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A LytM Domain Dictates the Localization of Proteins to the Mother Cell-Forespore Interface during Bacterial Endospore Formation

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A large number of proteins are known to reside at specific subcellular locations in bacterial cells. However, the molecular mechanisms by which many of these proteins are anchored at these locations remains unclear. During endospore formation in Bacillus subtilis, several integral membrane proteins are located specifically at the interface of the two adjacent cells of the developing sporangium, the mother cell and forespore. The mother cell membrane protein SpoIIIAH recognizes the cell-cell interface through an interaction with the forespore membrane protein SpoIIQ, and then the other proteins are positioned there by the SpoIIIAH-SpoIIQ complex. In this study, we investigated the molecular mechanisms underlying the formation of the SpoIIIAH-SpoIIQ complex. Using gel filtration chromatography and isothermal titration calorimetry, we measured the binding parameters that characterize the SpoIIIAH-SpoIIQ interaction in vitro. We also demonstrated that the interaction of SpoIIIAH and SpoIIQ is governed by their YscJ and degenerate LytM domains, respectively. Therefore, the LytM domain of SpoIIQ provides the positional cue that dictates the localization of mother cell membrane proteins to the mother cell-forespore interface.

The assembly of bacterial proteins at specific subcellular locations is important for numerous behaviors, including motility and chemotaxis, cell division, cell differentiation, multicellularity, and cell-cell interactions (reviewed in reference 25). The elucidation of the mechanisms that determine the subcellular localization of proteins is a fundamental issue in biology. Generally, a so-called founder protein recognizes a subcellular location, and then other proteins are anchored there through interactions (either direct or indirect) with the founder protein (reviewed in reference 23). One of the principle challenges is to understand what positional cues dictate the localization of founder proteins.

SpoIIIAH is an example of a founder protein that localizes to a cell-cell interface by interacting with a protein in the adjacent cell during endospore formation in the Gram-positive bacterium Bacillus subtilis. During sporulation, cells divide asymmetrically, giving rise to two adjacent daughter cells of dissimilar size, called the mother cell and forespore. As the peptidoglycan within the division septum is hydrolyzed, the mother cell septal membrane is wrapped around the forespore in a process called forespore engulfment. Once this process is completed, the forespore is released into the mother cytoplasm, where it matures into a dormant and resistant spore. The mother cell produces several membrane proteins that localize to the mother cell-forespore interface and control the transition from forespore engulfment to maturation. The mother cell membrane protein SpoIIIAH recognizes this location through the interaction of its extracellular domain with that of the forespore membrane protein SpoIIQ (5, 10). Although SpoIIIAH is inserted randomly into the mother cell membrane, it diffuses laterally and is captured at the mother cell-forespore interface by SpoIIQ (Fig. 1A). The SpoIIIAH-SpoIIQ complex then directly or indirectly affects the subcellular localization of three mother cell protein subcomplexes. One of the complexes (SpoIIIAB, SpoIIIAC, SpoIIIAD, SpoIIIAE, SpoIIIAF, or SpoIIIAI) is required for the activation of late forespore gene expression, whereas a second complex controls the activation of late mother cell gene expression (SpoIVFA, SpoIVFB, and BofA) (Fig. 1B) (10, 11, 24). SpoIIIAH-SpoIIQ and SpoIVFA-SpoIVFB provide a secondary pathway for affecting the septal localization of a third complex (SpoIIM, SpoIIP, and SpoIID), which mediates septal peptidoglycan hydrolysis (3). Since the SpoIIIAH-SpoIIQ complex defines the mother cell-forespore interface and controls forespore development, we sought to understand the molecular basis of the SpoIIIAH-SpoIIQ interaction.

SpoIIIAH is an integral membrane protein containing an N-terminal transmembrane segment and a C-terminal extracellular domain. The extracellular domain (residues 25 to 218) shares similarity with YscJ-FliF family proteins (PF01514) that form the inner membrane structures of the protein export apparatuses of type III secretion systems and flagella in Gram-negative bacteria (7, 15, 19, 29). The YscJ domain contains a three-stranded anti-parallel β-sheet (31). Since this fold is conserved in the other core component of the inner membrane structure (YscD), it was proposed to be a structural motif for the assembly of symmetric ring structures (27). In addition to a C-terminal YscI domain (residues 132 to 218), the extracellular domain has an N-terminal region (residues 25 to 95) lacking any predicted secondary structure followed by a predicted α-helix (residues 96 to 99) and a β-helix (residues 102 to 128) (Fig. 1C).

SpoIIQ is also an integral membrane protein containing an N-terminal transmembrane segment and a C-terminal extra-
cellular domain. The extracellular domain (residues 43 to 283) consists of a predicted LytM domain (residues 89 to 220) with a degenerate active site and extensions at both the N and C termini (residues 43 to 88 and 221 to 283, respectively) (Fig. 1D) (7, 17, 19, 28). Bacterial LytM proteins typically are metalloendopeptidases (peptidase M23 family) that cleave the peptide cross-bridges of the peptidoglycan cell wall (12). LytM domains consist of a core six-stranded β-sheet with four loops that form two walls that line either side of a central groove extending the length of the domain (13, 22). LytM active site is organized around a divalent metal cation (Zn$^{2+}$). The three Zn$^{2+}$ ligands, two histidines, and an aspartate (H210, D214, and H293 by Staphylococcus aureus LytM numbering) occur in two short sequence motifs, HXXXD and HXH (where X is any amino acid). These residues allow Zn$^{2+}$ to polarize the carbonyl carbon of the substrate for susceptibility to nucleophilic attack. A hydroxyl nucleophile generated by the His247-mediated deprotonation of water was proposed to facilitate hydrolysis, because His247 is close to the carbonyl group and in the same plane, while His291 is farther from the carbonyl group and off axis compared to His247 (S. aureus LytM numbering) (9). SpoIIQ contains only two of the three Zn$^{2+}$ ligands (D123 and H204 by B. subtilis SpoIIQ numbering) and lacks the proposed catalytic His (Fig. 1D; also see Fig. S1 in the supplemental material). Therefore, SpoIIQ contains a degenerate and probably catalytically inactive LytM domain.

Recently, an alternative function for degenerate LytM proteins was proposed. Escherichia coli produces two degenerate LytM proteins (EnvC and NlpD) that regulate peptidoglycan hydrolases (amidases AmiA, AmiB, and AmiC) (30). Although the LytM-amidase regulatory mechanism is unknown, a protein-protein interaction could either anchor the amidases at the cell division site or allosterically activate the amidase. A well-characterized example of a LytM domain that engages in a protein-protein interaction is the lysostaphin-type enzyme Staphylococcus aureus LytM. Lysostaphin-type enzymes are produced in a latent form that is held inactive by their N-terminal segments. In the X-ray crystal structure of latent S. aureus LytM, the Zn$^{2+}$ is tetrahedrally coordinated by the three residues mentioned above and an asparagine (N117) in the inhibitory segment, rendering the metal center catalytically inactive (22). The protein structure also shows that the inhibitory segment binds within the central LytM groove. Thus, degenerate LytM proteins may use their central grooves for...
protein-protein interactions similarly to latent lysostaphin-type enzymes.

In this study, we used purified proteins to test the hypothesis that the extracellular domains of SpoIIIAH and SpoIQ interact and form a complex through the interaction of their YscJ and LytM domains, respectively.

**MATERIALS AND METHODS**

**Protein expression and purification.** Various regions from spoIIIAH and spoIQ were amplified by PCR from B. subtilis (strain JH642) chromosomal DNA and ligated into pET14b (Novagen) at NdeI/BamHI or NdeI/Xhol restriction sites. The sequences of the oligonucleotides used to amplify spoIIIAH and spoIQ are listed in Table S1 in the supplemental material. Each plasmid contained the coding sequence for part of SpoIIIAH or SpoIQ fused at its N-terminal end to a hexahistidine (His) tag and thrombin cleavage site. SpoIQ and SpoIIIAH expression was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) in Escherichia coli BL21 Star (DE3) (Invitrogen) cultures grown at 37°C in LB medium supplemented with ampicillin (100 μg/ml). Cells were disrupted by passage through a French press (15,000 lb/ir), and the resulting lysates were centrifuged at 10,000 × g for 10 min. Histagged proteins were purified on a 1-ml HisTrap FF column (GE Healthcare) using an AKTA explorer 10 (GE Healthcare). His-tagged proteins were dialyzed at 4°C in 50 mM Tris, 100 mM NaCl, 2 mM β-mercaptoethanol (β-ME), pH 7.5. Proteins were then incubated with 100 μU of thrombin protease (GE Healthcare) at room temperature for 2 h to remove the His tags. Untagged proteins were purified on 1 ml HiTrap benzamidine FF and 1 ml HisTrap columns (GE Healthcare); followed by a HiLoad 16/60 Superdex 75 prep-grade gel filtration column (GE Healthcare). Protein concentrations were measured using a Bradford assay (Bio-Rad). The identity of each purified protein was confirmed by a tryptic peptide fingerprint generated by matrix-assisted laser desorption ionization mass spectrometry (Targeted Metabolomics and Proteomics Laboratory, University of Alabama, Birmingham).

**Gel filtration chromatography.** Proteins were concentrated to 100 to 200 μM using Amicon Ultra centrifugal filters (Millipore). A volume of 200 μl of each test protein was mixed or diluted into 200 μl of 50 mM Tris, 100 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5, and incubated on ice for 1 h. Proteins were then loaded into a 100-μl sample loop and injected onto a Superdex 200 10/300 GL high-resolution gel filtration column (GE Healthcare) in 50 mM Tris, 100 mM NaCl, 2 mM β-mercaptoethanol, pH 7.5. The elution of the proteins was monitored by UV absorbance at 280 and 230 nm. Fractions (500 μl) were collected and analyzed by SDS-PAGE and Coomassie blue staining.

**ITC, Isothermal titration calorimetry (ITC) experiments were performed with a VT-ITC instrument, and the data were processed with Origin 7.0 software (MicroCal). Both the SpoIIQ and SpoIIAHL proteins were extensively dialyzed together in the same buffer to minimize thermal effects from differences in solutions components. To minimize artifactual heats of buffer ionization, the experiments were performed in sodium phosphate buffer (at pH 7.5 with 100 mM NaCl), which has a low heat of ionization (ΔHion = −0.9 kcal/mol at 25°C). Proteins were placed into the sample cell at a concentration of 15 μM, while proteins were loaded into the syringe at 150 μM. Each experiment consisted of an initial test injection of 5 μl followed by 28 injections of 10 μl into the sample cell at 25°C (29 total injections). Each injection was made during a 10-s interval and spaced 180 s apart to allow baseline re-equilibration. Throughout the titrations, the sample cell was stirred at 300 rpm. The heat associated with the dilution of the injected protein was measured from the baseline at saturation and subtracted during data processing to correct the binding enthalpy for the heat of dilution.

**Peptidoglycan cosedimentation assay.** B. subtilis was grown in 100 ml LB medium at 37°C to mid-exponential phase (optical density at 600 nm [OD600] = 0.5), and vegetative peptidoglycan was purified according to the protocol provided by David Popham. The culture was chilled in an ice bath for 5 min and centrifuged, and the cell pellet was resuspended in 2 ml cold water. The suspension was dripped into 50 ml boiling, stirring 4% SDS during a period of 1 to 2 min. Boiling was continued for another 30 min, and water was added to maintain a volume of 50 ml. The solution was cooled to room temperature and centrifuged 7,000 × g for 6 min at room temperature. It was washed four times with 20 ml water at 60°C. The pellet was resuspended in 1 ml 100 mM Tris, pH 7.4, 10 μg DNase, 50 μg RNase, 20 mM MgSO4, and incubated at 37°C for 2 h. Trypsin (100 μl at 1 mg/ml) in 100 mM CaCl2 was added, and the solution was incubated at 37°C overnight. The peptidoglycan was washed three times with 1 ml water and resuspended in 1 ml water. Twenty-five μl peptidoglycan and 15 μl of 100 mM Tris, 100 mM NaCl, 2 mM β-ME, pH 7.5, was added to 10 μl of 100 μM SpoIIQ43-283, lysosome, or bovine serum albumin (BSA) (in 100 mM Tris, 100 mM NaCl, 2 mM β-ME, pH 7.5). The reaction mixtures were incubated on ice for 30 min and then centrifuged for 1 min. The supernatant was collected and the pellet was resuspended in 50 μl of 100 mM Tris, 100 mM NaCl, 2 mM β-ME, pH 7.5. Each fraction was analyzed by SDS-PAGE and Coomassie blue staining.

**RESULTS**

**SpoIIAH and SpoIQ interact directly in vitro.** To begin to understand the molecular basis of the SpoIIAH-SpoIQ interaction, we expressed and purified the extracellular domains of SpoIIAH and SpoIQ, which include the amino acid from position 25 through the amino acid at position 218 of SpoIIAH (SpoIIAH25-218) and the amino acid at position 43 through position 283 of SpoIQ (SpoIQ43-283), respectively, as described in Materials and Methods, and analyzed their interaction by gel filtration chromatography (Fig. 2A and B). The elution of the proteins from the column was monitored by UV absorbance and subsequently confirmed by SDS-PAGE. Apparent molecular masses of the proteins were determined by comparing their elution volumes to those obtained for several standard proteins. SpoIIAH25-218 eluted with an apparent molecular mass of 60 kDa. Whereas this protein has a predicted molecular mass of 21 kDa, its apparent molecular mass by SDS-PAGE is 32 kDa. Similarly, SpoIQ43-283 eluted with an apparent molecular mass of 67 kDa, while its predicted molecular and apparent molecular masses by SDS-PAGE are 26 and 35 kDa, respectively. Thus, both SpoIIAH25-218 and SpoIQ43-283 behaved as dimers in gel filtration chromatography. To determine the molecular mass of the protein-protein complex, SpoIIAH25-218 and SpoIQ43-283 were mixed and analyzed by gel filtration chromatography. The SpoIIAH25-218-SpoIQ43-283 complex eluted with an apparent molecular mass of 150 kDa, slightly larger than the sum of the molecular sizes observed for the individual proteins. Therefore, SpoIIAH25-218 and SpoIQ43-283 formed a heterotetrameric complex, a dimer of dimers, in gel filtration chromatography.

To confirm the stoichiometry of the SpoIIAH25-218-SpoIQ43-283 complex observed by gel filtration chromatography, we analyzed the complex by isothermal titration calorimetry (Fig. 2C). This experiment allowed the direct measurements of the protein complex in solution. The observed stoichiometry (n) was approximately 1:0. In addition to stoichiometry, we also measured an apparent binding constant (Kb) and enthalpy of about 5 × 106 M−1 (Kb [dissociation constant] = 2 × 10−7 M) and −38 kcal/mol, respectively. To gain insight into the mechanism of SpoIIAH-SpoIQ complex formation, we calculated the Gibbs free energy change (ΔG = −RT ln Kb = −9 kcal/mol) and entropy (ΔS = ΔG − ΔH) of the protein-protein interaction, where R is the gas constant S is entropy, and T is temperature in degrees kelvin. The favorable enthalpy and unfavorable entropy indicate that the complex appears to be driven by polar interactions and may involve conformation changes in one or both proteins.

**The YscJ domain of SpoIIAH interacts with SpoIQ.** The combination of gel filtration chromatography and isothermal titration calorimetry was used to assess which portions of the SpoIIAH extracellular domain were necessary and sufficient for the interaction with SpoIQ. First, we tested whether the
The N-terminal region, which lacks predicted secondary structure, was necessary for the interaction. To do so, we purified SpoIIAH90-218 (the numbers indicate the positions of amino acids included in the protein) and analyzed its interaction with SpoIIQ43-283 by gel filtration chromatography (Fig. 3A). SpoIIIAH90-218 interacted with SpoIIQ43-283. The analysis of these proteins by isothermal titration calorimetry indicated that the stoichiometry and binding affinity were indistinguishable from those observed with the full-length SpoIIIAH25-218 (Table 1). Therefore, the unstructured N-terminal region of SpoIIIAH (residues 25 to 89) was dispensable for the interaction with SpoIIQ.

We next tested whether the YscJ domain was sufficient for the interaction with SpoIIQ. We purified SpoIIIAH132-218 and analyzed its interaction with SpoIIQ43-283 by gel filtration chromatography (Fig. 3B). SpoIIIAH132-218 interacted with SpoIIQ43-283, indicating that the YscJ domain was sufficient for the interaction. The isothermal titration calorimetry data, however, showed a 4-fold reduction in the binding affinity of SpoIIIAH132-218 compared to that of SpoIIIAH25-218 (Table 1). Therefore, the unstructured N-terminal region of SpoIIIAH (residues 25 to 89) was dispensable for the interaction with SpoIIQ.

We next tested whether the YscJ domain was sufficient for the interaction with SpoIIQ. We purified SpoIIIAH132-218 and analyzed its interaction with SpoIIQ43-283 by gel filtration chromatography (Fig. 3B). SpoIIIAH132-218 interacted with SpoIIQ43-283, indicating that the YscJ domain was sufficient for the interaction. The isothermal titration calorimetry data, however, showed a 4-fold reduction in the binding affinity of SpoIIIAH132-218 compared to that of SpoIIIAH25-218 (Table 1). Therefore, while the YscJ domain is sufficient for the interaction with SpoIIQ, the adjacent N-terminal region (residues 90-131) also contributes to the interaction, although this contribution could be indirect.

The LytM domain of SpoIIQ is sufficient for the interaction with SpoIIIAH. In parallel with the experiments described above, we determined which regions of the SpoIIQ extracellular domain were necessary and sufficient for SpoIIIAH. To test whether the LytM domain was sufficient for the formation of the SpoIIAH-SpoIIQ complex, we purified a truncated...
SpoIIQ (SpoIIQ73-220) that lacked the N- and C-terminal extensions and analyzed its interaction with SpoIIIAH25-218 (Fig. 4A and B). We chose Gly73 at the N terminus of this truncation, because it is the site of SpoIVB-dependent proteolysis in vivo (8), which suggests that this region is not stably folded into the predicted LytM domain. SpoIIQ73-220 interacted with SpoIIIAH25-218 in gel filtration chromatography, and the binding affinity measured by isothermal titration calorimetry was reduced only 2.75-fold compared to that of full-length SpoIIQ43-283 (Table 2). Thus, the LytM domain was sufficient, and both extensions were dispensable for the interaction with SpoIIIAH.

To test whether the complete LytM domain was necessary for the formation of the complex, we analyzed the interaction

TABLE 1. Thermodynamic parameters determined by ITC for the interaction of truncated SpoIIIAH proteins (syringe) and SpoIIQ43-283 (sample cell)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>n</th>
<th>$K_d$ (M)</th>
<th>$\Delta G^\circ$ (kcal mol$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$-T\Delta S$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpoIIAH25-218</td>
<td>0.934</td>
<td>$1.96 \times 10^{-7}$</td>
<td>$-9.15$</td>
<td>$-40.4$</td>
<td>31.2</td>
</tr>
<tr>
<td>SpoIIAH90-218</td>
<td>1.06</td>
<td>$1.93 \times 10^{-7}$</td>
<td>$-9.16$</td>
<td>$-35.7$</td>
<td>26.6</td>
</tr>
<tr>
<td>SpoIIAH132-218</td>
<td>0.966</td>
<td>$7.81 \times 10^{-7}$</td>
<td>$-8.33$</td>
<td>$-20.8$</td>
<td>12.5</td>
</tr>
</tbody>
</table>

$^a$ Values are for 25 mM sodium phosphate buffer, 100 mM NaCl, pH 7.5, at 25°C.
$^b$ Calculated from $\Delta G = RT \ln K_d = \Delta H - T\Delta S$, where $R$ is the gas constant (1.987 kcal mol$^{-1}$) and $T$ is temperature in degrees kelvin.

FIG. 4. (A) Cartoon representation of the predicted tertiary structure of the SpoIIQ extracellular domain, emphasizing the organization of the core $\beta$-sheet of the LytM domain (filled blue objects). $\alpha$-Helices and $\beta$-strands are shown as cylinders and arrows, respectively. The predicted $\alpha$-helical insertion is shown in empty blue, and N- and C-terminal extensions are in empty gray. Residues at the N and C termini of truncated proteins are indicated in black. Filled circles show the positions of conserved active site residues, while empty circles show nonconserved (degenerate) residues. (B to D) Gel filtration chromatography of the interaction of SpoIIIAH25-218 and truncated versions of SpoIIQ proteins SpoIIQ73-220 (B), SpoIIQ114-220 (C), and SpoIIQ73-208 (D). Shown are Coomassie blue-stained SDS-PAGE analyses of the elution fractions from the gel filtration chromatography of the mixtures of SpoIIIAH25-218 and truncated SpoIIQ proteins (top) and truncated SpoIIQ proteins alone (bottom). (E) Peptidoglycan cosedimentation with SpoIIQ43-283, lysozyme, or bovine serum albumin (BSA). Peptidoglycan was added to each protein, incubated, and centrifuged as described in Materials and Methods. Coomassie blue-stained SDS-PAGE of the sedimentation fractions is shown.
of two truncated proteins, SpoIIQ114-220 and SpoIIQ73-208, lacking either N- or C-terminal portions of the LytM domain, respectively (Fig. 4A, C, and D). Both proteins were abundantly expressed, soluble, and eluted as discrete peaks in gel filtration chromatography, suggesting that they were stably folded. Neither SpoIIQ114-220 nor SpoIIQ73-208 interacted with SpoIIIAH25-218 by gel filtration chromatography, indicating that both loops are necessary for the complex to form. Consistently with these data, we did not detect the binding of either protein to SpoIIIAH25-218 by isothermal titration chromatography (Table 2). Thus, the complete LytM domain is necessary for the interaction with SpoIIIAH. Taken together with data from the SpoIIIAH truncations, these results suggest that the YscJ and LytM domains mediate the formation of the SpoIIIAH-SpoIIQ complex.

Typically, LytM proteins bind and hydrolyze peptidoglycan. We tested whether SpoIIQ was able to bind peptidoglycan. To this end, we used a simple sedimentation assay to test whether the SpoIIQ extracellular domain (SpoIIQ43-283) would bind peptidoglycan purified from vegetative *B. subtilis* (Fig. 4E). After coincubation and centrifugation, proteins that bind peptidoglycan should be found in the pellet, while those that do not bind should remain in the supernatant. SpoIIQ43-283 was observed in the supernatant. To confirm that binding can be detected under these conditions, we tested two control proteins. The positive-control protein lysozyme (a peptidoglycan hydrolase) was found in the pellet, while the negative-control protein bovine serum albumin (BSA) was found in the supernatant. Therefore, SpoIIQ does not bind to vegetative *B. subtilis* peptidoglycan.

The extracellular domain of SpoIIIAG does not interact with the SpoIIIAH-SpoIIQ complex. SpoIIIAG is a component of one of the protein subcomplexes that localizes to the mother cell-forespore interface (11). It is an integral membrane protein with an N-terminal transmembrane segment and a predicted C-terminal extracellular domain. Using the HHpred server (26), we and others noticed that this extracellular domain also shares weak similarity with the YscJ-FliF family proteins (Fig. 5A; also see Fig. S2 in the supplemental material) (11, 19). Since the localization of a CFP-SpoIIIAG fusion protein to the mother cell-forespore interface is dependent on SpoIIQ (11), it was proposed that SpoIIIAG interacts with SpoIIQ, as SpoIIIAH does. This would be consistent with the YscJ-LytM module in the SpoIIIAH-SpoIIQ complex. To test whether SpoIIIAG interacts with SpoIIQ, we purified the extracellular domain of SpoIIIAG (SpoIIIAG51-229) and analyzed its interaction with SpoIIQ43-283 by gel filtration chromatography (Fig. 5B). SpoIIIAG51-229 did not interact with SpoIIQ43-283. Since CFP-SpoIIIAG localization also is dependent on SpoIIIAH (11), we tested whether SpoIIIAG51-229 interacted with SpoIIIAH25-218 (Fig. 5C). Once again, we were unable to detect an interaction by gel filtration chromatography. We also were unable to detect an interaction be-

<table>
<thead>
<tr>
<th>Ligand</th>
<th>$n$</th>
<th>$K_d$ (M)</th>
<th>$\Delta G^o$ (kcal mol$^{-1}$)</th>
<th>$\Delta H$ (kcal mol$^{-1}$)</th>
<th>$-T\Delta S$ (kcal mol$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SpoIIQ43-283</td>
<td>0.909</td>
<td>2.08 x 10$^{-7}$</td>
<td>-9.12</td>
<td>-36.3</td>
<td>27.2</td>
</tr>
<tr>
<td>SpoIIQ73-220</td>
<td>1.13</td>
<td>5.78 x 10$^{-7}$</td>
<td>-8.51</td>
<td>-44.8</td>
<td>36.3</td>
</tr>
<tr>
<td>SpoIIQ114-220</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>SpoIIQ73-208</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
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</table>

a Values are for 25 mM sodium phosphate buffer, 100 mM NaCl, pH 7.5, at 25°C.

b Calculated from $\Delta G = RT \ln K_d = \Delta H - T\Delta S$, where $R$ is the gas constant (1.987 kcal mol$^{-1}$ mol$^{-1}$) and $T$ is temperature in degrees kelvin. ND indicates that binding was not detected and values were not determined.

![FIG. 5. (A) Cartoon representation of the predicted SpoIIIAG secondary structure. α-Helices and β-strands are shown as cylinders and arrows, respectively. The predicted YscJ domain is shown in filled light-green objects with the predicted four β-strand insertions in empty light green. Residues at the N and C termini of the protein are indicated in black. Gel filtration chromatography of the interaction of SpoIIIAG51-229 and SpoIIQ43-283 (B), SpoIIIAH25-218 (C), and the SpoIIQ43-283-SpoIIIAH25-218 complex (D). Shown are Coomassie blue-stained SDS-PAGE analyses of the elution fractions from the gel filtration chromatography.](image)
between SpoIIIAG51-229 and the SpoIIIAH25-218-SpoIIQ43-283 complex (Fig. 5D).

**DISCUSSION**

In this study, we demonstrated that the purified, unmodified SpoIIIAG and SpoIIQ extracellular domains directly interact in vitro. We measured an apparent dissociation constant of $2 \times 10^{-7}$ M. By comparison, the dissociation constants measured for homomeric eukaryotic cell adhesion proteins such as junction adhesion molecules (JAMs) and cadherins were approximately $10^{-4}$ to $10^{-5}$ M (2, 4, 16). The dissociation constant measured for heterotypic cell adhesion proteins such as integrin-laminin and P-selectin-PSGL1 were $10^{-2}$ to $10^{-3}$ M (14, 18). Thus, the binding affinity of the SpoIIIAG-SpoIIQ complex is within the range reported for other cell-cell junctions. Furthermore, we showed that the C-terminal YscJ domain was sufficient for the interaction with SpoIIQ. We also showed that the LytM domain is necessary and sufficient for interaction with SpoIIIAG in vitro. Thus, the LytM domain likely provides the positional cue that, when recognized by the YscJ domain, dictates the localization of several important mother cell proteins to the mother cell-forespore interface. As noted by others (6, 7), the LytM domain of SpoIIQ and the YscJ domain of SpoIIIAG are conserved among species of the genus Bacillus.

A direct interaction between SpoIIIAG and SpoIIQ was proposed based on several prior experiments using tagged proteins from cell lysates. GFP-SpoIIQ coimmunoprecipitated from a detergent-solubilized lysate of sporulating B. subtilis cells with FLAG-tagged SpoIIIAG (5). The transmembrane segments of both proteins were shown to be dispensable for the interaction when a nontagged SpoIIQ extracellular domain was pulled down from an E. coli lysate with a glutathione S-transferase-tagged SpoIIIAG extracellular domain (10).

Consistently with our results indicating that the LytM domain was necessary for the interaction with SpoIIIAG in vitro, Camp and Losick (6) showed that a truncation of the C-terminal 100 residues of SpoIIQ, leaving residues 1 to 183, blocked the localization of GFP-SpoIIIAG to the cell-cell interface. This truncation is expected to disrupt the SpoIIQ LytM domain (residues 89 to 220). They also showed that an internal LytM deletion (residues 202 to 216), expected to disrupt the LytM domain, prevented GFP-SpoIIIAG localization to the interface. In contrast, a truncation of the C-terminal 50 residues, which removes a portion of the C-terminal extension and preserves the LytM domain, produced a functional protein (SpoIIQ1-233) that anchored SpoIIIAG at the interface.

While LytM proteins are known to bind and hydrolyze peptidoglycan, they also may possess the capacity for protein-protein interactions. Although the precise molecular details of the SpoIIIAG-SpoIIQ interaction remain unclear, it is tempting to speculate that YscJ recognition occurs in the LytM central groove. In the structure of latent S. aureus lysostaphin, its inhibitory N-terminal segment binds within the LytM groove, making contacts with the loops to form the walls that line it. If SpoIIIAG binds to SpoIIQ in a similar manner, then those loops would be necessary for the interaction. In support of this model, our truncations, which are predicted to disrupt one of these walls, disrupted the interaction of SpoIIQ with SpoIIIAG. SpoIIQ also has an insertion of a predicted $\alpha$-helix (residues 100 to 108) in the first loop of its LytM domain that may be involved in the recognition of the YscJ domain. This protein-binding mode may be common among degenerate LytM proteins. Uehara et al. (30) recently showed that the amidases that mediate E. coli daughter cell separation are controlled by degenerate LytM proteins. Although the mechanism is unclear, it was proposed to involve a protein-protein interaction. It will be interesting to find out whether the protein-binding mode of latent lysostaphin is adapted for partner protein recognition in degenerate LytM proteins.

Peptidoglycan binding is another possible function of the LytM domain of SpoIIQ. The mother cell-forespore interface is a site where peptidoglycan remodeling occurs (1, 20, 21). Forespore engulfment is initiated by septal PG hydrolysis, and the migration of the mother cell membrane around the forespore is thought to be promoted by new peptidoglycan synthesis at the cell-cell interface. While the SpoIIQ extracellular domain did not bind to vegetative peptidoglycan in our cosedimentation assay, it is possible that it binds to a modified peptidoglycan (or muropeptides) found only at the cell-cell interface.

This study begins to elucidate how the mother cell founder protein SpoIIIAG recognizes the cell-cell interface. We currently know very little, however, about the mechanisms by which the SpoIIIAG-SpoIIQ complex affects the location of the three mother cell protein subcomplexes (SpoIIIAB-SpoIIIAC-SpoIIIAD-SpoIIIAE-SpoIIIAF-SpoIIIAG, SpoIVFA-SpoIVFA-BolA, and SpoIM-SpoIIP-SpoIID). Although SpoIIIAG and SpoIIQ are necessary for the localization of CFP-SpoIIIAG to the mother cell-forespore interface (11), we were unable to detect an interaction of the extracellular domain with SpoIIIAG, SpoIIQ, or the SpoIIIAG-SpoIIQ complex in vitro. Doan et al. (11) reported that neither SpoIIIAG nor SpoIIQ coimmunoprecipitated with SpoIIIAG from a detergent-solubilized lysate of sporulating B. subtilis cells. However, SpoIIIAG coimmunoprecipitated with FLAG-tagged SpoIIIAG from a lysate of sporulating B. subtilis cells prepared by mechanical disruption in the absence of a detergent. One possible explanation for these results is that the transmembrane segments of SpoIIIAG and SpoIIQ interact (directly or indirectly) in the plane of the mother cell membrane. This idea is consistent with the apparent detergent sensitivity of the interaction (direct or indirect) between SpoIIIAG and the SpoIIQ-SpoIIIAG complex. Another possibility is that SpoIIIAG interacts with another protein at the cell-cell interface. The putative SpoIIIAG YscJ domain has a 55-amino-acid segment, with four predicted $\beta$-strands inserted between the first and second $\beta$-strands of the core three-stranded $\beta$-sheet (Fig. 5A). Interestingly, this loop is unstructured and likely surface exposed in the Escl structure (31). Therefore, this flexible, surface-exposed loop may specify a protein-protein interaction by SpoIIIAG.

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