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Structural characterization of SpoIIIAB sporulation-essential protein in *Bacillus subtilis*

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ABSTRACT

Endospore formation in the Gram-positive bacterium *Bacillus subtilis* initiates in response to nutrient depletion and involves a series of morphological changes that result in the creation of a dormant spore. Early in this developmental process, the cell undergoes an asymmetric cell division that produces the larger mother cell and smaller forespore, the latter destined to become the mature spore. The mother cell septal membrane then engulfs the forespore, at which time an essential channel, the so-called feeding-tube apparatus, is thought to cross both membranes to create a direct conduit between the cells. At least nine proteins are required to form this channel including SpoIIQ under forespore control and SpoIIIAA-AH under the mother cell control. Several of these proteins share similarity to components of Type-II, -III and -IV secretion systems as well as the flagellum from Gram-negative bacteria. Here we report the X-ray crystallographic structure of the cytosolic domain of SpoIIIAB to 2.3 Å resolution. This domain adopts a conserved, secretion-system related fold of a six membered anti-parallel helical bundle with a positively charged membrane-interaction face at one end and a small groove at the other end that may serve as a binding site for partner proteins in the assembled apparatus. We analyzed and identified potential interaction interfaces by structure-guided mutagenesis *in vivo*. Furthermore, we were able to identify a remarkable structural homology to the C-subunit of a bacterial V-ATPase. Collectively, our data provides new insight into the possible roles of SpoIIIAB protein within the secretion-like apparatus essential to bacterial sporulation.

1. Introduction

Bacteria have evolved divergent transport systems to allow the specific selection and passage of small and large molecules across the plasma membrane for various purposes including cell homeostasis, virulence effector protein secretion, cell-cell communication, and pathogenesis (Burkinshaw and Strynadka, 2014; Christie et al., 2014; Costa et al., 2015; Korotkov et al., 2012; Kostakioti et al., 2005). Recently, a unique channel apparatus has been described to function during spore formation ("sporulation") by the Gram-positive bacterium *Bacillus subtilis* (reviewed in Crawshaw et al., 2014). Sporulation is an ancient developmental program that allows a starving cell to survive as a dormant cell type, a spore. Early in sporulation, an asymmetric septum divides the rod-shaped bacterium into two cells: a smaller "forespore", which will become the spore, and a larger "mother cell", which contributes to the development of the forespore but ultimately dies. At first these two cells lie side-by-side; later the mother cell membrane migrates around the forespore in a phagocytic-like process, resulting in the forespore being engulfed as a double-membraned protoplast within the mother cell cytosol. The forespore then acquires a protective peptidoglycan cortex and protein coat layers, and is finally released as a mature spore into the environment by lysis of the mother cell.

At around the time of engulfment, at least nine proteins assemble into a channel apparatus that spans the two opposing membranes separating the mother cell and forespore (Crawshaw et al., 2014). Eight of these proteins (SpoIIIAA-AH) are encoded in a single operon (*spoIIIA*) expressed in the mother cell under the control of the compartment-specific alternative sigma factor σ^E (Guillot and Moran, 2007; Illing and Errington, 1991). A ninth protein, SpoIIQ, is produced in the forespore under the control of σ^F (Londono-Vallejo et al., 1997). Mutants lacking any one of these channel genes display collapsed forespores that are unable to carry out gene expression directed by the late-acting sigma factor σ^G (Camp and Losick, 2008; Doan et al., 2009; Kellner et al., 1996; Londono-Vallejo et al., 1997). The block in gene expression is not specific to σ^G, however, given that the phage T7 RNA polymerase

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engineered to be expressed in the forespore also requires the channel genes for late activity (Camp and Losick, 2009). Together these data support a model in which the channel transports one or more substrates, yet to be identified, that generally support the physiology of the forespore as well as its capacity to carry out macromolecular synthesis. In one version of this model, the channel has been proposed to act as a "feeding tube" through which small molecule metabolites are transported to the developing spore from the mother cell (Camp and Losick, 2009).

Although many open questions remain regarding the SpoIIIAA-AH·SpoIIQ sporulation channel, recent studies have helped to bring into focus its evolutionary origins and some of its structural features. Intriguingly, the emerging picture is that this mother cell-to-forespore channel represents a novel, hybrid secretion system with proteins that share common elements with those of diverse secretion systems found in Gram-negative bacteria. Homology searches (Söding et al., 2005) reveal that SpoIIIAA resembles AAA + superfamily ATPases of the type II secretion system (T2SS) and type IV secretion system (T4SS) (Doan et al., 2009; Planet et al., 2001), while SpoIIIAB displays homology with the GspF/PilC family of proteins found in the T2SS and the related Type IV pilus (T4SP) secretion system (Abendroth et al., 2009; Karuppiah et al., 2010; Py et al., 2001). In contrast, SpoIIIAF, SpoIIIAG, and SpoIIIAH harbor domains that are evolutionarily related to the ring-forming PrgK/EscJ protein family of the type III secretion system (T3SS) (Bergeron et al., 2015; Levdikov et al., 2012; Meisner et al., 2012; Worrall et al., 2016; Yip et al., 2005). In the case of SpoIIIAH, structural studies have confirmed that its extracellular domain, which is anchored to the mother cell membrane by an N-terminal transmembrane domain, harbors the PrgK/EscJ "ring building motif" fold that helps to drive multimerization of the ring complex at the base of the T3SS apparatus (Bergeron et al., 2015; Worrall et al., 2016; Yip et al., 2005). This has led to a working model in which two stacked rings comprised of SpoIIIAH and the forespore membrane-anchored protein SpoIIQ, with which SpoIIIAH is known to interact (Blaylock et al., 2004; Doan et al., 2005; Levdikov et al., 2012; Meisner et al., 2012), serve as the "basal platform" upon which other channel components assemble. Recently we have determined the near-atomic resolution cryo-EM structure of SpoIIIAG in the assembled state, showing formation of ring building motif-mediated rings with interfacial packing similar to that observed in the T2SS and T3SS, but with unique stoichiometry and structural features critical to sporulation specific function (Zeytuni et al., 2017). Determining the structure of the remaining individual channel components, as well as how they fit together in a sporulation competent assembly, remains a crucial step toward a complete understanding of this unique channel apparatus.

In this study, we focus upon the essential SpoIIIAB sporulation channel protein. A mutant lacking *spoIIIAB*, as with other channel mutants, displays collapsed forespores that are unable to support late gene expression (Camp and Losick, 2009, 2008; Doan et al., 2009). Biochemical analysis has shown that SpoIIIAB resides in a complex with four other channel proteins: SpoIIIAD, SpoIIIAE, SpoIIIAF, and SpoIIIAG (Doan et al., 2009). Bioinformatics and structural prediction tools have suggested that SpoIIIAB is similar to regions of the GspF/PilC proteins of the T2SS and evolutionarily-related T4SP (Py et al., 2001; Abendroth et al., 2009; Karuppiah et al., 2010). These polytopic membrane proteins are localized to the inner membrane platform of the T2SS or T4SP although their specific functions remain unknown. Here we report the atomic structure of the cytosolic domain (residues 27–153) of SpoIIIAB protein to 2.3 Å resolution. In good agreement with structure prediction analysis, SpoIIIAB adopts a conserved, secretion-system related fold, comprised of an anti-parallel bundle of six helices. We have investigated potential interaction interfaces by structure-guided mutagenesis *in vivo* and discuss a surprising similarity to the C-subunit protein from the bacterial V-ATPase complex. Our data and structural analysis provides experimentally validated evidence that the sporulation channel is a hybrid secretion-like machinery.

Table 1
Data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

Protein	Native SpoIIIAB 27–153	SeMet derivative SpoIIIAB 27–153
Data collection	Home source	CLS
Space group	$P2_122_1$	$P22_12_1$
Cell dimensions		
a, *b*, *c* (Å)	74.54, 82.00, 84.75	75.01, 81.97, 83.49
α, β, γ (°)	90, 90, 90	90, 90, 90
Resolution (Å)	2.32	2.3
R sym or *R* merge	13.1 (60.2)	7.6 (36.7)
$I/\sigma I$	26.95 (2.81)	25.19 (4.26)
Completeness (%)	99.9 (98.1)	95 (1 0 0)
Redundancy	13.1	4.8
Wavelength (Å)	1.541	0.978
Refinement		
Resolution (Å)	2.32	2.3
No. reflections	23,068	22,403
Current Rwork/*R*free	20.7/25.8	
No. atoms		
Protein	4020	
Ligand/ion	40	
Water	114	
B-factors (Å^2)		
Protein	68.7	
Ligand/ion	46.1	
Water	42.9	
R.m.s. deviations		
Bond lengths (Å)	0.01	
Bond angles (°)	1.89	

2. Results

2.1. SpoIIIAB structure

SpoIIIAB is predicted to be a bitopic membrane protein with transmembrane helices at the N- and the C-termini and a soluble domain in between them (residues 27–153). To obtain structural and biochemical information for the soluble domain, recombinant $SpoIIIAB_{27\text{-}153}$ was over-expressed in BL21 *Escherichia coli* cells and found to be soluble and stable in solution. Crystallization trials using the sitting drop vapor diffusion resulted in the appearance of a single crystal form which diffracted to a resolution of 2.32 Å (see Table 1 for data collection and refinement statistics). Perhaps not unexpectedly, given the relatively low partial sequence identity of $SpoIIIAB_{27\text{-}153}$ to the T2SS/T4P GspF-family members of known structure (28.1% to *V. cholerae* EpsF, 22.6% to *V. cholerae* TcpE and 26.4% to *T. thermophilus* PilC), molecular replacement attempts were unsuccessful. To obtain phase information, SeMet labeled protein was prepared and used for single wavelength anomalous diffraction (SAD) phasing. The collected Se-Met derivative phasing datasets displayed similar resolution to the native crystal form and 8 selenium atoms were found in the crystal unit cell (Table 1). Although both Se-Met derivatized and native crystals were isomorphous with respect to the primitive orthorhombic space group including near identical unit cell dimensions, one of the space group screw axis has altered in unit cell direction a to b ($P2_122_1$in the native and $P22_12_1$ in the Se-Met derivative crystal forms) (Table 1). Accordingly, an initial model was built according to the SeMet derivative experimentally phased electron density map and then was used as an input for molecular replacement against the native dataset. After manual rounds of rebuilding and refinement the collected native data yielded a high-quality structure (Table 1) with close to all the protein sequence clearly resolved.

The asymmetric unit of our crystal contains four monomers that are arranged in an anti-parallel manner with the N- and C-termini of each monomer facing away from those of the adjacent monomer (Fig. S1A). Two distinct crystallographic packing interfaces are formed within the asymmetric unit; the first is the symmetrical interface between chain A/

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