Activity of a Bacterial Cell Envelope Stress Response Is Controlled by the Interaction of a Protein Binding Domain with Different Partners*

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The bacterial phage shock protein (Psp) system is a highly conserved cell envelope stress response required for virulence in *Yersinia enterocolitica* and *Salmonella enterica*. In non-inducing conditions the transcription factor PspF is inhibited by an interaction with PspA. In contrast, PspA associates with the cytoplasmic membrane proteins PspBC during inducing conditions. This has led to the proposal that PspBC exists in an OFF state, which cannot recruit PspA, or an ON state, which can. However, nothing was known about the difference between these two states. Here, we provide evidence that it is the C-terminal domain of *Y. enterocolitica* PspC (PspC<sup>CT</sup>) that interacts directly with PspA, both in vivo and in vitro. Site-specific photocross-linking revealed that this interaction occurred only during Psp-inducing conditions in vivo. Importantly, we have also discovered that PspC<sup>CT</sup> can interact with the C-terminal domain of PspB (PspC<sup>CT</sup>-PspB<sup>CT</sup>). However, the PspC<sup>CT</sup>-PspB<sup>CT</sup> and PspC<sup>CT</sup>-PspA interactions were mutually exclusive in vitro. Furthermore, in vivo, PspC<sup>CT</sup> contacted PspB<sup>CT</sup> in the OFF state, whereas it contacted PspA in the ON state. These findings provide the first description of the previously proposed PspBC OFF and ON states and reveal that the regulatory switch is centered on a PspC<sup>CT</sup> partner-switching mechanism.

Many cells monitor their cell envelope and respond to hostile conditions and events that can compromise it. Bacteria achieve this with specialized signaling pathways composed of envelope-associated sensory components and cytoplasmic transcriptional regulators, which work together to alter gene expression to combat the threat (1–4). These signaling pathways are known as extracytoplasmic/envelope stress responses. One is the phage shock protein (Psp)<sup>2</sup> system, so-named because it is induced when *Escherichia coli* is infected with a filamentous phage (5). However, the Psp system is not linked to phage infection exclusively. Instead, it is thought to promote bacterial survival during conditions that threaten the integrity of the cytoplasmic membrane (6–8). The Psp system is conserved in many Gram-negative bacteria, including several pathogens. It is essential for the virulence of the enteric pathogens *Yersinia enterocolitica* and *Salmonella enterica* serovar Typhimurium (9, 10). Furthermore, the *psp* genes are up-regulated during biofilm formation, macrophage infection, and the establishment of persister cells (11–14). Homologues of some Psp system components are also present in Gram-positive bacteria, archaea, and plant chloroplasts (1, 15).

The Psp system has been studied most in *E. coli* and *Y. enterocolitica*, where the *psp* genes are expressed from α<sup>a</sup>-dependent promoters that require the DNA-binding protein, PspF, for activity (16, 17). In both species the known PspF regulator consists of only the *pspA* operon and the unlinked *pspG* gene (9, 16, 18). However, the *pspA* operons of these two species differ at the distal ends, *pspABCDY-cjXF* in *E. coli* and *pspABCDY-cjXF* in *Y. enterocolitica*. Furthermore, only *pspA*, -B, -C, and -F have been linked to robust phenotypes, with PspA, -B, and -C each having dual regulatory and stress-mitigating functions (6–8, 19, 20). Thus, PspA, -B, -C, and -F are considered the core components of the Psp system. Expression of the *pspA* operon is induced by stimuli that include extreme heat and hyperosmotic shock, high ethanol concentration, and the mislocalization of outer membrane secretin proteins into the cytoplasmic membrane (6, 8, 21). Of these, only secretin mislocalization induces *psp* gene expression specifically in both *E. coli* and *Y. enterocolitica* (18, 22). Secretins are multimeric proteins with significance because they normally form the outer membrane channel of several types of bacterial protein export systems, many of which are involved in pathogenesis (23).

Regulation of *pspA* operon expression centers upon controlling the activity of the PspF transcription factor. In uninduced *Y. enterocolitica* cells, PspA inhibits PspF by interacting with it in the cytoplasm (24, 25). However, an inducing stimulus causes PspA to redistribute from the cytoplasm to the cytoplasmic membrane, releasing PspF to activate the *pspA* promoter (24).

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2 The abbreviations used are: Psp, phage shock protein; SOE, sewing overlap extension; DDM, n-dodecyl β-D-maltoside; IPTG, isopropyl 1-thio-β-D-galactopyranoside; MBP, maltose-binding protein; pBpa, p-benzoyl-L-phenylalanine.
The integral cytoplasmic membrane protein complex PspBC is thought to dissociate the PspA-PspF complex by sequestering PspA (25). Therefore, we proposed a model in which an inducing stimulus causes PspBC to switch from an OFF to an ON state, with only the latter able to interact with PspA (26). The C-terminal domain of PspC (PspCCT) has been proposed to mediate the interaction with PspA (26, 27). For example, PspCCT pulls PspA down from a complex cell lysate in vitro, although it is not known if PspCCT contacts PspA directly (26). Nor is it known what might prevent PspA from associating with PspBC in non-inducing conditions. In other words, what are the differences between the OFF and ON states of PspBC? In this work we have developed a combination of in vitro and in vivo approaches to investigate these questions. Our findings provide the first description of PspBC OFF and ON states and show that the regulatory switch is a PspCCT partner-switching mechanism.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains and Routine Growth**—Bacterial strains and plasmids are listed in Table 1, and primer sequences are listed in Table 2. Y. enterocolitica strains were grown at 26 °C or 37 °C as noted. E. coli strains were grown at 37 °C. Strains were grown in Luria-Bertani (LB) broth or on LB agar plates. Antibiotics were used as described previously (28) except that kanamycin was used at 40 μg/ml for E. coli strain MC3 and its derivatives.

**Plasmid Constructions**—All PCR-generated fragments were verified by DNA sequencing. Plasmids encoding GST-PspCCT with or without the V125D mutation or GST-PspBCT were

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**Table 1**

| Name | Genotype/Features | Reference/Source |
|------|------------------|------------------|
| E. coli strains | | |
| BL21 | F− ompT hsdS (r− m−) gal dcm | (49) |
| AJDE1770 | BL21Δ [pspF-F]:kan | This study |
| MC3 | F− araD139Δ (argF-lac)U169 relA1 rpsL150 fbbB5301 depC1 ptsF25 rbsR λ [pspAp-lacZY] | (50) |
| AJDE2892 | MC3 pspA-his, PspBC | This study |

| Y. enterocolitica strains | | |
| AJD3 | ΔsenR (R− M+) | (51) |
| AJD3298 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr | (24) |
| AJD3469 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr + ΔpspC | (24) |
| AJD3490 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr | (24) |
| AJD3701 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr + ΔpspC | (24) |
| AJD4739 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr + ΔpspC | (24) |
| AJD4741 | ΔsenR (R− M+): ΔpspF ΔpspA-ΔpspF-tacpPbr + ΔpspC | (24) |

**Plasmids**

| plasmids | | |
| p34S-Km | Ap′, Kmr, pUC18 ori | (52) |
| pBAD33 | Cm′, p15A ori, araB expression vector | (53) |
| pEVOL-pBpF | Cm′, pBSA aminocacyl-tRNA synthetase/ suppressor tRNA | (38) |
| pGEX-6P-1 | Ap′, pBR322 encoding GST | GE Healthcare |
| pMALc | Ap′, pBR322 ori, encoding MBP-LacZa | NAB |
| pRE112 | Cm′, R6K ori, mob− (RP4), sacB+ | (54) |
| pREP4-groEFL | Km′, p15A ori, groES groEL overexpression plasmid | (55) |
| pBL257 | Km′, R6K ori, mob− (RP4), sacB+ | (56) |
| pVT35 | Sm′, Sm′, RSF1010 ori, tac expression vector | (57) |
| pWSK29 | Ap′, pSCI101 ori, lacZp expression vector | (58) |
| pWSK129 | Km′, pSCI101 ori, lacZp expression vector | (58) |
| tacp-ycbC in pVT35 | tacp-ycbC in pVT35 | (9) |
| pAJD63 | pMALc derivative encoding MBP-PspBCT | (33) |
| pAJD935 | araBp-ysaC-His, in pBAD33 | (19) |
| pAJD1126 | Km′ derivative of pGEX-6P-1 | This study |
| pAJD1136 | pspBp-gsC+ in pWSK129 | (33) |
| pAJD1340 | pspBp-W23R pspC+ in pWSK129 | (33) |
| pAJD1659 | pspBp-V12SD in pWSK129 | (33) |
| pAJD2509 | E. coli pspA-His, PspBC in PBR375 | (33) |
| pAJD2135 | pspBp-G3FLAG in pRS112 | (33) |
| pAJD2164 | pspCp-3xFLAG in pR8112 | (33) |
| pAJD2344 | pGEX-6P-1 derivative encoding GST-PspBCT | This study |
| pAJD2355 | Km′ derivative of pAJD2544 | (33) |
| pAJD2510 | lacZp-psbBp-G13p-FLAG in pWSK129 | This study |
| pAJD2513 | psbBp-psbC-FLAG in pWSK129 | This study |
| pAJD2519 | psbBp-G3FLAG in pWSK129 | This study |
| pAJD2509 | E. coli pspBp-pspC+ in pWSK129 | This study |
| pAJD2514 | psbBp-G3FLAG in pWSK129 | This study |
| pAJD2546 | Km′ derivative of pAJD2544 | This study |
| pAJD2547 | Km′ derivative of pAJD2545 | This study |
| pAJD2600 | psbBp-G13p-V12SD-FLAG in pWSK129 | This study |
| pAJD2601 | psbBp-V12SD-G13p-FLAG in pWSK129 | This study |
| pAJD2602 | psbBp-V12SD-G13p-FLAG in pWSK129 | This study |

a AJD3 is a virulence plasmid-cured derivative of strain JB580v (51). All other Y. enterocolitica strains listed are derivatives of AJD3.
b Obtained from Addgene.
made by amplifying the region encoding the 49 C-terminal amino acids of PspC or the 51 C-terminal amino acids of PspB from the chromosome of an appropriate *Y. enterocolitica* strain with primers 1986/42 and 1794/107, respectively. The fragments were digested with BamHI/SalI and cloned into pGEX-6P-1. For use in *Y. enterocolitica*, which is naturally ampicillin-resistant, derivatives of these plasmids encoding kanamycin resistance were made by transferring the kanamycin resistance gene from p34S-Km into the SalI restriction site located downstream of the *gst* fusion gene.

A plasmid encoding PspB and PspC-FLAG was constructed by amplifying *pspBC* from plasmid pAJD1136 with primers 396/2085 and cloning it as a Sacl/XbaI fragment into pWSK129 to generate plasmid pAJD2510. pAJD2510 derivatives containing an amber codon (TAG) at different positions within *pspC*, the PspB-W23R mutation, or the *pspC*-V125D mutation were amplified by PCR to have a common 20-bp overlap. The upstream fragment was amplified with primers 396/2074, and the downstream fragment was amplified with primers 396/2074, and the downstream fragment was amplified with primers 2073/2085, both from pAJD1659. To generate plasmid pAJD2602 (encoding PspB-W23R, PspC-V125D and with codon 109 of *pspC* changed to TAG), the upstream fragment was amplified with primers 2073/2085 from pAJD2534, and the downstream fragment was amplified with primers 2073/2085 from pAJD1659. In all cases, each pair of upstream and downstream fragments was joined by SOE PCR with primers 396/2074 to generate an 0.6-kb fragment flanking the 3′ end of *pspC*.

### Table 2

| Name | 5′-to-3′ sequence |
|------|-------------------|
| 42   |GGCGATATCTACCTTTTCGTTTGGAGGACCCG| |
| 107  |GGCGGCTGAGCATCACCACCCATTTAGGAC| |
| 396  |GGCGGCGTCCGAGAAATAGCGGCGCTCATC| |
| 856  |GGGGCACTGAGAGTCTTTTGCTCTTACGTCGTC| |
| 857  |GGGGGACTCCTACTCTTAAAACGGCAATTAGCC| |
| 858  |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 881  |GGGGGACTCCTACTCTTAAAACGGCAATTAGCC| |
| 941  |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 942  |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1557 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1562 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1573 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1575 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1794 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 1986 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2026 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2027 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2028 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2029 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2032 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2067 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2068 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2073 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2074 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2083 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2084 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2085 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2086 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2087 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2088 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |
| 2089 |GGGGGACCTGACTCTTCGAGTTCCTATTCTA| |

Y. enterocolitica chromosome with primers 1573/1575 and cloning it as a Sacl/XbaI fragment. Plasmid pAJD2601, encoding PspC-V125D and with codon 131 of *pspC* changed to TAG, was made by amplifying *pspBC* from plasmid pAJD1136 with primers 396/2198 and cloning it as a Sacl/XbaI fragment into pWSK129. A plasmid encoding *E. coli* PspB and PspC was constructed by amplifying *pspBC* from plasmid pAJD1136 with primers 396/2198 and cloning it as a Sacl/XbaI fragment into pWSK129 to generate pAJD2524.
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~1-kb fragment with the 3×-FLAG-encoding region surrounded by ~0.5 kb on each side, and this fragment was cloned into sacB suicide plasmid pRE112. The suicide constructs were used to fuse the 3×-FLAG-encoding regions to the chromosomal Y. enterocolitica pspB or pspC genes by integration, selection for sucrose-resistant segregants, and confirmation by colony PCR and DNA sequencing.

The Δ(pspF-pspE) derivative of E. coli strain BL21 (AJDE1770) was made by introducing the Δ(pspF-pspE)::kan mutation (20) into strain BL21 by phage P1 vir transduction and confirming by colony PCR. To construct a ΔpspBC derivative of E. coli MC3 that also encodes PspA-His8 (AJDE2892), three fragments were amplified from E. coli genomic DNA by PCR. The first fragment encoding PspA without its first 40 codons (‘pspA’) was amplified with primers 2026/2067, which incorporated a SacI site upstream of ‘pspA’ and a His8 sequence immediately upstream of the pspA stop codon. The second fragment was amplified with primers 2068/2027 so that it had a BglII site preceded by the first 7 codons of E. coli pspB, the pspA-pspB intergenic region, and then a His8 sequence that matched the end of the first fragment. The third fragment was amplified with primers 2028/2029 so that it had a BglII site followed by the last 7 codons of E. coli pspC and ~550 bp of downstream DNA followed by an XbaI site. Then the first two fragments were joined by a SOE PCR reaction via their overlapping His8 sequence using primers 2026/2027, and the resulting fragment was ligated to the third fragment via their BglII sites to generate the in-frame pspBC deletion. The resulting fragment was digested with SacI/XbaI and ligated into sacB’ allelic exchange plasmid pSR47 to generate pAJD2509. Plasmid pAJD2509 was used to exchange the E. coli MC3 chromosomal ‘pspA-his8 ΔpspBC region with ‘pspA-his8 ΔpspBC’ by integration, selection for sucrose-resistant segregants, and confirmation by colony PCR and DNA sequencing.

Native Co-immunoprecipitation Assay—Strains were grown to saturation, diluted to an approximate absorbance of 0.08 (600 nm) in 500 ml of LB broth and shaken at 250 rpm for 2 h at 37 °C. Then 0.02% arabinose (final concentration) was added to induce ysaC expression, and growth continued at 37 °C for an additional 2 h. Cells from the equivalent of 300 ml at an absorbance of 1 (600 nm) were harvested by centrifugation and washed twice with 1 ml of 1.67% Triton X-100 in 1 liter of LB broth with 1 mM IPTG (final concentration). Cells were resuspended in 50 ml of PBS containing 250 μg/ml lysozyme and 0.1% (v/v) Triton X-100 and disrupted by sonication. Unbroken cells were removed by centrifugation at 20,000 x g for 25 min at 4 °C. 1 ml of the supernatant was incubated with 40 μl of glutathione-Sepharose beads (GE Healthcare), incubating them with solubilized DDM membrane lysates from Y. enterocolitica strains, and then washing before elution by boiling in SDS-PAGE sample buffer exactly as described previously except that 0.1% (v/v) Triton X-100 was added to the wash buffer (26).

In Vitro GST and Maltose-binding Protein (MBP) Fusion Protein Interaction Assays—E. coli strain AJDE1770 transformed with plasmid pREP4-groESL (to increase protein solubility) as well as a plasmid encoding GST alone (pGEX-6P-1) or GST-PspCCT (with or without the V125D mutation) was grown to mid-exponential phase at 20 °C in 1 liter of LB broth with 1 mM IPTG (final concentration). Cells were resuspended in 50 ml of PBS containing 250 μg/ml lysozyme and 0.1% (v/v) Triton X-100 and disrupted by sonication. Unbroken cells were removed by centrifugation at 20,000 x g for 25 min at 4 °C. 1 ml of the supernatant was incubated with 40 μl of glutathione-Sepharose (GE Healthcare) for 1 h at 4 °C with gentle rocking. The beads were recovered by centrifugation, washed 3 times with 1 ml of PBS, and then resuspended in 1 ml of PBS containing 5% (v/v) bovine serum albumin. The beads were recovered by centrifugation and washed twice with 1 ml of MBP column buffer (20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA). E. coli strain AJDE1770 transformed with a plasmid encoding MBP-LacZα or MBP-PspCCT was grown in LB broth supplemented with 0.2% (v/v) glucose to saturation at 30 °C. The culture was diluted 100-fold in 500 ml of LB broth supplemented with 0.2% (v/v) glucose and shaken at 250 rpm for 3 h at 37 °C. 0.3 mM IPTG (final concentration) was added, and growth was continued for an additional 2 h at 37 °C. Cells were harvested by centrifugation and resuspended in MBP column buffer and stored overnight at −20 °C. Cells were thawed and disrupted by sonication, and unbroken cells were removed by centrifugation at 20,000 x g for 20 min. The supernatant was incubated with 5 ml of amylose resin (New England Biolabs) for 2 h at 4 °C and then packed into a column. The column was washed with 20 volumes of MBP column buffer. Proteins were adding 30 μl of 50% protein A-Sepharose slurry equilibrated in PBS, rotating for 1 h at 4 °C, followed by removal of the protein A-Sepharose by centrifugation.

A protein A-Sepharose-anti-FLAG complex was made by mixing a 50% protein A-Sepharose slurry with anti-FLAG M2 monoclonal antibody (Sigma; 60 μl of 50% protein A-Sepharose slurry added to 1 μl of anti-FLAG antibody) and incubating for 3 h at 4 °C with gentle rocking. The complex was washed twice with membrane solubilization buffer and then resuspended in the same buffer to restore the 50% protein A-Sepharose slurry concentration. The precleared DDM solubilized membranes were added to 60 μl of the protein A-Sepharose-anti-FLAG complex and incubated overnight at 4 °C with gentle rocking. Proteins were immunoprecipitated by centrifugation, washed 2 times with membrane-solubilization buffer and 3 times with PBS containing 0.1% Triton X-100, resuspended in 60 μl of SDS-PAGE sample buffer, and then boiled for 10 min before analysis by SDS-PAGE and immunoblotting.

GST Fusion Protein Membrane Lysate Pulldown Assays—GST pulldown assays to detect interactions between the C terminus of PspC and various Psp proteins were done by purifying GST derivatives from E. coli strain AJDE1770 onto glutathione-Sepharose beads (GE Healthcare), incubating them with solubilized DDM membrane lysates from Y. enterocolitica strains, and then washing before elution by boiling in SDS-PAGE sample buffer exactly as described previously except that 0.1% (v/v) Triton X-100 was added to the wash buffer (26).
eluted with 5 ml of MBP column buffer supplemented with 10 mM maltose and ten 0.5-ml fractions were collected. Protein concentration in each fraction was determined by the bicinchoninic acid assay (BCA) method (Pierce), and the three fractions with the maximum yield were pooled.

15 μg of MBP-LacZa or MBP-PspBCT was mixed with the GST or GST-PspCCT (with or without the V125D mutation), immobilized onto glutathione-Sepharose, and then incubated for 3 h at 4 °C with gentle rocking. The beads were washed 5 times with 1 ml of 20 mM Tris-HCl, pH 7.4, 500 mM NaCl, 1 mM EDTA and collected by centrifugation. Proteins were recovered by resuspending the beads in 60 μl of 2× SDS-PAGE sample buffer and boiling for 10 min.

**Two-phase GST Fusion Protein Membrane Lysate Pulldown Assays**—GST or GST-PspCCT proteins were isolated from E. coli strain AJDE1770 transformed with plasmid pREP4-groESL and immobilized onto glutathione-Sepharose beads exactly as described above, except that 80 μl of glutathione-Sepharose was used, and the final preparation was washed with PBS instead of MBP-column buffer.

Solubilized membrane lysates were made from Y. enterocolitica strains by growing them to mid-exponential phase at 26 °C in 1 liter of LB broth containing 0.1 mM IPTG. Cells were harvested by centrifugation and resuspended in TBS (50 mM Tris-HCl, pH 8.0, 400 mM NaCl, 2 mM PMSF, 2× complete protease inhibitors) and stored overnight at −20 °C. Cells were thawed, 1 ml of 1.67 mg/ml DNase I per 5 g wet pellet weight was added, and cells were disrupted by sonication. Unbroken cells were removed by centrifugation at 20,000 × g for 20 min, and then the membrane fraction was isolated from the supernatant by centrifugation at 100,000 × g for 2.5 h. The membrane pellet was resuspended in the membrane solubilization buffer described above at a ratio of 10 ml/g, and membrane proteins were solubilized by adding 1% (w/v) DDM (final concentration). Insoluble material was removed by centrifuging for 30 min at 100,000 × g. The supernatant was diluted 10-fold in 50 mM Tris-HCl, pH 7.4, 10% (w/v) glycerol, 150 mM NaCl, and 3 mM β-mercaptoethanol.

50 μl of a solubilized membrane lysate was mixed with the GST or GST-PspCCT immobilized on glutathione-Sepharose beads and rocked gently for 2 h at 4 °C. The beads were washed 5 times with membrane solubilization buffer containing 500 mM NaCl and separated into two equal samples. One sample was mixed with a second DDM-solubilized membrane lysate and rocked gently for 2 h at 4 °C. Then both samples were washed 5 times with membrane solubilization buffer containing 500 mM NaCl, resuspended in 60 μl of 2× SDS-PAGE sample buffer, and boiled to recover all proteins. Equal volumes of the elution samples were analyzed by SDS-PAGE and immunoblotting.

In Vivo Photocross-linking—E. coli AJDE2892 was transformed with plasmid pEVOL-pBpF and either plasmid pAJD2510 or one of its derivatives. Each strain also contained tacp-yscC expression plasmid pAJD26 or the empty tacp expression vector pVLT35. The strains were grown in LB broth to saturation. They were then diluted to an absorbance of ~0.04 (600 nm) in 5 ml of LB broth supplemented with 0.2 mM p-benzoyl-l-phenylalanine (pBpa; Fisher) and grown for 2 h at 37 °C. Then 200 μM IPTG (final concentration) was added to induce pspBC and yscC expression, and growth was continued at 37 °C for an additional 2 h. Cells from the equivalent of 10 ml at an absorbance of 1 (600 nm) were harvested by centrifugation, washed with 1 ml of PBS, pH 7.3 (140 mM NaCl, 2.7 mM KCl, 10.1 mM Na2HPO4, 1.8 mM KH2PO4 adjusted to a pH of 7.3), and then resuspended in 0.45 ml of PBS, pH 7.3, supplemented with 200 μM IPTG (final concentration). The resuspended cells were divided into 2 samples of 0.2 ml each. One sample was transferred to a 96-well microtiter plate and irradiated with ultra violet light (UV) at a wavelength of 365 nm using a EA-240 lamp (Spectroline) for 30 min on ice. The second sample was treated similarly, except that it was not exposed to UV light. The cells were then harvested by centrifugation, resuspended in 80 μl of SDS-PAGE sample buffer, and boiled for 10 min before analysis by SDS-PAGE and immunoblotting.

**β-Galactosidase Assays**—Y. enterocolitica strains were grown to saturation and diluted into 5 ml of LB broth in 18-mm-diameter test tubes to an optical density (600 nm) of ~0.04. The cultures were grown on a roller drum at 37 °C for 2 h. Then, 200 μM IPTG or 0.02% arabinose (final concentration) was added to induce yscC and pspBC or to induce ysaC, respectively. Cells were grown for another 2 h at 37 °C before harvesting. β-Galactosidase enzyme activity was determined at room temperature in permeabilized cells as described (31). Activities are expressed in arbitrary Miller units (32). Individual cultures were assayed in duplicate, and average values from three independent cultures are reported. However, to monitor PsplacZ expression for the native co-immunoprecipitation and photocross-linking experiments, samples were taken directly from the experimental cultures before harvesting and assayed in duplicate with the average reported.

**Polyclonal Antiserum and Immunoblotting**—Proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes by semidy electroblotting. Enhanced chemiluminescent detection followed sequential incubation with polyclonal antiserum or monoclonal antibodies and then goat anti-rabbit IgG or goat anti-mouse IgG (Bio-Rad) horseradish peroxidase conjugate used at 1 in 5000 dilution. Dilutions of polyclonal antiserum were 1 in 10,000 for anti-PspA, anti-PspC, and anti-PspB (20, 24, 33). Monoclonal antibodies were used at 1 in 10,000 dilution for anti-DnaK (Assay Designs), 1 in 5000 for anti-FLAG (Sigma) and anti-His (GenScript), and 1 in 50,000 for anti-MBP (New England Biolabs) and anti-GST (GenScript).

**RESULTS**

The C-terminal Domain of Y. enterocolitica PspC Can Bind to PspA in Vitro Independently of Other Core Psp Proteins—Activation of the Psp system coincides with PspA switching its binding partner from PspF to PspBC (Refs. 24 and 25 and Fig. 1). The C-terminal cytoplasmic domain of PspC (PspCCT) is critical for PspA recruitment, but it is not known if PspCCT and PspA contact each other directly or if instead PspB bridges their association (26, 27, 33). Therefore, we began this work with an extensive PspCCT interaction analysis. The GST, GST-PspCCT, or GST-PspCCT-V125D proteins were bound to glutathione-Sepharose and used as bait to capture
proteins from a detergent-solubilized *Y. enterocolitica* Psp+ membrane lysate (Fig. 2). The PspC V125D mutation abolishes induction of *psp* gene expression *in vivo* and is in the hydrophobic face of a predicted amphipathic helix (Ref. 33; 125 is the amino acid position in full-length PspC). Consistent with our previous report, PspA was captured by GST-PspC<sub>CT</sub>, but this was almost abolished by the V125D mutation (Fig. 2 and Ref. 26). Next, we used a membrane lysate from an isogenic Δ*psspBC* strain in which PspA was the only core Psp protein present. PspA was still captured by GST-PspC<sub>CT</sub> (Fig. 2). Therefore, the association between PspA and GST-PspC<sub>CT</sub> does not require bridging by PspB or by the full-length PspC, that were both in the Psp<sup>+</sup> membrane lysate. Although these experiments cannot rule out bridging by another protein(s), they are consistent with a direct GST-PspC<sub>CT</sub>-PspA interaction, which is compromised by the V125D mutation.

The C-terminal Domain of PspC Can Bind to the Cytoplasmic Domain of PspB Directly *in Vitro*—During the preceding GST-PspC<sub>CT</sub> pulldown experiments with a Psp<sup>+</sup> membrane lysate, we also analyzed the samples by anti-PspB immunoblot. This revealed that PspB was retained by the glutathione-Sepharose-GST-PspC<sub>CT</sub> complex in addition to PspA (Fig. 2). We repeated the experiment with a membrane lysate from an isogenic strain in which PspB was the only core Psp protein present, and PspB was still bound by GST-PspC<sub>CT</sub> (Fig. 2). Furthermore, introduction of the V125D mutation into GST-PspC<sub>CT</sub> prevented PspB binding, implicating the hydrophobic face of the predicted PspC<sub>CT</sub> amphipathic helix as a binding determinant for PspB as well as for PspA. A likely explanation for these results is that PspC<sub>CT</sub> can interact with the cytoplasmic C-terminal domain of PspB (PspB<sup>CT</sup>) directly. Therefore, we tested this hypothesis using a purified *Y. enterocolitica* PspB<sup>CT</sup> fusion protein instead of a complex membrane lysate as the prey in our pulldown assay.

15 µg of MBP-PspB<sup>CT</sup> or MBP-LacZ (negative control) fusion protein was mixed with GST, GST-PspC<sub>CT</sub>, or GST-PspC<sub>CT-V125D</sub> proteins bound to glutathione-Sepharose. After washing, all proteins were eluted in SDS-PAGE sample buffer and analyzed by immunoblot. Consistent with our direct interaction hypothesis, MBP-PspB<sup>CT</sup> bound to GST-PspC<sub>CT</sub> but not to GST or to GST-PspC<sub>CT-V125D</sub> (Fig. 3). In contrast, MBP-LacZ did not bind to any of the GST proteins. Therefore, PspC<sub>CT</sub> can interact directly with PspB<sup>CT</sup> *in vitro*. Furthermore, the observation that the V125D mutation prevents PspC<sub>CT</sub> from interacting with both PspB<sup>CT</sup> and PspA suggests that their binding sites might overlap. This is consistent with a model in which PspC<sub>CT</sub> interacts with PspB<sup>CT</sup> in the OFF state, whereas it interacts with PspA in the ON state (Fig. 1). If all of this is correct, the binding of PspB<sup>CT</sup> or PspA to PspC<sub>CT</sub> might be mutually exclusive. Next, we tested this prediction using a modification of our *in vitro* pulldown assay.

**Evidence That the Interaction of the C-terminal Domain of PspC with PspA or PspB Is Mutually Exclusive *in Vitro*—**The GST pulldown assay was modified to include a second phase. In
PspCCT is mutually exclusive support the hypothesis that the binding of PspA or PspB to PspCCT specific consequence of mixing the GST-PspCCT and PspA complex with a membrane lysate because PspA was not displaced from a membrane lysate in which PspA is the only core Psp protein present; data not shown and see “Discussion”). Cells were grown in non-Psp inducing conditions, and β-galactosidase activities were determined.

When IPTG was not included in the growth medium, leaky expression was sufficient for the GST protein derivatives to be detected by immunoblot (Fig. 5). However, only GST-PspCCT induced Φ(pspA-lacZ) expression (Fig. 5). When IPTG was added, the level of the GST protein derivatives increased, but once again only GST-PspCCT induced Φ(pspA-lacZ) expression. Furthermore, as the GST-PspCCT level increased, Φ(pspA-lacZ) expression also increased, showing that the effect was concentration-dependent. This is consistent with GST-PspCCT sequestering the PspA protein away from PspF. The failure of GST-PspCCT-V125D to induce Φ(pspA-lacZ) expression suggests that the V125D mutation prevents PspA sequestration in vivo. This agrees with the V125D mutation preventing the GST-PspCCT-PspA interaction in vitro (Fig. 2). Therefore, these results support the model that PspCCT is directly involved in sequestering PspA from PspF.

The Activation Mechanism Does Not Involve Complete Dissolution of the PspBC Complex in Vivo—If the PspCCT-PspBCT interaction we detected in vitro occurs in the OFF state of full-length PspBC in vivo, then it must dissociate in inducing conditions so that PspA can bind to PspCCT. A simple hypothesis is that the entire PspB-PspC complex separates when an inducing signal is encountered. PspB and PspC interact in vivo, and it has been suggested that this complex is present in both OFF and ON conditions (20, 30). However, in those experiments the PspBC complex was identified only after treating cells with formaldehyde to cross-link proteins. Therefore, we were concerned that it might have been possible to cross-link PspB and PspC, which are close together but no longer in a stable complex. To address this we developed a co-immunoprecipitation assay to isolate a PspBC complex without using any cross-linker.

The low level of PspBC in uninduced cells makes complex detection difficult, and the massive increase in their concentrations when the Psp system is induced would complicate interpretation. Therefore, we used our Y. enterocolitica strain with the chromosomal pspA operon expressed from a tac promoter (24). Leaky tacp expression leads to higher levels of PspBC than their basal levels in a wild type strain, but they can still regulate Φ(pspA-lacZ) expression normally. However, Psp protein levels remain constant (Fig. 6; Refs. 19, 24, and 25). To facilitate the co-immunoprecipitation, a sequence encoding the 3×FLAG
epitope was fused to the end of the chromosomal pspB or pspC gene. The FLAG tags did not compromise regulation of pspA-lacZ expression (Fig. 6A).

Strains were grown in inducing (+YsaC secretin) or non-inducing (−YsaC secretin) conditions, and PspB-3×FLAG or PspC-3×FLAG was isolated from detergent-solubilized membrane lysate by anti-FLAG immunoprecipitation. PspC co-immunoprecipitated with PspB-3×FLAG regardless of the induction status (Fig. 6B). Similarly, PspB co-immunoprecipitated with PspC-3×FLAG. The control integral inner membrane protein FtsH did not co-immunoprecipitate with PspB-3×FLAG or with PspC-3×FLAG, and when PspB and PspC were not FLAG-tagged neither of them was present in the immunoprecipitates (Fig. 6B). These results suggest that PspB and PspC remain in complex when an inducing signal is encountered, perhaps via interactions between their transmembrane domains as has been suggested (27). If so, an inducing signal might dissociate or alter the interaction between only their C-terminal domains (Fig. 1).

Detection of a Contact between PspA and the C-terminal Domain of PspC upon Activation of the Psp Response in Vivo—Next, we wanted an in vivo test of the hypothesis that PspC<sup>CT</sup> interacts directly with PspA but only when an inducing signal is present. Photo-reactive site-specific cross-linking has been used to map interacting protein domains (e.g. Ref. 34 and 35). The photo-reactive amino acid analog pBpa forms cross-links when cells are exposed to UV light (36, 37). pBpa is incorporated at a specific location in a bacterial protein by introducing an internal amber codon into the gene and decoding it with an orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pair...
E. coli PspBC. Therefore, Y. enterocolitica E. coli 510/H9021 our analysis of the operon controlled by the lactosidase activity. Miller unit activities were 430 regulation. pspBC (w/v) arabinose, and empty vector. Cells were grown E. coli tion. To test this strain we introduced region to its chromosomal Y. enterocolitica PspBC proteins used in all preceding experiments. To facilitate this we modified E. coli Φ(pspa-lacZ) operon fusion strain MC3 by introducing a ΔpspBC in-frame deletion and fusing a His6 tag-encoding region to its chromosomal pspA gene to enhance PsP detection. To test this strain we introduced lacZp expression plasmids encoding E. coli PspBC, Y. enterocolitica PspBC, or the empty vector. Cells were grown +/−YscC secretin, and Φ(pspa-lacZ) expression was monitored by measuring β-galactosidase activity. Miller unit activities were 430 −YscC and 510 +YscC with the empty vector, 480 −YscC and 2400 +YscC with E. coli PspBC, and 510 −YscC and 2,600 +YscC with Y. enterocolitica PspBC. Therefore, Y. enterocolitica PspBC complemented the ΔpspBC mutation and communicated with E. coli PspA, and the His6 tag on PspA did not prevent normal regulation.

Next, we constructed derivatives of the Y. enterocolitica pspBC plasmid in which pspC had a single amber codon (denoted as X) at various positions within the region encoding its predicted C-terminal amphipathic helix. PspC also had a FLAG tag added to its C terminus for specific detection of the pBpa-suppressed protein (explained below). These plasmids were used to transform the E. coli host strain containing pEVOL-pBpF and screened for PspC functionality after growth with pBpa. In several cases, Φ(pspa-lacZ) expression was not induced by YscC, suggesting that the pBpa substitution destroyed PspC regulatory function or that suppression of the amber codon was insufficient (data not shown). However, a plasmid with an amber codon at position 131 (G131X) allowed induction of Φ(pspa-lacZ) expression in response to YscC, which was enhanced to the wild type level when pBpa was present (Fig. 7A). Suppression was also tested by immunoblot analysis. Without pBpa, the FLAG antibody did not detect any PspC-G131X (Fig. 7A). This was expected because the protein should be truncated at position 131, which precedes the region encoding the C-terminal FLAG tag. However, PspC-G131X was detected by FLAG antibody when pBpa was included in the growth medium (Fig. 7A). Furthermore, when anti-PspC polyclonal antiserum was used instead of FLAG antibody, only truncated PspC-G131X was detected without pBpa, whereas both truncated and full-length PspC-G131X were detected with pBpa (Fig. 7A).

To test if pBpa at position 131 of PspC would cross-link to PspA, cells were grown with or without YscC in media supplemented with pBpa and then exposed to UV light. Whole cell lysates were analyzed by SDS-PAGE and immunoblot (Fig. 7B). When YscC was overproduced (+YscC), two UV-dependent slower-migrating species were observed for PspC-G131X. One was also detected when PspC did not have the G131X mutation (but at a much lower level), which suggests that it was pBpa-independent. A similar UV-specific, but pBpa-independent phenomenon has been observed in photocross-linking analysis of another protein (41). Interestingly, the size of this complex was consistent with a PspC dimer (see “Discussion”). Regardless, the other UV-dependent band was specific to PspC-G131X, and its migration suggested that it might be a PspC-PspA complex (predicted mass of ~43 kDa). To test this, the samples were also analyzed by an anti-His6 immunoblot, and Xpa at position 131 of PspC would cross-link to YscC, whereas both derivatives pAJD935 (black bars). Strains were grown in medium containing 0.02% (w/v) arabinose, and β-galactosidase activities were determined. Error bars indicate the positive S.D. B, immunoblot analysis of input lysates and co-immunoprecipitates (Anti-FLAG IP) derived from the strains used in panel A. The polyclonal antisera used for detection are shown at the left.
Importantly, it also prevented the PspC-G131X-PspA cross-link (Fig. 7B). This corroborates the effect of the V125D mutation on the GST-PspC<sup>CT</sup>-PspA interaction in vitro (Fig. 2) and further demonstrates that a direct contact between PspC<sup>CT</sup> and PspA is essential for induction of the Psp response.

A Single Position within the C-terminal Domain of PspC Cross-links to PspB in the OFF State—Our in vitro experiments suggested that PspC<sup>CT</sup> interacted with PspB<sup>CT</sup> or PspA in a mutually exclusive manner (Fig. 4). This led to the hypothesis that the PspC<sup>CT</sup>-PspB<sup>CT</sup> interaction might represent the OFF state, whereas the PspC<sup>CT</sup>-PspA interaction is the ON state. In our final experiment we used photocross-linking to investigate if this predicted switch in the PspC<sup>CT</sup>-PspA interaction partner could be observed in vivo.

pBpa at position 131 in PspC cross-linked to PspA but not to PspB, making it unsuitable to test our hypothesis (Fig. 7 and data not shown). Therefore, we constructed additional PspC<sup>CT</sup>-pBpa substitution mutants and screened them for regulatory function and for photocross-linked complexes corresponding in size to both PspC-PspA and PspC-PspB (data not shown).

pBpa at position 109 met these criteria. However, the amount of the PspC-PspB complex was not very different in OFF (−YscC) and ON (+YscC) conditions (data not shown). Although this could be interpreted to mean that our model is incorrect, we noticed a phenomenon that might make such a conclusion unsafe. pspBC were expressed from the lacZp promoter to maintain constant expression +/−YscC. Despite this, the amount of PspB and PspC protein was higher in +YscC cells (e.g. Fig. 7Aii, and data not shown). We do not understand why, and this did not occur when the lacZp-pspBC plasmid was in Y. enterocolitica (data not shown). Regardless, a change in the total amount of PspBC complicates interpretation of any differences in the PspB-PspC complex level +/−YscC. To circumvent this we took advantage of our previous discovery of PspB and PspC mutations that cause constitutive Φ(pspA-lacZ) expression (33). Combining one of these mutations with PspC-Y109X should lock the PspBC regulatory complex into the ON state. Then, PspC-Y109X cross-linked complexes could be compared in the OFF state (i.e. without a constitutive mutation) to the ON state (i.e. with a constitutive mutation) but always without YscC so that PspBC protein levels would be similar in all strains. We chose the W23R mutation in PspB (33) so that the PspC protein would be identical in the strains being compared.

As expected, in cells with PspC-Y109X grown with pBpa, the PspB-W23R mutation increased pBpa/UV-dependent induction of the pspBC operon when compared with cells with wild type PspB (Fig. 8A). These strains were exposed to UV light, and cell lysates were analyzed by SDS-PAGE and immunoblot (Fig. 8B). In cells with PspC-Y109X and wild type PspB (OFF state), a pBpa/UV-dependent induction of the pspBC operon was not observed (Fig. 8B). This corroborates the effect of the V125D mutation on the GST-PspC<sup>CT</sup>-PspA interaction in vitro (Fig. 2) and further demonstrates that a direct contact between PspC<sup>CT</sup> and PspA is essential for induction of the Psp response.

**Figure 7.** In vivo photocross-linking analysis reveals an interaction between PspA and the C-terminal domain of PspC upon activation of the Psp response. A, panel i, functional analysis of PspC with pBpa incorporated at position 131. E. coli strain AJDE2892 contained a lacZp expression plasmid encoding Y. enterocolitica PspB and PspC-FLAG (PspC-FLAG) or derivatives with an amber mutation at position 131 of PspC (G131X) only or also with the V125D mutation. Strains also contained the empty tac promoter expression vector pVL135 (gray bars) or the yscC<sup>−</sup> derivative pAJD126 (black bars). All strains contained plasmid pEVOl-pBpF, which encodes a pBpa aminoacyl-tRNA synthetase/suppressor tRNA. Strains were grown in medium containing 200 µM IPTG with or without 200 µM pBpa as indicated, and β-galactosidase activities were determined. Error bars indicate the positive S.D. A, panel ii, immunoblot analysis of the strains used in A, panel i. Whole cell lysates of strains grown as described for A, panel i were analyzed by SDS-PAGE and immunoblotting with anti-FLAG monoclonal antibody or anti-PspC polyclonal antiserum. In our final experiment we used photocross-linking to investigate if this predicted switch in the PspC<sup>CT</sup>-PspA interaction partner could be observed in vivo.

B, panel i, functional analysis of PspC using anti-FLAG monoclonal antibody to determine PspC interaction partners. Whole cell lysates of strains grown as described for A, panel i were analyzed by SDS-PAGE and immunoblotting with anti-FLAG monoclonal antibody or anti-PspC polyclonal antiserum.

B, panel ii, immunoblot analysis of the strains used in A, panel ii. Whole cell lysates of strains grown as described for A, panel ii were analyzed by SDS-PAGE and immunoblotting with anti-FLAG monoclonal antibody or anti-PspC polyclonal antiserum.
complex was recognized by anti-FLAG (PspC) and by anti-PspB antibodies, and it corresponded in size to PspC/PspB (24 kDa; Fig. 8B). However, in PspC-Y109X PspB-W23R cells (ON state), the PspC/PspB complex was reduced to a barely detectable level, whereas a pBpa/UV-dependent complex that corresponded in size to PspC/PspA was recognized by anti-FLAG (PspC) and by anti-His6 (PspA) antibodies. Once again, we extended the experiment to test the effect of the PspC V125D mutation, which abolished both the PspC-Y109X-PspA and PspC-Y109X-PspB cross-links (Fig. 8B). This is completely consistent with the in vitro binding experiments (Figs. 2 and 3).

These experiments show that the same residue within PspCCT cross-links predominantly to PspB in the absence of a Psp-inducing signal but to PspA upon induction. The PspCCT-PspB interaction might be part of an inhibitory mechanism that occludes the PspA-binding site in non-Psp-inducing conditions. Most significantly, these data provide the first demonstration that PspBC do exist in different OFF and ON states in vivo.

### DISCUSSION

Activation of the Psp response requires PspA to interact with the PspBC complex at the cytoplasmic membrane (24, 25). Therefore, we proposed that PspBC alternate between OFF and ON states, with only the latter able to bind PspA (26). Here, our findings from both in vitro and in vivo approaches have supported this model and revealed that regulation of the Psp response is centered around a PspCCT partner-switching mechanism.

The GST-PspCCT membrane lysate pulldown experiments revealed that a PspCCT-PspA complex could form in vitro even in the absence of PspB (Fig. 2). This suggests a direct interaction, but intriguingly we have found that purified PspA is no longer captured by GST-PspCCT.3 The purification process might have inactivated its ability to bind to PspCCT, because purification is known to affect the physical state of PspA (42). Alternatively, PspA might require contact with membrane fragments in the lysate, in addition to PspCCT, because PspA can bind to phospholipid membranes directly (24, 43). Regardless, we confirmed a direct PspCCT-PspA interaction by in vivo photocross-linking (Fig. 7). This is the first demonstration of a direct contact between PspA and PspC. Other observations indicate that this interaction is essential to activate the Psp response. First, the V125D mutation prevents full-length PspC

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3 J. Flores-Kim and A. J. Darwin, unpublished data.
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from inducing the Psp response in vivo, prevents the PspC<sup>CT</sup>-PspA interaction in vitro, and also prevents PspC<sup>CT</sup> from cross-linking to PspA in vivo (Figs. 2 and 7–8 and Refs. 26 and 33). Second, overproduction of only the C-terminal domain of Y. enterocolitica PspC in vivo was sufficient to activate the Psp response in a concentration-dependent manner, and this was also prevented by the V125D mutation (Fig. 5). Consistent with our findings, it has also been reported that the C-terminal domain of E. coli PspC induced Φ<sub>pspA-lac2Δ</sub> operon fusion expression but only if pspB was co-expressed from the same plasmid (27). It is not clear why pspB co-expression was required in that case.

We also detected a direct interaction between PspB<sup>CT</sup> and PspC<sup>CT</sup> in vitro as well as a photocross-link between PspC<sup>CT</sup> and PspB in vivo (Figs. 3 and 8). Therefore, PspC<sup>CT</sup> can interact directly with both PspA and PspB. These interactions appeared to be mutually exclusive in vitro (Fig. 4) and occurred in different induction states in vivo (Fig. 8). This supports a model where the OFF state of PspBC involves an interaction between their C-terminal domains, which might help to block the access of PspA to its binding site. In other words, the PspC<sup>CT</sup>-PspB<sup>CT</sup> interaction might be an inhibitory complex. This means that negative regulation of the Psp system might involve two different inhibitory interactions, one between PspA and PspF and the other between PspB<sup>CT</sup> and PspC<sup>CT</sup>. Therefore, activation of the Psp system involves two partner switching mechanisms: PspA switching its partner from PspF to PspC and PspC<sup>CT</sup> switching its partner from PspB to PspA (Fig. 1B).

An alternative model for negative regulation has been proposed in which PspC is a bitopic membrane protein with its C-terminal domain in the periplasm in non-inducing conditions (27). An inducing signal flips the topology, positioning the C terminus in the cytoplasm to bind PspA. This is an exciting model, but so far several observations argue against it. First, analysis of Y. enterocolitica PspC showed that it is a polytopic membrane protein with both termini in the cytoplasm regardless of the Psp system induction status (26). Second, cysteine substitutions throughout the C-terminal domain of PspC did not form disulfide bonds without an oxidative catalyst, suggesting they were not in the oxidizing environment of the periplasm (33). Third, our finding that PspC<sup>CT</sup> photocross-links to PspB in non-inducing conditions suggests that it is not in the periplasm (Fig. 8).

PspC<sup>CT</sup> is predicted to form a conserved leucine zipper-like amphipathic helix (30, 44). If it does, the V125D mutation would place a charged amino acid into its hydrophobic face. The fact that this mutation prevents the binding of both PspA and PspB suggests that the hydrophobic face is an important binding determinant for both proteins. An obvious possibility is that the hydrophobic face is a common binding site for PspA and PspB. However, another possibility is that the hydrophobic property is important for dimerization of adjacent PspC<sup>CT</sup> domains. Indeed, previous oxidative cross-linking suggested that the hydrophobic face could be a PspC dimerization interface (30). In this scenario, PspB or PspA might only be able to bind to PspC<sup>CT</sup> dimers, which the V125D mutation might disrupt. Interestingly, our in vivo cross-linking detected a complex that might be a PspC dimer (Figs. 7B and 8B). A band of the same size was also barely detectable when PspC did not contain the cross-like pBpa (Fig. 7B). SDS-resistant PspC dimers have been reported in E. coli (45). Regardless, if the PspC-G131X complex we detected is a dimer, then dimerization of PspC<sup>CT</sup> might increase when the Psp system is induced (Fig. 7B). Whether or not this has any implications for the regulatory mechanism is not yet known.

Here, we have shown that one role played by PspB might be negative regulation, which is achieved by tethering PspC<sup>CT</sup>. However, PspB is also essential to activate the Psp system in response to most stimuli (e.g. Refs. 20, 46, and 47). A GST-PspB<sup>CT</sup> fusion protein does not pull PspA down from a membrane lysate in which PspA is the only core protein (45). This suggests that PspB and PspA might not interact directly. The requirement of PspB for activation might have a trivial explanation because it protects PspC from degradation by the FtsH protease (48). However, it remains possible that PspB plays a more active role in inducing the Psp system.

Another intriguing area for future investigation is the role of the N-terminal cytoplasmic domain of PspC (PspC<sup>NT</sup>). Deletion of PspC<sup>NT</sup> causes constitutive activation (33). This implies that PspC<sup>NT</sup> is required for negative regulation. Therefore, PspC<sup>NT</sup> could be part of the inhibitory complex containing PspB<sup>CT</sup> and PspC<sup>CT</sup>. We have attempted to investigate if PspC<sup>NT</sup> can interact with other Psp proteins. However, attempts to overproduce GST-PspC<sup>NT</sup>, MBP-PspC<sup>NT</sup>, and PspC<sup>NT</sup>-MBP fusion proteins in vivo were unsuccessful. Only a PspC<sup>NT</sup>-GST fusion protein could be produced and purified, but it did not capture any core Psp proteins in pulldown assays. Of course, more experiments are needed to investigate if these negative data are meaningful. Nevertheless, if PspC<sup>NT</sup> does not form stable interactions with other Psp proteins, how could it be involved in negative regulation? One possibility is steric hindrance, with PspC<sup>NT</sup> preventing PspA from approaching the PspC<sup>CT</sup>-PspB<sup>CT</sup> complex. An inducing signal might cause PspC<sup>NT</sup> to shift its position, allowing PspA to invade and displace PspB<sup>CT</sup> from PspC<sup>CT</sup>. This might explain why a random screen isolated numerous mutations throughout PspB and PspC (except for within PspC<sup>CT</sup>) that caused constitutive activation (33). Perhaps any mutation that causes even a slightly aberrant conformation of PspB or PspC interferes with the precise relative positioning of PspC<sup>NT</sup>

In summary, we have developed in vitro and in vivo approaches to provide important new insights into how the Psp response is controlled, including the first indication into how PspBC might be held in their OFF state. First, we have demonstrated a direct contact between PspA and PspC, which is essential for activation of the Psp response. Second, we have discovered a direct contact between the C-terminal domains of PspB and PspC. Third, our discoveries that these interactions are mutually exclusive in vitro and occur in different activation states in vivo provide the first description of OFF and ON states for the PspBC complex. Importantly, our findings have revealed that a PspC<sup>CT</sup> partner-switching mechanism controls the activation status of the Psp response.
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