeared in conflict with this model was the different requirement for these proteins in spore formation and in forespore gene expression under σ^G control. Cells lacking SpoIIQ or any of the SpoIIIA proteins, except SpoIIIAH, are severely impaired in sporulation (reduced 1000-fold) (Doan et al., 2009, Camp & Losick, 2008). By contrast, cells lacking SpoIIIAH have a mild defect in spore-formation (reduced 10-20-fold). Similarly, 10-20-fold more forespores exhibit σ^G-dependent gene expression in the SpoIIIAH mutant compared to mutants in SpoIIQ or the other SpoIIIA proteins (Doan et al., 2009) (Fig. 6B). The work presented here provides an explanation for this apparent paradox. Our data suggest that SpoIIIAH and a second mother-cell anchoring protein function together in building the transenvelope machine. Specifically, we imagine that in the absence of SpoIIIAH this second protein interacts with SpoIIQ across the double membrane septum and helps assemble the secretion complex (Fig. 8). The data presented here and previous localization studies in strains lacking SpoIIIAH (Doan et al., 2009) suggest that in the absence of SpoIIIAH fewer complexes form. However, those that do, presumably mediated by the second SpoIIQ anchor, appear to be sufficient to maintain forespore integrity and support σ^G activity and spore formation. A central challenge for the future is identifying this second mother-cell anchoring protein.

A role for the LytM groove in protein-protein interaction

The LytM domain was originally characterized on the lystostaphin D,D-endopeptidase from *Staphyloccocus aureus* and is a Zn^{2+} metallopeptidase (Odintsov et al., 2004). A large number of proteins contain this domain or a degenerate version that lacks the conserved residues involved in Zn^{2+} coordination. In the case of SpoIIQ, some of these residues are present but are not required for any of the known activities of this protein (Camp & Losick, 2009). The work presented here suggests that these domains could also serve as a protein-protein interaction surface. We suspect that many of the degenerate LytM domains could function in this capacity.

A role for SpoIID and SpoIIP in facilitating intermembrane protein-protein interaction and assembly of an active transenvelope complex

In addition to their role in promoting engulfment, this work and previous studies by the groups of Pogliano and Moran (Blaylock et al., 2004) reveal that SpoIID and SpoIIP play an important role in facilitating protein-protein interaction across the septum and in the assembly of an active secretion apparatus. Consistent with the idea that the SpoIIQ-SpoIIIAH interaction is weaker in the presence of septal PG, the septal localization of both proteins is reduced in cells lacking SpoIID and SpoIIP (Blaylock et al., 2004) (Fig. 1). Moreover, our data suggest that the second SpoIIQ anchoring protein is even more sensitive to the presence of the cell wall at the septum. One possibility is that this protein simply interacts more weakly with SpoIIQ. Alternatively, the extracytoplasmic domain of this protein could be shorter than that of SpoIIIAH and simply cannot "reach" across the space between the double-membrane prior to PG degradation (Fig. 8). Recent cryoelectron tomography of sporulating *B. subtilis* cells indicates that, prior to degradation of the septal PG, the distance between the two membranes is approximately 40 nm and after septal PG thinning, the intermembrane space is reduced to 20 nm (Tocheva *et al.*, 2013). Thus, the proximity of the apposing membranes after PG degradation might be sufficient to allow the second mother cell protein to interact with SpoIIQ (Fig. 8). A final possibility is that the extracellular domain of this second protein might not fit through the pores in the cell wall that are thought to have a radius of ~2 nm (Demchick & Koch, 1996) and thus the PG would serve as a true barrier to their interaction. These scenarios are not mutually exclusive and could all contribute to a requirement for cell wall removal in anchoring SpoIIQ.

Finally, we envision two scenarios for how PG degradation might influence the activity of the secretion complex (Fig. 8). In the first, SpoIIQ and SpoIIIAH interact prior to PG degradation but are unable to assemble into a secretion channel (Fig. 8Ai). In the second, these proteins form a channel in the presence of the septal PG but other proteins cannot assemble into the complex or the machinery remains inactive until the PG is degraded (Fig. 8Aii and Bi). Based on the co-crystal structure, the extracytoplasmic domains of SpoIIIAH and SpoIIQ span approximately 5 nm. Yet, the distance between the two membranes, prior to PG degradation, is approximately 40 nm (Tocheva *et al.*, 2013). Accordingly, for these proteins to interact, the regions of SpoIIIAH and SpoIIQ that connect the transmembrane segments to the folded domains, and that were disordered in the crystal structure (Levdikov et al., 2012), would have to adopt an extended conformation. Furthermore, upon PG thinning, these regions would need to accommodate the reduced space between the two membranes (Fig. 8Bi). We suspect that these changes in conformation are important in assembling an active complex and may also be critical to form the SpoIIIAH-SpoIIQ channel. Finally, as mentioned above, even after septal PG degradation the membranes remain ~20 nm apart (Tocheva *et al.*, 2013). This suggests that additional proteins are required to make up the channel that connects the mother cell and forespore. SpoIIIAG and SpoIIIAF share weak similarity to SpoIIIAH and the YscJ/FliF family (Meisner et al., 2008, Doan et al., 2009) and are therefore good candidates to assemble into connected ring-like structures to span the double membrane as has been observed in Type III secretion systems (Marlovits *et al.*, 2004, Schraidt *et al.*, 2010).

A variety of specialized secretion systems that are organized into operons typically encode peptidoglycan-degrading enzymes (Scheurwater & Burrows, 2011). It has been suggested that those that lack these enzymes utilize PG hydrolases associated with general PG metabolism. None of the proteins encoded in the *spoIIIA* operon resembles a peptidoglycan-degrading enzyme. Accordingly, we hypothesize that this sporulation-specific secretion system has evolved to utilize SpoIID and SpoIIP as a means to accommodate the assembly of its components within the PG between the mother cell and forespore.

Materials and Methods

General methods

All *B. subtilis* strains were derived from the prototrophic strain PY79 (Youngman *et al.*, 1983). Sporulation was induced by resuspension at 37°C according to the method of Sterlini-Mandelstam (Harwood & Cutting, 1990) or by exhaustion in supplemented DS medium (Schaeffer *et al.*, 1965). Sporulation efficiency was determined in 24-30 hour cultures as the total number of heat-resistant (80°C for 20 min) colony forming units (CFUs) compared with wild-type heat-resistant CFUs. Induction of *spoIID, spoIIP*, and *spoIIM* during the early stages of sporulation was with 1 mM IPTG. Deletion mutants were generated by isothermal assembly (Gibson, 2011) and direct transformation of assembled DNA products into *B. subtilis*. Tables of strains, plasmids and oligonucleotide primers and descriptions of plasmid construction and isothermal assembly deletion mutants can be found online as supplementary material.

Immunoblot analysis

Whole-cell lysates from sporulating cells (induced by resuspension) were prepared as described previously (Doan et al., 2009). Samples were heated for 10 min at 50°C prior to loading. Equivalent loading was based on OD_{600} at the time of harvest. Proteins were separated by SDS-PAGE on 12.5% polyacrylamide gels, electroblotted onto Immobilon-P membranes (Millipore) and blocked in 5% nonfat milk in phosphate-buffered saline (PBS)-0.5% Tween-20. The blocked membranes were probed with anti-SpoIID (1:10,000)

(Doan & Rudner, 2007), anti-SpoIIP (1:5,000) (Morlot et al., 2010), diluted into 3% BSA in 1X PBS-0.05% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated goat, anti-rabbit IgG (BioRad) and the Western Lightning reagent kit as described by the manufacturer (PerkinElmer).

Fluorescence microscopy

Fluorescence microscopy was performed with an Olympus BX61 microscope as previously described (Doan et al., 2009). Cells were mounted on a 2% agarose pad containing resuspension medium using a gene frame (BioRad). Fluorescent signals were visualized with a phase contrast objective UplanF1 100x and captured with a monochrome CoolSnapHQ digital camera (Photometrics) using Metamorph software version 6.1 (Universal Imaging). The membrane dye TMA-DPH (Molecular Probes) was used at a final concentration of 0.01 mM and exposure times were typically 200 ms. Images were analyzed, adjusted and cropped using Metamorph software.

Quantification of σ^G positive cells

^{G} activity was assessed in single cells at 3 and 3.5 hours after the onset of sporulation by microscopy using fluorescent reporters (P_{sspB}-*cfp* and P_{sspE}-*cfp*) (Doan et al., 2009). For the experiments in Figure 6, a forespore was considered ^{G} positive if it contained forespore fluorescence; it displayed normal forespore membrane morphology and was of normal size. The second and third criteria were included in the analysis to help assess ^{G} activity in forespores with faint forespore fluorescence. Faint forespore fluorescence in a normal-sized forespore with unperturbed membranes was scored as ^{G} positive. Faint forespore fluorescence in a small forespore with aberrant membrane morphology indicates forespore collapse (Doan et al., 2009) and was scored as ^{G} negative. The percentage of ^{G} positive cells was calculated based on the total number of cells that had completed engulfment. For the experiments in Figure 7, the cells examined were defective in engulfment. Accordingly, the percentage of ^{G} positive cells was determined based on the total number of cells that had an asymmetric septum.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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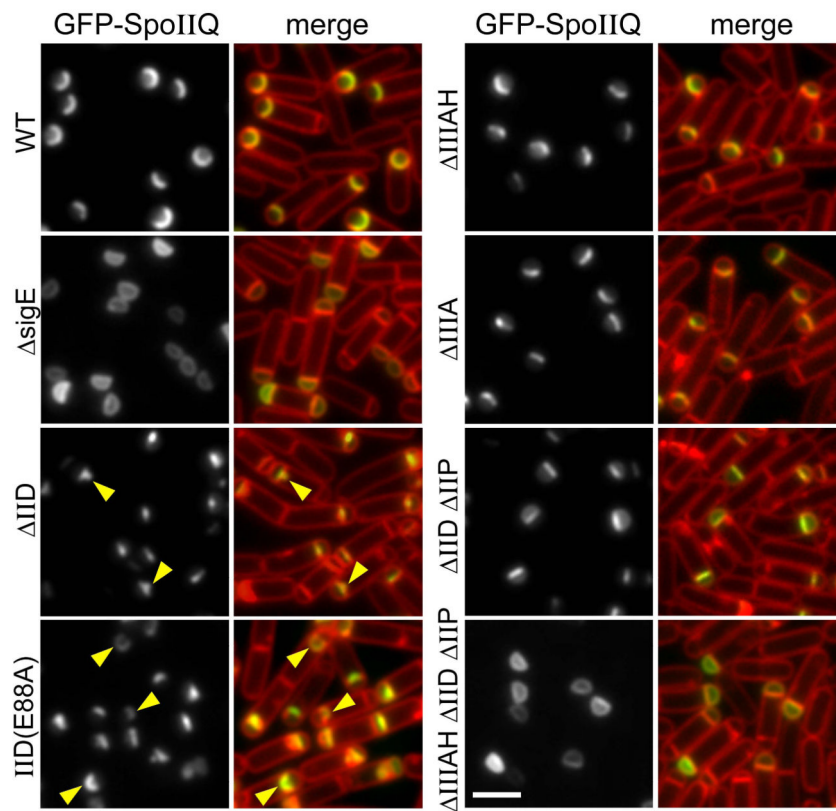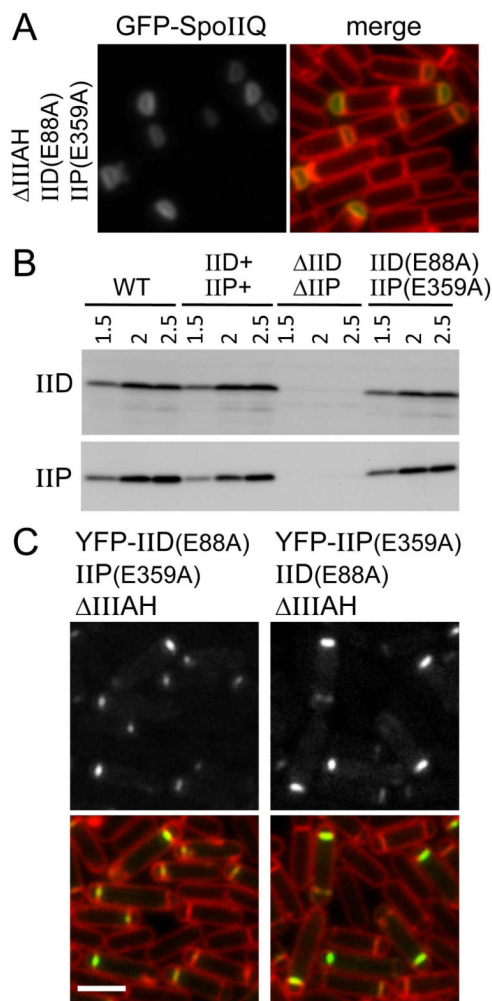
Figure 1. Localization of GFP-SpoIIQ to the forespore septal membrane requires SpoIID, SpoIIP and SpoIIIAH

Representative images of GFP-SpoIIQ localization in sporulating cells at hour 2 of sporulation. Images are from wild-type (BKM1897), *sigE* (BKM1930), *spoIIIAH* (BKM1905), *spoIID* (BKM1898), *spoIID*(E88A) (BKM1902), *spoIIIA* operon (BCR24), a *spoIID spoIIP* double mutant (BKM1928) and a *spoIID spoIIP spoIIIAH* triple mutant (BKM1929). Images are GFP-SpoIIQ (left) and merge of GFP-SpoIIQ with membranes stained with TMA-DPH. Localization of GFP-SpoIIQ to the bulged septal membrane is highlighted (yellow carets). Scale bar is 2 μm.
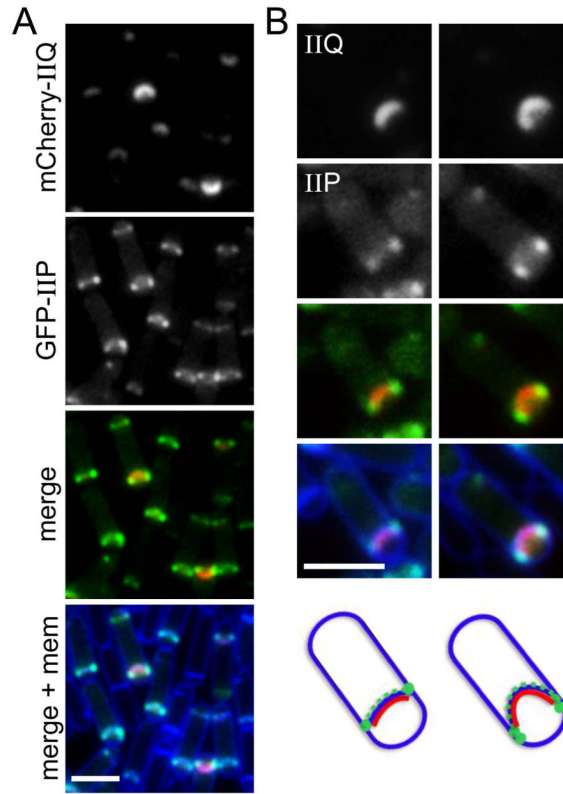
Figure 2. mCherry-SpoIIQ localizes in the wake of GFP-SpoIIP
Double labeling of mCherry-SpoIIQ and GFP-SpoIIP in sporulating cells (BCR188) imaged at hour 2 of sporulation. (A) Representative fields showing mCherry-SpoIIQ, GFP-SpoIIP, a merged image of the two fluorescent fusion proteins and a merged image including the membranes stained with TMA-DPH. (B) Larger images highlighting examples where mCherry-SpoIIQ and GFP-SpoIIP do not completely overlap. Schematic representations of mCherry-SpoIIQ (red), GFP-SpoIIP (green) and membranes (blue) are shown below. Scale bar is 2 μm. We note that mCherry-SpoIIQ localization is slightly delayed compared to GFP-SpoIIQ. We attribute this lag to the maturation times of the mCherry compared to GFP (Cormack *et al.*, 1996, Shaner *et al.*, 2005, Merzlyak *et al.*, 2007). Fig. S2B contains additional representative examples of cells from the same experiment.

Figure 3. The catalytic activities of SpoIID and SpoIIP are required for GFP-SpoIIQ localization to the forespore septal membrane

(A) Representative images of GFP-SpoIIQ localization in the *spoIIIAH,* SpoIID[E88A] SpoIIP[E359A] mutant (strain BCR12) at hour 2 of sporulation. Images are GFP-SpoIIQ (left) and merged with TMA-DPH-stained membranes (right). Scale bar is 2 μm. (B) Immunoblot analysis showing that cells harboring *spoIID*(E88A) and *spoIIP*(E359A) produce wild-type levels of protein. Cell lysates of wild-type (BKM1897), *spoIIIAH spoIID+ spoIIP+* (BCR13), *spoIIIAH spoIID spoIIP* (BKM1929) and *spoIIIAH spoIID*(E88A) *spoIIP*(E359A) (BCR12) were collected at hour 1.5, 2, and 2.5 after the onset of sporulation and analyzed for SpoIID and SpoIIP protein level. (C) SpoIID[E88A] and SpoIIP[E359A] localize to the polar septum. Representative images of YFP-SpoIID[E88A] and YFP-SpoIIP[E359A] at hour 2 of sporulation in a *spoIIIAH spoIIP*(E359A) background (BCR143) and *spoIIIAH spoIID*(E88A) background (BCR121), respectively. Scale bar is 2 μm.
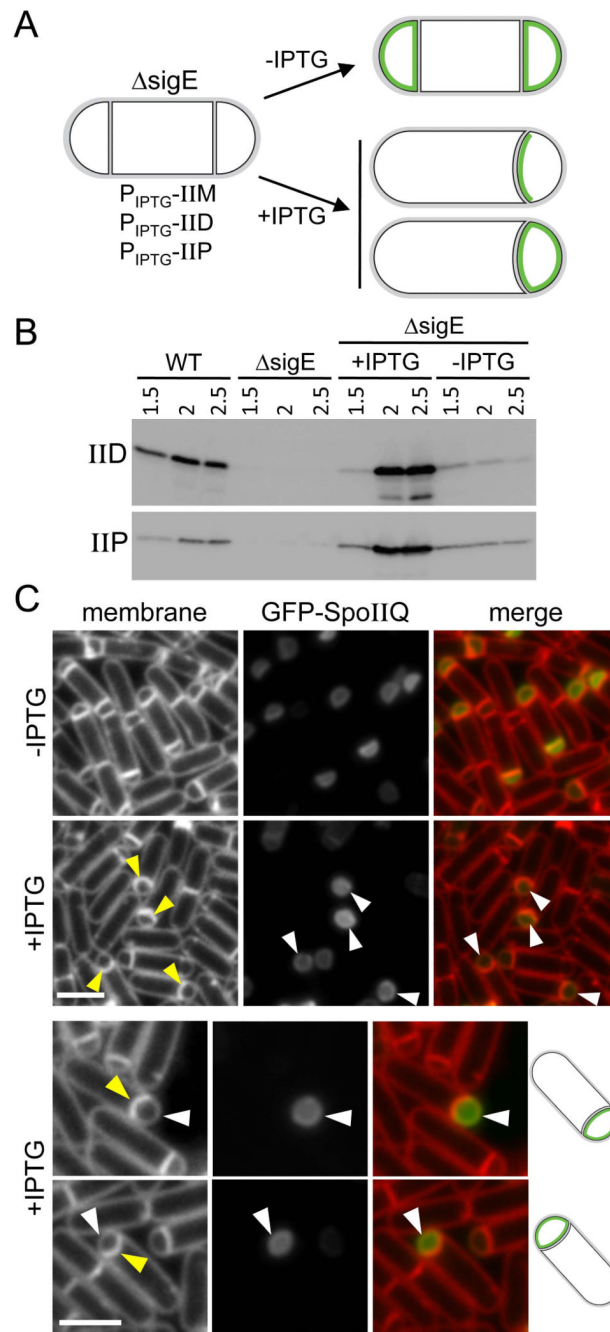
Figure 4. An additional ^E-controlled gene is required to localize GFP-SpoIIQ at the polar septum

(A) Experimental rationale and potential outcomes for GFP-SpoIIQ localization (in green) when SpoIID, SpoIIP and SpoIIM are artificially produced in a *sigE* mutant (strain BCR299). (B) Immunoblot analysis showing that SpoIID and SpoIIP are produced at wild-type levels upon addition of 1 mM IPTG to the culture medium 1.5 hours after the onset of sporulation. Cell lysates of strain BCR299 in the presence and absence of IPTG were analyzed for SpoIID and SpoIIP protein levels. As controls, a wild-type strain (BCR46) and a *sigE* mutant (BKM1930) were included. (C) Representative images of cells (strain BCR299) at hour 2 of sporulation from cultures with and without IPTG addition. Engulfing

septal membranes (yellow carets) and uniform GFP-SpoIIQ localization in the forespore membranes (white caret) are indicated. Images are TMA-DPH-stained membranes (left), GFP-SpoIIQ (middle) and a merge of the two (right). Scale bar is 2 μm. We note that in a subset of sporulating cells in which IPTG was added we observed a loss of GFP-SpoIIQ compartmentalization (see Fig. S5).
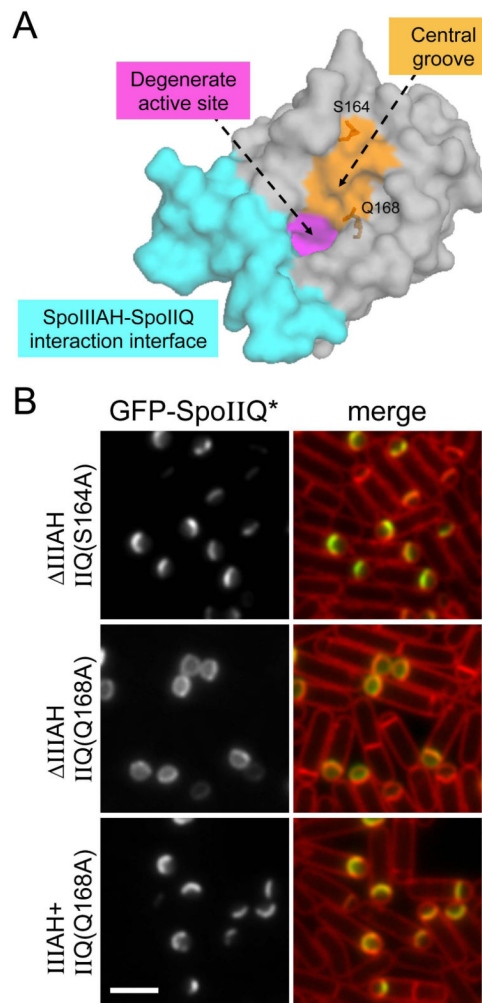
Figure 5. Point mutants in the LytM groove of SpoIIQ impair its localization to the forespore septal membrane

(A) Surface representation of the SpoIIQ crystal structure highlighting the SpoIIIAH-SpoIIQ interaction interface (cyan), the degenerate active site (magenta) and the characteristic LytM groove (orange). Representative residues in the LytM groove (Q168 and S164) that were mutated are shown. (B) Images of GFP-SpoIIQ localization at hour 2 of sporulation. A representative mutant that localized properly (GFP-SpoIIQ[S164A]) (BCR71) and one that failed to localize (GFP-SpoIIQ[Q168A])(BCR80). The loss of septal localization of GFP-SpoIIQ[Q168A] was dependent on the *spoIIIAH* mutant. GFP-SpoIIQ[Q168A] localized properly in an otherwise wild-type background (spoIIIAH[+], BCR87). Scale bar is 2 μm.
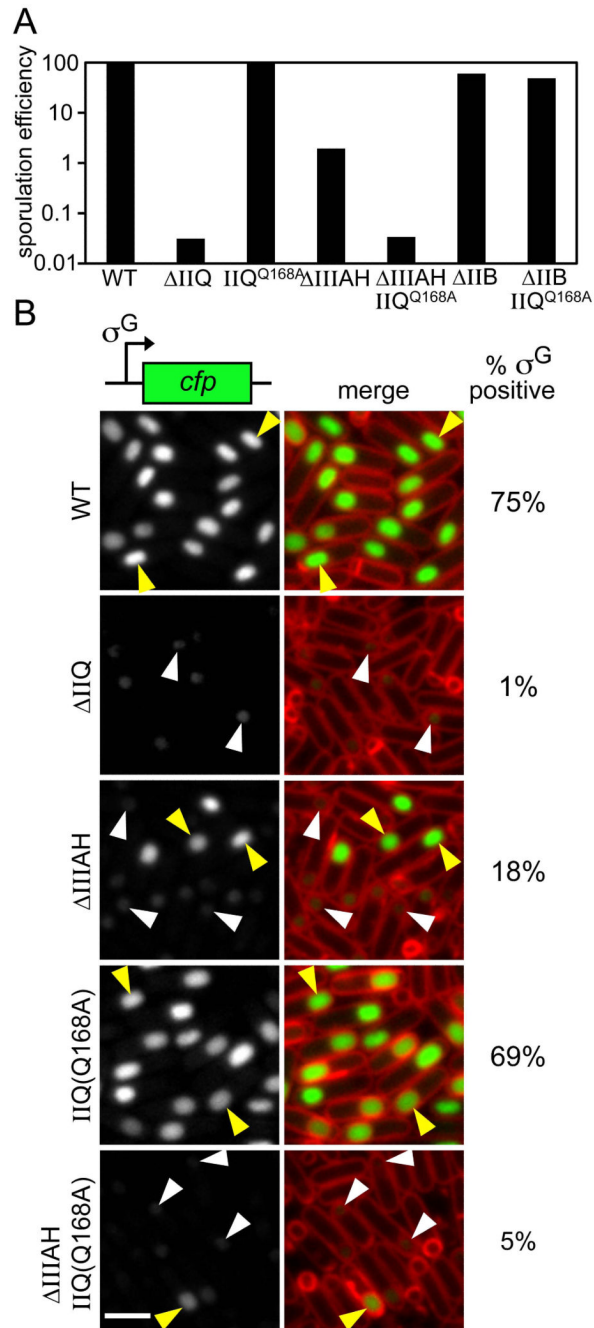
Figure 6. The interaction mediated by the LytM groove in SpoIIQ is required for efficient sporulation and σ^G activity

(A) Bar graph showing sporulation efficiency of wild-type (BCR163), *spoIIQ* (BTD1541), *spoIIQ*(Q168A) (BCR152), *spoIIIAH* (BCR161), *spoIIQ*(Q168A), *spoIIIAH* double mutant (BCR153), *spoIIB* (BCR162) and a *spoIIQ*(Q168A), *spoIIB* double mutant (BCR196). (B) Representative images of strains harboring the σ^G-responsive fluorescent reporter P~sspB~-*cfp* at hour 3.5 of sporulation. From top to bottom: wild-type (BCR186), *spoIIQ* (BCR151), *spoIIIAH* (BCR193), *spoIIQ*(Q168A) (BCR185) and *spoIIQ*(Q168A) *spoIIIAH* double mutant (BCR191). Forespores that display σ^G activity and are scored as positive (yellow carets) and those with weak activity and a collapsed forespore that are

scored as negative (white carets) are highlighted (see Materials and Methods). The percentages of ^{G} positive cells are indicated on the right. We note that ^{G}-dependent gene expression appears higher in our assays than in those reported previously using *lacZ* fusions (Camp & Losick, 2008). We suspect this reflects the difference between single-cell- and population-based assays and the threshold used to score ^{G} in an "on" vs. "off" state. Images are CFP (left) and merge with TMA-DPH-stained membranes (right). Scale bar is 2 μm. Fig. S6 contains larger fields of cells from the same experiment.
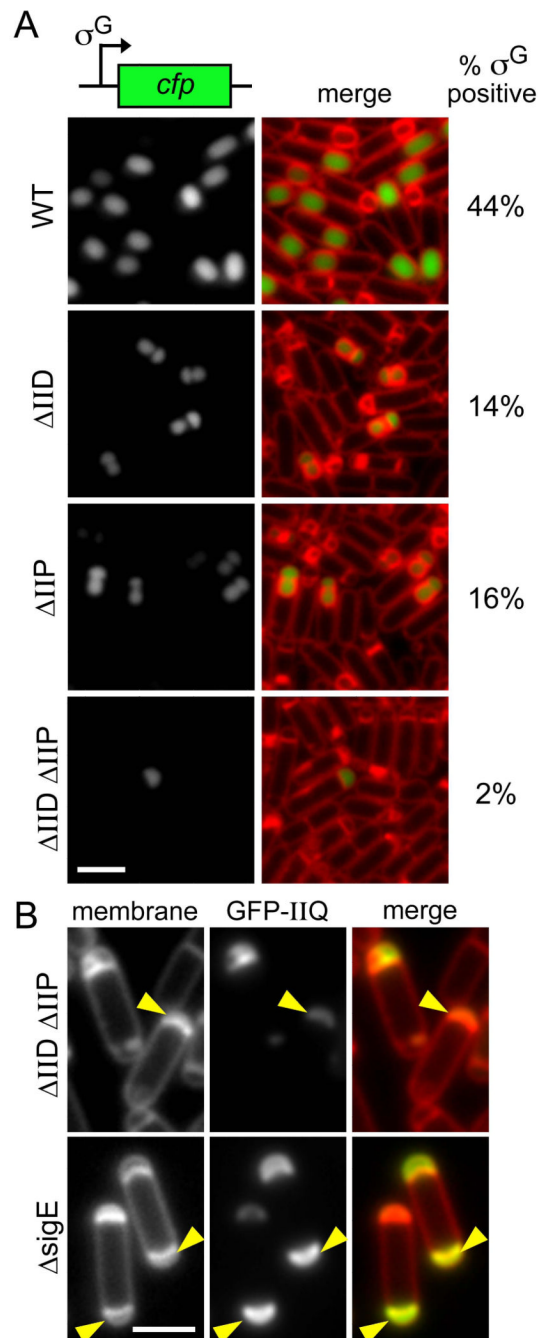
Figure 7. Septal peptidoglycan thinning by SpoIID and SpoIIP is required for σ^G activity
(A) Representative images of strains harboring the σ^G-responsive fluorescent reporter P_sspE-cfp at hour 3 of sporulation. From top to bottom: wild-type (BTD2583), spoIID (BTD2607), spoIIP (BCR527), and a spoIID, spoIIP double mutant (BCR529). The percentages (n>500) of σ^G positive cells are indicated on the right. Images are CFP (left) and merge with TMA-DPH-stained membranes (right). Scale bar is 2 μm. Fig. S7 contains larger fields of cells from the same experiment. (B) Representative images of forespores at hour 3 of sporulation in spoIID, spoIIP (BKM1928) and sigE (BKM1930). Examples of septal membranes that appear to have collapsed are indicated (yellow carets). Images are membranes stained with TMA-DPH (left), GFP-SpoIIQ (middle), and merged (right).
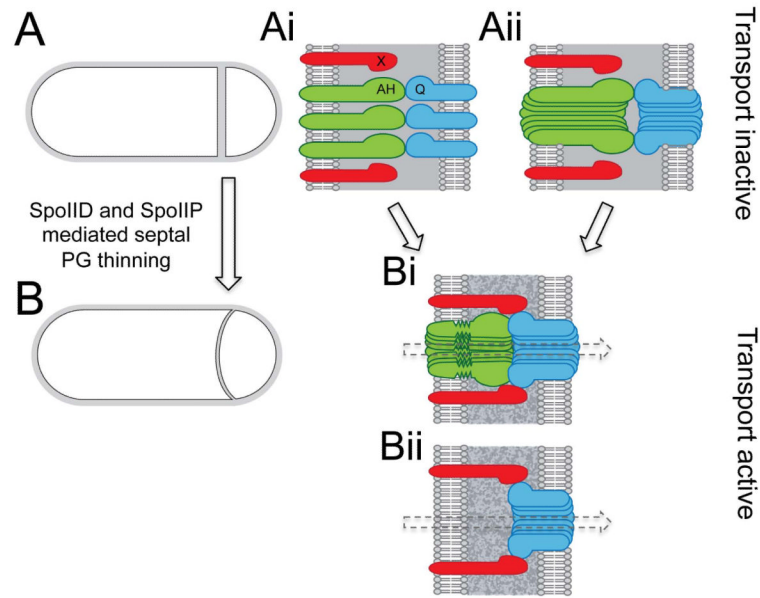
Figure 8. Septal peptidoglycan thinning facilitates the assembly and activity of the transenvelope secretion complex

(A) Schematic representation of a sporulating cell with a flat asymmetric septum that has yet to undergo thinning of the septal PG (grey). **(Ai and Aii)** SpoIIQ (blue) and SpoIIIAH (green) can interact across the septal PG. By contrast, septal PG or the distance between the two membranes (~40 nm) prevents the interaction between SpoIIQ and its other mother-cell partner protein (X, in red). In the absence of septal thinning, the transenvelope secretion complex is not properly assembled (Ai) and/or inactive (Aii) resulting in forespore collapse and loss of σ^G activity. **(B)** Schematic representation of a sporulating cell that has undergone septal PG thinning (speckled grey). **(Bi** and **Bii)** Hydrolysis of the septal PG layers decreases the distance between the mother-cell and forespore membranes (~20 nm) and facilitates the interaction between SpoIIQ and its other mother cell partner. Septal PG thinning also results in active transport and σ^G activity. **(Bi)** SpoIIIAH and/or SpoIIQ adopt an altered conformation as a result of the reduced distance between the two membranes. **(Bii)** In the absence of SpoIIIAH, the secretion apparatus retains some activity due to the interaction between SpoIIQ and its other mother cell partner. For simplicity, the other SpoIIIA proteins in the secretion apparatus are not shown.