A channel connecting the mother cell and forespore during bacterial endospore formation

Jeffrey Meisner*†, Xin Wang*†‡, Monica Serrano§, Adriano O. Henriques§, and Charles P. Moran, Jr.*¶

*Department of Microbiology and Immunology, Emory University School of Medicine, Atlanta, GA 30322; and §Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, Apartado 127, 2781-901 Oeiras Codex, Portugal

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At an early stage during *Bacillus subtilis* endospore development the bacterium divides asymmetrically to produce two daughter cells. The smaller cell (forespore) differentiates into the endospore, while the larger cell (mother cell) becomes a terminally differentiated cell that nurtures the developing forespore. During development the mother cell engulfs the forespore to produce a protoplast, surrounded by two bilayer membranes, which separate it from the cytoplasm of the mother cell. The activation of σ^G, which drives late gene expression in the forespore, follows forespore engulfment and requires expression of the *spoIIIA* locus in the mother cell. One of the *spoIIIA*-encoded proteins SpoIIIAH is targeted specifically to the membrane surrounding the forespore, through an interaction of its C-terminal extracellular domain with the C-terminal extracellular domain of the forespore membrane protein SpoIIQ. We identified a homologous relationship between the C-terminal domain of SpoIIIAH and the YscJ/FliF protein family, members of which form multimeric rings involved in type III secretion systems and flagella. If SpoIIIAH forms a similar ring structure, it may also form a channel between the mother cell and forespore membranes. To test this hypothesis we developed a compartmentalized biotinylation assay, which we used to show that the C-terminal extracellular domain of SpoIIIAH is accessible to enzymatic modification from the forespore cytoplasm. These and other results lead us to suggest that SpoIIIAH forms part of a channel between the forespore and mother cell that is required for the activation of σ^G.

Bacillus subtilis | flagellar protein export apparatus | sporulation | type III secretion system

Endospore formation in the gram-positive bacterium *Bacillus subtilis* involves a series of morphological changes that culminate in the production of a dormant spore (reviewed in refs. 1–3). In response to nutrient depletion, vegetative cells undergo an asymmetric cell division that yields two dissimilar progeny cells, the mother cell and forespore. Remarkably, the mother cell engulfs the forespore and nurtures it to maturity. Ultimately, the mother cell lyses, releasing the mature spore. The differentiation of these two cell types is governed by two parallel programs of gene expression controlled by a cascade of alternative RNA polymerase sigma (σ) factors. To synchronize the differentiation of the mother cell and forespore, the two programs of gene expression are coordinated through a series of intercellular signaling pathways. Following asymmetric cell division, σ^F is activated in the forespore. σ^F then signals the activation of σ^E in the mother cell. Together, σ^F and σ^E control expression of the genes responsible for forespore engulfment. After the completion of forespore engulfment, σ^G is activated in the forespore and signals the activation of σ^K in the mother cell. σ^G and σ^K control expression of the genes necessary for forespore maturation. While the intercellular signaling pathways that trigger the activation of σ^E and σ^K in the mother cell are well understood, the mechanisms that trigger the activation of σ^G in the forespore are not known.

In addition to the completion of forespore engulfment, the eight-gene *spoIIIA* locus (*spoIIIAA–AH*) is also necessary for the activation of σ^G. The *spoIIIA* locus is expressed under the control of σ^E in the mother cell. With the exception of *spoIIIAA*, each of the *spoIIIA* genes (*spoIIIAB–AH*) encodes a predicted integral membrane protein. SpoIIIAH contains an N-terminal transmembrane segment (TMS) and a C-terminal extracellular domain. Although SpoIIIAH is inserted randomly into the mother cell membrane, it is targeted to the sporulation septum, separating the mother cell and forespore, through an interaction of its C-terminal domain with the forespore integral membrane protein SpoIIQ (4, 5). SpoIIIAH and SpoIIQ colocalize along the engulfing membrane and form discrete foci surrounding the forespore. However, it is unclear why SpoIIIAH is necessary for σ^G activity in the forespore.

In this study, we identified a homologous relationship between SpoIIIAH and the proteins that form the export channel and structural scaffold of type III secretion and flagellar export systems. We hypothesized that if SpoIIIAH also forms a channel, then its C-terminal extracellular domain may be accessible to modification by an enzyme produced in the forespore. To test this hypothesis, we developed a compartmentalized biotinylation assay. Using this assay, we demonstrated that the C-terminal domain of SpoIIIAH is accessible to biotin ligase produced in the forespore. These and other results suggest that SpoIIIAH and SpoIIQ may form a channel between the mother cell and forespore.

Results

C-Terminal Extracellular Domain of SpoIIIAH Is Homologous to the YscJ/FliF Protein Family. Although no significant relationship to a protein of known function could be established using conventional search methods, the HHpred server (6, 7) identified a homologous relationship between the C-terminal extracellular domain of SpoIIIAH and the YscJ/FliF protein family (PF01514) (Fig. 1*A*). The sequence similarity covers a 78-amino-acid stretch with 23% identity, corresponding to the C-terminal domain of the YscJ proteins and the central domain of the FliF proteins. The members of the YscJ family are involved in type III secretion of virulence factors in gram-negative bacteria (8). The structure of a representative member, the enteropathogenic *Escherichia coli* EscJ protein, indicated that these proteins probably form ring structures with a central channel that is 75Å in diameter (9). The dimensions of the 24-subunit EscJ ring were similar to those estimated by electron microscopy (10). The YscJ ring is thought

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†J.M. and X.W. contributed equally to this work.

‡Present address: Meningitis Laboratory, Meningitis and Vaccine Preventable Diseases Branch, CDC/NCIRD/DBD, Atlanta, GA 30333.

¶To whom correspondence should be addressed. E-mail: moran@microbio.emory.edu.

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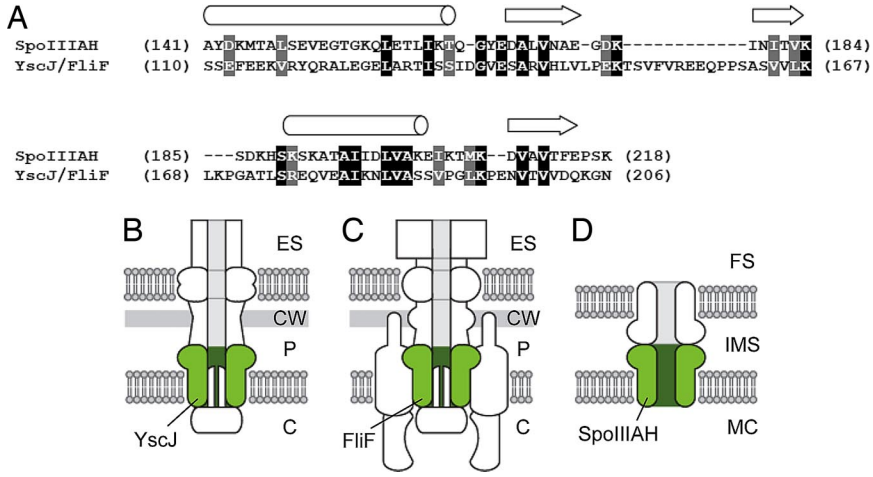

Fig. 1. Homology between SpoIIIAH and the YscJ/FliF protein family. HHpred alignment of SpoIIIAH and the YscJ/FliF family. (*A*) Identical (black boxes) and similar (gray boxes) amino acids are highlighted. Predicted secondary structure is indicated above the alignment. Cartoon representations of the type III secretion system (*B*), flagellar basal body (*C*), and SpoIIIAH channel model (*D*). YscJ, FliF, and SpoIIIAH are labeled and colored green. The extracellular space (ES), cell wall (CW), periplasm (P), and cytoplasm (C) of gram-negative bacteria, and mother cell (MC), intermembrane space (IMS), and forespore (FS) of bacterial endospores are indicated.

to form the inner membrane channel of the type III secretion system (Fig. 1*B*) (11). The FliF protein forms the bacterial flagellar protein export channel and the structural scaffold for flagellar assembly (Fig. 1*C*) (12). Therefore, we hypothesized that the C-terminal extracellular domain of SpoIIIAH forms a large, hollow ring with the N-terminal TMS anchored in the mother cell membrane (Fig. 1*D*). The SpoIIIAH ring may be part of a channel between the mother cell and forespore. The similarity of SpoIIIAH with the YscJ/FliF protein family and the hypothesis that SpoIIIAH forms a channel were independently proposed by Camp and Losick (13).

Compartmentalized Biotinylation Assay. If SpoIIIAH forms part of a channel between the mother cell and forespore, then the C-terminal extracellular domain may be accessible to modification by an enzyme produced in the forespore. To test this prediction, we developed a compartmentalized biotinylation assay, involving the biotinylation of target proteins by the *E. coli* biotin ligase, BirA, produced in either the mother cell or forespore (Fig. 2 *A* and *B*). BirA transfers biotin to a specific lysine in biotin-accepting proteins (14). Biotinylated proteins can then be detected by Western blot with a streptavidin-horseradish peroxidase (HRP) conjugate (15). We tagged proteins with a 14-amino-acid biotin acceptor peptide (BAP), defined as the minimal substrate required for *E. coli* BirA-catalyzed biotinylation (16). Then we tested whether the fusion proteins were biotinylated in *B. subtilis* strains producing the *E. coli* BirA in either the mother cell or forespore, under the control of the *spoIID* and *spoIIQ* promoters, respectively (17, 18). Immunoblot analysis of BirA indicated that similar amounts of BirA accumulated after the onset of sporulation in both the mother cell- and forespore-producing strains [supporting information (SI) Fig. S1].

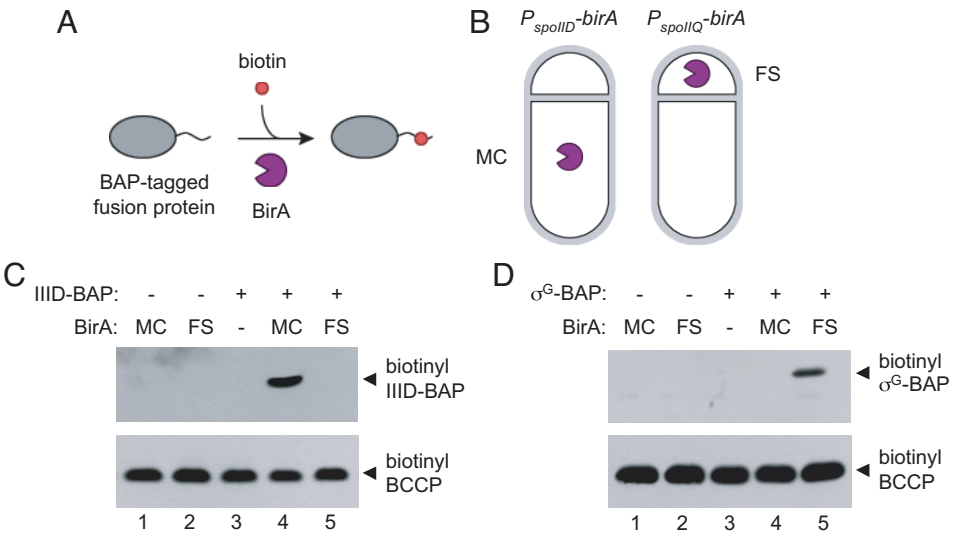

Fig. 2. Compartmentalized biotinylation assay. Cartoon representations of (*A*) BirA-catalyzed transfer of biotin to biotin acceptor peptide (BAP)-tagged fusion proteins, and (*B*) strains producing *E.coli* BirA in the mother cell (MC) and the forespore (FS). Compartmentalized biotinylation of SpoIIID-BAP (*C*) and σ^G-BAP (*D*). *Top* in *C* and *D* are streptavidin-HRP Western blots with lysates prepared at t_3 (SpoIIID-BAP) and t_4 (σ^G-BAP). Lysates were prepared from strains producing different combinations of the indicated BAP-tagged fusion protein and BirA in the mother cell, the forespore, or not at all. *C Lower* and *D Lower* show streptavidin Western blots, with the same lysates shown in *C Upper* and *D Upper*, comparing levels of biotinyl-biotin carboxyl carrier protein (BCCP).

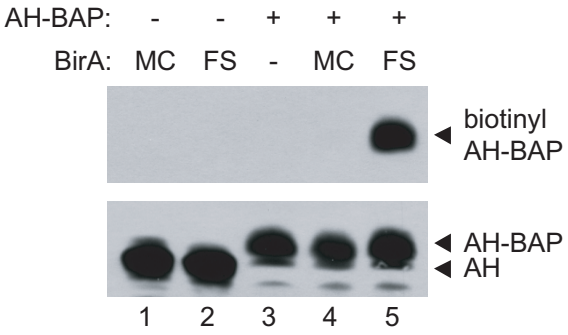

Fig. 3. Forespore-specific biotinylation of C-terminal BAP-tagged SpoIIIAH. (*Upper*) A streptavidin-HRP Western blot with lysates prepared at t_3 from strains producing combinations of SpoIIIAH-BAP and BirA produced in the mother cell, the forespore, or not at all, is shown. (*Lower*) An anti-SpoIIIAH Western blot with the same lysates as in *Upper* is shown.

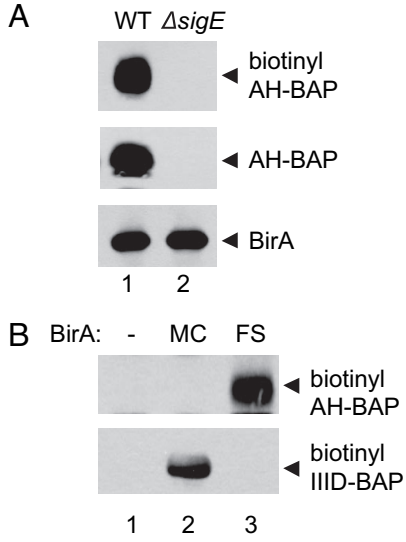

Fig. 4. Compartmentalization of SpoIIIAH-BAP production and BirA activity. (*A*) SpoIIIAH-BAP biotinylation in WT (lane 1) and *sigE* (lane 2) strains producing BirA in the forespore. Streptavidin-HRP (*Top*), anti-SpoIIIAH (*Middle*), and anti-BirA (*Bottom*) Western blots with lysates prepared at t_3. (*B*) Compartmentalized biotinylation of SpoIIIAH-BAP (*Top*) and SpoIIID-BAP (*Bottom*) in the same strains. Lysates were prepared at t_3 and analyzed by streptavidin-HRP Western blots.

To test whether BirA activity was compartmentalized in these strains, we analyzed the biotinylation of BAP-tagged SpoIIID and σ^G, produced exclusively in the mother cell and forespore, respectively (19, 20). The biotinylated BAP-tagged fusion proteins were detected by Western blot with a streptavidin-HRP conjugate. SpoIIID-BAP was only biotinylated in the strain that produced BirA in the mother cell and not in the strain that produced BirA in the forespore (Fig. 2*C*, lanes 4 and 5). Conversely, σ^G-BAP was biotinylated only in the strain that produced BirA in the forespore and not the strain that produced BirA in the mother cell (Fig. 2*D*, lanes 4 and 5). Neither biotinyl-SpoIIID-BAP nor biotinyl-σ^G-BAP was detected in the control strains that produced BirA or the BAP-tagged fusion proteins alone (Fig. 2 *C* and *D*, lanes 1–3). *B. subtilis* BirA-catalyzed biotinylation of the biotin carboxyl carrier protein (21) served as an internal control, showing that each lane contained similar amounts of protein (Fig. 2 *C* and *D*, *Bottom*). Each of the strains was sporulation proficient, indicating that the BAP tag did not interfere with normal protein function (data not shown). These results demonstrate that the compartmentalized biotinylation assay can be used to determine whether a protein is accessible to either the mother cell or forespore cytoplasm.

The C-terminal Domain of SpoIIIAH Is Accessible to Forespore-Produced BirA. To test whether the C-terminal extracellular domain of SpoIIIAH is accessible to the forespore cytoplasm, we assessed compartmentalized biotinylation in strains producing a C-terminal BAP-tagged SpoIIIAH. The SpoIIIAH-BAP fusion protein was biotinylated when BirA was produced in the forespore (Fig. 3, lane 5). Biotinyl-SpoIIIAH-BAP was not detected in the absence of *E. coli* BirA (Fig. 3, lane 3) or when it was produced in the mother cell (Fig. 3, lane 4). Immunoblots with anti-SpoIIIAH antiserum showed that SpoIIIAH-BAP accumulated to levels similar to that of wild-type SpoIIIAH in each of the strains (Fig. 3 *Lower*). Furthermore, these strains supported the production of heat-resistant spores similar to that of the wild-type strain, indicating that the BAP-tagged fusion protein was functional (data not shown). These results suggest that the C-terminal extracellular domain of the mother cell protein SpoIIIAH is accessible to the forespore cytoplasm.

Alternative explanations for the apparent forespore accessibility of the C-terminal BAP-tagged SpoIIIAH include noncompartmentalized production of SpoIIIAH-BAP in the forespore or noncompartmentalized BirA activity in the mother cell or intermembrane space. To test whether SpoIIIAH-BAP was produced in the forespore, we measured SpoIIIAH-BAP biotinylation in a mutant (*sigE*) that is defective in the mother cell but not forespore gene expression. If SpoIIIAH-BAP were produced in the forespore under the control of σ^F, then its expression would not be dependent on the mother cell specific activity of σ^E and therefore would be biotinylated even in a *sigE* mutant. However, we found that biotinyl-SpoIIIAH-BAP was not detected in the *sigE* mutant strain (Fig. 4*A*, *Top*). BirA production was unaffected in the mutant as expected, indicating that forespore gene expression was intact (Fig. 4*A*, *Bottom*). We also found that production of biotinyl-SpoIIIAH-BAP was not dependent on σ^G, the late forespore-specific factor (Fig. S2). Thus, SpoIIIAH-BAP production was completely dependent on σ^E and therefore restricted to the mother cell. To test for BirA activity in the mother cell in the strain that expressed BirA in the forespore, we analyzed biotinylation of SpoIIIAH-BAP and the mother cell protein SpoIIID-BAP in the same strain. As expected, SpoIIID-BAP was biotinylated only when BirA was produced in the mother cell but not in the strain that produced BirA in the forespore (Fig. 4*B Lower*). Conversely, SpoIIIAH-BAP was biotinylated only when BirA was produced in the forespore and not in the strain that produced BirA in the mother cell (Fig. 4*B Upper*). These results eliminate the possibility that biotinylation of SpoIIIAH-BAP occurred in the mother cell when BirA was produced in the forespore.

To test whether BirA produced in the forespore exhibited activity in the intermembrane space, we assessed biotinylation of C-terminal BAP-tagged control septal proteins SpoIID and SpoIVFA. SpoIID contains an extracellular peptidoglycan hydrolase domain that is necessary for septal thinning during forespore engulfment (22). SpoIVFA contains an extracellular domain that regulates late mother cell gene expression in response to a forespore protein that is secreted into the intermembrane space following engulfment (23). Neither SpoIID-BAP nor SpoIVFA-BAP was biotinylated (data not shown), while both supported the production of heat-resistant spores similar to wild type, indicating the fusion proteins were functional (data not shown). Therefore, BirA produced in the forespore is not active in the intermembrane space. This was not surprising because BirA-catalyzed biotinylation requires ATP (24), which is expected to be absent from the intermembrane

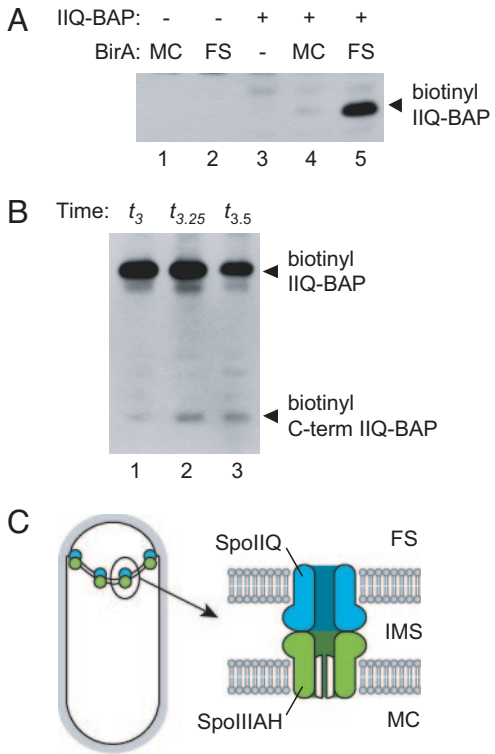

Fig. 5. Forespore-specific biotinylation of C-terminal BAP-tagged SpoIIQ. (*A*) Streptavidin-HRP Western blot with lysates prepared at t_3 from strains producing combinations of SpoIIQ-BAP and BirA produced in the mother cell, the forespore, or not at all. (*B*) Biotinyl-SpoIIQ-BAP proteolysis. Lysates prepared at t_3 (lane 1), $t_{3.25}$ (lane 2), and $t_{3.5}$ (lane 3). (*C*) SpoIIIAH-SpoIIQ channel model. (*Left*) SpoIIIAH and SpoIIQ track along the engulfing membrane and colocalize in discrete foci surrounding the forespore. (*Right*) The C-terminal extracellular domains of SpoIIIAH and SpoIIQ interact in the intermembrane space and form a channel that is open at the forespore end and closed (or gated) at the mother cell end.

space. Together these results indicate that SpoIIIAH-BAP is produced exclusively in the mother cell and accessible to biotinylation by BirA produced in the forespore cytoplasm. We note that the central diameter of the EscJ ring is large enough to accommodate BirA (9, 25). Therefore, these results are consistent with the hypothesis that SpoIIIAH forms a channel.

C-Terminal Extracellular Domain of SpoIIQ Is also Accessible to Forespore-Produced BirA. For the SpoIIIAH extracellular domain to be accessible to the forespore cytoplasm, it may interact with an integral membrane protein that forms a channel across the forespore membrane. Because SpoIIIAH interacts with the forepore protein SpoIIQ (4, 5), we tested whether the C-terminal extracellular domain of SpoIIQ was also accessible to the forespore cytoplasm. We introduced a C-terminal BAP-tagged *spoIIQ* allele into the compartmentalized biotinylation strains. These strains produced heat-resistant spores similar to wild type, indicating that the fusion protein was functional (data not shown). SpoIIQ-BAP was biotinylated only when BirA was produced in the forespore, suggesting that the C terminus of SpoIIQ is accessible to the forespore cytoplasm (Fig. 5*A*). After the completion of engulfment, SpoIIQ is processed by SpoIVB, a protease produced in the forespore and secreted into the intermembrane space, releasing the 30-kDa C-terminal domain from the full-length protein (26). To confirm that SpoIIQ-BAP was properly inserted into the membrane, we tested whether the biotinylated C-terminal proteolytic product accumulated after engulfment. Engulfment typically begins 2 h after the onset of

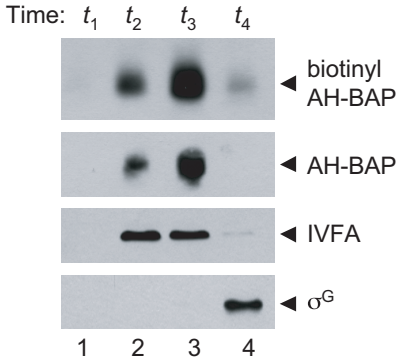

Fig. 6. Degradation of biotinyl-SpoIIIAH-BAP after engulfment. Lysates prepared hourly between t_1 and t_4 were analyzed by streptavidin-HRP (topmost), anti-SpoIIIAH antibody (second from top), anti-SpoIVFA (second from bottom), and anti-σ^G (bottommost) Western blots.

sporulation (t_2) and completes within 60 min (t_3) (27, 28). We detected the expected 30-kDa biotinylated product beginning at ~t_3 (Fig. 5*B*), indicating that the biotinyl-SpoIIQ-BAP was correctly inserted in the membrane with the C-terminal domain in the intermembrane space. Because the insertion of bacterial integral membrane proteins is cotranslational [reviewed in (29)], it is unlikely that C-terminal BAP-tagged SpoIIQ is biotinylated in the cytoplasm before membrane insertion. These results are consistent with the idea that SpoIIIAH and SpoIIQ form a channel that is open on the forespore end and closed (or gated) on the mother cell end (Fig. 5*C*).

Degradation of the Putative Channel after Engulfment. The interaction with SpoIIQ is necessary for the septal localization and stability of SpoIIIAH (4, 5, 30). This suggests that SpoIIQ should be necessary for the forespore accessibility and stability of SpoIIIAH-BAP. As expected, SpoIIIAH-BAP failed to accumulate in a *spoIIQ* mutant (data not shown). Septal thinning is necessary for the interaction of SpoIIIAH and SpoIIQ (4, 5). SpoIIIAH-BAP was undetectable (data not shown) in a mutant (*spoIIP spoIID*) that is completely defective in septal thinning (22).

Because the interaction with SpoIIQ was necessary for SpoIIIAH-BAP stability, we hypothesized that biotinyl-SpoIIIAH-BAP would be degraded upon the completion of engulfment and initiation of SpoIIQ proteolysis. We found that biotinyl-SpoIIIAH-BAP accumulated until t_3 and was degraded at t_4 (Fig. 6 topmost). To confirm that the degradation of biotinyl-SpoIIIAH correlated with the completion of engulfment, we analyzed the control proteins SpoIVFA and σ^G. SpoIVB cleaves SpoIVFA after engulfment, resulting in SpoIVFA degradation (and activation of pro-σ^K processing) (31, 32). After engulfment, σ^G activates expression of its own gene, promoting the accumulation of σ^G. While SpoIVFA was present at t_2–t_3 and lost at t_4 (Fig. 6 second from bottom), σ^G was detected by t_4 (Fig. 6 bottommost). These results suggest that the channel forms during engulfment and is degraded shortly after its completion.

Discussion

We identified a homologous relationship between the C-terminal extracellular domain of SpoIIIAH and the YscJ and FliF proteins, involved in type III secretion of virulence factors and flagellar protein export apparatus, respectively. We developed an *in vivo* compartmentalized biotinylation assay and showed that the C-terminal extracellular domain of the mother cell protein SpoIIIAH is accessible to biotinylation by BirA produced in the forespore, but not by BirA produced in the forespore. This result supports the hypothesis that SpoIIIAH forms a channel, analogous to those formed by YscJ and FliF, in

the engulfing mother cell membrane. An alternative explanation is that the C terminus of SpoIIIAH extends across the forespore membrane. This is unlikely, however, because the C terminus of SpoIIIAH does not contain a predicted transmembrane segment or amphipathic α-helix. Furthermore, the C terminus of SpoIIIAH is predicted to form a globular mixed α/β domain that is topologically similar to the YscJ/FliF domain and unlikely to extend across the forespore membrane (9). The fraction of SpoIIIAH that is biotinylated is unknown. Therefore, we don't know whether all molecules of SpoIIIAH participate in channel formation or whether only a fraction of the SpoIIIAH C termini within a channel is accessible to BirA. We also showed that the C-terminal extracellular domain of the forespore protein SpoIIQ, with which SpoIIIAH interacts, is accessible to BirA produced in the forespore. Therefore, we propose that SpoIIIAH and SpoIIQ form a channel that is open on the forespore end and closed (or gated) on the mother cell end.

The core components of the flagellar protein export apparatus, including three soluble proteins (ATPase regulator FliH, ATPase FliI, and chaperone FliJ) and six integral membrane proteins (FlhA, FlhB, FliO, FliP, FliQ, and FliR), are homologous to the type III secretion system (33, 34). FlhA, FlhB, FliO, FliP, FliQ, and FliR are thought to be located within the FliF ring (35, 36). In the type III secretion system, the YscJ ring is thought to be functionally analogous to the FliF ring, providing a scaffold for the assembly of YscR, YscS, YscT, YscU, and YscV. FlhA and FlhB (YscV and YscU) act as a docking platform for the FliI (YscN) ATPase (35–38). Although the functions of FliO, FliP, FliQ, and FliR (YscR, YscS, and YscT) are unknown, together with FlhA and FlhB, they are thought to form a protein-conducting channel (36).

By analogy with the type III secretion system and flagellar protein export apparatus, we propose that SpoIIIAH acts as a scaffold upon which the components of a novel export apparatus is assembled and, together with SpoIIQ, forms the channel through which substrate translocation between the mother cell and forespore occurs. In addition to SpoIIIAH, the *spoIIIA* locus is predicted to encode a cytoplasmic protein (SpoIIIAA) and six integral membrane proteins (SpoIIIAB–AG), several of which share similarity with the components of several protein secretion systems (J.M., unpublished data). SpoIIIAA is similar to the secretion superfamily ATPases involved in type II secretion, type IV pilus assembly, type IV secretion, DNA uptake, and archaeal flagellar assembly systems (39). The predicted cytoplasmic domain of SpoIIIAB is similar to the type II secretion system protein GspF, a component of the inner membrane platform to which the ATPase docks (38). Although SpoIIIAC and SpoIIIAD do not share similarity with proteins of known function, they resemble FliQ and FliP (YscS and YscR), respectively, in size, and number, and orientation of transmembrane segments. SpoIIIAE is similar to various electrochemical potential-driven transporters. SpoIIIAF shows similarity with FlhB (YscU), while SpoIIIAG has limited similarity to YscJ and FliF. Therefore, we hypothesize that SpoIIIAB–AG are assembled within the SpoIIIAH ring and form a novel export apparatus that gates the SpoIIIAH–SpoIIQ channel at the mother cell end. SpoIIIAB, and possibly SpoIIIAF, provide a docking platform for SpoIIIAA, which couples substrate translocation with ATP hydrolysis. Although the roles of SpoIIIAA–AG are not known, our preliminary results indicate that SpoIIIAA is not required for formation of the SpoIIIAH–SpoIIQ channel (data not shown). This result is consistent with those of Camp and Losick in which they identified a mutation that partially bypasses the requirement for *spoIIIAA–AG*, but not *spoIIIAH* and *spoIIQ*, for σ^G activation (13). They postulated that, under certain conditions, SpoIIIAH and SpoIIQ can constitute a minimal pathway for σ^G activation. However, in an otherwise wild-type strain SpoIIIAA–AG are necessary for efficient σ^G activation via the SpoIIIAH–SpoIIQ channel.

The export apparatus model predicts that a substrate(s) is produced in the mother cell and translocated into the forespore, where it triggers the activation of σ^G. The nature of the substrate is unclear. On the basis of the similarities of the SpoIIIA proteins to various protein secretion systems, we suspect that the substrate is a protein. If this were the case, then a mutation in the gene encoding the protein substrate would block σ^G activation. No such mutant has been isolated in decades of selection and screening. As noted by Camp and Losick, it is possible that the gene is essential for viability or necessary for a process earlier in sporulation (13). The substrate may also be a peptide encoded by a small ORF (ORF) missed by mutagenesis and overlooked in microarray analyses. Another possibility is that the substrate is a small molecule or metabolite, without which the forespore cannot sustain essential processes such as transcription or translation (13). While we acknowledge that the substrate might be a metabolite, this model does not account for the fact that, before engulfment, σ^G remains inactive while σ^F is active in the forespore. This argues that σ^G inactivity is not exclusively because of a general metabolic deficiency but rather a specific inhibition of σ^G that must also be relieved. Although the function of the SpoIIIAH–SpoIIQ channel remains to be elucidated, all of the SpoIIIA-encoded products are highly conserved among evolutionarily diverse endospore-forming *Bacillus* and *Clostridium* species (data not shown). Therefore, this novel hybrid secretion system may be an ancient, but specialized and essential structure for mother cell–forespore communication.

Materials and Methods

To construct *B. subtilis* strains expressing *E. coli birA* in the mother cell and forespore, C-terminal His-tagged *E. coli birA* was amplified by PCR from strain MG1655 chromosomal DNA with a reverse primer containing the His_6 coding sequence and the *spoIID* and *spoIIQ* promoters were amplified from *B. subtilis* strain JH642 chromosomal DNA. P_{spoIID}-*birA-his*$_6$ and P_{spoIIQ}-*birA-his*$_6$ were constructed by overlapping PCR, digested with BamHI and PstI, and ligated into the *amyE* gene replacement vector pDG1662. *B. subtilis* strain JH642 transformants were selected for chloramphenicol resistance and double-crossover recombinants were screened for spectinomycin sensitivity.

To construct strains expressing BAP-tagged alleles, the 3′ end of each coding sequence was amplified using gene-specific primers. Each reverse primer contained the BAP coding sequence followed by a stop codon. The *spoIID-BAP*, *sigG-BAP*, *spoIIAH-BAP*, *spoIID-BAP*, and *spoIIQ-BAP* fragments were digested with the appropriate restriction enzymes and ligated into an integration vector and used to transform *B. subtilis*. These strains contain the BAP-tagged alleles integrated at the native loci by Campbell recombination. *spoIVFA*-BAP was cloned into *thrC* gene replacement vector pDG1664 and used to transform *B. subtilis*. All *B. subtilis* strains, plasmids, and details of experimental procedures are described in *SI Materials and Methods* and Tables S1–S3.

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