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FtsH-Dependent Degradation of Phage Shock Protein C in *Yersinia enterocolitica* and *Escherichia coli*[▽]

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The widely conserved phage shock protein (Psp) extracytoplasmic stress response has been studied extensively in *Escherichia coli* and *Yersinia enterocolitica*. Both species have the PspF, -A, -B, and -C proteins, which have been linked to robust phenotypes, including *Y. enterocolitica* virulence. PspB and PspC are cytoplasmic membrane proteins required for stress-dependent induction of *psp* gene expression and for bacterial survival during the mislocalization of outer membrane secretin proteins. Previously, we reported that *Y. enterocolitica* PspB functions to positively control the amount of PspC by an uncharacterized posttranscriptional mechanism. In this study, we have discovered that the cytoplasmic membrane protease FtsH is involved in this phenomenon. FtsH destabilizes PspC in *Y. enterocolitica*, but coproduction of PspC with its binding partner PspB was sufficient to prevent this destabilization. In contrast, FtsH did not affect any other core component of the Psp system. These data suggested that uncomplexed PspC might be particularly deleterious to the bacterial cell and that FtsH acts as an important quality control mechanism to remove it. This was supported by the observation that toxicity caused by PspC production was reduced either by coproduction of PspB or by increased synthesis of FtsH. We also found that the phenomenon of FtsH-dependent PspC destabilization is conserved between *Y. enterocolitica* and *E. coli*.

The phage shock protein (Psp) system is a highly conserved extracytoplasmic stress response triggered by events likely to compromise the integrity of the cytoplasmic membrane (reviewed in references 5, 20, and 35). The system has been studied extensively in the Gram-negative bacteria *Escherichia coli* and *Yersinia enterocolitica*, but homologues of some of its components are also present in many Gram-negative/positive bacteria, as well as archaea and plants (e.g., see references 3, 29, 43, and 45). The Psp system is required for the virulence of *Y. enterocolitica* and *Salmonella enterica* serovar Typhimurium (6, 23) and for biofilm formation in *E. coli* (2), and its production is highly induced during macrophage infection by *S. enterica* serovar Typhimurium and *Shigella flexneri* (10, 30).

The complement of Psp proteins differs between species, but *E. coli* and *Y. enterocolitica* each have PspF, -A, -B, and -C. Removing any of these causes a robust phenotype, meaning that they can perhaps be considered the core Psp system in these two species. PspF is a transcription factor that binds to the *pspABC* control region and activates its σ^{54}-dependent promoter (12, 22). The PspA, -B, and -C proteins form a putative signal transduction pathway that modulates PspF activity. The integral cytoplasmic membrane proteins PspB and PspC respond to Psp-inducing stress by causing the sequestration of PspA to the cytoplasmic membrane (46). This presumably prevents PspA from forming an inhibitory complex with PspF in the cytoplasm (8, 9).

In addition to their regulatory roles, the increase in PspABC concentration after an inducing trigger is encountered reflects the fact that these proteins also have physiological roles in mediating stress tolerance. PspA has been linked to maintaining the proton motive force in *E. coli* (e.g., see references 26 and 27). In *Y. enterocolitica*, PspB and PspC (but not PspA) are essential for survival when outer membrane secretin proteins mislocalize within the cell envelope, which is a potent and specific Psp-inducing trigger (15, 32). Secretin sensitivity explains why a *pspC* null mutant is sensitive to native Ysc type 3 secretion system production and also avirulent in mice (6).

The critical roles of PspB and PspC have motivated us to investigate their function in *Y. enterocolitica*. During a genetic investigation, we reported that the steady-state concentration of PspC was higher when PspB was present, even when the genes were expressed from a non-*psp* promoter (15). We speculated that this phenomenon involved PspB protecting PspC from proteolysis, consistent with the fact that these two proteins interact *in vivo* (14, 32). In *E. coli*, the essential cytoplasmic membrane protein FtsH is a protease with several known targets (19). These targets include the integral cytoplasmic membrane proteins SecY and AtpB when they are produced in the absence of their normal binding partners (1, 24). Therefore, FtsH represented a promising candidate to destabilize PspC in the absence of its binding partner, PspB.

In this study, we tested the above hypothesis. Our data reveal that FtsH destabilizes PspC in *Y. enterocolitica* and that coproduction with PspB is sufficient to prevent this. FtsH does not affect any other core component of the Psp system. Therefore, we speculated that uncomplexed PspC is deleterious to the bacterial cell and that FtsH acts as a quality control mechanism to rapidly remove it. Consistent with this, toxicity caused by PspC production could be reduced by PspB coproduction or by increased production of FtsH. Our studies also indicate that the phenomenon of FtsH-mediated PspC destabilization is conserved between *Y. enterocolitica* and *E. coli*.

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TABLE 1. Strains and plasmids

Strain or plasmid	Genotype/features	Reference or source
Bacterial strains		
E. coli K-12		
MG1655	F$^-$ *rph-1*	16
AR3289	F$^-$ IN(*rrnD-rrnE*)*1 sfhC21 zad220*::Tn*10*	40
AR3291	F$^-$ IN(*rrnD-rrnE*)*1 sfhC21 zad220*::Tn*10* Δ*ftsH3*::*kan*	40
Y. enterocolitica		
AJD3[a]	Δ*yenR* (r$^-$ m$^+$)	25
AJD1171	Δ*yenR* (r$^-$ m$^+$) Δ(*pspF-ycjF*) Δ*pspG*	32
YVM859	Δ*yenR* (r$^-$ m$^+$) Δ*pspBC*	32
AJD4466[b]	Δ*yenR* (r$^-$ m$^+$) Δ*ftsH* [pAJD2105]	This study
AJD4490[b]	Δ*yenR* (r$^-$ m$^+$) Δ*pspBC* Δ*ftsH* [pAJD2105]	This study
AJD4625[b]	Δ*yenR* (r$^-$ m$^+$) Δ(*pspF-ycjF*) Δ*pspG* Δ*ftsH* [pAJD2105]	This study
Plasmids		
pBAD18-Kan	Km^r ColE1 *ori*, *araBp* expression vector	17
pBAD33	Cm^r p15A *ori*, *araBp* expression vector	17
pSR47S	Km^r R6K *ori*, *mob*$^+$ (RP4) *sacB*$^+$	33
pVLT35	Sm^r Sp^r RSF1010 *ori*, *tacp* expression vector	7
pWSK29	Ap^r pSC101 *ori*, *lacZp* expression vector	44
pAJD267	*araBp-pspF*$^+$ in pBAD18-Kan	6
pAJD268	*araBp-pspA*$^+$ in pBAD18-Kan	This study
pAJD1011	*araBp-pspBC*$^+$ in pBAD33	32
pAJD1014	*araBp-pspBC*$^+$ in pBAD18-Kan	This study
pAJD1041	*araBp-pspB*$^+$ in pBAD33	32
pAJD1085	*araBp*-Δ*pspB pspC*$^+$ in pBAD33	This study
pAJD2065	*lacZp*-Δ*pspB pspC*$^+$ in pWSK29	This study
pAJD2105	*tacp-ftsH*$^+$ in pVLT35	This study
pAJD2138	*araBp-pspB*$^+$ in pBAD18-Kan	This study
pAJD2139	*araBp*-Δ*pspB pspC*$^+$ in pBAD18-Kan	This study
pAJD2142[c]	*lacZp*-Δ*pspB*^{EC} *pspC*$^{EC+}$ in pWSK29	This study

[a] AJD3 is a virulence plasmid-cured derivative of strain JB580v (25). All other *Y. enterocolitica* strains listed are derivatives of AJD3.

[b] *Y. enterocolitica* Δ*ftsH* strains contained pAJD2105 and required IPTG for viability.

[c] pAJD2142 insert is *E. coli* DNA. All other plasmid inserts are *Y. enterocolitica* DNA.

MATERIALS AND METHODS

Bacterial strains, plasmids, and routine growth. Strains and plasmids are listed in Table 1. Primer sequences will be supplied upon request (please contact the corresponding author). PCR-generated fragments were verified by DNA sequencing. Strains were routinely grown in Luria-Bertani (LB) broth or on LB agar plates (34). Antibiotics were used as previously described (31).

Polyclonal antisera and immunoblotting. Lysates derived from equivalent amounts of bacterial cells (determined by optical density of cultures) were separated by SDS-PAGE on gels containing 12.5 to 15% polyacrylamide and then transferred to nitrocellulose by electroblotting. Equal loading was confirmed by total protein staining of the nitrocellulose with Ponceau S. Enhanced chemiluminescent detection followed sequential incubation with a diluted polyclonal antiserum followed by goat anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad) used at 1 in 5,000. Dilutions of previously described polyclonal antisera were 1 in 20,000 for anti-PspA and anti-PspF (46), 1 in 20,000 to 500,000 for anti-PspB (15), 1 in 5,000 to 40,000 for anti-PspC (32), and 1 in 10,000 for anti-FtsH (46). These antisera had been raised against *Y. enterocolitica* antigens but were also able to recognize the corresponding *E. coli* proteins.

Strain and plasmid constructions. *tacp-ftsH* expression plasmid pAJD2105 was constructed by amplifying the *ftsH* gene from *Y. enterocolitica* genomic DNA and cloning it into plasmid pVLT35. *araBp* (pBAD33 and pBAD18-Kan derivatives) and *lacZp* (pWSK29 derivative) expression plasmids carrying *Y. enterocolitica psp* genes were constructed by transferring inserts from previously described plasmids (6, 15, 32) using standard cloning procedures. pWSK29 carrying *E. coli* Δ*pspB pspC*$^+$ was made by amplifying the *pspB pspC* region from the chromosome of *E. coli* strain MG1655 using an upstream primer that introduced an in-frame deletion within *pspB* by loop-out mutagenesis and a second primer that annealed immediately downstream of *pspC*. The primers incorporated SacI (upstream) and XbaI (downstream) sites, which were used to clone the fragment into pWSK29. The design of this *E. coli* Δ*pspB pspC*$^+$ plasmid was analogous to the previously described expression plasmids with Δ*pspB pspC*$^+$ and *pspBC*$^+$ inserts from *Y. enterocolitica* (15). Including the *pspB* in-frame deletion ensures that both plasmids maintain the overlapping stop and start codons between the *pspB* and *pspC* open reading frames and the putative ribosome binding sites upstream of each gene. This avoids potential translational polarity effects on *pspC* expression, which is important when comparing PspC production from *pspC*$^+$ and *pspBC*$^+$ plasmids. The *pspBC* genes are arranged similarly in the genomes of *Y. enterocolitica* and *E. coli*.

The *Y. enterocolitica* Δ*ftsH* in-frame deletion mutation was made using the *sacB*$^+$ suicide plasmid pSR47S; plasmid construction and mutagenesis procedures were identical to those described previously (43a). Mutagenesis was done in strains containing the isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible *ftsH*$^+$ expression plasmid pAJD2105, which were grown in the presence of 50 μM IPTG. Deletion of the chromosomal *ftsH* gene was confirmed by colony PCR analysis and an IPTG-dependent growth phenotype.

Determination of *Y. enterocolitica* FtsH-dependent growth. Strains containing empty *tacp* expression plasmid pVLT35 or the derivative encoding FtsH (pAJD2105) were grown to saturation (optical density at 600 nm, >3) at 26°C in LB broth containing 1 μM IPTG. Four microliters of undiluted and serial 10-fold dilutions (10^{-1} to 10^{-7}) was spotted onto LB agar containing appropriate antibiotics with or without 50 μM IPTG. Plates were incubated at 26°C for 30 h.

Measurement of PspC steady-state level and *in vivo* degradation in *E. coli*. Saturated cultures were diluted into 6 ml of LB broth (+1 mM IPTG to induce *lacZp-pspC* expression) in 18-mm-diameter test tubes to an optical density (600 nm) of 0.1. The cultures were grown on a roller drum at 37°C for 3 h. Then, translation was blocked by adding 100 μg/ml chloramphenicol and incubation continued at 37°C. Samples for immunoblot analysis were harvested immediately before and 2 h after chloramphenicol addition.

FtsH depletion/overproduction and measurement of Psp protein steady-state level and *in vivo* degradation in *Y. enterocolitica*. Δ*ftsH* strains containing *tacp-ftsH* plasmid pAJD2105 were grown to saturation in LB broth plus 1 μM IPTG. These cultures were diluted into 5 ml of LB broth without IPTG in 18-mm-diameter test tubes to an optical density (600 nm) of 0.15. The cultures were grown on a roller drum at 26°C for 4 to 6 h. Overproduction of FtsH was achieved by including 50 to 100 μM IPTG in the growth medium. For experiments to measure PspB and PspC steady-state levels produced from *araBp-pspB/C* expression plasmids (pBAD33 derivatives), the growth medium contained 0.01% arabinose.

To monitor PspC degradation over time, strains contained *araBp-pspC* expression plasmid pAJD2139 (pBAD18-Kan derivative). Saturated cultures were diluted into 50 ml of LB broth in 125-ml flasks to an optical density (600 nm) of 0.15. The cultures were shaken at 200 rpm and 26°C for 4 h. *pspC* expression was then induced by addition of 0.02% arabinose. Thirty minutes after arabinose addition, translation was blocked by adding 100 μg/ml chloramphenicol. Samples were taken at different time points for immunoblot analysis. Experiments to compare PspA, -B, -C, and -F stabilities were done similarly except that after chloramphenicol addition a single sample was taken for analysis after 1 h.

Determination of the effect of FtsH overproduction on PspC-dependent toxicity. Saturated cultures were diluted into 5.5 ml of LB broth, containing 0.05% arabinose to induce *pspB/C* expression and 50 μM IPTG to induce *ftsH* expression, in 18-mm-diameter test tubes so that the initial optical density (600 nm) was approximately 0.2. The cultures were grown on a roller drum at 26°C for 8 h, and 0.1-ml samples were removed at hourly intervals for optical density measurement. At the 4-h time point, a 1-ml sample was removed for immunoblot analysis.

RESULTS

***Y. enterocolitica* and *E. coli* PspC proteins are stabilized in an *E. coli* Δ*ftsH* mutant.** We reported previously that PspB elevated the PspC protein concentration in *Y. enterocolitica* via an

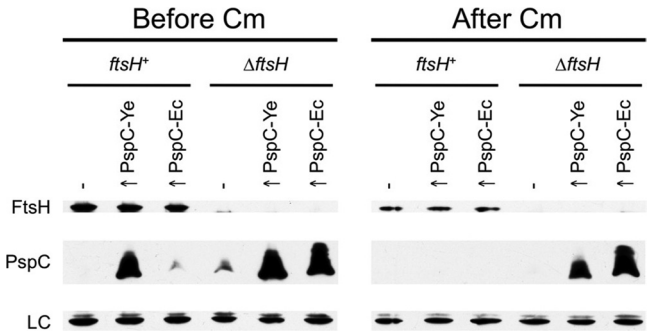

FIG. 1. Overproduced *Y. enterocolitica* and *E. coli* PspC proteins are stabilized in an *E. coli* Δ*ftsH* mutant. *E. coli* strain AR3289 (*ftsH*$^+$) and its isogenic Δ*ftsH* derivative AR3291 (Δ*ftsH*) contained empty *lacZp* expression plasmid pWSK29 (−) or derivatives encoding *Y. enterocolitica* PspC (↑ PspC-Ye) or *E. coli* PspC (↑ PspC-Ec). Cells were grown at 37°C for 3 h (Before Cm). Chloramphenicol was then added to block translation, followed by incubation for a further 2 h at 37°C (After Cm). Cell lysates were separated by SDS-PAGE and analyzed by anti-FtsH and anti-PspC immunoblotting. An unidentified *E. coli* protein that cross-reacted with the anti-FtsH serum served as a convenient loading control (LC).

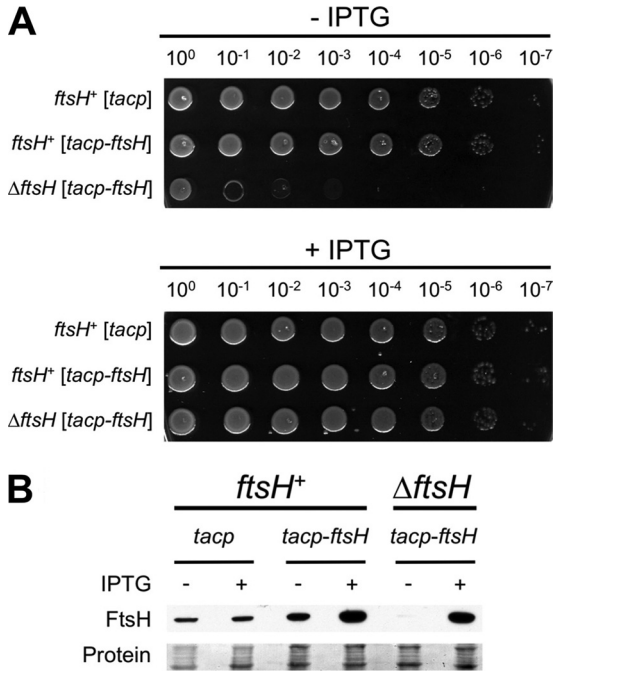

FIG. 2. FtsH depletion prevents growth of *Y. enterocolitica*. (A) Growth phenotypes of *ftsH*$^+$ and Δ*ftsH* strains. *ftsH*$^+$ and Δ*ftsH* strains contained empty *tacp* expression plasmid pVLT35 (*tacp*) or the derivative encoding FtsH (*tacp-ftsH*). Serial dilutions of saturated cultures were spotted onto LB agar with or without 50 μM IPTG and incubated at 26°C for 30 h. (B) Anti-FtsH immunoblot analysis of total cell lysates from the strains used in panel A. "Protein" indicates a Ponceau S stain of the nitrocellulose membrane used for immunoblot analysis (the region containing FtsH).

uncharacterized posttranscriptional mechanism (15). A likely explanation is that PspB protects PspC from proteolysis. This would be analogous to the *E. coli* integral cytoplasmic membrane proteins SecY and AtpB, which are degraded by the FtsH protease in the absence of their normal binding partners (1, 24). FtsH has not been studied in any *Yersinia* species. Therefore, as a preliminary test for a link between FtsH and PspC, we took advantage of *E. coli ftsH*$^+$ and Δ*ftsH* strains. Both strains have the *sfhC21* allele of *fabZ*, which suppresses the lethality caused by loss of FtsH (37), so that the only genetic difference between them is their *ftsH*$^+$ versus Δ*ftsH* genotype.

Plasmids with *Y. enterocolitica* or *E. coli pspC* genes expressed from the *lacZ* promoter were introduced into the *E. coli ftsH*$^+$ and Δ*ftsH* strains to overproduce PspC relative to the native *E. coli* Psp proteins encoded on the chromosome. Overproduced *E. coli* PspC was barely detectable in the *ftsH*$^+$ strain but was abundant in the Δ*ftsH* mutant (Fig. 1, left panel). In fact, even the endogenous *E. coli* PspC was detectable in the Δ*ftsH* strain but not in its *ftsH*$^+$ parent (compare empty vector lanes in the left panel of Fig. 1). The steady-state level of overproduced *Y. enterocolitica* PspC was perhaps only marginally higher in the Δ*ftsH* mutant (Fig. 1). This might be because *Y. enterocolitica* PspC is an imperfect substrate for *E. coli* FtsH (although a trivial explanation would be different efficiencies of the two expression plasmids). However, when translation was inhibited with chloramphenicol, both the overproduced *Y. enterocolitica* and *E. coli* PspC proteins were eliminated from the *ftsH*$^+$ strain but remained abundant in the Δ*ftsH* mutant for at least 2 h (Fig. 1, right panel).

These data indicated that *E. coli* FtsH destabilized both the *E. coli* and *Y. enterocolitica* PspC proteins. Therefore, we were motivated to proceed with an investigation into the role of FtsH in our model organism, *Y. enterocolitica*.

FtsH is essential in *Y. enterocolitica*. The *Y. enterocolitica ftsH* gene (originally annotated as YE0428) encodes a protein that is 92% identical to *E. coli* K-12 FtsH and is in a similar genomic context (data not shown). Predictions indicate that the *E. coli* and *Y. enterocolitica* FtsH proteins are both 647 amino acids in length, including a 26-amino-acid N-terminal Sec-dependent signal sequence, and that both *ftsH* genes have a TTG initiation codon (data not shown). FtsH is essential in *E. coli*, and we anticipated that this might be the case in *Y. enterocolitica*. Therefore, we used a standard *sacB*$^+$ suicide vector approach to delete *Y. enterocolitica ftsH* in a strain with a *tacp-ftsH* complementation plasmid. Growth of the resulting Δ*ftsH* mutant was dependent on *tacp-ftsH* expression, which confirmed that the gene is essential in *Y. enterocolitica* (Fig. 2A). Anti-FtsH immunoblot analysis revealed successful depletion of the FtsH protein when IPTG was omitted from the growth medium (Fig. 2B; note that a low level of FtsH protein was detectable upon prolonged exposure). These experiments were done at 26°C, which is the optimum growth temperature for *Y. enterocolitica* in the laboratory. At 37°C, depletion of FtsH was less efficient (data not shown), perhaps because the *tacp* promoter is more leaky at this temperature. Furthermore, overexpression of *ftsH* was toxic at 37°C, which made it impossible to find conditions under which the *tacp-ftsH* plasmid fully complemented the growth defect. Attempts to use an *araBp-ftsH* plasmid were also unsuccessful. Therefore, for our subsequent experiments we used the *tacp-ftsH* plasmid and a 26°C growth temperature, which allowed successful depletion or overproduction of FtsH and full restoration to a wild-type growth phenotype (Fig. 2). Importantly, *Y. enterocolitica psp*

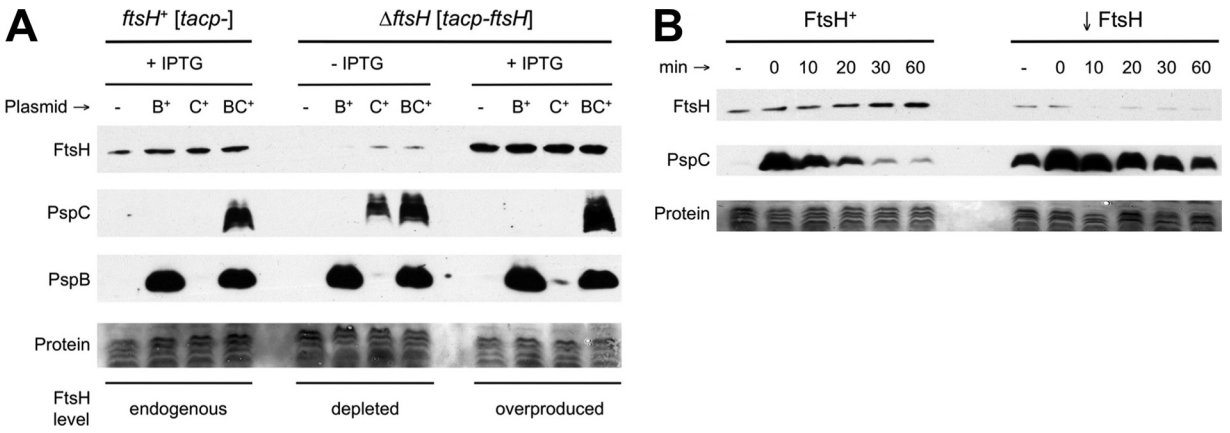

FIG. 3. FtsH-dependent degradation of PspC in *Y. enterocolitica*. (A) FtsH depletion increases PspC steady-state level when PspB is absent. Δ*pspBC ftsH*$^+$ and Δ*pspBC* Δ*ftsH* strains contained empty *tacp* expression plasmid pVLT35 (*tacp*-) or the derivative encoding FtsH (*tacp-ftsH*), respectively. Strains also contained empty *araBp* expression plasmid pBAD33 (−) or derivatives encoding PspB (B$^+$), PspC (C$^+$), or PspBC (BC$^+$). Strains were grown with or without 100 μM IPTG to induce *tacp-ftsH* expression, and cell lysates were analyzed by anti-FtsH, anti-PspC, and anti-PspB immunoblotting. "Protein" indicates a Ponceau S stain of the nitrocellulose membrane used for immunoblot analysis (the region containing PspC). (B) PspC degradation assay. Δ*pspBC ftsH*$^+$ (FtsH$^+$) and Δ*pspBC* Δ*ftsH* (↓ FtsH) strains contained *tacp-ftsH* plasmid pAJD2105 but were grown without IPTG to repress its expression. *araBp-pspC* expression from pAJD2139 was induced for 30 min by addition of 0.02% arabinose, after which translation was blocked by adding chloramphenicol. Samples were taken at different times for immunoblot analysis. "−" is a sample taken before inducing *araBp-pspC* expression. Subsequent samples were taken at the times indicated in minutes after adding chloramphenicol.

gene expression is regulated similarly at 26°C and 37°C and *psp* null strains are sensitive to secretin production at both temperatures (data not shown). Therefore, the Psp system apparently functions similarly under both temperature conditions.

PspB prevents FtsH-dependent degradation of PspC in *Y. enterocolitica*. Expression of *pspB* and/or *pspC* from a plasmid promoter that it does not control (*lacZp*) suggested that PspB might stabilize PspC in *Y. enterocolitica* (15). To determine the involvement of FtsH in this phenomenon, a similar set of pBAD33 derivative *araBp-pspB/C* plasmids was introduced into Δ*pspBC ftsH*$^+$ and Δ*pspBC* Δ*ftsH* strains containing empty *tacp* expression plasmid pVLT35 or the *tacp-ftsH* derivative, respectively. These strains were grown with or without IPTG to achieve endogenous, depleted, or overproduced levels of FtsH (Fig. 3A). *araBp-pspB/C* expression was induced with arabinose, and immunoblot assays were used to compare steady-state levels of PspB and PspC.

With endogenous or overproduced FtsH, the PspC protein was detected only when it was coproduced with PspB (Fig. 3A), consistent with the previous result (15). However, when FtsH was depleted, PspC was readily detected in the absence of PspB. This suggests that PspC is subject to FtsH-dependent degradation in *Y. enterocolitica* and that PspB can prevent this degradation, even when FtsH is overproduced.

Next, we did a PspC degradation assay in the absence of PspB as described in Materials and Methods. For this experiment, we used a pBAD18-Kan derivative encoding only PspC. The higher copy number than that of pBAD33 allowed detection of PspC in an *ftsH*$^+$ strain, and its kanamycin resistance allowed chloramphenicol to be used as a translation inhibitor. The results clearly indicated that depletion of FtsH significantly stabilized the PspC protein (Fig. 3B). Integrated density analysis of the PspC immunoblot signals with ImageJ software (http://rsb.info.nih.gov/ij) suggested that the half-life of PspC was extended from approximately 12 to 50 min upon FtsH depletion. Interestingly, in this experiment PspC was easily detected in the FtsH depletion strain even without arabinose addition (Fig. 3B). This is presumably due to a combination of leakiness of the *araBp* promoter and reduced proteolytic turnover of the PspC protein when FtsH was depleted.

PspC is the only core component of the Psp system affected by FtsH. Next, we tested whether FtsH destabilized any other core component of the Psp system. This was done in *ftsH*$^+$ and Δ*ftsH* strains with all chromosomal *psp* genes deleted and plasmids with individual *psp* genes expressed from the *araB* promoter (pBAD18-Kan derivatives). Deletion of the chromosomal *psp* genes eliminated potential complications caused by their differential expression in response to the various *araBp-psp* expression plasmids.

After translation was blocked with chloramphenicol, immunoblot analysis revealed that the stability of the PspF, PspA, and PspB proteins was unaffected by the FtsH status of the cell (Fig. 4). This indicates that FtsH does not affect any of these Psp proteins, even when FtsH is overproduced and all other Psp proteins are absent. In contrast, and consistent with the earlier results, depletion of FtsH stabilized PspC whereas FtsH overproduction destabilized it (Fig. 4). However, as before, coproduction of PspB and PspC together in this Δ(*pspF-ycjF*) Δ*pspG* strain protected PspC from FtsH destabilization (data not shown). Therefore, among all of the Psp regulon proteins, PspB is both necessary and sufficient for this protective effect.

PspC-dependent toxicity is alleviated by increased FtsH synthesis or by coproduction of PspB. The preceding experiments indicated that among the PspF, -A, -B, and -C proteins only PspC is subject to FtsH-dependent degradation. Furthermore, this occurs detectably only when PspC is made in the absence of its binding partner PspB. Therefore, we hypothesized that uncomplexed PspC might be particularly deleterious to the bacterial cell so that a specific FtsH-dependent mechanism has evolved to eliminate it. If this hypothesis was correct, we rea-

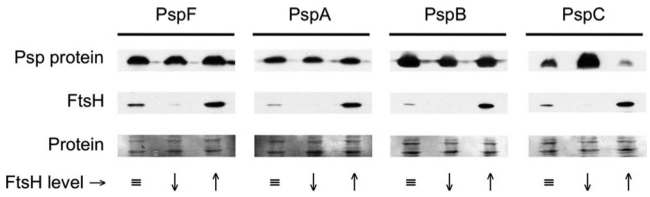

FIG. 4. PspC is the only core component of the Psp system affected by FtsH in *Y. enterocolitica*. Strains with a Δ(*pspF-ycjF*) Δ*pspG* genotype contained *araBp* expression plasmids (pBAD18-Kan derivatives) encoding PspF, PspA, PspB, or PspC as indicated at the top. All strains also contained *tacp-ftsH* expression plasmid pAJD2105. Approximately endogenous FtsH levels (≡) were achieved using an *ftsH*$^+$ strain grown without IPTG. FtsH depletion (↓) was achieved using an Δ*ftsH* strain grown without IPTG. FtsH overproduction (↑) was achieved using an Δ*ftsH* strain grown with 100 μM IPTG. Psp protein production was induced for 30 min by addition of 0.02% arabinose, after which translation was blocked by adding chloramphenicol. Samples were harvested 1 h after chloramphenicol addition, and cell lysates were analyzed by anti-FtsH and anti-PspF, anti-PspA, anti-PspB, or anti-PspC immunoblotting as appropriate. "Protein" indicates a Ponceau S stain of the nitrocellulose membrane used for immunoblot analysis (the region containing FtsH).

soned that conditions should be found where production of PspC is toxic and that this toxicity should be alleviated in one of two ways: first, by coproducing PspB, which would presumably allow formation of the normal and nontoxic PspBC complex, and alternatively, by increasing the synthesis of FtsH, which would more rapidly eliminate the abnormal, uncomplexed, and toxic PspC protein.

Again, these experiments were done in strains with all chromosomal *psp* genes deleted in order to eliminate complications caused by their differential expression. For example, in a previous study an *araBp-pspBC* plasmid caused overexpression of chromosomal *psp* genes whereas *araBp-pspB* and *araBp-pspC* plasmids did not (32). Each strain contained empty *araBp* expression plasmid pBAD18-Kan or *pspB*$^+$ and/or *pspC*$^+$ derivatives, together with either empty *tacp* expression plasmid pVLT35 or the *ftsH*$^+$ derivative. All strains were grown with arabinose and IPTG, and optical density was monitored over time.

Consistent with our hypothesis, *araBp-pspC* expression was toxic (reduced growth yield by approximately 35%) whereas *araBp-pspB* was not (Fig. 5, top). However, the toxicity caused by *pspC* expression was abolished either by FtsH overproduction or by coexpression of *pspB*. Immunoblot analysis confirmed that FtsH overproduction decreased PspC concentration whereas coproduction with PspB did not (Fig. 5, bottom). Therefore, the mechanism by which each alleviates PspC-dependent toxicity is different. This is fully consistent with FtsH eliminating toxic, uncomplexed PspC and with PspB promoting the formation of a stable, nontoxic PspBC complex.

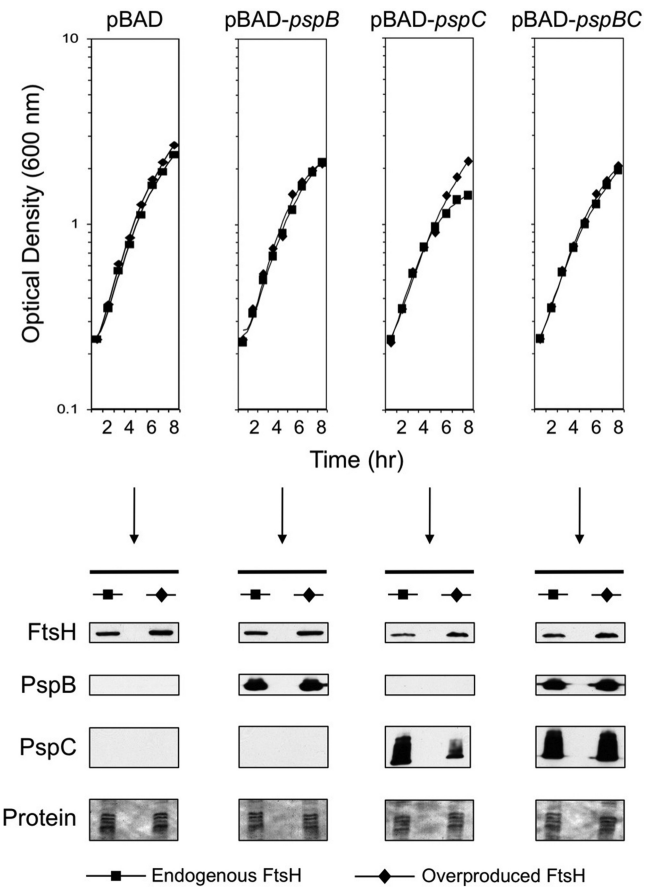

FIG. 5. PspC-dependent toxicity is alleviated by increased FtsH synthesis or by coproduction of PspB in *Y. enterocolitica*. Δ(*pspF-ycjF*) Δ*pspG ftsH*$^+$ strains contained the empty pVLT35 vector (Endogenous FtsH) or the derivative encoding FtsH (Overproduced FtsH). Strains also contained either the empty pBAD18-Kan vector (pBAD) or derivatives expressing *pspB*, *pspC*, or *pspBC* as indicated at the top. The strains were grown as described in Materials and Methods, and optical density was measured at hourly intervals. At the 4-h time point, samples were removed for the anti-FtsH, anti-PspB, and anti-PspC immunoblot analysis shown at the bottom. "Protein" indicates a Ponceau S stain of the nitrocellulose membrane used for immunoblot analysis (the region containing PspC).

DISCUSSION

This work has revealed a link between the phage shock protein system and the FtsH protease. FtsH destabilizes PspC produced in excess relative to its binding partner, PspB. The most likely explanation is that uncomplexed PspC is a degradation substrate of FtsH, although formal confirmation of that will require the establishment of an *in vitro* system to study PspBC. This would add PspC to the list of FtsH substrates, identified primarily in *E. coli*, which includes both integral cytoplasmic membrane and soluble cytoplasmic proteins (reviewed in references 19 and 36). Notably, the situation with PspC is strikingly analogous to the *E. coli* SecY and AtpB cytoplasmic membrane proteins, which are degraded by FtsH when produced in the absence of, or in excess over, their normal binding partners (1, 24).

SecY and AtpB function within complexes involved in moving proteins or protons, respectively, across the cytoplasmic membrane. It has been suggested that their unregulated subreactions, which might occur if they fail to assemble into their normal complexes, could be highly deleterious by compromising the permeability barrier (1). As such, FtsH-dependent degradation serves as an important quality control mechanism. Consistent with these ideas, AtpB overproduction is toxic (11, 42) and *E. coli ftsH* mutants are sensitized to SecY overpro-

duction (24). Similarly, PspC overproduction was toxic, but increased FtsH production, or PspB coproduction, alleviated this (Fig. 5). Perhaps the PspBC complex might also be involved in a transmembrane conductance function such that uncontrolled PspC activity can compromise the permeability barrier. Experiments in *E. coli* support part of this idea. In Δ*pspF E. coli* cells (i.e., with an uninducible Psp regulon), PspC overproduction decreases the proton motive force, but this does not happen when PspB and PspC are coproduced (21).

To our knowledge, this is the first dedicated investigation of FtsH function in any *Yersinia* species, and it has revealed a lot of similarity to *E. coli*. The FtsH proteins are predicted to be the same length, 92% identical, and translated from a TTG initiation codon. They also share at least one probable common substrate, PspC (Fig. 1), and FtsH is essential in both species. FtsH is essential in *E. coli* because one of its substrates, LpxC, catalyzes a committed step in the synthesis of lipopolysaccharide (LPS) (37). In the absence of FtsH, LpxC accumulation causes a lethal imbalance between phospholipid and LPS. An L85P substitution in FabZ (the *sfhC21* allele), which is involved in phospholipid biosynthesis, restores the balance and suppresses Δ*ftsH* lethality (37). We do not know if similar phenomena occur in *Y. enterocolitica*. *lpxC* (YE0678) and *fabZ* (YE3273) are conserved, and the predicted FabZ protein has leucine at position 85. Recent work showed that *E. coli* FtsH can degrade LpxC proteins from various species, including *Yersinia pseudotuberculosis* (28). Removal rather than depletion of FtsH might facilitate our *Y. enterocolitica* studies, and so we have attempted to use *sacB* suicide vector technology to exchange the wild-type *fabZ* gene for one encoding FabZ L85P (in an *ftsH*$^+$ strain). However, we could isolate wild-type segregants only from a *fabZ* (wild-type)/*fabZ* (L85P) merodiploid (S. Singh and A. J. Darwin, unpublished data). This might indicate that the L85P substitution is lethal in *Y. enterocolitica*, although further work will be needed to unequivocally confirm or deny that.

A link between FtsH and the Psp response fits well with several other connections between FtsH function and stress response reported in *E. coli*. First, *ftsH* expression is partially dependent on the alternate sigma factor σ^{32} (RpoH), which controls the cytoplasmic heat shock response, and σ^{32} itself is also an FtsH degradation substrate (18). Second, FtsH might be involved in shutting off the oxidative stress response by degrading the transcription factor SoxS (13). Third, compromised FtsH function leads to induction of the Cpx and RpoE extracytoplasmic responses, probably due to the proposed role of FtsH in quality control of aberrant membrane proteins (38).

We have detected FtsH-dependent PspC destabilization in *Y. enterocolitica* when PspC is produced in excess of PspB. Similarly, *E. coli* AtpB has been shown to be degraded by FtsH only when produced in excess of its binding partners (1). For some time, SecY was also thought to be degraded by FtsH only when produced in excess of its binding partner, SecE. However, it has now been found that FtsH degrades SecY when aberrant proteins block Sec translocation complexes, which might be part of a defense mechanism against such events (41). Therefore, we speculate that a situation probably arises where FtsH targeting of endogenous PspC occurs and is important. In fact, some data already support this. In an *E. coli* Δ*ftsH* strain, we detected the endogenous PspC protein but not in the *ftsH*$^+$ parental strain (Fig. 1). Although small, this effect is consistent with destabilization of endogenous PspC by FtsH (but cause and effect are hard to establish; see below). We did not observe this in *Y. enterocolitica*, but that might be because we only depleted FtsH whereas in the *E. coli* Δ*ftsH* strain it is absent. FtsH-dependent degradation of σ^{32} and SoxS in *E. coli* is implicated in shutoff of the cytoplasmic heat shock and oxidative stress responses, respectively. Similarly, PspC degradation could influence shutoff of the Psp response because PspC positively controls *psp* gene expression. We have found that depletion of FtsH in *Y. enterocolitica* can slightly increase basal-level Φ(*pspA-lacZ*) operon fusion expression (S. Singh and A. J. Darwin, unpublished data). However, compromised FtsH function might itself cause membrane stress, at least in *E. coli* (38), which could explain any elevated *psp* gene expression. Thus, we cannot precisely establish cause and effect for this phenomenon. Similarly, extended experiments to examine effects of FtsH depletion on Psp response shutoff would be complicated by the membrane stress and eventual growth arrest caused by the FtsH depletion itself.

A 1999 publication suggested that loss of FtsH in *E. coli* deactivated σ^{54} activity by an unknown mechanism, abolishing expression of σ^{54}-dependent promoters, including *pspAp* (4). Such a phenomenon would not impact our work with *pspC* expressed from the *lacZp* and *araBp* promoters. Furthermore, as mentioned above, FtsH depletion in *Y. enterocolitica* does not reduce basal Φ(*pspA-lacZ*) expression, nor does it prevent secretin-dependent induction (S. Singh and A. J. Darwin, unpublished data). Other work has argued against global loss of σ^{54} activity in an *E. coli* Δ*ftsH* strain (39).

In summary, this work has uncovered a role for FtsH in destabilizing a component of the Psp stress response. This further solidifies connections between FtsH function and the bacterial cell envelope, with FtsH being linked to the biosynthesis of cell membrane components (LPS and phospholipids), quality control of integral membrane proteins, and the functioning of bacterial cell envelope stress responses.

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