

## Septal localization of forespore membrane proteins during engulfment in *Bacillus subtilis*

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In *Bacillus subtilis*, many membrane proteins localize to the sporulation septum, where they play key roles in spore morphogenesis and cell-specific gene expression, but the mechanism for septal targeting is not well understood. SpoIIQ, a forespore-expressed protein, is involved in engulfment and forespore-specific gene expression. We find that SpoIIQ dynamically localizes to the sporulation septum, tracks the engulfing mother cell membrane, assembles into helical arcs around the forespore and is finally degraded. Retention of SpoIIQ in the septum requires one or more mother cell-expressed proteins. We also observed that any forespore-expressed membrane protein initially localizes to the septum and later spreads throughout the forespore membrane, suggesting that membrane protein insertion occurs at the forespore septal region. This possibility provides an attractive mechanism for how activation of mother cell-specific gene expression is restricted to adjacent sister cells, since direct insertion of the signaling protein SpoIIR into the septum would spatially restrict its activity. In keeping with this hypothesis, we find that SpoIIR localizes to the septum and is transiently expressed.

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### Introduction

It is becoming increasingly clear that bacteria share with eucaryotes the ability to specifically and dynamically localize proteins within the cell. The best-studied example is FtsZ, a divergent tubulin homolog required for cell division, which assembles into rings at midcell during vegetative growth, moving to new division sites via spiral intermediates (Addinall and Holland, 2002; Ben-Yehuda and Losick, 2002; Lutkenhaus, 2002; Margolin, 2002). In fact, the number of bacterial proteins with dynamic localization is ever increasing: *Escherichia coli* MinCD oscillate at a rapid rate from pole to pole within the cell to promote correct placement of the division machinery (Marston and Errington, 1999; Justice *et al.*, 2000; Pichoff and Lutkenhaus, 2001; Johnson *et al.*,

2002); DivK of *Caulobacter crescentus*, a signal transduction protein required for polar morphogenesis and cell cycle progression, whose localization alternates between the cell pole and even dispersal throughout the membrane (Jacobs *et al.*, 2001; Ausmees and Jacobs-Wagner, 2003); and *Bacillus subtilis* SpoIIIE, required for DNA translocation and membrane fusion during sporulation, relocalizes from the septal midpoint to the cell pole (Sharp and Pogliano, 1999, 2002; Bath *et al.*, 2000). While the number of proteins with dynamic localization increases, the mechanism for the specific targeting of these proteins is still not well understood, particularly for membrane proteins. Most membrane proteins utilize the Sec translocation machinery for entry into the cytoplasmic membrane, but it has recently been shown that in *E. coli*, SecYEG are uniformly distributed throughout the membrane, further complicating the mechanism for membrane protein targeting (Brandon *et al.*, 2003).

Sporulation in *B. subtilis* provides an excellent system to study dynamic protein localization, since it involves the dramatic rearrangement of the bacterial cell (Stragier and Losick, 1996), with specifically localized proteins mediating many key steps. The first morphological change of sporulation is the switch from medial to polar division (Figure 1A), resulting in the formation of two distinct cell types: a smaller forespore that eventually becomes the spore and a larger mother cell that lyses upon release of the spore. Relocalization of the division site is mediated by movement of the FtsZ ring from midcell to both poles (Levin and Losick, 1994; Ben-Yehuda and Losick, 2002), allowing recruitment of proteins required for cell-specific gene expression to the sporulation septum (Arigoni *et al.*, 1999), which is ultimately synthesized at only one of the potential division sites. Following the onset of forespore and mother cell-specific gene expression, the mother cell membrane migrates around the forespore in a phagocytosis-like event known as engulfment (Figure 1A). During engulfment, the required mother cell-expressed membrane proteins localize to the leading edge of the engulfing membrane (Abanes-De Mello *et al.*, 2002), while mother cell-expressed proteins required for coat assembly (Pogliano *et al.*, 1995; Driks, 2002) and intracellular signal transduction (Rudner and Losick, 2002) localize to the mother cell-derived outer forespore membrane. With one exception, described below, little is known about the mechanism through which membrane proteins are localized during sporulation.

Two possible mechanisms can be envisioned for the specific localization of membrane proteins within the bacterial cell to the polar septum (Figure 1B and C). First, membrane proteins could be directly inserted into the membrane at the sporulation septum (Figure 1B, directed insertion). Alternatively, membrane protein insertion could occur elsewhere, with the proteins diffusing through the membrane until they are retained at the septum by a specific ligand (Figure 1C, lateral sorting or diffusion and capture). Recently, evidence has been provided that the mother cell-expressed

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SpoIVFB, required for activation of the late mother cell-specific  $\sigma$  factor,  $\sigma^K$ , inserts into the mother cell cytoplasmic membrane, diffusing to the septum where it is retained (Rudner *et al.*, 2002).

Here we present evidence that in the forespore, membrane proteins can reach their correct destination by directed insertion and capture, as when non-native membrane proteins, such as those from *E. coli*, are expressed in the forespore, they initially localize to the sporulation septum and later diffuse throughout the forespore membrane (Figure 1D). In contrast, localized proteins can be selectively retained by interacting with a septum-specific ligand (Figure 1C), which in the case of SpoIIQ is a mother cell-expressed protein required to couple movement of SpoIIQ around the forespore to the engulfing mother cell membrane. Interestingly, SpoIIQ regulates the late forespore-specific transcription factor,  $\sigma^G$  (Sun *et al.*, 2000), whose activity is tightly regulated both by the completion of engulfment and by an intracellular signal transduction cascade (Illing and Errington, 1991; Stragier and Losick, 1996; Serrano *et al.*, 2003). Our cell biological studies thereby further support a role for SpoIIQ in this process, as it interacts with a mother cell-specific ligand, and displays a dynamic localization pattern coordinated with the completion of engulfment. We propose that the directed insertion of forespore-specific proteins into the septum delivers the SpoIIR signaling protein to the sporulation septum thereby restricting activity to its adjacent mother cell and preventing it from inappropriately activating  $\sigma^E$  in neighboring cells.

## Results

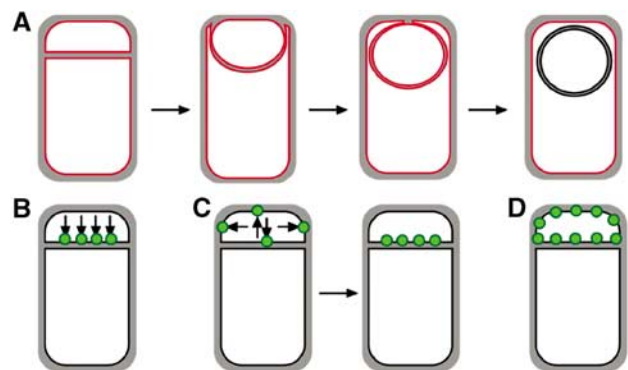
### Localization of heterologous GFP fusion proteins in the forespore

We were interested in identifying signals required for localization of integral membrane proteins to the forespore septal membrane, and therefore compared localization of membrane proteins not normally expressed during sporulation to that of sporulation-specific membrane proteins, such as SpoIIQ. Forespore-expressed proteins without localization signals should diffuse throughout the forespore membrane (Figure 1D), whereas localized proteins might first localize to the septal region of the forespore membrane, and later move around the forespore membrane together with the engulfing mother cell membrane (Figure 1C). The mechanism by which a forespore-expressed protein might track the engulfing mother cell membrane remains unclear, as the obvious morphological transformation of engulfment involves only the mother cell membrane (Sun *et al.*, 2000; Abanes-De Mello *et al.*, 2002).

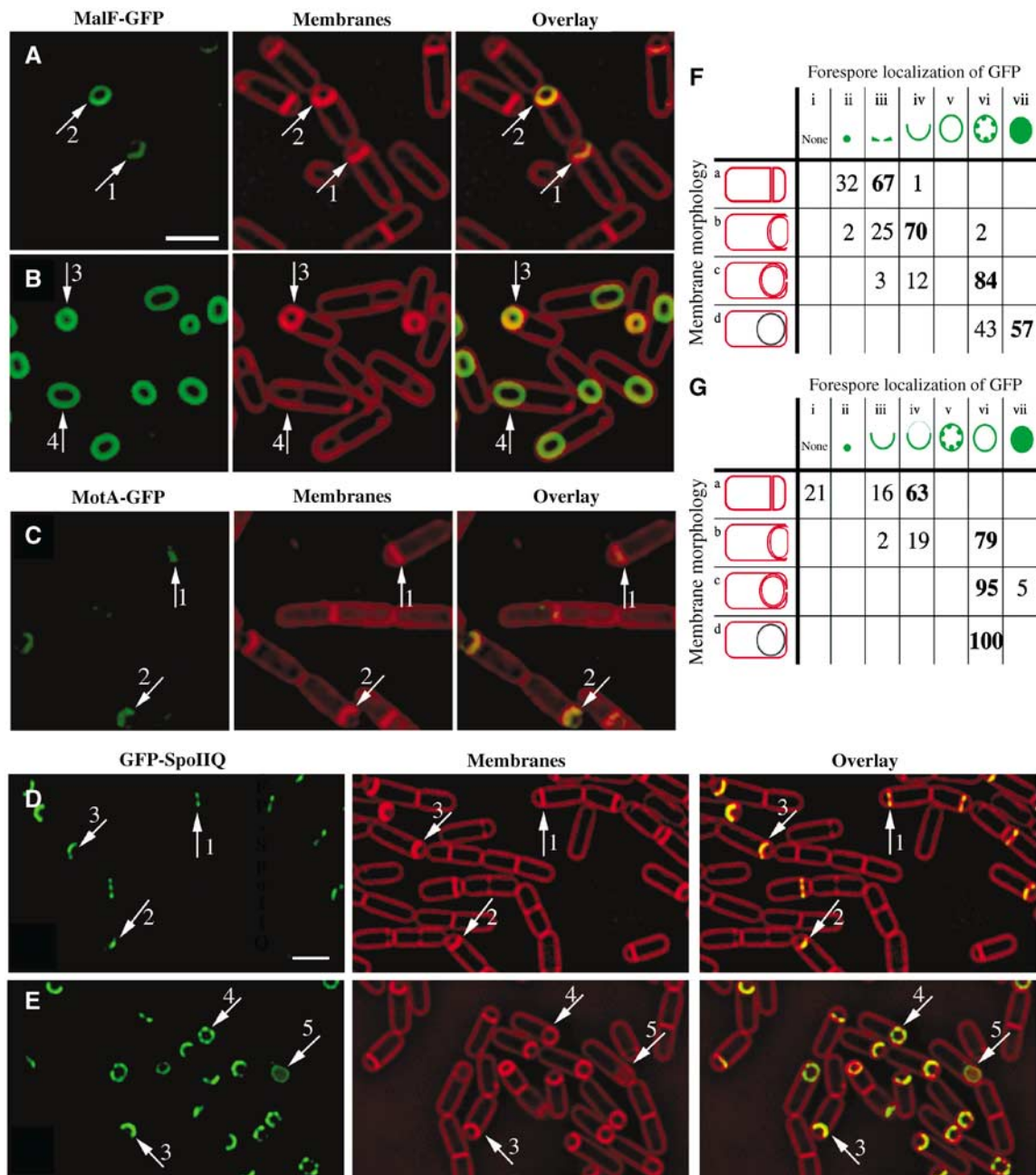
We therefore constructed GFP fusions to sporulation-specific proteins, as well as to heterologous membrane proteins, and used the promoters and translational initiation sites from forespore-specific genes to mediate their expression. For the heterologous proteins, the first two membrane-spanning segments of five different membrane proteins (*E. coli* MalF, LacY, MotA and SecY, and *B. subtilis* AraP) were expressed from the promoter and translational initiation sites from one of four different forespore-specific promoters (*spoIIQ*, *spoIIR*, *katX* and *csfB*), with *gfp* fused to either the 5' or 3' end of the membrane protein-encoding region. For the native proteins, we constructed GFP fusions to the membrane proteins SpoIIQ and SpoIIR (at the N-terminus, since the C-termini are

extracellular), to the soluble proteins CsfB and KatX and utilized native promoters for expression. Western blot analysis showed that these GFP fusion proteins were stable early in sporulation (data not shown), with the exception of GFP-SpoIIQ and GFP-SpoIIR (discussed further below).

Cells containing the GFP fusion proteins were induced to sporulate via resuspension (Materials and methods) and visualized using deconvolution fluorescence microscopy using fluorescent membrane stains to visualize the dramatic changes in membrane morphology associated with engulfment, providing a convenient method to discriminate between sporangia at different stages of sporulation since engulfment typically initiates 2 h after the onset of sporulation ( $t_2$ ) and completes within 60 min ( $t_3$ ) (Figure 1A) (Pogliano *et al.*, 1999; Sharp and Pogliano, 1999). In early sporangia, with flat polar septa and faint GFP fluorescence, every heterologous membrane protein constructed localized first to the sporulation septum (MalF-GFP is shown in Figure 2A and B; MotA-GFP in Figure 2C; other proteins in Supplementary Figure 1; see arrows 1). We quantified this apparent septal enrichment for the most highly expressed protein (MalF-GFP, AR4) comparing fluorescence intensity of GFP and the membrane-specific stain FM 4-64 at the septum, forespore pole and mother cell cytoplasmic membrane (Materials and methods). As expected, the membrane stain was approximately twice as bright at the septum as at the cell pole or mother cell cytoplasmic membrane (79 700, 38 800 and 43 300 photons/ $\mu\text{m}^2$ , respectively), since the septum is comprised of two parallel membranes (Figure 1A). In contrast, the GFP fluorescence was 5–30 times brighter at the septum than at the forespore pole, indicating that enrichment of the heterologous membrane fusion proteins is not due to a difference in the volume of membrane at the septum versus cell pole. In sporangia at early stages of engulfment (with curved sporulation septa), and slightly brighter GFP fluorescence, the membrane proteins remained enriched



**Figure 1** Engulfment pathway of sporulation in *B. subtilis* and models for targeting of proteins synthesized in the forespore. (A) Cartoon showing *B. subtilis* engulfment. Following polar septation, the membrane of the larger mother cell migrates around the forespore, until it is completely enclosed. Membranes stained by FM 4-64 are shown in red; following membrane fusion, FM 4-64 no longer stains the forespore membranes, providing an assay for the completion of engulfment. (B–D) Models for targeting forespore-expressed membrane proteins. Proteins (green) can either be directly inserted into the forespore septal region (B) or inserted elsewhere and migrate to the septal region (C). Then, they can either be retained during engulfment (C) or freely diffuse throughout the forespore membrane (D).



**Figure 2** Localization patterns of forespore-expressed heterologous membrane proteins and the native forespore protein SpoIIQ. (A, B) MalF-GFP (green) 2 h (A, hereafter  $t_2$ ) and 3 h (B, hereafter  $t_3$ ) after the onset of sporulation stained with FM 4-64 (red). In most sporangia, engulfment is underway at  $t_2$  and complete by  $t_3$ . (C) MotA-GFP (green) at  $t_2$  stained with Mitotracker Red (red). In panels A and C, arrows 1 and 2 represent sporangia with flat or slightly curving polar septa. In panel B, arrows 3 and 4 represent sporangia before and after membrane fusion, respectively. (D) GFP-SpoIIQ (green) at  $t_2$  and (E)  $t_3$  stained with FM 4-64 (red). In panels D and E, arrows 1, 2 and 3 represent early sporangia and arrows 4 and 5 represent sporangia late in engulfment. The scale bar in (A, D) is 2  $\mu$ m. (F, G) Quantitation of GFP localization at various stages of engulfment (a-d). Numbers refer to the percentage of sporangia in each engulfment class with the indicated localization pattern; those in bold represent the predominant class. (F) GFP-SpoIIQ (AR126) localization. Seven subclasses of GFP localization were scored: (i) none; (ii) medial focus; (iii) ring; (iv) tracking mother cell engulfing membrane; (v) freely diffusible; (vi) punctate; (vii) soluble. A total of 504 sporangia were scored from  $t_2$  and  $t_3$ . (G) MalF-GFP (AR4) localization. Seven subclasses for GFP localization were scored: (i) none; (ii) focus formation; (iii) tracking mother cell engulfing membrane; (iv) enriched at septum but ahead of engulfing membranes; (v) punctate; (vi) freely diffusible; (vii) soluble. A total of 296 sporangia were scored from  $t_2$  and  $t_3$ .

at the septum with faint fluorescence clearly visible at the cell pole (Figure 2A and C and Supplementary Figure 1, arrows 2), while late in engulfment each fusion protein was uniformly distributed throughout the forespore membrane (Figure 2B, arrows 3 and 4).

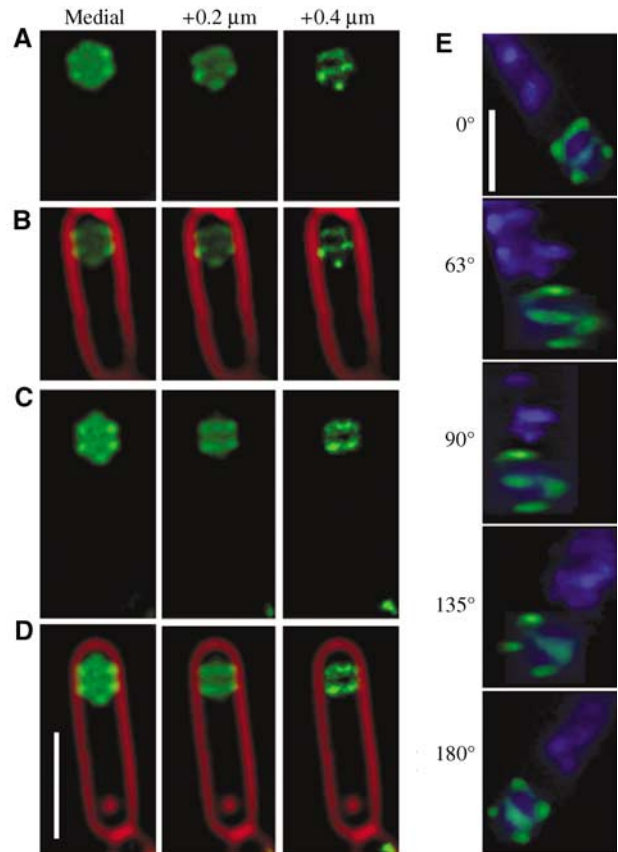
Thus, at least five different membrane proteins not normally expressed in the forespore localize to the sporulation septum in early sporangia, but appear uniformly distributed throughout the forespore membrane in later sporangia. To reduce the possibility that either the untranslated sequences

in the 5' end of the mRNA or the first six amino acids of SpoIIQ directed septal localization, we expressed these fusion proteins from a total of four different promoters (including translational initiation signals and the first six codons), two of which encoded nonlocalized cytoplasmic proteins (*csfB* and *katX*; Supplementary Figure 1 and data not shown). In each case, the heterologous fusion proteins showed identical localization patterns regardless of the expression cassettes used, suggesting that any forespore-expressed membrane protein initially localizes to the sporulation septum, later diffusing throughout the forespore membrane. One possible explanation for these findings is that in the forespore, membrane proteins are directly inserted into the septal membrane region, but in the absence of specific localization signals, they later diffuse throughout the forespore membranes.

### Dynamic localization of a native membrane protein, SpoIIQ

We compared the localization of the heterologous GFP fusions described above to two well-characterized  $\sigma^F$ -dependent membrane proteins, SpoIIQ and SpoIIR (described below). Transcriptional profiling experiments have identified only 66 genes under  $\sigma^F$  (and  $\sigma^F/\sigma^G$ ) control (Fawcett *et al.*, 2000) in contrast to the 253 genes controlled by  $\sigma^E$  (Eichenberger *et al.*, 2003), with only a few membrane proteins of known function. We constructed a functional fusion of GFP to the cytoplasmic N-terminus of SpoIIQ, a protein essential for the synthesis and activation of the late forespore-specific transcription factor  $\sigma^G$  (Sun *et al.*, 2000), and integrated this into a *spoIIQ* null strain (Materials and methods). The resulting strain (AR126) was similar to wild type for both engulfment and spore production. In early sporangia with very faint GFP fluorescence, GFP-SpoIIQ localized as a ring at the septum or a focus at the septal midpoint (Figure 2D, arrows 1 and 2, respectively). In sporangia that had initiated engulfment, GFP-SpoIIQ lined the septum, and closely followed the engulfing mother cell membrane as it moved around the forespore (Figure 2D and E, arrows 3). Subsequently, GFP-SpoIIQ displayed punctate membrane-localized fluorescence in sporangia near the completion of engulfment (Figure 2E, arrow 4). After the completion of membrane fusion, cytoplasmic GFP fluorescence was observed (Figure 2E, arrow 5), suggesting that GFP was released from the forespore membrane. A striking three-dimensional structure was observed at the transition between the last two stages. In the medial focal plane, we observed both soluble GFP fluorescence and six GFP-SpoIIQ foci, which appeared to mark the corners of a hexagonal forespore (Figure 3 and Supplementary Figure 2). In the uppermost focal plane, four of these foci resolved into two lines, which traversed the width of the forespore, suggesting that at the time that GFP-SpoIIQ is being degraded, it is arranged into two or more hoops that surround the forespore (Figure 3, +0.4  $\mu\text{m}$  column). Indeed, three-dimensional modeling shows that SpoIIQ forms helical arcs around the forespore (Figure 3E; Supplementary movie) similar to those of the actin-like proteins MreB and Mbl (Jones *et al.*, 2001; Carballido-Lopez and Errington, 2003; Daniel and Errington, 2003).

The localization of GFP-SpoIIQ correlated well with the various stages of engulfment (Figure 2F), and was dramatically different from that of MalF-GFP. First, in early sporangia, we never observed foci at the septal midpoint or ring



**Figure 3** Three-dimensional visualization of GFP-SpoIIQ rings after membrane fusion. Optical sectioning deconvolution microscopy was used to obtain images from different focal planes within the specimen (A–D) and used to reconstruct the three-dimensional model of GFP-SpoIIQ (E). In all, 20 images were obtained from focal planes 0.1  $\mu\text{m}$  (A–D) or 0.15  $\mu\text{m}$  (E) apart. (A–D) Different focal planes of GFP-SpoIIQ (green) from two sporangia, starting with the medial GFP focal plane (far left) and +0.2 and +0.4  $\mu\text{m}$ . For reference, the medial focal plane of FM 4-64-stained membrane (red) is included as an overlay (B, D). (E) Three-dimensional model of GFP-SpoIIQ. Z-stacks each separated by 0.15  $\mu\text{m}$  were loaded into the volume builder function of the Applied Precision SoftWorx program (version 3.3) with modeled projections around the Y-axis shown and DAPI-stained chromosomes shown as a reference point (also shown in the Supplementary movie). Scale bar, 2  $\mu\text{m}$ .

formation with MalF-GFP or any other heterologous membrane protein. Second, MalF-GFP moved ahead of the engulfing mother cell membrane in 63% of sporangia (Figure 2A, arrow 2; Figure 2G, Class a-iv), whereas GFP-SpoIIQ never did. Finally, MalF-GFP remained associated with the forespore membrane even after the completion of engulfment (Figure 2B, arrow 4), in contrast to cytoplasmic GFP fluorescence observed for GFP-SpoIIQ (in 57% of sporangia; see Figure 2F, Class d-vii). Overall, most sporangia displayed uniform rings of MalF-GFP fluorescence (79% for sporangia with curving septa, 100% for sporangia that have completed engulfment; shown in Figure 2G, Class b-vi, c-vi and d-vi, respectively).

### SpoIIQ is degraded during sporulation

The cytoplasmic GFP fluorescence visualized in late GFP-SpoIIQ sporangia suggested proteolytic release of GFP from the SpoIIQ membrane-spanning segment. To test this

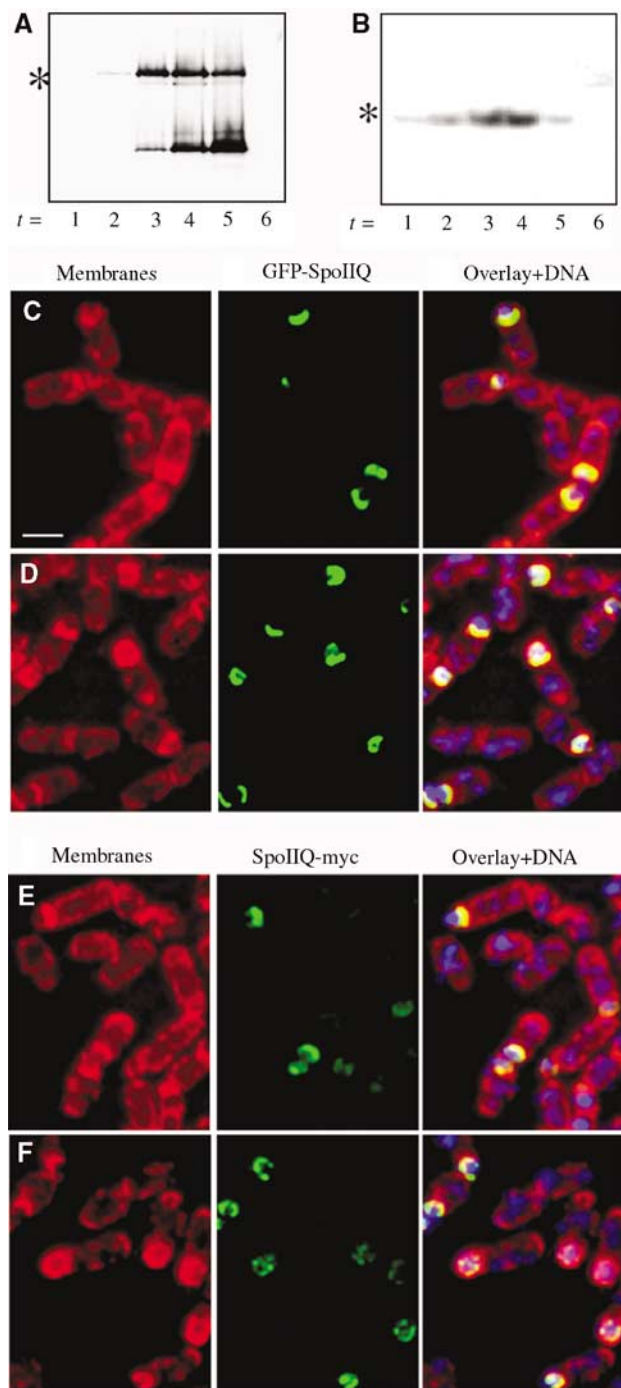
hypothesis, we performed immunofluorescence and Western blot analysis of GFP-SpoIIQ, SpoIIQ-myc, SpoIIQ-flag and GFP-SpoIIQ-flag (Figure 4 and data not shown) using GFP-, c-Myc- or Flag-specific antibodies, respectively. GFP-SpoIIQ was first detected at  $t_2$  and was markedly unstable, producing one major breakdown product approximately the size of soluble GFP (Figure 4A). This degradation product accumulated during sporulation, coincident with a decrease in the level of full-length protein (Figure 4A (\*)). These results are in contrast to the more stable levels of MalF-GFP and its smaller GFP-sized band (data not shown). Similarly, SpoIIQ-myc was first detected at  $t_1$ , with full-length protein peaking around  $t_3$  and decreasing with time, with no smaller degradation products detected (Figure 4B). Immunofluorescence data for GFP-SpoIIQ closely correlate with the localization observed in live cells (Figure 4C and D). In contrast, immunofluorescence analysis of SpoIIQ-myc revealed similar early localization patterns, but little soluble signal was detected within the forespore at later stages of engulfment (Figure 4E and F). Together, these results suggest that SpoIIQ is cleaved near the extracellular domain, producing a smaller GFP-sized product.

#### Movement of GFP-SpoIIQ around the forespore depends on engulfment

GFP-SpoIIQ appeared to track the engulfing mother cell membrane as it moved around the forespore; however, it might move around the forespore by an engulfment-independent mechanism and only coincidentally track the engulfing membrane. To determine more precisely if GFP-SpoIIQ tracked the engulfing membranes, we tested if its movement around the forespore depended on the mother cell-expressed engulfment proteins SpoIIM, SpoIIP and SpoIID. Mutants lacking these proteins fail to initiate engulfment, and ultimately the forespore breaks through the septum into the mother cell (Lopez-Diaz *et al.*, 1986; Smith *et al.*, 1993; Frandsen and Stragier, 1995), forming bulges at the septum that are readily visualized by fluorescence microscopy (Pogliano *et al.*, 1999; Perez *et al.*, 2000; Abanes-De Mello *et al.*, 2002). We constructed strains expressing either GFP-SpoIIQ or MalF-GFP in *spoIIP* (AR139 or AR154), *spoIID* (AR137 or AR152) or *spoIIM* (AR140 or AR153), and also in a strain lacking all three engulfment proteins. In strains lacking any one or all three of the engulfment proteins, GFP-SpoIIQ localized to the septum as in wild type, forming a focus and lining the septum (Figure 5C; quantitation shown in Supplementary Figure 3). However, movement around the forespore was abolished, and GFP-SpoIIQ stayed in the septum without moving into the bulge (Figure 5D; 69% in *spoIIP*, 58% in *spoIID* and 79% in *spoIIM*). In contrast, MalF-GFP was initially enriched in the septum (Figure 5I, arrow), but then moved around the forespore and into the bulge (Figure 5J; 72% in *spoIIP*, 63% in *spoIID* and 83% in *spoIIM*). Thus, movement of SpoIIQ, but not MalF, throughout the forespore membrane is coupled to migration of the mother cell membrane during engulfment.

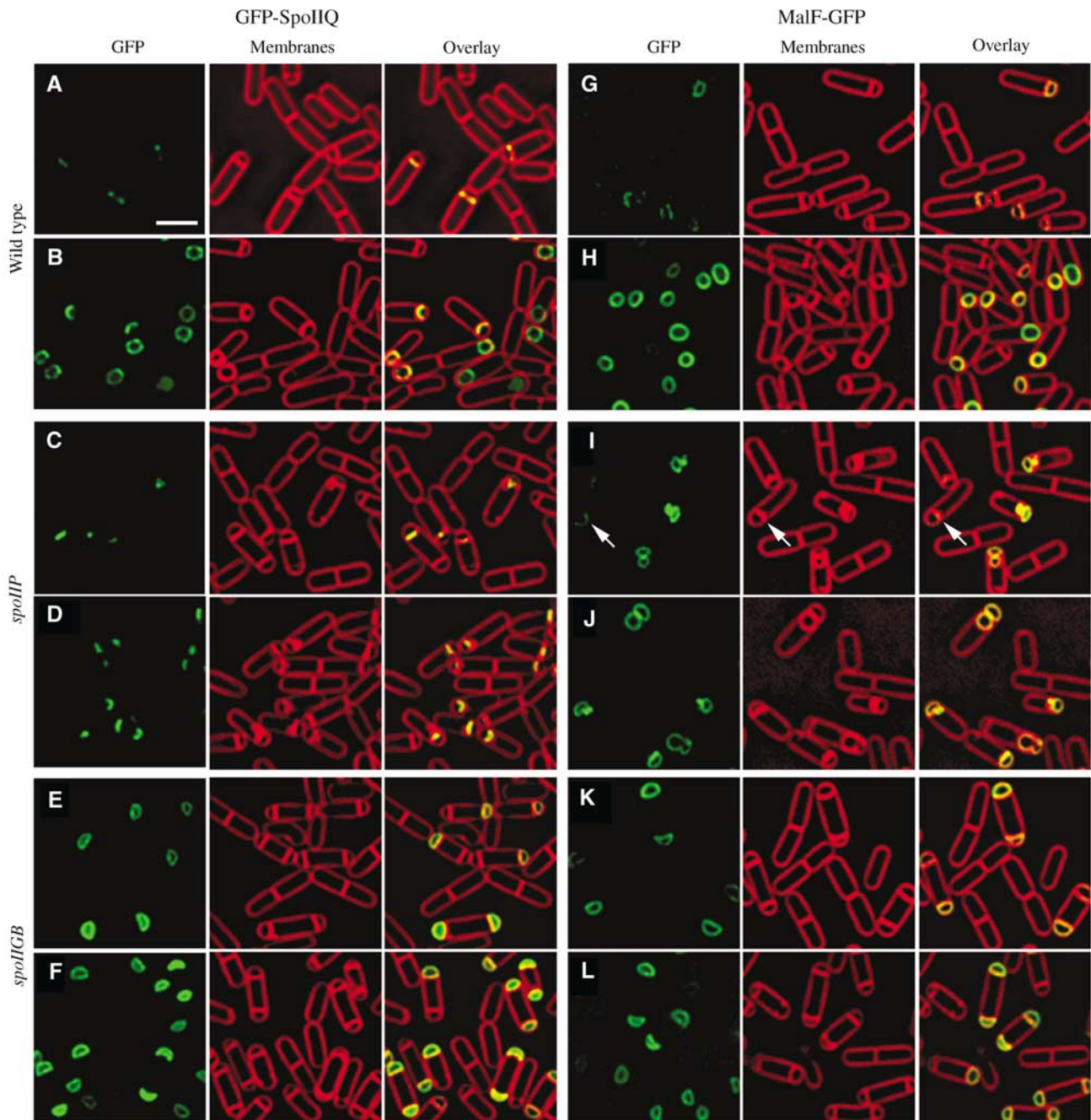
#### Retention of GFP-SpoIIQ in the septum requires a mother cell-expressed protein

One simple mechanism for a forespore-specific protein to track the engulfing mother cell membrane would be for its extracellular domain to interact with that of a mother cell-



**Figure 4** Western blot analysis and immunofluorescence of GFP-SpoIIQ and SpoIIQ-myc. (A) Immunoblot of GFP-SpoIIQ depicting specific proteolytic cleavage during engulfment. Full-length GFP-SpoIIQ is marked with an asterisk (\*). (B) Western blot of SpoIIQ-myc shows no smaller degradation product. Full-length SpoIIQ-myc is marked with an asterisk (\*). Immunofluorescence samples were taken at (C, E)  $t_2$  and (D, F)  $t_3$  and stained with Mitotracker Red (membranes in red), DAPI (DNA in blue) and SpoIIQ (GFP or Myc in green). (C, D) Localization patterns for GFP-SpoIIQ observed for fixed cells are the same as with live cells. (E, F) Localization of SpoIIQ-myc suggests that degradation occurs at the C-terminus of the protein since early patterns are similar to GFP-SpoIIQ, but little signal is observed in late sporangia. Scale bar, 2  $\mu$ m.

specific membrane protein. This zipper-like interaction would allow both proteins to localize to the boundary between the two cells, the septum. We therefore tested if



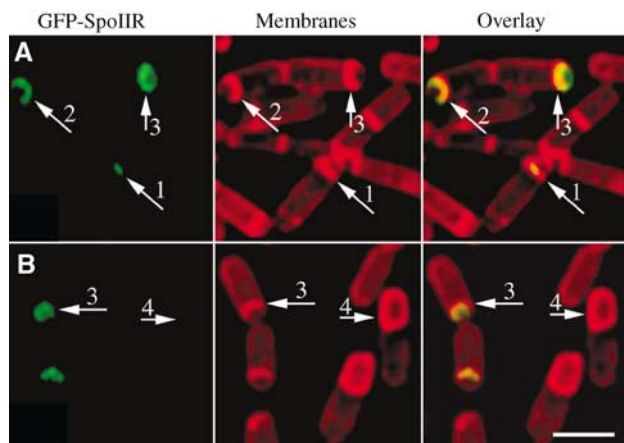
**Figure 5** Localization of GFP-SpoIIQ and MalF-GFP in different *spo* backgrounds. Samples were taken at  $t_2$  (A, C, E, G, I, K) and  $t_3$  (B, D, F, H, J, L) after resuspension and stained with FM 4-64 (red, middle column). GFP images (green) are on the left with overlaid images on the right. (A) AR126 (*spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ $\Omega$ cat*) at  $t_2$  and (B)  $t_3$ . (C) AR139 (*spoIIQ::spc spoIIIP::tet amyE::P<sub>spoIIQ</sub>gfp-spoIIQ $\Omega$ cat*) at  $t_2$  and (D)  $t_3$ . GFP-SpoIIQ remains at the septum. (E) AR156 (*spoIIQ::spc spoIIGB::erm amyE::P<sub>spoIIQ</sub>gfp-spoIIQ $\Omega$ cat*) at  $t_2$  and (F)  $t_3$ . GFP-SpoIIQ diffuses throughout the forespore membrane. (G) AR4 (*amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp $\Omega$ cat*) at  $t_2$  and (H)  $t_3$ . (I) AR154 (*spoIIIP::tet amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp $\Omega$ cat*) at  $t_2$  and (J)  $t_3$ . The arrow in (I) shows enrichment at the septum. (K) AR16 (*spoIIGB::erm amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp $\Omega$ cat*) at  $t_2$  and (L)  $t_3$ . Scale bar, 2  $\mu$ m.

retention of SpoIIQ in the septum required mother cell-specific gene expression, by localizing GFP-SpoIIQ in a strain lacking  $\sigma^E$ . Such mutants fail to initiate engulfment and divide at a second potential division site within the mother cell, producing sporangia with two forespores and a single anucleate mother cell (Kenney and Moran, 1987). Strikingly, in the absence of  $\sigma^E$ -directed gene expression, GFP-SpoIIQ freely diffused throughout the forespore membrane, identical to MalF-GFP (Figure 5E, F, K and L; quantitation in

Supplementary Figure 3). These results suggest that a  $\sigma^E$ -dependent protein is required to tether GFP-SpoIIQ to the sporulation septum, as well as to mediate its assembly into foci and hoops.

### SpoIIIR

The initial localization of integral membrane proteins expressed in the forespore to the septum suggests that membrane protein insertion is limited to this site, and that there is



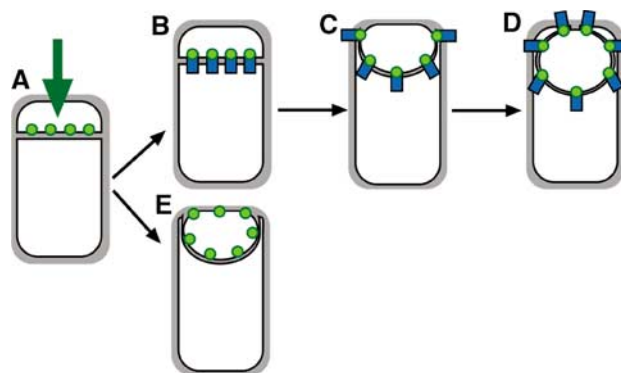
**Figure 6** Localization of GFP-SpoIIR. Samples were taken at  $t_2$  (A) and  $t_3$  (B) and stained with Mitotracker Red (red). Arrows 1 and 2 represent early sporangia, whereas arrows 3 and 4 represent late sporangia. GFP-SpoIIR (green) first localizes as a focus in the middle of the sporulation septum, and tracks with the engulfing mother cell membrane, but fluorescence is rapidly lost. Scale bar, 2  $\mu$ m.

a barrier to diffusion away from the septal membrane domain. This possibility could explain a puzzling aspect of cell-specific gene expression: mother cell-specific gene expression requires activation of a sigma factor produced before polar septation,  $\sigma^E$ , as well as a secreted protein produced in the forespore, SpoIIR (Karow *et al.*, 1995), and yet only adjacent daughter cells activate  $\sigma^E$  (Fujita and Losick, 2002). While purified SpoIIR can be added to cultures to allow premature activation of  $\sigma^E$  in cells that have not yet synthesized the polar septum, this normally never occurs (Hofmeister *et al.*, 1995). It has been proposed, but never demonstrated, that SpoIIR secretion is restricted to the sporulation septum, thereby preventing release of SpoIIR into the culture supernatant and the consequent aberrant activation of  $\sigma^E$  in neighboring cells.

We therefore localized SpoIIR, using a functional N-terminal GFP fusion to SpoIIR (Materials and methods), which fully complemented a *spoIIR* null mutation. Sporulation was induced by resuspension and GFP-SpoIIR localized at  $t_2$  and  $t_3$  (Figure 6A and B), respectively. In early sporangia, GFP-SpoIIR first localized as a focus at the septal midpoint (Figure 6A, arrow 1), similar to GFP-SpoIIQ. Later, GFP-SpoIIR lined the septum, moving along with the mother cell membranes (Figure 6A, arrows 2 and 3). GFP-SpoIIR was only transiently present during sporulation, and as engulfment neared completion no GFP fluorescence was observed, suggesting that GFP-SpoIIR is completely degraded (Figure 6B, arrow 4). We were unable to detect GFP-SpoIIR by Western blot analysis, likely because of its low expression relative to SpoIIQ (Karow *et al.*, 1995).

## Discussion

The results presented here provide evidence that localization of proteins to the bacterial septum can be mediated by an extremely simple mechanism involving an interaction between extracellular domains of proteins located on either side of the septum (Figure 7). During sporulation, these



**Figure 7** Model for localization of forespore membrane proteins. (A) Any membrane protein (green) synthesized by a  $\sigma^F$ -dependent promoter inserts directly into the forespore septal domain. (B–D) Localized proteins (such as SpoIIQ) are retained through an interaction with a partner protein (blue) made in the mother cell or the septal peptidoglycan. (E) Nonlocalized proteins (such as MalF-GFP) freely diffuse throughout the forespore membrane.

zipper-like interactions provide an effective means for forespore proteins to track the engulfing mother cell membrane, and for mother cell proteins to be retained in the septum (Figure 7A–D). If not, as is the case with GFP-SpoIIQ in the absence of its mother cell-specific ligand or a non-native protein (MalF-GFP), the protein is freely diffusible in the forespore membrane (Figure 7E). The case we describe here likely involves at least three different proteins, since septal localization of SpoIIQ requires an unidentified mother cell-expressed protein, and we have recently found that localization of the mother cell-expressed protein SpoIIIAH to the septum requires SpoIIQ. Although the extracellular domains of SpoIIIAH and SpoIIQ directly interact, this interaction is not essential for the SpoIIQ septal retention, suggesting that the primary tether for SpoIIQ is another mother cell-expressed protein (B Blaylock, X Jiang, A Rubio, C Moran and K Pogliano, in preparation). We note that a similar mechanism could mediate septal localization in nondifferentiating bacterial cells, since interactions between the extracellular domains of cytoplasmic membrane proteins would be restricted to membrane invaginations, such as the septum.

It does not appear that this zipper-like interaction directs the initial localization of forespore-expressed membrane proteins to the septum, since we have found that any membrane protein expressed in the forespore is initially localized to the septum (Figure 7A), prior to diffusing throughout the rest of the forespore membrane. Indeed, there seem to be only two requirements for the initial localization of proteins to the forespore septum: a  $\sigma^F$ -dependent promoter and one or more membrane-spanning segments. These results seem most consistent with directed insertion of membrane proteins into the forespore septum followed by their slow diffusion throughout the forespore membrane (Figure 7A and E) since we found that the heterologous GFP fusion proteins (i.e. MalF-GFP), after their direct insertion into the septum, freely diffuse in the forespore membrane. However, it remains possible that membrane protein insertion occurs throughout the forespore membrane and newly synthesized proteins either quickly diffuse to the septum and are degraded everywhere except the septum, or that the folding of GFP occurs most rapidly at the septal membrane domain. While further experiments are

necessary to discriminate among these models, we favor the directed insertion hypothesis, because the forespore is a unique and tiny bacterial cell that likely consists of one cell pole and a newly synthesized septum. Prior studies have demonstrated that bacterial cell poles are static structures, with little turnover of surface proteins, membrane or cell wall material compared to the longitudinal envelope (Anderson *et al*, 1978; Mobley *et al*, 1984; Koch and Burdett, 1986; Clarke-Sturman *et al*, 1989; Merad *et al*, 1989). Thus, if the forespore has only a small volume of longitudinal envelope, the septum might comprise the bulk of its metabolically active cell envelope, thereby becoming the preferred site of membrane protein insertion. Regardless of the precise mechanism, our results suggest that in the forespore newly synthesized membrane proteins are initially restricted to the sporulation septum and later either captured at a specific location or released.

One clear mechanism by which membrane proteins could be restricted to the septum would be if the membrane protein biogenesis machinery were localized to the septum, or if there were a sporulation-specific protein insertion complex. We tested if the known or likely components of the membrane protein insertion machinery are localized to the septum by constructing a functional GFP fusion to SecDF however, this protein was uniformly distributed throughout the cell membrane during both vegetative growth and sporulation, with no quantifiable enrichment at either vegetative or sporulation septa (A Rubio, X Jiang and K Pogliano, in preparation). Recently, it has been shown by Brandon *et al* (2003) that in *E. coli*, SecYEG is uniformly distributed throughout the membrane. We found the same to be true of two candidates for a sporulation-specific protein insertion complex, SpoIIIJ and YqjG, which share homology with YidC from *E. coli*, a protein required for membrane protein insertion (Samuelson *et al*, 2000; Scotti *et al*, 2000). However, while SpoIIIJ is essential for sporulation, the available evidence suggests that SpoIIIJ and YqjG are involved in membrane protein folding rather than insertion (Murakami *et al*, 2002; Serrano *et al*, 2003).

We do not believe it is likely that newly synthesized membrane proteins are restricted to the sporulation septum in the much larger mother cell, which has a large area of longitudinal cell envelope. Our attempts to test directly the localization of mother cell-expressed membrane proteins, such as MalF, failed, because the hybrid proteins failed to reach high steady-state levels. However, Rudner and Losick have presented evidence that in the mother cell, septal localization is mediated by the diffusion of randomly inserted membrane proteins followed by their capture at the septum (Rudner *et al*, 2002). In addition, we have also obtained evidence that SpoIIIAH is randomly inserted into the mother cell membrane, and only later retained in the septum by its interaction with SpoIIQ (B Blaylock, X Jiang, A Rubio, C Moran and K Pogliano, in preparation), in support of the diffusion and capture model.

We propose that initial localization of forespore-expressed exported proteins to the septum plays two crucial roles in the establishment of polarity. First, the initial restriction of newly synthesized membrane proteins to the septum would increase the local concentrations of proteins (such as SpoIIQ) required for septal localization of mother cell-expressed proteins (such as SpoIIIAH), thereby increasing the affinity

of the mother cell proteins for the septum. Second, restriction of forespore membrane proteins to the septum could also limit the forespore to mother cell signaling event necessary for the onset of mother cell-specific gene expression. This crucial event requires the forespore-expressed and secreted protein SpoIIQ, which can be added to the culture medium to mediate the premature activation of mother cell transcription in sporangia lacking a sporulation septum (Hofmeister *et al*, 1995). Based on our localization studies of GFP-SpoIIQ, we propose that the restriction of SpoIIQ secretion to the sporulation septum prevents its release into the culture supernatant, which would have devastating effects on the timing of mother cell-specific gene expression.

Our results also demonstrate that SpoIIQ displays a remarkably dynamic and complex localization pattern consistent with its multiple roles during sporulation. It is interesting that during the punctate and soluble stages of GFP-SpoIIQ localization, the forespore is hexagonal, with GFP-SpoIIQ assembling helical arcs at the vertices. This structure is reminiscent of the actin-like proteins MreB and Mbl, and the helical incorporation of peptidoglycan dictated by Mbl (Jones *et al*, 2001; Carballido-Lopez and Errington, 2003; Daniel and Errington, 2003). Therefore, it is possible that the assembly of SpoIIQ into arcs is mediated either by MreB or Mbl, or the peptidoglycan itself, along which the engulfing membrane has been proposed to track (Illing and Errington, 1991; Kellner *et al*, 1996; Pogliano *et al*, 1999; Abanes-De Mello *et al*, 2002).

Previous studies demonstrated that under certain growth conditions SpoIIQ is required for the phagocytosis-like process of engulfment, with mutants unable to allow membrane migration across the cell pole. However, SpoIIQ is essential for forespore-specific gene expression under all conditions, as it is required for both the synthesis and activation of the second forespore-specific transcription factor  $\sigma^G$  (Londono-Vallejo *et al*, 1997; Sun *et al*, 2000). Interestingly, both of these latter functions require a signal from the mother cell to the forespore: expression of the gene encoding  $\sigma^G$  requires an unidentified mother cell-expressed protein (Evans *et al*, 2003), while the activation of  $\sigma^G$  requires both the completion of engulfment, a process that depends on several mother cell-expressed proteins (SpoIID, M, P), and the eight mother cell-expressed proteins encoded by the *spoIIIA* operon (Illing and Errington, 1991; Kellner *et al*, 1996; Pogliano *et al*, 1999; Abanes-De Mello *et al*, 2002). Clearly, SpoIIQ is poised to play a key role in both of these functions, as it interacts with at least two mother cell-expressed proteins: its unidentified tether and SpoIIIAH (tethered by SpoIIQ). We postulate that the striking SpoIIQ assemblage described here represents an intracellular signal transduction system comprised of proteins made in both the mother cell and the forespore, coupling the completion of engulfment to cell-specific gene expression.

## Materials and methods

### **Bacterial strains, genetic manipulations and growth conditions**

*B. subtilis* strains listed in Table I are derivatives of wild-type strain PY79 (Youngman *et al*, 1984) and were constructed by standard methods (Dubnau and Davidoff-Abelson, 1971). Construction of GFP fusions is described below with plasmids listed in Table I; all plasmids were sequenced by the Shared Resource UCSD Cancer Center (funded in part by NCI Cancer Center Support Grant #2 P30



CA23100-18). The GFP fusion protein encoding plasmids were introduced into the *B. subtilis* chromosome by marker replacement at the *amyE* locus, selecting chloramphenicol resistance (5 µg/ml) and confirmed by spectinomycin sensitivity (100 µg/ml). Strains carrying these fusions produced wild-type levels of heat-resistant spores (~10<sup>8</sup> spores/ml). Sporulation was induced by resuspension with *t* representing the hours after onset of sporulation (Sterlini and Mandelstam, 1969). Sporulation efficiency was determined after 24 h of sporulation at 37°C, by heating samples to 80°C for 20 min, plating on rich media and quantifying the number of heat-resistant spores per milliliter.

### GFP fusion constructions

Heterologous GFP fusions were constructed by amplification of membrane-spanning segments 1 and 2 from *E. coli* membrane proteins MalF, LacY, SecY and MotA and *B. subtilis* membrane protein AraP, as predicted by HMMTOP and TMpred programs (Tusnady and Simon, 2001). All PCR products contained flanking *Bam*HI restriction sites for ease of cloning into existing GFP-containing plasmids (Sharp and Pogliano, 2002) and resulted in plasmids with membrane-spanning segments upstream and in frame with *gfp* (see Table I). GFP-SpoIIQ was constructed by PCR

amplification from PY79 using primers 5'-CAGAATGTTGGGGCCGCGGGTGAACAATGAGAGAG-3' and 5'-GATAGACGTGGGGCCCAATTAAGACTGTTCACT-3' (*Not*I restriction site shown in bold). This fragment was then digested with *Not*I and ligated to an existing GFP-containing plasmid (pMDS13) digested with *Eag*I (Sharp and Pogliano, 2002) to yield pAR47. This plasmid, which has the *spoIIQ* promoter, translational initiation site and first six codons driving expression of *gfp* fused in frame with the rest of *spoIIQ*, was transformed into KP575, producing AR126. GFP-SpoIIR was constructed by PCR amplification using primers 5'-CACGGACGGCCGCGGTGGGAGCGATGAAAAAACA-3' and 5'-TTTTCGAGACTGTTCACTGTTAGGAAAAAG-3' (*Eag*I restriction site shown in bold). This fragment and pMDS78 were digested with *Eag*I and ligated to yield pAR46, which has the *spoIIR* promoter driving expression of *gfp* fused in frame with *spoIIR*. pAR46 was transformed into AR130, producing AR132.

### Construction of myc-tagged SpoIIQ

Primers 5'-AAGGAATTCAGTCTAGCTGAGAAAAGACG-3' (*Eco*RI restriction site shown in bold) and 5'-TTTTCGAGACTGTTCACTGTTCTTC-3' (*Ava*I restriction site shown in bold) were used to amplify a 486 bp fragment from PY79 corresponding to the 3' end of *spoIIQ*.

**Table I** *B. subtilis* strains and plasmids used in this study

Strain	Genotype	Source
PY79		Youngman <i>et al</i> (1984)
KP8	<i>spoIID::Tn917</i> ΩHU298	Lopez-Diaz <i>et al</i> (1986)
KP161	<i>spoIIGB::erm</i>	Kenney and Moran (1987)
KP298	<i>amyE::sspE(2G)-lacZ</i> Ωcat <i>spoIIIG::neo</i>	Oke and Losick (1993)
KP513	<i>spoIIP::tet</i>	Frandsen and Stragier (1995)
KP519	<i>spoIIM::Tn917</i> ΩHU287	Smith <i>et al</i> (1993)
KP575	<i>spoIIQ::spc</i>	Londono-Vallejo <i>et al</i> (1997)
AR4	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
AR6	<i>amyE::P<sub>spoIIQ</sub>gfp</i> Ωcat	This work
AR16	<i>spoIIGB::erm amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
AR29	<i>amyE::P<sub>spoIIQ</sub>lacY<sub>12</sub>-gfp</i> Ωcat	This work
AR31	<i>amyE::P<sub>spoIIQ</sub>araP<sub>12</sub>-gfp</i> Ωcat	This work
AR77	<i>amyE::P<sub>spoIIR</sub>gfp</i> Ωcat	This work
AR78	<i>amyE::P<sub>spoIIR</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
AR90	<i>amyE::P<sub>csfB</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
AR92	<i>amyE::P<sub>csfB</sub>gfp</i> Ωcat	This work
AR104	<i>amyE::P<sub>spoIIQ</sub>secY<sub>12</sub>-gfp</i> Ωcat	This work
AR111	<i>amyE::P<sub>spoIIQ</sub>motA<sub>12</sub>-gfp</i> Ωcat	This work
AR126	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat	This work
AR130	<i>spoIIR::kan</i>	Karow <i>et al</i> (1995)
AR132	<i>spoIIR::kan amyE::P<sub>spoIIR</sub>gfp-spoIIR</i> Ωcat	This work
AR137	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat	This work
	<i>spoIID::Tn917</i> ΩHU298	
AR139	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat <i>spoIIP::tet</i>	This work
AR140	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat	This work
	<i>spoIIM::Tn917</i> ΩHU287	
AR151	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat <i>spoIIIG::neo</i>	This work
AR152	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat <i>spoIID::Tn917</i> ΩHU298	This work
AR153	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat <i>spoIIM::Tn917</i> ΩHU287	This work
AR154	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat <i>spoIIP::tet</i>	This work
AR156	<i>spoIIQ::spc amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat <i>spoIIGB::erm</i>	This work
AR161	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat <i>spoIIIG::neo</i>	This work
Plasmid	Insert	Source
pMDS12	<i>amyE::gfp</i> Ωcat	Sharp and Pogliano (2002)
pMDS13	<i>amyE::P<sub>spoIIQ</sub>gfp</i> Ωcat	Sharp and Pogliano (2002)
pMDS78	<i>amyE::P<sub>spoIIR</sub>gfp</i> Ωcat	Sharp and Pogliano (2002)
pAR1	<i>amyE::P<sub>spoIIQ</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
pAR5	<i>amyE::P<sub>spoIIQ</sub>lacY<sub>12</sub>-gfp</i> Ωcat	This work
pAR7	<i>amyE::P<sub>spoIIQ</sub>araP<sub>12</sub>-gfp</i> Ωcat	This work
pAR22	<i>amyE::P<sub>spoIIR</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
pAR23	<i>amyE::P<sub>csfB</sub>gfp</i> Ωcat	This work
pAR28	<i>amyE::P<sub>csfB</sub>malF<sub>12</sub>-gfp</i> Ωcat	This work
pAR34	<i>amyE::P<sub>spoIIQ</sub>secY<sub>12</sub>-gfp</i> Ωcat	This work
pAR39	<i>amyE::P<sub>spoIIQ</sub>motA<sub>12</sub>-gfp</i> Ωcat	This work
pAR46	<i>amyE::P<sub>spoIIR</sub>gfp-spoIIR</i> Ωcat	This work
pAR47	<i>amyE::P<sub>spoIIQ</sub>gfp-spoIIQ</i> Ωcat	This work

The vector, pKL94 (Perez *et al*, 2000) and this PCR fragment were digested with *EcoRI* and *AvaI* and ligated to yield pAR50, with the 3' end of *spoIIQ* in frame with the c-myc epitope. Transformation of pAR50 into PY79 via a single homologous recombination event integrating the *spoIIQ-myc*-containing plasmid at the chromosomal *spoIIQ* locus, resulted in strain AR190.

#### Microscopy and image analysis

For GFP visualization, live cells were stained with 4',6'-diamidino-2-phenylindole (DAPI) (0.2 µg/ml; Molecular Probes) and Mitotracker Red (0.1 µg/ml; Molecular Probes) or with DAPI (0.2 µg/ml) and FM 4-64 (5 µg/ml; Molecular Probes) as described (Sharp and Pogliano, 1999), and images were collected with an Applied Precision optical sectioning microscope. GFP enrichment at the forespore septal membrane was quantified using the 'edit polygon' function of Delta Vision software. Briefly, GFP fluorescence at the septum, pole and mother cell cytoplasmic membrane was compared to the FM 4-64- or Mitotracker Red-stained membranes (Pogliano *et al*, 1999, 2002), after manually drawing polygons. The integrated intensity of each region from 15 cells was determined using the 'polygon statistics' function. Immunofluorescence microscopy was performed as described (Perez *et al*, 2000).

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#### Western blot analysis

Samples were prepared as described (Pogliano *et al*, 1997), heated at 50°C for 10 min, loaded on a 12.5% SDS-polyacrylamide gel and transferred to nitrocellulose (Perez *et al*, 2000). A 1:500 dilution of mouse monoclonal anti-GFP antibodies (Roche) was used, followed by a 1:500 dilution of horseradish peroxidase-labeled anti-mouse antibodies, and enhanced chemiluminescence (ECL, Amersham) was used for detection.

#### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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