Trends in Microbiology

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REVIEWS

Review

The New Kid on the Block: A Specialized Secretion System during Bacterial Sporulation

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The transport of proteins across the bacterial cell envelope is mediated by protein complexes called specialized secretion systems. These nanomachines exist in both Gram-positive and Gram-negative bacteria and have been categorized into different types based on their structural components and function. Interestingly, multiple studies suggest the existence of a protein complex in endospore-forming bacteria that appears to be a new type of specialized secretion system. This protein complex is called the SpoIIIA-SpoIIQ complex and is an exception to the categorical norm since it appears to be a hybrid composed of different parts from well-defined specialized secretion systems. Here we summarize and discuss the current understanding of this complex and its potential role as a specialized secretion system.

Highlights

The A-Q complex likely represents a new class of specialized secretion system that is highly conserved in endospore-forming bacteria.

The A-Q complex is required for endospore formation. Mutants exhibit morphological defects and lack physiological potential.

The A-Q complex appears to be a hybrid specialized secretion system composed of proteins with homology to those found in type II, type III, and type IV specialized secretion systems.

The structure and function of the A-Q complex is not conserved in endospore-forming bacteria, and differences exist between members of the Bacillales and Clostridiales.

The A-Q complex highlights the functional diversity of specialized secretion systems.

Specialized Secretion Systems in Bacteria

Specialized secretion systems (see Glossary) are large multiprotein complexes that bacteria employ to transport proteins, other molecules, and sometimes DNA across their cell envelope [1–8]. The diversity of specialized secretion systems is related to the variety of functions they mediate in bacterial biology, ranging from and not limited to: flagellar assembly, host infection and symbiosis, adhesion to host cells or surfaces, and killing of bacterial competitors [1–5]. Although found in both Gram-positive and Gram-negative bacteria, they are most commonly associated with Gram-negative species. In these organisms, specialized secretion systems traverse the inner and outer membranes and utilize ATP hydrolysis in the cytoplasm to energize secretion of proteins across the outer membrane [3,5]. Interestingly, in Gram-positive bacteria that produce endospores (spores henceforth), the problem of transporting proteins across two membranes is also apparent as the spore is surrounded by two lipid bilayers (Figure 1A). This review focuses on a highly conserved protein complex required for spore development, the SpoIIIA-SpoIIQ complex (called the A-Q complex for simplicity), that spans these two membranes and resembles a specialized secretion system [8–14].

Sporulation

A subset of bacteria belonging to the phylum Firmicutes are known to enter a developmental pathway called **sporulation** [15–17]. In *Bacillus subtilis*, a Gram-positive model organism, sporulation is induced by starvation stress and culminates in the formation of a metabolically dormant and stress-resistant spore. Spores possess characteristics that allow them to be resistant to a variety of stresses, including desiccation, destruction by cell-wall-degrading enzymes, UV radiation, high temperatures, digestion by protozoans, detergents, and acids [6,7]. Importantly, because they are dormant, spores are also inert to antibiotics [6,7]. These resistance properties not only make spores one of the hardiest cell types on Earth, but also

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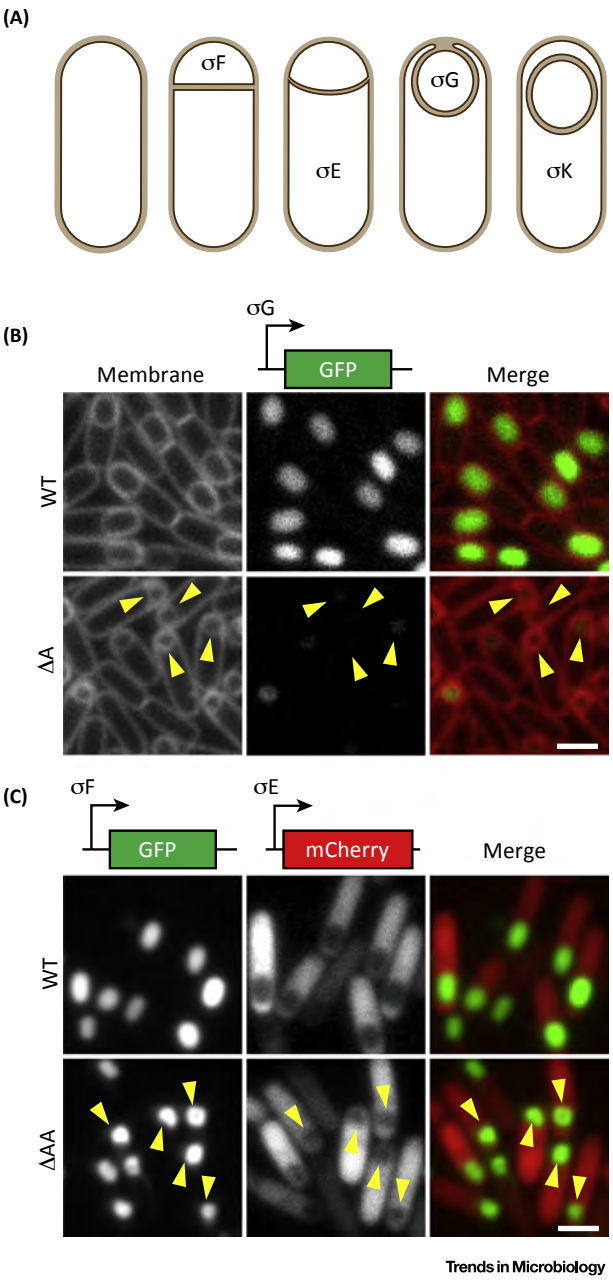


Figure 1. Sporulation and Phenotypes of A-Q Complex Mutants. (A) Schematic representation of the sporulation program in *Bacillus subtilis*, illustrating cell-specific gene transcription governed by σ factors and the morphological process of engulfment. (B) σG activity in wild-type (WT) and A-Q complex mutant cells lacking the *spoIIIA* locus (ΔA). Images, left to right, are membranes stained with the fluorescent membrane dye TMA-DPH, a fluorescent reporter for σG activity (P_{sspB}-*gfp*), and composite image displaying both fluorescent signals. Yellow arrowheads point to forespores that display reduced σG activity and spore size. (C) Spore size in wild-type (WT) and A-Q complex mutant cells lacking the *spoIIIAA* gene (ΔAA). Images, left to right, are forespores expressing a fluorescent reporter for σF activity (P_{spoIIQ}-*gfp*), mother cells expressing a fluorescent reporter for σE activity (P_{spoIID}-*mCherry*), and composite image displaying both fluorescent signals. Yellow arrowheads point to forespores that have reduced spore size. Scale bar is 2 μm. Abbreviations: GFP, green fluorescent protein; mCherry, a fluorescent reporter.

Glossary

LytM domain (Peptidase_M23): a protein domain that is broadly distributed in bacteria and has been implicated in a variety of processes, including cell division and cell shape. Canonical LytM proteins are metalloendopeptidases that cleave cross-links in the peptidoglycan cell wall. Degenerate LytM domains lack catalytic activity and appear to take on other roles in bacterial biology.
Metalloendopeptidase: an enzyme that requires a metal to break peptide bonds at nonterminal aminoacids.
Protomer: the subunit of a larger oligomeric protein assembly.
Ring-building motif (RBM): a structural arrangement with a wedge-shaped fold, composed of two α-helices folding against a three-stranded β-sheet, that is postulated to allow for assembly of the symmetrical oligomeric ring structures present in the basal platform of type III specialized secretion systems.
Sigma factor (σ factor): a protein that promotes the binding of RNA polymerase to the promoters of specific genes and mediates the initiation of RNA synthesis.
Specialized secretion systems: protein complexes that mediate ATP-driven translocation of proteins, and sometimes DNA, from the bacterial cytoplasm to the extracellular environment. These systems promote specific biological processes in bacteria that include flagellar assembly, host infection and symbiosis, adhesion to host cells or surfaces, and killing of bacterial competitors.
Sporulation: a cell differentiation process that culminates in the formation of dormant and stress-resistant cells called spores.

underlie aspects of the epidemiology of spore-forming pathogens, such as *Bacillus cereus* (food poisoning), *Bacillus anthracis* (anthrax disease), and multiple *Clostridium* species of which *Clostridium difficile* (infectious diarrhea) is the best known [18–20].

Under the microscope, the beginning of spore formation is evidenced by the formation of an asymmetric septum (Figure 1A). This morphological event generates two cells of unequal size and distinct developmental fates. The smaller cell (called the forespore) develops into the

dormant spore. The larger cell (designated the mother cell) contributes to forespore development but then lyses upon spore maturation. The different stages of forespore development are controlled by cell–cell signalling pathways that lead to the activation of cell-type-specific **sigma factors** controlling gene expression in the mother cell or in the forespore (Figure 1A). Upon polar division, σF is activated in the forespore, which then signals to activate σE in the mother cell. Later in the process, activation of σG in the forespore triggers σK activation in the mother cell. The early stages of this gene-expression cascade are accompanied by morphological changes to the double-membrane polar septum that divides the forespore and mother cell. The cell wall between these two membranes is remodelled, promoting the movement of the mother cell membrane around the forespore in a phagocytic-like manner (Figure 1A). Collectively, this process is called engulfment [21,22]. At the end of engulfment, the mother cell membranes undergo fission, and the forespore is released into the mother cell cytoplasm as a double-membrane protoplast, with an inner membrane derived from the forespore and an outer membrane derived from the mother cell [23]. At this stage, the forespore undergoes further maturation processes, including the deposition of spore cortex peptidoglycan and external protective coat layers around it [24]. Once the spore is mature, the mother cell lyses and releases it into the environment, where it remains dormant until favourable nutrient conditions arise.

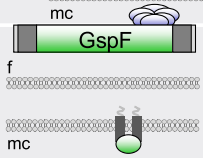
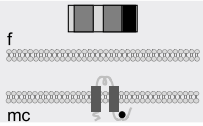
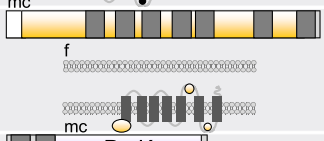
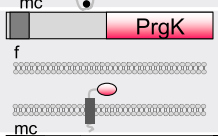
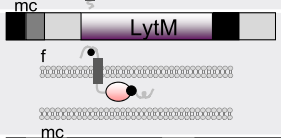
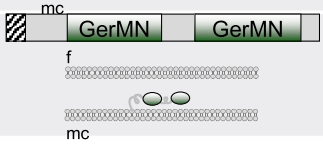
During the engulfment process, the A-Q complex assembles in the membranes surrounding the spore. This complex is composed of at least nine proteins (Table 1): the forespore protein SpoIIQ (Q) and eight mother-cell proteins (SpoIIIAA–SpoIIIAH) encoded in the *spoIIIA* operon [25], transcribed under the control of σF and σE, respectively [25,26]. Two additional factors, GerM and SpoIIIL (under σE and σF control, respectively), have been recently implicated in the A-Q complex [27,28]. In the absence of any of these factors, the sporulating cells produce small forespores with membrane deformities [9,27,28]. Moreover, these forespores have limited transcriptional potential and fail to activate gene expression under σG control [9,29,30]. Consequently, A-Q complex mutants are severely defective in spore formation [9,29–32].

Several of the SpoIIIA proteins have sequence and structural similarity to components of type II, III, and IV secretion systems found in Gram-negative bacteria [8–12,33,34]. These homologies suggest that the A-Q complex could function as a new type of specialized secretion apparatus (see Outstanding Questions). This review focuses on summarising what we know, and still do not know, about this broadly conserved protein complex. Although most of the review hinges on studies performed in the spore-forming model *Bacillus subtilis,* some focus will be placed on recent studies in the important spore-forming pathogen, *C. difficile*.

Early Studies on the A-Q Complex

The earliest studies on the A-Q proteins date back to the 1970s, where the advent of chemical mutagenesis led to the discovery and characterization of mutations within the *spoIIIA* locus [35,36]. Studies followed that refined the structure and transcriptional regulation of the *spoIIIA* operon [25,37,38]. Decades later, the *spoIIQ* locus was described and SpoIIQ was found to reside in the forespore membranes by immunofluorescence microscopy [26]. At the beginning of the millennium, protein localization and biochemical studies performed by the Pogliano and Rudner laboratories provided evidence that SpoIIIAH and SpoIIQ establish a transenvelope interaction between the mother and the forespore [39–41]. It was shown that SpoIIIAH localization in the forespore outer membranes (derived from the mother) depends on SpoIIQ located in the forespore inner membrane (derived from the forespore) [39,40]. In support of these observations, protein interaction assays revealed that SpoIIIAH and SpoIIQ establish a direct interaction [39,40].

Table 1. Topology of Known Components of the A-Q Complex and Their Similarities with Protein Homologues Found in Known Specialized Secretion Systems

	Size (residues)	Topology and homology regions[a]	Protein homologue (% of sequence identity)[b]	Function of the homologue
SpoIIIAA	307		VirB11 (18%) from T4P/GspE from T2SS (18%)	VirB11/GspE: ATPase involved in substrate export and pilus biogenesis
SpoIIIAB	171		GspF from T2SS (16%)	ATPase membrane anchor
SpoIIIAC	68		NI	NI
SpoIIIAD	133		NI	NI
SpoIIIAE	399		Permease domain of the multidrug transporter ABCG2 (11%)	Transport of various drugs through the cytoplasmic membrane
SpoIIIAF	206		RBM2 of PrgK from T3SS (12%)	Ring-building motif, part of the inner-membrane platform
SpoIIIAG	229		RBM2 of PrgK from T3SS (17%)/RBM3 of FliF from flagella (14%)	Ring-building motif, part of the inner-membrane platform
SpoIIIAH	218		RBM2 of PrgK from T3SS (17%)	Ring-building motif, part of the inner-membrane platform
SpoIIQ	283		LytM from the *S. aureus* cell wall synthesis machinery (20%)	Hydrolysis of the Gly-Gly bond in the pentaglycine inter-peptide link found in the staphylococcal cell wall
GerM	366		NI Belongs to the GerMN protein family (PF10646)	NI

[a]The presence of transmembrane segments and topology of proteins was predicted using the TMHMM server[iii]. Homology regions were predicted using the HHpred[iv] or Phyre2[v] servers.
[b]The percentage of sequence identity is based on ClustalW[vi].

Insights into the Function of the A-Q Complex

The possibility that the A-Q complex could function as a specialized secretion system was put forward by studies from the Losick, Moran, and Rudner laboratories. These studies identified weak homologies between the SpoIIIA proteins and proteins involved in specialized secretion systems [8–10,29] (Table 1). Furthermore, they provided models invoking secretory roles for the A-Q complex in spore development (Box 1).

Moran and colleagues used a compartmentalized biotin ligase accessibility assay to test the hypothesis that SpoIIIAH and SpoIIQ could form a channel between the mother cell and forespore [10]. They demonstrated that a fusion between SpoIIIAH and the biotin acceptor peptide (BAP), SpoIIIAH-BAP, is accessible to biotinylation if BirA (biotin ligase) is produced in the forespore but not when it is produced in the mother cell; the same trend was also observed with SpoIIQ-BAP [10]. Based on the homology of SpoIIIAH to inner-membrane ring-forming proteins of type III secretion systems (Table 1), the authors hypothesized that SpoIIIAH and SpoIIQ could form a channel or conduit between the mother cell and forespore. Furthermore, because they observed no biotinylation of SpoIIIAH-BAP or SpoIIQ-BAP when BirA was produced in the mother cell, they suggested that this channel is open on the forespore side and possibly gated on the mother cell side by the other SpoIIIA proteins [10]. Whether or not the A-Q complex is involved in the translocation of proteins between the mother and the forespore remains to be demonstrated (secretion of proteins model, Box 1).

Box 1. Models for the Secretion Function of the A-Q Complex

Model 1 – Piliation

The A-Q complex assembles a short pilus that attaches the outer and inner forespore membranes [11]. Although no pilin-like proteins have been identified so far as being involved in the A-Q complex, electron micrographs showing membrane deformities and engulfment defects in A-Q complex mutant forespores (including mutants with point mutations in the Walker motifs of SpoIIIAA) support this model [9,31]. Against this model are two observations. Firstly, only SpoIIIAH and SpoIIQ, but not the remaining SpoIIIA proteins, have been implicated in attaching the outer and inner forespore membrane [60]. The transenvelope, zipper-like interaction between SpoIIIAH and SpoIIQ is sufficient to attach the mother and the forespore in protoplasted sporulating cells, mediating engulfment by a ratchet-like mechanism [40,60]. Secondly, high-copy-numbers of the A-Q complex are not required to execute its role in sporulation, which might be expected for a secretion complex with roles in membrane attachment. A tenfold decrease in SpoIIIAE levels does not impact spore formation [9].

Model 2 – Feeding-tube Model

The A-Q complex acts as a gap-like junction, allowing the mother cell to 'feed' small molecules required for biosynthetic activity, like ATP, to the forespore [29]. The main premise of this model is that engulfment isolates the forespore from the external environment, making it depend on the mother cell to obtain small molecules required for macromolecular synthesis [29,32]. Against this model is the observation that the A-Q complex plays a role in spore development even when the forespore has access to the outside environment: σG activity in *B. subtilis* engulfment-defective mutants (the forespore is not internalized inside the mother cell) still depends on the A-Q complex [9].

Model 3 – Secretion of Proteins

The A-Q complex functions to translocate protein(s) between the mother and forespore [10]. Studies examining compartment-specific gene expression, using fluorescent fusions or biotin labelling, indicate that there is no cross-compartment leakiness. Nevertheless, if SpoIIIAG is in fact an inner component of the A-Q transenvelope conduit, then the diameter of its pore (7.6 nm) would allow the passage of globular proteins weighing up to 150 kDa between the two cells [11,12]. If SpoIIIAG rather constitutes a bushing module surrounding a protein conduit, then the diameter of the pore will be smaller than 7.6 nm and limit the size of the transported molecules [11]. In this case, it would be more plausible for the transported molecule(s) to be a small metabolite(s).

Camp and Losick found that the forespores of A-Q complex mutants are defective in their capacity to sustain transcriptional activity [29]. In one experiment, they showed that *lacZ* transcription from a T7-dependent promotor, using T7 RNA polymerase produced in the forespore, is severely compromised in A-Q complex mutants. In a second experiment, they showed that a late phase of σF activity that is unmasked in *sigG* mutants is also dependent on the A-Q complex. These observations suggest that A-Q complex mutants are defective in their ability to sustain all transcription in the forespore, not just that associated with σG. Why A-Q complex mutant forespores lose their transcriptional capacity remains unclear; one model is that the A-Q complex mediates the translocation of small molecules between the mother and the forespore [29] (feeding-tube model, Box 1).

In addition to demonstrating that several of the A-Q complex proteins reside in a large membrane complex by coimmunoprecipitation [9], Rudner and colleagues also identified a morphological defect in fully engulfed forespores of A-Q complex mutants using fluorescence and electron microscopy. This morphological defect was designated forespore collapse. While wild-type forespores are large in size, with ellipsoid shape and uniform membranes, the collapsed forespores of A-Q mutants are smaller in size and have distinct membrane deformities (Figure 1B,C) [9]. Importantly, the authors demonstrated that collapse does not occur in forespores lacking *sigG*. This observation suggests that forespore collapse in A-Q complex mutants does not occur because of failure to activate σG; instead it appears that forespore collapse leads to defects in σG activation. The authors suggest that forespore collapse could represent an altered physiological and/or energetic state that compromises forespore morphological development and all biological activities depending of it, including σG-dependent transcription [9]. What causes forespore collapse is unclear, but this phenotype may point to a structural role for the A-Q complex (pilliation model, Box 1). Alternatively, forespore collapse may also result from a failure to maintain membrane potential as a result of an altered physiological and/or energetic state.

The function of the A-Q complex appears to be short-lived and tied to the completion of the engulfment process. In wild-type cells, σG activity occurs after engulfment completion and results in increased levels of the forespore-produced protease SpoIVB, which degrades SpoIIQ and causes the disassembly of A-Q complex [42,43]. Thus, the function of the A-Q complex occurs throughout engulfment and is then terminated once engulfment is completed and σG is activated in the forespore. The significance of this coupling between A-Q complex function and engulfment remains unclear (Box 1).

Structural Insights into the A-Q Complex

Recent structural studies of core A-Q components strongly suggest that the complex might contain a set of stacked rings spanning the intermembrane space and resembling those found in specialized secretion systems.

The SpoIIIAH-SpoIIQ Heterodimer

Two independent studies by the Moran and Wilkinson laboratories determined the crystal structure of the SpoIIIAH-SpoIIQ heterodimer and revealed important structural features of these proteins, as well as the structural nature of their interaction [33,34].

The structure of the globular domain of AH (residues 104–218) revealed the presence of the canonical $\alpha_1\beta_1\beta_2\alpha_2\beta_3$ **ring-building motif (RBM)** (residues 134–218) found in the EscJ/PrgK family of ring-forming proteins from type III secretion systems, as well as an additional N-terminal helix (called helix α_0 henceforth) packing against helix α_1 and the three-stranded

β-sheet (Figure 2A,B) [33,34]. SpoIIQ folds into a **LytM domain** but displays a degenerate active site lacking two of the catalytic histidines present in the LytM **metalloendopeptidase** from *Staphylococcus aureus*, including one zinc-coordinating histidine [33,34,44]. Another

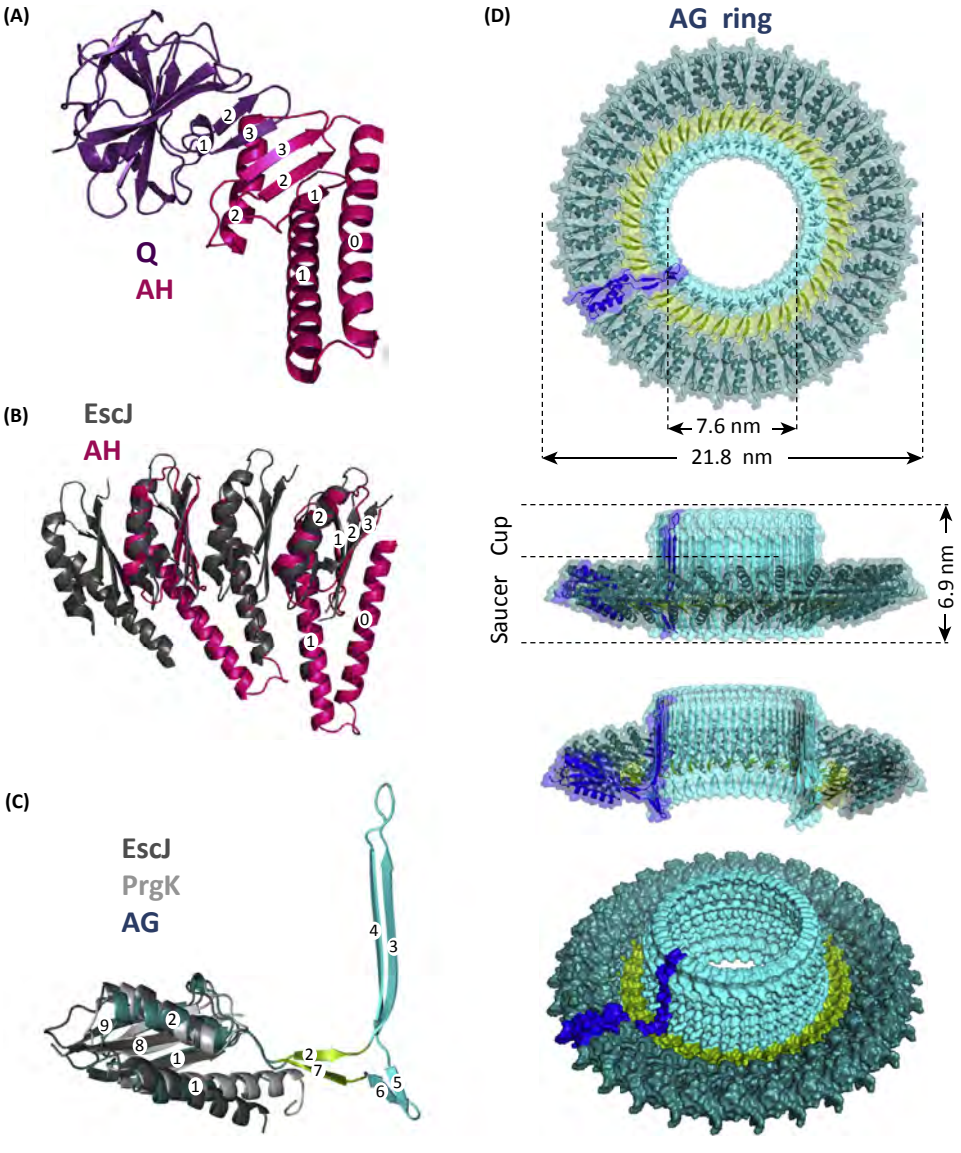


Figure 2. Structure of Core Proteins of the A-Q Complex. (A) Ribbon representation of the SpoIIIAH-SpoIIQ (AH-Q) dimer showing the intermolecular β-sheet. Helix α_1 and strands β_2 and β_3 of SpoIIQ are annotated. SpoIIIAH α-helices and β-strands are numbered 0–1 and 1–3, respectively. (B) Ribbon representation of two SpoIIIAH ring-building motifs (RBMs) (with numbered α-helices and β-strands) superimposed onto the tetramer formed by RBM2 of EscJ. (C) Ribbon representation of the SpoIIIAG (AG) protomer superimposed onto EscJ and PrgK RBM2. SpoIIIAG α-helices and β-strands are numbered 1–2 and 1–9, respectively. In the SpoIIIAG model, the RBM is in teal, the two-stranded β-sheet (β_2-β_7) forming the planar β-ring in green and the two two-stranded β-sheets (β_3-β_4 and β_5-β_6) forming the vertical β-barrel in cyan. (D) Ribbon and surface representation of the SpoIIIAG ring shown in top, side, cropped and tilted views, from top to bottom. The color code is identical to panel (C), with one SpoIIIAG protomer shown in dark blue. PDB entries used to generate the figure panels are 3UZ0 for SpoIIIAH and SpoIIQ (residues 104–217 and 78–220, respectively), 1YJ7 for EscJ (residues 96–186), 4OYC for PrgK (residues 94–176) and 5WC3 for SpoIIIAG (residues 89–227).

main structural difference between SpoIIQ and LytM is a region of 14 residues in SpoIIQ that forms a short helix (α_1) and a β-hairpin (β_2-β_3). This region partially obstructs the degenerate active site of SpoIIQ and is engaged in the interaction with SpoIIIAH (Figure 2A) [33,34]. The heterodimerization of SpoIIIAH and SpoIIQ is achieved through antiparallel pairing of the third β-strands of the two partners, resulting in a continuous, five-stranded intermolecular mixed β-sheet. The heteromeric interface is strengthened by the packing of the short α_2 helix of SpoIIIAH against helix α_1 of SpoIIQ. Although neither validated *in vitro* nor *in vivo*, this interface, which involves hydrophobic and electrostatic interactions, most likely reflects the physiological SpoIIIAH-SpoIIQ interface since the complex was crystallized from its soluble form isolated by size-exclusion chromatography [33,34].

Modelling the SpoIIIAH-SpoIIQ Heterodimer into Rings

The RBM of SpoIIIAH superimposes onto the second RBM of EscJ (PDB entry 1YJ7, residues 98–186) and PrgK (PDB entry 4OYC, residues 98–200) with a root-mean-squared deviation (r.m.s.d.) of 1.8 Å and 1.6 Å, respectively [45,46]. Although the crystallized SpoIIIAH construct did not oligomerize in solution, its structural similarity with EscJ/PrgK RBMs provides support for the idea that SpoIIIAH might form an oligomeric ring, and prompted the modelling of SpoIIIAH into rings. Wilkinson and colleagues generated a model of the SpoIIIAH-SpoIIQ ring based on the alignment of the SpoIIIAH-SpoIIQ dimer onto the crystal structure of the EscJ tetramer [34]. Superposition of one SpoIIIAH **protomer** onto one of the four EscJ molecules shows that helix α_0 of SpoIIIAH overlays helix α_1 from the neighbouring EscJ protomer. Consequently, only two SpoIIIAH protomers can be superposed onto four EscJ protomers (Figure 2B), leading to a 12-mer ring model in which stacked rings of SpoIIIAH and SpoIIQ form a channel of 6- and 10-nm diameter on the SpoIIIAH and SpoIIQ sides, respectively [34]. By contrast, *ab initio* ring modelling of the SpoIIIAH-SpoIIQ complex by Moran and colleagues generated compact ring models with C15 and C18 symmetry [33]. In the 15-mer model, the SpoIIIAH and SpoIIQ rings display inner diameters of 8 and 13 nm, respectively, while these diameters measure 11 and 15 nm in the 18-mer model. In all of these models of the SpoIIIAH-SpoIIQ complex, the SpoIIIAH-SpoIIIAH interface diverges from the EscJ/PrgK RBM2 ones: the SpoIIIAH-SpoIIIAH interface is much smaller and mainly involves residues carried by helices α_0 and α_1, while the EscJ–EscJ interface involves residues carried by the β-sheet of one protomer and residues carried by helices α_1 and α_2 of the neighbouring protomer.

The SpoIIIAG Ring

SpoIIIAG is anchored in the outer forespore membrane through an N-terminal transmembrane segment [11]. Its soluble domain resides in the intermembrane space and is made of a disordered N-terminal region of low sequence conservation (residues 47–88) followed by a longer C-terminal domain displaying higher sequence conservation (residues 89–229) [11] (Table 1). Recently, the Morlot and Rudner laboratories demonstrated that SpoIIIAG is capable of forming ring-like oligomers *in vitro*, in three endospore-forming bacteria [11]. Cryo-EM reconstruction of *B. subtilis* SpoIIIAG at low resolution revealed a 'cup-and-saucer' architecture with a large central pore and dimensions similar to the ring-forming proteins observed in the inner membrane platform of type III secretion systems and flagella. Furthermore, they provided evidence that SpoIIIAG ring formation *in vivo* is required for the function of the A-Q complex in *B. subtilis* [11]. More recently, Strynadka and colleagues reported a cryo-EM structure of the SpoIIIAG ring at ~3.5 Å, revealing a 30-fold symmetry and near-atomic details of the SpoIIIAG protomer and oligomerization interfaces [12]. In agreement with the predicted model of SpoIIIAG [11], regions encompassing residues 89–127 and 181–227 fold into the canonical RBM of the EscJ/PrgK/FliF family of type III secretion/flagellar systems ($\alpha_1\beta_1\beta_8\alpha_2\beta_9$ topology, Figure 2C) [12]. Similar to EscJ/PrgK rings, the oligomerization interface of the SpoIIIAG RBM

involves hydrophobic and electrostatic interactions allowed by the packing of helices α_1 and α_2 against the three-stranded β-sheet of the adjacent RBM [12,45,46]. In support of this experimental model of the SpoIIIAG ring, mutation of key interface residues (I107R, V120R, R185D, R209E, R217A, and K223E) impaired oligomerization *in vitro* and decreased sporulation efficiency *in vivo* [11,12]. Mutations R209E and K223E were further shown to result in reduced σG activity and collapsed forespores [11]. The 30-mer RBM oligomer, which forms the saucer region of the SpoIIIAG rings, displays an outer diameter of 21.8 nm (Figure 2D) close in size to those of the 24-mer EscJ/PrgK and 26-mer FliF rings (~18 nm, ~19 nm, and ~24 nm for EscJ, PrgK, and FliF, respectively) [12,45,47,48].

Within the $\beta_1\beta_8$ hairpin of the RBM lies a region (residues 128–180) that folds into a new motif with no similarity to any known structure. This motif contains three two-stranded antiparallel β-sheets arranged as a planar β-triangle (Figure 2C). Oligomerization of this motif results into an unprecedented 60-stranded vertical β-barrel (made of $\beta_3\beta_4$ and $\beta_5\beta_6$) and a horizontal β-ring (made of $\beta_2\beta_7$) (Figure 2D) [12]. In agreement with the predicted SpoIIIAG ring model, the two longest β-strands ($\beta_3\beta_4$) make up the major part of the cup region and form a hollow channel with an inner diameter of 7.6 nm [11,12]. This region is reminiscent of the cup-like structure formed by domain R in the FliF rings, with similar inner diameter (9 nm for FliF domain R) and height values (6.9 nm for SpoIIIAG versus 7 nm for FliF). Interestingly, the third RBM in FliF also has an insertion region (longer than the SpoIIIAG β-triangle motif by 58 residues) predicted to contain a high proportion of β-structures [46]. The structure of the SpoIIIAG ring thus suggests that, in the FliF rings, the R region might also be made of this insertion region that would form a large β-barrel. By analogy with FliF, for which the cup region was experimentally shown to face the outer membrane [48,49], the cup region in SpoIIIAG is thus likely oriented toward the forespore. This model also places the SpoIIIAG RBM ring (forming the saucer region) in an orientation similar to that of the EscJ/PrgK RBM rings [47,50].

The cup region in FliF is thought to contact the flagellar bushing formed by FlgI and to surround the lower region of the cylindrical rod of the flagellum (formed by FlgB, FlgC, FlgF, and FlgG) [51]. The similar dimensions of the SpoIIIAG and FliF cup regions suggest that SpoIIIAG might also accommodate an inner rod complex, but no protein candidates have been identified so far. Alternatively, the SpoIIIAG cup region might not shelter any protein but constitute part of a channel connecting the mother cell and forespore cytoplasm. Since a channel only made of SpoIIIAG would not be long enough to cross the intermembrane space (~20 nm) [52], other proteins are expected to participate in this putative transenvelope conduit. Based on the facts that SpoIIIAH shares structural similarities with EscJ/PrgK, and that it interacts directly with SpoIIQ, the other channel-forming candidates so far are SpoIIIAH and SpoIIQ.

Assembly and Architecture of the A-Q Complex

What is known about the assembly of the A-Q complex relates to the localization of SpoIIQ, SpoIIIAH, GerM, and SpoIIIAG [9,28,53,54], and insertion of SpoIIIAE into the mother cell membrane [8,55].

SpoIIQ localization to the forespore septal membrane depends on SpoIIIAH and GerM produced in the mother cell [28,39,40,53]. These proteins in turn require SpoIIQ for their localization to the septal membrane on the mother cell side. The establishment of this tripartite complex then appears to allow for efficient localization of SpoIIIAG to the mother cell septal membrane, since the absence of any of these proteins affects SpoIIIAG localization [9,28].

Similar to what has been observed in other specialized secretion systems, the cell wall between the mother and the forespore appears to act as a barrier for the assembly of the A-Q complex [53,54]. Although SpoIIQ and SpoIIIAH establish their interaction in the presence of septal cell wall, the interaction between SpoIIQ and GerM only takes place once the septal cell wall has been cleaved [28,53]. The cleavage of the septal cell wall reduces the distance between the mother cell and forespore membrane from 40 nm to 20 nm [52], likely facilitating the interaction between SpoIIQ and GerM [28]. Thus, cleavage of the septal cell wall by the engulfment cell-wall-degrading SpoIID-SpoIIM-SpoIIP machinery facilitates the assembly and activity of the A-Q complex [28,53].

How these proteins could come together to build a conduit between the mother cell and forespore remains an open question. If a stacked-ring arrangement is considered, similar to those observed in type III secretion systems, then the SpoIIIAG ring could possibly stack against a SpoIIIAH ring, which would be in direct contact with a putative SpoIIQ ring [11,12] (Figure 3, Key Figure). Regarding GerM, it contains two GerMN (Germane) domains, a family of domains with unknown function [56]. Although the structure of GerM and its molecular interaction network remain to be characterized, it is tempting to speculate that it might form a ring through interaction(s) with SpoIIIAG, SpoIIIAH, and/or SpoIIQ [28] (Figure 3).

Importantly, although SpoIIQ has been viewed as the secretin of the A-Q complex, transmembrane swapping experiments suggest that it is not (Figure 3): replacing the transmembrane segment of SpoIIQ with one from another protein (MalF – maltose permease) did not compromise A-Q complex function [29]. Thus, additional, unidentified proteins are required for the formation of a secretin pore in the forespore inner membrane.

Finally, although the exact role of SpoIIIAE in the A-Q complex remains elusive, this large multispan membrane protein was shown to interact with SpoIIIJ, a membrane protein translocase of the YidC/Oxa1p/Alb3 family. This suggests an important role for SpoIIIJ in the insertion and/or folding of SpoIIIAE in the mother cell membrane [8,55].

The A-Q Complex in *Clostridium difficile*

Recent studies from the Shen and Henriques laboratories suggest that the A-Q complex of *C. difficile* may have a different basal structure, as well as play different roles in sporulation, compared to its relative *B. subtilis* [31,32]. These differences are not surprising considering how the sporulation program is triggered, how sporulation unfolds at the level of sigma factor activation, and more globally the ecological niches occupied by these bacteria [57–59].

As is the case for *B. subtilis*, SpoIIQ and SpoIIIAH from *C. difficile* were shown to interact and localize in the membranes surrounding the forespore, throughout engulfment. Interestingly, a deletion of SpoIIIAH in *C. difficile* abolishes the production of heat-resistant spores to the same degree as deletions of the other SpoIIIA proteins [32]. This is not the case for $SpoIIIAH^{Bs}$ [28,53]; thus, $SpoIIIAH^{Cd}$ plays a more critical role in this organism. Indeed, in *B. subtilis* GerM appears to partially compensate for the absence of SpoIIIAH in the localization of SpoIIQ. Moreover, phylogenetic analysis further shows that while GerM homologs are present in the Bacillaceae family, they are absent in the Clostridiaceae [28].

Another difference between the A-Q complex of *C. difficile* and *B. subtilis* resides in SpoIIQ. While $SpoIIQ^{Bs}$ and $SpoIIQ^{Cd}$ contain a LytM-endopeptidase domain, $SpoIIQ^{Bs}$ lacks the critical histidine residues involved in Zn^{2+} binding that are required for endopeptidase activity. In several clostridial species, including *C. difficile*, these histidine residues are present,

Key Figure

Model for the Architecture of the A-Q Complex

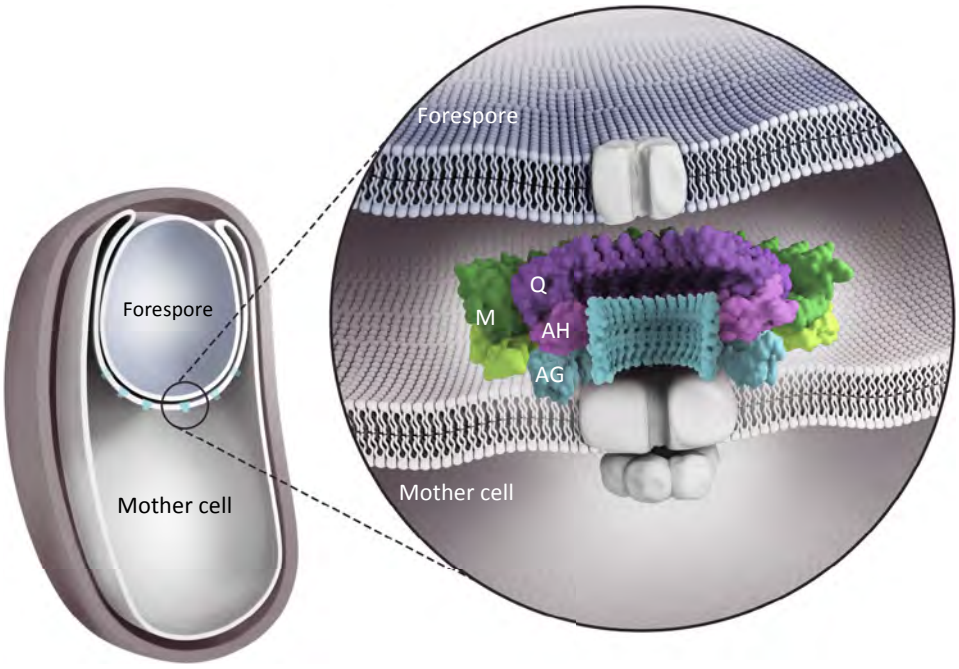


Figure 3. Schematic showing a stacked-ring arrangement for the A-Q complex in *Bacillus subtilis*. SpoIIQ (Q, purple), SpoIIIAH (AH, pink), SpoIIIAG (AG, teal) and GerM (M, green) are hypothesized to stack upon each other to generate a conduit that connects the mother cell and forespore. The structure of GerM and its molecular interaction network remain to be characterized, but it is tempting to speculate that it might form a ring through interaction(s) with SpoIIIAG, SpoIIIAH, and/or SpoIIQ [28]. The other membrane proteins of the A-Q complex (AB–AF) are shown schematically as a single complex (gray) with a predicted membrane pore. SpoIIIAA is shown as a hexamer by analogy to ATPases of other specialized secretion systems. While evidence supports the existence of a pore in the forespore membrane (shown in grey), the identity of this protein is unknown. The PDB entry used for the 30-mer SpoIIIAG ring is 5WC3, the 15-mer model of the SpoIIIAH-SpoIIQ rings was generously provided by C.P. Moran Jr., and the 30-mer model of the GerM ring was generated using the SymmDock server[i] based on *in silico* models of the two GerMN domains of GerM using the Phyre2 server[ii].

suggesting that SpoIIQ^{Cd} may be involved in cell wall remodelling during engulfment [14]. However, mutation of the H120 residue, required for Zn^{2+} binding, did not affect sporulation to the same degree as a *spoIIQ* null allele, suggesting that the endopeptidase activity of SpoIIQ^{Cd} is not absolutely required for sporulation in *C. difficile* [31,32].

Importantly, both studies identified striking engulfment defects in A-Q complex mutants of *C. difficile*. Null mutants of *spoIIQ*, *spoIIIAH*, or the entire *spoIIIA* operon reduced the frequency of fully engulfed forespores, suggesting that the A-Q complex is critical for the engulfment process in *C. difficile.* This is in stark contrast to *B. subtilis*, where only SpoIIIAH and SpoIIQ have been implicated in the engulfment process in certain conditions and not to the same degree as that observed in *C. difficile* [30,60]. Furthermore, *C. difficile* A-Q complex mutants also failed to

Box 2. Other Roles for the A-Q Complex Proteins in Sporulation

The transenvelope interaction between SpoIIIAH and SpoIIQ is not only required for the assembly and function of the A-Q complex, it also appears to serve as an important protein–protein interaction hub for proteins with diverse roles in sporulation, on both sides of the septum [28]. The complexity of this protein–protein interaction network has been elucidated by studies examining the localization requirements of many sporulation proteins. The theme emerging from these studies is that SpoIIIAH and SpoIIQ are located upstream of this interaction network, and mutants lacking *spoIIQ* or *spoIIIAH* are defective in the localization of proteins involved in: engulfment (SpoIID and SpoIIP) [62]; the activation of the late mother cell transcription factor σK (SpoIVFA) [39,62]; the formation of the spore coat (CotE) [61]; spore germination (GerM) [28]; the regulation of σF and σG (SpoIIE) [63] and the accumulation of dipicolinic acid (DPA) in the forespore (SpoVV) [64]. While it remains unclear whether or not all of of these other interactions result from direct contacts with SpoIIIAH or SpoIIQ, it is clear that establishment of the SpoIIIAH-SpoIIQ interaction is a key step in the sporulation developmental program. How SpoIIIAH and SpoIIQ manage to keep all of these interactions in check, and at the same time assemble into the A-Q complex, is an interesting question.

adhere the spore coat to the outer forespore membrane, causing accumulations of coat material in the mother cell cytoplasm [31]. Although SpoIIIAH and SpoIIQ in *B. subtilis* appear to play some role in coat deposition through their role in localising CotE [61] (Box 2), no defects in coat deposition have been described. These data support the idea that the A-Q complex may play a structural role during the engulfment process (Box 1).

In further support of the idea that the A-Q complex could play a different role in spore development in *C. difficile,* Shen and colleagues demonstrated that the A-Q complex is not required to activate σG, at least prior to engulfment completion [31]. Interestingly, the study of Henriques and colleagues demonstrated that the A-Q complex is required for σG activity once engulfment is completed [32]. Interestingly, they showed that the A-Q complex is also required for σK activity in the mother cell [32]. Why the A-Q complex is required for σG activity only after completion of engulfment in *C. difficile* is unclear, but could reflect a necessity of the forespore as it becomes internalized in the mother cell and isolated from the external environment [32].

Collectively, the study of the A-Q complex in *C. difficile* raises even more questions about its role in spore development. Furthermore, these studies highlight the importance of studying the A-Q complex in other spore-formers.

Concluding Remarks

The idea that the A-Q complex could function as a specialized secretion system is relatively recent. While the exact secretory role of this complex during sporulation remains elusive, structural data clearly support the idea that the A-Q complex is a new type of specialized secretion system required to connect the mother cell and spore. Its hybrid-like composition and unique role during sporulation clearly warrant future investigations that will likely reveal novel and intriguing insights about the relationship between structure and function of specialized secretion systems (see Outstanding Questions).

Outstanding Questions

Is the A-Q complex a *de facto* specialized secretion system and, if so, what does it secrete? There is currently no evidence to suggest that the A-Q complex is involved in the translocation of molecules across the spore membranes. Several models invoking secretion have been put forward, and distinguishing between these models will likely shed light on the function of the A-Q complex.

What does the A-Q complex look like? The A-Q complex proteins exhibit homology to proteins of type II, type III, and type IV secretion systems. How these proteins come together to build this hybrid complex, and what their combined architecture looks like, could reveal intricacies of building a molecular machine from apparently different parts.

Have all components of the A-Q complex been identified? At least nine proteins are thought to constitute the core of the A-Q complex but there are likely other accessory components remaining to be identified and characterized.

What is the evolutionary origin of the A-Q complex? The unusual collection of protein homologies exhibited by the A-Q complex proteins begs the following questions: was the A-Q complex cobbled together using parts of different specialized secretion systems, or does it reflect an ancient module of specialized secretion from which other systems evolved? The answer to these questions will require in-depth phylogenetic analysis of the A-Q proteins.

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Resources

[i] http://bioinfo3d.cs.tau.ac.il/SymmDock/
[ii] www.sbg.bio.ic.ac.uk/phyre2/
[iii] www.cbs.dtu.dk/services/TMHMM/

[iv]https://toolkit.tuebingen.mpg.de/#/tools/hhpred

[v]www.sbg.bio.ic.ac.uk/phyre2/

[vi]https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_clustalw.html

References

1. Chandran, V. (2013) Type IV secretion machinery: molecular architecture and function. *Biochem. Soc. Trans.* 41, 17–28
2. Portaliou, A.G. *et al.* (2016) Type III secretion: building and operating a remarkable nanomachine. *Trends Biochem. Sci.* 41, 175–189
3. Dalbey, R.E. and Kuhn, A. (2012) Protein traffic in Gram-negative bacteria – how exported and secreted proteins find their way. *FEMS Microbiol. Rev.* 36, 1023–1045
4. Buttner, D. (2012) Protein export according to schedule: architecture, assembly, and regulation of type III secretion systems from plant- and animal-pathogenic bacteria. *Microbiol. Mol. Biol. Rev.* 76, 262–310
5. Costa, T.R. *et al.* (2015) Secretion systems in Gram-negative bacteria: structural and mechanistic insights. *Nat. Rev. Microbiol.* 13, 343–359
6. Setlow, P. (2007) I will survive: DNA protection in bacterial spores. *Trends Microbiol.* 15, 172–180
7. Setlow, P. (2006) Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *J. Appl. Microbiol.* 101, 514–525
8. 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
9. Doan, T. *et al.* (2009) Novel secretion apparatus maintains spore integrity and developmental gene expression in *Bacillus subtilis*. *PLoS Genet.* 5, e1000566
10. Meisner, J. *et al.* (2008) A channel connecting the mother cell and forespore during bacterial endospore formation. *Proc. Natl. Acad. Sci. U. S. A.* 105, 15100–15105
11. Rodrigues, C.D. *et al.* (2016) A ring-shaped conduit connects the mother cell and forespore during sporulation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 113, 11585–11590
12. Zeytuni, N. *et al.* (2017) Near-atomic resolution cryoelectron microscopy structure of the 30-fold homooligomeric SpoIIIAG channel essential to spore formation in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 114, E7073–E7081
13. Galperin, M.Y. *et al.* (2012) Genomic determinants of sporulation in Bacilli and Clostridia: towards the minimal set of sporulation-specific genes. *Environ. Microbiol.* 14, 2870–2890
14. Crawshaw, A.D. *et al.* (2014) A mother cell-to-forespore channel: current understanding and future challenges. *FEMS Microbiol. Lett.* 358, 129–136
15. Errington, J. (2003) Regulation of endospore formation in *Bacillus subtilis*. *Nat. Rev. Microbiol.* 1, 117–126
16. Piggot, P.J. and Hilbert, D.W. (2004) Sporulation of *Bacillus subtilis*. *Curr. Opin. Microbiol.* 7, 579–586
17. Higgins, D. and Dworkin, J. (2012) Recent progress in *Bacillus subtilis* sporulation. *FEMS Microbiol. Rev.* 36, 131–148
18. Gomez, S. *et al.* (2017) Clinical, epidemiological and microbiological characteristics of relapse and re-infection in *Clostridium difficile* infection. *Anaerobe* 48, 147–151
19. Carroll, K.C. and Bartlett, J.G. (2011) Biology of *Clostridium difficile*: implications for epidemiology and diagnosis. *Annu. Rev. Microbiol.* 65, 501–521
20. Seekatz, A.M. and Young, V.B. (2014) *Clostridium difficile* and the microbiota. *J. Clin. Invest.* 124, 4182–4189
21. Morlot, C. *et al.* (2010) A highly coordinated cell wall degradation machine governs spore morphogenesis in *Bacillus subtilis*. *Genes Dev.* 24, 411–422
22. Abanes-De Mello, A. *et al.* (2002) A cytoskeleton-like role for the bacterial cell wall during engulfment of the *Bacillus subtilis* forespore. *Genes Dev.* 16, 3253–3264
23. Doan, T. *et al.* (2013) FisB mediates membrane fission during sporulation in *Bacillus subtilis*. *Genes Dev.* 27, 322–334
24. McKenney, P.T. *et al.* (2013) The *Bacillus subtilis* endospore: assembly and functions of the multilayered coat. *Nat. Rev. Microbiol.* 11, 33–44
25. Guillot, C. and Moran, C.P., Jr (2007) Essential internal promoter in the *spoIIIA* locus of *Bacillus subtilis*. *J. Bacteriol.* 189, 7181–7189
26. Londono-Vallejo, J.A. *et al.* (1997) SpoIIQ, a forespore-expressed gene required for engulfment in *Bacillus subtilis*. *Mol. Microbiol.* 24, 29–39
27. Meeske, A.J. *et al.* (2016) High-throughput genetic screens identify a large and diverse collection of new sporulation genes in *Bacillus subtilis*. *PLoS Biol.* 14, e1002341
28. Rodrigues, C.D. *et al.* (2016) GerM is required to assemble the basal platform of the SpoIIIA-SpoIIQ transenvelope complex during sporulation in *Bacillus subtilis*. *Mol. Microbiol.* 102, 260–273
29. 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
30. Sun, Y.L. *et al.* (2000) A dispensable role for forespore-specific gene expression in engulfment of the forespore during sporulation of *Bacillus subtilis*. *J. Bacteriol.* 182, 2919–2927
31. Fimlaid, K.A. *et al.* (2015) Regulation of *Clostridium difficile* spore formation by the SpoIIQ and SpoIIIA proteins. *PLoS Genet.* 11, e1005562
32. Serrano, M. *et al.* (2016) The SpoIIQ-SpoIIIAH complex of *Clostridium difficile* controls forespore engulfment and late stages of gene expression and spore morphogenesis. *Mol. Microbiol.* 100, 204–228
33. Meisner, J. *et al.* (2012) Structure of the basal components of a bacterial transporter. *Proc. Natl. Acad. Sci. U. S. A.* 109, 5446–5451
34. Levdikov, V.M. *et al.* (2012) Structure of components of an intercellular channel complex in sporulating *Bacillus subtilis*. *Proc. Natl. Acad. Sci. U. S. A.* 109, 5441–5445
35. Piggot, P.J. (1973) Mapping of asporogenous mutations of *Bacillus subtilis*: a minimum estimate of the number of sporeulation operons. *J. Bacteriol.* 114, 1241–1253
36. Piggot, P.J. and Coote, J.G. (1976) Genetic aspects of bacterial endospore formation. *Bacteriol. Rev.* 40, 908–962
37. Illing, N. and Errington, J. (1990) The *spoIIIA* locus is not a major determinant of prespore-specific gene expression during sporulation in *Bacillus subtilis*. *J. Bacteriol.* 172, 6930–6936
38. Illing, N. and Errington, J. (1991) The *spoIIIA* 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
39. Doan, T. *et al.* (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
40. Blaylock, B. *et al.* (2004) Zipper-like interaction between proteins in adjacent daughter cells mediates protein localization. *Genes Dev.* 18, 2916–2928
41. Rubio, A. and Pogliano, K. (2004) Septal localization of forespore membrane proteins during engulfment in *Bacillus subtilis*. *EMBO J.* 23, 1636–1646
42. Chiba, S. *et al.* (2007) Impact of membrane fusion and proteolysis on SpoIIQ dynamics and interaction with SpoIIIAH. *J. Biol. Chem.* 282, 2576–2586
43. Jiang, X. *et al.* (2005) Engulfment-regulated proteolysis of SpoIIQ: evidence that dual checkpoints control sigma activity. *Mol. Microbiol.* 58, 102–115

44. 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
45. Yip, C.K. *et al.* (2005) Structural characterization of the molecular platform for type III secretion system assembly. *Nature* 435, 702–707
46. Bergeron, J.R. *et al.* (2015) The modular structure of the inner-membrane ring component PrgK facilitates assembly of the type III secretion system basal body. *Structure* 23, 161–172
47. Worrall, L.J. *et al.* (2016) Near-atomic-resolution cryo-EM analysis of the Salmonella T3S injectisome basal body. *Nature* 540, 597–601
48. Suzuki, H. *et al.* (2004) Structure of the rotor of the bacterial flagellar motor revealed by electron cryomicroscopy and single-particle image analysis. *J. Mol. Biol.* 337, 105–113
49. Francis, N.R. *et al.* (1994) Isolation, characterization and structure of bacterial flagellar motors containing the switch complex. *J. Mol. Biol.* 235, 1261–1270
50. Schraidt, O. and Marlovits, T.C. (2011) Three-dimensional model of *Salmonella*'s needle complex at subnanometer resolution. *Science* 331, 1192–1195
51. Minamino, T. and Imada, K. (2015) The bacterial flagellar motor and its structural diversity. *Trends Microbiol.* 23, 267–274
52. Tocheva, E.I. *et al.* (2013) Peptidoglycan transformations during *Bacillus subtilis* sporulation. *Mol. Microbiol.* 88, 673–686
53. Rodrigues, C.D. *et al.* (2013) Peptidoglycan hydrolysis is required for assembly and activity of the transenvelope secretion complex during sporulation in *Bacillus subtilis*. *Mol. Microbiol.* 89, 1039–1052
54. Fredlund, J. *et al.* (2013) The SpoIIQ landmark protein has different requirements for septal localization and immobilization. *Mol. Microbiol.* 89, 1053–1068
55. Serrano, M. *et al.* (2008) Processing of a membrane protein required for cell-to-cell signaling during endospore formation in *Bacillus subtilis*. *J. Bacteriol.* 190, 7786–7796
56. Rigden, D.J. and Galperin, M.Y. (2008) Sequence analysis of GerM and SpoVS, uncharacterized bacterial 'sporulation' proteins with widespread phylogenetic distribution. *Bioinformatics* 24, 1793–1797
57. Pereira, F.C. *et al.* (2013) The spore differentiation pathway in the enteric pathogen *Clostridium difficile*. *PLoS Genet.* 9, e1003782
58. Saujet, L. *et al.* (2014) The regulatory network controlling spore formation in *Clostridium difficile*. *FEMS Microbiol. Lett.* 358, 1–10
59. Saujet, L. *et al.* (2013) Genome-wide analysis of cell type-specific gene transcription during spore formation in *Clostridium difficile*. *PLoS Genet.* 9, e1003756
60. Broder, D.H. and Pogliano, K. (2006) Forespore engulfment mediated by a ratchet-like mechanism. *Cell* 126, 917–928
61. McKenney, P.T. and Eichenberger, P. (2012) Dynamics of spore coat morphogenesis in *Bacillus subtilis*. *Mol. Microbiol.* 83, 245–260
62. Aung, S. *et al.* (2007) Dual localization pathways for the engulfment proteins during *Bacillus subtilis* sporulation. *Mol. Microbiol.* 65, 1534–1546
63. Flanagan, K.A. *et al.* (2016) A membrane-embedded amino acid couples the SpoIIQ channel protein to anti-sigma factor transcriptional repression during *Bacillus subtilis* sporulation. *J. Bacteriol.* 198, 1451–1463
64. Ramirez-Guadiana, F.H. *et al.* (2017) A two-step transport pathway allows the mother cell to nurture the developing spore in *Bacillus subtilis*. *PLoS Genet.* 13, e1007015