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RESEARCH ARTICLE

Envelope Stress and Regulation of the *Salmonella* Pathogenicity Island 1 Type III Secretion System

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ABSTRACT *Salmonella enterica* serovar Typhimurium uses a type three secretion system (T3SS) encoded on the *Salmonella* pathogenicity island 1 (SPI1) to invade intestinal epithelial cells and induce inflammatory diarrhea. The SPI1 T3SS is regulated by numerous environmental and physiological signals, integrated to either activate or repress invasion. Transcription of *hilA*, encoding the transcriptional activator of the SPI1 structural genes, is activated by three AraC-like regulators, HilD, HilC, and RtsA, that act in a complex feed-forward loop. Deletion of *bamB*, encoding a component of the β-barrel assembly machinery, causes a dramatic repression of SPI1, but the mechanism was unknown. Here, we show that partially defective β-barrel assembly activates the RcsCDB regulon, leading to decreased *hilA* transcription. This regulation is independent of RpoE activation. Though Rcs has been previously shown to repress SPI1 when disulfide bond formation is impaired, we show that activation of Rcs in a *bamB* background is dependent on the sensor protein RcsF, whereas disulfide bond status is sensed independently. Rcs decreases transcription of the flagellar regulon, including *fliZ*, the product of which indirectly activates HilD protein activity. Rcs also represses *hilD*, *hilC*, and *rtsA* promoters by an unknown mechanism. Both *dsbA* and *bamB* mutants have motility defects, though this is simply regulatory in a *bamB* background; motility is restored in the absence of Rcs. Effector secretion assays show that repression of SPI1 in a *bamB* background is also regulatory; if expressed, the SPI1 T3SS is functional in a *bamB* background. This emphasizes the sensitivity of SPI1 regulation to overall envelope homeostasis.

IMPORTANCE *Salmonella* causes worldwide foodborne illness, leading to massive disease burden and an estimated 600,000 deaths per year. *Salmonella* infects orally and invades intestinal epithelial cells using a type 3 secretion system that directly injects effector proteins into host cells. This first step in invasion is tightly regulated by a variety of inputs. In this work, we demonstrate that *Salmonella* senses the functionality of outer membrane assembly in determining regulation of invasion machinery, and we show that *Salmonella* uses distinct mechanisms to detect specific perturbations in envelope assembly.

KEYWORDS Bam, RcsCDB, SPI1, *Salmonella*, pathogenesis

Salmonella serovars are common foodborne pathogens, causing over 1 million cases of disease per year in the United States (1). *Salmonella enterica* serovar Typhimurium typically causes self-limiting gastroenteritis, but in some immunocompromised individuals infection can result in septicemia and death. Infection is initiated by activating the *Salmonella* pathogenicity island 1 type 3 secretion system (SPI T3SS) in the distal ileum of the small intestine. This system is tightly controlled by a large number of inputs that regulate the production of the transcriptional activator, HilA. The *hilA* gene is directly activated by three AraC-like regulators, HilD, HilC, and RtsA, which act in a feed-forward loop to activate expression of themselves, each other, and *hilA* (Fig. 1) (2). This regulatory circuit responds to a plethora of environmental signals and regulatory

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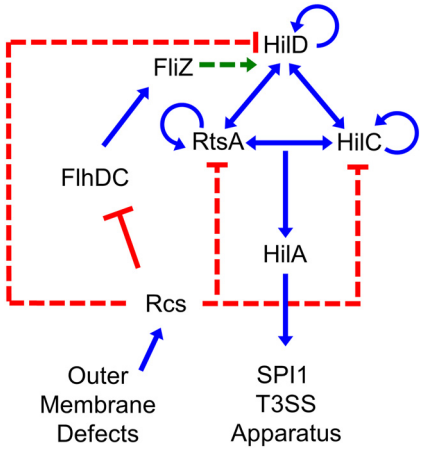

FIG 1 SPI1 T3SS regulatory circuit. Simplified regulatory model of the SPI1 T3SS and related regulators. Blue lines indicate transcriptional activation, red lines indicate transcriptional repression, and green lines indicate regulation at the protein level. Dotted lines indicate that the exact mechanism of regulation is not known and is likely indirect.

systems, leading to precise control of expression and coordination with other cellular systems.

One signal that has been shown to repress *hilA* expression is a defect in the β-barrel assembly machinery (BAM) complex (3, 4), required for proper assembly of outer membrane β-barrel proteins (OMPs) (5–11). OMPs are synthesized in the cytoplasm, secreted into the periplasm through the Sec translocase (12), and delivered to the multisubunit BAM complex via chaperones (13). The BAM complex consists of the β-barrel OMP BamA, which is highly conserved and essential, and multiple accessory proteins that aid in OMP assembly (6, 8, 14–16). These include BamBCDE, which are all OM lipoproteins whose precise functions are unknown (6, 8, 17). When the system is strained, unfolded OM proteins accumulate in the periplasm, leading to activation of RpoE, which upregulates BAM complex components and outer membrane homeostasis proteins to reduce outer membrane stress (18, 19).

RpoE can negatively regulate genes as well, mainly through small RNAs (sRNAs) that rapidly alter expression, binding mRNAs to block their translation or stimulate degradation (20). The RpoE-regulated sRNAs MicA and RybB reduce OMP synthesis by repressing *ompA*, *lamB*, *ompC*, and *ompX* translation (21–24). MicC represses translation of *ompC* and *ompD* mRNAs (25, 26), while the more recently characterized MicL (RyeF) represses the major OM lipoprotein Lpp (25, 27).

Though RpoE could sense a defective BAM complex and repress *hilA*, there are other envelope stress responses that could be responsible. PhoPQ and RcsBCD both respond to OM insults (28–30), while EnvZ/OmpR responds to osmotic stress (31) and periplasmic stress (32); all of these systems are known to regulate SPI1 (2, 3, 33–36). The Rcs system responds to β-lactam antibiotics (37–40), lysozyme (41, 42), LPS charge/membrane fluidity (28, 43, 44), and lipoprotein trafficking (45). The Rcs system is activated via a phosphorylation cascade from the sensor kinase RcsC, through the phosphotransfer protein RcsD, to the response regulator RcsB (46). When phosphorylated, RcsB can form homodimers and activate various targets in the cell, including the sRNA RprA, known mainly for increasing translation of the general stationary-phase stress response sigma factor RpoS (47, 48). RcsB can also form heterodimers with several other proteins (49), including RcsA, to activate colonic acid capsule genes and repress flagellar regulatory genes, leading to a biofilm lifestyle (49–51).

Deletion of the nonessential β-barrel assembly protein *bamB* leads to repression of *hilA* expression through an unknown mechanism (3, 4). In this work, we show that, although deletion of *bamB* does activate the RpoE stress response in *Salmonella*, repression of *hilA* occurs independently of known RpoE-regulated sRNAs. Rather, a

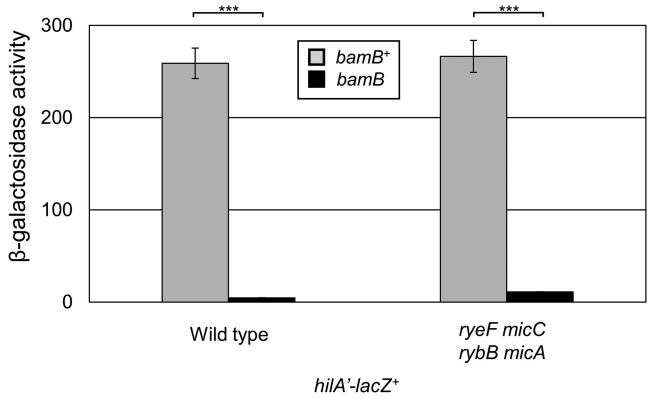

FIG 2 Loss of BamB represses SPI1 expression independent of RpoE-activated sRNAs. β-Galactosidase activity was measured from cells containing a *hilA'-lacZ*$^+$ transcriptional fusion in the indicated backgrounds. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. Strains used: JS749, JS1040, JS2367, and JS2368.

dysfunctional BAM complex stimulates the Rcs response to repress *hilA* through the flagellar regulatory protein FliZ, which affects HilD protein activity, as well as through transcriptional control of *hilD*, *hilC*, and *rtsA*.

RESULTS

Loss of BamB leads to repression of *hilA* independently of RpoE. Deletion of *bamB* (*yfgL*), encoding a component of the β-barrel assembly machinery, has been shown to repress expression of the SPI1 T3SS (3, 4, 52). We first wanted to confirm that deletion of *bamB* leads to repression of the main SPI1 activator *hilA* using a *hilA'-lacZ*$^+$ transcriptional fusion. In the *bamB* mutant background, *hilA* expression decreased dramatically, demonstrating that loss of BamB leads to loss of SPI1 expression (Fig. 2).

The BAM complex is required to properly fold and assemble outer membrane β-barrel proteins (5–11). Misfolded outer membrane proteins induce the RpoE regulon, which includes periplasmic chaperones, periplasmic proteases, and small RNAs that block translation of certain outer membrane protein mRNAs, relieving stress on the BAM system (19). Mutations affecting the BAM complex are known to stimulate the RpoE response in *E. coli* (53). We first hypothesized that the repression of SPI1 seen in the *bamB* mutant was mediated through RpoE. We confirmed that that deletion of *bamB* activates RpoE in *Salmonella* by monitoring expression from an *rpoE'-lacZ*$^+$ transcriptional fusion (see Fig. S1 in the supplemental material).

Previous data had suggested that deletion of *bamB* leads to repression of *hilA* through repression of *hilD* translation (3), and because negative regulatory effects from RpoE tend to be sRNA mediated (12), we hypothesized that *hilA* is repressed through an RpoE-activated sRNA. We deleted the known RpoE-regulated sRNA genes *micA*, *micC*, *micL* (*ryeF*), and *rybB* and examined *hilA* expression in the presence and absence of BamB (Fig. 2). Deletion of RpoE-activated sRNAs did not alleviate repression of *hilA* expression, demonstrating that deletion of *bamB* integrates into SPI1 regulation via some other mechanism.

To test if RpoE could repress SPI1 expression independently of known regulated sRNAs, we monitored expression of the *hilA'-lacZ*$^+$ fusion in a strain overexpressing OmpX, known to induce the RpoE regulon (54). Expression of OmpX led to activation of RpoE, as demonstrated by increased expression from an *rpoE'-lacZ*$^+$ fusion (Fig. S1), but this had no effect on SPI1 expression, suggesting that loss of *bamB* must repress SPI1 independently of RpoE.

Rcs mediates repression of SPI1 in the absence of *bamB*. To identify the mediator of *hilA* repression in the absence of *bamB*, we performed a transposon mutagenesis in a *hilA'-lacZ*$^+$ *bamB* background, screening for mutants with increased expression of *hilA*

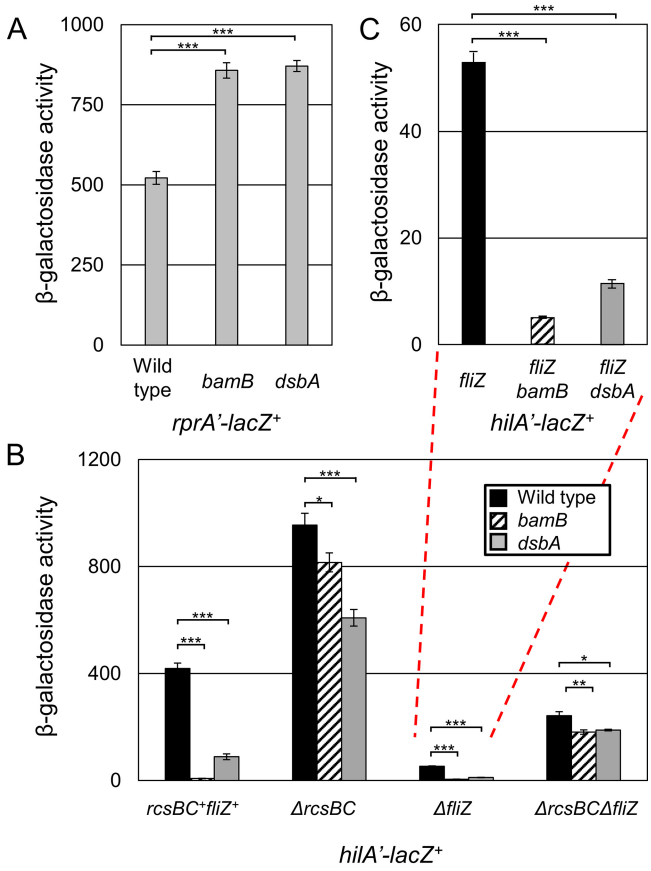

FIG 3 BAM dysfunction stimulates Rcs, which represses SPI1. β-Galactosidase activity was measured from cells containing the indicated transcriptional fusion. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB medium. Panel C represents a replotting of the indicated data in panel B. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. P values are indicated as follows from unpaired t tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Strains used were JS2376, JS2377, and JS2378 (A) and JS749, JS1040, JS754, JS775, JS776, JS2379, JS778, JS2380, JS780, JS782, JS2381, and JS783 (B).

on LB agar containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal). Subsequent β-galactosidase assays identified four MudCm insertions (55) that conferred greater than a 5-fold increase in *hilA* expression in the *bamB* background (Fig. S2). DNA sequence analyses (56) indicated that two of the insertions were in *topA*, encoding topoisomerase I, one was in *yjiY*, encoding an inner membrane transporter (57), and one was in *rcsD* (*yojN*), encoding part of the RcsBCD signal transduction system (58). To confirm these phenotypes, we transduced the MudCm insertions into strains containing the *hilA'-lacZ*$^+$ with or without *bamB*. The *yjiY*-associated insertion had the most modest effect and functions through an unknown mechanism. MudCm insertions associated with *topA* and *rcsD* alleviated repression most dramatically. The mutations in *topA* are more likely causing indirect increases in *hilA* expression and we did not follow up with this mutation (59). Given the role of the Rcs system in sensing envelope stress (46) and the fact that this system had been previously shown to affect SPI1 expression, it seemed likely that the *bamB* effect could be mediated primarily through Rcs.

To confirm that the Rcs regulon was induced in the absence of *bamB*, we fused the promoter of the Rcs-regulated sRNA gene *rprA* to *lacZ* and integrated that fusion into the lambda attachment site. In the absence of *bamB*, *rprA* expression roughly doubled, confirming activation of the Rcs regulon (Fig. 3A). We previously demonstrated that *Salmonella* uses Rcs to sense disulfide bond status in the periplasm and repress *hilA* expression when disulfide bond formation is defective (35). We confirmed that Rcs is

activated in the absence of *dsbA*; deletion of *dsbA* also led to doubling of *rprA* expression, similar to effects seen in the *bamB* deletion background.

Disulfide bond status and β-barrel assembly affect SPI1 expression through Rcs. The *dsbA* mutant was shown to repress SPI1 both through activation of Rcs, which represses *flhDC*, encoding the main transcriptional regulator of the flagellar regulon, and by preventing proper assembly of the flagellar apparatus, thereby blocking export of the anti-sigma factor FlgM (35). Both of these effects decreased expression of *fliZ*, encoding a protein that indirectly controls HilD activity (60, 61).

To explore how Rcs senses β-barrel protein assembly compared to disulfide bond status, we deleted *bamB* or *dsbA* in the presence and absence of *rcsBC* and tested expression of a *hilA′-lacZ*$^+$ fusion. Deletion of *rcsBC* led to increased *hilA* expression, demonstrating that in SPI1-inducing conditions, Rcs partially represses *hilA* (Fig. 3B). In the absence of *rcsBC*, deletion of *dsbA* repressed *hilA*, but much less than in an *rcsBC*$^+$ background, because the *dsbA* effect functions through Rcs and FlgM (35). Most importantly, in the absence of *rcsBC*, deletion of *bamB* had only a minor effect on *hilA* expression. This demonstrates that the Rcs system does mediate repression of *hilA* when the BAM complex is not fully functional.

The Rcs system represses the flagellar operon by downregulating the master flagellar regulator FlhDC (51). Flagellar regulation has been shown to integrate into SPI1 regulation, mainly through the FlhDC-activated flagellar regulator FliZ (60, 61). To test if the *bamB* effect is integrated through FliZ similarly to *dsbA*, we tested the effects of *dsbA* and *bamB* mutants on *hilA* expression in the absence of *rcsBC* and *fliZ*. In the absence of *fliZ*, deletion of either *dsbA* or *bamB* significantly decreased *hilA* expression (Fig. 3C). This shows that *bamB* and *dsbA* repressive effects integrate into SPI1 at least in part independently of FliZ. When both *rcsBC* and *fliZ* are deleted, we see almost no further repression of SPI1 in the *bamB* and *dsbA* mutant backgrounds, proving that these systems are the main integration point for SPI1 regulation. Together, these data show that, although FliZ is a key link between Rcs and SPI1 regulation, Rcs regulates SPI1 through some additional mechanism.

Rcs senses disulfide bond status and β-barrel assembly through distinct mechanisms. Lin et al. showed that the *dsbA* effect is amplified in the presence of a reducing agent that further disrupts disulfide bond formation in *Salmonella* (35). If the mechanism of sensing disulfide status and BAM function are the same, we would expect dithiothreitol (DTT) to amplify the effects of the *bamB* mutant as well. The addition of the reducing agent DTT further decreased *hilA* expression in the *dsbA* deletion background, as expected (Fig. 4). In contrast, DTT partially suppressed the repression of *hilA* expression conferred by loss of BamB. These data strongly suggest that the mechanism of SPI1 regulation, and perhaps the mechanism of Rcs activation, differs in the *bamB* and *dsbA* backgrounds.

The *bamB* effect depends on the signaling protein RcsF, whereas the *dsbA* effect is RcsF independent. We originally proposed that deletion of *dsbA* affected disulfide bond formation in the sensor protein RcsF, which contains two disulfide bonds (62) and senses a subset of signals that activate RcsBCD. To sense outer membrane stress, RcsF is threaded through abundant β-barrel proteins, which require BAM for proper folding (63, 64). To test if the *dsbA* effect is functioning at the level of RcsF, we deleted *dsbA* in the absence of *rcsF* and monitored expression of *hilA*. Deletion of *dsbA* led to repression of *hilA* regardless of *rcsF* (Fig. 5), showing that *dsbA* affects expression independently of the disulfide bonds in RcsF. In contrast, deletion of *rcsF* completely suppressed the decrease in *hilA* expression conferred by loss of BamB. Thus, the outer membrane defect caused by deletion of *bamB* is sensed by RcsF, whereas disulfide bond status appears to activate Rcs through some other mechanism.

Flagellar gene expression responds to β-barrel assembly. To confirm that the *bamB* effect functions partially through flagellar regulation, we monitored expression of both *flhDC* and *fliZ*. Expression from both the *fliZ′-lacZ*$^+$ and *flhDC′-lacZ*$^+$ fusions was significantly reduced in the absence of *bamB* (Fig. 6). This repression was completely

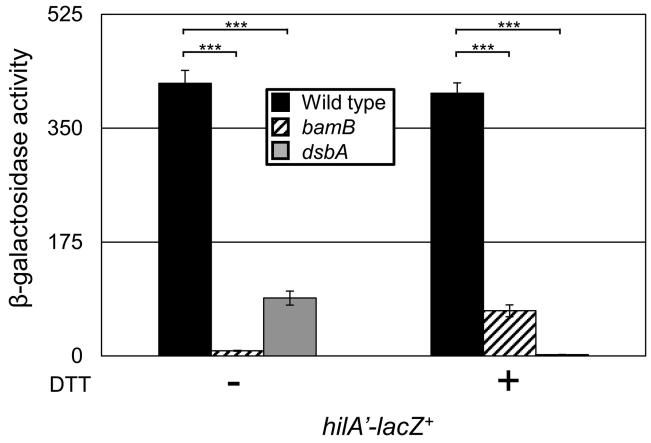

FIG 4 Rcs senses disulfide bond status and BAM functionality through distinct mechanisms. β-Galactosidase activity was measured from cells containing a *hilA'-lacZ*$^+$ transcriptional fusion in the designated backgrounds. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB medium in the presence or absence of 2 mM DTT. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. *P* values are indicated as follows from unpaired *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Strains used: JS749, JS1040, and JS754.

alleviated in the absence of *rcsBC*, demonstrating that all regulatory effects are dependent on RcsBC. In contrast, deletion of *dsbA* had an additional slight repressive effect on *fliZ* expression independent of RcsBC, whereas *flhDC* expression was not affected. These data suggest that *bamB* represses SPI1 by activating Rcs to repress *flhDC* and, therefore, *fliZ*. The *dsbA* mutation also affects *fliZ* transcription independently of Rcs, presumably due to the inability to export FlgM, leading to decreased expression from the FliA-dependent promoter (35).

To test how BamB and DsbA affect flagellar motility, we inoculated various *Salmonella* strains on motility agar (Fig. 7). Loss of BamB conferred a modest motility defect. In contrast, loss of DsbA conferred a complete loss of motility, apparently equivalent to deletion of *flgI*, encoding a flagellar structural protein previously shown to require DsbA to fold appropriately (65). Further deletion of either *rcsBC* or *rcsF* completely suppressed

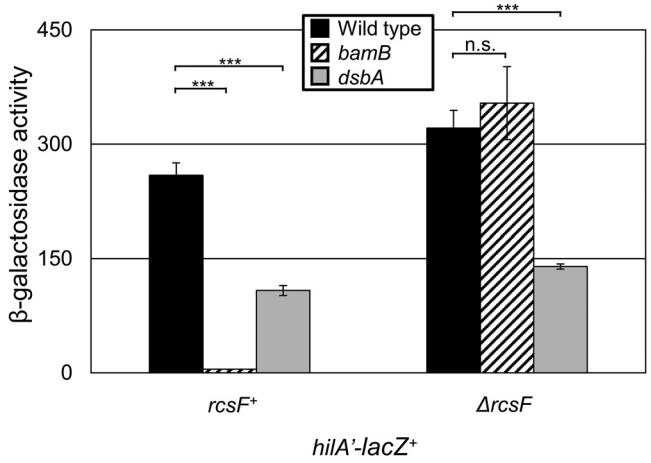

FIG 5 RcsF senses β-barrel assembly but not disulfide bond status. β-Galactosidase activity was measured from cells containing a *hilA'-lacZ*$^+$ transcriptional fusion in the designated backgrounds. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. *P* values are indicated as follows from unpaired *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Strains used: JS749, JS1040, JS754, and JS2383-JS2385.

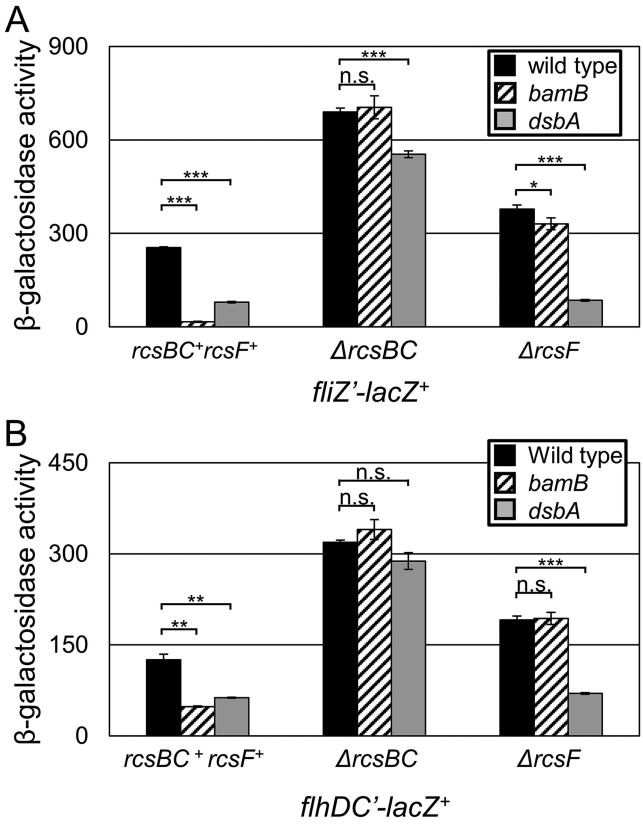

FIG 6 Rcs repression of flagellar regulators. β-Galactosidase activity was measured from cells containing the indicated *lacZ* transcriptional fusions. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB medium. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. *P* values are indicated as follows from unpaired *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Strains used were JS696, JS785, JS794, JS788, and JS2392-JS2396 (A) and JS2347 and JS2405-JS2412 (B).

the *bamB* motility defect but had no effect in the *dsbA* background (Fig. 7B and C). This suggests that the effect on motility caused by loss of BamB is due to activation of the Rcs system via RcsF, and thus the Bam complex, or at least BamB, is not required to build a functional flagellum. Loss of DsbA, on the other hand, activates Rcs, leading to repression of flagellar expression (Fig. 6), but also leads to an inability to assemble a functional apparatus.

***β*-Barrel assembly affects *hilD*, *hilC*, and *rtsA* transcription.** Because repression of SPI1 expression caused by loss of *bamB* is partially independent of FliZ (Fig. 3B) and because Lin et al. suggested that part of the *dsbA* effect is HilD dependent, we tested if the *bamB* effect also functions through *hilD*, *hilC*, or *rtsA* transcription. Because HilD, HilC, and RtsA activate expression of themselves and each other, we monitored expression of the promoters in strains deleted for all three regulators. Surprisingly, expression from all three promoters was significantly decreased in both the *bamB* and *dsbA* backgrounds (Fig. 8A). This is the first case of significant signal integration at the level of *hilC* transcription that we have observed. To test if this effect was dependent on Rcs, we monitored expression from a *hilD'-lacZ*$^+$ fusion and examined both *bamB* and *dsbA* effects in the presence and absence of *rcsBC* or *rcsF*. Because this fusion is a deletion of *hilD*, we expect no expression from *hilC* or *rtsA*, so any effect on *hilD* transcription should be specific to the *hilD* promoter (33). Expression of *hilD* was repressed about 2-fold in an RcsBC-dependent manner (Fig. 8B). As above, loss of RcsF suppressed the decreased expression in the *bamB* background but had no effect in the *dsbA* mutant background. We observed a similar pattern on a *hilC'-lacZ*$^+$ transcriptional

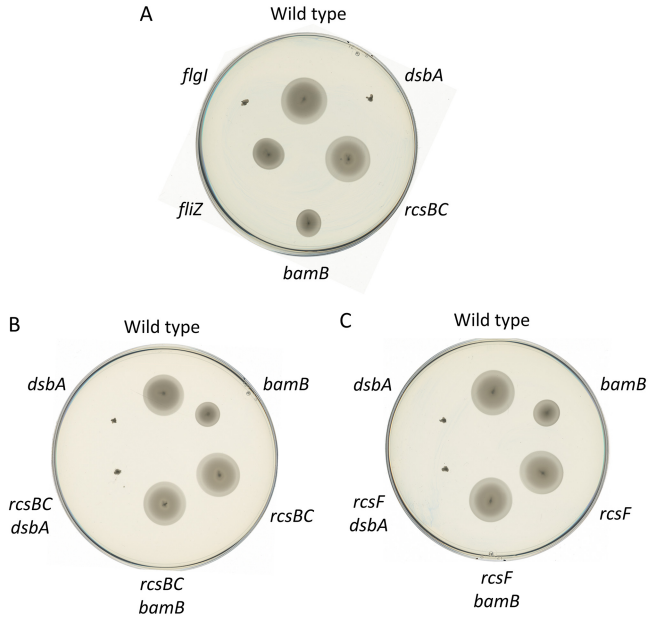

FIG 7 Rcs represses motility. Colonies of the indicated strains were stabbed into motility agar and incubated at 37C for 11 h. Strains used were as follows: wild type, JS1039, JS326, JS745, JS746, and JS747 (A); wild type, JS1039, JS745, JS2413, JS2414, and JS326 (B); and wild type, JS1039, JS2382, JS2415, JS326, and JS2416 (C).

fusion (Fig. 8C). Together, these data demonstrate that Rcs integrates into SPI1 regulation through *hilD*, *hilC*, and *rtsA* transcription, a combinatorial effect repressing SPI1 expression that could explain the remaining repressive phenotype observed in the absence of *fliZ* (Fig. 3).

SPI1 can assemble in the absence of *bamB*. Effects on β-barrel assembly could lead to outer membrane stress that affects proper assembly of the SPI1 T3SS. Alternatively, repression of SPI1 may just be regulatory and if bypassed, a functional T3SS could assemble and secrete effectors. To test this, we deleted *hilD* and expressed *hilA* from a plasmid to bypass regulatory repression in a *bamB* background. As expected, we observed SopB secretion in the *hilD* pHilA background (Fig. 9). To confirm the functionality of this assay, we deleted *invG*, a structural protein of the SPI1 T3SS that is required for a functional apparatus (66–68). As expected, SopB was produced but not secreted in the absence of InvG. Deletion of *bamB* had no effect on SopB production or secretion in the pHilA background. When we deleted *invG* in the *bamB* background, secretion was abolished, demonstrating that this is SPI1-dependent export. Together, these data suggest that defects in β-barrel assembly lead to repression of the SPI1 T3SS but do not destroy its functionality (4).

DISCUSSION

Expression of the SPI1 T3SS is tightly controlled in response to various regulatory inputs. This allows *Salmonella* to activate or repress the invasion apparatus depending on environmental conditions that can indicate location within the host, as well as the physiological status within the bacterium (33–35, 60, 69–71). Here, we show that *Salmonella* specifically senses outer membrane β-barrel protein assembly through RcsF, the outer membrane lipoprotein sensor. RcsF stimulates the RcsCDB system, leading to repressed expression of *flhDC*, encoding the master flagellar regulator. This in turn represses SPI1 by decreasing expression of the flagellar regulator FliZ that indirectly activates HilD protein activity (35, 60). We also show that Rcs represses transcription of *hilD*, *hilC*, and *rtsA* by an unknown mechanism. Although the SPI1 T3SS can function if expressed in a *bamB* null strain, it is presumably deleterious to assemble the machine and invade while dealing with significant outer membrane stress, or, perhaps, when

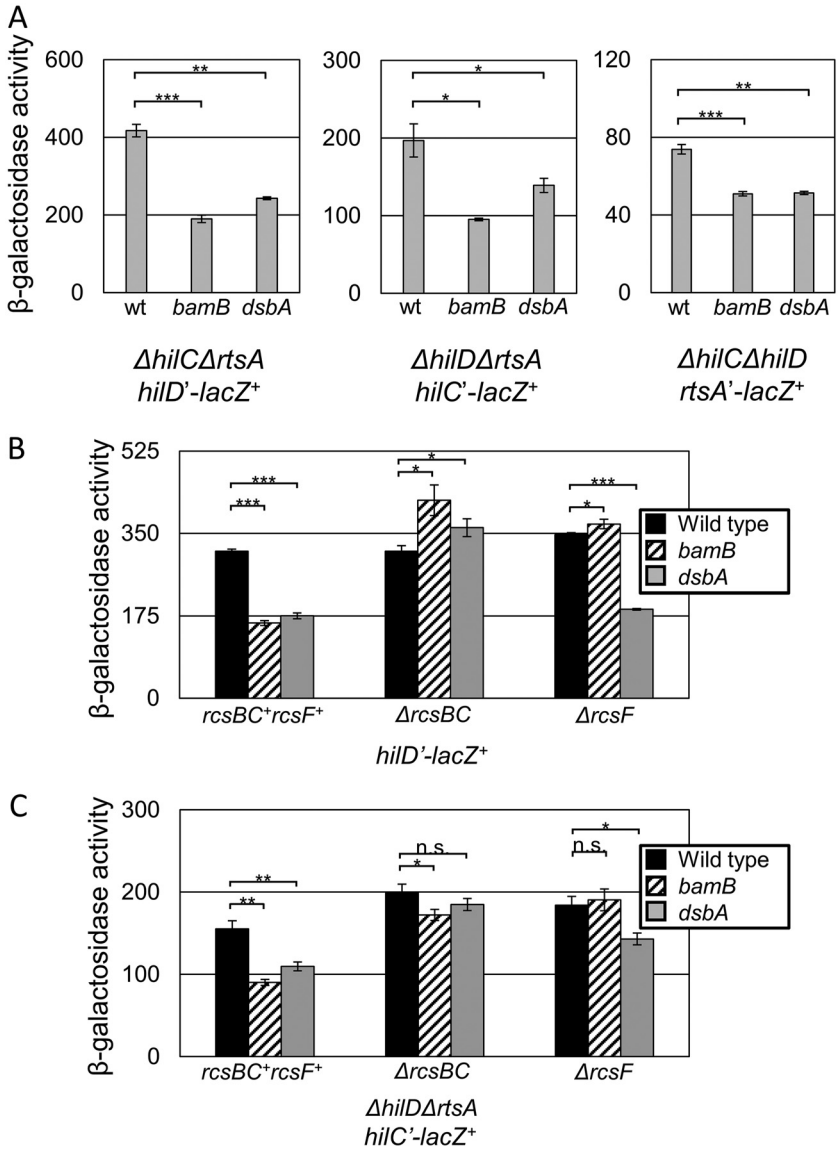

FIG 8 Rcs represses *hilD*, *hilC*, and *rtsA* transcription. β-Galactosidase activity was measured from cells containing the indicated *lacZ* transcriptional fusions in the designated backgrounds. Cells were grown overnight, subcultured 1:100, and then grown with mild aeration for 6 h at 37°C in HSLB medium. β-Galactosidase activity units are defined as (micromoles of ONP formed per minute) × 10^6/(OD_{600} × milliliters of cell suspension) and are reported as mean ± standard deviation where $n = 3$. *P* values are indicated as follows from unpaired *t* tests: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. Strains used were JS2201, JS2386-JS2391, JS2198, and JS2191 (A); JS488 and JS2397-2404 (B); and JS2198, JS2388-JS2389, and JS2417-JS2422 (C).

some particular protein or aspect of outer membrane integrity is deficient. *Salmonella* has clearly evolved complex mechanisms for appropriate activation of the invasion apparatus and senses critical bacterial functions via several different mechanisms.

Though RcsCDB has previously been shown to sense hindered disulfide bond status and repress SPI1 through flagellar regulatory pathways, *bamB* and *dsbA* deletions activate Rcs using clearly different mechanisms. We originally proposed that loss of *dsbA* activates through RcsF, which contains multiple disulfide bonds (62). It was subsequently shown that mutating the cysteine residues in RcsF leads to decreased stimulation of the Rcs response (72). Here, we show that deletion of *dsbA* stimulates Rcs through an unknown RcsF-independent mechanism. This is in stark contrast to a *bamB* mutant, in which activation of Rcs is strictly RcsF dependent. Consistent with indepen-

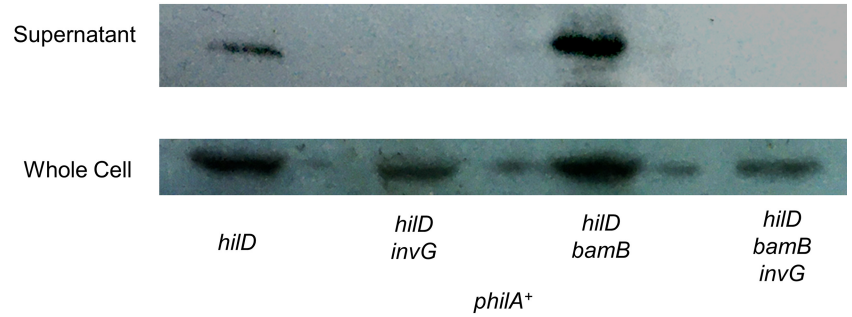

FIG 9 The SPI1 T3SS can function in the absence of *bamB*. Cells were grown overnight, subcultured 1:100 into 10 ml HSLB-Amp and grown standing for 18 h at 37°C. Cells were pelleted and supernatant was TCA precipitated. Western blotting was performed using anti-FLAG to identify SopB levels within the cell and the cell culture supernatant as indicated. The strain used was JS2425-JS2428 containing plasmid pHilA^{+}.

dent mechanisms of activation, the addition of the reducing agent DTT partially suppresses the *bamB* phenotype. DTT presumably disrupts the key disulfide bonds in RcsF, reducing its signal transduction role and alleviating stimulation of Rcs in the absence of *bamB*. In contrast, the addition of DTT exacerbates the *dsbA* phenotype to stimulate Rcs, suggesting that disulfide bond status in some other protein(s) may be affected to activate signaling. One enticing hypothesis is that the repressor of Rcs, IgaA, which contains four conserved periplasmic cysteines (73), becomes dysfunctional in the *dsbA* background. This is difficult to test because *igaA* is an essential gene, but disruption of its disulfide bonds inhibits its ability to repress Rcs (73, 74). Further studies will be required to demonstrate this mechanism conclusively.

Another key difference between *dsbA* and *bamB* mutants is motility. Both stimulate Rcs, which represses flagellar regulatory pathways, leading to clear motility defects in both backgrounds. In the case of defective β-barrel protein assembly, motility is restored in the absence of RcsBC, meaning that the motility defects are solely regulatory. In contrast, in the absence of *dsbA*, we see Rcs-independent motility defects. This is likely due to key disulfide bonds in FlgI that are required for proper assembly of the flagellar apparatus (75). Without proper assembly, flagellar regulatory systems are repressed by the anti-sigma factor FlgM, which remains intracellular and bound to the sigma factor FliA, leading to further repression of SPI1 via decreased expression of *fliZ* from the FliA-dependent promoter (35, 76, 77).

In addition, we expand upon how Rcs controls SPI1 regulation. Most signals that integrate into SPI1 regulation have previously been shown to function at the level of *hilD* translation or through HilD protein, as in the case of FliZ (60). However, Rcs activation also represses *hilD*, *hilC*, and *rtsA* transcription. This is a rare example of a regulatory input acting through *hilC*. Decreased expression of these critical activators certainly contributes to downregulation of SPI1. The mechanism of this regulation is unknown, although we do not believe that it is direct. Rcs could, for example, be functioning through H-NS, which contributes to silencing of *hilD*, *hilC*, *rtsA*, and *hilA* (33, 78–80).

The role of the Rcs system in pathogenesis is complex. The negative regulatory protein IgaA was originally identified by partial loss of function mutations that allowed *Salmonella* to proliferate in fibroblasts, normally nonpermissive for replication (81). However, in mice, activation of the system by point mutations in *igaA* (82) or *rcsC* (30) attenuates *Salmonella* virulence. Knocking out capsule production partially, but not completely, suppressed this loss in virulence (30). These studies included intraperitoneal infections, so the virulence defect is not simply due to decreased expression of flagella or the SPI1 T3SS, which are neither expressed nor required systemically (2, 83). Deleting downstream Rcs components also suppressed the virulence defect caused by the respective activating mutations (30, 82). These results would suggest that the Rcs

system is normally "off" during *Salmonella* infection. However, Detweiler et al. (84) showed that *rscC* loss of function mutants were attenuated after several weeks of infection in NRAMP$^{+/+}$ mice. Thus, while inappropriate activation of RcsCDB is clearly detrimental, proper regulation is important during infection.

MATERIALS AND METHODS

Media, reagents, and enzymatic assays. Strains were routinely grown at 37°C in Luria-Bertani (LB) medium containing 1% salt, termed HSLB. SOC medium was used to recover all transformants (85). Strains containing temperature-sensitive plasmids pCP20 and pKD46 were grown at 30°C. Motility agar contained 0.3% Bacto agar, 1% tryptone, and 0.5% NaCl. When necessary, antibiotics were used as follows: ampicillin 50 μg/ml, chloramphenicol 20 μg/ml, and kanamycin 50 μg/ml. Primers were purchased from IDT; plasmids were constructed using NEBuilder HiFi DNA Assembly and Invitrogen restriction enzymes. Strains for β-galactosidase assays were grown overnight in LB containing 0% salt (NSLB) at 37°C, and subcultured 1:100 in HSLB with or without 2 mM dithiothreitol (DTT) and then grown for 6 h, at which point the β-galactosidase was performed as previously described (86). β-Galactosidase activity units are defined as (micromoles of *o*-nitrophenol formed per minute) $\times$ 10^6/(optical density at 600 nm [OD_{600}] $\times$ millilters of cell suspension). The data are expressed as means $\pm$ standard deviations with three biological replicates ($n = 3$).

Strain and plasmid construction. All strains and plasmids used in this study are described in Table S1 in the supplemental material. All strains are derivatives of *Salmonella enterica* serovar Typhimurium ATCC 14028 (American Type Culture Collection). Deletions and simultaneous insertion of antibiotic resistance cassettes were constructed using λ-red-mediated recombination as previously described (87, 88), with the indicated endpoints. All deletions were checked by PCR analysis and transduced into clean wild-type backgrounds using P22 HT105/1 *int*-201 transduction (85). When necessary, antibiotic cassettes were removed using the FLP recombinase, encoded on the temperature-sensitive pCP20 plasmid (89).

To build the *rprA-lacZ* fusion, the promoter of *rprA* was cloned into pDX1 (35) using primers *rprA* promoter forward (5′-TCT AGA GGA TCC CCG GGT ACT TAT CAA TTC AAC GCA CAC-3′) and *rprA* promoter reverse (5′-TTG TCG GAT CCC CGG GAA TTT GTG CTA ATA GTA GGC ATG-3′). PDX1 was digested with EcoRI and KpnI. The fusion was then integrated into the lambda attachment site using λInt produced from the CRIM helper plasmid pINT-ts (90). Integration of a single copy was confirmed by PCR.

The SopB-3$\times$FLAG fusion was constructed using primers pSUB11 sopB fw (5′-TTG GCA GTC AGT AAA AGG CAT TTC TTC ATT AAT CAC ATC TGA CTA CAA AGA CCA TGA CGG-3′) and pSUB11 sopB rev (5′-TAA ACG ATT TAA TAG ACT TTC CAT ATA GTT ACC TCA AGA CCA TAT GAA TAT CCT CCT TAG-3′) as previously described (91).

MudCm insertions. An estimated 40,000 MudCm insertions were created in a *hilA′-lacZ*$^+$ *bamB* mutant background and plated on LB agar containing 50 μg/ml X-Gal and 20 μg/ml chloramphenicol (55). Dark blue colonies were isolated and β-galactosidase assays were performed as described above. MudCm insertions were located by semirandom PCR, first amplifying using primers INSEQB (5′-GGC CAC GCG TCG ACT AGT CAN NNN NNN NNN ACG CC-3′) and MudL1 (5′-CCC ATC AGA TCC CGA ATA ATC C-3′) (56). A 2.5-μl aliquot of that product was used as a template for primers INSEQD (5′-GGC CAC GCG TCG ACT AGT AC-3′) and MudL2 (5′-GCA AGC CCC ACC AAA TCT AAT CC-3′). The final PCR product was sequenced using primer MudL3 (5′-CGA CTA AAA TTT GCA CTA CAG GC-3′).

Western blotting of secreted proteins. SopB was detected by Western blotting as previously described (35). Strains were grown for 10 h with mild aeration at 37°C, then subcultured 1:100 in 10 ml of HSLB containing ampicillin at a concentration of 50 μg/ml, and grown standing for 18 h at 37°C. The culture supernatant was then filter sterilized using a 0.22-μm filter and concentrated to 1 ml using an Amicon-15 filter with a nominal molecular weight limit of 30,000. Proteins were precipitated with a final concentration of 10% cold trichloroacetic and by incubating them on ice for 30 min. Precipitated proteins were collected by centrifugation at 20,000 $\times$ *g* for 30 min at 4°C. The supernatant was then removed, and the trichloroacetic acid precipitate was washed with 1 ml of ice-cold 95% isopropanol. The samples were then centrifuged for 20 min at 20,000 $\times$ *g* and the supernatant was removed. The pellet was washed a final time in 95% isopropanol and allowed to air dry. The pellet was then suspended in 15 μl of 50 mM Tris (pH 8), and 15 μl of 2 $\times$ SDS loading buffer was added. The primary antibody used was monoclonal anti-FLAG BioM2 antibody produced in mouse (Sigma-Aldrich, St. Louis, Missouri), and the secondary was Pierce high-sensitivity streptavidin-horseradish peroxidase (Thermo Fisher Scientific, Waltham, MA).

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.3 MB.

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