MINIREVIEW

A mother cell-to-forespore channel: current understanding and future challenges

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Abstract

Formation of endospores allows some bacteria to survive extreme nutrient limitation. The resulting dormant cell, the spore, persists in the environment and is highly resistant to physical and chemical stresses. During spore formation, cells divide asymmetrically and the mother cell engulfs the developing spore, encasing it within a double membrane and isolating it from the medium. Communication between mother cell and isolated forespore involves a specialised connection system that allows nurturing of the forespore and continued macromolecular synthesis, required to finalise spore maturation. Here, we review current understanding of this feeding channel formed by a forespore protein, SpoIIQ, and a mother cell protein, SpoIIIAH, in the model organism *Bacillus subtilis* and the important human pathogen *Clostridium difficile*. We also analyse the presence of this channel across endospore-forming bacteria and highlight the main questions still remaining.

Introduction

Some bacteria can initiate a process of endosporulation under conditions of nutrient limitation. Formation of endospores proceeds through a series of well-defined morphological stages that culminates in the production of the spore (Hilbert & Piggot, 2004; Higgins & Dworkin, 2012). Spores are highly resistant to physical and chemical insults and remain in the environment in a metabolically dormant state. Once conditions are favourable, the spores germinate into a vegetative cell state, re-establishing the bacterial population (Setlow, 2006). Endosporulation is widespread amongst the *Bacilli* and *Clostridia* and is associated with the ability of some of these bacteria to spread infection in different hosts, including humans (Setlow, 2006; Deakin *et al.*, 2012). Importantly, spores have recently been shown to be the key infectious agent in *Clostridium difficile* infections (CDIs) and are responsible for disease transmission and recurrence (Deakin *et al.*, 2012). *Clostridium difficile* is a major cause of human morbidity and mortality in hospitals across the developed world, and CDIs place considerable economic pressure on healthcare systems. New, effective methods for infection treatment and management are required, and sporulation is therefore a promising therapeutic target for CDIs, as well as other infections caused by endosporeformers.

Most of our knowledge of sporulation is derived from studies in the Gram-positive model organism *Bacillus subtilis*, in which over 500 genes are expressed during the process (Fawcett *et al.*, 2000; Eichenberger *et al.*, 2003; Eichenberger *et al.*, 2004; Steil *et al.*, 2005; Wang *et al.*, 2006). Detailed studies of endosporulation have only recently been extended to other organisms, particularly to clostridial species. As such, the general mechanisms herein described have been identified in *B. subtilis*, unless otherwise specified. Due to its importance as a human pathogen and to the recent advances in understanding sporulation in *C. difficile*, our discussion of the clostridial

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mechanisms will focus on this organism. This review also focuses on the structure and function of a specialised secretion system used for cell–cell communication during spore development.

Sporulation cycle in *B. subtilis*

Following asymmetric division, the mother cell and the forespore follow different but interdependent programmes of gene expression that are also coupled to the course of morphogenesis (detailed in Fig. 1). Gene expression during sporulation is largely controlled by a cascade of cell type-specific RNA polymerase sigma (σ) factors, whose production and activation is under tight temporal and spatial control. After asymmetric septation, σ^F becomes active in the forespore (Hilbert & Piggot, 2004; Higgins & Dworkin, 2012). An intercompartment signal transduction pathway emanating from the forespore then activates σ^E in the mother cell. Following engulfment completion, σ^F and σ^E are replaced by σ^G and σ^K, respectively. The onset of σ^G activity coincides with engulfment completion and requires the activity of σ^E in the mother cell. The activity of σ^G, in turn, triggers the activation of σ^K (Hilbert & Piggot, 2004; Higgins & Dworkin, 2012). The cell–cell signalling pathways result in the successive activation of the sporulation sigma factors alternating between the two cells. Because these pathways operate at key intermediate stages in the process, they maintain the programmes of gene expression of the two cells in close register and tied to the course of morphogenesis (Hilbert & Piggot, 2004; Higgins & Dworkin, 2012).

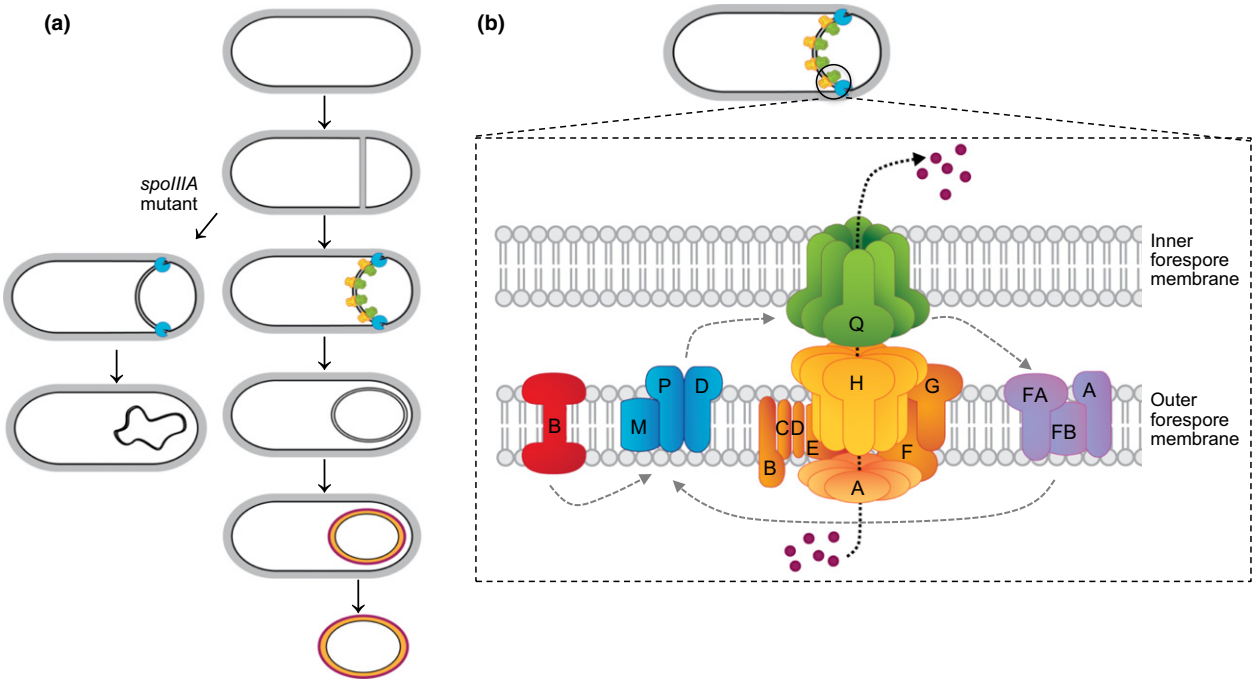

Fig. 1. (a) Cartoon representation of the morphological stages of sporulation. First, the cell divides near one of the poles, giving rise to a small forespore and a larger mother cell. Initially, the cells lay side by side. Then, the mother cell wraps itself around the smaller forespore in a process called engulfment. When engulfment is completed, the forespore is released into the mother cell cytoplasm surrounded by two membranes. At latter stages of morphogenesis, the engulfed forespore becomes encased in several protective layers. Finally, the mother cell lyses and the mature spore is released. The localisation of SpoIIIAH (orange), SpoIIQ (green) and the SpoIID-SpoIIM-SpoIIP complex (blue) during engulfment is represented. In the absence of SpoIIIA proteins (or SpoIIQ), the forespore looses metabolic potential and collapses. (b) Schematic representation of the SpoIIIA-Q secretion complex in the membranes that surround the forespore. SpoIIQ (Q) and SpoIIIAH (AH) are shown as multimeric rings. The actual stoichiometry of the complex is still unknown. Other products of the *spoIIIA* operon are also represented (SpoIIIAA to SpoIIIAG, denoted A–G). Some of these membrane-associated proteins share similarity with components of secretion systems: SpoIIIAA (A) with Type II and IV ATPases; SpoIIIAB (B) with the GspF and TadB/C family from Type II and IV systems; SpoIIIAG (G) with AH and with the YscJ/FliF family of Type III secretion systems; SpoIIIAF (F) with the FlhB (YscU) family from type III systems; and SpoIIIAE (E) with ABC-type permeases of Type I secretion systems (Camp & Losick, 2009; Doan *et al.*, 2009; Meisner & Moran, 2011). In addition, SpoIIB (B, red), the SpoIID-SpoIIM-SpoIIP (D, M and P, blue) and the SpoIVFA-SpoIVFB-BofA (FA, FB and A, purple) complexes are also represented. The grey arrows represent the dependencies of the various subcomplexes for localisation. The 'feeding tube model' is also illustrated. The complex between the SpoIIIA proteins and Q transports an unknown metabolite (red circle) into the forespore that maintains the potential for macromolecular synthesis and forespore integrity.

Recently, the gene regulatory network that controls spore formation in *C. difficile* was described (Fimlaid *et al.*, 2013; Pereira *et al.*, 2013; Saujet *et al.*, 2013; Paredes-Sabja *et al.*, 2014). The main morphological stages of sporulation are conserved, as are the main periods of activity and functions in morphogenesis of the cell type-specific sigma factors. However, the temporal segregation between the activities of the early and late sigma factors of *C. difficile* is less defined than in *B. subtilis*. Moreover, the activity of σ^E is partially independent of σ^F, σ^G is not dependent on σ^E, and σ^K is active independently of σ^G. Therefore, *C. difficile* does not appear to rely on the tightly coupled cell–cell signalling pathways found in the *B. subtilis* model (Fimlaid *et al.*, 2013; Pereira *et al.*, 2013; Saujet *et al.*, 2013).

SpoIIQ and SpoIIIAH as basal components of a bacterial transporter

Several studies have identified a core of conserved endosporulation genes (Galperin *et al.*, 2012; Traag *et al.*, 2013), and a genomic signature was defined (Abecasis *et al.*, 2013), which includes 48 genes and is dominated by genes with an established function in spore formation.

The endosporulation genomic signature includes the products of the *spoIIIA* operon. These proteins form a complex at the spore outer membrane that is essential for sporulation and communication between the forespore and the mother cell. Importantly, the protein encoded by the last gene in the operon, SpoIIIAH (AH) together with SpoIIQ (Q), a forespore protein, forms a channel connecting the two cells, which appears to be gated from the mother cell side (Londono-Vallejo *et al.*, 1997; Meisner *et al.*, 2008, Camp & Losick, 2009; Doan *et al.*, 2009). Q is a forespore membrane protein expressed under the control of σ^F, containing an N-terminal transmembrane segment and a C-terminal extracellular domain (Londono-Vallejo *et al.*, 1997). The extracellular domain (residues 43–283) contains a degenerate LytM endopeptidase (peptidase M23 family) domain, flanked by both N- and C-terminal extensions (residues 43–88 and 221–283; Meisner & Moran, 2011). The C-terminal extension was shown to be dispensable for Q function and is not conserved amongst Q homologues (Doan *et al.*, 2009). Q uses this degenerate LytM domain as a surface for protein–protein interaction with the mother cell membrane protein AH (Meisner & Moran, 2011; Levdikov *et al.*, 2012; Meisner *et al.*, 2012).

The AH protein, expressed under the control of σ^E (Illing & Errington, 1991), also has an N-terminal transmembrane segment and a C-terminal extracellular domain. The C-terminal domain (residues 25–218) shows similarity to the YscJ/FliF family of Type III secretion proteins commonly found in Gram-negative bacteria (Camp & Losick, 2008; Meisner *et al.*, 2008). Proteins of this family form large multimeric rings that serve as the scaffold for the assembly of the secretion system. The YscJ-like domain of AH is involved in the interaction with Q (Meisner & Moran, 2011).

The other products of the *spoIIIA* operon code for membrane-associated proteins that also share also similarity with components of secretion systems (details in Fig. 1). It is possible that *spoIIIA*-encoded proteins other than AH are involved in the regulation of the channel from the mother cell side (Doan *et al.*, 2009). Several of the SpoIIIA proteins are detected in a complex with Q and at least one, SpoIIIAG, localises to the outer forespore membrane in an AH- and Q-dependent manner (Doan *et al.*, 2009).

SpoIIQ:SpoIIIAH localisation

Soon after asymmetric division, Q localises to the sporulation septum, tracks the engulfing membranes and assembles into helical arcs around the forespore (Rubio & Pogliano, 2004). Correct localisation of Q is achieved by two pathways: via AH and through the SpoIID, SpoIIM and SpoIIP mother cell proteins (hereafter referred to as the DMP complex) (Fredlund *et al.*, 2013; Rodrigues *et al.*, 2013). Interestingly, one pathway seems to be able to compensate for the other as Q only shows diffuse localisation when both pathways are absent.

AH helps anchoring Q through direct protein–protein interactions, whilst the DMP complex may act indirectly. This complex is involved in hydrolysis of the septal peptidoglycan (PG) present between the two membranes, thereby helping the movement of the mother cell membrane around the forespore (see below) (Abanes-De Mello *et al.*, 2002; Broder & Pogliano, 2006). Point mutations affecting the activities of D and P, but not D alone, impair the localisation of Q in the absence of AH (Fredlund *et al.*, 2013; Rodrigues *et al.*, 2013). Therefore, without AH, the enzymatic activity of the DMP machine appears to be required for Q localisation, potentially via interaction of the LytM domain of Q with a yet unidentified mother cell protein that would act as an additional anchor for Q (Fredlund *et al.*, 2013; Rodrigues *et al.*, 2013).

AH localisation is primarily achieved by interaction with Q and takes place by a diffusion and capture mechanism (Blaylock *et al.*, 2004; Doan *et al.*, 2005). The interaction of the extracellular domain of AH with Q retains AH in the sporulation septum and directs its assembly into helical arcs and foci around the forespore during engulfment (Blaylock *et al.*, 2004; Doan *et al.*, 2005). The DMP complex is dispensable for the localisation of AH, suggesting that septal PG does not provide a barrier for

the interaction between AH and Q (Fredlund *et al.*, 2013).

Once engulfment is complete, Q is cleaved by the protease SpoIVB, releasing the LytM-like domain from the N-terminal membrane anchor; this cleavage, however, does not appear to be essential for spore formation (Chiba *et al.*, 2007). As Q is also required for AH stability (Blaylock *et al.*, 2004; Doan *et al.*, 2005), this cleavage may lead to AH degradation. The Q:AH channel does not appear to be present following engulfment completion (Meisner *et al.*, 2008).

SpoIIQ:SpoIIIAH channel function

spoIIIA mutants of *B. subtilis* are blocked just after completion of engulfment of the forespore by the mother cell (Illing & Errington, 1991). Q is the only forespore-produced protein required for engulfment, although in certain culturing conditions, *spoIIQ* mutants are able to efficiently complete engulfment (Sun *et al.*, 2000). Importantly, the *spoIIIA* products and Q are required for the activity of σ^G in the engulfed forespore under all conditions, and therefore also for the activation of the mother cell-specific sigma factor σ^K (Illing & Errington, 1991; Sun *et al.*, 2000; Serrano *et al.*, 2004). Thus, consistent with their signature status, Q and the *spoIIIA*-encoded products are essential for sporulation.

The Q:AH channel is required for late forespore-specific activity not only of σ^G but also of σ^F, as well as activity of the heterologous T7 RNA polymerase (Camp & Losick, 2009). Based on this evidence, it was proposed that this channel does not specifically lead to σ^G activation, but rather conveys specific metabolite(s) that allow macromolecular synthesis in the forespore. Thus, the channel may work as a 'feeding tube', providing small molecules needed to maintain the metabolic potential in the forespore (Camp & Losick, 2009; Doan *et al.*, 2009). In support of this model, in the absence of *spoIIIA* or *spoIIQ*, the forespore membranes lose their integrity, resulting in an apparent collapse of the forespore (Fig. 1; Serrano *et al.*, 2004; Camp & Losick, 2009).

In *C. difficile,* σ^G is active prior to engulfment completion. This early activity of σ^G does not require the activity of σ^E in the mother cell; however, the activity of σ^G increases in the forespore following engulfment completion (Pereira *et al.*, 2013). As the *sigE* mutant is blocked at an early stage, we do not presently know whether σ^E or the Q:AH channel are required for σ^G activity at late stages of sporulation and, more generally, to maintain the potential for macromolecular synthesis in the forespore, as suggested for *B. subtilis* (Camp & Losick, 2009; Doan *et al.*, 2009). Conservation amongst sporeformers of *spoIIIA* (Galperin *et al.*, 2012; Abecasis *et al.*, 2013) and *spoIIQ* (see below) suggests that Q:AH is essential across endosporulators. Testing the channel function in *C. difficile* with an analysis of *spoIIQ* and *spoIIIAH* mutants is therefore required to clarify this hypothesis.

In *B. subtilis*, Q:AH complex was also shown to be important, directly or indirectly, for correct localisation of other proteins to the forespore outer membrane. SpoIVFA, a mother cell membrane protein involved in σ^K activation, fails to localise to the forespore outer membrane in the absence of AH, Q (Doan *et al.*, 2005; Jiang *et al.*, 2005) or the DMP complex (Doan *et al.*, 2005). Localisation of DMP to the sporulation septum is primarily mediated by the SpoIIB protein (Aung *et al.*, 2007), but its recruitment can also depend on the SpoIVFA/B proteins (Aung *et al.*, 2007). As such, DMP localisation is also indirectly mediated by the Q:AH complex.

The membrane movement during engulfment relies on pulling and push forces, driven by hydrolysis and synthesis of PG (Meyer *et al.*, 2010). The enzymatic activity of the DMP complex mediates membrane migration in only one direction by degrading the PG, thereby pulling the mother cell membrane around the forespore (Abanes-De Mello *et al.*, 2002; Broder & Pogliano, 2006). Engulfment relies heavily on gene expression in the mother cell where SpoIID and SpoIIM of the DMP complex are produced, whilst SpoIIP is produced in both cells (Hilbert & Piggot, 2004; Dworkin & Losick, 2005; Higgins & Dworkin, 2012). However, when the DMP activity is reduced, the interaction of Q with AH across the sporulation septum becomes essential, facilitating engulfment by a ratchet-like mechanism (Broder & Pogliano, 2006). Hence, the Q:AH complex works as a secondary machinery for engulfment and also provides a redundant mechanism to localise the DMP proteins to the septum. This redundancy confers robustness to the engulfment process.

SpoIIQ:SpoIIIAH structure

Structures of a complex of the extracellular domains, lacking the transmembrane regions, of Q and AH of *B. subtilis* have been determined recently (Levdikov *et al.*, 2012; Meisner *et al.*, 2012; Fig. 2). The complex determined corresponds to a heterodimeric structure in solution. Interaction of the two proteins is established by the C-terminal regions, which contain the LytM-like (Q) and the YscJ/FliF-like (AH) domains. Each protein contributes a β-strand to the binding site, creating an extended β-sheet spanning both proteins (Fig. 2). Based on the ring-forming properties of the AH YscJ/FliF-like domain, Levdikov *et al.* suggest that Q and AH form individual dodecamers that then interact. By contrast, Meisner and co-authors suggest the formation of 15 or 18mer rings, based on modelling simulations. The inner diameter of

the channel would therefore range from *c.* 60 Å (12mers) to *c.* 140 Å (18mers).

Q:AH across endosporeformers

Whilst *spoIIIA* is a universal feature of endosporeformers (Galperin *et al.*, 2012; Abecasis *et al.*, 2013), whether Q orthologues are conserved in *Clostridia* is still not established (Galperin *et al.*, 2012). In *C. difficile*, for example, expression of the *spoIIIA* operon during sporulation was found to be under the control of σ^E (Fimlaid *et al.*, 2013; Saujet *et al.*, 2013). Interestingly, these studies also found that gene CD0125, coding for a LytM-containing protein, is under the control of σ^F. It has been suggested that this gene represents a nonorthologous gene replacement of *spoIIQ* but that its presence and role might not be conserved in other clostridia (Galperin *et al.*, 2012). To further understand the presence and potential relevance of the Q:AH complex in other endospore formers, we used hidden Markov model (HMM)based searches to try to identify potential orthologues of Q and AH (for details, see Figs 2 and 3).

AH HMM sequence readily identifies orthologues amongst other spore-forming *Bacilli* and *Clostridia* in BLASTP searches. It is important to note that conservation is mostly maintained within the YscJ/FliF containing C-terminal domain, with low conservation seen in the N-terminal membrane-spanning region. Interestingly, when the search is carried out across all bacteria, excluding the *Bacilli* and *Clostridia*, no clear orthologues can be identified.

A similar strategy was used to identify Q orthologues. Importantly, apart from proteins annotated as Q, this search retrieved proteins annotated simply as 'peptidase M23', even though no peptidase domain is included. This observation strengthens the idea that these are likely to be true Q orthologues, as they contain an orthologous non-peptidase domain within the LytM-like enzymatic context.

Conservation of function of Q:AH is further supported by the observation that residues in the β-strands that create the binding interface are highly conserved in both AH and Q (Fig. 2), indicating that function and structure are likely to be maintained across endosporeformers.

Importantly, not all Q-like proteins identified seem to have a degenerate LytM catalytic site. Endopeptidase activity requires the presence of a Zn^{2+} ion, coordinated by two conserved motifs, HxxxD and HxH (Odintsov *et al.*, 2004; Firczuk *et al.*, 2005). In *B. subtilis,* the first histidine is replaced by a serine residue and Q does not interact with PG or Zn^{2+} (Meisner & Moran, 2011; Levdikov *et al.*, 2012; Meisner *et al.*, 2012; Rodrigues *et al.*, 2013). Conversely, *C. difficile* CD0125 has an intact HxxxD motif and is potentially an active endopeptidase. Strikingly, most *Bacilli* Q orthologues share the degeneration characteristic of the *B. subtilis* protein and only a few maintain a histidine in the required position (Fig. 3, top). The situation is more complex in the *Clostridia*,

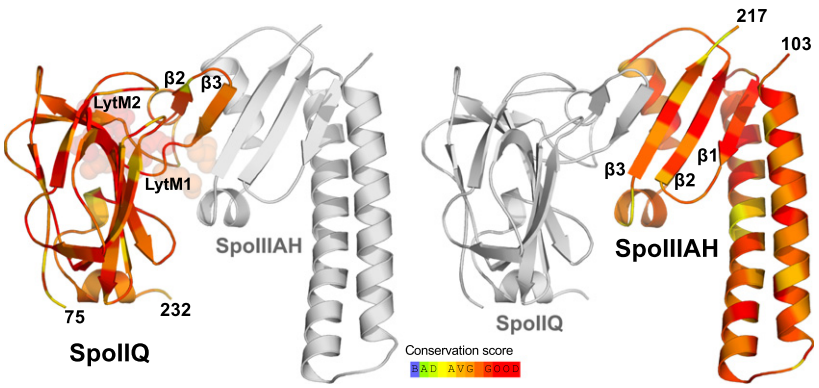

Fig. 2. Cartoon representation of *Bacillus subtilis* Q:AH soluble domains structure (Levdikov *et al.*, 2012; Meisner *et al.*, 2012). Left: Q is coloured according to sequence conservation score, from blue (bad) to red (good). AH is coloured grey. The LytM catalytic residues are represented by spheres (transparent). Right: AH coloured by conservation score, Q: grey. Residues that form the β-sheet at the binding interface are well conserved (dark orange) in Q and average to well conserved (light orange to light red) in AH. This conservation suggests that the interaction between the two proteins is maintained across endospore formers, indicating that both function and structure of the channel are conserved. Conservation scores were calculated by T-coffee, based on the sequence alignment of Q and AH orthologues identified in the representative endosporeformers as defined by de Hoon *et al.* (2010). AH orthologues were identified by BLASTP (Altschul *et al.*, 1990) searches using the *B. subtilis* AH sequence. Due to the ubiquitous presence of the LytM domain in unrelated peptidases, many false positives were initially retrieved when searching for Q orthologues using a similar strategy. Therefore searches were carried out using the non-LytM region only, with, potential orthologues only included if the sequence spanned both the LytM and the N-terminal regions and corresponded to the full protein. Furthermore, their genetic context was found to match gene localisation observed in *B. subtilis* – downstream of *spoIID*, under σ^E-control.

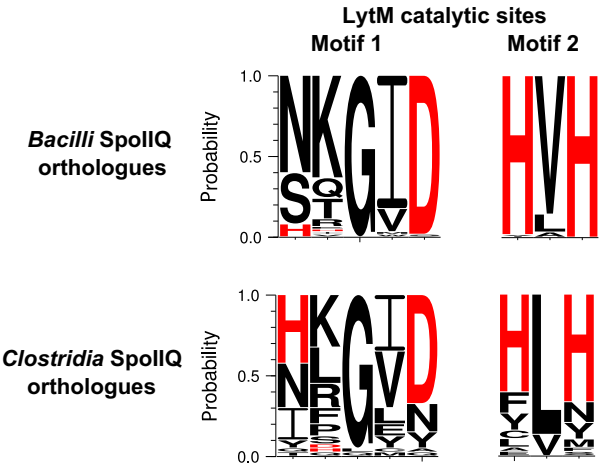

Fig. 3. Sequence logo of the two catalytic LytM motifs in *Bacilli* (top) and *Clostridia* (bottom). The catalytic residues (HxxxD, motif 1 and HxH, motif 2) are highlighted in red. The presence of an intact LytM motif 1 in the majority of *Clostridia* suggests that clostridial Q might have endopeptidase activity that could play a role in forespore engulfment. Importantly, only a small part of *Bacilli* seems to retain the required catalytic residues, indicating that this activity is either not necessary or performed by other proteins. Investigating enzymatic activity in both genera would provide important clues about the functional differences between the channel and constituent proteins in *Bacilli* and *Clostridia*. Sequences were retrieved after BLASTP searches of nonredundant databases using a HMM, created using HMMER3 (Eddy, 1998) based on the sequence alignment described in Fig. 2. The search for SpoIIQ orthologues based on the HMM was carried out using only the non-LytM domain, to avoid false positives, as mentioned in Fig. 2. The sequence logo was created using WEBLOGO (Crooks *et al.*, 2004).

with many having an intact catalytic motif, but some also having a degenerate sequence (Fig. 3, bottom). We can therefore speculate that many, if not all, clostridial Q proteins will bind Zn^{2+} and exhibit endopeptidase activity. These observations hint at potential differences between the functionality of the Q:AH complex in different bacteria, particularly between *Bacilli* and *Clostridia*. Clearly, more detailed studies of the function, structure and potential enzymatic activity of Q:AH in other endosporeformers are required.

Outlook

Although extensive studies in *B. subtilis* have provided us with important information regarding the role and importance of the *spoIIIA* operon and, in particular, the Q:AH channel, in effective forespore-to-mother cell communication, many questions still remain. The exact nature of the molecules transported across the two membranes is still unknown. Moreover, whether that transport is active and requires energy, possibly provided by the SpoIIIAA ATPase or passive remains to be investigated. Details of the precise composition of the pore forming oligomers and interactions within and across the bi-membrane system are also lacking, which are crucial for the full characterisation and understanding of this channel.

Does the Q:AH complex or either protein have other functions beyond those described to date? The presence of intact LytM catalytic motifs in at least some Q orthologues clearly raises the possibility that they might be active endopeptidases. This enzymatic activity could be important for correct engulfment and/or protein localisation at the septum, complementing and/or replacing, at least partially, the function of the DPM complex. Further investigation is clearly required to probe this possibility.

Our analysis reported here indicates that both Q and AH are widely spread across endosporeformers, and it is reasonable to assume that the complex will be important in forespore-to-mother cell communication in different species. Therefore, only detailed studies in other representative species can reveal the intricate molecular details of this communication channel. Of particular interest will be the function and architecture of the Q:AH complex in spore-forming human pathogens, as understanding the determinants of sporulation in these bacteria is of paramount importance to develop new therapeutic strategies.

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