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GerM is required to assemble the basal platform of the SpoIIIA–SpoIIQ transenvelope complex during sporulation in *Bacillus subtilis*

Christopher D. A. Rodrigues, Fernando H. Ramírez-Guadiana, Alexander J. Meeske, Xindan Wang and David Z. Rudner*
Department of Microbiology and Immunobiology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA.

Summary

Sporulating *Bacillus subtilis* cells assemble a multimeric membrane complex connecting the mother cell and developing spore that is required to maintain forespore differentiation. An early step in the assembly of this transenvelope complex (called the A–Q complex) is an interaction between the extracellular domains of the forespore membrane protein SpoIIQ and the mother cell membrane protein SpoIIIAH. This interaction provides a platform onto which the remaining components of the complex assemble and also functions as an anchor for cell–cell signalling and morphogenetic proteins involved in spore development. SpoIIQ is required to recruit SpoIIIAH to the sporulation septum on the mother cell side; however, the mechanism by which SpoIIQ specifically localizes to the septal membranes on the forespore side has remained enigmatic. Here, we identify GerM, a lipoprotein previously implicated in spore germination, as the missing factor required for SpoIIQ localization. Our data indicate that GerM and SpoIIIAH, derived from the mother cell, and SpoIIQ, from the forespore, have reciprocal localization dependencies suggesting they constitute a tripartite platform for the assembly of the A–Q complex and a hub for the localization of mother cell and forespore proteins.

Accepted 2 July, 2016. *For correspondence. E-mail rudner@hms.harvard.edu; Tel. (617) 432-4455; Fax (617) 738-7664.

Introduction

Bacteria possess highly organized internal architectures that are intimately linked to essential biological processes (Shapiro *et al.*, 2009; Rudner and Losick, 2010; Lenz and Sogaard-Andersen, 2011). The mechanisms by which proteins and protein complexes localize to specific subcellular sites remain incompletely understood (Rudner and Losick, 2010). In many cases, the localization of one protein, or a set of proteins, to a specific subcellular position depends on others and this dependency underlies the ordered assembly of large macromolecular complexes (Goehring *et al.*, 2006; Gamba *et al.*, 2009; Diepold *et al.*, 2010; Li and Sourjik, 2011). An example of this ordered assembly can be found in specialized secretion systems (Lybarger *et al.*, 2009; Diepold *et al.*, 2010; Chandran, 2013). These multicomponent nano-machines are involved in the transport of proteins (and sometimes DNA) between bacterial cells or between bacteria and host cells (Buttner, 2012; Dalbey and Kuhn, 2012; Chandran, 2013; Portaliou *et al.*, 2016). Their assembly typically involves the formation of a basal platform followed by the ordered association of the remaining components (Lybarger *et al.*, 2009; Diepold *et al.*, 2010; Diepold *et al.*, 2011; Morimoto *et al.*, 2014). Here, we define the molecular basis for the localization of the basal platform of a novel transenvelope complex that resembles a specialized secretion system connecting two daughter cells during the developmental process of sporulation.

In response to starvation, *Bacillus subtilis* enters a developmental pathway that culminates in the formation of a stress-resistant spore (Errington, 2003; Higgins and Dworkin, 2012; Tan and Ramamurthi, 2014). The first morphological event in this process is the formation of an asymmetric septum, generating two cells of unequal size and distinct developmental fates. The smaller cell (called the forespore) differentiates into the dormant spore while the larger cell (referred to as the mother cell) nurtures the forespore and prepares it for dormancy. During this developmental process, the mother cell and forespore follow cell-type-specific programs of

gene expression that are linked to each other by cell–cell signalling pathways. Polar division triggers the activation of the σF transcription factor in the forespore, which, in turn, leads to the activation of σE in the mother cell. At a later stage, transcription under σG control in the forespore triggers σK activation in the mother cell. Initially, the forespore and mother cell lie side-by-side separated by a double membrane septum. However, shortly after polar division, cell wall hydrolases produced in the mother cell degrade the septal peptidoglycan and aid in the migration of the mother cell membranes around the forespore in a phagocytic-like process called engulfment. Upon completion of engulfment, the forespore resides as a free protoplast in the mother cell. At this late stage, the mother cell packages the spore in protective layers while the spore prepares for dormancy. Finally, mother cell lysis releases the mature spore into the environment.

During the morphological process of engulfment the mother cell and forespore assemble a multimeric complex that spans the double membrane between them (Blaylock *et al*., 2004; Doan *et al*., 2005; Doan *et al*., 2009). This transenvelope complex (called the A–Q complex) is composed of eight mother cell proteins (SpoIIIAA–SpoIIIAH, referred to as AA–AH, for simplicity) encoded in the *spoIIIA* operon (Illing and Errington, 1991) and one forespore protein SpoIIQ (Q) (Londono-Vallejo *et al*., 1997). Sporulating cells lacking any of these factors produce forespores that are smaller in size, develop membrane invaginations, and in some instances lose their integrity (Doan *et al*., 2009; Rodrigues *et al*., 2013). In addition, these forespores are unable to maintain transcriptional potential including gene expression under σG control (Sun *et al*., 2000; Camp and Losick, 2009; Doan *et al*., 2009). Thus, this complex is essential to maintain forespore development. Several of the SpoIIIA proteins share remote homology to components of specialized secretion systems found in Gram-negative bacteria, suggesting that the A–Q complex functions as a novel secretion apparatus (Doan *et al*., 2009; Camp and Losick, 2008; Meisner *et al*., 2008). In support of this idea, the extracellular domain of SpoIIIAH (AH) has been shown to share structural homology with the PrgK/EscJ ring-forming proteins found in Type III secretion systems (Yip *et al*., 2005; Levdikov *et al*., 2012; Meisner *et al*., 2012). It remains unclear what the A–Q complex transports; however, the mother cell protein SpoIIIAA (AA) resembles a secretion ATPase suggesting that if this complex is a secretion system then transport likely occurs from mother cell to forespore (Doan *et al*., 2009). In the context of this model, the secreted factor(s) would be necessary to maintain the metabolic and/or transcriptional potential of the forespore (Camp and Losick, 2009).

One of the earliest steps in the assembly of the A–Q complex is the interaction between the forespore membrane protein Q and the mother cell membrane protein AH. The extracellular domains of these two proteins associate in the space between the double membrane septum (Blaylock *et al*., 2004; Doan *et al*., 2005). This transenvelope interaction is required for the assembly of the rest of the complex and is thought to function as a basal platform (Camp and Losick, 2008; Doan *et al*., 2009). However, protein localization studies indicate that the assembly of this platform requires additional factors (Rubio and Pogliano, 2004; Fredlund *et al*., 2013; Rodrigues *et al*., 2013). In particular, although the specific localization of AH to the septal membrane on the mother cell side depends on Q, the septal localization of Q on the forespore side is largely unaffected by the absence of AH (Fredlund *et al*., 2013; Rodrigues *et al*., 2013). We have previously shown that in addition to AH, proper localization of Q requires degradation of the septal peptidoglycan and an additional unidentified protein produced in the mother cell under σE control (Rodrigues *et al*., 2013).

Here, we report that GerM, a lipoprotein previously implicated in spore germination (Sammons *et al*., 1987; Slynn *et al*., 1994) is the missing mother cell protein required for Q localization. We show that GerM and AH are required to anchor Q at the septum and in their absence Q becomes uniformly distributed in the forespore membranes. Furthermore, forespores in the double mutant fail to thrive, do not maintain σG activity, and exhibit a synergistic sporulation defect. Consistent with the idea that GerM is Q's elusive partner, we show that GerM is sufficient to localize Q to the septal membrane in the absence of all other σE-dependent proteins, provided the septal peptidoglycan is thinned. Furthermore, protein localization studies reveal that GerM is surface-exposed and, like AH (Blaylock *et al*., 2004; Doan *et al*., 2005), localizes to the septal membrane in a manner that depends on Q. Finally, we show that GerM is required for the proper localization of SpoIIIAG (AG), an essential component of the A–Q complex. Collectively, our results suggest that AH, Q and GerM form a tripartite basal platform in the assembly of the A–Q transenvelope complex.

Results

GerM is required for σG activity and forespore development

In the course of our analysis of new sporulation genes identified by transposon-sequencing (Meeske *et al*., 2016), we characterized a subset of previously identified sporulation mutants that had not been examined cytologically. One of these was *gerM*, a gene identified almost 30 years ago in a screen for germination mutants (Sammons *et al*.,

1987). Cells lacking *gerM* were reported to have pleiotropic defects in sporulation and impaired germination (Sammons *et al*., 1987; Slynn *et al*., 1994). We introduced the *gerM* null mutant into a strain that harboured fluorescent reporters for all four sporulation-specific sigma factors (Meeske *et al*., 2016) and analyzed the cells during a sporulation time course (Figs 1 and S1). No obvious defects were observed at the early stages of sporulation (Fig. S1); however, at 3.5 h (T3.5) after the onset of sporulation, a subset of sporulating cells lacking GerM had reduced gene expression under the control of the late-acting forespore transcription factor σG (Fig. 1A and quantified in Fig. 2). Furthermore, in many cases these forespores appeared smaller in size (Fig. 1A and B). Introduction of *gerM* at an ectopic locus restored σG activity, forespore morphology and wild-type levels of sporulation (Fig. S2). The mutant phenotypes associated with the absence of GerM were similar to those observed in cells lacking proteins in the A–Q transenvelope complex (Doan *et al*., 2009; Rodrigues *et al*., 2013), suggesting that *gerM* might function in the same genetic pathway.

gerM *is in the A–Q pathway*

To investigate whether *gerM* is in the *A–Q* pathway, we took advantage of the partially penetrant phenotypes of the *AH* mutant. Unlike all other components of the A–Q complex, cells lacking AH have a relatively mild sporulation defect (1–10% sporulation efficiency compared to 0.01–0.001% for mutants in any other component) and ~95% of the forespores are smaller in size and have reduced σG activity (Fig. 2) (Doan *et al*., 2009). The Δ*gerM* mutant had a similar reduction in sporulation efficiency but a less penetrant defect in σG activity and forespore size (Fig. 2). If the role of GerM in maintaining forespore morphology and σG activity is related to that of the A–Q complex, then we would expect a Δ*gerM* Δ*AH* double mutant to display synergistic phenotypes and this is what we observed (Fig. 2). The Δ*gerM* Δ*AH* double mutant had a sporulation efficiency of 0.03%, similar to the Δ*Q* mutant (Fig. 2). Furthermore, almost all of the sporulating cells lacking GerM and AH had smaller forespores and reduced σG activity (Fig. 2). Introduction of *gerM* at an ectopic locus in the Δ*gerM* Δ*AH* double mutant restored sporulation efficiency and σG activity to levels observed in the Δ*AH* null (Fig. S2). These results are consistent with the idea the GerM functions in the A–Q pathway and raised the possibility it is part of this transenvelope complex.

GerM and AH are required for Q localization

Our previous work indicated that Q localization requires AH and a second unidentified protein synthesized in the mother cell under σE control (Rodrigues *et al*., 2013). *gerM* is predicted to encode a secreted lipoprotein [PRED-LIPO; reliability score of 0.995, (Bagos *et al*., 2008)] and expression profiling, 5′ end mapping (Feucht

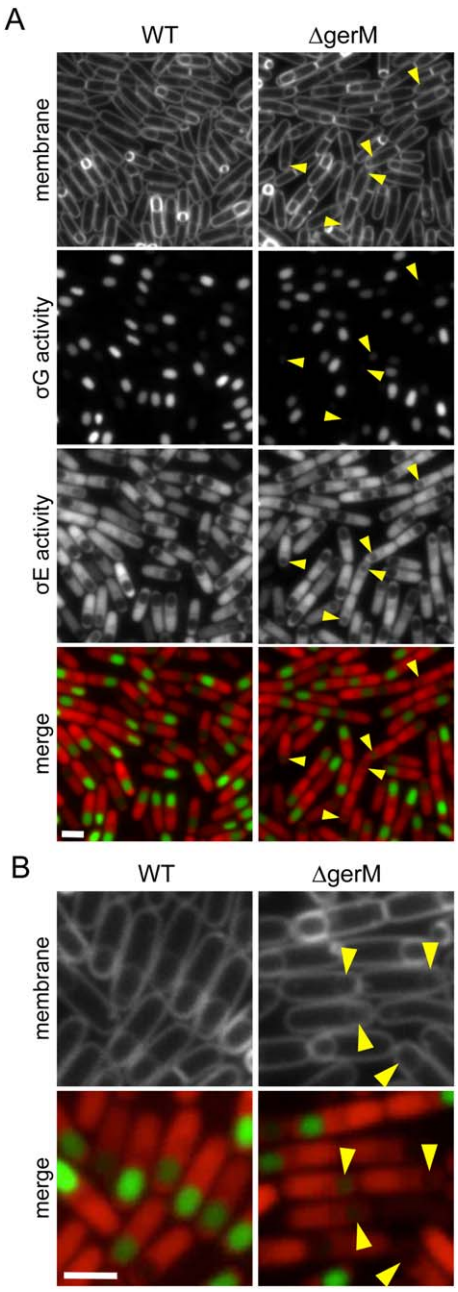

Fig. 1. GerM is required for σG activity and forespore morphology.
A. Representative images of wild-type (WT, BCR1071) and Δ*gerM* (BAM833) sporulating cells at hour 3.5 (T3.5) of sporulation. Images (from top to bottom) are membrane staining with TMA-DPH, σG activity (P_{sspB}-*cfp*), σE activity (P_{spoIID}-*mCherry*) and merge of σG activity (green) and σE activity (red). Small and/or collapsed forespores with reduced σG activity are highlighted (yellow carets). Scale bar indicates 2 μm. A complete sporulation time-course comparing wild-type and Δ*gerM* can be found in Fig. S1.
B. Larger images highlighting the defects in σG activity and forespore morphology in the Δ*gerM* mutant.

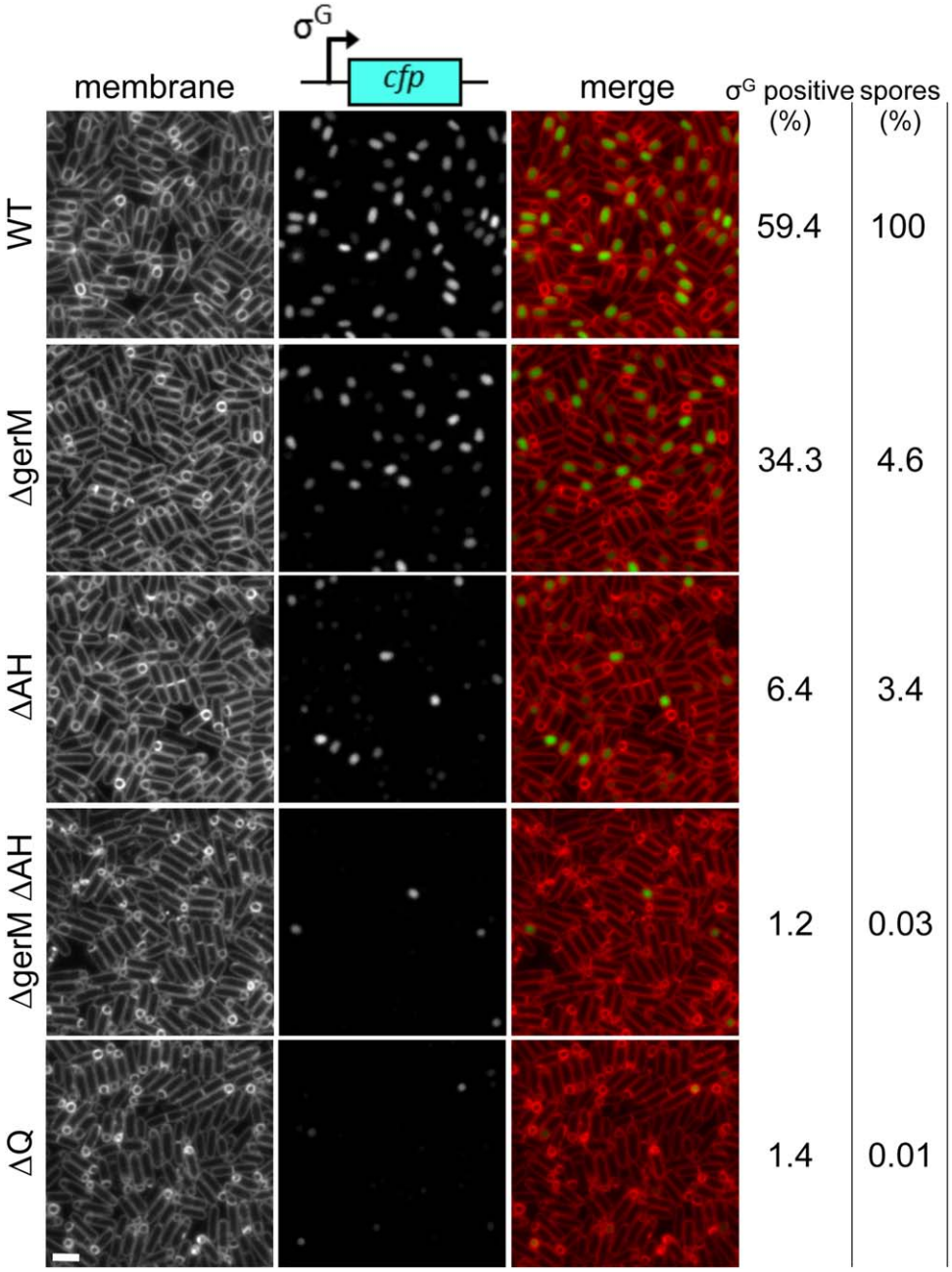

Fig. 2. Synergistic defects in the *gerM AH* double mutant. Representative images of sporulating cells harboring the σG-dependent reporter P_{sspB}-*cfp* at hour 4 after the onset of sporulation. Images are wild-type (WT, BTD1609), Δ*gerM* (BCR1190), Δ*AH* (BCR1233), Δ*gerM* Δ*AH* double mutant (BCR1200) and Δ*Q* (BCR151). TMA-DPH-stained membranes (left), σG activity (middle) and a merged image (right) are shown. Scale bar represents 2 μm. The percentage of σG positive cells ($n > 600$) at hour 4 are shown (see *Experimental procedures* section for details). Spore titers relative to wild-type at hour 30 are indicated on the right. The data are representative of two biological replicates.

et al., 2003; Eichenberger *et al*., 2004; Steil *et al*., 2005) and our fluorescence microscopy indicate that *gerM* is part of the σE regulon (Fig. S3). Accordingly, we investigated whether GerM was the missing mother cell protein required to localize Q to the septal membranes on the forespore side. In the absence of AH or GerM, a functional GFP–Q fusion retained much of its localization in the septal membranes with weaker and heterogenous signal in the peripheral membranes (Fig. 3A). Strikingly, in cells lacking both GerM and AH, GFP–Q was evenly distributed in all forespore membranes (Fig. 3A). The extent of mislocalization was indistinguishable from that observed in cells lacking the mother cell transcription factor σE (Fig. 3A) (Rubio and Pogliano, 2004). Finally and as expected, introduction of *gerM* at an ectopic locus in the Δ*gerM* Δ*AH* double mutant restored proper Q localization (Fig. S4).

The extracellular domain of Q contains a degenerate LytM domain (Meisner and Moran, 2011). Functional LytM domains cleave peptide crossbridges that link the glycan strands in peptidoglycan (Odintsov *et al*., 2004). Although Q's LytM domain lacks the catalytic residues

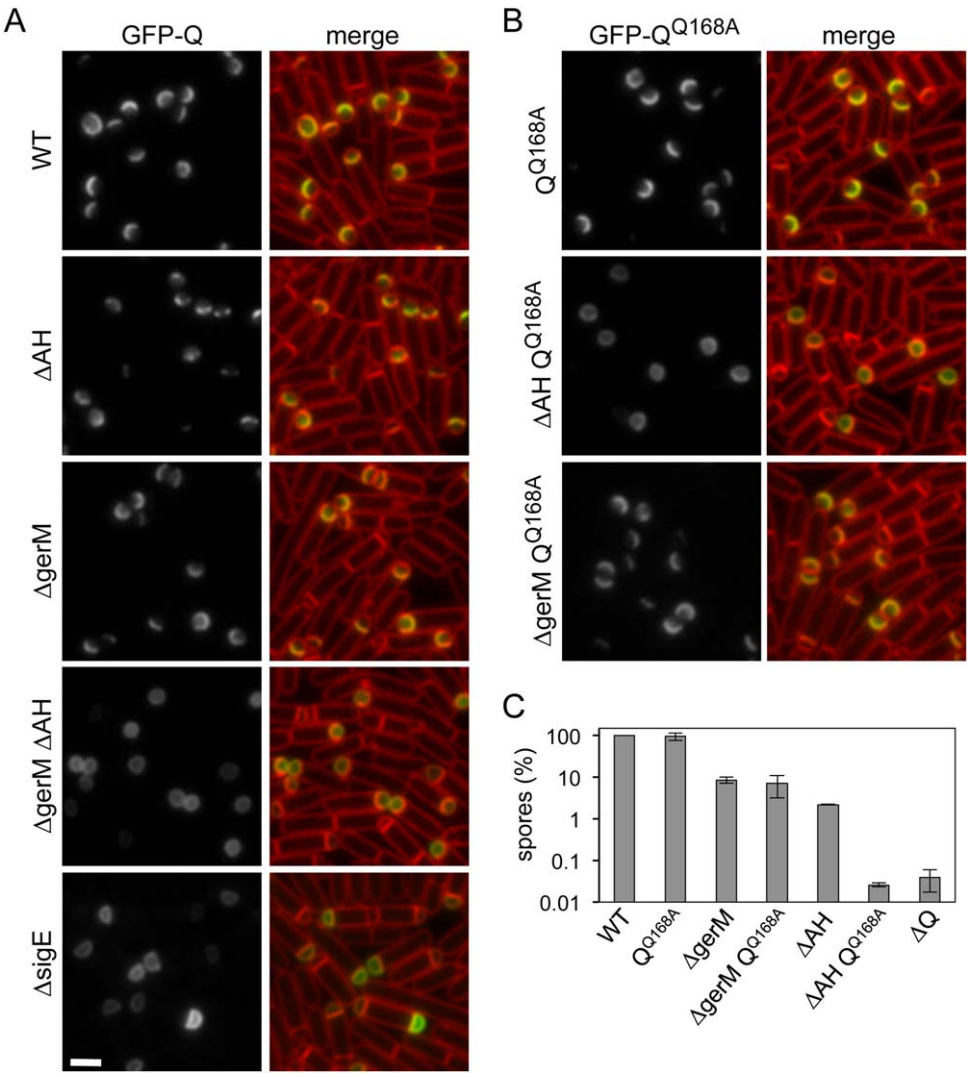

Fig. 3. GFP–Q localization requires GerM and AH.
A. Representative images of GFP–Q localization in sporulating cells at hour 2 of sporulation. Images are from wild-type (BCR46), Δ*AH* (BCR56), Δ*gerM* (BCR1211), the Δ*AH* Δ*gerM* double mutant (BCR1197) and Δ*sigE* (BKM1930). Scale bar represents 2 μm.
B. Representative images of GFP–Q^{Q168A} localization in sporulating cells at hour 2 of sporulation. Images show GFP–Q^{Q168A} in an otherwise wild-type background (BCR87), Δ*AH* (BCR80) or Δ*gerM* (BCR1313).
C. Bar graph showing sporulation efficiencies of wild-type (BCR163), Q^{Q168A} (BCR152), Δ*gerM* (BCR1314), the Δ*gerM* Q^{Q168A} double mutant (BCR1313), Δ*AH* (BCR1335), the Δ*AH* Q^{Q168A} double mutant (BCR1334) and Δ*Q* (BTD1541). Error bars represent standard deviations ($n = 2$).

required for endopeptidase activity, the domain adopts a similar fold (Levdikov *et al*., 2012; Meisner *et al*., 2012). We have previously shown that the substrate-binding groove in the degenerate LytM domain of Q is likely to function as the interaction surface for its second anchoring protein (Rodrigues *et al*., 2013). Specifically, we showed that a point mutation in this groove (Q168A) had no discernable impact on the localization of Q or on sporulation efficiency (Fig. 3B and C). However, in combination with an *AH* null mutant, GFP–Q^{Q168A} was almost completely mislocalized (Fig. 3B) (Rodrigues *et al*., 2013). Moreover, the Q^{Q168A} Δ*AH* double mutant had a synergistic sporulation defect (Fig. 3C). Accordingly, if GerM is the second anchoring protein for Q, our data predict it would act through the LytM groove. Consistent with this idea, GFP–Q^{Q168A} retained most of its septal localization in the absence of GerM and the sporulation efficiency of the Δ*gerM* Q^{Q168A} double mutant was no worse than the Δ*gerM* single mutant (Fig. 3B and C).

GerM localizes to the outer forespore membrane in a manner that depends on the Q's LytM groove and AH

GerM is predicted to be a lipoprotein that is anchored in the outer leaflet of the mother cell membrane. In support of this idea, we found that a functional GerM–His6 fusion (Figs S2 and S4) was membrane-associated and susceptible to trypsin cleavage in a protease accessibility assay (Fig. 4C). To determine the subcellular localization of GerM in the mother cell membranes, we generated a functional GerM–mCherry fusion (Fig. S5) and monitored its localization during a sporulation time course. Because of the slow maturation of mCherry (Shaner *et al*., 2005; Merzlyak *et al*., 2007), the earliest time point at which we could detect the fluorescent

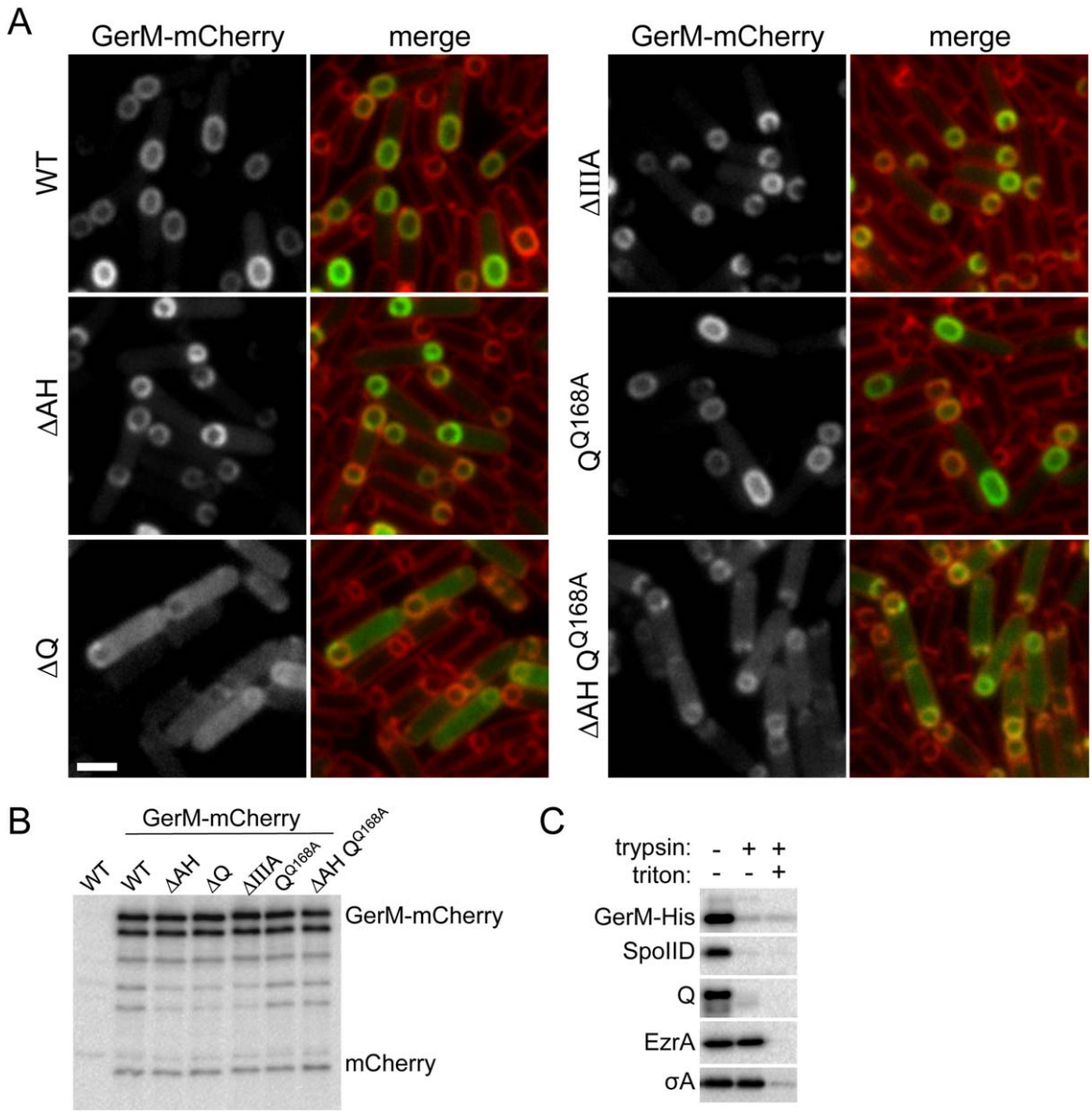

Fig. 4. GerM–mCherry localizes to septal membrane in a manner that depends on Q.
A. Representative images of GerM–mCherry localization at 2.5 h after the onset of sporulation. Images are from wild-type (BCR1332), Δ*AH* (BCR1344), Δ*Q* (BCR1345), Δ*spoIIIA* (BCR1346), Q^{Q168A} (BCR1348) and the Δ*AH* Q^{Q168A} double mutant (BCR1353). Scale bar represents 2 μm.
B. GerM–mCherry levels and proteolytic products are similar in all mutant backgrounds tested. Immunoblot analysis using anti-mCherry antibodies of sporulating cells from the same strains described above and with a true wild-type control (PY79, no mCherry) harvested at hour 2.5 of sporulation. The nature of the GerM–mCherry doublet is currently unknown.
C. GerM–His6 is surface exposed and thus accessible to trypsin digestion. Immunoblot analysis using anti-His antibodies of protoplasted sporulating cells (strain BCR1306) treated with Trypsin in the presence and absence of TritonX-100. Consistent with the idea that GerM is a lipoprotein, it remained cell-associated after the generation of protoplasts. As controls, the immunoblot was probed for two membrane proteins with extracellular domains (SpoIID and Q), a membrane-anchored cytoplasmic protein (EzrA) and a cytoplasmic protein (SigA).

fusion was hour 2.5. However, consistent with the idea that GerM is part of the A–Q complex, GerM–mCherry localized to the outer forespores membrane during and after the completion of engulfment (Fig. 4A). Moreover, GerM localization to the forespore membrane was significantly reduced in the absence of Q (Fig. 4A). The mislocalized GerM–mCherry appeared as a diffuse cytoplasmic haze as if the fusion was cleaved releasing soluble mCherry. However, immunoblot analysis revealed that most of the fusion remained full-length and that the small degree of proteolysis was similar in all backgrounds examined (Fig. 4B). A similar diffuse localization has been observed for the polytopic membrane protein SpoIIE (King *et al.*, 1999) and CFP–AH in the

absence of Q (Doan *et al.*, 2005). The molecular basis of this localization pattern is currently unknown.

To investigate whether Q's LytM groove was required for GerM localization, we monitored GerM–mCherry in the Q^{Q168A} mutant. The localization of the mCherry fusion in this background was similar to wild-type (Fig. 4A). One possible explanation for the absence of a localization defect is that GerM also interacts with AH, which associates with a distinct interface on Q (Levdikov *et al.*, 2012; Meisner *et al.*, 2012). To test this idea, we examined GerM–mCherry in a *ΔAH, Q^{Q168A}* double mutant. Under these conditions, GerM–mCherry had a pronounced mislocalization phenotype (Fig. 4A) although it was not as strong as in the Q null suggesting that the Q168A substitution is not sufficient to completely disrupt Q-dependent localization of GerM. Altogether, our results are consistent with the idea that GerM, Q and AH form the basement layer of the A–Q transenvelope complex.

GerM localization requires septal peptidoglycan hydrolysis

Our previous studies revealed that the unidentified mother cell protein that helps anchor Q in the forespore membrane requires degradation of septal peptidoglycan (PG), presumably to bring the two membranes into close apposition to allow for efficient interaction with Q (Rodrigues *et al.*, 2013). If GerM is indeed this anchor, its interaction with Q and by extension its localization, should depend on thinning of the septal PG. To test this, we monitored GerM–mCherry localization in a strain lacking the two cell wall hydrolases SpoIID and SpoIIP that degrade the septal PG after polar division (Abanes-De Mello *et al.*, 2002; Chastanet and Losick, 2007; Morlot *et al.*, 2010). In the absence of both enzymes, GerM–mCherry had a diffuse membrane localization phenotype similar to what was observed in the absence of Q (Fig. 5A). Furthermore, consistent with the idea that septal PG hydrolysis allows GerM to interact with Q, in sporulating cells lacking either SpoIID or SpoIIP, GerM–mCherry was enriched at sites where the PG was thinned and the septal membranes bulged into the mother cell cytoplasm (Figs 5A and S6). Finally, immunoblot analysis indicates that GerM–mCherry was predominantly full-length with similar amounts of smaller proteolytic products in all backgrounds examined (Fig. 5B).

GerM is sufficient to localize Q in the absence of all other σE-dependent proteins

All of our data thus far are consistent with the idea that GerM is the missing σE-dependent protein that, together with AH, anchors Q in the septal membrane on the forespore side. However, despite extensive effort using *in vitro* and *in vivo* protein-protein interaction assays, we were unable to detect a direct interaction between GerM and the extracellular domains of Q, AH or both. We reasoned that if GerM is in fact Q's missing σE-dependent anchor then expression of GerM in the absence of all other σE-dependent proteins should be sufficient to localize Q. To test this, we took advantage of a strain we used previously to investigate whether the second Q anchor is a protein under σE control (Fig. 6A)

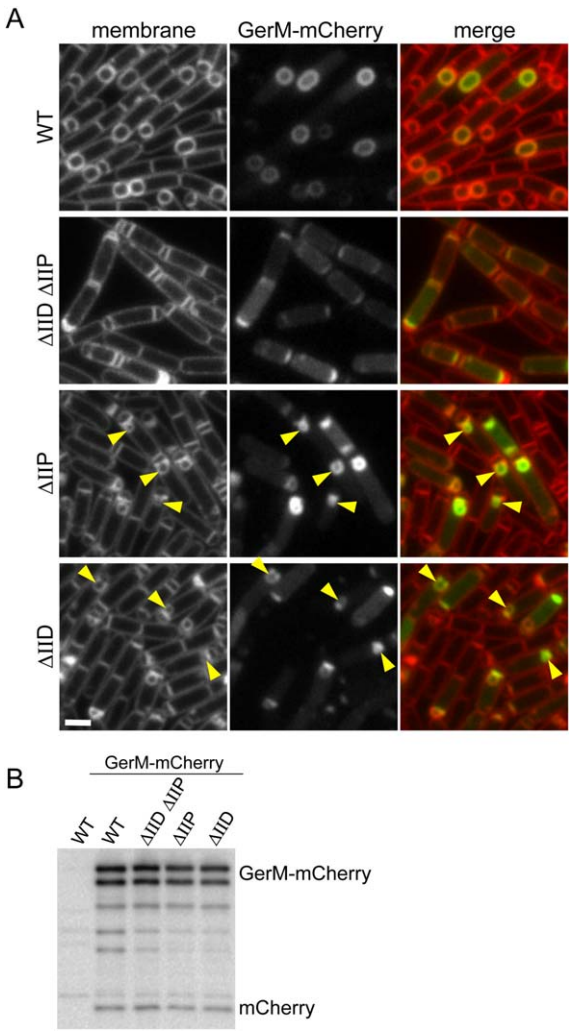

Fig. 5. GerM–mCherry localization to the septal membrane requires thinning of the septal peptidoglycan.
A. Representative images of GerM–mCherry localization at hour 2.5 of sporulation. Images are from wild-type (BCR1332), the Δ*spoIID* Δ*spoIIP* double mutant (BCR1381), Δ*spoIIP* (BCR1347) and Δ*spoIID* (BCR1414). Enrichment of GerM–mCherry at septal bulges is highlighted (yellow carets). Larger fields of cells can be found in Fig. S6. Scale bar represents 2 μm.
B. GerM–mCherry levels and proteolytic products are similar in all mutant backgrounds tested. Immunoblot analysis using anti-mCherry antibodies of sporulating cells from the same strains described above and with a true wild-type control (PY79, no mCherry) harvested at hour 2.5 of sporulation.

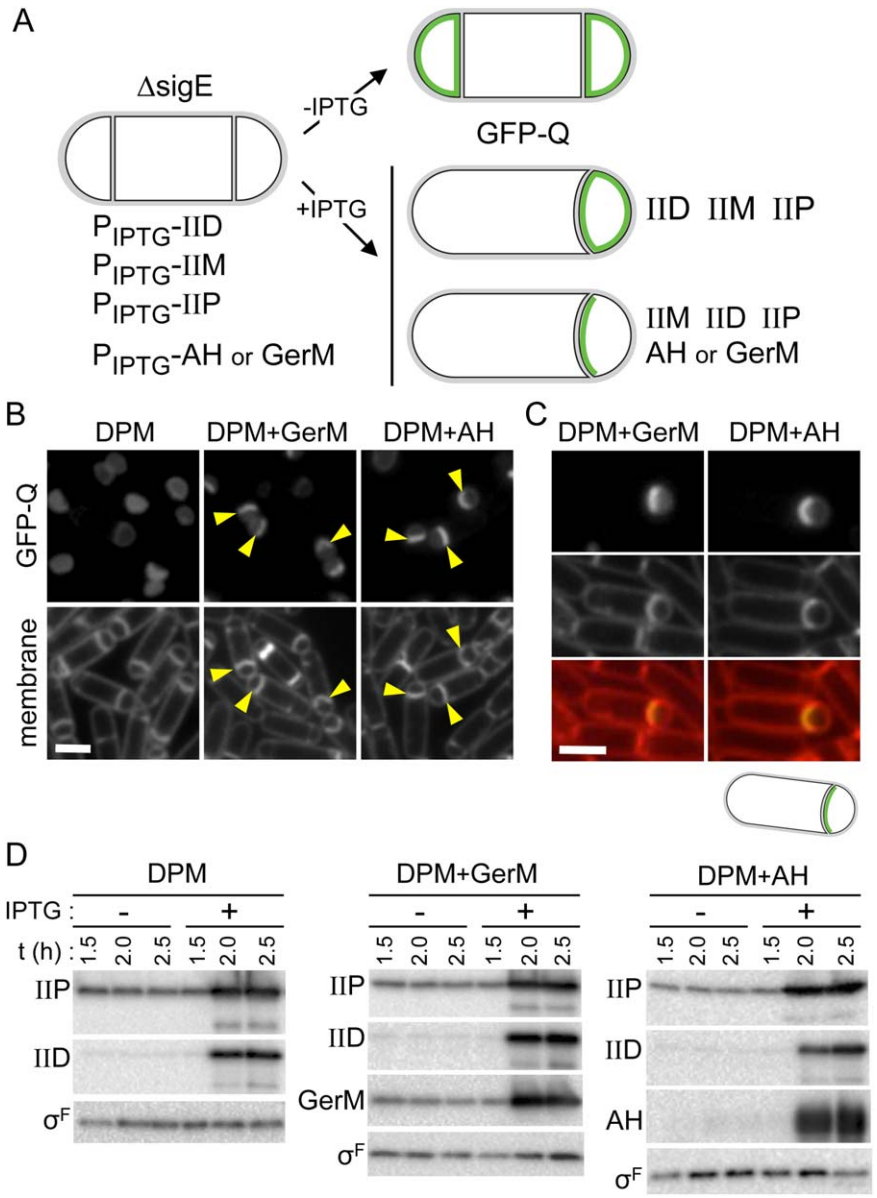

Fig. 6. GerM is sufficient to localize GFP–Q at the sporulation septum.
A. Experimental rationale and schematic outcomes for GFP–Q localization (in green) when SpoIID, SpoIIP and SpoIIM, alone (BCR1444) or together with either GerM (BCR1447) or AH (BCR1446) are artificially produced in a Δ*sigE* mutant.
B. Representative images of cells at hour 2.5 of sporulation in which IPTG (1 mM final) was added 1.5 h after the onset of sporulation. Examples of engulfing septal membranes with enrichment of GFP–Q in the forespore membranes are indicated (yellow carets). Scale bar represents 2 μm. As described previously (Rodrigues *et al.*, 2013), in a subset of sporulating cells (in strain BCR1444 and derivatives) in which IPTG was added GFP–Q lost compartmentalization (not shown). Images of the same strains from the same time point in the absence of IPTG can be found in Fig. S8.
C. Larger images highlighting GFP–Q enrichment in the engulfing membrane when either GerM or AH was induced.
D. Immunoblot analysis monitoring SpoIID, SpoIIP, GerM–His6 and AH accumulation upon the addition of IPTG. σF was used to control for loading.

(Rodrigues *et al.*, 2013). This strain lacks *sigE* but contains IPTG-inducible alleles of *spoIID*, *spoIIP* and *spoIIM* encoding the cell wall degrading machinery to ensure thinning of the septal PG. GFP–Q fails to localize to the septal membranes in this background (Fig. 6A and B) (Rodrigues *et al.*, 2013). To test if GerM is sufficient to localize Q, we introduced an IPTG-inducible allele of *gerM-his6* into this strain (Fig. 6A). As a control for this experiment, we constructed a complementary strain containing an IPTG-inducible allele of *AH*. When GerM was expressed in addition to SpoIID, SpoIIP and SpoIIM, GFP–Q was enriched at the septum (Figs 6B–D and S7A–C). As expected, a similar enrichment of GFP–Q was observed when AH was expressed (Figs 6B–D and S7A–C). These results indicate that GerM or AH alone is sufficient to localize Q. Collectively, the data presented here and our previous analysis of Q localization (Rodrigues *et al.*, 2013) indicate that GerM and AH are the two σE-controlled proteins required to anchor Q in the septal membrane.

GerM and AH are necessary for SpoIIIAG localization

In previous work, we showed by co-immunoprecipitation that at least five of the SpoIIIA proteins (SpoIIIAB, SpoIIIAD, SpoIIIAE, SpoIIIAF and SpoIIIAG) reside in a multimeric membrane complex (Doan *et al.*, 2009). Furthermore, we found that the localization of a partially functional CFP–SpoIIIAG (CFP–AG) fusion in the outer forespore membrane was substantially reduced in cells lacking Q and was

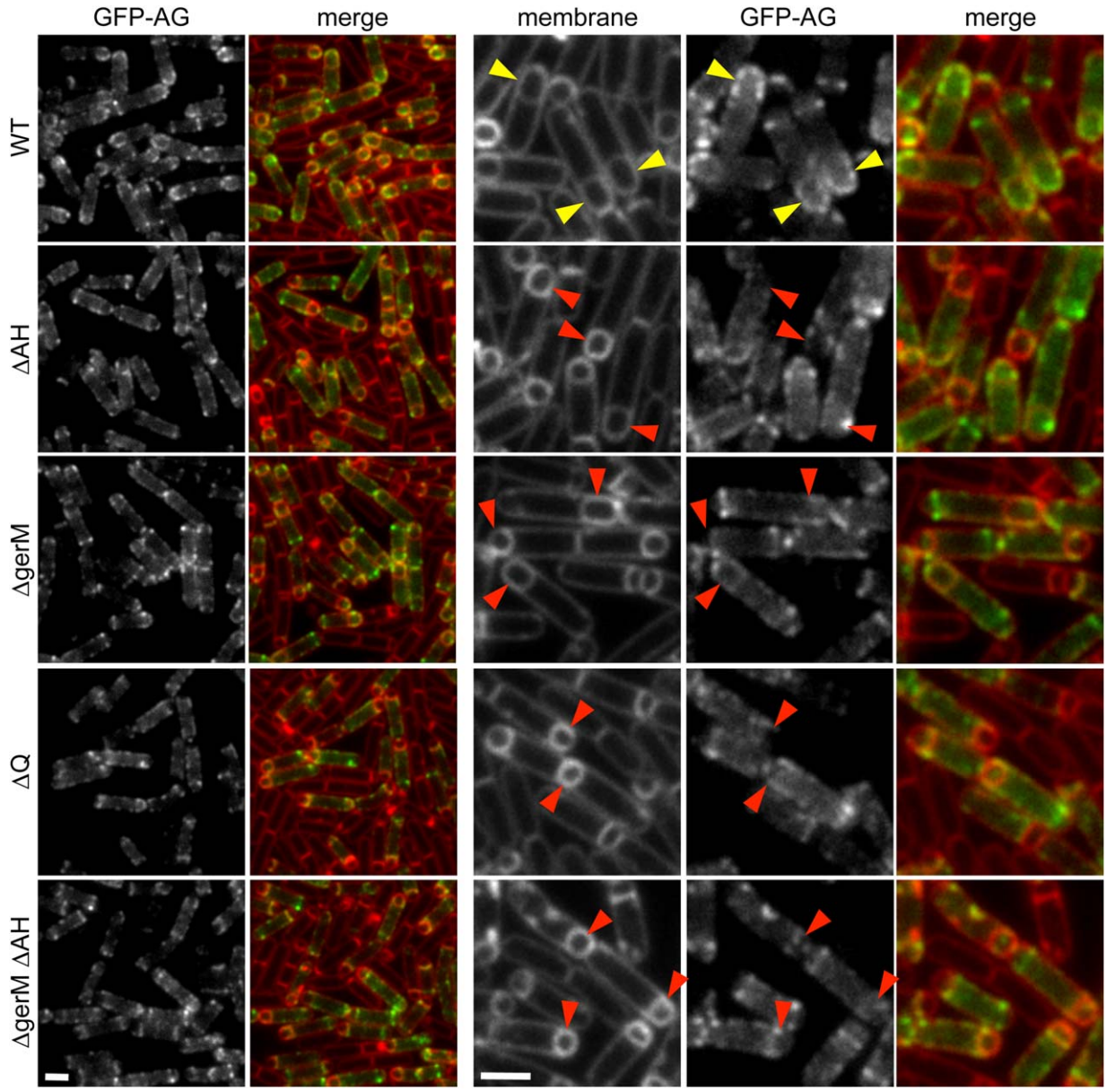

Fig. 7. GerM is required for efficient localization of GFP–AG.
Representative images of GFP–AG localization at hour 2.5 of sporulation. Images are from wild-type (BCR1193), Δ*AH* (BCR1228), Δ*gerM* (BCR1328), Δ*Q* (BCR1340) and the Δ*AH* Δ*gerM* double mutant. (BCR1343). Larger images are shown on the right. Wild-type forespores with GFP–AG puncta are highlighted with yellow carets. Mutant forespores with reduced accumulation of GFP–AG puncta around the forespore are highlighted with red carets. Scale bar represents 2 μm.

impaired in the absence of AH (Doan *et al*., 2009). Since our data suggest that GerM functions as part of the basement layer of the A–Q complex, we investigated whether GerM was required for the localization of SpoIIIAG (AG). We monitored CFP–AH (Doan *et al*., 2005) and separately a partially functional GFP–AG fusion during a sporulation time course in the presence and absence of GerM. Consistent with our finding that GerM–mCherry retains its proper localization in the absence of AH (Fig. 4A), in the *gerM* mutant, CFP–AH localized to the outer forespore membrane in a manner indistinguishable from wild-type (Fig. S9). In a wild-type background, GFP–AG localized as bright puncta predominantly in the membranes surrounding the forespore as previously reported (Fig. 7) (Doan *et al*., 2009). In the *gerM* mutant, we observed a reduction in the number of forespores with bright GFP–AG puncta; instead, the GFP–AG signal was more dispersed in the peripheral membranes (Fig. 7). This mislocalization phenotype was enhanced by the loss of AH and qualitatively resembled the *Q* mutant (Fig. 7). These results suggest that GerM, AH

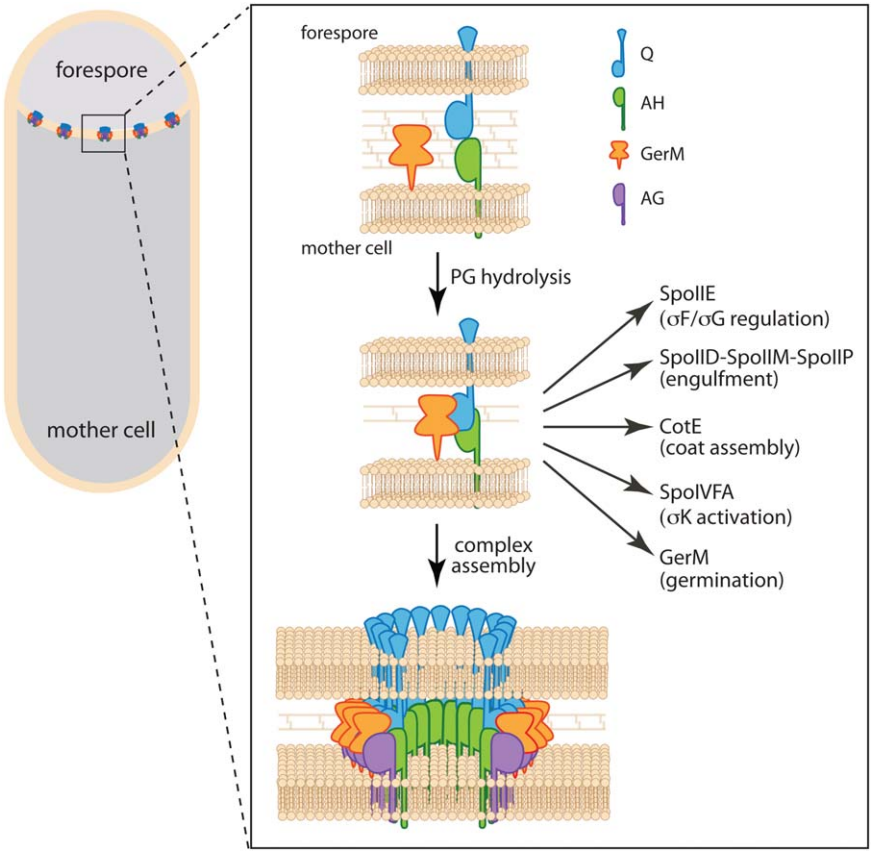

Fig. 8. Schematic model showing the assembly of the basal platform of the A–Q complex and its localization network. Upon production of Q in the forespore and AH in the mother cell the two proteins associate across the sporulation septum. Degradation of the septal peptidoglycan by SpoIID and SpoIIP brings the two septal membranes closer together (Tocheva *et al.*, 2013) allowing for stable association with GerM either through direct interaction as shown here or mediated by an unidentified bridging protein. This transenvelope complex directly or indirectly anchors morphogenetic and signalling proteins on both sides of the sporulation septum (see *Discussion* section). The complex also serves as the basal platform for the assembly of AG and the rest of the proteins in the *spoIIIA* operon (not shown). The complex is shown as a ring-shaped conduit based on the structural similarity between AH and the EcsJ/PrgK family. By analogy to specialized secretion systems, it is hypothesized that one or several membrane proteins encoded in the *spoIIIA* operon assemble a pore in the mother cell membrane (not shown). Evidence suggests the existence of a pore in the forespore membrane (Meisner *et al.*, 2008) (not shown), however the identity of this protein is unknown.

and Q function as a scaffold for AG and likely the entire SpoIIIA complex.

Discussion

Here, we have identified GerM as the missing σE-controlled protein required to localize Q in the septal membrane. Our data suggest that Q, AH and GerM constitute a basal platform upon which the A–Q complex is assembled (Fig. 8). In addition to its role in the A–Q complex, this platform has been found to function as an interaction hub, contributing to the localization of a diverse set of sporulation proteins on both sides of the septal membrane (Doan *et al.*, 2005; Aung *et al.*, 2007; Chastanet and Losick, 2007; Campo *et al.*, 2008; McKenney and Eichenberger, 2012; Flanagan *et al.*, 2016; Dworkin, 2014) (Fig. 8). Thus, by defining how this founder complex is localized to the septal membrane, our findings reveal how cell–cell signalling complexes and morphogenetic proteins achieve their proper localization.

Evidence for a tripartite complex

In previous work we proposed the existence of an additional σE-dependent protein that anchors Q in the septal membranes (Rodrigues *et al.*, 2013). While we were unable to detect a direct interaction between GerM and the extracellular domains of Q (or the AH–Q complex), by all other measures GerM is the missing σE-dependent protein and fits the requirements for Q's elusive partner: (i) GerM is specifically expressed under the control of σE in the mother cell; (ii) the mutant has a synergistic sporulation defect with ΔAH; (iii) cells lacking GerM and AH fail to localize Q; (iv) GerM's localization requires septal PG hydrolysis and Q; and finally (v) GerM is sufficient to localize Q in the absence of all other σE-dependent genes (provided the cell wall has been sufficiently thinned). Our inability to detect a direct interaction between Q and GerM leaves open the possibility that a protein produced earlier during sporulation or during vegetative growth functions as an additional component of the basal platform bridging GerM and Q. It is also formally possible that GerM indirectly affects assembly of the complex, for example by acting as a chaperone. However, we favour a simpler model in which GerM directly contacts Q and does so, in part, through its LytM groove. In the context of this model, the interaction between GerM and Q is weak and might require a specific conformation that is favoured in the context of full-length Q and its transmembrane segment

and/or when GerM is anchored in the membrane. Alternatively, some feature of the intermembrane space, like partially degraded peptidoglycan, might be necessary to stabilize the interaction between these proteins.

The data presented here and our previous findings and those of the Pogliano, Moran, Henriques and Losick groups (Blaylock *et al*., 2004; Doan *et al*., 2005; Camp and Losick, 2008; Meisner *et al*., 2008; Camp and Losick, 2009; Doan *et al*., 2009; Fredlund *et al*., 2013; Rodrigues *et al*., 2013) lead us to propose a working model for the assembly of the A–Q complex (Fig. 8). Assembly initiates with the activation of σF in the forespore. σF controls the production of Q (Londono-Vallejo *et al*., 1997) and is also responsible for triggering the activation of σE in the mother cell (Londono-Vallejo and Stragier, 1995). σE, in turn, directs the synthesis of the cell wall degrading machine (SpoIID/SpoIIM/SpoIIP) (Chastanet and Losick, 2007; Abanes-De Mello *et al*., 2002; Morlot *et al*., 2010), AH and the rest of the proteins in the *spoIIIA* operon, and GerM. Thinning of the septal peptidoglycan allows GerM to contact Q, reinforcing the association between AH and Q (Fig. 8). This tripartite complex then serves as the basal platform for the assembly of the remaining components of the A–Q complex. Based on remote homologies and the structural similarity between AH and EcsJ/PrgK family (Levdikov *et al*., 2012; Meisner *et al*., 2012), this complex is thought to assemble into a ring-shaped conduit that connects mother cell and forespore (Fig. 8). The complex could function as a specialized secretion system or feeding tube allowing the mother cell to nurture the forespore (Camp and Losick, 2009; Doan *et al*., 2009). Defining the structure of this complex and its role in maintaining forespore differentiation are exciting challenges for the future.

GerM is a novel component of the A–Q complex

That GerM's role in the assembly of the A–Q complex was missed by us, and others, for so many years, highlights the power of a name. The *gerM* mutant was originally defined as having pleiotropic defects during sporulation and a delay in spore germination (Sammons *et al*., 1987). Since the gene was identified in a germination screen it was given a '*ger*' designation rather than a '*spo*' name. Had it been called *spoIIT* or *spoIIIL*, its role in the A–Q pathway would likely have been discovered over a decade ago. In the original characterization of GerM (Sammons *et al*., 1987; Slynn *et al*., 1994), it was reported that a subset of sporulating cells lacking *gerM* arrest after polar division and the mutant had a reduction in glucose dehydrogenase activity, an activity associated with a late stage of sporulation. Our cytological analysis failed to detect a stage II block or a defect in engulfment. However, since the time of the original publication on *gerM*, the gene encoding glucose dehydrogenase (*gdh*) was found to be under σG control (Nakatani *et al*., 1989). Accordingly, the reduced activity in the mutant is fully consistent with our finding that a subset of sporulating cells lacking GerM have weak or undetectable σG activity. GerM's role in germination is currently unclear but our data are fully consistent with the idea that GerM has a second function in sporulation and/or germination beyond its role in the A–Q complex. Specifically, the defects in σG activity and forespore morphology were weaker in cells lacking GerM compared to the AH mutant (Fig. 2). Yet, the sporulation efficiencies in the two mutants were similar. Furthermore, unlike AH and Q that are degraded shortly after engulfment is complete (Chiba *et al*., 2007), GerM persists through late stages of sporulation (Fig. S10). GerM's second function could be related to the accumulation of dipicolinic acid in the spore and/or cortex hydrolysis upon germination (Slynn *et al*., 1994). Alternatively, GerM could influence germination indirectly by promoting proper coat assembly (McKenney and Eichenberger, 2012).

Bioinformatics analyses of *B. subtilis* GerM indicate that GerM homologs are present in virtually all the endospore formers of the Bacillaceae family but are absent in the Clostridiacaea (Fig. S11). Accordingly, if Q and the proteins in the *spoIIIA* operon assemble a similar transenvelope complex in the Clostridiacaea, the basal platform must differ in protein composition. Consistent with this idea, studies on the A–Q complex in *Clostridium difficile* point to the possibility that AH may be the sole anchor for Q. This hypothesis is a based on the observation that an *AH* mutant in *C. difficile* has a sporulation defect comparable to mutations in the other *spoIIIA* genes (Fimlaid *et al*., 2015; Serrano *et al*., 2015).

GerM contains two tandem copies of a novel domain designated GERMN (Rigden and Galperin, 2008). While this domain organization is restricted to a subset of endospore formers (Fig. S11), the GERMN domain is also present in isolation or fused to other protein domains in a diverse collection of bacterial phyla, including *Actinobacteria*, *Cyanobacteria*, *Proteobacteria* and in *Deinococcus-Thermus* group (Rigden and Galperin, 2008). Intriguingly, in the bacterium *Halothermothrix orenii* the GERMN domain is fused to an amidase domain involved in cell wall remodelling, suggesting GERMN could be a PG binding domain (Rigden and Galperin, 2008). If it is, GerM could stabilize the A–Q complex through an interaction with the remodelled cell wall in the intermembrane space. Future biochemical and

structural analysis will be required to define the extent of GerM's role in the A–Q complex.

Experimental procedures

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 and Cutting, 1990) or by exhaustion in supplemented DS medium (Schaeffer *et al.*, 1965). Sporulation efficiency was determined in 24–30 h cultures as the total number of heat-resistant (80°C for 20 min) colony forming units (CFUs) compared with wild-type heat-resistant CFUs. Deletion mutants were generated by isothermal assembly (Gibson, 2011) and direct transformation into *B. subtilis*. Tables of strains, plasmids and oligonucleotide primers and descriptions of plasmid construction and isothermal assembly deletion mutants can be found in Supporting Information.

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 (1:10000) (Doan and Rudner, 2007), anti-SpoIIQ (1:10000) (Doan *et al.*, 2005), anti-SpoIIIAH (1:10000) (Doan *et al.*, 2005), anti-SpoIIP (1:10000) (Morlot *et al.*, 2010), anti-σA (1:10000) (Fujita, 2000), anti-EzrA (1:10000) (Levin *et al.*, 1999), anti-His (Genscript) (1:4000), anti-mCherry (1:10000), diluted into 3% BSA in 1X PBS–0.05% Tween-20. Primary antibodies were detected using horseradish peroxidase-conjugated goat, anti-rabbit IgG (1:20000, Bio-Rad) 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 resupsension 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.

Protease susceptibility

Protease susceptibility assays were preformed as described previously (Doan and Rudner, 2007) in a *spoIIIAH* mutant (strain BCR1306) to ensure that membrane proteins present in the inner and outer forespore membrane would not be artificially inaccessible due to protoplast engulfment (Broder and Pogliano, 2006). Twenty-five millilitre of sporulating cells (induced by resuspension) were harvested by centrifugation at 2 h after the onset of sporulation, washed and resuspended in 2 ml of 1X SMM buffer (0.5 M sucrose, 20 mM $MgCl_2$ and 20 mM maleic acid pH 6.5) (Harwood and Cutting, 1990). The cells were then protoplasted by lysozyme (5 mg ml^{-1} final) for 10 min. The protoplasts were harvested by centrifugation and resuspended in 1 ml of 1X SMM. One-hundred microliter protoplasts were incubated with Trypsin (30 μg ml^{-1}) (Worthington), Trypsin and Triton X-100 (2%), or 1XSMM for 15 min. Reactions were terminated by the addition of 100 μl of 2X SDS-sample buffer and boiling for 5 min at 95°C. Five microliter from each reactions was analyzed by immunoblot.

Quantification of σG positive cells

σG activity was assessed in single cells as described previously (Rodrigues *et al.*, 2013) at hour 4 after the onset of sporulation using the fluorescent reporter P_{sspB}-*cfp* (Doan *et al.*, 2009). A forespore was considered σG 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 ensure that those forespores that had just activated σG and therefore had faint forespore fluorescence were scored appropriately. Faint forespore fluorescence in normal-sized forespores with unperturbed membranes was scored as σG positive. Faint forespore fluorescence in small forespores with aberrant membrane morphologies indicative of arrested development (Doan *et al.*, 2009) was scored as σG negative. The percentage of σG positive cells was calculated based on the total number of cells.

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Supporting information

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