The SpoIIQ-SpoIIIAH complex of *Clostridium difficile* controls forespore engulfment and late stages of gene expression and spore morphogenesis

Mónica Serrano,1 Adam D. Crawshaw,2† Marcin Dembek,2,3† João M. Monteiro,4 Fátima C. Pereira,1‡ Mariana Gomes Pinho,4 Neil F. Fairweather,3 Paula S. Salgado2* and Adriano O. Henriques1*
1 Microbial Development, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, Estação Agronômica Nacional, Avenida da República, 2780-157, Oeiras, Portugal.
2 Institute for Cell and Molecular Biosciences, Faculty of Medical Sciences, Newcastle University, Newcastle upon Tyne, UK.
3 MRC Centre for Molecular Bacteriology and Infection, Department of Life Sciences, Imperial College London, London, UK.
4 Bacterial Cell Biology Laboratory, Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, Estação Agronômica Nacional, Avenida da República, 2780-157, Oeiras, Portugal.

Summary

Engulfment of the forespore by the mother cell is a universal feature of endosporulation. In *Bacillus subtilis*, the forespore protein SpoIIQ and the mother cell protein SpoIIIAH form a channel, essential for endosporulation, through which the developing spore is nurtured. The two proteins also form a backup system for engulfment. Unlike in *B. subtilis*, SpoIIQ of *Clostridium difficile* has intact LytM zinc-binding motifs. We show that *spoIIQ* or *spoIIIAH* deletion mutants of *C. difficile* result in anomalous engulfment, and that disruption of the SpoIIQ LytM domain via a single amino acid substitution (H120S) impairs engulfment differentially. SpoIIQ and SpoIIQH120S interact with SpoIIIAH throughout engulfment. SpoIIQ, but not SpoIIQH120S, binds Zn2+, and metal absence alters the SpoIIQ-SpoIIIAH complex *in vitro*. Possibly, SpoIIQH120S supports normal engulfment in some cells but not a second function of the complex, required following engulfment completion. We show that cells of the *spoIIQ* or *spoIIIAH* mutants that complete engulfment are impaired in post-engulfment, forespore and mother cell-specific gene expression, suggesting a channel-like function. Both engulfment and a channel-like function may be ancestral functions of SpoIIQ-SpoIIIAH while the requirement for engulfment was alleviated through the emergence of redundant mechanisms in *B. subtilis* and related organisms.

Introduction

Intercellular communication, crucial for the coordinated behavior of cells, often involves gap junctional protein channels that span the lipid bilayers of adjacent cells allowing them to exchange ions and small water-soluble molecules to maintain cellular homeostasis. These intercellular connections are formed by oligomers of membrane-anchored channel-forming protein subunits, with each of the two contacting cells contributing half of the channel (Houghton, 2005). Gap-junctions are found in virtually all animal cells, and resemble analogous structures found in plant cells, called plasmodesmata (Brunkard et al., 2015). Gap-junction-mediated transport can, for example, allow metabolic cooperation, whereby a cell transfers nutrients or intermediate metabolites to an adjacent cell, itself unable to synthesize or acquire them. A membrane protein complex formed during endospore development by the bacterium *Bacillus subtilis*, is used by the mother cell as a conduit to nurture the developing endospore in a gap junction-type function (Blaylock et al., 2004; Camp and Losick, 2008; Meisner et al., 2008; Doan et al., 2009).
Endospore differentiation in *B. subtilis* involves two cells formed at the onset of the process through polar division of the rod-shaped cell (Fig. 1A). The smaller cell, or forespore, will become the future spore, while the larger mother cell nurtures spore development but ultimately lyses to release the mature spore into the environment (Hilbert and Piggot, 2004; Stragier and Losick, 1996). At the time of division, the two cells are in direct contact with the external medium (Fig. 1A). However, soon after division, the mother cell membrane begins to engulf the forespore, eventually transforming it into a free protoplast surrounded by two membranes of opposing polarity. Engulfment completion marks the transition to the last stages in development, during which spore morphogenesis is completed and the spore is prepared for dormancy (Fig. 1A). The forespore and the mother cell follow different programs of gene expression, largely defined by a cascade of cell type-specific RNA polymerase sigma (σ) factors, but cell-cell communication pathways coordinate and keep the two programs in harmony with the sequence of morphogenesis (Losick and Stragier, 1992; Stragier and Losick, 1996; Kroos and Yu, 2000; Rudner and Losick, 2001). σ^F_ is activated in the forespore soon after polar division and it controls early stages of development in this cell. σ^F_ is also responsible for the activation of the mother cell-specific regulatory protein σ^E_. The onset of σ^G_ activity coincides with engulfment completion, and leads to activation of σ^K_, which replaces σ^E_ in the mother cell. Moreover, σ^G_ activity requires expression of the σ^F_-controlled forespore gene spoIIQ (Londono-Vallejo et al., 1997) and of the mother cell σ^F_-controlled spoIIA operon (Ilting and Errington, 1991b) (Fig. 1B). In the absence of any of the spoIIA-encoded proteins or SpolIIQ the forespore collapses and gene expression in the forespore halts (Serrano et al., 2004; Doan et al., 2005; 2009; Camp and Losick, 2008, 2009). SpolIIAH and SpolIIQ localize to the asymmetric septum and to the engulfing membranes, and the two proteins interact in the intermembrane space via their extracytoplasmic domains (Rubio and Pogliano, 2004; Blaylock et al., 2004; Doan et al., 2005; 2009). This interaction localizes SpolIIAH to the septum and to the engulfing membranes but the primary tether for SpolIIQ, although dependent on mother cell gene expression, is still unclear (Blaylock et al., 2004; Rubio and Pogliano, 2004; Fredlund et al., 2013; Rodrigues et al., 2013). SpolI Q and SpolIIAH are thought to form a scaffold for the assembly of a larger complex that includes the remaining spoIIA-encoded proteins (Chiba et al., 2007; Doan et al., 2009). Evidence suggests that SpolIIAH and SpolIIQ form a direct channel between the cytoplasm of the mother cell and the forespore (Meisner et al., 2008). SpolIIAH is related to the YcsJ/FliF family of proteins that form oligomeric rings in Type III secretion systems and the flagellar basal body, and the structure of a SpolIIQ-SpolIIAH co-crystal confirms the potential of the complex to form two apposed ring-like structures (Meisner et al., 2012; Levdikov et al., 2012). Importantly, the C-terminus of SpolIIAH can be labelled by a forespore-produced heterologous biotin ligase in a SpolIIQ-dependent manner, providing direct evidence for a channel (Meisner et al., 2008). The strong similarity of SpolIIA to transport ATPases, and the varying degrees of similarity of the remaining SpolIIA proteins with components of Type II/IV secretion systems, together with the phenotypes caused by mutations in the ATP binding motifs of SpolIIA, suggest a specialized system for transport into the forespore (Blaylock et al., 2004; Camp and Losick, 2008; Meisner et al., 2008; Doan et al., 2009). In *B. subtilis* spoIIQ or spoIIAH mutants, the activity of σ^G_ and the continued activity of σ^F_, as well as the activity of the heterologous single chain RNA polymerase from phage T7, produced from a σ^F_-dependent promoter, is severely curtailed (Camp and Losick, 2009). This led to a model in which the SpolIIQ-SpolIIAH complex functions as a gap-junction-like feeding tube, through which the mother cell supplies the forespore with small molecules required to maintain its metabolic potential following insulation from the external medium (Camp and Losick, 2009; Doan et al., 2009). The molecules conveyed to the forespore by the SpolIIQ-SpolIIAH complex remain unknown. Isolation of the forespore upon engulfment completion is a hallmark of endosporeulation. Accordingly, the spoIIIQ operon and spoIIQ are conserved and part of a signature for endosporeulation (Galperin et al., 2012; Abecasis et al., 2013; Crawshaw et al., 2014) (Fig. 1C). The prediction that the function of spoIIIQ and spoIIQ is conserved has not been tested.

Engulfment is mainly controlled by a complex of three proteins produced under the control of σ^E_ (Abanes-De Mello et al., 2002; Chastanet and Losick, 2007; Aung et al., 2007; Gutierrez et al., 2010; Morlot et al., 2010) (Fig. 1A and B). Within this complex, termed the DMP machine, SpolID and SpolIP are peptidoglycan (PG) hydrolases that act coordinateably to generate the main force for advancing the leading edge of the mother cell membrane (Abanes-De Mello et al., 2002; Aung et al., 2007; Chastanet and Losick, 2007; Morlot et al., 2010; Gutierrez et al., 2010). spoIIID, spoIII M or spoIIIP mutants are blocked in the initial stage of engulfment, septal PG thinning (Lopez-Diaz et al., 1986; Smith et al., 1993; Bylund et al., 1994; Frandsen and Stragier, 1995; Ilting and Errington, 1991a; Aung et al., 2007) (Fig. 1A). However, when the activity of the DMP machine is compromised, two redundant processes gain importance. One is synthesis of PG at the leading edges of the engulfing membranes (Meyer et al., 2010). The second is a
Fig. 1. Sporulation and the SpoIIQ-SpoIIIAH channel.

A. Schematic representation of the main stages of sporulation. Asymmetric cell division at one of the poles gives rise to the small daughter cell or forespore, side-by-side with the mother cell (I). As sporulation progresses, the mother cell engulfs the forespore within its cytoplasm. In *B. subtilis*, the DMP protein complex (green) is essential for hydrolysis of PG within the septum during the initial stages of engulfment (II-IV) (see text for details; see also panel B). In *B. subtilis* and presumably other sporeformers, SpoIIQ (blue) and SpoIIIAH (yellow), together with additional spoIIA-encoded proteins (not represented), are also involved in engulfment and required for sporulation (III-V). Spore maturation involves the formation of two main protective layers (the cortex PG and the coats) in the intermembrane space (VI). It is not known whether the SpoIIQ-SpoIIIAH channel is maintained at these late stages in Clostridia and other endosporeformers, but the two proteins are degraded following engulfment completion in *B. subtilis* (Jiang *et al.*, 2005; Meisner *et al.*, 2008). Finally, the mother cell lyses, releasing the mature spore into the media (VII).

B. SpoIIQ-SpoIIIAH and DMP organization. The proposed organization in *B. subtilis*, of SpoIIQ (blue) and SpoIIIAH (yellow) multimeric rings in the inner (IFM) and outer (OFM) forespore membranes is shown schematically. The interaction of the two rings is proposed to form a channel that allows communication between the mother cell and the forespore. The DMP complex (SpoIID, D in green; SpoIIP, P in light blue; and SpoIIM, M, in brown) required for PG remodeling during engulfment in *B. subtilis* is also represented (see text for details).

C. Schematic representation of the octacistronic *spoIIIA* (top) and *spoIID*-spoIIQ operons (bottom) of *C. difficile*. The position of two σE-dependent promoters identified in the *spoIIIA* operon of *B. subtilis* is shown by bent arrows. The figure also shows the percentage of sequence similarity between the indicated proteins of *C. difficile* and their counterparts in *B. subtilis* (Meisner *et al.*, 2005, 2008). Finally, the mother cell lyses, releasing the mature spore into the media (VII).

D. LytM motifs in Firmicutes. *B. subtilis* SpoIIQ, with the LytM domain and the position of the conserved residues in motifs 1 and 2 indicated, with the secondary structure elements observed in the structural models labelled (Meisner *et al.*, 2012; Levdkov *et al.*, 2012) (cylinders: α helices; arrows: β strands). A sequence alignment of SpoIIQ orthologues found in representative *Bacilli* (top group) and *Clostridia* (bottom group) (Galperin *et al.*, 2012) when compared to the Hidden Markov Model sequence (HMM) previously described (Crawshaw *et al.*, 2014) shows the relative conservation of the LytM catalytic residues (HinxD, motif 1 and HxH, motif 2, highlighted). Sequences were retrieved and the HMM sequence created as previously described (Crawshaw *et al.*, 2014). Colors indicate conservation when compared to the HMM sequence.
backup mechanism provided by the strong SpoIIQ-SpoIIIAH interaction across the intermembrane space. Importantly, the SpoIIQ-SpoIIIAH interaction is also capable of driving engulfment independently of the DMP machine in protoplasts generated by enzymatic removal of the PG (Broder and Pogliano, 2006). The SpoIIQ-SpoIIIAH interaction may facilitate engulfment by a Brownian ratchet-like mechanism, in which thermal fluctuations of the engulfing membrane create new sites of SpoIIQ-SpoIIIAH interaction that prevent membrane retraction (Broder and Pogliano, 2006; Meyer et al., 2010; Gutierrez et al., 2010; Rodrigues et al., 2013; Ojkic et al., 2014). While the essential function of the two proteins during B. subtilis sporulation appears to be in the assembly of the feeding channel, several observations suggest that SpoIIQ-SpoIIIAH may in fact represent an ancestral mechanism for engulfment, masked by redundancy (Ojkic et al., 2014). Firstly, the similarity of the C-terminal domain of SpoIIIAH to the YscJ family suggests an old origin for the protein (Ojkic et al., 2014). Secondly, SpoIIQ has a LytM endopeptidase domain found in several cell wall hydrolases (Ramadurai and Jayaswal, 1997; Firczuk et al., 2005; Camp and Losick, 2008; Meisner et al., 2008; Rodrigues et al., 2013). While in B. subtilis and most Bacilli, this domain lacks a critical histidine residue required for Zn\(^{2+}\) binding and the formation of a catalytically active enzyme, in several clostridial species the domain is intact, suggesting that it could contribute directly to PG degradation during engulfment (Crawshaw et al., 2014) (Fig. 1D). It is also interesting that while SpoIIAH and SpoIIQ interact via their YscJ and LytM domains respectively, a second surface in the LytM domain may be involved in an interaction with another protein(s), possibly the DMP machine or the products of its activity. This interaction seems to maintain the localization of SpoIIQ even in the absence of SpoIIIAH (Rodrigues et al., 2013; Fredlund et al., 2013). Finally, under certain nutritional conditions, SpoIIQ and SpoIIIAH are required for engulfment, even in the presence of the DMP machine, suggesting that SpoIIQ and SpoIIIAH could have more important roles in engulfment in other organisms (Sun et al., 2000; Broder and Pogliano, 2006; Aung et al., 2007).

Recent improvements in the available genetic tools have spurred studies of spore development in clostridial species, including C. difficile, a major nosocomial pathogen (Fimlaid et al., 2013; Pereira et al., 2013; Saujet et al., 2013). In C. difficile, although the main stages of sporulation and the principal functions of the cell type-specific sigma factors appear largely maintained, the cell-cell communication pathways are less conserved. Importantly, the activity of $\sigma^G$ was shown to be partially independent of $\sigma^E$ (Saujet et al., 2013; Pereira et al., 2013; Fimlaid et al., 2013). Together with the presence of an intact LytM domain in the C. difficile orthologue of SpoIIQ, this raised questions about the function of SpoIIQ-SpoIIIAH in this organism.

Here, we have analyzed the function of the spoIIQ and spoIIIAH genes in C. difficile, with hopes that novel insights into the function of the SpoIIQ-SpoIIIAH complex would emerge. We show that, in C. difficile, SpoIIQ and SpoIIIAH are required for engulfment, but also for later stages in spore morphogenesis. We also show that an intact LytM domain in SpoIIQ is at least partially dispensable for normal engulfment. Our study shows that SpoIIQ and SpoIIIAH interact during engulfment and form a complex in vitro. We establish that SpoIIQ binds Zn\(^{2+}\), and absence of this metal alters the SpoIIQ-SpoIIIAH interaction. spoIIQ or spoIIIAH mutants are impaired in late gene expression and we propose that Zn\(^{2+}\)-binding by SpoIIQ facilitates a late channel-like function of the complex. Evidence is presented that the activity of SpoIIQ-SpoIIIAH is required for late transcription in both sporangial chambers. Our study also suggests that both control of engulfment and a channel-like function are ancestral functions of the SpoIIQ-SpoIIIAH complex.

### Results

**spoIIQ and spoIIIAH in-frame deletion mutants are blocked in sporulation**

A recent study used mutagenesis with a mariner transposon followed by transposon-directed insertion site sequencing (TraDIS) to identify a set of 798 genes whose disruption reduced the efficiency of sporulation by C. difficile (Dembek et al., 2015). Among the genes identified and studied in more detail was the orthologue (CD630_01250) of the B. subtilis spoIIQ gene. An in-frame deletion mutant of spoIIQ, lacking codons 42–195, was generated by allele-coupled exchange, or ACE (Fig. 2A) in the background of the widely used 630Δerm strain, modified to bear a truncated pyrE gene (Ng et al., 2013). The ΔspoIIQ mutant (spoIIQ for simplicity) was shown to initiate sporulation in a rich medium (Brain Heart Infusion Supplement, BHIS) but to be severely impaired in heat resistant spore formation; however, the stage of block was not examined in detail (Dembek et al., 2015). Introduction of the wild type (WT) spoIIQ gene at the pyrE locus restored sporulation (Dembek et al., 2015). Here, we used ACE to similarly construct a spoIIIAH allele, in which codons 23–196 were removed in frame (Fig. 2A), and to transfer the mutation to the spoIIIAH locus of strain 630Δerm. The steps involved in the construction of the ΔspoIIIAH mutant, hereinafter referred to as spoIIIAH for simplicity (strain AHCD812; Supporting Information Table S1), are outlined in

© 2015 The Authors. *Molecular Microbiology* published by John Wiley & Sons Ltd., *Molecular Microbiology* **100**, 204–228
Supporting Information Fig. S1 (see also the Supporting Experimental procedures). To facilitate the identification of the stage of block imposed by the spoIIQ or spoIIIAH mutations, as well as their impact on cell type-specific gene expression at the single cell level (see below), we used liquid sporulation medium (SM), in which sporulation was more synchronized than in other growth conditions tested (Pereira et al., 2013).

The spoIIQ mutant (Dembek et al., 2015) and the newly constructed spoIIIAH mutant, along with the parental WT strain 630Δerm were grown in SM, and the titer of heat resistant spores (spores/ml of culture) was determined 24, 48 and 72 h after inoculation. In line with earlier results (Pereira et al., 2013), the titer of spores for the WT strain increased from $1.7 \times 10^5 \pm 2.9 \times 10^4$ spores/ml of culture at hour 24, to $1.5 \times 10^6 \pm 4.5 \times 10^5$ spores/ml at hour 48 and $2.1 \times 10^6 \pm 3.7 \times 10^5$ spores/ml at hour 72 (Table 1). In contrast, while the spoIIQ or spoIIIAH mutations did not affect cell viability, no heat resistant spores were detected...
for either mutant, at any sampling time (Table 1). Thus, the spoIIQ mutation had a more severe impact on the titer of heat resistant spores in SM, as compared to BHIS (Dembek et al., 2015). In any event, under our culturing conditions, spoIIQ and spoIIIAH are essential for heat resistant spore formation.

Even though the spoIIIAH mutation is an in-frame deletion, we tested for polar effects by in trans complementation analysis. We constructed a plasmid in which spoIIIAH, expressed from the promoter for the spoIIIA operon, was fused to its 3'-end to the SNAPCd reporter (Pereira et al., 2013). In parallel, a similar plasmid was produced bearing a spoIIQ-SNAPCd fusion expressed from the spoIIQ promoter. The ability of the spoIIQ-SNAPCd or spoIIIAH-SNAPCd alleles to complement the spoIIQ or spoIIIAH mutations was assessed by measuring the titer of heat resistant spores at hours 24, 48 and 72 of growth in SM. The spoIIQ mutant complemented with the spoIIQ-SNAPCd allele gave a titer of $4.8 \times 10^5 \pm 1.3 \times 10^4$ spores/ml of culture at hour 24 (as compared with $1.7 \times 10^5 \pm 2.9 \times 10^4$ spores/ml for the WT), $4.7 \times 10^5 \pm 1.6 \times 10^4$ at hour 48 (1.5 $\times 10^6 \pm 4.5 \times 10^5$ for the WT) and $2.9 \times 10^5 \pm 4.4 \times 10^4$ at hour 72 (2.1 $\times 10^6 \pm 3.7 \times 10^5$ for the WT) (Table 1). Although largely restoring sporulation to the spoIIQ mutant, the spoIIQ-SNAPCd allele seemed to perform better at early time points. The spoIIIAH mutant complemented with the fusion showed titers of $1.2 \times 10^5 \pm 2.9 \times 10^4$ spores/ml of culture at hour 24, $1.5 \times 10^6 \pm 2.8 \times 10^5$ at hour 48, and $1.9 \times 10^6 \pm 1.0 \times 10^5$ at hour 72 of growth in SM (Table 1). Thus, spoIIIAH-SNAPCd fully restored sporulation to the spoIIIAH mutant. We conclude that the sporulation phenotype of the two mutants is thus due to loss of function of the spoIIQ or spoIIIAH genes (see also (Dembek et al., 2015)). Together, the results confirm the requirement for sporulation of the spoIIQ and spoIIIAH genes, suggest that the impact of the spoIIQ mutation may be medium-dependent as also found for B. subtilis (Sun et al., 2000; Broder and Pogliano, 2006; Aung et al., 2007), and show that the SpolIIQ-SNAPCd and SpolIIIAH-SNAPCd fusions are largely functional.

spoIIQ and spoIIIAH mutants are often blocked at an intermediate stage in the engulfment sequence

To determine the stage at which the spoIIQ and spoIIIAH mutations caused a defect in the spore differentiation pathway, cultures of the two mutants, in parallel with the parental strain, were grown in SM and imaged after 14 hours of growth, following staining with the membrane dye FM4-64 and with the DNA marker Hoechst 33342. Previous work has shown that at this
time of incubation in SM medium, all of the main stages of sporulation, including free mature (phase bright) spores, are represented in a culture of the WT (Pereira et al., 2013); therefore these conditions were used throughout this study, unless otherwise stated. The cells were imaged using Super Resolution Structured Illumination Microscopy (SR-SIM) in which the lateral resolution is increased to about 110 nm, as compared with the diffraction limit of about 250 nm of conventional light microscopy (Schermelleh et al., 2010). As described previously (Pereira et al., 2013), the forespore chromosome stains strongly following asymmetric division and engulfment, while the more dispersed mother cell chromosome shows a less intense signal. The spoIIQ and spoIIIAH mutants completed asymmetric division and showed strong staining of the forespore chromosome (Supporting Information Fig. S2, blue and red arrows; compare A with B and C). Thus, neither asymmetric division nor segregation and condensation of the forespore chromosome are blocked in spoIIQ or spoIIIAH sporangia. Asymmetric division and forespore chromosome segregation were also observed for the spoIIQ mutant in BHIS medium (Dembek et al., 2015). We then scored cells based on morphological classes that can be visualized by phase contrast and fluorescence microscopy (Fig. 2B and C). To better visualize the asymmetric septum and the forespore membranes during engulfment in spoIIQ and spoIIIAH sporangia, the cells were imaged by SR-SIM following FM4-64 staining, but omitting the DNA labelling step (Fig. 2B).

Class a, defined as cells with flat septa, was scored individually as was class d, defined as completed engulfment. However, cells with curved septa, i.e. at intermediate stages of engulfment were scored together as class b-c. About 43% of the sporulating cells of the WT strain 630Δerm showed straight, flat, septa (class a), 14% showed curved septa (class b-c), and about 43% of the cells had completed the engulfment sequence (sum of classes d to f) (Fig. 2C); 6% showed discernible spores inside the mother cell (class e; which comprises sporangia with phase dark, phase grey or phase bright spores), and free spores represented 8% of the population of sporulating cells (class f) (Fig. 2C). In contrast, no cells scored as class e or f for the spoIIQ mutant and only 1% of the cells was scored as class e for the spoIIIAH mutant (Fig. 2C). Rather, 36% of spoIIQ sporangia and 49% of spoIIIAH sporangia were scored as class b-c (Fig. 2C). As a result, the number of class d cells (engulfment completed) decreased to 10% for the spoIIQ mutant, and to 3% for the spoIIIAH mutant (Fig. 2C). We conclude that the spoIIQ or spoIIIAH mutants are both impaired in forespore engulfment.

spoIIQ and spoIIIAH mutants form bulges and vesicles during engulfment

Class b-c sporangia of both mutants (i.e., scored at an intermediate stage in engulfment) were often seen in which the two edges of the engulfing membranes were asymmetrically located or had bulges or vesicles protruding from the septal region into the mother cell cytoplasm (Supporting Information Fig. S2, red arrows in panels B and C) and that contained DNA (Supporting Information Fig. S2, yellow arrows in the overlay figures of panels B and C). To better characterize these phenotypes, b-c cells were sub-divided into two additional classes (Fig. 2C and D). Sub-class b1 includes sporangia showing bulges, vesicles or asymmetrically located edges of the engulfing membranes (Fig. 2B and C; yellow and green arrows). Sub-class b2 (Fig. 2C and D) groups sporangia that show septa with a zig-zag shape where bulge formation seems to occur off the center of the septum (Fig. 2B, blue arrows) and septa curved towards the forespore distal pole rather than into the mother cell cytoplasm (Fig. 2B, white arrows). Class b1 sporangia were found at a frequency of about 38% for spoIIQ and of 31% for spoIIIAH mutants (Fig. 2B and D; note that the numbers refer to the percentage of cells in class b-c showing the indicated phenotypes). Class b2 sporangia were seen at a frequency of 0.5% for the spoIIQ mutant but at a frequency of 69% for the spoIIIAH mutant (Fig. 2B and D). Thus, a distinctive phenotypic characteristic of the spoIIIAH mutant is the presence of septa that curved inward, into the forespore, and the formation of bulges off center (Fig. 2B and D).

Bulges and vesicles are not seen for spoIIQ or spoIIIAH mutants of B. subtilis. They are however formed by mutants in the spoIID, spoIM or spoIP genes, coding for the DMP complex (Lopez-Diaz et al., 1986; Smith et al., 1993; Margolis et al., 1993; Frandsen and Stragier, 1995; Aung et al., 2007; Gutierrez et al., 2010) (Fig. 2D), or in spoIB mutants, where the DMP proteins fail to localize (Perez et al., 2000). Septa that curve towards the forespore pole are also seen in B. subtilis cells lacking both septal hydrolases SpoIID and SpoIP or in cells unable to produce αC and hence lacking any of the DMP proteins (Illing and Errington, 1991a; Rodrigues et al., 2013). Moreover, asymmetrically positioned leading edges of the engulfing membranes has also been noted for a B. subtilis mutant with impaired activity of the SpoID lytic transglycosylase (Gutierrez et al., 2010). The bulges/vesicles phenotype of DMP mutants in B. subtilis is due to the absence of PG hydrolytic activity within the septum that, together with continued synthesis of PG, causes growth of the bulges and eventually formation of the vesicles (Meyer et al., 2010). We speculate that the bulges and vesicles...
seen for the spoIIQ and spoIIIAH mutants of C. difficile may arise by a similar mechanism, i.e., impaired PG hydrolytic activity within the septum whilst biosynthetic activity is maintained (Meyer et al., 2010). We suggest that PG degradation within the septum is impaired in spoIIQ and spoIIIAH mutants of C. difficile.

The H120S substitution in the LytM domain does not cause formation of bulges or vesicles during engulfment

We considered the possibility that the bulges/vesicles phenotype of the spoIIQ and spoIIIAH mutants of C. difficile was caused, at least in part, by the absence of an activity associated with the SpoIIQ protein, which possesses a LytM domain with all the conserved residues needed for the formation of a functional endopeptidase catalytic site (Fig. 1D). To test for a role of the SpoIIQ LytM domain in C. difficile, the conserved His120 residue in motif 1 was changed to a serine, found at the homologous position in the enzymatically inactive B. subtilis SpoIIQ orthologue (Fig. 1D; see also the Experimental procedures). The spoIIQ H120S-SNAPCd allele, under the control of its normal promoter, was introduced in a plasmid, which was transferred to the spoIIQ mutant strain (Fig. 2A). Asymmetric septation and forespore chromosome segregation proceeded normally in the mutant (Supporting Information Fig. S2, compare A with C and D). The analysis of the spoIIQ H120S mutant by SR-SIM following FM4-64 staining shows that 13% of the sporangia complete engulfment (sum of classes d and e) as compared to 14% (classes d to f) for the spoIIQ-SNAPCd strain (Fig. 2C). This does not differ much from the percentage of class d cells seen for a spoIIQ deletion mutant (10%; Fig. 2C). Strikingly, however, no bulges, vesicles or inverted septa were seen for the spoIIQ H120S-SNAPCd strain (Fig. 2C, sub-classes b1 and b2). In experiments detailed below, we found that SpoIIQH120S-SNAPCd accumulates to reduced levels as compared to the WT protein, and localizes less efficiently (section on the localization of SpoIIQ-SNAPCd). Thus, the cells of the spoIIQ H120S mutant able to complete engulfment appear to do so independently of an intact LytM domain, or at least of the presence of His120.

At least one component of the DMP machine localizes independently of SpoIIQ or SpoIIIAH

As an intact LytM domain in SpoIIQ may not be essential for engulfment, it seemed unlikely that the bulges/vesicles phenotype of the spoIIQ deletion mutant was due to the loss of an enzymatic activity. Moreover, the bulges/vesicles phenotype is also seen for the spoIIIAH mutant, which is not predicted to exhibit any enzymatic activity. Still, the bulges/vesicles phenotype of spoIIQ and spoIIIAH mutants, reminiscent of the phenotypes associated with DMP mutants of B. subtilis (Lopez-Diaz et al., 1986; Smith et al., 1993; Margolis et al., 1993; Frandsen and Stragier, 1995; Aung et al., 2007; Gutierrez et al., 2010), suggests that SpoIIQ and SpoIIIAH are somehow required for a PG hydrolytic activity during engulfment. In B. subtilis, the primary landmark protein for localization of the DMP machinery is SpolIB, produced in pre-divisional cells (Perez et al., 2000; Aung et al., 2007). However, SpoIIQ and SpoIIIAH can localize the DMP proteins in the absence of SpolIB via an alternative pathway involving SpolVFA, a membrane protein required for the activation of αK (Aung et al., 2007). As both SpolIB and SpolVFA are absent from the Clostridia (Galperin et al., 2012), it seemed possible that SpoIIQ and SpoIIIAH could be involved in the correct localization of the DMP proteins in C. difficile. As a first test of this idea, we constructed a spoIID-SNAPCd fusion and examined the localization of the protein by SR-SIM. Control experiments showed that under our experimental conditions no release of the SNAPCd domain by proteolysis was detected (Supporting Information Fig. S3A). In WT cells, SpoIID-SNAPCd localizes along the mother cell membranes, but it is enriched at the septal region (Supporting Information Fig. S4A, red and green arrows). SpoID-SNAPCd was found lining flat septa, and as faint foci at the leading edges of the engulfing membranes (Supporting Information Fig. S4A, yellow arrows). The signal from SpoID-SNAPCd remained following engulfment completion (Supporting Information Fig. S4A). Surprisingly, SpoID-SNAPCd remained at the septum in spoIIQ or spoIIIAH sporangia and in the latter mutant, enrichment at the leading edges of the engulfing membranes was more apparent (Supporting Information Fig. S4B and C). Thus, at least one of the components of the DMP PG hydrolytic machine is recruited to the septal region independently of SpoIIQ or SpoIIIAH. Possibly then, SpoIIQ and SpoIIIAH are directly or indirectly required for proper activity of the DMP complex or of a yet unknown factor required for engulfment (see also the Discussion).

SpoIIQ- and SpoIIQH120S-SNAP localize to the septum and forespore membranes

The analysis of the localization of SpoID-SNAPCd in spoIIQ or spoIIIAH sporangia suggested that the recruitment and maintenance of SpoIID to the septum and engulfing membranes was essentially independent of SpoIIQ and SpoIIIAH. To gain further insight into the function of SpoIIQ and SpoIIIAH, we first studied the
subcellular localization of the SpoIIQ-SNAP\textsuperscript{Cd} and SpoIIQ\textsuperscript{H120S}-SNAP\textsuperscript{Cd} fusion proteins by SR-SIM. As shown above, SpoIIQ-SNAP\textsuperscript{Cd} is largely functional (Table 1). In WT cells, SpoIIQ-SNAP\textsuperscript{Cd} first localized uniformly along flat septa; then, in cells with curved septa, the fusion proteins form foci at the edges and at the center of the septum (Fig. 3A). In sporangia that had completed the engulfment sequence, SpoIIQ-SNAP\textsuperscript{Cd} was found around the entire contour of the forespore (Fig. 3A). This pattern did not differ from that seen for SpoIIQ-SNAP\textsuperscript{Cd} in the absence of spoIIQ, except that in the deletion mutant, the frequency of cells decorated by the fusion protein was higher (Fig. 3, compare the percentages shown for cells at different stages in the engulfment sequence in panels A and B, left column). Presumably, SpoIIQ-SNAP\textsuperscript{Cd} is slightly impaired for assembly at least in the presence of the WT protein. Immunoblotting and fluoroimaging analysis of proteins in whole cell extracts before and after labelling with the TMR-Star substrate shows that more SpoIIQ-SNAP\textsuperscript{Cd} accumulates in the absence of WT SpoIIQ than in WT cells (Supporting Information Fig. S3B). Thus, it seems possible that, in the presence of WT SpoIIQ, the SpoIIQ-SNAP\textsuperscript{Cd} protein that fails to localize is degraded.

The localization of the SpoIIQ\textsuperscript{H120S}-SNAP\textsuperscript{Cd} fusion protein followed the same pattern as that of SpoIIQ-SNAP\textsuperscript{Cd} (along flat septa, foci at the edges of the curved septa, and full encasement of the engulfed forespore) (Fig. 3A and B, right panels). However, the percentage of cells showing localization of SpoIIQ\textsuperscript{H120S}-SNAP\textsuperscript{Cd} in spoIIQ mutant or WT cells was lower than for SpoIIQ-SNAP\textsuperscript{Cd} (Fig. 3; compare the percentages shown in panels A and B, right). Fluorescence intensity profiles suggest that the levels of SpoIIQ\textsuperscript{H120S}-SNAP\textsuperscript{Cd} localized to the septum and engulfing membranes are lower than for the WT fusion protein (Fig. 3B, bottom).
SpoIIIAH and the DMP components are not produced, sigE fully restore sporulation to a spoIIQ mutant. Because SpoIIQ-SNAPCd was dependent on the presence of WT SpoIIQ (Supporting Information Fig. S3B). The H120S substitution may have rendered the fusion protein slightly unstable in vivo. It is also possible that SpoIIQ-H120S-SNAPCd is partially impaired in its ability to correctly localize and that mislocalized protein is degraded (see also below). However, since the bulges and vesicles phenotype is not seen for the spoIIQ H120S-SNAPCd strain, it seems likely that once at its subcellular address, the protein is largely functional during engulfment. While the highest spore titer obtained for the complementation of the spoIIQ mutation with the spoIIQ-SNAPCd allele was of $2.9 \times 10^5 \pm 4.4 \times 10^4$ spores/ml of culture 72 h after inoculation in SM (see above), the highest spore titer observed for the spoIIQ spoIIQ H120S-SNAPCd strain, also at hour 72, was of only $7.83 \times 10^3 \pm 2.4 \times 10^2$ spores/ml (Table 1). Thus, spoIIQ H120S-SNAPCd does not support sporulation efficiently. Impaired localization and/or instability of the SpoIIQ-H120S-SNAPCd protein may explain its failure to fully restore sporulation to a spoIIQ mutant. Because the spoIIQ H120S-SNAPCd strain sometimes completes the engulfment sequence, it appears that SpoIIQ bearing an intact LytM domain is required for a late, post-engulfment completion, stage of sporulation.

**Co-dependent localization of SpoIIQ/SpoIIQ$^{H120S}$ and SpoIIIAH-SNAP**

We then wanted to determine whether the localization of SpoIIQ-SNAPCd was dependent on the presence of SpoIIIAH. For that, the plasmid expressing spoIIQ-SNAPCd was introduced into the spoIIIAH mutant, and sporulating cells imaged by SR-SIM, following MTG staining. In the absence of SpoIIIAH, SpoIIQ-SNAPCd was still detected at the septum, but the protein also showed dispersed localization around the forespore, even in cells with flat septa (Fig. 3C, red and blue arrows). Fluorescence intensity profiles confirmed the presence of SpoIIQ-SNAPCd ahead of the engulfing membranes (Fig. 3C, bottom). The mislocalization of SpoIIQ-SNAPCd was particularly evident in the abortive disporic sporangia formed by sigE mutants, in which SpoIIIAH and the DMP components are not produced, and a second polar septum is formed (Fig. 3D). The localization of SpoIIQ in B. subtilis is partly dependent on spoIIIAH (Rubio and Pogliano, 2004; Blaylock et al., 2004; Doan et al., 2009; Fredlund et al., 2013; Rodrigues et al., 2013).

Next, we examined the localization of SpoIIIAH-SNAPCd. The fusion protein decorated a smaller percentage of sporangia in the presence of the WT spoIIIAH allele in cells that had just initiated engulfment but not in sporangia at later stages in the sequence (Fig. 4A, compare the percentages in the top and bottom set of panels). As assessed by its ability to restore sporulation to the spoIIIAH mutant, SpoIIIAH-SNAPCd is largely functional (Table 1). Under our conditions, no release of the SNAPCd domain from SpoIIIAH-SNAPCd was detected (Supporting Information Fig. S3C). Reminiscent of the localization pattern of SpoIIQ-SNAPCd, the SpoIIIAH-SNAPCd fusion protein localized along flat septa and was found around the forespore in sporangia in which engulfment was completed (Fig. 4B). However, at intermediate stages in engulfment, while SpoIIQ-SNAPCd formed foci at the leading edges of the engulfment membranes in the presence of the WT spoIIQ allele, SpoIIIAH-SNAPCd formed a continuous shell or arc lining the curved, migrating membrane, in both the presence or in the absence of the spoIIIAH WT allele (Fig. 4B). In agreement, fluorescence intensity profiles show little enrichment of SpoIIIAH-SNAPCd at the leading edges of the engulfing membranes (Fig. 4B, bottom panels). Thus, the data suggests that not all SpoIIIAH co-localizes with SpoIIQ.

Consistent with this inference, while SpoIIIAH-SNAPCd mislocalized along the mother cell membrane in the absence of SpoIIQ (Fig. 4C, white arrows), it was still found at the septal plate (yellow arrows). Fluorescence intensity profiles reveal a pattern of strong SpoIIIAH-SNAPCd signals along the mother cell membrane, suggesting that some of the delocalized protein remains associated (Fig. 4C, bottom). Integration of the fluorescence signal suggests that about 42% of the fusion protein remains at the septum (Fig. 4C, bottom panel). Therefore, although SpoIIQ and SpoIIIAH are largely co-dependent for localization, some SpoIIIAH can localize to asymmetric septa and the engulfing membranes independently of SpoIIQ. This contrasts with the situation in B. subtilis, where the localization of SpoIIIAH is dependent on SpoIIQ, but SpoIIQ localizes in the absence of SpoIIIAH (Rubio and Pogliano, 2004; Rodrigues et al., 2013; Fredlund et al., 2013). It suggests that in addition to SpoIIQ, tethering of SpoIIIAH at the septum and engulfing membranes involves additional factors.

**SpoIIQ and SpoIIIAH form a complex in vivo**

Both the pattern and the partial co-dependency for localization of SpoIIQ and SpoIIIAH suggested that the two proteins interact, as is the case for B. subtilis (Blaylock...
et al., 2004; Doan et al., 2009). To test for formation of a complex between SpoIIQ and SpoIIIAH, we took advantage of the strains producing the SpoIIQ-SNAP<sup>Cd</sup> or SpoIIQ<sup>H120S</sup>-SNAP<sup>Cd</sup> translational fusions, and the SNAP-Capture resin in a modified pull-down assay (see the Experimental procedures section). The SNAP-Capture resin carries the SNAP substrate benzylguanine covalently cross-linked to agarose beads, which allows the SNAP tag to be covalently bound to the resin. We prepared whole cell lysates from SM cultures of the strains producing the various fusion proteins in our standard sampling conditions (Figs. 3 and 4). As a control, we also prepared a lysate from a strain expressing the isolated SNAP<sup>Cd</sup> domain under the control of the mother cell-specific P<sub>spoIIA</sub> promoter (Supporting Information Fig. S5, ‘SNAP’). The lysates were incubated with the SNAP capture resin, the beads were washed and the proteins interacting with the immobilized SNAP fusion released by boiling and analysed by immunoblotting with anti-SNAP or anti-SpoIIIAH antibodies. Note that in these experiments the immobilized ‘Spo’-SNAP<sup>Cd</sup> fusion is not released from the column by boiling and therefore is not resolved by SDS-PAGE. Using an anti-SNAP antibody, we verified accumulation of the SNAP<sup>Cd</sup> domain, of SpoIIQ-SNAP<sup>Cd</sup> and of SpoIIQ<sup>H120S</sup>-SNAP<sup>Cd</sup> in the whole cell extracts (Supporting Information Fig. S5, bottom panel). However, SpoIIQ<sup>H120S</sup>-SNAP<sup>Cd</sup> (but not SpoIIQ-SNAP<sup>Cd</sup>) remained undetected (Supporting Information Fig. S4). This is in line with the inference that the H120S substitution may impair the in vivo
stability of the fusion protein. Following incubation of the beads with the lysates, the mixture was washed. Some SNAPCd or SpoIIQ-SNAPCd was detected in the wash fractions for the two assays indicating incomplete capture by the beads (Supporting Information Fig. S5, bottom). After boiling, no SNAP or SpoIIQ-SNAPCd was detected by immunoblotting, consistent with covalent binding of the proteins to the SNAP substrate immobilized on the beads.

Importantly, SpoIIIAH, detected with a specific antibody raised against the purified extracytoplasmic domain of the protein (Supporting Information Fig. S6; see also the Experimental procedures section), was pulled down by SpoIIQ-SNAPCd but not by the isolated SNAPCd domain, indicating binding to the immobilized fusion protein (Supporting Information Fig. S5, top). However, the presence of SpoIIIAH in the wash fraction of the assay suggests that not all of the SpoIIIAH present in the extract binds to the immobilized SpoIIQ-SNAPCd, or that not all SpoIIIAH molecules bind with equal strength to the fusion protein. SpoIIIAH was not detected in the pull-down fraction of the SpoIIQH120S, SNAPCd-producing strain (Supporting Information Fig. S5, top, last lane), possibly due to the low levels of accumulation of the fusion protein. Also, the levels of SpoIIIAH in the extracts and wash fraction of the strain producing SpoIIQH120S-SNAPCd were reduced compared to the strain producing the WT SpoIIQ-SNAPCd fusion (Supporting Information Fig. S5, top). It is possible that a less stable complex is formed between SpoIIQH120S, SNAPCd and SpoIIIAH, as suggested by our in vitro experiments described below, and that unbound SpoIIIAH (and also SpoIIQH120S, see above) is less stable. Our failure to produce an antibody against the purified SpoIIQ protein precluded a pull-down experiment with immobilized SpoIIIAH-SNAPCd. In any event, the results show that at least a fraction of the SpoIIIAH molecules forms a complex with SpoIIQ-SNAPCd in sporulating cells of C. difficile.

C. difficile SpoIIQ is a Zn2+ binding protein

Unlike its B. subtilis counterpart, the SpoIIQ protein of C. difficile has all the residues required to form a LytM domain able to bind Zn2+ ([Crawshaw et al., 2014]; Fig. 1D and Supporting Information Fig. S7). Moreover, the observed phenotypes of the spoIIQ H120S mutant indicate that an intact LytM domain is important for the function of SpoIIQ, at least following engulfment completion (above). As a first step to investigate the behavior of SpoIIQ and SpoIIIAH in vitro, we wanted to establish whether SpoIIQ in C. difficile has the ability to coordinate Zn2+. We analyzed the Zn2+ content of pure protein samples by inductively coupled plasma mass spectrometry (ICP-MS). The soluble domains of the WT and H120S variants of SpoIIQ (sSpoIIQ and sSpoIIQH120S, residues 31–222; i.e. where the N-terminal transmembrane domain is replaced by a TEV–tobacco etch virus protease-cleavable His6 tag; Supporting Information Fig. S6), were overproduced in E. coli and purified by Ni2+-affinity chromatography, followed by tag removal (see Material and Methods for details). Samples of purified protein were then analyzed by size exclusion chromatography (SEC) immediately after affinity chromatography (Fig. 5A, untreated, grey trace), as well as after incubation with a fivefold Zn2+ excess (Fig. 5A, 5x Zn2+, black trace) or in the presence of 1mM EDTA (Fig. 5A, EDTA, red trace). Fractions from the SEC column were collected and the metal content quantified. In order to establish the relative occupancy with Zn2+, the protein concentration for each fraction was also determined (Fig. 5A, top, sSpoIIQ, blue trace and bottom panel, sSpoIIQH120S, purple trace). Addition of an excess of Zn2+ shows that sSpoIIQ can bind to the metal to approximately 80% occupancy, assuming that each sSpoIIQ molecule binds one Zn2+ ion (Fig. 5A, compare the blue and black traces). As expected, the H120S substitution abolishes binding to the metal, even in the presence of excess Zn2+ (Fig. 5A, bottom panel, compare purple and black traces). The absence of detectable metal in the untreated samples is likely due to the affinity purification steps, where imidazole would have chelated the metal, as well as partial metal chelation by the Superdex resin. These results show that C. difficile SpoIIQ is a Zn2+ binding protein and that His120 is required for metal coordination.

The interaction between SpoIIQ and SpoIIIAH in vitro is influenced by Zn2+

To determine whether C. difficile SpoIIQ and SpoIIIAH interact directly, the purified proteins were analysed by size exclusion chromatography multi-angle laser light scattering (SEC-MALLS), alone or in combination. The soluble domain of SpoIIIAH (sSpoIIIAH, residues 29–229) was overproduced and purified as described above for sSpoIIQ and sSpoIIQH120S (Supporting Information Fig. S6; for details see the Experimental procedures section). We first analyzed the behaviour of the individual proteins, which eluted as single peaks with calculated molecular masses of 22.3 kDa for sSpoIIIAH, 20.9 kDa for sSpoIIQ, and 20.8 kDa for sSpoIIQH120S (Fig. 5B, top, yellow, blue and purple dotted lines respectively, which correspond to the weight average molecular mass, calculated from refractive index and light
scattering measurements). As the predicted MW of these proteins is 23.0 kDa for sSpoIIIAH and 21.4 kDa for both forms of sSpoIIQ, the calculated masses indicate that all proteins are monomeric in solution. Analysis by SDS-PAGE of the elution fractions (Fig. 5B, bottom panel, first three rows) confirms the presence of a single species in solution.

Next, purified sSpoIIQ and sSpoIIIAH were incubated for one hour in a 1:1 molar ratio, in the presence of a fivefold excess of Zn$^{2+}$ to allow for maximum metal binding, and analyzed by SEC-MALLS. This analysis revealed a new species eluting at 10.0 ml (Fig. 5B, green trace), with a calculated mass of 43.1 kDa (Fig. 5B, green dotted line) and containing both proteins, as verified by SDS-PAGE (Fig. 5B, bottom panel, row 4). These results confirm that SpoIIQ and SpoIIIAH interact via their soluble domains, as observed for the *B. subtilis* orthologues (Meisner et al., 2012; Levdikov et al., 2012). The calculated molecular mass of the complex suggests the presence of a heterodimer of sSpoIIQ and sSpoIIAH. An additional peak was observed with a calculated mass of 23.6 kDa (Fig. 5B, second peak between 10.5 and 11 ml, green trace and dotted line). Analysis by SDS-PAGE indicates that this peak corresponds mostly to sSpoIIQ, with only traces of sSpoIIIAH detectable in the gel (Fig. 5B, bottom, row 4). This could be a result of an excess of sSpoIIQ in the original incubation due to differences in protein concentration calculations and/or some partial dissociation of the complex.

Surprisingly, incubation of sSpoIIQH120S with sSpoIIIAH at a 1:1 molar ratio, yielded a different elution profile (Fig. 5B, top, light purple trace), although a complex was still formed, as detected by SDS-PAGE (Fig. 5B, bottom, last lane). This complex elutes with a calculated MW of 27.3 kDa (Fig. 5B,
top, light purple dotted line), a value between that expected for a heterodimeric species and for monomeric sSpoIIQH120S or sSpoIIIAH, indicating the presence of a partially dissociating complex. Consistent with the localization of SpoIIQH120S-SNAPCd, which depends on SpoIIIAH, this result confirms that sSpoIIQH120S still interacts with sSpoIIIAH but that the complex formed seems to be less stable. Together, these observations imply that His120 and/or coordination of Zn\(^{2+}\) impacts on the interaction of SpoIIQ with SpoIIIAH.

To test whether the observed effect is due to the ability of SpoIIQ to bind Zn\(^{2+}\) and not to an indirect effect of the H120S substitution, the SEC-MALLS analysis of purified WT sSpoIIQ and sSpoIIIAH was repeated after incubation of the proteins in the presence of 1 mM EDTA. As shown by ICP-MS experiments, purified sSpoIIQ contains no detectable zinc and addition of EDTA ensures no metal ions would be present in solution. Importantly, the presence of EDTA does not alter the elution profile of sSpoIIQ alone (Supporting Information Fig. S6C). In the absence of metals, the sSpoIIQ-sSpoIIIAH complex now exhibits an elution profile identical to that of the sSpoIIQH120S-sSpoIIIAH sample, with a calculated mass of 27.6 kDa (Fig. 5B, top, light green dashed trace and line; bottom row 5). This confirms that the presence of Zn\(^{2+}\) alters the characteristics of the complex formed between sSpoIIQ and sSpoIIIAH, and that when sSpoIIQ is unable to bind the metal, the interaction of the two proteins is affected.

**SpoIIQ and SpoIIIAH interact throughout engulfment**

In an attempt to localize the interaction between SpoIIQ and SpoIIIAH in sporulating cells, we made use of a Split-SNAP reporter (Mie et al., 2012). The crystal structure of the SNAP-tag shows that the protein consists of two domains (Daniels et al., 2000). Earlier studies have shown that the 128-residue SNAP-tag can be split into an N-terminal fragment (residues 1–91) and a C-terminal fragment (residues 92–182) carrying the reactive Cys145 residue, that do not re-associate efficiently unless fused to two interacting proteins (Fang et al., 2005; Mie et al., 2012). We fused the C-terminus of SpoIIQ to the N-terminal moiety of SNAPCd (or SNAPCd-N) and the C-terminus of SpoIIIAH to the C-terminal moiety of the reporter (SNAPCd-C) (Fig. 6A). Under these conditions and based on the *B. subtilis* model, the interaction of SpoIIQ and SpoIIIAH would allow association of the SNAPCd N- and C-terminal domains in the intermembrane space, formation of the substrate-binding site, which includes Cys145 in the C-terminal moiety, and labelling of the reconstituted reporter (Fig. 6A).

Co-production of SpoIIQ-SNAPCd-N with SpoIIIAH-SNAPCd-C resulted in a fluorescence signal from the reconstituted SNAPCd domain along flat septa (Fig. 6B, red arrows). Fluorescence from the reconstituted SNAPCd was also seen in sporangia with curved septa as a punctate pattern along the engulfing membranes (Fig. 6B, yellow arrows), reminiscent of the pattern of SpoIIQ- and SpoIIIAH-SNAPCd localization (Figs. 3 and 4; above). These foci, which are more evident on the higher magnification images shown as the bottom row of figure 6B, presumably represent SpoIIQ-SpoIIIAH complexes. In the higher magnification images, nearly circular signals are seen, whose size (<120 nm across) is too large to represent SpoIIQ-SpoIIIAH oligomers, assuming that the structure proposed for the *B. subtilis* channel is conserved (Meisner and Moran, 2011; Levskov et al., 2012; Meisner et al., 2012.) (Fig. 6A). The origin of these structures is presently unclear. Decoration of the entire contour of the forespore following engulfment completion was also detected and the punctate pattern persisted (Fig. 6B, last column of the set of panels on the left). We also examined the interaction between SpoIIQH120S-SNAPCd-N and SpoIIIAH-SNAPCd-C. A punctate pattern of the fluorescence signal from the reconstituted SNAPCd was also found at septa, engulfing membranes and around the engulfed forespore but only in about 43% of the sporangia, as compared to 71% for the strain producing SpoIIQ-SNAPCd-N (Fig. 6B, set of panels on the right).

In whole cell extracts prepared from the samples used for fluorescence microscopy, SpoIIQ-SNAPCd-N or SpoIIQH120S-SNAPCd-N accumulate as a species of approximately 37 kDa, as detected with an anti-SNAP monoclonal antibody, consistent with the predicted size of the fusions (Fig. 6C). SpoIIIAH was detected with an anti-SpoIIIAH antibody as a species of about 25 kDa, and SpoIIIAH-SNAPCd as a species of about 40 kDa, consistent with their predicted size (Fig. 6C). SpoIIIAH-SNAPCd-C was not detected with the anti-SNAP monoclonal antibody, suggesting that this antibody only recognizes the N-terminal domain of the SNAP tag. The difference in mobility between the SpoIIQ-SNAPCd-N (or SpoIIQH120S-SNAPCd-N) and SpoIIIAH-SNAPCd-C fusions is also consistent with the slower migration of purified SpoIIIAH relative to SpoIIQ in SDS-PAGE (Supporting Information Fig. S6B). Neither SpoIIQ-SNAPCd-N nor SpoIIIAH-SNAPCd-C was labelled by the TMR-Star substrate when independently produced, as detected by fluoroimaging (Fig. 6C, last two lanes) or fluorescence microscopy (Fig. 6D). In contrast, SpoIIIAH-SNAPCd-C, which carries Cys145, was labelled when the fusion was co-produced with
either SpoIIQ-SNAPCd or SpoIIQH120S-SNAPCd (Fig. 6C, first two lanes).

Thus, SpoIIQ-SNAPCd and SpoIIQH120S-SNAPCd interact with SpoIIIAH-SNAPCd in the septum, appear to form discrete foci during engulfment, and remain associated upon engulfment completion. The observed interaction of SpoIIQH120S-SNAPCd-N with SpoIIIAH-SNAPCd-C in the forespore membranes is consistent with formation of a complex between sSpoIIQH120S and sSpoIIIAH, albeit altered, in vitro (see above).

**spoIIQ and spoIIIAH are required for late forespore- and mother cell-specific gene expression**

A critical function of the mother cell to forespore channel formed by SpoIIQ and SpoIIIAH in *B. subtilis* is to allow continued transcription in the forespore when, following engulfment completion, it becomes isolated from the external environment (Camp and Losick, 2009). As at least some cells of the *spoIIQ H120S* mutant complete engulfment without the accumulation of abnormal forms
such as bulges, vesicles and inverted septa (Fig. 2C), but the mutant is still impaired in sporulation (Table 1), we reasoned that SpoIIQ and possibly SpoIIIah could also have a post-engulfment function in sporulation. To test this possibility, we examined whether the spoIIQ and spoIIIah mutations had an effect on early and late gene expression in C. difficile. We monitored the activity of $\sigma^F \text{ and } \sigma^E$ using transcriptional fusions of the gpr and spoIIA promoters to the SNAPCd tag respectively (Saujet et al., 2013; Pereira et al., 2013). Expression of P_gpr-SNAPCd was detected in 87% of the sporulating cells of the WT, in 82% of spoIIah sporangia, and in 84% of the spoIIQ sporangia (Fig. 7A). Expression of P_spoIia-SNAPCd was detected in 88% of the sporulating cells in the WT, in 93% of the cells in the spoIIah mutant and 83% of the spoIIQ sporangia (Fig. 7A). A quantitative analysis revealed an increase in the intensity of the fluorescence signal for the P_gpr-SNAPCd fusion in individual cells of the spoIIQ or spoIIah mutants, prior to engulfment completion (Fig. 7B, left column of plots). This increase may result from augmented activity of $\sigma^F$ in sporangia impaired in the engulfment sequence and/or from a reduction in the activity of $\sigma^G$ prior to engulfment completion ([Pereira et al., 2013b]; see also below). In contrast, no effect of the mutations was detected for the P_spoIia-SNAPCd fusion (right column of plots). We conclude that deletion of spoIIIah or spoIIQ does not curtail the activity of the early forespore- and mother cell-specific $\sigma$ factors during engulfment. Nevertheless, we found whole cell expression of the P_gpr-SNAPCd fusion in 25% of the spoIIIah sporangia and in 19% of the spoIIQ cells, and of the P_spoIia-SNAPCd fusion in 3% and 7% of the spoIIIah and spoIIQ sporangia respectively (Fig. 7C). This loss of compartmentalization of the activities of $\sigma^F \text{ and } \sigma^E$ is most likely due to instability and eventual collapse of the forespore membranes in either mutant (as seen in Fig. 2B and Supporting Information Fig. S2). Instability of the forespore membranes in spoIIQ and spoIIIah sporangia has also been reported for B. subtilis (Doan et al., 2009).

To monitor the activity of $\sigma^G \text{ and } \sigma^K$ we used the previously characterized P_sspa-SNAPCd and P_cote-SNAPCd transcriptional fusions respectively (Pereira et al., 2013). While $\sigma^G \text{ and } \sigma^K$ show some activity during engulfment, their main period of activity begins with engulfment completion (Pereira et al., 2013). Expression of the $\sigma^G$-dependent P_sspa-SNAPCd fusion was detected in 57% of the sporangia in the WT, in 70% of the spoIIIah cells and in 53% of the spoIIQ cells prior to engulfment completion (Fig. 8A). Moreover, neither mutation significantly affected the distribution of the fluorescence signal measured in individual cells during engulfment; the average signal intensity was of 1.3 ± 0.4 for the WT and of 1.2 ± 0.5 for the spoIIIah mutant ($P = 0.2$) and of 1.4 ± 0.6 for spoIIQ cells in comparison with 1.7 ± 0.4 for the WT, in a parallel experiment ($P = 0.109$) (Fig. 8B). The slight reduction in the activity of $\sigma^G$ in spoIIQ or spoIIIah sporangia prior to engulfment completion, as assessed using the P_sspa-SNAPCd reporter, may nevertheless contribute to the increased activity of $\sigma^K$, as suggested above. However, in cells of the spoIIIah mutant in which engulfment was completed, the distribution of the fluorescence signal in individual cells was strongly reduced (Fig. 8B; average intensity, in arbitrary units, of 2.4 ± 1.0 for the WT, and of 1.1 ± 0.3 for the mutant; $P < 0.0001$). We did not quantify the intensity of the signal for the fraction of spoIIQ cells expressing the P_sspa-SNAPCd fusion, as they were rare in the population. In addition, for the spoIIIah mutant, 3% of the cells scored during engulfment and 25% of the cells scored following engulfment completion P_sspa-SNAPCd displayed a whole cell expression pattern (Fig. 8C). This whole cell pattern of fluorescence was also seen in 6% of the spoIIQ sporangia during engulfment and in 13% of the cells that had completed engulfment (Fig. 8C). Whole cell fluorescence is presumably due to collapse of the forespore membranes and loss of compartmentalization (Fig. 8C) (Doan et al., 2009).

As shown in figure 8A, the number of pre-engulfment cells expressing the $\sigma^K$ reporter fusion P_cote-SNAPCd fusion only represented 8% of the sporulating cells in the spoIIIah mutant and 10% of the spoIIQ cells (as compared with 15% for the WT), and we did not quantify the intensity of the fluorescence signal in those cells (Fig. 8A, right set of panels). A whole cell pattern of P_cote-SNAPCd expression was detected for 10% of the spoIIIah cells and 4% of the spoIIQ cells prior to engulfment completion, most likely due to instability of the forespore membranes as suggested above for a similar pattern observed for the P_sspa-SNAPCd fusion (Fig. 8C). The spoIIQ and spoIIIah mutations thus appear to reduce slightly the fraction of pre-engulfment cells in which $\sigma^K$ is active. Strikingly, however, 84% of the WT cells that had concluded the engulfment sequence expressed the P_cote-SNAPCd reporter, whereas in the spoIIIah mutant only 15% of the cells expressed the fusion (Fig. 8A). Scoring was not possible for cells of the spoIIQ mutant in which very few cells showing complete engulfment were seen (see also above).

We conclude that the spoIIQ and spoIIIah mutations severely compromise the activity of the forespore-specific $\sigma^G$ factor following engulfment completion (Fig. 8D). Furthermore, while both the spoIIQ and spoIIIah deletion mutations reduced the activity of $\sigma^K$ during engulfment, at least the spoIIIah deletion mutation strongly curtailed the activity of $\sigma^K$ following engulfment completion (Fig. 8D).
We show that the spoIIQ and spoIIIAH genes of C. difficile are required for sporulation because they have a role during engulfment of the forespore by the mother cell and are additionally needed for late stages of morphogenesis, following engulfment completion. Our observations bear important implications for our understanding of the engulfment process, and the control of gene expression during endospore development.
Engulfment

In *B. subtilis*, forespore engulfment is mainly controlled by the DMP machine, while PG synthesis and the SpoIIQ-SpoIIIAH interaction play non-essential, redundant roles (Crawshaw et al., 2014). The contribution of the SpoIIQ-SpoIIIAH interaction for engulfment and its more stringent requirement under certain nutritional conditions suggests that redundancy confers environmental robustness to the process (Broder and Pogliano, 2006). It has also been suggested that the SpoIIQ-SpoIIIAH interaction may represent an original core mechanism for engulfment, present before the emergence of the more complex DMP-based process (Ojkic et al., 2014). Our results are in line with this inference, as we found that *spoIIQ* or *spoIIIAH* mutants of *C. difficile* are impaired in engulfment. However, *spoIIQ* and *spoIIIAH*...
mutants show phenotypes reminiscent of those associated with DMP mutants of *B. subtilis* (Lopez-Diaz et al., 1986; Margolis et al., 1993; Smith et al., 1993; Frandsen and Stragier, 1995; Aung et al., 2007). While the function of the DMP machine in *C. difficile* remains to be characterized, it seems possible that both the DMP and the SpolIQ-SpoIIAH modules are essential for engulfment in *C. difficile* and that these two engulfment modules have become functionally separated in *B. subtilis* and presumably in related organisms. In both spolIQ and spoIIAH mutants of *C. difficile*, bulges and vesicles are formed at the asymmetric septum, reminiscent of similar structures formed in *B. subtilis* by spoIIID and spoIIIP mutants which are blocked in the initial stage of engulfment, septal thinning (Lopez-Diaz et al., 1986; Ilbing and Errington, 1991a; Frandsen and Stragier, 1995; Abanes-De Mello et al., 2002). Bulges and vesicles are sites of active cell wall synthesis and their formation is prevented by cell wall-active antibiotics as well as by disruption of the gene coding for the SpoVD transpeptidase (which itself localizes to the bulges and vesicles) (Meyer et al., 2010). We presume that the bulges and vesicles arise in the *C. difficile* mutants by impaired cell wall degradation activity and continued synthesis of new cell wall as described for *B. subtilis* (Meyer et al., 2010). Since the DMP proteins are conserved in *C. difficile*, one possibility is that deletion of spolIQ or spoIIAH may somehow impair the activity of the DMP machine. This control may not operate at the level of the localization of the DMP complex as at least a SpoIID-SNAPCd translational fusion localized to the asymmetric septum in *spoIIQ* or *spoIIAH* sporangia (Supporting Information Fig. S4). However, it remains to be tested whether the localization of the SpoIM and SpolIP protein is affected in *spoIIQ* or *spoIIAH* mutants. In *B. subtilis*, the sepal localization of the DMP proteins is mainly controlled by SpoIB, with SpolIQ and SpolIIAH proteins making an indirect contribution. A SpoIB orthologue is absent from most *Clostridia* including *C. difficile* (Galperin et al., 2012) and how SpoIID-SNAPCd localizes to the septum in the absence of SpolIQ or SpolIIAH is not known. Consistent with a role in engulfment, SpolIQ-SNAPCd and SpolIIAH-SNAPCd fusions localize to the septum and the engulfing membranes. Moreover, SpolIQ-SNAPCd formed a complex with SpolIIAH in *vivo*, and Split-SNAPCd fusions suggest a direct interaction between the two proteins during engulfment. However, while the localization of SpolIQ-SNAPCd is dependent on SpolIIAH, the localization of SpolIIAH-SNAPCd to the septum is only partially dependent on SpolIQ. In *B. subtilis*, it is the localization of SpolIQ that is partially independent of SpolIIAH, and requires the DMP machine, or a product generated by its activity (Rubio and Pogliano, 2004; Blaylock et al., 2004; Doan et al., 2005; Fredlund et al., 2013; Rodrigues et al., 2013). Possibly, the localization of SpolIIAH in *C. difficile* requires, in addition to SpolIQ, the DMP proteins or a product of their activity. The bulges/vesicles phenotype is more pronounced in *spolIIAH* cells, in which septa that curve towards the spore pole are a distinctive phenotype. That this particular phenotype was also seen for *B. subtilis* spoIID/spoIIIP double mutants or in sigE sporangia, unable to produce the DMP proteins (Ilbing and Errington, 1991a; Rodrigues et al., 2013) suggests that, in *C. difficile*, SpolIIAH may have a more prominent role, partly independent of SpolIQ, in controlling the activity of the DMP machine.

In *B. subtilis*, engulfment is much faster in the absence (1-2 min; dependent on SpolIQ/SpolIIAH) than in the presence of the cell wall (about 45 min; dependent on DMP) (Ojkic et al., 2014). However, in the absence of the cell wall, about 40% of the engulfing membranes retract (Ojkic et al., 2014). Since in *C. difficile* the SpolIQ-SpoIIAH complex has a more central role in engulfment, it will be interesting to investigate the mechanics and kinetics of engulfment in this organism.

**Role of the LytM domain of SpolIQ in spore morphogenesis**

SpolIQ has an intact LytM endopeptidase domain suggesting that the protein could directly participate in PG hydrolysis during engulfment. However, in *B. subtilis* and in most other *Bacillus* species and closely related organisms (Crawshaw et al., 2014) (Fig. 1D), the LytM domain of SpolIQ lacks a conserved histidine residue required for coordination of Zn$^{2+}$ as part of the catalytic center (Supporting Information Fig. S7A). At least in *B. subtilis* SpolIQ, where a serine replaces the conserved histidine residue, the protein no longer coordinates the metal and seems to be catalytically inactive (Supporting Information Fig. S7B, compare top and bottom; see also the Supporting Results and Discussion). The observation that in *C. difficile* and in several other *Clostridia*, the histidine residue is present raised the possibility that, in these organisms, an enzymatically active SpolIQ protein directly contributed to PG hydrolysis during engulfment. Importantly, we show that WT SpolIQ, but not a form in which the conserved histidine was replaced by a serine, coordinates Zn$^{2+}$ and therefore could exhibit enzymatic activity. However, the spoIIQ H120S allele did not...
completely impair progress through the engulfment sequence, and in particular, did not cause a bulge/vesicle phenotype. Thus, intact LytM motifs able to bind Zn$^{2+}$ may not be essential during engulfment in *C. difficile*.

In *B. subtilis*, the LytM domain is involved in the interaction with SpoIIIAH and also with a yet unidentified protein that contributes to the septal localization of SpoIIA, at least in the absence of SpoIIIAH (Rodrigues et al., 2013). Using SEC-MALLS, we show that *C. difficile* C-terminal domains sSpoIIQ and sSpoIIIAH form a heterodimeric complex in solution. Importantly, we show that Zn$^{2+}$ binding by SpoIIQ plays a currently uncharacterized role in the interaction with SpoIIIAH. Firstly, the complex formed by sSpoIIQ$^{H120S}$ or WT sSpoIIQ in the presence of EDTA is less stable, as seen by SEC-MALLS. Secondly, an interaction between SpoIIQ$^{H120S}$ and SpoIIIAH is still detected in vivo using Split-SNAP fusions, although in a reduced number of cells.

In *B. subtilis*, SpoIIQ and SpoIIIAH interact via their degenerate LytM and YscJ domains respectively (Meisner and Moran, 2011; Levdikov et al., 2012; Meisner et al., 2012), with the noncatalytic serine downstream from the last β-sheet of the complex interface (Supporting Information Fig. S7C, see also the Supporting Results and Discussion). Since the H120S substitution affects the stability of the SpoIIQ and SpoIIIAH complex, it seems possible that the LytM catalytic region is involved in the interaction with SpoIIIAH also in *C. difficile*. This hypothesis is further supported by the fact that secondary structure predictions for *C. difficile* SpoIIQ indicate that the protein is less structured than its *B. subtilis* orthologue, particularly in the region around the complex interface (Supporting Information Fig. S7D and Supporting Results and Discussion). Importantly, an α-helix in *B. subtilis* SpoIIQ shown to stabilize the complex interface is absent in the *C. difficile* protein (Supporting Information Fig. S7C and D; Supporting Results and Discussion). Moreover, based on channel topology models proposed (Meisner and Moran, 2011; Levdikov et al., 2012; Meisner et al., 2012), it is possible that these structural elements upstream of the LytM domain might also be involved in the interactions between heterodimers required to form a ring (for details, see Supporting Information S1 text). It is therefore possible that the less structured *C. difficile* SpoIIQ requires coordination by the Zn$^{2+}$ ion to provide a stable platform for interaction with SpoIIIAH and the formation of a stable ring (Supporting Information Fig. S7 and Supporting Results and Discussion).

Work in *B. subtilis* has shown the involvement of SpoIIQ and the spoIIIA-encoded proteins in formation of a specialized secretion machine, through which the mother cell nurtures the developing spore, providing small molecules required for continued transcription in the forespore following engulfment completion (Meisner et al., 2008; Camp and Losick, 2009; Doan et al., 2009). The second, later function of the SpoIIQ-SpoIIAH complex of *C. difficile* in spore morphogenesis may be similar and a functional LytM domain and/or a stable complex could be crucial for this later activity. Further work, including structural determination of WT and SpoIIQ$^{H120S}$ complexes with SpoIIIAH, would help elucidate the exact functional and/or structural role of both Zn$^{2+}$ coordination and an intact LytM domain.

**Expanding the mother cell-to-forespore channel model**

The specialized secretion system formed by the spoIIIA-encoded proteins and SpoIIQ maintains the stability of the forespore membranes and the potential for macromolecular synthesis in the forespore during late stages of morphogenesis (Serrano et al., 2004; Meisner et al., 2008; Doan et al., 2009; Camp and Losick, 2009). In *B. subtilis*, the transcriptional activity in the forespore following engulfment completion is severely curtailed in spoIIQ or spoIIIAH mutants, and in these mutants the forespore membranes collapse and compartmentalized gene expression is lost (Serrano et al., 2004; Camp and Losick, 2009; Doan et al., 2009). Two lines of evidence suggest that the SpoIIQ and SpoIIIAH proteins of *C. difficile* may also support a channel-like function. Firstly, among the cells of spoIIQ or spoIIIAH mutants that reach late stages in engulfment or complete the sequence, many show disorganized forespore membranes (Fig. 2 and Supporting Information Fig. S2), and studies with cell type-specific transcriptional fusions to the SNAP fluorescent reporter show that in those cells compartmentalized gene expression is lost (Figs. 7 and 8). Therefore, like in *B. subtilis*, SpoIIQ and SpoIIIAH are both required for stability of the forespore at late stages of morphogenesis. Secondly, we have shown that mutations in either spoIIQ or spoIIIAH impair the activity of the late forespore-specific regulatory protein σ$^{G}$, consistent with the model that the SpoIIQ and SpoIIIAH proteins are required for continued transcription in the forespore. Importantly, the early activities of σ$^{E}$, σ$^{F}$ or σ$^{G}$ were not affected. In *B. subtilis* the onset of σ$^{G}$ activity coincides with engulfment completion. In *C. difficile* however, although the main period of σ$^{G}$ activity follows engulfment completion, σ$^{G}$-dependent transcription is also detected in the forespore before engulfment is completed (Pereira et al., 2013; Saujet et al., 2013).

The channel model predicts transport of yet unidentified small molecules into the forespore, required for continued macromolecular synthesis and transcriptional syntheses.
activity in this cell following engulfment completion. In *B. subtilis*, assembly of the channel is also required for late, σ^K-dependent transcriptional activity in the mother cell for at least two reasons. Not only is the assembly of the channel required for proper localization of the pro-σ^K processing machinery to the forespore outer membrane, but also the activation of the pro-σ^K processing machinery requires the production, under the control of σ^G, of a protein secreted to the intermembrane space. Thus, in *B. subtilis*, the activity of σ^K is tightly coupled to engulfment completion. In contrast, the activity of σ^K in *C. difficile* is detected during engulfment, although it increases following engulfment completion (Fimlaid *et al*., 2013; Saujet *et al*., 2013; Pereira *et al*., 2013). However, the σ^K protein of *C. difficile* lacks a pro-sequence, and the activity of σ^K in either pre- or post-engulfment sporangia is independent of σ^G (Saujet *et al*., 2013; Pereira *et al*., 2013; Fimlaid *et al*., 2013). Consistent with the absence of a requirement for post-translational processing by sporulation-specific proteins, σ^K is active upon induction in vegetative cells of *C. difficile* (Pishdadian *et al*., 2015). An important finding of our investigation is that the activity of σ^K in *C. difficile*, in spite of its independence on σ^G, is also impaired before or after engulfment completion in spolIQ or spolIIAH sporangia. In the framework of the channel model, one possible explanation for this observation is that, in the absence of channel activity, a metabolite accumulates in the mother cell that is inhibitory for σ^K or alternatively, that the forespore produces a compound required in the mother cell. If so, why σ^E, which is highly similar to σ^K, is immune to a putative inhibitory signal or otherwise does not require a forespore-produced compound, is unclear.

We cannot presently rule out a direct role of SpolIQ and SpolIIAH in the regulation of σ^K activity. Importantly, it was previously noticed that disruption of the sigF gene had an impact on the expression of σ^K dependent genes, whereas mutation of σ^G essentially had no effect (Saujet *et al*., 2013). Presumably, the impact of the sigF mutation on the expression of σ^K genes may be accounted for by the lack of spolIQ expression in the mutant, as we now show. How SpolIQ and SpolIIAH influence the activity of σ^K in *C. difficile* appears to imply a novel regulatory mechanism and is an important goal for future research. In any case, the observation that proper gene expression in the mother cell is also impaired in the absence of SpolIQ or SpolIIAH, expands on the channel model, suggests an unexpected degree of metabolic cooperation between the two cells throughout development and may lead to experimental strategies to identify the compound or compounds that are transferred through the channel. As highlighted by this and other recent studies (reviewed in (Paredes-Sabja *et al*., 2014; Al-Hinai *et al*., 2015; Fimlaid and Shen, 2015), work in the ancient Clostridia group of organisms will continue to provide novel insights into the evolution and mechanism governing endosporulation by the Firmicutes. On the other hand, the SpolIQ-SpolIIAH complex of *C. difficile* represents a promising target for strategies aiming at interfering with sporulation and therefore with the transmission and environmental persistence of this pathogen.

**Experimental procedures**

**Growth conditions and general methods**

Bacterial strains and their relevant properties are listed in Supporting Information Table S1. The *Escherichia coli* strain DH5α (Bethesda Research laboratories) was used for molecular cloning and strain HB101 (RP4) was used as the donor in conjugation experiments (Hussain *et al*., 2005). Luria-Bertani medium was routinely used for growth and maintenance of *E. coli*. When indicated, ampicillin (100 μg/ml) or chloramphenicol (15 μg/ml) was added to the culture medium. The *C. difficile* strains used in this study are congenic derivatives of the wild-type strain 630erm (Hussain *et al*., 2005) and were routinely grown anaerobically (5% H_2, 15% CO_2, 80% N_2) at 37°C in Brain Heart Infusion (BHI) medium (Difco) or SM medium (for 1l: 90 g Bacto-tryptone, 5 g Bacto-peptone, 1 g (NH_4)_2SO_4 and 1.5 g Tris base) (Wilson *et al*., 1982). When necessary, cefoxitin (25 μg/ml), thiampenicol (15 μg/ml), or erythromycin (5 μg/ml) was added to *C. difficile* cultures. The efficiency of sporulation was determined as described before (Pereira *et al*., 2013b); see also the Supporting Experimental Procedures.

**Mutants and SNAPCd fusion**

The construction of spolIQ, spolIQH120S and spolIIAH mutants and the construction of transcriptional and translational SNAPCd fusions are described in detail in the Supporting Experimental Procedures.

**Split-SNAPCd fusion**

The SNAPCd was divided between amino acid residues 91 and 92 (Daniels and Tainer, 2000; Mie *et al*., 2012). The N- and C-terminal fragments are referred to as SNAPCd^-N and SNAPCd^-C respectively. To construct a spolIQ-SNAPCd^-N translational fusion, the spolIQ gene fused to SNAPCd^-N was PCR-amplified from pMS480 using primers spolIQ-40D and nSNAPR (all primers used in this study are listed in Supporting Information Table S2) producing a 1288 bp product. A fusion of spolIIAH to SNAPCd^-C was constructed by overlap extension. The spolIIAH and cSNAPCd^- were amplified separately from pMS481, using primer pairs spolIIAAD/P4, and SpolIIAH-cSNAPCd^- D/cSNAPCd^- R. The resulting 1222 bp spolIIAH fragment (which includes its native promoter) was mixed with the 275 bp cSNAPCd^-
fragment and the mixture amplified using primers spoIIIAH and cSNAPCD, R, a step that yielded a fragment of 1497 bp. The spoIIQ-nSNAPCD fragment (which includes the native spoIIQ promoter) was digested with EcoRI and NotI, mixed with the spoIIIAH-cSNAPCD fragment digested with EcoRI and Xhol, and both fragments introduced via a triple ligation into pMTL84121 (Heap et al., 2009), digested with NotI and Xhol, creating pMS490. We used pMS490, containing the spoIIQ gene (see above) and spoIIIAH-specific primers to convert the histidine codon 120 to a serine codon, producing pMS498. All plasmids were introduced into E. coli HB101 (RP4) and then transferred to C. difficile 630erm and derivatives by conjugation (Heap et al., 2007) (Supporting Information Table S1).

SNAP imaging

Whole cell extracts were obtained by withdrawing 5 ml samples from C. difficile cultures 14 hours after inoculation in SM medium. The extracts were prepared immediately following labelling with 250 nM of the TMR-Star substrate (New England Biolabs), for 30 min in the dark. Following labelling, the cells were collected by centrifugation (4000 × g, for 5 min at 4°C), the cell sediment was washed with phosphate-buffered saline (PBS) and re-suspended in 1 ml French press buffer (10 mM Tris pH 8.0, 10 mM MgCl2, 0.5 mM EDTA, 0.2 mM NaCl, 10% Glycerol, 1 mM PMSF). The cells were lysed using a French pressure cell (18000 lb/in²). Proteins in the extracts were resolved on 15% SDS-PAGE gels. The gels were first scanned in a Fuji TLA-5100 fluorimager, and then subject to immunoblot analysis as described previously (Pereira et al., 2013). The anti-SNAP antibody (New England Biolabs) was used at a 1:1000 dilution, and a rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) was used at a 1:10000 dilution, to horseradish peroxidase (Sigma) was used at dilution 1:1000. The immunoblots were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

SNAP-capture pull-down experiments

Whole cell extracts were obtained by withdrawing 5 ml samples from C. difficile cultures 14 hours after inoculation in SM medium. The cell pellets were re-suspended in 1 ml portions of buffer A [100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10% glycerol] and lysed in a French pressure cell (18000 lb/in²). The lysate was cleared by centrifugation. One milliliter of cleared lysate was bound to 80 µl of a 50% slurry of SNAP-Capture pull down resin (NEB) at room temperature for 30 min. The resin was washed three times in buffer B (same as A but with 200 mM NaCl) and re-suspended in a final volume of 40 µl. The samples were subjected to SDS-PAGE and immunoblotting.

Microscopy

Samples of 1 ml were withdrawn from SM cultures 14 h after inoculation, and the cells collected by centrifugation (4000 × g for 5 min). The cells were washed with 1 ml of PBS, and re-suspended in 0.1 ml of PBS supplemented with the lipophilic styryl membrane dye N-(3-triethylammoniumprpyl)-4-(p-diethy lamino phenyl-hexatrienyl) pyridinium dibromide (FM4-64; 10 µg/ml) (Vida and Emr, 1995; Pogliano et al., 1999) and the DNA stains DAPI (4',6-diamidino-2-phenylindole; 50 µg/ml) (from Molecular Probes, Invitrogen) or Hoechst 33342 (2'-[4-ethoxyphenyl]-5-[4-methyl-1-piperazinyl]-2,5'-bi-1H-benzimid azole trihydrochloride trihydrate) (from Pierce). For SNAP labelling experiments, 200 µl of cells in culture samples were labelled with TMR-Star (as above), collected by centrifugation (4000 × g, 3 min, at room temperature), washed four times with 1 ml of PBS, and finally re-suspended in 1 ml of PBS containing the membrane dye Mitotracker Green (0.5 µg/ml) (Molecular Probes, Invitrogen). For the quantification of gene expression at the single cell level, we used conventional fluorescence microscopy as detailed in the Supporting Experimental Procedures. For the characterization of mutant phenotypes, the cells were imaged using Super-resolution Structured Illumination Microscopy (SR-SIM) performed in an Elyra PS.1 Microscope (Zeiss), using a Plan-Apochromat 63x/1.4 oil DIC M27 objective and a Pco. edge 5.5 camera. Images were acquired using 405 nm (50 mW), 488 nm (100 mW) or 561 nm (100 mW) laser lines, at 5–20% of total potency. The grid periods used were 23 µm, 28 µm or 34 µm for acquisitions with the 405 nm, 488 nm or 561 nm lasers respectively. For each SIM acquisition the corresponding grating was shifted five times and rotated five times, giving a total of 25 acquired frames. Final SIM images were reconstructed employing the ZEN software (black edition, 2012, version 8.1,0.484; Zeiss), using synthetic, channel-specific Optical Transfer Functions (OTFs). Fluorescence intensity profiles were generated from the microscopy images (red channel, corresponding to TMR-Star labelling of the various SNAP fusion proteins) using the 3D Surface plotter function of ImageJ (http://imagej.nih.gov/ij/).

SEC-MALLS

The overproduction and purification of soluble (denoted by the prefix s) versions of WT SpoIIQ, its variant SpoIIQH120S and SpoIIIAH are described in the Supporting Experimental Procedures. Protein concentration was determined by A280 and adjusted to a total concentration of 2.5 mg/ml. Complex samples were formed by pre-incubation at 1:1 ratio for 1 h at 4°C. Fivemfold excess of ZnCl2 or 1 mM EDTA was added to individual or complex samples as necessary. Individual and complex samples were then injected on a Wyatt SEC WTC-050S5 column primed with 50 mM MES pH 6.0, 250 mM NaCl buffer and analyzed using a Wyatt DAWN HELEOS 8 light scattering detector and Optilab Tr-EX refractive index monitor (Wyatt). 0.5 ml fractions were collected and peak fractions were resolved in 15% SDS-PAGE gels. Weight average molecular mass was calculated based on refractive index and light scattering measurements using Astra 6 software (Wyatt).

Metal content analysis

Protein samples in 50 mM MES pH 6.0, 250 mM NaCl were diluted to a concentration of 10 µM (untreated
samples). When appropriate, 50 μM (5X) ZnCl₂ or 1mM EDTA was added to the samples before size exclusion chromatography. Each sample was then loaded individually to a Superdex 200 GL 10/300 Increase column (GE Healthcare). To avoid contamination with metal and/or EDTA, the samples were analyzed in the order: EDTA, untreated, 5X EDTA as appropriate. 0.5 ml fractions were collected during elution. ICP-MS samples (3 ml) were prepared by adding 300 μl sample of each fraction to 2.7 ml of 2.5% HNO₃ containing 20 ppb Ag as an internal standard. Metal standards run before and after analysis of the fractions contained Mg, Mn, Fe, Cu, Ni, Co, Zn at 0, 1, 5, 10, 25, 50, 75 and 100 ppb. The protein concentration of each fraction was determined by absorbance at A280. Three technical replicates were analyzed by SEC followed by ICP-MS.

Acknowledgements

We thank K. Waldron and O. Davies for expert help with ICP-MS and SEC-MALLS experiments respectively. We also thank C.P, Moran, Jr. and A. Shen for helpful discussions and A. Shen for sharing her results prior to publication. This work was supported by Medical Research Council UK New Investigator Research Grant MR/M000923/1 to PS, by Fundação para a Ciência e a Tecnologia through Grant Pest-C/EQB/LA0006/2011 to AOH and through programme IF (IF/00268/ 2013/CP1173/CT0006) to MS, by the European Union Marie Skłodowska Curie Innovative Training Networks contract number 642068 to AOH and by the European Research Council grant ERC-2012-STG-310987 to MGP. ADC is funded by that part of the support of the European Union Marie Skłodowska Curie Innovative Training Networks contract number 642068 to AOH and by the European Research Council grant ERC-2012-STG-310987 to MGP. ADC is funded by the Newcastle/Durham/Liverpool BBSRC Doctoral Training Programme.

Note added in proof:

While this manuscript was under revision, Fimlaid and co-authors published an analysis of the role of spoIIQ and spoIIIAH in C. difficile (Fimlaid KA, Jensen O, Donnelly ML, Siegrist MS, Shen A (2015) Regulation of Clostridium difficile Spore Formation by the SpoIIQ and SpoIIIA Proteins. PLoS Genet 11(10): e1005562. doi:10.1371/journal.pgen.1005562).

While the main conclusions in the two studies are in general agreement, this study further expands our insights into the function of spoIIQ and spoIIIAH.

References

Abanes-De Mello, A., Sun, Y.L., Aung, S., and Pogliano, K. (2002) A cytoskeleton-like role for the bacterial cell wall during engulfment of the Bacillus subtilis forespore. Genes Dev 16: 3253–3264.

Abecasis, A.B., Serrano, M., Alves, R., Quintais, L., Pereira-Leal, J.B., and Henriques, A.O. (2013) A genomic signature and the identification of new sporulation genes. J Bacteriol 195: 2101–2115.

Al-Hinaï, M.A., Jones, S.W., and Papoutsakis, E.T. (2015) The Clostridium sporulation programs: diversity and preservation of endospore differentiation. Microbiol Mol Biol Rev 79: 19–37.

Aung, S., Shum, J., Abanes-De Mello, A., Broder, D.H., Fredlund-Gutierrez, J., Chiba, S., and Pogliano, K. (2007) Dual localization pathways for the engulfment proteins during Bacillus subtilis sporulation. Mol Microbiol 65: 1534–1546.

Blaylock, B., Jiang, X., Rubio, A., Moran, C.P., Jr., and Pogliano, K. (2004) Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. Genes Dev 18: 2916–2928.

Broder, D.H., and Pogliano, K. (2006) Forespore engulfment mediated by a ratchet-like mechanism. Cell 126: 917–928.

Brunkard, J.O., Runkel, A.M., and Zambryński, P.C. (2015) The cytosol must flow: intercellular transport through plasmodesmata. Curr Opin Cell Biol 35: 13–20.

Bylund, J.E., Zhang, L., Haines, M.A., Higgins, M.L., and Piggot, P.J. (1994) Analysis by fluorescence microscopy of the development of compartment-specific gene expression during sporulation of Bacillus subtilis. J Bacteriol 176: 2898–2905.

Camp, A.H., and Losick, R. (2008) A novel pathway of intercellular signalling in Bacillus subtilis involves a protein with similarity to a component of type III secretion channels. Mol Microbiol 69: 402–417.

Camp, A.H., and Losick, R. (2009) A feeding tube model for activation of a cell-specific transcription factor during sporulation in Bacillus subtilis. Genes Dev 23: 1014–1024.

Chastanet, A., and Losick, R. (2007) Engulfment during sporulation in Bacillus subtilis is governed by a multi-protein complex containing tandemly acting autolysins. Mol Microbiol 64: 139–152.

Chiba, S., Coleman, K., and Pogliano, K. (2007) Impact of membrane fusion and proteolysis on SpolIIQ dynamics and interaction with SpoIIAH. J Biol Chem 282: 2576–2586.

Crashaw, A.D., Serrano, M., Stanley, W.A., Henriques, A.O., and Salgado, P.S. (2014) A mother cell-to-forespore channel: current understanding and future challenges. FEMS Microbiol Lett 358: 129–136.

Daniels, D.S., Mol, C.D., Arvai, A.S., Kanugula, S., Pegg, A.E., and Tainer, J.A. (2000) Active and alkylated human AGT structures: a novel zinc site, inhibitor and extrahelical base binding. EMBO J 19: 1719–1730.

Daniels, D.S., and Tainer, J.A. (2000) Conserved structural motifs governing the stoichiometric repair of alkylated DNA by O(6)-alkylguanine-DNA alkyltransferase. Mutat Res 460: 151–163.

Dembek, M., Barquist, L., Boineau, C.J., Cain, A.K., Mayho, M., Lawley, T.D., Fairweather, N.F., and Fagan, R.P. (2015) High-throughput analysis of gene essentiality and sporulation in Clostridium difficile. MBio 6: e02383.

Doan, T., Marquis, K.A., and Rudner, D.Z. (2005) Subcellular localization of a sporulation membrane protein is achieved through a network of interactions along and across the septum. Mol Microbiol 55: 1767–1781.

Doan, T., Morlot, C., Meisner, J., Serrano, M., Henriques, A.O., Moran, C.P., Jr., and Rudner, D.Z. (2009) Novel secretion apparatus maintains spore integrity and...
developmental gene expression in Bacillus subtilis. PLoS Genet 5: e1000566.
Fang, Q., Kanugula, S., and Pegg, A.E. (2005) Function of domains of human O6-alkylguanine-DNA alkyltransferase. Biochemistry 44: 15396–15405.
Fimlaid, K.A., Bond, J.P., Schutz, K.C., Putnam, E.E., Leung, J.M., Lawley, T.D., and Shen, A. (2013) Global analysis of the sporulation pathway of Clostridium difficile. PLoS Genet 9: e1003660.
Fimlaid, K.A., and Shen, A. (2015) Diverse mechanisms regulate sporulation sigma factor activity in the Firmicutes. Curr Opin Microbiol 24: 88–95.
Firczuk, M., Mucha, A., and Bochtler, M. (2005) Crystal structures of active LytM. J Mol Biol 354: 578–590.
Frandsen, N., and Stragier, P. (1995) Identification and characterization of the Bacillus subtilis spoIIP locus. J Bacteriol 177: 716–722.
Fredlund, J., Broder, D., Fleming, T., Claussin, C., and Pogliano, K. (2013) The SpoIIQ landmark protein has different requirements for septal localization and immobilization. Mol Microbiol 89: 1053–1068.
Galperin, M.Y., Mekhedov, S.L., Puigbo, P., Smirnov, S., Wolf, Y.I., and Rigden, D.J. (2012) Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. Environ Microbiol 14: 2870–2890.
Gutierrez, J., Smith, R., and Pogliano, K. (2010) SpoIIQ-mediated peptidoglycan degradation is required throughout engulfment during Bacillus subtilis sporulation. J Bacteriol 192: 3174–3186.
Heap, J.T., Pennington, O.J., Cartman, S.T., Carter, G.P., and Minton, N.P. (2007) The Clostron: a universal gene knock-out system for the genus Clostridium. J Microbiol Methods 70: 452–464.
Heap, J.T., Pennington, O.J., Cartman, S.T., and Minton, N.P. (2009) A modular system for Clostridium shuttle plasmids. J Microbiol Methods 78: 79–85.
Hilbert, D.W., and Piggot, P.J. (2004) Compartmentalization of gene expression during Bacillus subtilis spore formation. Microbiol Mol Biol Rev 68: 234–262.
Houghton, F.D. (2005) Role of gap junctions during early embryo development. Reproduction 129: 129–135.
Hussain, H.A., Roberts, A.P., and Mullany, P. (2005) Generation of an erythromycin-sensitive derivative of Clostridium difficile strain 630 (630Deltaerm) and demonstration that the conjugal transposon Tn916DeltaE enters the genome of this strain at multiple sites. J Gen Microbiol 151: 137–141.
Illing, N., and Errington, J. (1991a) Genetic regulation of morphogenesis in Bacillus subtilis: roles of sigma E and sigma F in prespore engulfment. J Bacteriol 173: 3159–3169.
Illing, N., and Errington, J. (1991b) The spoIID operon of Bacillus subtilis defines a new temporal class of mother-cell-specific sporulation genes under the control of the sigma E form of RNA polymerase. Mol Microbiol 5: 1927–1940.
Jiang, X., Rubio, A., Chiba, S., and Pogliano, K. (2005) Engulfment-regulated proteolysis of SpoIIQ: evidence that dual checkpoints control sigma activity. Mol Microbiol 58: 102–115.
Kroos, L., and Yu, Y.T. (2000) Regulation of sigma factor activity during Bacillus subtilis development. Curr Opin Microbiol 3: 553–560.
Levdikov, V.M., Blagova, E.V., McFeat, A., Fogg, M.J., Wilson, K.S., and Wilkinson, A.J. (2012) Structure of components of an intercellular channel complex in sporulating Bacillus subtilis. Proc Natl Acad Sci USA 109: 5441–5445.
Londono-Vallejo, J.A., Frehel, C., and Stragier, P. (1997) SpoIIQ, a forespore-expressed gene required for engulfment in Bacillus subtilis. Mol Microbiol 24: 29–39.
Lopez-Diaz, I., Clarke, S., and Mandelstam, J. (1986) spoIID operon of Bacillus subtilis: cloning and sequence. J Gen Microbiol 132: 341–354.
Losick, R., and Stragier, P. (1992) Crisscross regulation of cell-type-specific gene expression during development in B. subtilis. Nature 355: 601–604.
Margolis, P.S., Driks, A., and Losick, R. (1993) Sporulation gene spoIIB of Bacillus subtilis. J Bacteriol 175: 528–540.
Meisner, J., Maehigashi, T., Andre, I., Dunham, C.M., and Moran, C.P., Jr. (2012) Structure of the basal components of a bacterial transporter. Proc Natl Acad Sci USA 109: 5446–5451.
Meisner, J., and Moran, C.P., Jr. (2011) A LytM domain dictates the localization of proteins to the mother cell-forespore interface during bacterial endospore formation. J Bacteriol 193: 591–598.
Meisner, J., Wang, X., Serrano, M., Henriques, A.O., and Moran, C.P., Jr. (2008) A channel connecting the mother cell and forespore during bacterial endospore formation. Proc Natl Acad Sci USA 105: 15100–15105.
Meyer, P., Gutierrez, J., Pogliano, K., and Dworkin, J. (2010) Cell wall synthesis is necessary for membrane dynamics during sporulation of Bacillus subtilis. Mol Microbiol 76: 956–970.
Mie, M., Naoki, T., Uchida, K., and Kobatake, E. (2012) Development of a split SNAP-tag protein complementation assay for visualization of protein-protein interactions in living cells. Analyst 137: 4760–4765.
Morlot, C., Uehara, T., Marquis, K.A., Bernhardt, T.G., and Rudner, D.Z. (2010) A highly coordinated cell wall degradation machine governs spore morphogenesis in Bacillus subtilis. Genes Dev 24: 411–422.
Ng, Y.K., Ehsaan, M., Philip, S., Collery, M.M., Janoir, C., Collignon, A., Cartman, S.T., and Minton, N.P. (2013) Expanding the repertoire of gene tools for precise manipulation of the Clostridium difficile genome: allelic exchange using pyrE alleles. PLoS One 8: e56051.
Ojkic, N., Lopez-Garrido, J., Pogliano, K., and Endres, R.G. (2014) Bistable forespore engulfment in Bacillus subtilis by a zipper mechanism in absence of the cell wall. PLoS Comput Biol 10: e1003912.
Paredes-Sabja, D., Shen, A., and Sorg, J.A., (2014) Clostridium difficile spore biology: sporulation, germination, and spore structural proteins. Trends Microbiol.
Pereira, F.C., Saujet, L., Tome, A.R., Serrano, M., Monot, M., Couture-Tosi, E., Martin-Verstraete, I., Dupuy, B., and Henriques, A.O. (2013) The spore differentiation pathway in the enteric pathogen Clostridium difficile. PLoS Genet 9: e1003782.
Perez, A.R., Abanes-De Mello, A., and Pogliano, K. (2000) SpoIIIB localizes to active sites of septal biogenesis and spatially regulates septal thinning during engulfment in bacillus subtilis. J Bacteriol 182: 1096–1108.
Pishdadian, K., Fimlaid, K.A., and Shen, A. (2015) SpolIID-mediated regulation of sigmaK function during Clostridium difficile sporulation. *Mol Microbiol* 95: 189–208.

Pogliano, J., Osborne, N., Sharp, M.D., Abanes-De Mello, A., Perez, A., Sun, Y.L., and Pogliano, K. (1999) A vital stain for studying membrane dynamics in bacteria: a novel mechanism controlling septation during Bacillus subtilis sporulation. *Mol Microbiol* 31: 1149–1159.

Ramadurai, L., and Jayaswal, R.K. (1997) Molecular cloning, sequencing, and expression of lytM, a unique autolytic gene of Staphylococcus aureus. *J Bacteriol* 179: 3625–3631.

Rodrigues, C.D., Marquis, K.A., Meinsner, J., and Rudner, D.Z. (2013) Peptidoglycan hydrolysis is required for assembly and activity of the transenvelope secretion complex during sporulation in Bacillus subtilis. *Mol Microbiol* 89: 1039–1052.

Rubio, A., and Pogliano, K. (2004) Septal localization of forespore membrane proteins during engulfment in Bacillus subtilis. *EMBO J* 23: 1636–1646.

Rudner, D.Z., and Losick, R. (2001) Morphological coupling in development: lessons from prokaryotes. *Dev Cell* 1: 733–742.

Saujet, L., Pereira, F.C., Serrano, M., Soutourina, O., Monot, M., Shelyakin, P.V., Gelfand, M.S., Dupuy, B., Henriques, A.O., and Martin-Verstraete, I. (2013) Genome-wide analysis of cell type-specific gene expression during spore formation in Clostridium difficile. *PLoS Genet.*

Schermelleh, L., Heintzmann, R., and Leonhardt, H. (2010) A guide to super-resolution fluorescence microscopy. *J Cell Biol* 190: 165–175.

Serrano, M., A. Neves, Soares, C.M., Moran, C.P., Jr., and Henriques, A.O., (2004) Role of the anti-sigma factor SpolIAB in regulation of sigmaG during Bacillus subtilis sporulation. *J Bacteriol* 186: 4000–4013.

Smith, K., Bayer, M.E., and Youngman, P. (1993) Physical and functional characterization of the Bacillus subtilis spolIM gene. *J Bacteriol* 175: 3607–3617.

Stragier, P., and Losick, R. (1996) Molecular genetics of sporulation in Bacillus subtilis. *Annu Rev Genet* 30: 297–241.

Sun, Y.L., Sharp, M.D., and Pogliano, K. (2000) A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of Bacillus subtilis. *J Bacteriol* 182: 2919–2927.

Vida, T.A., and Emr, S.D. (1995) A new vital stain for visualizing vacuolar membrane dynamics and endocytosis in yeast. *J Cell Biol* 128: 779–792.

Wilson, K.H., Kennedy, M.J., and Fekety, F.R. (1982) Use of sodium taurocholate to enhance spore recovery on a medium selective for Clostridium difficile. *J Clin Microbiol* 15: 443–446.

**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web-site.