Envelope Stress Activates Expression of the Twin Arginine Translocation (Tat) System in *Salmonella*

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ABSTRACT The twin arginine translocation system (Tat) is a protein export system that is conserved in bacteria, archaea, and plants. In Gram-negative bacteria, it is required for the export of folded proteins from the cytoplasm to the periplasm. In *Salmonella*, there are 30 proteins that are predicted substrates of Tat, and among these are enzymes required for anaerobic respiration and peptidoglycan remodeling. We have demonstrated that some conditions that induce bacterial envelope stress activate expression of a Δ*tatABC-lacZ* fusion in *Salmonella enterica* serovar Typhimurium. Particularly, the addition of bile salts to the growth medium causes a 3-fold induction of a Δ*tatABC-lacZ* reporter fusion. Our data demonstrate that this induction is mediated via the phage shock protein (Psp) stress response system protein PspA. Further, we show that deletion of *tatABC* increases the induction of *tatABC* expression in bile salts. Indeed, the data suggest significant interaction between PspA and the Tat system in the regulatory response to bile salts. Although we have not identified the precise mechanism of Psp regulation of *tatABC*, our work shows that PspA is involved in the activation of *tatABC* expression by bile salts and adds another layer of complexity to the *Salmonella* response to envelope stress.

IMPORTANCE *Salmonella* species cause an array of diseases in a variety of hosts. This research is significant in showing induction of the Tat system as a defense against periplasmic stress. Understanding the underlying mechanism of this regulation broadens our understanding of the *Salmonella* stress response, which is critical to the ability of the organism to cause infection.

KEYWORDS *Salmonella*, bile, Psp, envelope stress, stress response

The twin arginine translocation (Tat) system is a protein export system present in the cytoplasmic membrane of many bacteria and archaea. In Gram-negative organisms, the Tat translocon transports substrate proteins from the cytoplasm to the periplasmic space where the protein can undergo further export out of the cell or remain in the periplasm. Substrates of Tat are proteins that must be folded in the cytoplasm, often because they contain essential cofactors that are limiting in the periplasmic space (1, 2). This process is independent of ATP and requires the use of proton motive force (PMF) to drive substrate translocation (3, 4). The secretion complex is made of three proteins: TatA or TatE, TatB, and TatC (2, 5, 6). Much work has been done to understand the assembly of the Tat system at the cytoplasmic membrane. These data show that either TatA or TatE are recruited to a TatB/TatC complex bound to a Tat substrate, and TatA or TatE seem to form translocation channels for the substrate (7, 8). TatA or TatE is recruited to the complex in variable numbers to allow for translocation of substrates of various sizes (8). Despite being encoded in a single *tatABC* operon, there are about 25 copies of TatA per TatBC, suggesting posttranscriptional regulation. Indeed, published evidence suggests that this variable number of Tat structural components is due, at least in part, to mRNA decay-based regulation (9). Tat is named after the N-terminal twin arginine signal sequence commonly associated with Tat substrates. Secreted proteins carry the twin arginine signal sequence, (S/T)RRxFLK, which binds to the translocon and initiates movement to the

periplasm (1, 10, 11). While loss of the Tat system is lethal in some organisms, it is not lethal to *Salmonella enterica* serovar Typhimurium (*S.* Typhimurium); however, there are pleiotropic effects on metabolism, virulence, and proper cell envelope development (6, 12, 13). Tat mutants are also more susceptible than wild-type *S.* Typhimurium to antimicrobial agents, such as detergents and β-lactam antibiotics (14–17).

The main structural components of the Tat system are encoded in the gene *tatABC* operon (referred to here as the *tat* operon) (18). TatE, which is separately encoded from other Tat complex proteins, is functionally equivalent to TatA (18).

The total number of Tat substrates varies by species. *S.* Typhimurium has 30 proteins that are either experimentally confirmed or predicted to be exported via Tat (12). These Tat substrates include enzymes necessary for anaerobic respiration, hydrogenases, and cell wall amidases, among other critical cell processes (12). Indeed, the Tat system has been shown to be critical for the virulence of *S.* Typhimurium and other pathogenic species (12, 15, 19–21) but is not essential for *in vitro* growth. In *S.* Typhimurium, this virulence defect is due to the combined loss of *amiA*, *amiC*, and *sufI* (12, 21). AmiA and AmiC are *N*-acetylmuramyl-L-alanine amidases that remove cross-links in peptidoglycan during cell division (22, 23), while SufI (FtsP) is important for stabilization of the divisome (22, 24). The combined data from *Escherichia coli* and *S.* Typhimurium suggest a very important role of the Tat system in maintenance of the Gram-negative cell envelope. Strains deleted for genes encoding the Tat apparatus experience several growth phenotypes, including elongated cells, septal defects during division, and altered proton motive force (PMF) (17, 22, 25). While little is known about the regulation of *tat* genes, previous work has demonstrated a role for CpxR in directly binding the *tat* promoter and activating *tat* gene expression in response to protamine (26).

Envelope or extracytoplasmic stress responses (ESRs) detect stressors to the envelope and initiate actions to repair or prevent damage. There are several well-studied ESRs identified in *S.* Typhimurium, including Cpx, Bae, Rcs, σ^E, and Psp (27–29). Evidence from *Escherichia coli* is clear that these systems demonstrate a substantial amount of overlap both in what stressors they sense and in what genes they control in response to stress (29). For example, each of the pathways listed have been shown to be induced at least somewhat in response to 4 mM indole, but the Bae response seems to be most dramatic (29). The Cpx system responds to misfolded proteins in the envelope and regulates a wide array of genes, including Tat substrates *amiA* and *amiC* (30). Additionally, CpxR has been shown to directly induce *tatABC* expression (26). The BaeSR two-component system controls a small subset of genes involved in production of efflux pumps (31). The Rcs system is a phosphorelay system that controls colanic acid production and aids in the maintenance of PMF (32). The alternative sigma factor, σ^E, recruits RNA polymerase to the promoters of genes encoding chaperones, proteases, and outer membrane biogenesis factors needed to respond to envelope stress (33, 34). These stress response systems are reviewed in detail by Macritchie and Raivio (27).

The phage shock protein (Psp) stress response system is a variation on the two-component system, which has multiple resident membrane components (35) and plays a role in maintenance of PMF (36). Primary components of the Psp system include the transcriptional enhancer protein PspF, which interacts with the alternate sigma factor RpoN (σ^{54}) to activate transcription of a small subset of the σ^{54} regulon. PspF is only known to directly activate expression of the *pspABCDE* operon *pspF* and *pspG* (29). PspA is an antagonist to PspF, binding PspF so it cannot interact with σ^{54} to activate gene expression (35). PspB and PspC are membrane sensor components that sense mislocalized or misfolded membrane proteins and recruit PspA to the membrane, freeing PspF to activate transcription (37, 38). PspA has several other additional roles in stress response in the cell. In *E. coli*, it has been shown that PspA is able to form large oligomers to bind areas on the cytoplasmic membrane where there is an increase in stress, and PspA accumulation at the membrane seems to stabilize the membrane to help maintain PMF (39). PspA has recently been described as a member of the endosomal sorting complexes required for transport (ESCRT)-III membrane remodeling family of proteins with homologs to eukaryotes and archaea (40). Several links between the

TABLE 1 Effect of periplasmic stress agents on *tatABC-lacZ* expression

Growth condition	Relative β-galactosidase activity[a]	P value vs LB control[b]
LB control	100.00 ± 4.34	NA
42°C	73.09 ± 4.35	NS
0.6 mM NaCl	86.36 ± 0.81	NS
0.5 mM Dibucaine	99.35 ± 4.79	NS
4% Ethanol	160.84 ± 9.37	0.005
5% Bile salts	174.79 ± 2.61	<0.001
4 mM Indole	177.14 ± 6.78	<0.001
0.10% SDS	180.64 ± 9.70	<0.001
9% Bile salts	397.81 ± 43.87	<0.001

[a]LB with no additives grown at 37°C set to 100 U of β-galactosidase activity. Strain JRE143 was used under each condition.
[b]Significance was determined by one-way ANOVA; NS, not significant; NA, not applicable.

Psp and Tat systems have already been established. TatA and PspA have been demonstrated to interact in the membrane by copurification and electron microscopy (41). This effect is apparently independent of PspF, suggesting a basal level of PspA in the cell (41). Additional work has shown that overproduction of Tat substrates SufI and CueO actually cause inefficient Tat secretion by outcompeting other Tat substrates for use of the Tat machinery, but concurrent overexpression of PspA somehow relieves this saturation and allows more substrates out of the cytoplasm (42). Additionally, previous work has shown that a deletion of *tatC* acts as a stressor on the cell, leading to activation of *psp* gene expression (43).

Given the critical role of Tat in virulence and maintenance of the *S.* Typhimurium envelope, we sought to determine if there was environmental regulation of the *tat* operon. In this work, we show that some environmental conditions that induce envelope stress activate expression of the *tat* operon in *S.* Typhimurium. This regulation is dependent on both the Tat structural components and the Psp system, likely through the known interaction of TatA and PspA.

RESULTS

Envelope stress conditions activate *tat* expression. Given the critical nature of the twin arginine translocation (Tat) system in proper cell envelope biogenesis, we predicted that at least some conditions that induce envelope stress in *S.* Typhimurium would activate expression of *tatABC* to compensate for envelope damage. We constructed a transcriptional Δ*tatABC-lacZ* fusion and monitored expression under different conditions. The data show (Table 1) that 5% and 9% bile salts, 4 mM indole, 0.1% SDS, and 4% ethanol induced expression of Δ*tatABC-lacZ* to various degrees, while 0.5 mM dibucaine, 0.6 mM NaCl, and heat shock at 42°C had no significant impact on *tatABC-lacZ* activity. Given the relatively large 4-fold change in Δ*tatABC-lacZ* expression, we chose to focus primarily on bile salts as a mechanism for studying the induction of *tatABC*. To determine if bile salt activation of Δ*tatABC-lacZ* is physiologically significant, we looked at translocation of the artificial Tat substrate TorA-mCherry-SsrA (44). In this instance, the signal sequence from the Tat substrate TorA is engineered in front of mCherry to translocate the protein to the periplasm via Tat. The SsrA tag leads to degradation of cytoplasmic mCherry; thus, fluorescence indicates translocation of the construct (44). We then assayed for mCherry fluorescence in 0%, 5%, and 9% bile salts. Indeed, the data show that TorA-mCherry-SsrA is translocated to the periplasm at a higher level when cells are grown in bile salts (Fig. S1 in the supplemental material). As expected, deletion of *tatABC* leads to little TorA-mCherry-SsrA translocation and low fluorescence.

It has been repeatedly shown that pathogenicity of *S.* Typhimurium is responsive to a variety of environmental signals that alter the expression of genes driving invasion of intestinal epithelial cells (45, 46), survival within macrophages (47, 48), and an array of other critical bacterial processes. *S.* Typhimurium encounters bile as it transits from the stomach to the small intestine (49), and it plays an important role in the regulatory
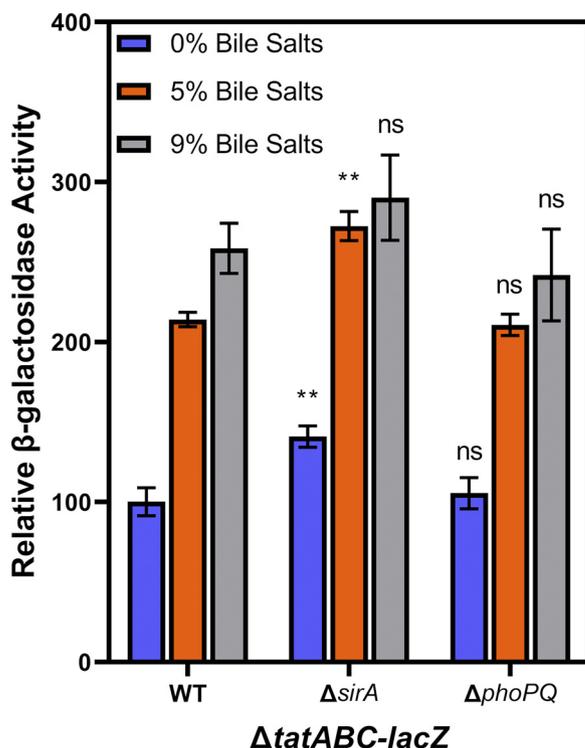
FIG 1 Bile activates expression of *tatABC-lacZ* independently of BarA/SirA and PhoPQ. All strains contain transcriptional *lacZ* fusions to *tatABC* and are otherwise wild-type (WT) or deleted for *sirA* or *phoPQ* as indicated. All strains were grown in LB with 0%, 5%, or 9% bile salts added. LB with no (0%) bile salts was set to 100 U of β-galactosidase activity. Strains used were JRE143, JRE237, and JRE275. Significance was determined by one-way analysis of variance (ANOVA) with strains compared to WT at the same concentration of bile salts; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

network of *Salmonella* (49–51). The BarA/SirA two-component system has been implicated in the *S.* Typhimurium response to bile (49, 52), although that effect has recently been attributed to other mechanisms (53). To determine if BarA/SirA is involved in bile regulation of *tat*, we moved a *sirA*::cm allele into the Δ*tatABC-lacZ* background and grew the strains in Luria-Bertani (LB) with no additive or 9% bile salts. Bile salts contain roughly equal amounts of sodium cholate and sodium deoxycholate but are missing many components of physiologically produced bile. The data show that disruption of *sirA* has no impact on bile activation of *tat* (Fig. 1). Furthermore, when SirA is overexpressed in the vector pBAD30, it does not activate Δ*tatABC-lacZ* over the vector-only control in any concentration of bile (data not shown). The PhoP/PhoQ two-component system is primarily known for controlling the cellular response to Mg^{2+} and Ca^{2+} ions and antimicrobial peptides (54–56). Additionally, it has been demonstrated that the PhoP/PhoQ system does play a role in the overall cellular bile response in *S.* Typhimurium (50, 57). Work from the Finlay lab demonstrated that physiological bile induces *tat* expression about 2-fold in a *Salmonella enterica* serovar Typhi (*S.* Typhi) microarray, and this is at least partially via PhoPQ (50). Therefore, we moved a deletion of *phoPQ* into the Δ*tatABC-lacZ* construct and monitored *tat* expression in LB with no additive or 9% bile salts. The data show that Δ*phoPQ*::Cm had no impact on *tatABC-lacZ* expression at any level of bile salts (Fig. 1).

Tat response to bile salts is dependent on the Psp system. *S.* Typhimurium encodes several well-studied systems that respond to stress in the cell envelope, including σ^E, BaeSR, Cpx, Rcs, and Psp (27). Each of these plays a specific role in response to a set of envelope stressors, although these are often overlapping among the different systems. To test the effect of known stress response systems on *tatABC* expression, we deleted genes associated with each and monitored effects on Δ*tatABC-lacZ* via β-galactosidase assays. We deleted genes associated with the Cpx (*cpxR*), σ^E (*rpoE*), BaeSR (*baeR*), and Rcs (*rcsB*
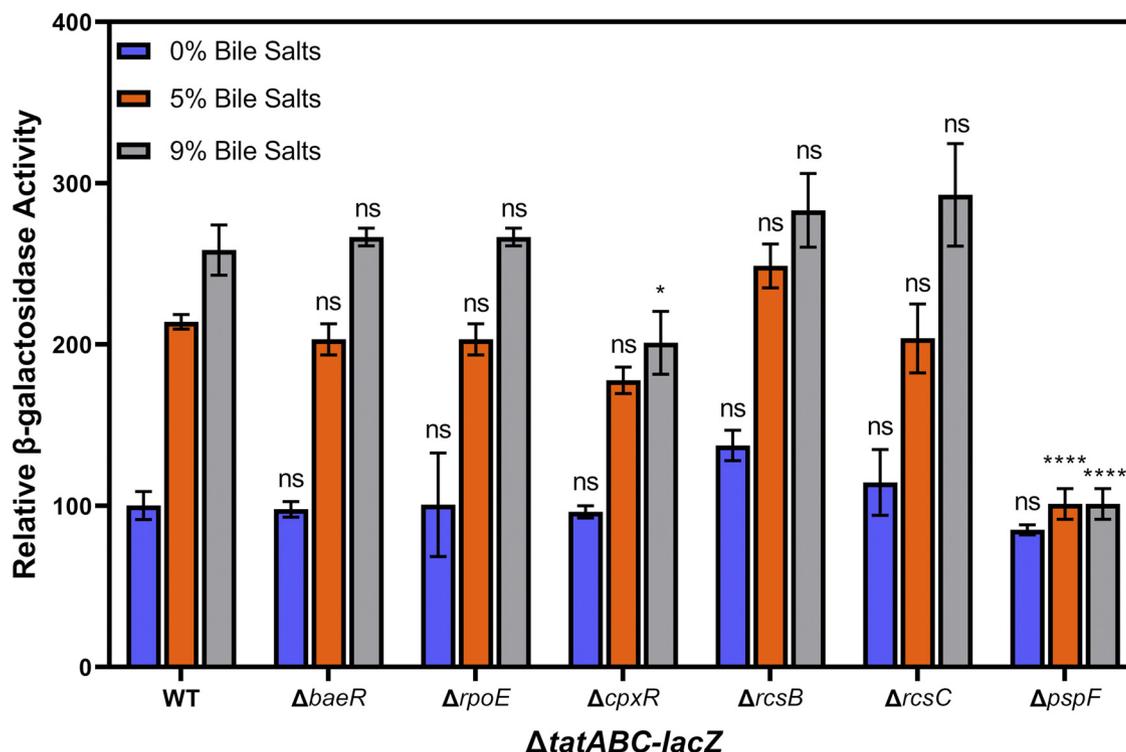
FIG 2 Bile activates expression of *tatABC-lacZ* independently of the σE, Cpx, Rcs, and BaeRS systems but is dependent on Psp. All strains contain transcriptional *lacZ* fusions to *tatABC* and are otherwise wild-type (WT) or are deleted for *rpoE*, *cpxR*, *baeR*, *rcsB*, *rcsC*, or *pspF* as indicated. All strains were grown in LB with 0%, 5%, or 9% bile salts added as indicated. LB with no (0%) bile salts was set to 100 U of β-galactosidase activity. Strains used were JRE143, JRE269, JRE271, JRE273, JRE322, JRE324, and JRE325. Significance was determined by one-way ANOVA with strains compared to WT at the same concentration of bile salts; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

and *rcsC*) systems. CpxR is the response regulator in the Cpx system and is phosphorylated by CpxA (27, 29). σ^E is an alternative sigma factor that is sequestered at the cytoplasmic membrane by RseA. Envelope stress leads to release of σ^E via proteolytic cleavage of RseA by DegS (27, 58). BaeRS is a classic two-component regulatory system with a small regulon. BaeR is the response regulator and is phosphorylated by BaeS when the cell is exposed to toxic compounds (59). RcsA is an auxiliary factor in the Rcs system, as RcsB is the most critical transcriptional activator and can function in the absence of RcsA. RcsC is an unusual sensor kinase that phosphorylates RcsD, which in turn phosphorylates RcsB. Phosphorylated RcsB can form a homodimer or a heterodimer with RcsA. Each complex regulates a subset of the Rcs regulon (27, 32, 60). Single deletions of *rpoE*, *baeR*, *rcsB*, and *rcsC* had no significant impact on bile activation of Δ*tatABC-lacZ* (Fig. 2). CpxR has previously been shown to bind directly to the *tatABC* promoter (26); however, Δ*tatABC-lacZ* shows only a small (1.3-fold), although statistically significant, change in expression in the Δ*cpxR* background (Fig. 2).

We next deleted components of the Psp system (*pspF*). PspF is a transcriptional regulator that binds to σ^{54} and activates gene expression in response to stress conditions (29, 35). PspA inhibits PspF function by direct protein-protein interaction and is also required for membrane integrity (35, 37). Thus, if the Psp system was activating Δ*tatABC-lacZ*, we would expect the Δ*tatABC-lacZ* fusion to be nonresponsive to bile salts in a Δ*pspF* background. Indeed, the data show that in a Δ*pspF* background, addition of bile salts no longer induces Δ*tatABC-lacZ* expression (Fig. 2), demonstrating that bile salt activation of Δ*tatABC-lacZ* occurs via the Psp system. We further deleted *pspA*, *pspB*, and *pspC* to determine the effect on Δ*tatABC-lacZ*. Given the role of PspA as an antagonist of PspF, we predicted that a deletion of *pspA* would result in an increase in *tatABC* expression. The data show that a *pspA* deletion behaves identically to a deletion of *pspF*, and Δ*tatABC-lacZ* no longer responds to bile
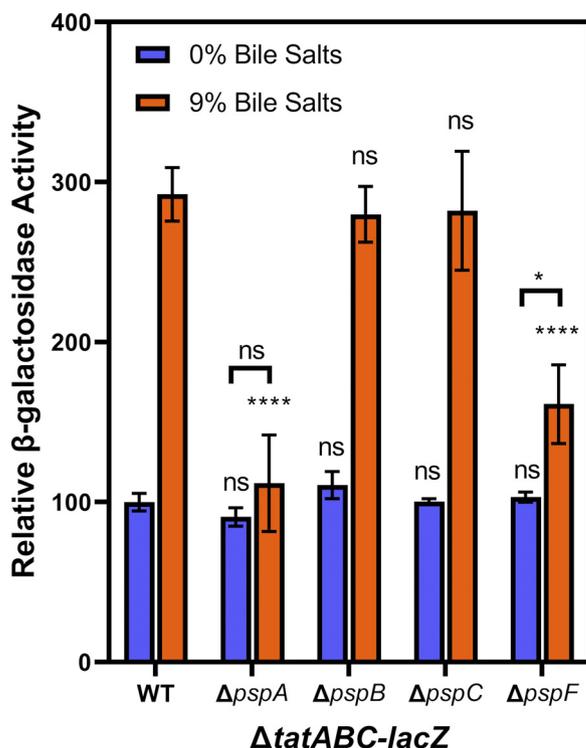
FIG 3 Bile activation of *tatABC-lacZ* expression is dependent on PspA and PspF. All strains contain transcriptional *lacZ* fusions to *tatABC* and are otherwise wild-type (WT) or are deleted for *pspF*, *pspA*, *pspB*, or *pspC* as indicated. All strains were grown in LB with 0% or 9% bile salts added. LB with no (0%) bile salts was set to 100 U of β-galactosidase activity. Strains used were JRE143, JRE 567, JRE322, JRE904, and JRE1033. Significance was determined by one-way ANOVA with strains compared to WT at the same concentration of bile salts; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

salts. Given the polar nature of the Δ*pspA*::Cm allele, we moved deletions of *pspB* and *pspC* into the Δ*tatABC-lacZ* background to confirm that the phenotype is caused by loss of *pspA* and not disruptions to the rest of the downstream operon. The data show that Δ*pspB* and Δ*pspC* have no impact on Δ*tatABC-lacZ* expression, and regulation by bile salts is dependent on PspA (Fig. 3).

To confirm the involvement of the Psp system in the regulation of *tatABC*, we cloned the entire *psp* region from *pspF* to *pspE* into pDX1, an apramycin-resistant derivative of the plasmid pAH125. The resulting plasmid (pJE229) was integrated into the *Salmonella* chromosome at the *attB*$_\lambda$ site (61, 62). In this instance, *psp* genes are all under the control of their native promoters in single copy; therefore, any abnormal effects of PspA overproduction are mitigated while still providing data on complementation. The data show that pJE229 integrated at *attB*$_\lambda$ complements the *pspA* and *pspF* deletions (Fig. 4), confirming that the Psp system plays a role in activation of *tatABC* expression in response to bile salts.

Deletion of *tatABC* induces *tatABC* expression in high bile salts. We sought to determine the minimal promoter region necessary for *tatABC* activation by bile salts. To do this, we cloned promoter fragments of various length in front of the promoterless *lacZ* in pAH125 for integration in the *S.* Typhimurium chromosome at the *attB*$_\lambda$ site and monitored *lacZ* activity via β-galactosidase assays. We cloned fragments of 1,000 bp upstream of the TatA start codon, 750 bp, 500 bp, and 250 bp. Our data demonstrate that there is no difference between the 1,000-bp, 750-bp, and 500-bp fragments in terms of an overall response to bile salts (Fig. 5). There is a slightly lower level of *tatABC* induction in the 250-bp fragment. Additionally, we moved deletions of *pspA* and *tatABC* into the P_{tatABC}-*lacZ* fusion background. These data show that deletion of *tatABC* causes an induction of P_{tatABC}-*lacZ* expression in 9% bile salts, and a deletion of both *pspA* and *tatABC* together causes a decrease in the expression of *tatABC* in 9% bile salts. The 1,000-bp, 750-bp, and
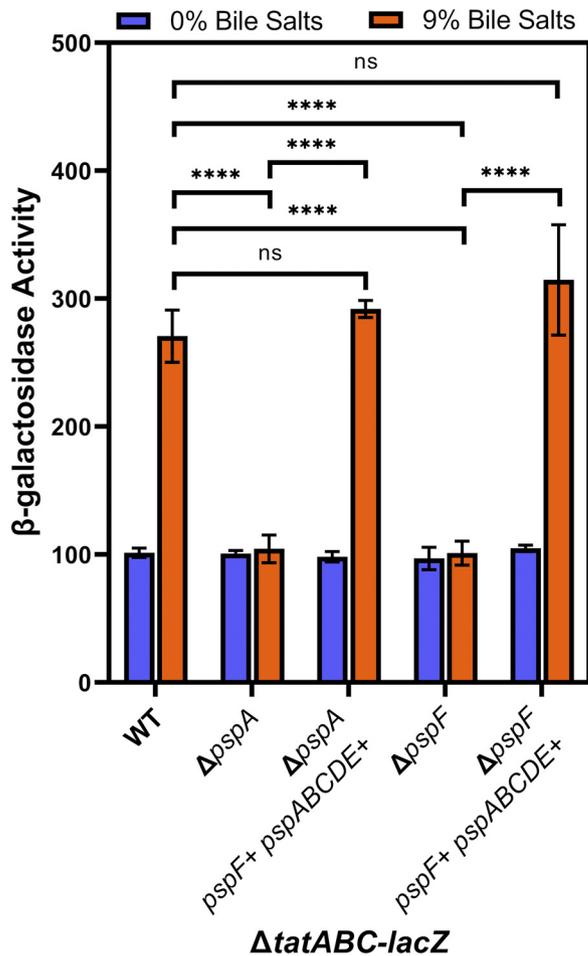
FIG 4 Bile activation of *tatABC-lacZ* is restored with *pspF* and *pspABCDE* complemented in *trans*. All strains contain transcriptional *lacZ* fusions to *tatABC* and are otherwise wild-type (WT) or are deleted for *pspA* or *pspF* as indicated. Strains also contain pJE229 (*psp* region) integrated at the *attB*$_\lambda$ site for complementation of deletions as indicated. All strains were grown in LB with 0% or 9% bile salts added. Strains used were JRE143, JRE567, JRE568, JRE973, and JRE1010. Significance was determined by one-way ANOVA; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

500-bp fragments show regulatory effects of deleting *pspA* and *tatABC*. Further, the 250-bp fragment still responds to bile, albeit at a slightly lower level, but does not show a Δ*tatABC* or Δ*pspA* effect. This suggests that disruption to the proper formation of the Tat complex activates expression of *tatABC* genes and this regulatory effect occurs somewhere between 250 bp and 500 bp upstream of the TatA start codon. Published work has shown that CpxR binds directly to P_{tatABC} at bases 96 to 110 from the TatA start codon (26). Thus, it is possible that the residual effect of bile salts on the 250-bp fragment is due to CpxR regulation. To determine if this is the case, we made targeted mutations to the CpxR binding box on the 250-bp *tatABC* promoter fragment to inhibit CpxR binding and activation (Fig. S2). The data show that the altered 250-bp promoter fragment is still activated by bile salts and is unaffected by deletions of *tatABC* and *pspA* (Fig. S3).

Further, we moved a deletion of *cpxR* into the promoter fusion fragments in the *tatABC*$^+$ and Δ*tatABC* backgrounds. The data show that deletion of *cpxR* has a small but significant impact on the expression of the P_{tatABC}-*lacZ* fusion in response to bile salts at any length of promoter (Fig. 6); thus, while CpxR binds to the *tatABC* promoter, it seems to have a minor role in the activation of *tatABC* in response to bile salts.

Overproduction of Tat substrates does not induce *tat* expression. With the importance of PspA in regulation of the Tat system and the known interaction between PspA and Tat during overproduction of Tat substrates (41, 63), we predicted that
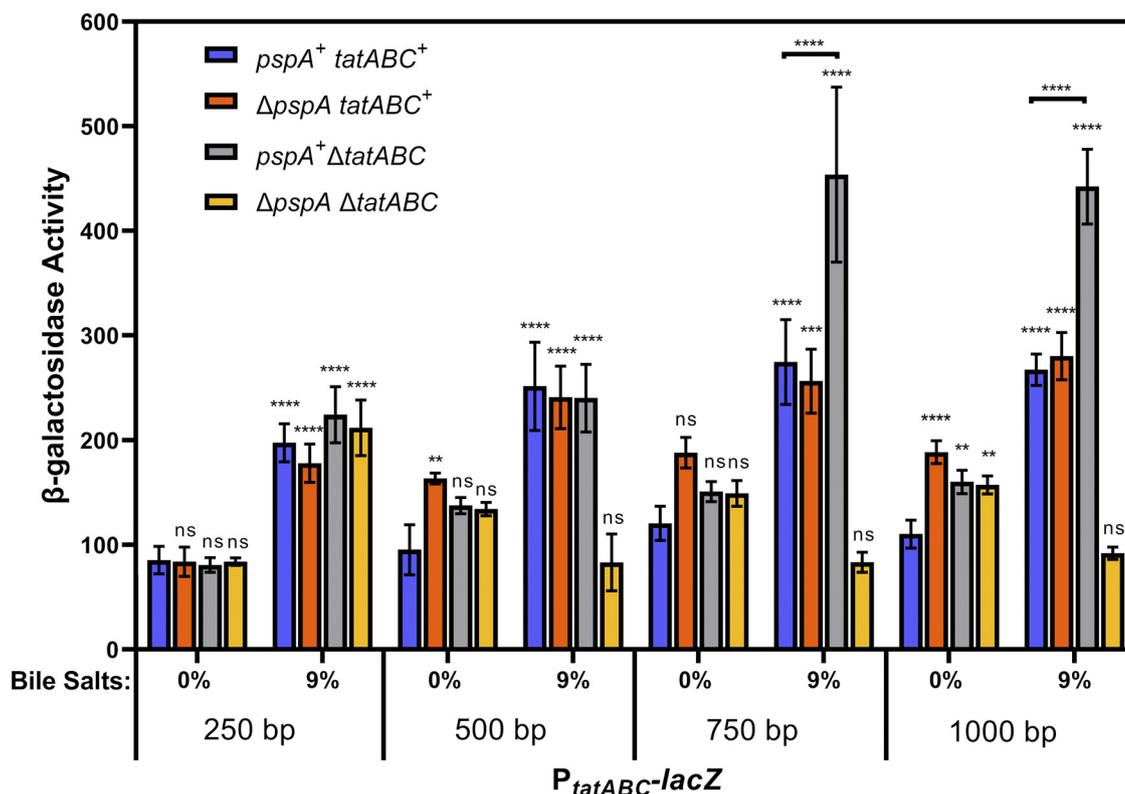
FIG 5 Deletion of *pspA* abrogates induction of *tatABC-lacZ* expression by bile salts in a Δ*tatABC* background. All strains contain transcriptional *lacZ* fusions to different lengths of the *tatABC* promoter integrated at the *attB*$_\lambda$ site. Strains are otherwise wild-type (WT) or are deleted for *pspA*, *tatABC*, or *pspA tatABC*. All strains were grown in LB with 0% or 9% bile salts added. Strains used were JRE651, JRE656, JRE659, JRE661, JRE663, JRE667, JRE701 through JRE705, JRE708, JRE722, JRE735, JRE774, and JRE824. Significance was determined by one-way ANOVA with comparisons made between WT of the same promoter length at 0% bile salts, unless noted; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

overproduction of Tat substrates would induce *tatABC* gene expression as well. To test this, we cloned genes encoding several Tat substrates, *sufI*, *amiA*, *amiC*, *fhuD*, *wcaM*, and *cueO*, into the arabinose inducible vector pBAD33 and assayed expression of the 1,000-bp P$_{tatABC}$-*lacZ* fusion in *tatABC*⁺ and Δ*tatABC* backgrounds. The data show that overproduction of the tested substrates had no significant effect on P$_{tatABC}$-*lacZ* expression over the vector control in either the *tatABC*⁺ or the Δ*tatABC* backgrounds (Table 2). Given these results, we did not pursue the cloning of other Tat substrates.

DISCUSSION

The Tat system is required for proper maintenance of the bacterial cell envelope; thus, proper expression of the components of the Tat translocon is critical to the bacterium (12, 16). Our data demonstrate that some conditions, including high concentrations of bile salts, that stress the *Salmonella* envelope also activate *tatABC* gene expression. This activation seems to be dependent on the presence of both TatABC structural proteins and the phage shock protein PspA. *β*-Galactosidase assays on a *S.* Typhimurium strain with a *lacZ* reporter fusion to the *tatABC* promoter indicate that bile salts, indole, and SDS are compounds that activate *tatABC* expression. The strongest *tatABC*-activating condition that we have identified is 9% bile salts (Table 1). Craig et al. demonstrated that deletion of *tatC* dramatically attenuates *S.* Typhimurium virulence in mice and that this is due to the loss of three critical proteins involved in cell wall biogenesis during cell division: AmiA, AmiC, and SufI (12). Additionally, *in vitro* evidence demonstrates that deletions of *tat* are highly susceptible to cell wall-damaging agents, such as bile, SDS, and *β*-lactam antibiotics (16, 17). Thus, it makes sense that bile would induce expression of *tatABC*, as more substrates of the Tat apparatus are needed to manage potential damage in the cell envelope. Indeed,
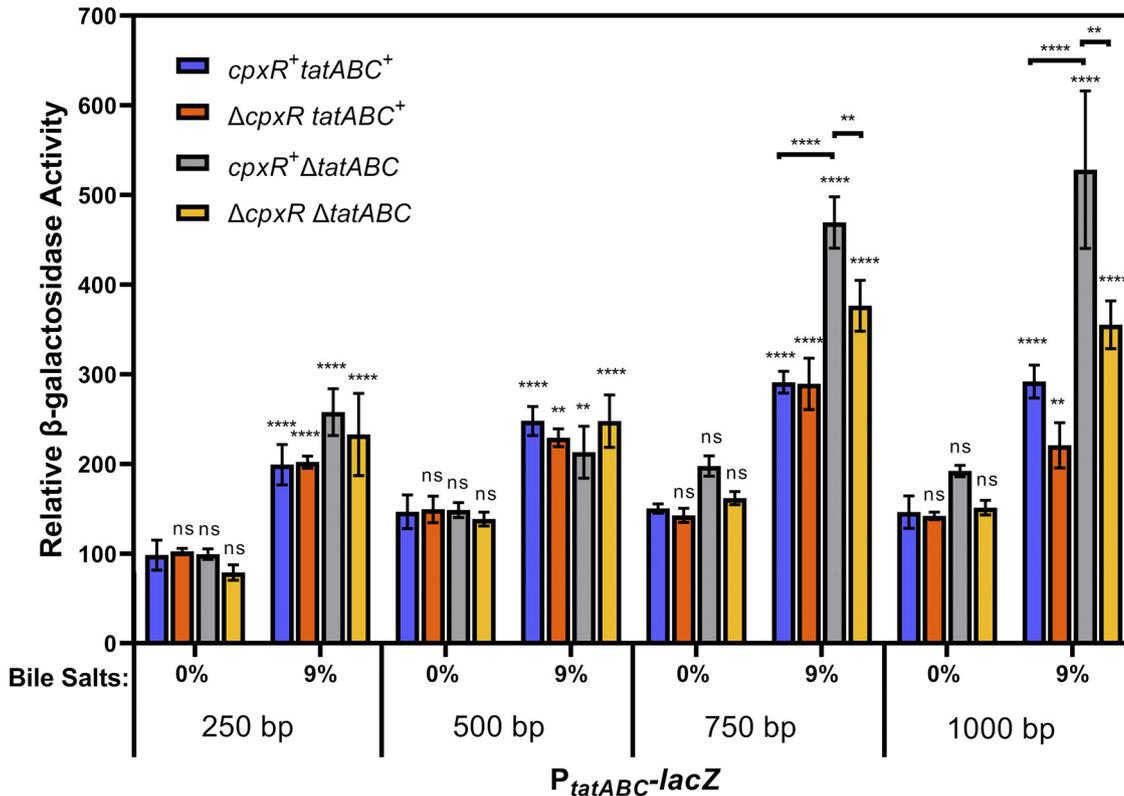
FIG 6 The Cpx system plays a minor role in bile salt activation of *tatABC-lacZ* expression. All strains contain transcriptional *lacZ* fusions to different lengths of the *tatABC* promoter integrated at the *attB$_λ$* site. Strains are otherwise wild-type (WT) or are deleted for *cpxR*, *tatABC*, or *cpxR tatABC*. All strains were grown in LB with 0% or 9% bile salts added. Strains used were JRE651, JRE656, JRE659, JRE661, JRE663, JRE667, JRE722, JRE735, JRE743 through JRE745, JRE775, JRE1031, and JRE1032. Significance was determined by one-way ANOVA with comparisons made between WT of the same promoter length at 0% bile salts, unless noted; NS, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

we also show that increasing bile salts in the growth medium also increases translocation of the artificial Tat substrate TorA-mCherry-SsrA, indicating that a 3-fold increase in transcription of *tatABC* has a significant impact on the amount of Tat substrate translocated (Fig. S1 in the supplemental material). It has been previously shown that the Cpx stress response system activates known Tat substrates *amiA* and *amiC* (30) and binds the *tatABC* promoter (26), although Cpx has only a minor role in the activation of *tatABC* in response to bile salts (Fig. 2 and 6). Additionally, the virulence phenotype of an Δ*amiA* Δ*amiC* strain was attributed to the growth defect in deoxycholate (21).

TABLE 2 Effect of Tat substrate overproduction on P_{tatABC}-*lacZ* expression

	Relative *β*-galactosidase activity			
Plasmid[a]	*tatABC*⁺	*P* value[b] vs pBAD33 *tatABC*⁺	Δ*tatABC*	*P* value[b] vs pBAD33 *tatABC*⁺
pBAD33	100.00 ± 5.36	NA	103.86 ± 2.21	NS
pJE216 (AmiA)	96.11 ± 7.06	NS	99.21 ± 3.29	NS
pJE217 (AmiC)	94.18 ± 2.84	NS	101.38 ± 2.53	NS
pJE218 (SufI)	97.87 ± 2.93	NS	114.01 ± 9.67	NS
pJE234 (CueO)	86.21 ± 4.59	NS	104.42 ± 2.34	NS
pJE235 (FhuD)	112.03 ± 3.84	NS	82.07 ± 6.57	NS
pJE236 (WcaM)	96.15 ± 6.31	NS	92.05 ± 6.71	NS

[a]Overproduction of Tat substrates does not induce P_{tatABC}-*lacZ* expression. All strains contain transcriptional *lacZ* fusions to the 1,000-bp *tatABC* promoter integrated at the *attB$_λ$* site and indicated plasmids, which are all derived from pBAD33. Strains are otherwise wild-type (WT) or are deleted for *tatABC* as indicated. All strains were grown in LB with chloramphenicol included for plasmid maintenance and 0.2% arabinose included for induction of the P_{BAD} promoter. Strains used were JRE1070 through JRE1081, JRE1101, and JRE1102.
[b]Significance was determined by one-way ANOVA; NS, not significant; NA, not applicable.

These data are supported by microarray experiments in *S.* Typhi, showing that physiological bile purified from mice works partially via the PhoPQ regulatory system (50). Data from Antunes et al. (50) indicate that physiological bile induces *tatABC* expression about 2-fold, although our data show that the PhoPQ system is not involved in activation of Δ*tatABC-lacZ* by bile salts (Fig. 1). The BarA/SirA two-component regulatory system has also been previously implicated in the *Salmonella* bile response (64, 65); however, our data demonstrate that a *sirA*-null mutant has no impact on *tat* expression (Fig. 1). The PhoP/PhoQ two-component regulatory system had an impact in the bile regulation of *Salmonella* Typhi (50); however, deleting *phoPQ* had no impact on *tatABC-lacZ* expression in *Salmonella* Typhimurium. It should be noted that the study by Antunes et al. used physiological bile, and our study uses bile salts; thus, it is possible that some component of physiological bile that is not present in bile salts is responsible for the PhoP/PhoQ effect observed by Antunes et al. (50).

We constructed Δ*tatABC-lacZ* strains with deletions of five well-studied stress response systems in *S.* Typhimurium: σ^E, BaeSR, CpxRA, RcsBCD, and Psp (29). Given the established binding of CpxR to the *tatABC* promoter, we predicted Cpx to be the most likely candidate for regulation of *tatABC* in response to bile salts. Our data indicate that the Cpx system has a small but significant effect on *tatABC* activation by bile salts (Fig. 2 and 6). This small effect on *tatABC-lacZ* expression could account for the small residual induction in the Δ*pspF* background (Fig. 2 and 3). Unfortunately, it is difficult to create a *cpxR pspA tat* deletion strain, as this is lethal in even low concentrations of bile salts. In the 250-bp fragment of the *tatABC* promoter, we see induction in response to bile salts but no effect of deleting either *tatABC* or *pspA* (Fig. 5). We hypothesized that if we mutate the known CpxR binding site in the 250-bp *tatABC* promoter fragment then CpxR would no longer bind, and induction by bile salts would be abrogated. However, in the mutated 250-bp fragment (Fig. S2), we saw no effect of the altered CpxR binding site on regulation, and bile salts still induced the *lacZ* fusion (Fig. S3). This suggests a possible secondary mechanism for regulation of *tatABC* that is independent of the Psp and Cpx systems. Additionally, σ^E, BaeSR, and Rcs do not play any significant role in bile salt activation of *tatABC* expression (Fig. 2). The data show that the Psp system is required for the activation of *tatABC* in response to bile salts (Fig. 2 and 3). PspF is an enhancer for σ^{54} binding to promoters it controls, and our current understanding suggests that the Psp regulon is limited, controlling the *pspABCDE* operon and *pspG* (35). We confirmed the role of Psp in Δ*tatABC-lacZ* regulation by complementation of the *pspA*-deletion mutant with *pspF* and *pspABCDE* cloned onto pDX1 and integrated into the *S.* Typhimurium chromosome (Fig. 4). We do not have any data that suggest that PspF regulates *tatABC* outside its normal role in controlling production of PspA. PspA seems to be the critical player in bile salt activation of *tatABC*. Indeed, our model (Fig. 7) proposes that high levels of PspA are leading to induction of *tatABC*.

Data from DeLisa et al. has previously demonstrated a critical role for PspA in Tat substrate translocation, somehow easing the burden on the system when Tat substrates CueO and SufI are overproduced (63). We predicted that overproduction of Tat substrates would induce expression of the *tat* operon via PspA. Our data demonstrate that overproduction of Tat substrates has no significant impact on *tatABC-lacZ* expression (Table 2). Additionally, our data show that deletion of *tatABC* genes themselves causes an increase in *tatABC* expression in response to the addition of bile salts and that the effect of Δ*pspA* only occurs in the Δ*tatABC* background, suggesting that there is a critical mechanistic relationship between Psp and Tat. There is no evidence that PspA is a transcriptional regulator outside its antagonism of PspF, and the structure of PspA does not contain any likely DNA-binding motifs (66); thus, action at the level of *tat* transcription is very likely indirect. The mechanism of action behind this regulation is currently unknown; however, the data provide compelling evidence that the Psp system has an important role in the transcription of *tatABC*. The relationship between the Tat and Psp systems, and that of TatA and PspA in particular, plays an important role in *Salmonella* physiology, and this work adds another layer to this complex relationship.

Given the apparent functional overlap between TatA and TatE (18), it would also be interesting to see if *tatE*, which is encoded independently of the *tatABC* operon, is
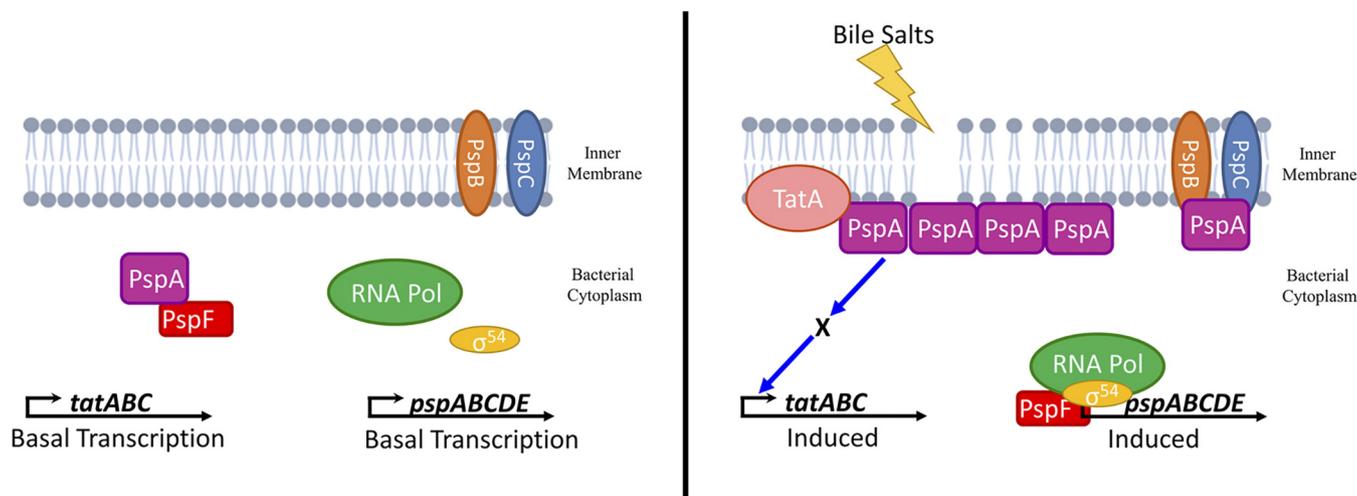
FIG 7 Proposed model of Psp regulation of *tatABC-lacZ* in response to bile salts. Under low bile salt conditions, PspA antagonizes PspF to prevent transcription of the *psp* operon. Under high bile salt conditions, the membrane is stressed, and PspB and PspC recruit PspA to the membrane, freeing PspF to activate the *psp* operon. Accumulation of PspA at the membrane induces expression of *tatABC* via an unknown mechanism; RNA Pol, RNA polymerase.

activated by any of the same signals as *tatABC*. It is possible that *tatE* expression is tied to other conditions. Although there is still much work to be done to understand the specific mechanisms of how envelope stress is inducing expression of the *tat* operon, we provide evidence of transcriptional regulation of this critical protein translocation system and demonstrate a role for the Psp system in this transcriptional activation.

MATERIALS AND METHODS

Media, reagents, and enzymatic assays. Luria-Bertani (LB) medium was used in all experiments for growth of bacteria, and super optimal broth with catabolite repression (SOC) was used for the recovery of transformants (67), unless otherwise noted. Bacterial strains were routinely grown at 37°C except for strains containing the temperature-sensitive plasmids pINT-ts, pCP20, or pKD46, which were grown at 30°C. Antibiotics were used at the following concentrations: 50 μg/mL ampicillin (Amp), 20 μg/mL chloramphenicol (Cm), 50 μg/mL kanamycin (Km), and 50 μg/mL apramycin (Apr). Enzymes were purchased from New England Biolabs (Ipswich, MA) and were used according to the manufacturer's recommendations. Primers were purchased from Integrated DNA Technologies (Coralville, IA). Bile salts number 3 and other reagents were purchased from Research Products International (Mt. Prospect, IL). Antibiotics were purchased from MilliporeSigma. (St. Louis, MO). β-Galactosidase assays were performed using a microtiter plate assay as previously described on strains grown under the indicated conditions (68, 69). β-Galactosidase activity units are defined as (micromoles of ortho-nitrophenol (ONP) formed min^{-1}) $\times$ 10^6/(optical density at 600 nm [OD$_{600}$] $\times$ milliliters of cell suspension) and are reported as mean $\pm$ standard deviation, where n = 4. Cultures used in the β-galactosidase assay were initially inoculated into LB with no additives and grown for 16 h. Cultures were then subcultured 1:100 in either LB or LB with indicated additive and grown for 5 h at 37°C in a shaking incubator at 225 rpm. Heat-shocked cultures were grown for 5 h at 42°C.

Strain and plasmid construction. Bacterial strains and plasmids are described in Table S1 in the supplemental material. All *Salmonella enterica* serovar Typhimurium strains used in this study are isogenic derivatives of strain 14028 (American Type Culture Collection) and were constructed using P22 HT105/1 *int*-201 (P22)-mediated transduction (67). Deletion of various genes and concomitant insertion of an antibiotic resistance cassette were performed using Lambda Red-mediated recombination as previously described (70). In all cases, the appropriate insertion of the antibiotic resistance marker was checked by P22 linkage to known markers and/or PCR analysis. The constructs resulting from this procedure were moved into a clean, wild-type background (14028) by P22 transduction. In some strains, the antibiotic resistance cassettes were removed using the temperature-sensitive plasmid pCP20 carrying FLP recombinase (71). For construction of the Δ*tatABC-lacZ* reporter fusion strain, a kanamycin-resistant deletion of *tatABC* was generated using primers P106 and P107 (Table S2). The insertion mutation generated by Lambda Red-mediated recombination was converted to transcriptional *lacZ* fusions using an FLP/FLP recombination target (FRT)-mediated site-specific recombination method, as previously described (72). Plasmids constructed in this work were verified by sequencing analysis at the Arizona State University Genomics Facility. Primers used for the deletions and cloning are described in Table S2. Plasmid pJE229 was constructed by amplification of the entire region from *pspF* to *pspE* using primers P395 and P396 (Table S2). The amplified product and pDX1 were digested with SphI and NheI. This digest removes *lacZ* while maintaining *oriRg*, apramycin resistance, and *attP*. The pDX1 backbone was gel purified and ligated to the *psp* region and transformed into electrocompetent *pir*$^+$ *E. coli*. Different lengths of the *tatABC* promoter were cloned 5' to the promoterless *lacZ* gene in pAH125 (see Table S2 for detailed primer information) to generate *tatABC*+ P$_{tatABC}$-*lacZ* constructs. After sequence verification, all of the resulting pAH125-

or pDX1-derived plasmids were integrated into the *S.* Typhimurium chromosome at the *attB*$_\lambda$ site using λ Int produced from the conditional replication, integration, and modular (CRIM) helper plasmid pINT-ts (61). The integrated plasmids were tested by PCR to confirm that a single copy was integrated. pAT6::Cm was made by amplifying *cat* from pBAD33 and digesting the PCR product and pAT6 with PvuI to remove β-lactamase. Digested pAT6 was treated with rSAP to prevent self-ligation, purified, and ligated to the *cat* cassette.

TorA-mCherry-ssrA translocation assay. Overnight cultures were subcultured 1:100 in LB with ampicillin and 0.1% arabinose and grown for 5 h at 37°C; 1:100 subcultures were made again in LB with ampicillin and 0.1% arabinose in a 96-well plate. Cells were grown for 14 h at 37°C with shaking and continuous orbital rotation at 180 rpm in a Biotek Cytation 3 plate reader. Every 15 min, cells were excited at 570 nm, and emission was measured and recorded at 610 nm. At each time point, the OD$_{600}$ was also recorded. The following formula was used to calculate normalized mCherry values: (mCherry − mCherry background)/OD$_{600}$. Because bile salts also emit a fluorescent signal at 610 nm, mCherry background was calculated by reading the media-only control wells and taking the mean fluorescence at 610 nm at each of the bile concentrations. The corrected mCherry signal was then divided by OD$_{600}$ to generate a relative fluorescent mCherry signal in each well.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.
SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

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