Phage Shock Protein C (PspC) of *Yersinia enterocolitica* Is a Polytopic Membrane Protein with Implications for Regulation of the Psp Stress Response

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Phage shock proteins B (PspB) and C (PspC) are integral cytoplasmic membrane proteins involved in inducing the *Yersinia enterocolitica* Psp stress response. A fundamental aspect of these proteins that has not been studied in depth is their membrane topologies. Various *in silico* analyses universally predict that PspB is a bitopic membrane protein with the C terminus inside. However, similar analyses yield conflicting predictions for PspC: a bitopic membrane protein with the C terminus inside, a bitopic membrane protein with the C terminus outside, or a polytopic protein with both termini inside. Previous studies of *Escherichia coli* PspB-LacZ and PspC-PhoA fusion proteins supported bitopic topologies, with the PspB C terminus inside and the PspC C terminus outside. Here we have used a series of independent approaches to determine the membrane topologies of PspB and PspC in *Y. enterocolitica*. Our data support the predicted arrangement of PspB, with its C terminus in the cytoplasm. In contrast, data from multiple independent approaches revealed that both termini of PspC are located in the cytoplasm. Additional experiments suggested that the C terminus of PspC might be the recognition site for the FtsH protease and an interaction interface with PspA, both of which would be compatible with its newly proposed cytoplasmic location. This unexpected arrangement of PspC allows a new model for events underlying activation of the Psp response, which is an excellent fit with observations from various previous studies.

The phage shock protein (Psp) system is a well-conserved extracytoplasmic stress response in Gram-negative bacteria thought to be important during events that can compromise cytoplasmic membrane integrity. It has been most studied in *Yersinia enterocolitica* (reviewed in references 12 and 57) and *Escherichia coli* (reviewed in reference 31). However, homologues of some of its components have been identified in Gram-positive bacteria, archaea, and plant chloroplasts (4, 41, 54, 56). Several important phenotypes have been associated with the Psp system. It is essential for virulence in *Y. enterocolitica* and *Salmonella enterica* serovar Typhimurium (13, 37), important during biofilm formation in *E. coli* (2), highly induced during macrophage infection by *S. enterica* and *Shigella flexneri* (19, 42), and implicated in bacterial persistence (53).

The divergently transcribed *pspF* and *pspABC* genes in *Y. enterocolitica* and *E. coli* encode the core of the Psp system, having both regulatory and physiological roles (12, 31, 57). Since its discovery, activation of the Psp system has been associated with the production of secretin proteins (7). Secretins are ring-like outer membrane pore-forming proteins of various secretion systems, including type 2 and type 3 secretion, type IV pilus biogenesis, and filamentous phage extrusion (22, 40). Secretins promote the specific and potent activation of the Psp system when they mislocalize into the cytoplasmic membrane (e.g., see references 26 and 30). Secretin mislocalization is also toxic to *Y. enterocolitica*, *S. enterica* serovar Typhimurium, and *E. coli* mutants with defective Psp systems (e.g., 50). In *Y. enterocolitica*, it has been shown that this toxicity results from a profound breach in the cytoplasmic membrane permeability barrier of a *psp* null mutant, which can be prevented by PspB and PspC (30).

Induction of *pspA* operon expression is thought to be mediated by interactions between Psp proteins and the relocalization of PspA from the cytoplasm to the cytoplasmic membrane (58).

PspF is a DNA-binding protein that activates the *pspA* promoter (23, 35) but is inhibited by an interaction with PspA in the cytoplasm (17, 18, 58). The integral cytoplasmic membrane proteins PspB and -C play a critical role in inducing the Psp system. They are excellent candidates to sense the inducing signal and transduce it to the cytoplasm, perhaps by interacting with PspA and sequestering it from PspF (34, 58). Consistent with this, *Y. enterocolitica* PspC mutants that are unable to induce *pspA* operon expression in response to secretin induced-stress have been isolated (25). The mutations were predicted to disrupt a leucine zipper-like amphipathic helix in the putative periplasmic domain (C terminus) of PspC, which fitted the proposal that this region might be a stress-responsive sensory domain (1, 25).

Critical to formulating models for the regulatory function of PspB and -C is an understanding of their membrane topologies. Prediction programs agree that PspB is a bitopic membrane protein with its C terminus in the cytoplasm, and β -galactosidase activity of an *E. coli* PspB-LacZ fusion protein supports this (32). However, the situation is less clear for PspC. Jovanovic et al. noticed that different topology prediction programs did not agree on the topology of *E. coli* PspC (34). Some predicted the C terminus to be outside, others inside, and some predicted two transmembrane domains with both termini in the cytoplasm. The topology of PspC has been addressed experimentally in just one study, where the alkaline phosphatase activity of a PspC-PhoA fusion

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protein supported the bitopic arrangement with the C terminus in the periplasm (39). However, it has been suggested that the C terminus of PspC might interact with PspA to activate the Psp system (34). To reconcile this suggestion with the apparent location of the PspC C terminus in the periplasm, Jovanovic et al. proposed that PspC might change its membrane topology in response to an inducing signal (34). All of this uncertainty surrounding the membrane topology of PspC prompted us to investigate it in our model organism *Y. enterocolitica* using a variety of independent approaches. A previously unrecognized polytopic arrangement with both termini in the cytoplasm was revealed for PspC, but no evidence that this topology changes in response to an inducing stimulus was found. This allows us to propose a new working model for the events underlying PspBC-dependent activation of the Psp system.

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth. Bacterial strains and plasmids are listed in Table 1, and primer sequences are listed in Table 2. The DNA sequences of all PCR-generated fragments were checked. Growth medium was Luria-Bertani (LB) broth or LB agar. Antibiotics were used as described previously (45).

Plasmid constructions. Plasmids encoding PspC-PhoA or PspB-PhoA were made by a PCR splicing by overlap extension (SOEing) strategy (29). A downstream fragment encoding PhoA without its initiation codon and signal sequence was amplified from the chromosome of *E. coli* MG1655 with the primers 1752 and 1530, which incorporated an XbaI site downstream of 'phoA'. Upstream fragments encoding *Y. enterocolitica* PspC or PspB without their termination codons were amplified with the primer pairs 345/1751 and 396/1753, respectively, which incorporated a SacI site upstream and a 20-nucleotide (nt) overlap with the upstream end of the 'phoA' fragment downstream. The 'pspB' or 'pspC' and 'phoA' fragments were joined in a PCR SOEing reaction with the primers 345/1530 (PspC-PhoA) and 396/1530 (PspB-PhoA) and cloned as SacI/XbaI fragments into pBAD18-Kan to make pAJD2294 (PspC-PhoA) and pAJD2295 (PspB-PhoA). As negative controls, similar plasmids in which the upstream fragments fused to the 'phoA' fragment terminated immediately after the PspB or PspC start codons and were generated using the primers 1754/1755 for pAJD2329 [PspC(ATG)-PhoA] and 1756/1757 for pAJD2331 [PspB(ATG)-PhoA] were constructed. For the plasmid encoding CpxP-PhoA, the upstream fragment encoding CpxP was amplified from the chromosome of AJD3 with the primers 1535/1772, fused to the 'phoA' fragment in a PCR SOEing reaction, and cloned as a SacI/XbaI fragment into pBAD18-Kan (pAJD2327). Plasmids encoding PspB-green fluorescent protein (GFP) (pAJD2322), PspC-GFP (pAJD2323), and GFP-PspC (pAJD2324) were made by amplifying the respective gene fusions to *gfp*⁺ (48) from the chromosomes of unpublished *Y. enterocolitica* strains in our collection using the primers 345/1789 for the *pspC* fusions and 396/1789 for the *pspB* fusion.

For the PspC-T25 and PspC-T18 fusions, the *pspC* gene was amplified by PCR with the primers 32/34 and cloned into the BamHI site of pKNT25 or pUT18 as a BamHI-BglIII fragment to make pAJD2258 or pAJD2260, respectively. For the PspB-T25 fusion, the *pspB* gene was amplified by PCR with the primers 30/33 and cloned into the BamHI site of pKNT25 as a BamHI-BglIII fragment to make pAJD2298. The PspA-T18 (pAJD1088) and T25-PspC (pAJD2110) plasmids were described previously (24, 58). For plasmids encoding the T25-PspC mutant derivatives, BglIII/BamHI PCR fragments were amplified with the primers 38/M13F(−40) from pAJD1658 (PspC with the L118P substitution [PspC-L118P]), pAJD1659 (PspC-V125D), pAJD1563 (PspC-S127P), pAJD1569 (PspC-F130S), pAJD1527 (PspCΔ123-139), and pAJD1314 (PspC-V33E) and cloned into the unique BamHI site of pAJD1668 (24) to make pAJD1857, pAJD1676, pAJD1677, pAJD1678, pAJD1679, and pAJD1675, respectively.

Plasmids encoding the PspB cysteine substitutions E56C (pAJD1944), I64C (pAJD2033), and E68C (pAJD2035) and the PspC cysteine substitutions S26C (pAJD1926) and G112C (pAJD1990) were described previously (24). Plasmids encoding the PspC cysteine substitutions L103C and R119C were constructed by a PCR SOEing approach as described previously (24). The upstream fragments were generated with the primers 396/1331 (L103C) and 396/1363 (R119C), and the downstream fragments with primers 397/1330 (L103C) and 397/1362 (R119C). The fragments were joined in a PCR SOEing reaction with the primers 396/397 and cloned into pWSK129 to generate pAJD1983 and pAJD1997. The plasmid encoding CpxP-3xFLAG (pAJD2163) was constructed by amplifying a fragment from the chromosome of AJD3 with the primers 1535/1536. Primer 1535 incorporated a SacI site upstream of *cpxP*, and primer 1536 incorporated a 3×FLAG sequence immediately upstream of the *cpxP* stop codon and an XbaI site downstream of the stop codon. The SacI/XbaI fragment was cloned into pBAD33.

Plasmids encoding the PspC C terminus fused to glutathione S-transferase (GST) were made by amplifying the region encoding the C-terminal 55 amino acids of PspC (wild type or with the V125D mutation) from the chromosome of the appropriate *Y. enterocolitica* strain (25) with the primers 42/925. The fragments were digested with BamHI-SalI and cloned into pGEX-6P-1 digested with the same enzymes.

Subcellular fractionation. Cell pellets were resuspended in 5 ml of 50 mM sodium phosphate buffer (pH 7.0) containing complete protease inhibitors (Roche) and separated into soluble and insoluble (including membrane) fractions exactly as described previously (58). For each individual strain, samples of each fraction derived from an equivalent amount of whole cells were analyzed so that the relative amount of the protein in each could be assessed. However, fractions derived from different strains were not normalized to one another. Detection of the cytoplasmic membrane protein FtsH and the cytoplasmic protein DnaK validated the fractionation procedure.

Alkaline phosphatase assays. Saturated cultures were diluted into 5 ml of LB broth in 18-mm-diameter test tubes to an optical density (600 nm) of 0.1. Cultures were grown on a roller drum at 37°C for 2 h. Then, 0.2% (wt/vol) arabinose was added to induce production of the fusion proteins, and growth continued for an additional 2 h at 37°C. One milliliter of each culture was harvested and washed in 1 ml 0.88% (wt/vol) NaCl that contained a 1 mM concentration of the sulfhydryl alkylating agent iodoacetamide to prevent alkaline phosphatase located in the cytoplasm from forming disulfide bonds in cells that are not actively growing (15). Cells were resuspended in 1 ml 1 M Tris-HCl (pH 8.0) containing 1 mM iodoacetamide, permeabilized with chloroform-SDS, and equilibrated at 37°C for 5 min. A 0.1-ml volume of 0.4% (wt/vol) *p*-nitrophenyl phosphate (PNPP) in 1 M Tris-HCl (pH 8.0) was added, and samples were incubated at 37°C until a yellow color was visible, when the assay was terminated by adding 0.1 ml of 1 M KH₂PO₄. After absorbances at 420 nm and 550 nm were determined, alkaline phosphatase activities were calculated in arbitrary Miller units as described previously (6, 47). Individual cultures were assayed in duplicate, and values from three independent cultures were averaged.

Fluorescence measurement. Saturated cultures were diluted into 20 ml of LB broth to an optical density (OD) at 600 nm of 0.1. Cultures were grown for approximately 15 h at 26°C in LB broth containing 0.2% (wt/vol) arabinose to induce production of the fusion proteins. Cells were resuspended in 2 ml of 50 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 15 mM EDTA (21) so that OD at 600 nm was 4 for all samples. Cells were incubated at room temperature for 20 min and transferred to cuvettes, and fluorescence was detected using a Spectra Max M5 microplate/cuvette reader (excitation, 491 nm; emission, 512 nm). Values reported were averaged from three independent cultures.

Adenylate cyclase reconstitution. To test for adenylate cyclase reconstitution, pairs of plasmids encoding T18/T25 fusion proteins were introduced simultaneously into *E. coli* BTH101 by calcium chloride transformation. Transformants were streaked onto MacConkey-Maltose plates

TABLE 1 Strains and plasmids

Name	Genotype/features	Reference/source
<i>E. coli</i> strains		
MG1655	F ⁻ <i>rph-1</i>	27
BL21	F ⁻ <i>ompT hsdS</i> (<i>r_B</i> ⁻ <i>m_B</i> ⁻) <i>gal dcm</i>	52
BTH101	<i>cya-99 araD139 galE15 galK16 rpsL</i> (Sm ^r) <i>hsdR2 mcrA1 mcrB1</i>	D. Ladant ^a
<i>Y. enterocolitica</i> strains		
AJD3 ^b	Δ yenR (R ⁻ M ⁺)	38
AJD1204	Δ yenR (R ⁻ M ⁺) <i>araGFB::</i> [ϕ (<i>pspAp-lacZY</i>)] Δ pspBC	46
AJD3490	Δ yenR (R ⁻ M ⁺):: <i>[pspF⁺] ΔaraGFB::</i> [ϕ (<i>pspAp-lacZY</i>)] Δ pspF Δ pspAp:: <i>[lacI^q-tacp]</i>	58
Plasmids		
pBAD18-Kan	Km ^r , Col E1 <i>ori</i> , <i>araBp</i> expression vector	28
pBAD33	Cm ^r , p15A <i>ori</i> , <i>araBp</i> expression vector	28
pWSK129	Km ^r , pSC101 <i>ori</i> , <i>lacZp</i> expression vector	55
pVLT35	Sm ^r Sp ^r , RSF1010 <i>ori</i> , <i>tacp</i> expression vector	14
pKT25	Km ^r , p15A <i>ori</i> , vector for fusion to C terminus of Cya-T25	36
pKNT25	Km ^r , p15A <i>ori</i> , vector for fusion to N terminus of Cya-T25	D. Ladant
pUT18	Ap ^r , Col E1 <i>ori</i> , vector for fusion to N terminus of Cya-T18	36
pGEX-6P-1	Ap ^r , Col E1 <i>ori</i> , GST fusion vector	GE Healthcare
pREP4-groESL	Km ^r , p15A <i>ori</i> , <i>groES groEL</i> overexpression plasmid	10
pAJD1668	pKT25 derivative containing <i>cyaT25</i> and <i>pspB</i>	24
pAJD126	<i>tacp-yscC</i> in pVLT35	13
pAJD1134	<i>lacZp-pspB⁺</i> in pWSK129	25
pAJD1135	<i>lacZp-pspC⁺</i> in pWSK129	25
pAJD1136	<i>lacZp-pspBC</i> in pWSK129	25
pAJD1088	pUT18 derivative containing <i>pspA-cyaT18</i>	58
pAJD1349	pAJD1136 derivative containing <i>pspB⁺ pspC-L69P</i>	25
pAJD1468	pAJD1135 derivative containing <i>pspC-V33E</i>	25
pAJD1471	pAJD1135 derivative containing <i>pspC-L69P</i>	25
pAJD1507	pGEX-6P-1 derivative encoding GST-PspC ^{CT}	This study
pAJD1569	pAJD1136 derivative containing <i>pspB⁺ pspC-F130S</i>	25
pAJD1658	pAJD1136 derivative containing <i>pspB⁺ pspC-L118P</i>	25
pAJD1659	pAJD1136 derivative containing <i>pspB⁺ pspC-V125D</i>	25
pAJD1665	pAJD1136 derivative containing <i>pspB⁺ pspC-S127P</i>	25
pAJD1675	pAJD1668 derivative containing <i>cyaT25-pspC-V33E</i>	This study
pAJD1676	pAJD1668 derivative containing <i>cyaT25-pspC-V125D</i>	This study
pAJD1677	pAJD1668 derivative containing <i>cyaT25-pspC-S127P</i>	This study
pAJD1678	pAJD1668 derivative containing <i>cyaT25-pspC-F130S</i>	This study
pAJD1679	pAJD1668 derivative containing <i>cyaT25-pspC(Δ123–139)</i>	This study
pAJD1845	pAJD1136 derivative containing <i>pspB⁺ pspC-C43S</i>	25
pAJD1857	pAJD1668 derivative containing <i>cyaT25-pspC-L118P</i>	This study
pAJD1894	pAJD1135 derivative containing <i>pspC-L118P</i>	25
pAJD1895	pAJD1135 derivative containing <i>pspC-V125D</i>	25
pAJD1897	pAJD1135 derivative containing <i>pspC-F130S</i>	25
pAJD1926	pAJD1136 derivative containing <i>pspB⁺ pspC-S26C-C43S</i>	24
pAJD1944	pAJD1136 derivative containing <i>pspB-E56C pspC-C43S</i>	24
pAJD1983	pAJD1136 derivative containing <i>pspB⁺ pspC-C43S-L103C</i>	This study
pAJD1990	pAJD1136 derivative containing <i>pspB⁺ pspC-C43S-G112C</i>	24
pAJD1997	pAJD1136 derivative containing <i>pspB⁺ pspC-C43S-R119C</i>	This study
pAJD2033	pAJD1136 derivative containing <i>pspB-I64CpspC-C43S</i>	24
pAJD2035	pAJD1136 derivative containing <i>pspB-E68C pspC-C43S</i>	24
pAJD2060	pAJD1135 derivative containing <i>pspC-C43S</i>	24
pAJD2110	pAJD1668 containing <i>cyaT25-pspC</i>	24
pAJD2163	<i>araBp-cpxP-3XFLAG</i> in pBAD33	This study
pAJD2258	pKNT25 containing <i>pspC-cyaT25</i>	This study
pAJD2260	pUT18 containing <i>pspC-cyaT18</i>	This study
pAJD2294	<i>araBp-pspC-phoA</i> in pBAD18-Kan	This study
pAJD2295	<i>araBp-pspB-phoA</i> in pBAD18-Kan	This study
pAJD2298	pKNT25 containing <i>pspB-cyaT25</i>	This study
pAJD2322	<i>araBp-pspB-gfp</i> in pBAD18-Kan	This study
pAJD2323	<i>araBp-pspC-gfp</i> in pBAD18-Kan	This study
pAJD2324	<i>araBp-gfp-pspC</i> in pBAD18-Kan	This study
pAJD2327	<i>araBp-cpxP-phoA</i> in pBAD18-Kan	This study
pAJD2329	<i>araBp-pspC(atg)-phoA</i> in pBAD18-Kan	This study
pAJD2331	<i>araBp-pspB(atg)-phoA</i> in pBAD18-Kan	This study
pAJD2364	pGEX-6P-1 derivative encoding GST-PspC ^{CT-V125D}	This study

^a Institut Pasteur.^b AJD3 is a virulence plasmid cured derivative of strain JB580v (24). AJD1204 and AJD3490 are derivatives of AJD3.

TABLE 2 Primers used in this study

Name	5'-to-3' sequence with restriction sites underlined
M13F(-40)	GTTTCCAGTCACGAC
30	GGGAGATCTGACTGCCTCCAATTTGGATG
32	GGGAGATCTAACTGACGAAAACGACTCTG
33	CGCGGATCCAGCTTGAATATACTTAAGG
34	CGCGGATCCAAATTGGAGGCAGTCA
38	GGGAGATCTCATGGGCGATATGTTTAAAAAC
42	GGGCGTCTGACTCACAACGACGAAAACGAC
345	CCGGAGCTCATCCAAATTGGAGGCAGTC
396	CGGGAGCTCAGCTTGAATATACTTAAGGAG
397	GGCTCTAGAGTCATATCACAACGACG
925	CGCGGATCCGAACCCGCGCCTGCATCCAG
1330	CGCCAGTGTCTTGATCAGTTAGAAT
1331	GATCAAGACACTGGCGCGCGCTCGC
1362	CGTTTGTGCCAGGTCGAGCGCTATG
1363	CGACCTGGCACAACGTTGCTCTCC
1530	GGCTCTAGAGCAGCGAAAATCACTGCCG
1535	CGGGAGCTCGCAGAGCAGTAAATCGC
1536	GGCTCTAGATTACTTGTCTATCGTCATCCTTGT AATCGATATCATGATCTTTATAATCACCCTG ATGGTCTTTGTAGTCCTTCTGGGCAGAAG AGGGTTGT
1751	GCCCGGTTTTCCAGAACAGGCAACTGACGA AAACGACTCTGCACCCC
1752	CCTGTTCTGGAAAACCGGGC
1753	GCCCGGTTTTCCAAGAACAGGTGACTGCCTC CAATTGGATGC
1754	CGGGAGCTCGCAGTTAACTGACGATGCAC
1755	GCCCGGTTTTCCAGAACAGGCATTATGACTG CTCCAATTT
1756	CGGGAGCTCGCCAGGCCGCCGGCAT
1757	GCCCGGTTTTCCAGAACAGGCATTATTCTC CTTAAGTATATTC
1772	GCCCGGTTTTCCAGAACAGGCTTCTGGGCAG AAGAGGGTTGT
1788	GGCTCTAGAAATTGTTTTTAAACATATCGCCC
1789	GGCTCTAGAGGCTTAGCGTTAATTGAATGAG

containing 1 mM isopropyl-D-thiogalactopyranoside (IPTG) and incubated at 26°C for approximately 40 h before being imaged with a scanner.

Chemical labeling of cysteines. The protocol was based on that of Bogdanov et al. (5). Saturated cultures were diluted in 200 ml of LB broth to an optical density of approximately 0.1 (600 nm) and shaken at 225 rpm for 2 h at 37°C. Then, 0.2 mM IPTG or 0.2% arabinose was added, and growth continued at 37°C for another 2 h. Cells from the equivalent of 100 ml at an optical density (600 nm) of 0.6 were harvested by centrifugation, resuspended in 1.5 ml of 100 mM HEPES-KOH, 250 mM sucrose, 25 mM MgCl₂, and 0.1 mM KCl, pH 7.0, and divided into two samples (A and B). To label cysteines located on the periplasmic side of the inner membrane, sample A was treated with 0.1 mM N α -(3-maleimidylpropionyl) biocytin (MPB) (Santa Cruz Biotechnology) and incubated at room temperature for 5 min with gentle shaking. To label cysteines located on either side of the inner membrane, sample B was sonicated before the cell lysate was treated with 0.1 mM MPB. For both samples, labeling of reactive cysteines was quenched by the addition of 20 mM β -mercaptoethanol, and then the cell envelope of sample A was also disrupted by sonication. Unbroken cells were removed by centrifugation at 16,000 $\times$ g for 2 min, and then the supernatant was separated into membrane (pellet) and soluble (supernatant) fractions by ultracentrifugation at 65,000 $\times$ g for 30 min.

For isolation of PspB and PspC, membranes were resuspended in 0.1 ml of 1% (wt/vol) SDS, 50 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM dithiothreitol (DTT), complete protease inhibitors, and 10 U/ml DNase I

(New England BioLabs) and shaken vigorously at 37°C for 20 min (CpxP-FLAG was isolated directly from the supernatant after ultracentrifugation). Denaturing SDS solubilization was used to maximize recovery of PspB and PspC and so increase the detection limit of the assay. Next, samples were diluted 10-fold with 1% Triton X-100, 50 mM Tris-HCl (pH 7.5), 300 mM NaCl, 5 mM EDTA, and 1 $\times$ complete protease inhibitors to sequester the SDS into Triton X-100 micelles so that antibodies would not be denatured in the subsequent immunoprecipitation (IP). Unsolubilized material was removed by centrifugation (16,000 $\times$ g for 2 min), and the supernatants were precleared by adding 20 μ l of 50% protein A Sepharose slurry and inverting slowly at 4°C for 1 h. Proteins were isolated from the precleared lysates by IP with anti-PspB or -PspC polyclonal antisera or anti-FLAG monoclonal antibodies and incubation overnight at 4°C with gentle inversion. Immune complexes were isolated by adding 60 μ l of 50% protein A Sepharose slurry and inverting gently at 4°C for 3 h. After centrifugation, the immune complexes were washed three times with 1.37 M NaCl, 27 mM KCl, 43 mM Na₂HPO₄, 14 mM KH₂PO₄, and 0.1% (vol/vol) Triton X-100 and resuspended by vortexing in 30 μ l of SDS-PAGE sample buffer. Proteins were denatured by heating at 95°C and separated by SDS-PAGE. Biotinylated proteins were detected by immunoblotting as described below. To control for efficient immunoprecipitation, membranes were stripped and reprobed to detect total PspB, PspC, or CpxP-FLAG using polyclonal antisera or monoclonal antibodies.

GST fusion protein pulldown assay. A solubilized membrane lysate from *Y. enterocolitica* overexpressing the *pspA* operon was made by growing strain AJD3490 to mid-exponential phase at 26°C in 1 liter of LB broth containing 0.1 mM IPTG. Cells were suspended in TBS (50 mM Tris-HCl, pH 8.0, 400 mM NaCl) containing 2 mM phenylmethylsulfonyl fluoride (PMSF) and 2 $\times$ complete protease inhibitors. Following storage overnight at -20°C, 1 ml of 1.67-mg/ml DNase I per 5 g wet weight of cells was added, and cells were disrupted by sonication. Unbroken cells were removed by centrifugation at 20,000 $\times$ g for 20 min, and then the membrane fraction was isolated by centrifuging the supernatant at 65,000 $\times$ g for 2.5 h. The membrane pellet was resuspended in 50 mM Tris-HCl, pH 7.4, 10% (wt/vol) glycerol, 150 mM NaCl, 3 mM β -mercaptoethanol, 2 mM PMSF, and 2 $\times$ complete protease inhibitors (10 ml per g of membrane pellet) and homogenized with a tissue grinder. Membrane proteins were solubilized by adding 1% *n*-dodecyl β -D-maltoside (DDM) (final concentration). Insoluble material was removed by centrifugation at 65,000 $\times$ g for 30 min, and the supernatant was diluted 10-fold in 50 mM Tris-HCl, pH 7.4, 10% (wt/vol) glycerol, 150 mM NaCl, and 3 mM β -mercaptoethanol.

E. coli strain BL21 containing plasmid pREP4-groESL and also pGEX-6P-1, pAJD1507, or pAJD2364 was grown to mid-exponential phase at 20°C in a liter of LB broth containing 1 mM IPTG. Cells were suspended in 50 ml of phosphate-buffered saline (PBS) (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂HPO₄, and 1.8 mM KH₂PO₄) containing 250 μ g/ml lysozyme and 0.1% (vol/vol) Triton X-100. Cells were disrupted by sonication, and unbroken cells were removed by centrifugation at 20,000 $\times$ g for 20 min at 4°C. One milliliter of the supernatant was incubated with 40 μ l of glutathione Sepharose (GE Healthcare) for 1 h at 4°C with gentle rocking. The Sepharose beads were collected by centrifugation at 500 $\times$ g for 1 min and washed three times with 1 ml of PBS. They were then incubated overnight at 4°C with 1 ml of PBS containing 5% (wt/vol) bovine serum albumin (BSA) and washed twice with 1 ml PBS before being incubated with 50 μ l of the *Y. enterocolitica* solubilized membrane lysate for 3 h at 4°C with gentle rocking. The beads were washed five times with gentle rocking for 10 min at 4°C with 1 ml of 50 mM Tris-HCl, pH 7.4, 10% (wt/vol) glycerol, 500 mM NaCl, 3 mM β -mercaptoethanol. The beads were resuspended in 60 μ l of 2 $\times$ SDS-PAGE sample buffer, boiled for 10 min, and then analyzed by SDS-PAGE and immunoblotting.

Polyclonal antisera and immunoblotting. Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by electroblotting. Enhanced chemiluminescence detection followed sequential incubation with polyclonal antiserum or monoclonal antibodies and then

FIG 1 Generally accepted topologies of PspB and PspC prior to this study. Arrows indicate the approximate locations of cysteine substitutions used in this study. TM, transmembrane domain; P, periplasm; C, cytoplasm; M, cytoplasmic membrane. Numbers indicate amino acid positions within each protein, and the N and C termini are labeled N and C, respectively.

goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) horseradish peroxidase conjugate used at a 1-in-5,000 dilution. Dilutions of polyclonal antisera were 1 in 20,000 to 500,000 for anti-PspB (25), 1 in 10,000 to 500,000 for anti-PspC (46), 1 in 40,000 to 80,000 for anti-PhoA (Novus Biologicals), 1 in 500,000 for anti-PspA (58), and 1 in 10,000 for anti-FtsH (58). Monoclonal antibodies were used at a 1-in-10,000 dilution for both anti-DnaK (Assay Designs) and anti-FLAG (Sigma). For detection of biotinylated proteins enhanced chemiluminescence detection followed incubation with streptavidin-horseradish peroxidase conjugate only (1-in-8,000 dilution; Pierce).

β -Galactosidase assays. Bacterial two-hybrid strains were grown in LB broth containing 1 mM IPTG at 30°C for 15 h. β -Galactosidase enzyme activity was determined at room temperature in permeabilized cells as described previously (43). Activities are expressed in arbitrary Miller units (47). Individual cultures were assayed in duplicate, and values from three independent cultures were averaged.

RESULTS

PhoA fusion analysis. The PspB and PspC topologies that are widely accepted in the literature are shown in Fig. 1. To test these, we began with C-terminal PhoA (alkaline phosphatase) fusion analysis (PhoA is active only in the periplasm; see reference 44). For this, we constructed arabinose-inducible expression plasmids encoding the PspB-PhoA and PspC-PhoA fusion proteins, which retained the ability to induce PspA protein synthesis (data not shown). PhoA was also fused to the C terminus of the known periplasmic protein CpxP (11). Fusion proteins of the expected sizes were detected in whole cells at approximately equivalent levels (Fig. 2A). Furthermore, CpxP-PhoA and PspB-PhoA localized to the soluble and membrane fractions, respectively, as expected (Fig. 2A). However, although PspC-PhoA appeared stable in intact cells, it was not detected in either fraction after cell breakage. We suspected that the PspC-PhoA fusion protein was degraded upon cell breakage, a phenomenon we have observed with other PspC fusion proteins in our laboratory (data not shown). Consistent with this, upon increasing the concentration of PhoA antiserum and prolonged exposure of the immunoblot, apparent degradation products were observed, but only in the membrane fraction (Fig. 2A). This should not affect the analysis because the protein remained intact unless cells were fractionated.

Next, we measured alkaline phosphatase activities of cells producing each fusion protein (Fig. 2A). The CpxP-PhoA protein had

high activity, as expected (approximately 11,300 Miller units). No alkaline phosphatase activity was detected in cells with PspB-PhoA, which is consistent with the predicted location of its C terminus in the cytoplasm. Cells with the PspC-PhoA protein did have detectable alkaline phosphatase activity. This agrees with the previous report that fusion of PhoA to the C terminus of *E. coli* PspC produced a protein with alkaline phosphatase activity (39). Thus, it supports the topology shown in Fig. 1. However, the alkaline phosphatase activity of PspC-PhoA (approximately 240 Miller units) was almost 50-fold lower than that of CpxP-PhoA despite the two proteins being produced at comparable levels (Fig. 2A). This raised concern about the physiological significance of this result and prompted us to use other independent means to investigate PspC topology.

GFP fusion analysis. To address our concern with the PhoA analysis, we used a reciprocal fusion approach. This was fusion to green fluorescent protein (GFP), which has been used in topology analyses because it is fluorescent only in the cytoplasm (16, 20; some recently described “superfolder” derivatives of GFP are active in the periplasm, but they were not used in our study). We constructed plasmids encoding PspB-GFP, PspC-GFP, and GFP-PspC fusion proteins. All of these proteins retained the ability to induce PspA protein synthesis (data not shown) and localized to the membrane fraction (Fig. 2B).

As expected, robust fluorescence was detected in cells containing the PspB-GFP fusion protein (Fig. 2B). This is consistent with our reciprocal PhoA fusion analysis and the β -galactosidase activity of an *E. coli* PspB-LacZ fusion protein (32), further supporting the universally predicted arrangement of PspB (Fig. 1). Similarly, cells with the GFP-PspC protein were fluorescent, which is consistent with the prediction of the PspC N terminus being in the cytoplasm (Fig. 1). However, cells containing PspC-GFP were also fluorescent. In fact, the fluorescence of cells with the PspC-GFP fusion was comparable to that of cells with PspB-GFP. This suggests that the C terminus of PspC might also be located in the cytoplasm, but it conflicts with the conclusion from PspC-PhoA analysis. Therefore, to investigate this paradox further, we decided to employ a third independent fusion approach.

Adenylate cyclase reconstitution analysis suggests that both ends of PspC are located in the cytoplasm. We took advantage of a bacterial two-hybrid system based on reconstitution of *Bordetella pertussis* adenylate cyclase activity (BACTH) (36). Proteins are fused to T18 and T25 fragments of the catalytic domain of adenylate cyclase (CyaA). Interaction of the proteins results in functional complementation between T25 and T18 and the synthesis of cAMP from ATP. This induces cAMP-dependent genes, such as those required for maltose catabolism. Functional complementation of CyaA activity can occur only if the T18/T25 fragments are located in the cytoplasm. Therefore, this approach can be used to monitor where the termini of interacting membrane proteins are located. When we and others have used this system to investigate PspC interactions, T18 or T25 were fused only to the N terminus of PspC because of its widely accepted topology (Fig. 1) (e.g., see references 33 and 46). However, now that the GFP fusion analysis suggested that the PspC C terminus could also be in the cytoplasm, we decided to investigate the effect of fusing T18 or T25 to this end of PspC. For this, derivatives of the BACTH vectors were constructed encoding PspC-T18 or PspC-T25 fusion protein and used in conjunction with other plasmids encoding previously

FIG 2 PhoA and GFP fusion protein analysis. (A, panel i) Anti-PhoA, anti-DnaK, and anti-FtsH immunoblot analysis of subcellular fractions from strains with the indicated PhoA fusion protein. Shown at the bottom is immunoblot analysis of subcellular fractions of the strain with PspC-PhoA in which a higher concentration of anti-PhoA polyclonal antiserum was used (1 in 40,000) to detect degradation products in the membrane fraction (in the upper part of the figure, the anti-PhoA polyclonal antiserum was used at 1 in 80,000). (A, panel ii) Alkaline phosphatase activities of whole cells of strains with the indicated PhoA fusion protein. The PspC(ATG)-PhoA and PspB(ATG)-PhoA proteins were negative controls that had only the initiation codon of PspC or PspB and the same upstream noncoding DNA present in the PspC-PhoA and PspB-PhoA proteins, respectively. (B, panel i) Anti-PspB, anti-PspC, anti-DnaK, and anti-FtsH immunoblot analysis of subcellular fractions from strains with the indicated GFP fusion protein ("–" indicates the empty vector). (B, panel ii) Fluorescence of whole cells of strains with the indicated GFP fusion protein.

studied T25-PspC and PspB-T25 proteins (24, 46). All of these fusion proteins were detected in the membrane fraction (Fig. 3).

Previous BACTH analysis has supported PspC-PspC and PspC-PspB interactions (e.g., see references 24 and 46). Therefore, we tested for the ability of PspC-T18 to reconstitute adenylate cyclase activity when combined with PspB-T25, PspC-T25, or T25-PspC protein (Fig. 3). The PspC-T18-plus-PspB-T25 combination gave a positive result, supporting the contention that the C termini of PspB and PspC are located in the cytoplasm. Furthermore, the PspC-T18 protein reconstituted adenylate cyclase activity when combined with either PspC-T25 or T25-PspC. This further supports the GFP fusion analysis suggesting that both PspC termini can be located in the cytoplasm.

Accessibility of cysteine residues to a non-membrane-permeating reagent suggests that PspC has only one topological arrangement. GFP and T18/T25 fusion analysis agreed that both termini of PspC could be in the cytoplasm. However, it did not distinguish between PspC being arranged as a polytopic protein with both termini inside or two populations of PspC in opposite bitopic membrane topologies (N terminus inside, C terminus out-

side, and *vice versa*). Furthermore, the low but detectable alkaline phosphatase activity of the PspC-PhoA protein (Fig. 2A) raised the possibility that a small proportion of PspC might adopt a topology with its C terminus in the periplasm. Therefore, we needed to address whether both termini being inside is likely to be the only topology of PspC or if bitopic arrangements might occur instead or in addition. For this, we chose to assay the accessibility of cysteine residues to the thiol-modifying reagent α -(3-maleimidylpropionyl) biocytin (MPB) under conditions where MPB does not pass through the cytoplasmic membrane.

Previously, we described cross-linking analysis of numerous PspB and PspC cysteine substitution mutants encoded by low-copy-number *lacZ* promoter expression plasmids (24). However, cysteines at many positions of a protein cannot be labeled with MPB even after membrane disruption due to the properties of their local environment (5). Therefore, we first had to screen our collection of cysteine substitution mutants for those that retained wild-type function and could be labeled by MPB when cells had been disrupted by sonication (data not shown). This led to us selecting E56C, I64C, and E68C, located in the C terminus of

FIG 3 Adenylate cyclase domain fusion protein analysis. (A) Reconstitution of adenylate cyclase activity. *E. coli* strain BTH101 contained a plasmid encoding PspC-T18 and a second plasmid encoding either T25-PspC, PspC-T25, PspB-T25, or the empty T25 vector as indicated. Strains were grown on MacConkey-maltose agar at 26°C for approximately 40 h. (B) Anti-PspB, anti-PspC, and anti-DnaK immunoblot analysis of subcellular fractions from *E. coli* BTH101 strains producing the fusion proteins used in panel A [the relevant fusion protein(s) present is indicated at the top]. Arrows indicate the approximate positions of the proteins with PspC fused to either T25 or T18. Detection of the cytoplasmic DnaK protein was used to confirm that the membrane fractions were not significantly contaminated with cytoplasmic proteins. For the experiment involving detection of the PspC fusion proteins (right side), all samples were run on the same gel, but some irrelevant intervening lanes have been excised, as indicated by separately boxed areas.

PspB, S26C, located in the N terminus of PspC, and G112C, located in the C terminus of PspC, for the topology analysis (Fig. 1) (24). In addition, we also used L103C and R119C, in the C terminus of PspC, and the resulting mutants, like the other mutants, retained their Psp system regulatory function (24; data not shown). CpxP, which has a single cysteine, was used as a periplasmic control. Cells were grown to logarithmic phase and treated with MPB before or after membrane disruption by sonication, and then biotinylated PspB/PspC/CpxP proteins were isolated and detected by immunoprecipitation and immunoblotting. At lower concentrations and with shorter incubation periods, MPB is non-permeable for the inner membrane (5). Therefore, labeling of a cysteine residue with MPB before sonication is indicative of a periplasmic cysteine residue. Conversely, failure to label before sonication but success after sonication indicates a cytoplasmic location.

CpxP was biotinylated robustly before sonication, consistent with its periplasmic location and confirming that MPB could cross the *Y. enterocolitica* outer membrane (Fig. 4A). In contrast, all of the PspB cysteine substitution mutants were labeled by MPB only after cell disruption (Fig. 4B), consistent with the location of its C terminus in the cytoplasm. This result also confirms that MPB is nonpermeable for the *Y. enterocolitica* inner membrane under the conditions we used. Similarly, all of the PspC cysteine substitution mutants were labeled only after sonication, regardless of whether the cysteine was in the N or C terminus (Fig. 4C). This further confirms that both ends of PspC are on the cytoplasmic side of the membrane and suggests that this is the only arrangement of PspC. However, it remains possible that a minor proportion of PspC adopts a bitopic membrane topology with one of its termini in the periplasm, but it is below the limit of detection by this assay. Finally, when the experiments were done with cells grown under a Psp-inducing condition (secretin protein overproduction), the results were the same (data not shown). This argues against a topological change for PspC (or PspB) upon induction of the Psp system.

In silico analysis of *Y. enterocolitica* PspC topology using TOPCONS. The TOPCONS software program has been reported to be a more reliable method of topology prediction than some

other commonly used programs (3). In brief, this method predicts membrane topology by forming a consensus from five individual prediction methods. We applied this method to *Y. enterocolitica* PspC, and the consensus prediction agrees with our experimental analysis (two transmembrane domains with both termini in the cytoplasm) (Fig. 5A). The transmembrane domains are separated by only a single glycine residue, an arrangement that has been confirmed for at least one other bacterial membrane protein (59). However, of the five individual predictions used to form the consensus, three predicted this polytopic arrangement, but the PRODIV and PRO predictions did not (Fig. 5A). The TOPCONS prediction server allows the user to do the analysis with one or both termini restrained to be inside or outside if experimental data are available to support it. When we repeated the analysis with both termini restrained inside, as our data had revealed, the PRODIV and PRO predictions now also identified two putative transmembrane domains, albeit with slightly different start and end points compared to the other predictions (Fig. 5A). Regardless of whether or not restraint was applied, the consensus predictions were identical. TOPCONS analysis of *E. coli* PspC also predicts the polytopic membrane arrangement, with both termini inside (34). Therefore, TOPCONS prediction agrees with the arrangement of PspC revealed by our experimental analysis.

The C terminus of PspC might be the recognition site for the FtsH protease. The preceding experiments had demonstrated that the previously accepted topology of PspC (Fig. 1) is incorrect. In particular, most if not all PspC is arranged with its C terminus in the cytoplasm and not the periplasm. Therefore, we reasoned that there should be some biological phenomena consistent with a cytoplasmically located C terminus. Recently, we reported FtsH-dependent degradation of PspC when it was produced without its binding partner, PspB (51). FtsH is a protease that identifies integral inner membrane protein targets via exposed N- or C-terminal cytoplasmic tails of at least approximately 20 amino acids in length (8, 9). We were interested to investigate whether the N or C terminus of PspC might fulfill this role. If this was the case, we predicted that some mutations in the N or C terminus of PspC might disrupt the FtsH recognition site and so stabilize the protein in the absence of PspB.

FIG 4 Determination of PspB and PspC topology by chemical modification of cysteine residues. For all panels, whole cells were incubated with the cysteine biotinylation reagent MPB either before (-) or after (+) sonication. (A) Analysis of cells with the CpxP-3xFLAG expression plasmid pAJD2163. After labeling, CpxP-3xFLAG was isolated from the soluble cell fraction by immunoprecipitation with anti-FLAG monoclonal antibodies. Immunoprecipitates were analyzed by immunoblotting using streptavidin-horseradish peroxidase to recognize biotinylated protein (Biotin). The membrane was then stripped and reprobed with anti-FLAG monoclonal antibodies to detect total CpxP-3xFLAG (FLAG). (B) Analysis of cells with plasmids encoding wild-type PspB (WT), the indicated cysteine substitution mutant, or no PspB (-). After labeling, PspB was isolated from the membrane fraction by immunoprecipitation with anti-PspB polyclonal antiserum. Immunoprecipitates were analyzed by immunoblotting using streptavidin-horseradish peroxidase to recognize biotinylated protein (Biotin). The membrane was then stripped and reprobed with anti-PspB polyclonal antiserum to detect total PspB. (C) Analysis of cells with plasmids encoding PspC with the native cysteine replaced by serine (C43S), derivatives that also had the indicated cysteine substitution mutation, or no PspC (-). After labeling, PspC was isolated from the membrane fraction by immunoprecipitation with anti-PspC polyclonal antiserum. Immunoprecipitates were analyzed by immunoblotting using streptavidin-horseradish peroxidase to recognize biotinylated protein (Biotin). The membrane was then stripped and reprobed with anti-PspC polyclonal antiserum to detect total PspC.

We have a large collection of PspC amino acid substitution mutants, including many that were isolated due to their altered regulatory function (25). As a nonbiased approach to determine whether any of these mutations might interfere with FtsH-dependent degradation, we selected a collection of 16 different mutations located throughout the protein (data not shown). Low-copy-number *lacZp* expression plasmids encoding wild-type or mutant PspC were introduced into the Δ *pspBC* strain AJD1204 to produce PspC without PspB, which makes it subject to FtsH-dependent degradation (51). Then, the steady-state level of PspC was monitored by immunoblotting. This preliminary analysis revealed that only the L118P, V125D, and F130S mutations, which are all located within the C-terminal 22 amino acids, increased the steady-state level of PspC (data not shown). To characterize this phenotype further, the experiment was repeated with a subset of the original group of mutants produced with or without PspB (Fig. 6). In the presence of PspB, when PspC is not subject to FtsH-dependent degradation (51), the steady-state level of all of the PspC mutants was comparable to that of wild-type PspC. The only exception was PspC-L69P, which was apparently inherently less stable/abundant than wild-type PspC. However, in the absence of PspB, when PspC is subject to FtsH-dependent degradation (51), the PspC-L118P, -V125D, and -F130S mutants were much more abundant than PspC-C43S, PspC-L69P, or wild-type PspC (Fig. 6).

This result suggests that FtsH might recognize uncomplexed PspC through its C terminus. Consequently, some C-terminal

mutations in PspC might blind FtsH from the recognition region in PspC, leading to increased abundance of these mutants in the absence of its binding partner, PspB. The C-terminal tail of PspC could only be the FtsH recognition site if it is in the cytoplasm, consistent with our topological analysis.

C-terminal PspC mutations reduce its association with PspA. In response to secretin stress, PspBC promotes the relocation of PspA to the inner membrane in order to free cytoplasmic PspF from inhibition by PspA (58). This probably involves PspBC-PspA interactions (1, 34, 58). It was suggested that the C-terminal leucine zipper-like amphipathic helix of *E. coli* PspC could bind PspA if PspC changed its topology from C terminus outside to inside in response to a Psp-inducing signal (34). In support of this, the BACTH system described above provided evidence that *E. coli* PspA interacted with the isolated C terminus of PspC when it was produced in the cytoplasm (34). Our data suggest that the PspC C terminus is always in the cytoplasm. Nevertheless, this is still consistent with the C terminus acting as the PspA interaction interface. In fact, when we identified PspC mutant proteins unable to induce *pspA-lacZ* expression, all of the mutations were in the C terminus and predicted to affect the amphipathic helix (25). If this is the PspA binding site, the mutant PspC proteins should be defective for association with PspA. Therefore, in the final experiments we tested this prediction.

We have used the BACTH approach to demonstrate an association between the PspA-T18 and T25-PspC proteins (58). Therefore, we constructed plasmids encoding T25-PspC with one of the

FIG 5 TOPCONS analysis of the *Y. enterocolitica* PspC primary sequence. (A) Outputs from the TOPCONS server (<http://topcons.cbr.su.se/>). On the left is the output with no restraint applied. On the right is the output when both termini are restrained to be in the cytoplasm, which is based on experimental data from this study. On both sides, the individual outputs of the five TOPCONS component predictions are shown at the top and the final consensus prediction based on those five outputs is shown at the bottom (consensus predictions were identical with or without restraint). (B) Primary sequence of PspC with the two transmembrane helices predicted by TOPCONS shown in red and the region predicted to form an amphipathic helix labeled.

mutations that prevent activation of the Psp system (L118P, V125D, S127P, F130S, and Δ 123–139; see reference 25). As an additional control, we also included a mutation that had the opposite phenotype of constitutive Psp system activation (V33E; see reference 25). Plasmids encoding PspA-T18 and a T25-PspC variant were introduced into an *E. coli* *cytA* null strain, BTH101, and the association between them was monitored by measuring β -galactosidase activity (Fig. 7A). High β -galactosidase activity was observed with wild-type PspA and PspC, supporting the close association reported previously (58). The PspC-V33E mutation did not affect this association. However, the C-terminal mutations reduced the association between PspA and PspC (Fig. 7A).

The BACTH experiment suggested that disruption of the PspC

C terminus reduces PspA-PspC association. Next, we used a pull-down assay to focus solely on the C terminus of PspC. GST or derivatives fused to the C terminus of either wild-type PspC (GST-PspC^{CT}) or the V125D mutant (GST-PspC^{CT-V125D}) were bound to glutathione Sepharose and incubated with a DDM-solubilized membrane lysate from a *Y. enterocolitica* PspABCD-YcjXF-over-producing strain. After washing, all proteins were eluted and analyzed by SDS-PAGE and anti-PspA immunoblotting. Some non-specific binding of PspA to the GST-only control was observed (Fig. 7C), which was not unexpected because the same thing was reported for *E. coli* PspA (1). However, much more efficient binding of PspA occurred with the GST-PspC^{CT} protein. Furthermore, this specific binding of PspA was abolished by the V125D mutation (Fig. 7C).

Together, the BACTH and pull-down data support the contention that the amphipathic helix in the C terminus of PspC might be a site of interaction with PspA. Once again, this could only occur with full-length PspC if the C terminus is located in the cytoplasm.

DISCUSSION

Activation of the Psp system involves relocation of PspA from the cytoplasm to the inner membrane, an event that requires PspB and PspC (58). Although the mechanism by which PspB and PspC promote the relocation of PspA has not been established, it is reasonable to hypothesize that it involves a direct interaction with PspA (1). PspA can be cytoplasmic or peripherally associated with the inner membrane (58). This means that any interaction

FIG 6 Mutations close to the C terminus of PspC have an increased steady-state level in the absence of PspB. Anti-PspC immunoblot analysis of total cell lysates from Δ pspBC strain AJD1204 with a plasmid encoding wild-type PspC (WT), various mutants with the indicated amino acid substitution, or no PspC (-) is shown. On the right, the plasmid also encoded wild-type PspB (+ PspB; pAJD1136 derivatives), whereas on the left it did not (- PspB; pAJD1135 derivatives). “Protein,” a Ponceau S stain of the nitrocellulose membrane used for immunoblot analysis (the region containing PspC).

FIG 7 Mutations close to the C terminus of PspC reduce its association with PspA. (A) Bacterial two-hybrid assay. *E. coli* strain BTH101 contained two different plasmids. One encoded PspA-T18, and the other encoded T25-PspC with wild-type PspC (WT), the indicated PspC mutant, or T25 only (-). β-Galactosidase activities were determined as described in Materials and Methods. (B) Anti-PspC and anti-PspA immunoblot analysis of the strains from panel A. "Protein," a Ponceau S stain of the nitrocellulose membrane used in each immunoblot (the region containing the protein detected in the corresponding immunoblot). (C) GST fusion protein pulldown assay. GST, GST fused to the wild-type PspC C terminus (GST-PspC^{CT}), or a derivative with a V125D mutation (GST-PspC^{CT-V125D}) was bound to glutathione-Sepharose (Beads) and incubated with detergent-solubilized membrane lysate from *Y. enterocolitica* strain AJD3490. After washing, proteins were recovered by resuspension in SDS-sample buffer and heating. Recovered proteins (Elution) were analyzed by SDS-PAGE and immunoblotting with PspA antiserum. The GST fusion protein in each elution was detected by Ponceau S staining the immunoblot membrane.

with PspB/C might be through their cytoplasmic domains. Therefore, the development of even the most basic models of regulation of the Psp system requires an understanding of the membrane topology of these proteins. However, uncertainty about the topology of PspC drove us to investigate it experimentally. Our results reveal that PspC is a polytopic membrane protein with both ends in the cytoplasm. This has significant implications when devising a working model for how activation of the Psp system might occur.

This study focused primarily on PspC rather than PspB. This was because all topology prediction programs agree that PspB is likely to be a bitopic membrane protein with a single transmembrane domain close to its N terminus and with most of the protein, including its C terminus, in the cytoplasm (Fig. 1). However, there is only one report of experimental validation of this topology (analysis of a PspB-LacZ fusion protein; reference 32), and so we included a limited analysis of PspB here. All of our data supported the predicted topology with a cytoplasmic C terminus. We did not directly address the location of the PspB N terminus here, but at least two observations support its location close to the periplasmic side of the membrane. First, no topology prediction program identifies more than a single transmembrane helix (data not shown). Second, when the serine at position 2 was replaced by cysteine, abundant disulfide-bonded PspB dimers formed spontaneously *in vivo* (24). This strongly suggests that the N terminus is exposed to the oxidizing environment in the periplasm.

Our data provided unanimous support for the contention that the N terminus of PspC is in the cytoplasm (fluorescent GFP-PspC, ability of T25-PspC to reconstitute adenylate cyclase activity, and inability to label an N-terminal cysteine with a non-membrane-permeating reagent). Furthermore, the catalyzed oxidative cross-linking between a cysteine in the N terminus of PspC and either of two cysteines in the cytoplasmic C terminus of PspB reported previously provides further support for this arrangement (24).

Prior to this work, it was generally accepted that the C terminus of PspC was in the periplasm (Fig. 1). This was based on only one experiment, for which alkaline phosphatase activity of an *E. coli* PspC-PhoA fusion protein was reported (39). We supported this finding with a *Y. enterocolitica* PspC-PhoA fusion (Fig. 2A). However, the alkaline phosphatase activity was much lower than that when PhoA was fused to the known periplasmic protein CpxP. This caused us concern that the result might be an artifact of the approach. This might be the case, because all of the other independent approaches we used revealed that the C terminus is in the cytoplasm (fluorescent PspC-GFP, ability of PspC-T18 and PspC-T25 to reconstitute adenylate cyclase activity, and inability to label three different C-terminal cysteines with a non-membrane-permeating reagent). Furthermore, in a previous report, 12 different amino acids in the C terminus of PspC were replaced by cysteine (24). Several of these cysteine substitution mutants formed disulfide-bonded dimers when an oxidative catalyst was added. However, none of them formed a disulfide bond spontaneously without the catalyst. Perhaps this was the first indication that the C terminus of PspC is not in the oxidative environment of the periplasm. Therefore, when taken together, all of these data support the location of the PspC C terminus in the cytoplasm. Nevertheless, the outlying result with PspC-PhoA could indicate that a very minor amount of the total PspC adopts the bitopic membrane topology shown in Fig. 1. However, it is also possible that addition of the large PhoA enzyme to PspC artificially alters its arrangement.

Analysis of a series of inner membrane proteins in *E. coli* suggested that PhoA/GFP fusion proteins that produced both alkaline phosphatase and GFP activity could be explained by proteins that can be inserted into the inner membrane in two different orientations (21). However, the inability to detect labeling of N- or C-terminal cysteines with a non-membrane-permeating reagent, even when films were overexposed (Fig. 4C and data not shown), sug-

FIG 8 New model for the mechanism of PspBC-dependent activation of the Psp response. In the uninduced state, the cytoplasmic domain of PspB and/or the N terminus of PspC might tether the C terminus of PspC (alternatively, the C-terminal amphipathic helix of PspC could interact with the cytoplasmic face of the inner membrane in the uninduced state; not shown). This blocks the ability of the leucine zipper-like amphipathic helix within the C-terminal domain of PspC to interact with PspA. Under inducing conditions, cytoplasmic membrane stress (wavy arrows) might be sensed by PspB and/or PspC, perhaps via their transmembrane domains. This causes a conformational change that exposes the C terminus of PspC, allowing its amphipathic helix (stripes) to recruit PspA away from PspF. PspF then activates the *pspA* operon promoter.

gests that this does not happen with PspC. Instead, both termini are in the cytoplasm. The possibility remains that a small proportion of PspC adopts a topology with one of its termini in the periplasm but it was below the limit of detection in the cysteine-labeling assay. However, even if that were the case, it would be only a very minor part of the total PspC population. Finally, we also obtained similar cysteine labeling results when cells were grown under Psp-inducing or Psp-noninducing conditions (Fig. 4 and data not shown). This argues against any change in PspC topology as part of the Psp activation mechanism.

In silico analysis with TOPCONS also supports a polytopic arrangement of PspC with both termini in the cytoplasm for both *Y. enterocolitica* and *E. coli* PspC (Fig. 5) (34). Interestingly, the first putative transmembrane domain in the consensus prediction (TM-1, 47 to 67) (Fig. 7) is in close proximity to a glycine zipper motif (GxxxG; reviewed in reference 49). In fact, when the TOPCONS analysis is done with both termini restrained to be cytoplasmic, the PRODIV and PRO programs predict that this motif is within the first transmembrane domain (Fig. 5A). Glycine zipper motifs are thought to promote helix packing between membrane proteins (49).

New model for activation of the Psp system. The PspC topology uncovered here allows us to propose a new working model for activation of the Psp system (Fig. 8). Under noninducing conditions, PspB and PspC are in their “off” state such that the cytoplasmic domain of PspB and/or the N terminus of PspC might work to tether the C terminus of PspC (although an equally plausible hypothesis is that the C-terminal amphipathic helix of PspC might interact with the cytoplasmic face of the inner membrane in the “off” state). This prevents the amphipathic helix in the PspC C terminus from binding to PspA. PspB and/or PspC might sense an inducing signal related to altered cytoplasmic membrane properties. The lack of a PspC periplasmic domain argues against this being the sensory region as was hypothesized previously (1, 25). Instead, we now suspect that the transmembrane domains of PspB and/or PspC might sense the inducing signal. Regardless of the mechanism, the inducing signal switches PspBC to an “on” state, exposing the C terminus of PspC so that it sequesters PspA from

PspF. It is also possible that PspB binds to PspA under the induced condition, because evidence for a PspB-PspA interaction, facilitated by PspC, has been reported for *E. coli* (1). In addition to being consistent with the findings reported here, this model is particularly satisfying because it also fits well with published observations. First, using cysteine substitution mutants, the cytoplasmic domain of PspB could be cross-linked by a disulfide bond to the N terminus of PspC, suggesting they might interact (24). Second, in a random screen, a wide variety of amino acid substitutions throughout PspB and in the N terminus and predicted transmembrane domains of PspC caused constitutive activation, as did deletion of the PspC N terminus (25). This implicates these regions in negative regulation (Fig. 8). Third, in the same random screen, no mutations in the C terminus of PspC caused constitutive activation. In contrast, C-terminal mutations prevented activation, indicating a role in positive regulation (e.g., binding to PspA) (Fig. 8). Fourth, BACTH analysis supported a possible interaction between the isolated C terminus of *E. coli* PspC and PspA (34), and this is now further supported by our pulldown experiment (Fig. 7C).

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