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(p)ppGpp-Dependent Regulation of the Nucleotide Hydrolase PpnN Confers Complement Resistance in *Salmonella enterica* Serovar Typhimurium

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ABSTRACT The stringent response is an essential mechanism of metabolic reprogramming during environmental stress that is mediated by the nucleotide alarmones guanosine tetraphosphate and pentaphosphate [(p)ppGpp]. In addition to physiological adaptations, (p)ppGpp also regulates virulence programs in pathogenic bacteria, including *Salmonella enterica* serovar Typhimurium. *S.* Typhimurium is a common cause of acute gastroenteritis, but it may also spread to systemic tissues, resulting in severe clinical outcomes. During infection, *S.* Typhimurium encounters a broad repertoire of immune defenses that it must evade for successful host infection. Here, we examined the role of the stringent response in *S.* Typhimurium resistance to complement-mediated killing and found that the (p)ppGpp synthetase-hydrolase, SpoT, is required for bacterial survival in human serum. We identified the nucleotide hydrolase, PpnN, as a target of the stringent response that is required to promote bacterial fitness in serum. Using chromatography and mass spectrometry, we show that PpnN hydrolyzes purine and pyrimidine monophosphates to generate free nucleobases and ribose 5′-phosphate, and that this metabolic activity is required for conferring resistance to complement killing. In addition to PpnN, we show that (p)ppGpp is required for the biosynthesis of the very long and long O-antigen in the outer membrane, known to be important for complement resistance. Our results provide new insights into the role of the stringent response in mediating evasion of the innate immune system by pathogenic bacteria.

KEYWORDS *Salmonella*, complement, innate immunity, serum, virulence

Salmonella enterica is a bacterial pathogen with a broad host range for both animals and humans. Nontyphoidal strains of *Salmonella*, such as serovar Typhimurium, are acquired through contaminated food or water and cause gastrointestinal disease that is usually self-limiting (1). However, in developing countries, invasive, nontyphoidal serovars have been linked to severe clinical complications due to systemic bacteremia (2). One of the hallmarks of *S.* Typhimurium pathogenesis is its capacity to transition between the extracellular and intracellular environment due, in part, to the evolved ability of the bacteria to evade innate host defenses (3, 4).

A prominent arm of innate immunity that *S.* Typhimurium can encounter during infection is the complement system. Complement is composed of more than 30 proteins that are present in the circulation and at mucosal surfaces where frequent contact with microorganisms is made (5, 6). Activation of complement results in a proteolytic cascade that leads to the coating of target surfaces for phagocytic uptake

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or perturbation of bacterial membranes by the membrane attack complex (MAC) (6–8). Mechanisms of complement resistance by pathogenic bacteria have been described as proteolytic inactivation of complement proteins or the expression of cell surface structures to prevent complement deposition (9). For example, *S.* Typhimurium can express long variants of lipopolysaccharide (LPS) O-antigen, and this prevents the integration of the MAC onto the outer membrane (10, 11). Bacteria also undergo metabolic changes to ensure optimal use of resources upon exposure to serum (12–14). However, the role of these metabolic adaptations in promoting survival in serum is largely underexplored.

The stringent response is a mechanism of cellular reprogramming that allows bacteria to survive environmental stressors. It is characterized by the generation of the nucleotide alarmones ppGpp and pppGpp, collectively referred to as (p)ppGpp, and leads to rapid alterations in bacterial physiology (15, 16). The synthetase RelA produces (p)ppGpp during amino acid starvation through the sensing of uncharged tRNAs in the ribosomal A site (17–19). (p)ppGpp is also synthesized by SpoT in response to fatty acid, phosphate, iron, or carbon source limitation (20–22). In contrast to RelA, SpoT also has a hydrolase domain to balance cellular (p)ppGpp levels (23). Accumulation of (p)ppGpp inhibits the synthesis of ribosomal proteins, rRNA and tRNA, and activates stress-specific genes (15, 16). (p)ppGpp also regulates various metabolic pathways, including nucleotide biosynthesis, which occurs through allosteric regulation of enzymes such as PurF and PpnN in *Escherichia coli* (24–27). Bacterial pathogens often couple (p)ppGpp signaling to virulence gene expression (15, 16). In *S.* Typhimurium, (p)ppGpp helps activate genes required for invasion of the intestinal epithelium and intracellular survival (28–31). Thus, the stringent response mediates both physiological changes and expression of virulence factors in bacteria for successful host infection.

In this study, we examined the cross talk between the stringent response, metabolic reprogramming, and evasion of innate immunity. We found that SpoT is required for the survival of *S.* Typhimurium against human complement. Furthermore, we show that (p)ppGpp-mediated regulation of a nucleosidase called PpnN (formerly SL1344_2949) and biosynthesis of the LPS O-antigen promotes the survival of *S.* Typhimurium in serum. Our data provides insight into the role of nucleotide metabolism in bacterial resistance against complement.

RESULTS

The stringent response is required for complement resistance in *S.* Typhimurium. The stringent response is activated in response to environmental insults and is characterized by the production of (p)ppGpp. Mutants lacking the regulators, *relA* and *spoT*, are devoid of (p)ppGpp and have pleiotropic phenotypes, including the inability to grow in defined minimal media due to amino acid auxotrophies (32, 33). Consistent with this, we found that the growth of the Δ*relA* Δ*spoT* mutant was severely attenuated in M9-glucose minimal medium compared to growth in nutrient-rich LB broth, whereas the Δ*relA* mutant grew similarly to the wild type under both conditions (see Fig. S1 in the supplemental material). We were unable to generate a single Δ*spoT* mutant, because the (p)ppGpp hydrolase domain of SpoT is essential for bacterial viability in the presence of RelA (34). Given the role of the stringent response in enabling bacteria to adapt to changes in the environment, we next tested whether the stringent response was required for resistance against complement-mediated killing. We grew *S.* Typhimurium strains lacking *relA* or both *relA* and *spoT* in LB until stationary phase to mimic stringent response conditions and tested their survival in pooled human serum. The Δ*relA* mutant survived similarly to the wild-type strain, whereas the Δ*relA* Δ*spoT* mutant was highly susceptible to killing in normal human serum (Fig. 1A). Consistent with these data, complementation of the Δ*relA* Δ*spoT* mutant with SpoT expressed in *trans* from its native promoter completely rescued bacterial viability to wild-type levels. In contrast, the double mutant remained highly susceptible to serum-mediated killing when it was complemented with *spoT*^{G955C}, which encodes for a catalytically inactive SpoT mutant containing an E319Q point mutation that abrogates (p)ppGpp synthase activity

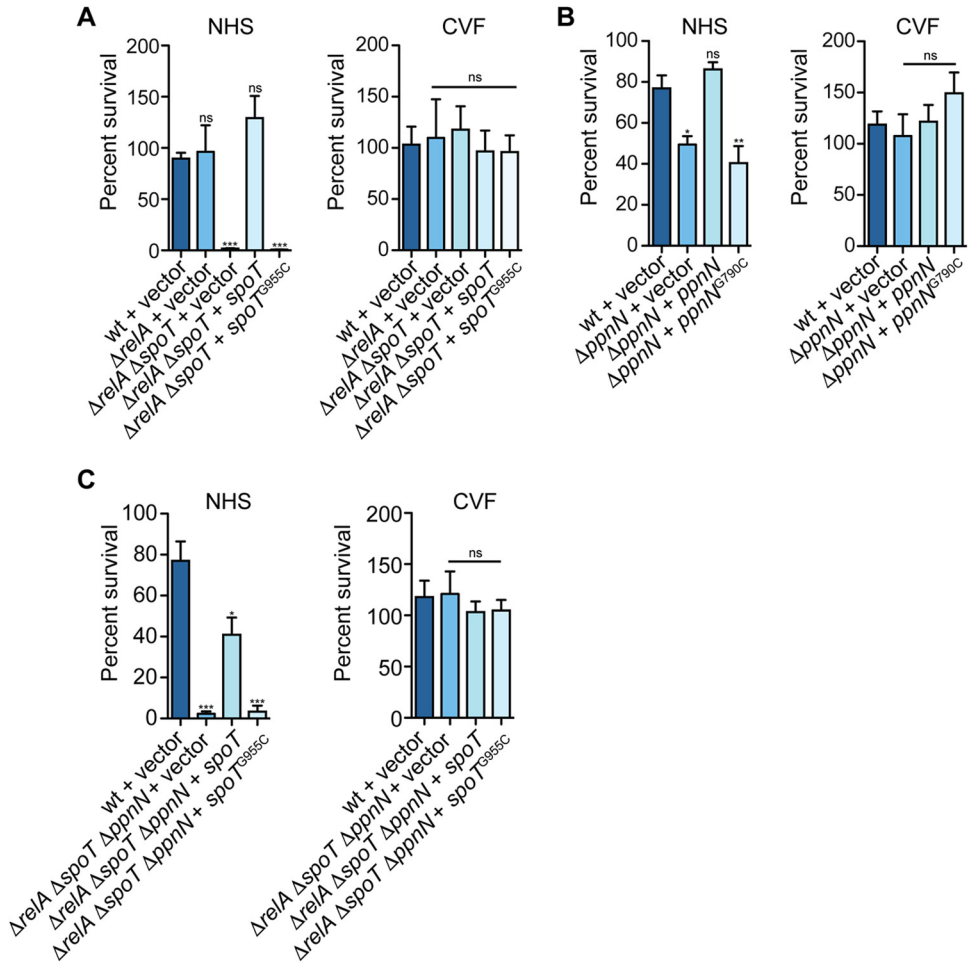

FIG 1 Stringent response is required for complement resistance in *S.* Typhimurium. (A) Δ*relA* Δ*spoT* mutant is susceptible to killing in pooled normal human serum (NHS) and complementation with *spoT*, or inhibition of complement with cobra venom factor (CVF) is sufficient to rescue bacterial survival. (B) Δ*ppnN* mutant is susceptible to killing in pooled NHS. Complementation with *ppnN* or inhibition of complement with CVF is sufficient to rescue bacterial viability, whereas complementation with *ppnN*^{G790C} does not recover the survival of the bacteria. (C) Δ*relA* Δ*spoT ppnN*::*cat* mutant complemented with *spoT* or *spoT*^{G955C} is susceptible to killing in pooled NHS, and inhibition of complement with CVF is sufficient to rescue bacterial viability. Data are the means ± standard errors of the means (SEM; error bars) from at least three independent experiments. Strains are carrying the empty pGEN-MCS vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.0001$.

(35). These data confirmed that the (p)ppGpp synthase activity of SpoT is important for *S.* Typhimurium survival in normal human serum. Moreover, we could recover bacterial survival of the wild-type and mutant strains following pretreatment of the serum with cobra venom factor (CVF), which depletes the serum of human C3 and prevents downstream activation of the membrane attack complex (Fig. 1A) (36). These data demonstrate that killing was due to the activation of the complement system (36, 37).

It is well established that (p)ppGpp signaling results in global regulatory changes in bacteria, including nucleotide metabolism (15). Recent work in *Escherichia coli* has shown that (p)ppGpp can allosterically activate the nucleotide hydrolase, PpnN, to degrade nucleotide 5′-monophosphates to the corresponding free bases and ribose 5′-phosphate (25, 26, 38, 39). PpnN is part of the Lonely Guy (LOG) protein family (Pfam PF03641), which are mainly single-domain phosphohydrolases that share a conserved "PGG$_x$GT$_{xx}$E" motif that is important for the biosynthesis of cytokinins in plant growth and development (39–43) (Fig. S2A). Furthermore, an amino acid sequence alignment between PpnN from *E. coli* and SL1344_2949 (PpnN hereafter) in *S.* Typhimurium showed that these proteins share 94% pairwise identity in amino acids (Fig. S2B). We

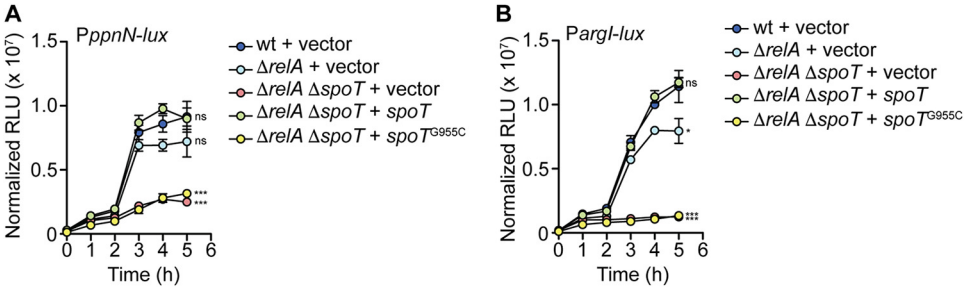

FIG 2 PpnN is regulated in a (p)ppGpp-dependent manner. (A and B) Transcriptional reporter of the full-length *ppnN* promoter (A) and *argI* promoter (B), a canonical gene target regulated during the stringent response, showed (p)ppGpp-dependent up-regulation. RLU, relative light units. Data are the means ± SEM (error bars) from three independent experiments. Strains are carrying the empty pWSK129 vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; ***, $P < 0.0001$.

also found that *S*. Typhimurium *ppnN* is coregulated with genes, such as *pagP* and *pgtE*, that are involved in bacterial resistance against innate immunity (4, 44–46). To study the role of nucleotide metabolism in promoting serum resistance, we deleted *ppnN* in *S*. Typhimurium and tested the viability of the mutant in human serum. Although the Δ*ppnN* mutant grew similarly to the wild-type strain in both LB and M9-glucose medium (Fig. S1), it showed a significant ~30% reduction in viability upon exposure to human serum compared to wild-type *S*. Typhimurium. Expression of PpnN in *trans* or pretreatment of the serum with CVF restored bacterial survival back to wild-type levels, indicating a direct connection of this phenotype to complement (Fig. 1B). To determine whether the $PGG_xGT_{xx}E$ motif was required for PpnN-mediated complement resistance in *S*. Typhimurium, we generated a *ppnN*^{G790C} variant using site-directed mutagenesis, which mutates residue E264 to glutamine. A Δ*ppnN* mutant expressing PpnN^{E264Q} remained susceptible to killing in human serum, confirming that the function of PpnN is required for complement resistance (Fig. 1B).

PpnN is regulated in a (p)ppGpp-dependent manner. Based on our findings that the Δ*relA* Δ*spoT* and Δ*ppnN* mutants were compromised for serum survival, we hypothesized that (p)ppGpp-mediated regulation of PpnN is required for complement resistance. To test this, we generated a deletion of *ppnN* in the Δ*relA* Δ*spoT* background and tested the survival of the Δ*relA* Δ*spoT ppnN*::*cat* mutant in human serum. Consistent with our previous data with the Δ*relA* Δ*spoT* mutant, the Δ*relA* Δ*spoT ppnN*::*cat* mutant was highly susceptible to complement killing (Fig. 1A and C). Complementation of Δ*relA* Δ*spoT ppnN*::*cat* with *spoT* partially recovered *S*. Typhimurium survival in human serum compared to the wild-type strain, whereas expression of *spoT* fully restored the survival of the Δ*relA* Δ*spoT* double mutant (Fig. 1A and C). Similar to the Δ*relA* Δ*spoT* mutant, expression of SpoT^{E319Q} was unable to rescue the viability of the Δ*relA* Δ*spoT ppnN*::*cat* mutant (Fig. 1A and C). In contrast, pretreatment of the serum with CVF rescued the survival of both mutants to wild-type levels (Fig. 1A and C). Taken together, these data support that (p)ppGpp production by SpoT acts through *ppnN* and other unknown factor(s) to confer resistance to complement killing by *S*. Typhimurium.

Previously it was shown that PpnN in *E. coli* K-12 is allosterically regulated by (p)ppGpp (25, 26, 39). This prompted us to investigate the regulation of PpnN by (p)ppGpp in *S*. Typhimurium. First, we tested whether the activation of the *ppnN* promoter was dependent on (p)ppGpp. *ppnN* was activated to similar levels in wild-type *S*. Typhimurium and the Δ*relA* mutant, but its expression was significantly reduced in the Δ*relA* Δ*spoT* mutant that is defective for (p)ppGpp synthesis (Fig. 2A). Complementation with *spoT* was sufficient to recover activation of *ppnN*, whereas complementation with *spoT*^{G955C} was unable to restore activity of the *ppnN* promoter. Notably, the activation of the *ppnN* promoter between the wild type and the Δ*relA* Δ*spoT* mutant was significantly different at 5 h of growth in LB when the bacteria are in stationary phase and (p)ppGpp levels increase (47, 48). As a control, we also tested the expression

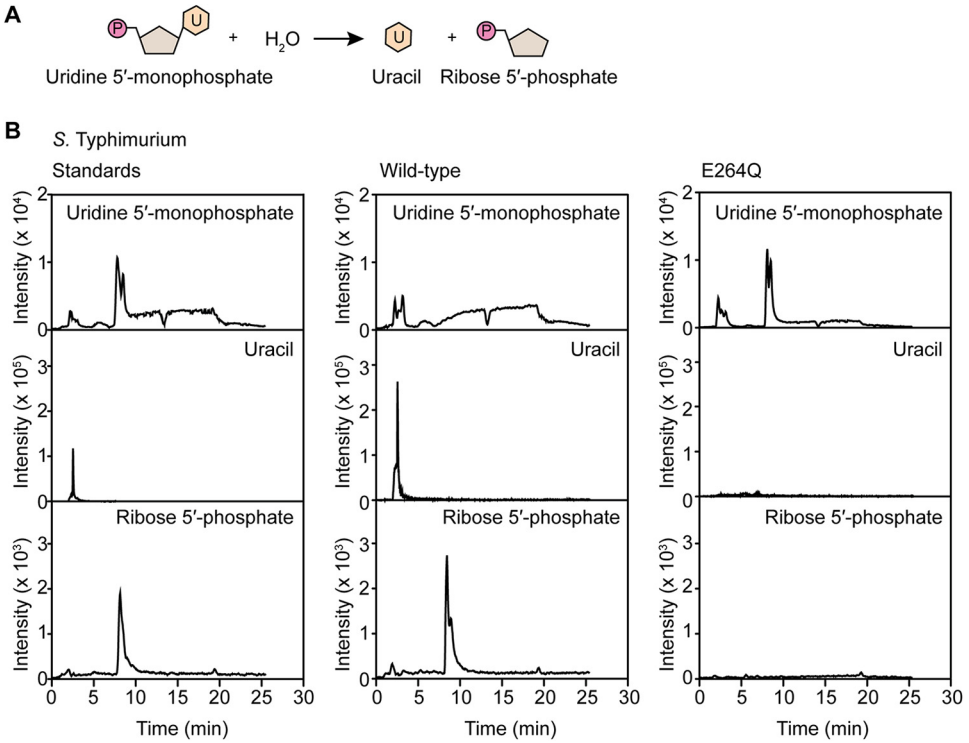

FIG 3 PpnN from *S.* Typhimurium strain SL1344 is a pyrimidine/purine nucleosidase. (A and B) PpnN hydrolyzes uridine 5′-monophosphate (UMP) to uracil and ribose 5′-phosphate. Introduction of an E264Q catalytic site mutation in PpnN abrogates protein function. Data are representative of two replicates.

of a known (p)ppGpp-regulated target, *argI*, which is involved in L-arginine biosynthesis (49). The *argI* promoter was partially repressed in the *relA* mutant compared to the wild type, which is consistent with the role of RelA in responding to amino acid stress during bacterial growth in stationary phase (Fig. 2B) (47). Furthermore, *argI* promoter activity was completely abolished in the Δ*relA* Δ*spoT* mutant, and complementation with *spoT*, but not *spoT*^{G955C}, was sufficient to restore *argI* activation to wild-type levels (Fig. 2B). The amino acid sequence alignment of PpnN in *E. coli* and *S.* Typhimurium also showed that the (p)ppGpp binding residues, R68, R70, K73, R341, and Y347, are conserved between the two homologues, strongly suggesting that (p)ppGpp regulates PpnN in *S.* Typhimurium post-translationally (Fig. S2B) (25).

PpnN is a pyrimidine/purine nucleosidase. Our data indicated that PpnN promotes the survival of *S.* Typhimurium in human serum. In *E. coli*, PpnN is a cytoplasmic hydrolase that degrades purine and pyrimidine nucleotide 5′-monophosphates to the corresponding free bases and ribose 5′-phosphate (25, 26, 38, 39). To compare PpnN in *S.* Typhimurium to its *E. coli* homologue, we tested their enzymatic activity by purifying PpnN from *S.* Typhimurium and *E. coli* using affinity chromatography and incubated it with the nucleotide substrate, uridine 5′-monophosphate (UMP) (38). Hydrophilic interaction chromatography combined with mass spectrometry (HILIC-MS) was used to monitor the presence of the substrate and the potential products. Similar to the *E. coli* homologue, PpnN from *S.* Typhimurium degraded UMP to uracil and ribose 5′-phosphate (Fig. 3A and B and Fig. S3). As a control, we also purified and incubated a catalytic inactive variant of PpnN containing the E264Q point mutation with UMP, and, as expected, the function of the protein was abrogated (Fig. 3B). To determine the nucleotide specificity of PpnN, we also tested its ability to hydrolyze purines such as guanosine 5′-monophosphate (GMP). Similar to UMP, PpnN hydrolyzed GMP to guanine and ribose 5′-phosphate (Fig. S4). UMP and GMP were used as representative substrates, as the detection of the reaction standards was optimal using HILIC-MS.

Together, these data confirm that PpnN in *S*. Typhimurium is a cytosolic nucleotide 5′-phosphate nucleosidase with broad substrate specificity.

PpnN does not confer complement resistance by contributing to cell wall biogenesis. The cytosolic localization and function of PpnN suggested that it mediates complement resistance through its metabolic activity. A common metabolite that PpnN produces from the hydrolysis of purine and pyrimidine monophosphates is ribose 5′-phosphate. We hypothesized that ribose 5′-phosphate could be entering carbon metabolism and conferring complement resistance by contributing to LPS biosynthesis (Fig. 4A) (50, 51). To investigate whether PpnN contributes to maintaining the integrity of the cell wall, we determined the MIC of ampicillin and colistin, which are antibiotics that target peptidoglycan biosynthesis and LPS, respectively, for wild-type *S*. Typhimurium and the Δ*ppnN* and Δ*relA* Δ*spoT* mutants (52). As negative controls, we also tested rifampin and ciprofloxacin, which affect the synthesis of RNA and DNA, respectively (8, 53). The Δ*ppnN* mutant did not display an increase in susceptibility to any of these antibiotics, whereas the Δ*relA* Δ*spoT* mutant showed an 8-fold increase in susceptibility to colistin (Fig. 4B). These data suggested that the lack of (p)ppGpp production compromises the ability of the bacteria to synthesize the full-length variants of LPS. To investigate this possibility, we extracted whole LPS from wild-type and mutant strains of *S*. Typhimurium and used gel electrophoresis to examine the O-antigen polymers produced by each strain. The Δ*relA* Δ*spoT* mutant showed a decreased production of the very long and long O-antigen relative to the wild-type strain, and expression of SpoT in *trans* completely restored its production (Fig. 4C). However, complementation of the Δ*relA* Δ*spoT* mutant with SpoT^{E319Q} was unable to restore the O-antigen, demonstrating that the (p)ppGpp synthetase activity of SpoT is required for regulating its biosynthesis (Fig. 4C). We next engineered bioluminescence reporters to confirm the regulatory input of (p)ppGpp in the production of the LPS O-antigen. Using *lux*-transcriptional fusions for *wzy* and *wzz*ST, which encode the O-antigen polymerase and the long O-antigen chain length regulator, respectively, we found that both genes are expressed in a (p)ppGpp-dependent manner (Fig. 4D and E) (54). In contrast to the Δ*relA* Δ*spoT* mutant, the O-antigen profile of the Δ*ppnN* mutant was similar to wild type (Fig. 4C). Consistent with our previous data, we also found that the outer membrane protein composition of the Δ*ppnN* mutant was similar to that of the wild-type strain, whereas the Δ*relA* Δ*spoT* mutant showed significant differences (Fig. S5). These findings demonstrate that PpnN promotes the survival of *S*. Typhimurium in human serum in a manner independent of cell wall integrity and suggest that the regulation of LPS O-antigen biosynthesis by (p)ppGpp is another mechanism contributing to complement resistance during the stringent response.

DISCUSSION

Here, we showed that the stringent response is required for *S*. Typhimurium survival against the complement system, a component of innate immunity that bacteria encounter during host infection. By using bioinformatics to guide mechanistic predictions, we identified the nucleotide monophosphate nucleosidase, PpnN, as being important for complement resistance through the SpoT-dependent second messenger, (p)ppGpp.

Our findings share some parallels with SpoT in *Helicobacter pylori* and *Borrelia burgdorferi*, where SpoT is required for optimal growth in the presence of serum (55). An *H. pylori* Δ*spoT* mutant exhibits relaxed growth over wild-type bacteria in serum-free medium, suggesting that SpoT is able to sense serum starvation. There have also been other studies relating the stringent responses to bacterial survival either in whole blood or in serum (56–59). For example, a (p)ppGpp^0 mutant in *Enterococcus faecalis* is attenuated in serum due to dysregulated metal homeostasis. Supplementation of the serum with iron or manganese was able to restore bacterial viability (59). In *Salmonella* Typhi, (p)ppGpp regulates the expression of the (Vi) capsular polysaccharide, which prevents complement deposition and formation of the MAC on the cell surface (58, 60). In contrast to *S*. Typhi, *S*. Typhimurium does not express a capsule, which suggests that (p)ppGpp mediates complement resistance through a different mechanism.

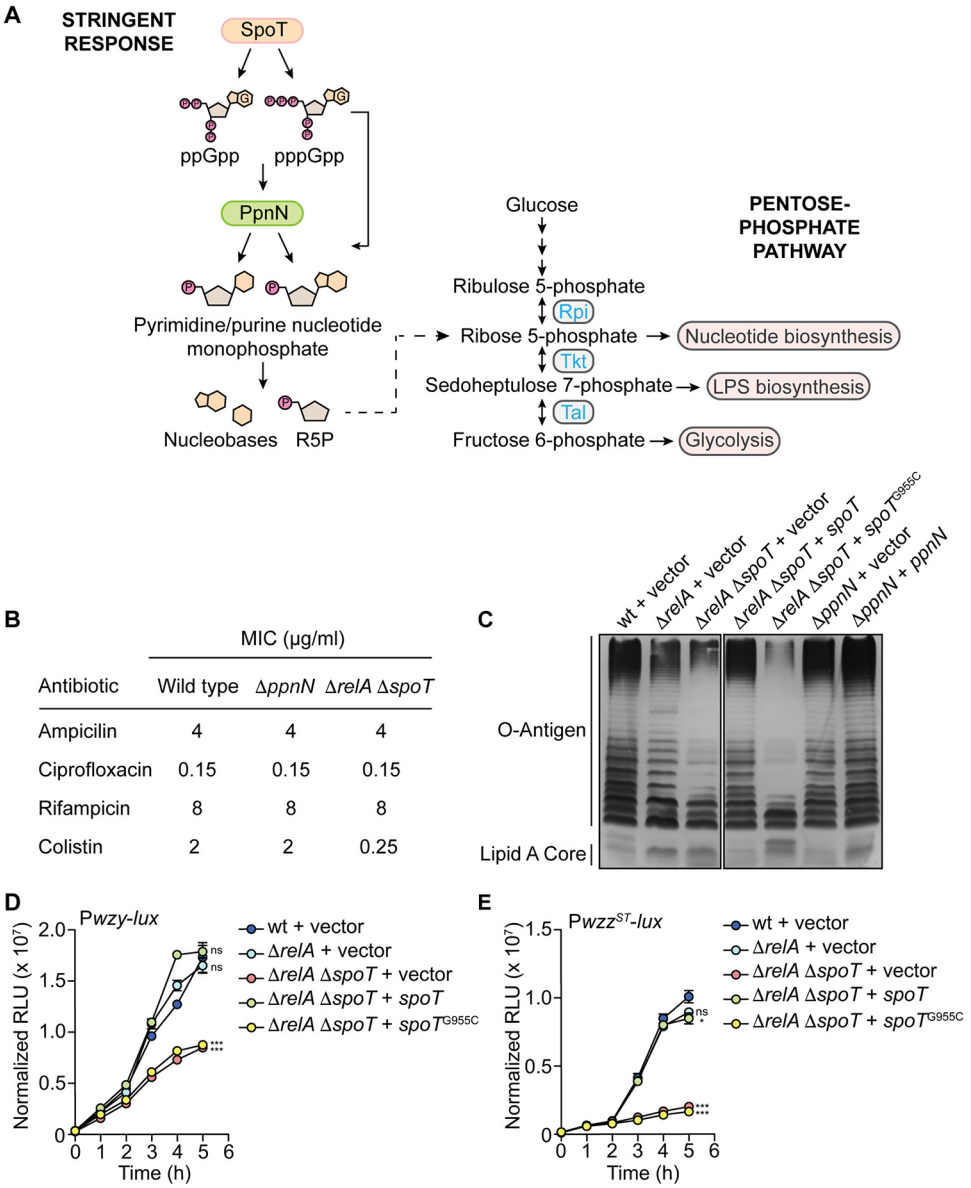

FIG 4 PpnN does not confer complement resistance by contributing to cell wall biogenesis. (A) Model showing (p)ppGpp-mediated regulation of *ppnN*. PpnN produces free nucleobases and ribose 5′-phosphate (R5P), the latter of which could enter the pentose phosphate pathway and regulate carbon metabolism. (B) MIC of ampicillin, ciprofloxacin, rifampin, and colistin for wild-type *S.* Typhimurium strain SL1344 and Δ*ppnN* and Δ*relA* Δ*spoT* mutants. The Δ*ppnN* mutant does not show increased susceptibility to any of the tested antibiotics relative to wild-type *S.* Typhimurium, whereas the Δ*relA* Δ*spoT* mutant is 8-fold more susceptible to colistin. Data are representative of three replicates. (C) LPS analysis of the Δ*relA* Δ*spoT* and Δ*ppnN* mutants compared to wild-type *S.* Typhimurium. The Δ*relA* Δ*spoT* mutant expresses significantly lower levels of very long and long O-antigen, whereas the LPS O-antigen of the Δ*ppnN* mutant is similar to that of the wild-type strain. Data are representative of two replicates. Strains carry the empty pGEN-MCS vector control unless otherwise specified. Transcriptional reporter of the full-length *wzy* promoter (D) and wzz^{ST} promoter (E) showed (p)ppGpp-dependent up-regulation. Data are the means ± SEM (error bars) from three independent experiments. Strains are carrying the empty pWSK129 vector control unless otherwise specified. ns, not significant; *, $P < 0.05$; ***, $P < 0.0001$.

The role of a LOG protein in mammalian pathogens has only been investigated in *Mycobacterium tuberculosis*. In *M. tuberculosis*, the LOG protein Rv1205 produces cytokinins that are maintained at basal levels by a proteasome system. The function of the cytokinins is unclear, but it has been shown that proteasome-deficient *M. tuberculosis* is susceptible to killing due to synergy between host nitric oxide and cytokinins that accumulate (41, 61). To our knowledge, our study is the first to report that a LOG-like

protein aids in resistance to complement killing. Previously it was shown that the biosynthesis of purines and pyrimidines is needed for bacterial growth in serum (62). However, this study used heat-inactivated serum where complement was presumably inactive, suggesting that nucleotides were not serving to resist the bactericidal activity of complement. Several biochemical screens have identified that nucleotide metabolism is a key output of the stringent response (24, 25). For example, (p)ppGpp inhibits the purine biosynthesis enzymes Gpt, Hpt, GuaB, PurA, and PurF (24, 63). It has also been shown that (p)ppGpp enhances the activity of PpnN in *E. coli* to degrade nucleotides (25, 26, 38, 39). Deletion of *ppnN* in *S.* Typhimurium did not affect the integrity of the cell wall, suggesting that PpnN promotes complement resistance through its metabolic roles in the cytoplasm. It is possible that the generation of nucleotide precursors by PpnN allows for the reallocation of resources to other pathways relevant for survival in serum. In addition, PpnN may facilitate the transition from serum to more favorable conditions by increasing the cellular pool of nucleotide metabolites (26, 64, 65). This is consistent with the finding that a Δ*ppnN* mutant is outcompeted by wild-type *E. coli* when the bacteria transition between nutrient-rich and -poor media (26). Although the mechanism of PpnN-mediated complement resistance is not fully understood, it is unlikely that it interacts directly with complement proteins, as it is not secreted (66). Exploring the contributions of PpnN to metabolism and how this influences complement resistance in *S.* Typhimurium will be the focus of future research. A functional genomics approach, such as RNA sequencing of wild-type *S.* Typhimurium and the Δ*ppnN* mutant exposed to human serum, may reveal insights into the potential pathways affected by PpnN. This may be followed by the systematic deletion of metabolic enzymes in a Δ*ppnN* mutant to test which pathways enhance complement resistance.

Our data suggest that there are other genetic targets in addition to *ppnN* that contribute to (p)ppGpp-mediated resistance to complement killing. Another possible mechanism is through the regulation of cell wall biogenesis genes by the alternative sigma factor, RpoS, and (p)ppGpp during bacterial growth in stationary phase (33, 47, 67). In particular, bacteria exhibit an increase in LPS, cross-linking of outer membrane lipoproteins, and an increase in thickness of the peptidoglycan layer under nutrient-limited conditions (10, 47, 68). This is supported by our findings that an *S.* Typhimurium Δ*relA* Δ*spoT* mutant displays significantly lower levels of the very long and long O-antigen and increased susceptibility to complement. The decreased production of O-antigen by the Δ*relA* Δ*spoT* mutant was corroborated by lower expression levels of *wzy* and wzz^{ST}. Our LPS silver staining also suggests that wzz^{fepE} from the O-antigen biosynthesis pathway is regulated in a (p)ppGpp-dependent manner (54). We and others have also shown that the LPS O-antigen of *S.* Typhimurium is involved in colistin resistance (52). Furthermore, RNA sequencing of *S.* Typhimurium grown to stationary phase in LB showed that genes such as *pagC* and *pgtE* are activated by (p)ppGpp (30). PagC and PgtE are outer membrane proteins that have been directly implicated in complement resistance in *Salmonella* (45, 46, 69). Together, these data suggest that the stringent response coordinates resistance to complement in *S.* Typhimurium by remodeling the cell membrane and reprogramming metabolism.

In summary, our findings highlight the role of nucleotide metabolism and the biosynthesis of the LPS O-antigen during the stringent response in mediating evasion of innate immunity by pathogenic bacteria. (p)ppGpp signaling is an essential mechanism of the bacterial stress response that involves the regulation of virulence gene expression for successful host infection. Identifying the genetic factors that allow bacteria to evade the immune system will reveal new therapeutic targets that could be integral in informing drug discovery efforts.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Salmonella enterica* serovar Typhimurium (SL1344) and isogenic derivatives were used in the study and are listed in Table 1. Bacteria were grown in LB or M9 minimal medium supplemented with 1% glucose and 0.135 mM L-histidine. Antibiotics were added

TABLE 1 Bacterial strains used in the study

Strain	Source or reference
Wild-type *S.* Typhimurium strain SL1344	Coombes et al. (79)
Δ*ppnN S.* Typhimurium strain SL1344	This study
ppnN::*aph S.* Typhimurium strain 14028s	Porwollik et al. (70)
Δ*relA S.* Typhimurium strain SL1344	This study
Δ*relA* Δ*spoT S.* Typhimurium strain SL1344	This study
Δ*relA* Δ*spoT ppnN*::*cat S.* Typhimurium strain SL1344	This study
Wild-type *E. coli* strain K-12	Baba et al. (80)
BL21 (DE3) *E. coli*	Agilent Technologies
TOP10 *E. coli*	Invitrogen

where appropriate (streptomycin, 100 μg/ml; kanamycin, 50 μg/ml; ampicillin, 200 μg/ml; chloramphenicol, 34 μg/ml), and strains were grown at 37°C with shaking.

Cloning and mutant generation. *ppnN*::*aph* from the *Salmonella* single-gene deletion (SGD) library was transduced to *S.* Typhimurium strain SL1344 as previously described, with modifications (70, 71). The donor mutant strain was grown overnight in LB supplemented with 0.5× E-Salts and 0.2% D-glucose and mixed with ~1.5×10^5 PFU of P22 HT phage at 37°C. Next, 1 ml of the donor strain was pelleted and the supernatant was mixed with 100 μl of chloroform to lyse any remaining live bacteria. Two microliters of the donor lysate was then mixed with 200 μl of a wild-type *S.* Typhimurium strain SL1344 overnight culture and incubated at 37°C for 1 h before plating on LB supplemented with 50 μg/ml kanamycin. Colonies were restreaked onto green indicator agar to screen for the absence of phage. To ensure that the colonies were free of phage lysogen, white colonies were streaked across a line of P22 H5 phage on green agar and incubated overnight at 37°C. Successful transduction of the mutant was verified by PCR. The *aph* cassette was removed using pCP20, resulting in Δ*ppnN*::FRT in *S.* Typhimurium strain SL1344.

Lambda Red recombination was used to generate an in-frame, marked mutant of *S.* Typhimurium *relA*::*cat* (72). Wild-type *S.* Typhimurium carrying pKD46 was transformed with linear PCR products amplified using Phire Hot Start II DNA polymerase (Thermo Fisher) and primers (Sigma-Aldrich) containing gene-specific regions of homology and flanking the *cat* cassette carried by pKD3. Transformants were selected on LB agar supplemented with chloramphenicol (34 μg/ml), and knockouts were verified by PCR. The *cat* cassette was removed using pCP20, resulting in the Δ*relA*::FRT strain, and confirmed by PCR. Lambda Red treatment was repeated in the Δ*relA*::FRT background to generate the Δ*relA*::FRT Δ*spoT*::FRT double mutant and in the Δ*relA*::FRT Δ*spoT*::FRT double mutant background to generate the Δ*relA*::FRT Δ*spoT*::FRT *ppnN*::*cat* triple mutant.

For the cloning of *spoT*, primers EC215F (GTA TCA TAT GGC ACG CGT AAC TGT TCA GGA CGC TG) and EC215R (AGT CCC ATG GCT AGT TTC GGT TAC GGG TGA CTT TA) or EC224F (GTA TGA GCT CGC ACG CGT AAC TGT TCA GGA CGC TG) and EC224R (AGT CGC GGC CGC CTA GTT TCG GTT ACG GGT GAC TTT A) were used to PCR amplify the coding sequence of *spoT* and 291 bp upstream of the start codon. PCR-amplified products were then cloned into pGEN-MCS or pWSK129 after digestion with NdeI/NcoI (Thermo Fisher) or SacI/NotI (Thermo Fisher), respectively. For the cloning of *ppnN*, the coding sequence and 800 bp upstream of the start codon of *ppnN* in SL1344 was PCR amplified using primers EC100F (ATG CGA ATT CGG ATA TCT GGA CGT TGT ATG AAC TT) and EC100R (GTA TCA TAT GTT AAG CGC AGA TCT CGT AAC AGG GG) and then cloned as an EcoRI/NdeI (Thermo Fisher) DNA product into pGEN-MCS. pGEN-MCS-*ppnN*^{G790C} (encoding PpnN^{E264Q}) and pGEN-MCS-*spoT*^{G955C} (encoding SpoT^{E319Q}) were generated using the Q5 site-directed mutagenesis kit (NEB) and primers EC186F (TAC GGC GGA ACA GCT GCT TTA TTT GCT G) and EC186R (CCC ACG CCG CCC GGG AAG ATG ATG ATA C) or EC320F (GGC GTT CCT GTT CAA GTC CAG ATC CGT A) and EC320R (GGC GTT CCT GTT CAA GTC CAG ATC CGT A), respectively. pWSK129-*spoT*^{G955C} was cloned by PCR amplifying EC224F (GTA TGA GCT CGC ACG CGT AAC TGT TCA GGA CGC TG)/EC325R (ACG GAT CTG GAC TTG AAC AGG AAC G) and EC326F (GGC GTT CCT GTT CAA GTC CAG ATC C)/EC224R (AGT CGC GGC CGC CTA GTT TCG GTT ACG GGT GAC TTT A) and then performing splicing by overlap extension (SOE) PCR using EC224F and EC224R. The product of the SOE PCR was digested with SacI/NotI (Thermo Fisher) and cloned into pWSK129.

Bioluminescence reporters were generated using primers EC97F (ATG CGG ATC CGG ATA TCT GGA CGT TGT ATG AAC TT) and EC97R (GCC ATA CGT AGT AAA CTC CTT ATG GGA CGC AAC AC) to PCR amplify 800 bp upstream of the start codon of *ppnN*, EC188F (ATG CGG ATC CCA ATG GTG GCT TTC GCC AGG) and EC188R (GCC ATA CGT AAT AGA GCC TTT AGA AAA AAT GCT TA) to amplify 1,000 bp upstream of the start codon of *wzy*, EC212F (AGT CGG ATC CGT GAC GCA CGC CGT CGT CAT) and EC212R (CGG CTA CGT AAC TTC CCT CAC ATG GCT TAG GCC TC) to PCR amplify 579 bp upstream of the start codon of *argI*, and EC321F (ATG CGG ATC CGT GAT CAG CAT CAA CCC CGC) and EC321R (GCC ATA CGT AAG ATA CCC TAA CTA AAA AAA GGA TG) to PCR amplify 1,000 bp upstream of the start codon of *wzz*ST. PCR-amplified products were then cloned into pGEN-*luxCDABE* after digestion with SnaBI/BamHI (Thermo Fisher) (73).

All plasmid constructs were sequence verified by Sanger sequencing (GENEWIZ) and then transformed by electroporation (Bio-Rad) into the appropriate strain backgrounds for downstream experiments. Plasmids used in the study are listed in Table 2, and primers (Sigma-Aldrich) are listed in Table 3.

Bacterial growth curves. Bacteria grown overnight in LB medium were harvested and normalized to an optical density at 600 nm (OD_{600}) of 0.5. Cells were washed and resuspended in phosphate-buffered saline (PBS) and diluted to an OD_{600} of 0.05 in fresh LB or M9 minimal medium supplemented with 1%

TABLE 2 Plasmids used in the study

Plasmid[a]	Description	Source or reference
pGEN-MCS	Low-copy-no. cloning vector	Lane et al. (73)
pGEN-*luxCDABE*	Lux transcriptional reporter plasmid	Lane et al. (73)
pWSK129	Low-copy-no. cloning vector	Wang and Kushner (81)
pEXT20	Expression vector	Dykxhoorn et al. (82)
pET-24a	Expression vector	Novagen
pKD3	Template plasmid for Lambda Red recombination	Datsenko and Wanner (72)
pKD46	Lambda Red recombinase expression plasmid	Datsenko and Wanner (72)
pCP20	Lambda Red flippase expression plasmid	Datsenko and Wanner (72)
pGEN-MCS-*ppnN*ST	*ppnN*ST with 800 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*ppnN*$^{ST(G790C)}$	*ppnN*ST containing G790C point mutation with 800 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*spoT*	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pGEN-MCS-*spoT*^{G955C}	*spoT* containing G955C point mutation with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pWSK129-*spoT*	*spoT* with 291 bp upstream of coding sequence cloned into pGEN-MCS for complementation experiments	This study
pWSK129-*spoT*^{G955C}	*spoT* containing G955C point mutation with 291 bp upstream of coding sequence cloned into pWSK129 for complementation experiments	This study
pEXT20-*ppnN*ST-6HIS	PpnNST expression plasmid, C-terminal His6 tagged	This study
pEXT20-*ppnN*^{EC}-6HIS	PpnN^{EC} expression plasmid, C-terminal His6 tagged	This study
pET-24a-*ppnN*ST	PpnNST expression plasmid, C-terminal His6 tagged	This study
pET-24a-*ppnN*$^{ST(G790C)}$	PpnNST expression plasmid, C-terminal His6 tagged containing G790C point mutation in *ppnN*ST coding sequence	This study
pGEN-*luxCDABE-ppnN*ST	Lux transcriptional reporter for *ppnN*ST promoter	This study
pGEN-*luxCDABE-argI*	Lux transcriptional reporter for *argI* promoter	This study
pGEN-*luxCDABE-wzy*	Lux transcriptional reporter for *wzy* promoter	This study
pGEN-*luxCDABE-wzz*ST	Lux transcriptional reporter for *wzz*ST promoter	This study

[a]ST and EC denote *ppnN* from *S.* Typhimurium strain SL1344 and *E. coli* strain K-12, respectively.

glucose and 0.135 mM L-histidine. Bacteria were then grown in 96-well flat, clear-bottom polystyrene plates (Corning) at 37°C with shaking, and the A_{600} was measured every 1 h using the BioTek Epoch 2 plate reader.

Serum bactericidal assay. Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 0.5. Cells were washed and resuspended in PBS and diluted (1:10) further for the assay. The equivalent OD_{600} of 0.005 of each strain was incubated in 90% pooled normal human serum (Innovative Research) at 37°C. Serum treated with 5 U/ml cobra venom factor (Quidel) for 30 min at 37°C was used as a negative control for each strain. The number of viable bacteria was determined by plating on LB agar supplemented with 200 μg/ml of ampicillin to select for strains carrying the pGEN-MCS empty vector control or complementation plasmid, and percent survival was calculated as the number of CFU/ml at 30 min relative to 0 min.

Bioluminescence reporter assay. *S.* Typhimurium strains containing *lux* transcriptional fusions were subcultured (1:50) and grown to mid-exponential phase (OD_{600} of 0.4 to 0.5) in LB and then subcultured (1:50) again into LB in black 96-well flat, clear-bottom polystyrene plates (Corning). Plates were incubated at 37°C with shaking, and luminescence and A_{600} were measured every 1 h up to 5 h using the PerkinElmer Plate Reader. Luminescence was normalized to A_{600}.

Protein purification. BL21(DE3) *E. coli* harboring pET-24a-*ppnN*-6HIS or pET-24a-*ppnN*^{G790C}-6HIS was subcultured (1:50) into LB broth at 37°C with shaking and grown until an OD_{600} of 0.2. Cells were induced with 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) until the culture reached mid-logarithmic phase (OD_{600} of 0.5), after which the culture was left to incubate overnight at 18°C. Cells were pelleted by centrifugation for 10 min at 5,000 $\times$ *g* and resuspended in lysis buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, 10 mM imidazole, 1 mM 2-mercaptoethanol, 300 mM NaCl, 4 mM phenylmethanesulfonyl fluoride, pH 7.5). Cells were lysed using a Continuous Cell Disruptor (Constant Systems Ltd.) operated at 20,000 lb/in^2 and then centrifuged at 30,000 $\times$ *g* for 30 min to pellet cellular debris. Lysates were applied to a nickel-nitrilotriacetic acid affinity column and washed with a gradient buffer (100 mM Tris-HCl, 5 mM $MgCl_2$, 500 mM NaCl, pH 7.5) containing 20 mM, 40 mM, and 60 mM imidazole. Proteins were eluted in 100 mM Tris-HCl, 5 mM $MgCl_2$, 500 mM NaCl, 500 mM imidazole, pH 7.5. Eluted fractions were run on a 12% SDS-PAGE gel and stained with Coomassie brilliant blue to verify isolation of the target protein. In a second purification step, elution fractions were combined and applied to a HiLoad 16/60 Superdex 200-pg filtration column (GE Healthcare). Proteins were eluted in 1-ml fractions in buffer containing 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5. Fractions containing the target proteins (as determined by SDS-PAGE) were pooled and stored at −80°C with 20% glycerol.

Enzymatic assay. Purified proteins (50 μg/ml) were incubated with 0.2 mM of substrate at 37°C in buffer (10 mM Tris-HCl, 1 mM $MgCl_2$, pH 7.5) for 1.5 h. At 0 h and 1.5 h, 150 μl of each reaction mixture

TABLE 3 Primers used in the study

Primer	Gene[a]	Direction	Destination	Sequence (5′–3′)
EC85	*ppnN*ST	F	pET24-a	CGAACATATGTTGATTACACATATTAGCCCGCTTG
EC85	*ppnN*ST	R	pET24-a	GTATCTCGAGAGCGCAGATCTCGTAACAGGGGATG
EC96	*ppnN*ST	F	pEXT20	CGAAGGATCCTTGATTACACATATTAGCCCGCTTG
EC98	*ppnN*ST	R	pEXT20	GTATAAGCTTTTAATGGTGGTGGTGATGATGAGCG CAGATCTCGTAACAGGGG
EC97	*ppnN*ST	F	pGEN-*luxCDABE*	ATGCGGATCCGGATATCTGGACGTTGTATGAACTT
EC97	*ppnN*ST	R	pGEN-*luxCDABE*	GCCATACGTAGTAAACTCCTTATGGGACGCAACAC
EC100	*ppnN*ST	F	pGEN-MCS	ATGCGAATTCGGATATCTGGACGTTGTATGAACTT
EC100	*ppnN*ST	R	pGEN-MCS	GTATCATATGTTAAGCGCAGATCTCGTAACAGGGG
EC167	*ppnN*^{EC}	F	pEXT20	CGAAGAATTCTTGATTACACATATTAGCCCGCTTG
EC167	*ppnN*^{EC}	R	pEXT20	GTATGGTACCTTAATGGTGGTGGTGATGATGCGTGCAGATTTCGTAGCAAGGG
EC186	*ppnN*ST	F	*ppnN*ST	TACGGCGGAACAGCTGCTTTATTTGCTG
EC186	*ppnN*ST	R	*ppnN*ST	CCCACGCCGCCCGGGAAGATGATGATAC
EC188	*wzy*	F	pGEN-*luxCDABE*	ATGCGGATCCCAATGGTGGCTTTCGCCAGG
EC188	*wzy*	R	pGEN-*luxCDABE*	GCCATACGTAATAGAGCCTTTAGAAAAAATGCTTA
EC200	*relA*	F	Δ*relA*	CGCATGTAATGATTACCGGCTTACCGACTTCGGTAGGCCTGGTCCCTTAAGGAGAGGACGATG GTCGCGGTAAGAAGTGCACATATTAATGTGTAGGCTGGAGCTGCTTCG
EC200	*relA*	R	Δ*relA*	GTTGCTAATGCGGCTTTGCTGAACGAGTAGCAAAGCCGCTACATGATTACTGTCTGGGGTTTAC CCCCCGTGCAGTCGCCGTGCATCAATCATATGAATATCCTCCTTAG
EC204	*spoT*	F	Δ*spoT*	GAATTACAAGCCGTTACCGCTATTGCTGAAGGTCGTCGTTAATCACAAAGCGGGTCGCCCTTGT ATCTGTTTGAAAGCCTGAATCAACTGGTGTAGGCTGGAGCTGCTTCG
EC204	*spoT*	R	Δ*spoT*	TCAGGCTGACGCCTGGCGAGCATTTCGCATATACGCGCATAACGTTTTGGATTCATAGCGCTAG TTTCGGTTACGGGTGACTTTAATGACCATATGAATATCCTCCTTA
EC212	*argI*	F	pGEN-*luxCDABE*	AGTCGGATCCGTGACGCACGCCGTCGTCAT
EC212	*argI*	R	pGEN-*luxCDABE*	CGGCTACGTAACTTCCCTCACATGGCTTAGGCCTC
EC215	*spoT*	F	pGEN-MCS	GTATCATATGGCACGCGTAACTGTTCAGGACGCTG
EC215	*spoT*	R	pGEN-MCS	AGTCCCATGGCTAGTTTCGGTTACGGGTGACTTTA
EC224	*spoT*	F	pWSK129	GTATGAGCTCGCACGCGTAACTGTTCAGGACGCTG
EC224	*spoT*	R	pWSK129	AGTCGCGGCCGCCTAGTTTCGGTTACGGGTGACTTTA
EC245	*ppnN*ST	F	Δ*ppnN*ST	ATCAGCCAGGGCTATTGTAATCAACAGGGAATGGCGTGTTGCGTCCCATAAGGAGTTTACTTGA TTACACATATTAGCCCGCTTGGCTCAGTGTAGGCTGGAGCTGCTTCG
EC245	*ppnN*ST	R	Δ*ppnN*ST	ATACCGCAATGAAAGGAATGGGAGAAGCGCCCGGCTGCTGGCGGCAACCGGGCATAAGCGTT AAGCGCAGATCTCGTAACAGGGGATGTACATATGAATATCCTCCTTAG
EC320	*spoT*	F	pGEN-MCS	GGCGTTCCTGTTCAAGTCCAGATCCGTA
EC320	*spoT*	R	pGEN-MCS	GTGCGGGCCGATCATTGAGGTGTGCAAA
EC321	*wzz*ST	F	pGEN-*luxCDABE*	ATGCGGATCCGTGATCAGCATCAACCCCGC
EC321	*wzz*ST	R	pGEN-*luxCDABE*	GCCATACGTAAGATACCCTAACTAAAAAAAGGATG
EC325	*spoT*	R	pWSK129	ACGGATCTGGACTTGAACAGGAACG
EC326	*spoT*	F	pWSK129	GGCGTTCCTGTTCAAGTCCAGATCC

[a]ST and EC denote *ppnN* from *S.* Typhimurium strain SL1344 and *E. coli* strain K-12, respectively.

was inactivated with 150 μl of methanol cooled at −80°C. For negative-control reactions, a catalytic inactive protein variant was used or wild-type protein was heat inactivated at 95°C for 60 min. Relative abundance of reactant compounds and products was measured using hydrophilic chromatography and mass spectrometry (HILIC-MS) on an LTQ Orbitrap XL (Thermo Fisher).

Antibiotic susceptibility testing. MIC determinations of ampicillin, colistin, rifampin, and ciprofloxacin (BioShop Canada) were performed using broth microdilution in 96-well flat, clear-bottom polystyrene plates (Corning) (74). Antibiotics were 2-fold serially diluted in LB medium from 512 μg/ml down to ~1e-3 μg/ml. Bacterial cultures grown overnight were diluted to 10^6 CFU/ml in fresh LB medium and then diluted further to 10^5 CFU/ml in the microplates containing the antibiotics. Plates were incubated at 37°C in sealed plastic bags. The A_{600} was read after overnight incubation using a PerkinElmer plate reader.

LPS analysis. Bacteria grown overnight in LB medium were harvested and normalized to an OD_{600} of 3.0. Two milliliters of each strain under investigation was pelleted by centrifugation at 16,000 × *g* for 2 min, and the LPS was extracted as previously described (75), with some modifications. Cells were resuspended in 200 μl of TRIzol reagent (Thermo Fisher) and then incubated for 10 min at room temperature. Next, 20 μl of chloroform was added for every milliliter of culture. The resulting TRIzol-chloroform mixture was vortexed vigorously and incubated at room temperature for an additional 10 min, followed by centrifugation at 16,000 × *g* for 10 min to separate the aqueous and organic phases. The aqueous phase was transferred to a new microcentrifuge tube, and 100 μl of distilled water was added to the organic phase, vortexed briefly, incubated for an additional 10 min, and centrifuged again for 10 min at 16,000 × *g* to create phase separation. Two additional water extractions were performed to ensure complete removal of the LPS. The combined aqueous phases were then dried at 45°C for ~2 h using a Vacufuge (Eppendorf). Dried pellets were resuspended in 500 μl of 0.375 M $MgCl_2$ dissolved in 95% ethanol that had been cooled at −20°C and then centrifuged for 15 min at 16,000 × *g* (76). The final pellets were normalized by weight/volume (grams per microliter) and resuspended in distilled water. LPS was run on 16% SDS-PAGE gels and stained with silver nitrate as previously described (77) and imaged using the ChemiDoc MP imaging system (Bio-Rad).

Outer membrane profiling. The outer membrane of different *S.* Typhimurium strains was isolated as previously described (78), with some modifications. Bacterial cell pellets were harvested from overnight cultures and resuspended in 50 mM Tris-HCl (pH 8.0), 50 mM $MgCl_2$, 150 mM NaCl, and then lysed by sonication. Lysates were centrifuged at 8,000 $\times$ *g* for 10 min and then filtered through 0.2-μm low-protein-binding filters to remove insoluble debris. Total membranes were harvested from cell-free lysates by ultracentrifugation at 100,000 $\times$ *g* for 1 h. To isolate the outer membrane, total membrane pellets were solubilized in the buffer described above supplemented with 1.5% Triton X-100 and EDTA-free protease inhibitor cocktail (Roche Applied Science) for 24 h at 4°C. Outer membranes were pelleted by ultracentrifugation at 100,000 $\times$ *g* for 1 h and then resuspended in sterile 1$\times$ PBS buffer. Outer membrane preparations were normalized by protein content following quantification using the 2-D Quant kit (GE Healthcare) and then separated on 10% SDS-PAGE gels. Coomassie brilliant blue R-250 dye (BioShop) was used to stain the gels, followed by imaging using the ChemiDoc MP imaging system (Bio-Rad).

Statistical analysis. Data were analyzed using GraphPad Prism 5.0a software (GraphPad Inc., San Diego, CA) using one-way analysis of variance. *P* values of <0.05 were considered significant.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 1.9 MB.

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We have no conflicts of interest with the contents of this article to declare.

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