A Family of Small Intrinsically Disordered Proteins Involved in Flagellum-Dependent Motility in *Salmonella enterica*

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ABSTRACT By screening a collection of *Salmonella* mutants deleted for genes encoding small proteins of ≤ 60 amino acids, we identified three paralogous small genes (*ymdF*, *STM14_1829*, and *yciG*) required for wild-type flagellum-dependent swimming and swarming motility. The *ymdF*, *STM14_1829*, and *yciG* genes encode small proteins of 55, 60, and 60 amino acid residues, respectively. A bioinformatics analysis predicted that these small proteins are intrinsically disordered proteins, and circular dichroism analysis of purified recombinant proteins confirmed that all three proteins are unstructured in solution. A mutant deleted for *STM14_1829* showed the most severe motility defect, indicating that among the three paralogs, *STM14_1829* is a key protein required for wild-type motility. We determined that relative to the wild type, the expression of the flagellin protein *FliC* is lower in the $\Delta STM14_1829$ mutant due to the downregulation of the *flhDC* operon encoding the *FlhDC* master regulator. By comparing the gene expression profiles between the wild-type and $\Delta STM14_1829$ strains via RNA sequencing, we found that the gene encoding the response regulator *PhoP* is upregulated in the $\Delta STM14_1829$ mutant, suggesting the indirect repression of the *flhDC* operon by the activated *PhoP*. Homologs of *STM14_1829* are conserved in a wide range of bacteria, including *Escherichia coli* and *Pseudomonas aeruginosa*. We showed that the inactivation of *STM14_1829* homologs in *E. coli* and *P. aeruginosa* also alters motility, suggesting that this family of small intrinsically disordered proteins may play a role in the cellular pathway(s) that affects motility.

IMPORTANCE This study reports the identification of a novel family of small intrinsically disordered proteins that are conserved in a wide range of flagellated and non-flagellated bacteria. Although this study identifies the role of these small proteins in the scope of flagellum-dependent motility in *Salmonella*, they likely play larger roles in a more conserved cellular pathway(s) that indirectly affects flagellum expression in the case of motile bacteria. Small intrinsically disordered proteins have not been well characterized in prokaryotes, and the results of our study provide a basis for their detailed functional characterization.

KEYWORDS small proteins, intrinsically disordered proteins, flagella, motility, *Salmonella*, *PhoP*

The flagellum is a cell-bound multiprotein complex that in combination with chemotaxis (1) enables the bacterial cell to move toward nutrients or away from toxic chemicals (2). *Salmonella enterica* serovar Typhimurium is one of the best-studied

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model bacteria for genetics and structural analysis of flagella, and it produces three to eight peritrichous flagella, each consisting of the subunit protein called flagellin (3, 4). *Salmonella* possesses two functionally equivalent but antigenically distinct flagellins, FliC and FliB, encoded by *fliC* and *fliB*, respectively, which are subject to antigenic phase variation (5). While normal levels of flagellum expression enable individual *Salmonella* cells to swim in liquid or semisolid 0.3% agar (swimming motility), hyperflagellation enables a group of *Salmonella* cells to move as a raft on solid surfaces (swarming motility) (6). Flagella also play important roles in *Salmonella* pathogenesis. For example, flagellum-dependent motility promotes efficient colonization by *Salmonella* cells in the intestine (7) and invasion into intestinal epithelial cells (8); *Salmonella* mutants defective in flagellum expression are attenuated in both survival inside macrophages (9–11) and virulence in murine models of infection (10). Moreover, flagella mediate the attachment of *Salmonella* cells to cholesterol to initiate biofilm formation (12), and hyperflagellated swarming cells exhibit enhanced resistance to a broad range of antibiotics (13). Therefore, flagellum-dependent motility provides *Salmonella* with advantages to survive better in natural and host environments.

Although advantageous, the production of flagella is costly, consuming about 2% of the total energy in a cell (3, 14), and it must be tightly regulated in coordination with other cellular needs under given environmental conditions. In *Salmonella* and the closely related *Escherichia coli*, approximately 40 genes are known to encode proteins for the structural parts (basal body, hook, and filament), transport, assembly, and regulatory proteins of flagella; their hierarchical expression is grouped into three classes (3). Class I genes, which are at the top of the hierarchy, comprise the *flhDC* operon encoding the master regulator FlhDC. FlhDC activates the transcription of class 2 genes that include the *fliA* gene encoding the sigma factor FliA (σ^{28}), which subsequently promotes the expression of class 3 genes, including the *fliC* gene. There are at least four transcriptional activators (15–18) and eight repressors (19–31) that are known to control the transcription of the *flhDC* operon either directly or indirectly. Some, if not all, of these regulators are activated or inhibited in response to changes in intracellular or extracellular conditions. For example, the response regulator PhoP, which is activated under low Mg^{2+} and/or low pH conditions, is a repressor (20, 29), whereas the adenylate cyclase receptor protein CRP, which is activated by binding to intracellular cyclic 3',5'-AMP (cAMP), is an activator of *flhDC* transcription (18). Therefore, the transcription (levels) of *flhDC*, and subsequently, of downstream flagellum genes, reflects the conditions inside and/or outside the cell.

In addition to these transcriptional regulators, the small molecule cyclic diguanosine monophosphate (c-di-GMP), which is a universal second messenger in bacteria (32), controls flagellum-dependent motility. Several transcriptional regulators that bind to c-di-GMP, such as FleQ in *Pseudomonas aeruginosa* (33) and VpsT in *Vibrio cholerae* (34), are known to control flagellum-dependent motility. While no such transcriptional regulator has been identified in *Salmonella* to date, two c-di-GMP-binding proteins are known to inhibit the function of flagella: BcsA and YcgR. An increase in intracellular c-di-GMP stimulates the cellulose synthase BcsA, resulting in increased production of the exopolysaccharide cellulose, which in turn slows down flagellar rotation (35, 36). Concomitantly, a complex of the YcgR protein with c-di-GMP inhibits the action of the motor protein MotA that controls flagellar rotation speed (37–39). In addition, the YdiV (also known as CdgR) protein, which neither binds to nor degrades c-di-GMP (40), appears to be involved in controlling intracellular c-di-GMP levels, although opposing results have been reported with regard to intracellular c-di-GMP levels in $\Delta ydiV$ mutants (40, 41). YdiV functions as a posttranscriptional repressor of flagellum-dependent motility by inhibiting the DNA binding of FlhD₄C₂ (42, 43) and promoting ClpXP-mediated degradation of FlhD (42). Whether and how YdiV is involved in c-di-GMP-mediated motility control remain to be further investigated.

In a recent study, a collection of 1,023 single gene deletion mutants in *Salmonella* was screened for altered motility, which uncovered nine genes not previously known to be associated with motility (44). These newly identified genes include those

FIG 1 Identification of three paralogs small genes (*ymdF*, *yciG*, and *STM14_1829*) required for wild-type swimming motility in *Salmonella*. (A) Representative swim agar plate. Swimming motility assay was performed as described in Materials and Methods. (B) Swimming motility in WT (HL10), $\Delta 1829$ ($\Delta STM14_1829$; HL1233), $\Delta yciG$ (HL1369), $\Delta ymdF$ (HL1232), $\Delta 1829 \Delta yciG$ (HL1384), $\Delta 1829 \Delta ymdF$ (HL1380), $\Delta yciG \Delta ymdF$ (HL1382), $\Delta 1829 \Delta ymdF \Delta yciG$ (HL1402), $\Delta fljB$ (HL1640), $\Delta fljC$ (HL1438), $\Delta fljB \Delta fljC$ (HL1441), and $\Delta yhjH$ (HL1388) strains. Statistical significance shown is relative to WT. (C) Complementation test. A $\Delta 1829$ mutant was complemented with pUC19 plasmid carrying a wild-type copy of *STM14_1829* (p1829): WT/vector (HL1927), $\Delta 1829$ /vector (HL1928), and $\Delta 1829$ /p1829 (HL1930). Shown are the means $\pm$ standard deviations (SDs) from three independent experiments. Error bars indicate SDs. Statistical significance was determined by one-way analysis of variance (ANOVA) followed by *post hoc* Tukey's test. ****, $P \leq 0.0001$.

encoding a transcription regulator (TctD), c-di-GMP-metabolizing enzymes (STM0343 and STM0551), and a 53-amino-acid (aa) small protein (STM2010) of unknown function. These results suggested that our knowledge about the regulation of flagellum-dependent motility is far from complete and that additional motility-involved genes are likely to exist.

In this study, we identified a family of three paralogs small genes (*ymdF*, *STM14_1829*, and *yciG*) previously unknown to be required for flagellum-dependent motility in *Salmonella*. Small genes here are defined as those encoding proteins equal to or smaller than 60 aa. *ymdF*, *STM14_1829*, and *yciG* encode 55-aa, 60-aa, and 60-aa small proteins of unknown function, respectively. Our preliminary characterization of purified recombinant YmdF, STM14_1829, and YciG small proteins indicated that they are unstructured in solution and are intrinsically disordered proteins (IDPs). Genetic and phenotypic analyses indicated that *STM14_1829* is a key small protein required for full motility and that a swimming motility defect in a $\Delta STM14_1829$ mutant is due to the increased activity of PhoP, which consequently downregulates the transcription of the *flhDC* operon and, subsequently, of downstream flagellum genes such as *fliC*. Orthologs of YciG, STM14_1829, and YmdF are conserved in a wide range of both flagellated and nonflagellated bacteria, and the numbers of paralogs within bacteria vary from one to three depending on the species, suggesting that these orthologs may play larger roles in fundamental cellular processes that, in motile bacteria, affect flagellar expression.

RESULTS

Identification of three paralogs small genes required for wild-type swimming motility in *Salmonella*. To gain functional insights on uncharacterized small genes, we screened an in-house collection of *Salmonella* mutants, each carrying a deletion of a small gene with unknown function, for altered phenotypes compared to the wild-type *Salmonella* strain 14028s. As a result, we identified the *STM14_1829* gene, whose deletion renders *Salmonella* defective in swimming motility (Fig. 1A).

FIG 2 YciG, YmdF, and STM14_1829 (1829) proteins are intrinsically disordered. (A) Amino acid sequences of three paralogs in *S. Typhimurium* 14028s. A repeated motif is marked with an arrowed line, and the amino acid residues conserved in the repeat of each peptide are in bold. (B) Genetic organizations of neighboring genes of *ymdF*, *STM14_1829*, and *yciG* are shown. Promoter positions shown are based on our previous analysis of transcriptomes in the wild-type 14028s (46). (C) CD spectra obtained with purified YciG, STM14_1829, and YmdF recombinant proteins. The α -helical antimicrobial peptide LL-37 was used as a control.

STM14_1829 encodes a 60-aa small protein of unknown function, and we found that the genome of strain 14028s harbors two additional paralogs, *ymdF* and *yciG*, which encode a 55-aa and a 60-aa small protein, respectively. These three small proteins share approximately 85% identity at the amino acid level (Fig. 2A). The *ymdF* and *STM14_1829* genes appear to be monocistronic, while *yciG* is the first of the four-gene operon *yciGEF-katN* (Fig. 2B) (45). Note that *STM14_1273* and *ycdF*, upstream and overlapping genes of *yciG* (Fig. 2B), respectively, are likely pseudogenes or false annotations; our previous transcriptome and translato-me analyses of the wild-type strain 14028s cells grown in four different media showed no detectable expression of *STM14_1273* and *ycdF* (46).

To determine whether the three paralogous genes are functionally redundant, we constructed single and combined deletion mutants and tested them for swimming motility. As controls, we included $\Delta fliC$, $\Delta fliB$, $\Delta fliC \Delta fliB$, and $\Delta yhjH$ mutants. *FliC* is the main flagellin produced by *Salmonella* under our experimental condition. The *yhjH* gene encodes c-di-GMP-degrading phosphodiesterase, the loss of which results in

increased intracellular c-di-GMP levels and decreased swimming motility (38). As expected, the control strains exhibited a complete loss of swimming motility ($\Delta fliC \Delta fliB$), defective swimming ($\Delta fliC$ and $\Delta yhjH$), and a wild-type phenotype ($\Delta fliB$). Of the mutants deleted for the three paralogous genes alone or in combination, those commonly lacking *STM14_1829* exhibited similar levels of swimming defects, suggesting that among the three paralogous genes, *STM14_1829* encodes a key small protein required for wild-type swimming motility (Fig. 1A and B). Despite the high sequence identity, no swimming defects were seen in a single deletion mutant of either *yciG* or *ymdF* and only a minor swimming defect in a $\Delta yciG \Delta ymdF$ double mutant under this test condition (Fig. 1B). However, the $\Delta yciG \Delta ymdF$ double mutant, as well as those harboring a *STM14_1829* deletion in common, exhibited a more severe swimming defect in glucose-containing M9 minimal swim agar (see Fig. S1A in the supplemental material). This was in contrast to the $\Delta yciG$ and $\Delta ymdF$ single mutants, which still displayed the wild-type swimming motility in the same medium, suggesting that YciG and YmdF likely play redundant roles in swimming motility. Single, double, and triple paralogous gene mutants all grew as well as the wild type (see Fig. S1B), indicating that their swimming motility defects are not due to a growth defect. Furthermore, the swimming motility defect in the $\Delta STM14_1829$ mutant was partially restored by complementation with a plasmid expressing the wild-type copy of *STM14_1829* but not with the empty plasmid (Fig. 1C). The single, double, and triple mutants also displayed swarming motility defects, with trends matching their swimming motility defects (see Fig. S2). Altogether, these results demonstrate that three paralogous small genes (*yciG*, *STM14_1829*, and *ymdF*) are required for full flagellum-dependent motility in *Salmonella*, with *STM14_1829* being the most crucial and YciG and YmdF being required only under certain growth conditions.

YciG, STM14_1829, and YmdF are intrinsically disordered proteins. The amino acid sequences of the three small proteins (YmdF, *STM14_1829*, and YciG) share ~85% identity, contain repeated motifs, and exhibit variations only in the last 6 to 10 residues of their C-terminal regions (Fig. 2A). The repeated motif in the three proteins belongs to a family of KGG-containing domains (called stress-induced bacterial acidophilic repeat motif), which are found in bacterial and eukaryotic proteins expressed under stressful conditions (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cddsrv.cgi?uid=313819>). We also noted that the amino acid compositions of the three small proteins are severely biased. All three proteins have high contents of amino acids (3.3 to 5 mol% of P and Q; 7.2 to 23.3 mol% of A, E, G, K, and S) that are known to promote disorder and very few (hydrophobic) amino acids (C, W, V, F, Y, L, I, and M) that promote the folding of a globular protein (47). Consistent with this observation, algorithms such as DISOPRED (48) and PHYRE (49) that predict intrinsic disorder indicated that all three proteins are IDPs (data not shown). To verify these structural predictions experimentally, we expressed each of the three proteins in *E. coli* as a chimera with N-terminal His₆-SUMO and purified the recombinant proteins. After the removal of the His₆-SUMO tag, the final purified recombinant proteins (see Fig. S3) were analyzed by circular dichroism (CD) (Fig. 2B). We used the α -helical antimicrobial peptide LL-37 (50) as a control and comparison for CD measurements. As expected, the CD spectrum of LL-37 showed double dichroic minima at 208 and 222 nm, diagnostic for α -helical secondary structure (50). In contrast, the CD spectra of YciG, *STM14_1829*, and YmdF (Fig. 2B) all showed a minimum CD signal at ~198 nm, indicating random coils (51, 52). These results show that YciG, *STM14_1829*, and YmdF are intrinsically disordered proteins.

YciG, STM14_1829, and YmdF are required for wild-type flagellum expression. To determine whether the swimming motility defect in the $\Delta STM14_1829$ and other mutants was due to the decreased production of flagella, we first examined cell-bound FliC levels in the wild-type and mutant strains (Fig. 3A). The control $\Delta yhjH$ mutant with a known swimming motility defect was confirmed to produce wild-type levels of FliC (53), and as expected, $\Delta fliC$ and $\Delta fliB$ mutants produced only a single type of flagellin (Fig. 3A). The FliC levels of both the $\Delta yciG$ and $\Delta ymdF$ single mutants were similar to that of the wild type, consistent with their wild-type swimming motility (Fig. 1B).

FIG 3 Decreased expression of the FliC flagellin in the $\Delta STM14_1829$ ($\Delta 1829$) mutant. (A) Evaluation of cell-bound flagellum levels. Flagellin proteins prepared as described in Materials and Methods were run on a 12% SDS-PAGE gel and visualized by Coomassie brilliant blue staining. Shown is representative of two independent experiments, and the strains used are identical to those in Fig. 1B. (B) *fliC* promoter (P_{fliC}) activity was determined using a chromosomal $P_{fliC-lac}$ transcriptional fusion. Overnight cultures were diluted 100-fold in fresh LB medium and grown for 3 h, at which time cells were harvested for β -galactosidase assays. Shown are the means $\pm$ SDs from three independent experiments performed in duplicate. Error bars indicate SDs. Statistical significance was determined by one-way ANOVA followed by *post hoc* Dunnett's test. ns, not significant; ****, $P < 0.0001$. Strains carrying a chromosomal $P_{fliC-lac}$: WT (HL1625), $\Delta 1829$ (HL1627), $\Delta yciG$ (HL2561), $\Delta ymdF$ (HL2563), $\Delta 1829 \Delta yciG$ (HL1652), $\Delta 1829 \Delta ymdF$ (HL1648), $\Delta yciG \Delta ymdF$ (HL1650), $\Delta 1829 \Delta ymdF \Delta yciG$ (HL1629), and $\Delta yhjH$ (HL2527) mutants.

Mutant strains carrying the *STM14_1829* deletion all produced FliC at similar levels, much lower than that of the wild type (Fig. 3A), consistent with their decreased swimming motility (Fig. 1B). Unexpectedly, the $\Delta yciG \Delta ymdF$ double mutant with only a minor swimming defect (Fig. 1B) produced FliC at a level similar to *STM14_1829* deletion mutants.

Next, we examined the *fliC* promoter (P_{fliC}) activity in the wild-type and mutant strains by using a chromosomal *lac* transcriptional fusion to *fliC* ($P_{fliC-lac}$) and measuring β -galactosidase activity. Consistent with the pattern of the cell-bound FliC levels (Fig. 3A), the P_{fliC} activity in both the $\Delta yciG$ and $\Delta ymdF$ single mutants was only slightly lower than that of the wild type, whereas the P_{fliC} activity in the $\Delta STM14_1829$ mutant was 5.4-fold lower than that of the wild type (Fig. 3B). In fact, for all mutants with *STM14_1829* deletions, their P_{fliC} activities roughly correlated with both their reduced cell-bound FliC levels (Fig. 3) and decreased swimming motility (Fig. 1A and B). On the other hand, similar to the pattern of the decreased cell-bound FliC levels (Fig. 1B), the P_{fliC} activity in the $\Delta yciG \Delta ymdF$ double mutant was ~ 7.4 -fold lower than that of the wild type (Fig. 3B). Note that the FliC level of the $\Delta yciG \Delta ymdF$ double mutant was similar to those of other mutants carrying the *STM14_1829* deletion (Fig. 3A). Given that the swimming motility of the $\Delta yciG \Delta ymdF$ double mutant is $\sim 80\%$ of that of the wild type (Fig. 1B),

this result suggests that STM14_1829 may also be required for other mechanisms of motility control in addition to its requirement for the wild-type transcription of *fliC*. Despite the high similarity of YciG and YmdF to STM14_1829, a plasmid expressing either *yciG* or *ymdF* did not suppress the motility defect in the $\Delta STM14_1829$ mutant (data not shown), supporting this notion. Consistent with the lowered expression of *fliC* in the $\Delta STM14_1829$ mutant, an electron microscopic analysis showed that the $\Delta STM14_1829$ mutant produced fewer flagella than the wild type (see Fig. S4). Altogether, these results indicate that while all three paralogous small proteins are likely involved in transcriptional regulation of *fliC* expression, STM14_1829 may play an additional role in controlling swimming motility.

Deletion of STM14_1829 alone or in combination with its paralogs does not alter intracellular c-di-GMP levels. In *Salmonella*, an increase in intracellular c-di-GMP levels results in decreased swimming motility, and this effect is known to be mediated through BcsA (35) and YcgR (37, 38) proteins that are activated upon binding to c-di-GMP. To examine the possible involvement of c-di-GMP in the swimming motility defect in the $\Delta STM14_1829$ mutant, we constructed double mutants in which the STM14_1829 gene was deleted in combination with either *bcsA* or *ycgR* and tested them for swimming motility. If the motility defect in the $\Delta STM14_1829$ mutant was due to an increase in intracellular c-di-GMP levels, either or both of the double mutants ($\Delta STM14_1829 \Delta bcsA$ and/or $\Delta STM14_1829 \Delta ycgR$ mutants) would be expected to exhibit a partial or full restoration of wild-type motility. However, both the $\Delta STM14_1829 \Delta bcsA$ and the $\Delta STM14_1829 \Delta ycgR$ double mutants exhibited defects in swimming motility similar to the single $\Delta STM14_1829$ mutant (Fig. 4A and B). To test whether an increase in intracellular c-di-GMP levels would worsen the swimming motility defect observed in the $\Delta STM14_1829$ mutant, we constructed a $\Delta STM14_1829 \Delta yhjH$ double mutant and examined its swimming motility. Indeed, we observed a more severe swimming motility defect in the $\Delta STM14_1829 \Delta yhjH$ double mutant than in either the $\Delta STM14_1829$ or $\Delta yhjH$ single mutants (Fig. 4C), suggesting that the swimming motility defect in the $\Delta STM14_1829$ mutant is independent of increased intracellular c-di-GMP levels. To further rule out the involvement of c-di-GMP, we determined intracellular c-di-GMP levels in the wild-type, $\Delta STM14_1829$ single, and $\Delta STM14_1829 \Delta yciG \Delta ymdF$ triple mutant cells grown on swarming agar. As shown in Fig. 4D, the $\Delta STM14_1829$ mutant, as well as the mutant deleted for all three paralogous genes (*yciG*, STM14_1829, and *ymdF*), produced wild-type levels of c-di-GMP, while the control $\Delta yhjH$ mutant produced ~ 3.5 -fold higher c-di-GMP levels than the wild type (38). Altogether, these results demonstrate that under our test conditions, the motility defect in the $\Delta STM14_1829$ mutant was unlikely due to increased intracellular levels of c-di-GMP.

Expression of flagellum-related genes is reduced in the $\Delta STM14_1829$ mutant. Since the deletion of STM14_1829 alone or in combination with the other small genes produced a similar extent of swimming motility defect, we focused on the $\Delta STM14_1829$ single mutant. To probe for a potential regulatory system that explained the decreased *fliC* expression in the $\Delta STM14_1829$ mutant, we performed RNA sequencing (RNA-seq) experiments comparing the gene expression profiles between the wild-type and $\Delta STM14_1829$ mutant strains. After overnight cultures were diluted 100-fold, cells of the respective strains were grown in Luria-Bertani (LB) medium for ~ 3 h and harvested for the preparation of total RNA. cDNA libraries prepared from three independent RNA samples from both strains were sequenced on an Illumina platform, and the resulting sequencing data were processed to obtain a list of differentially expressed genes in the $\Delta STM14_1829$ mutant, with an artificial cutoff (≥ 2 -fold change, $\Delta STM14_1829$ versus wild type [WT], $P < 0.01$).

The analysis of RNA-seq data revealed that more than 1,600 genes were differentially expressed in the $\Delta STM14_1829$ mutant compared to that in the wild type, indicating that the deletion of the STM14_1829 gene results in drastic changes in gene expression (see Table S3). However, we immediately noticed that most, if not all, flagellum-related genes were downregulated in the $\Delta STM14_1829$ mutant. These include the genes that encode the master regulator (*flhDC*), the flagellum structural/export proteins (*flgNMA-L*

FIG 4 Swimming motility defect in the $\Delta 1829$ ($\Delta STM14_1829$) mutant is not due to increased intracellular c-di-GMP levels. Deletion of *STM14_1829* in combination with either *bcsA* (A) or *ycgR* (B) does not further decrease swimming motility. (C) Swimming motility defect in a $\Delta 1829 \Delta yhjH$ double mutant is worse than that of a $\Delta 1829$ or a $\Delta yhjH$ single mutant. (D) Both a $\Delta 1829$ single and a triple ($\Delta 1829 \Delta yciG \Delta ymdF$) mutant produced wild-type levels of intracellular c-di-GMP. Shown are the means $\pm$ SDs from three independent experiments performed in duplicate. Error bars indicate SDs. Statistical significance was determined by one-way ANOVA followed by *post hoc* Tukey's test. *, $P < 0.05$; ****, $P \leq 0.0001$. WT (HL10), $\Delta 1829$ (HL1233), $\Delta bcsA$ (HL2368), $\Delta 1829 \Delta bcsA$ (HL2370), $\Delta ycgR$ (HL2222), $\Delta 1829 \Delta ycgR$ (HL2510), $\Delta yhjH$ (HL2508), $\Delta 1829 \Delta yhjH$ (HL2504), $\Delta 1829 \Delta ymdF \Delta yciG$ (HL1558).

operon, *flhCD*, *flhEAB*, *fliBC*, *fliZA*, *fliDST*, and *fliE-R* operon), and the motor proteins (*motAB*) (Fig. 5A). To validate the downregulated transcription of *flhDC* in the $\Delta STM14_1829$ mutant, we used a luciferase reporter called *P_{flhDC}-lux* and introduced it into both wild-type and mutant strains. The resulting strains, in addition to that carrying the wild-type copy of the intact *flhDC* operon, harbor single chromosomally integrated luciferase operons (*luxCDBAE*) fused to the native promoter of *flhDC* (*P_{flhDC}*) (54). Consistent with the RNA-seq result (Fig. 5A), the *P_{flhDC}-lux* activity was ~ 2 -fold lower in the $\Delta STM14_1829$ mutant than in the wild type (Fig. 5B), suggesting that the downregulation of *fliC* and other flagellum-related genes in the $\Delta STM14_1829$ mutant was due at least in part to the decreased expression of the master regulator FlhDC.

Transcriptional regulator PhoP is upregulated in the $\Delta STM14_1829$ mutant.

The RNA-seq data described above in "Expression of flagellum-related genes is reduced in the $\Delta STM14_1829$ mutant" suggested that the expression or activity of one or more transcriptional regulators controlling the transcription of the *flhDC* operon may be altered in the $\Delta STM14_1829$ mutant. In *S. Typhimurium* and the closely related *E. coli*, there are at least four transcriptional activators (15–18) and 12 repressors (19–31) known to directly or indirectly regulate the transcription of the *flhDC* operon. Our

FIG 5 Expression of flagellar genes is downregulated in the $\Delta 1829$ ($\Delta STM14_1829$) mutant. (A) RNA-seq results show downregulation of flagellar genes in the $\Delta 1829$ mutant (HL1233) relative to that in the WT (HL10). All downregulated genes shown are statistically significant ($P < 0.0001$). (B) WT (HL2555) and $\Delta 1829$ (HL2557) strains carrying a chromosomal P_{thcD} -*luxCDABE* were grown in LB medium at 37°C for 3 h with shaking, and luciferase activity was measured. Shown are the means $\pm$ SDs from three independent experiments performed in duplicate. Error bars indicate SDs. Statistical significance was determined by one-way ANOVA followed by *post hoc* Tukey's test. **, $P < 0.01$; ***, $P < 0.001$.

RNA-seq data revealed that in the $\Delta STM14_{1829}$ mutant, the transcription of all four transcriptional activator genes was downregulated compared to that in the wild type, whereas four transcriptional repressors were downregulated, seven were unchanged, and one was upregulated (see Table S4). This RNA-seq result suggested that decreased levels of *flhDC* transcription in the $\Delta STM14_{1829}$ mutant may result from the combined effects of several differentially expressed transcriptional regulators.

However, one upregulated repressor PhoP did stand out (see Table S4). PhoP is a response regulator, forming a two-component regulatory system with its cognate sensor kinase, PhoQ. The active form of phosphorylated PhoP (PhoP~P) controls the expression of target genes (55, 56), including those encoded on *Salmonella* pathogenicity islands 1 and 2 (SPI-1 and SPI-2). In general, the expression of SPI-1 is negatively (57) and that of SPI-2 genes is positively controlled by PhoP~P (58). In our RNA-seq data, we found that in the $\Delta STM14_1829$ mutant, many known PhoP-activated genes, including SPI-2 genes, were upregulated (Fig. 6A; see Fig. S5A for SPI-2 genes), whereas SPI-1 genes were downregulated (see Fig. S5B). These RNA-seq results indicated that PhoP~P activity is higher in the $\Delta STM14_1829$ mutant than in the wild type. Using quantitative real-time PCR (qRT-PCR), we validated the RNA-seq data for several PhoP-regulated genes and others that are differentially expressed in the $\Delta STM14_1829$ mutant, including upregulated genes (*phoP* and PhoP-activated *ugtL* and *pagD*) and downregulated genes (*rflM* and *rtsB*, both encoding transcriptional repressors of the *flhDC* operon) (Fig. 6B). Specifically, using a β -galactosidase reporter strain in which the promoter of the PhoP-activated *pagC* gene (P_{pagC}) is transcriptionally fused to *lac* (P_{pagC} -*lac*) on the chromosome, we determined that the P_{pagC} activity was ~ 4 -fold higher in the $\Delta STM14_1829$ mutant than in the wild type and that this upregulation appeared to require PhoP (Fig. 6C). The P_{pagC} activity in the $\Delta STM14_1829$ mutant was restored to wild-type levels when complemented with a plasmid (p1829) expressing *STM14_1829* (see Fig. S6). This result supports the notion that PhoP activity is elevated in the $\Delta STM14_1829$ mutant.

If the swimming motility defect in the $\Delta STM14_1829$ mutant was due to the higher PhoP activity, the deletion of the *phoP* gene would be expected to restore the swimming motility of the $\Delta STM14_1829$ mutant to the wild type level. Indeed, the $\Delta STM14_1829 \Delta phoP$ double mutant exhibited wild-type swimming motility (Fig. 7A). Moreover, the lowered P_{flhDC} -*lux* (Fig. 7B) and P_{flhC} -*lac* (Fig. 7C) activities in the $\Delta STM14_1829$ mutant were all restored to wild-type levels in this double mutant. Altogether, these results indicate that the deletion of *STM14_1829* results in hyperactivation of PhoP, which in turn leads to the downregulation of the *flhDC* operon and other flagellum-related genes, including *flhC*.

FIG 7 Deletion of the *phoP* gene restores swimming motility and P_{flhDC} and P_{flcC} promoter activity in the $\Delta 1829$ ($\Delta STM14_1829$) mutant to wild-type levels. (A) Swimming motility in WT (HL10), $\Delta 1829$ (HL1233), $\Delta phoP$ (HL2114), and $\Delta 1829 \Delta phoP$ (HL2116) strains was determined as described in Materials and Methods. (B) Luciferase (P_{flhDC} -lux) activity in WT (HL2555), $\Delta 1829$ (HL2557), $\Delta phoP$ (HL2567), and $\Delta 1829 \Delta phoP$ (HL2559) strains. (C) β -Galactosidase (P_{flcC} -lac) activity in WT (HL1625), $\Delta 1829$ (HL1627), $\Delta phoP$ (HL2565), and $\Delta 1829 \Delta phoP$ (HL2529) strains. Overnight cultures of respective strains in LB medium were diluted 100-fold in fresh LB medium and grown for 3 h, at which point cells were harvested for β -galactosidase and luciferase assays. Shown are the means $\pm$ SDs from three independent experiments performed in duplicate. Statistical significance was determined by one-way ANOVA followed by *post hoc* Tukey's test. Statistically insignificant is not shown. *, $P < 0.05$; **, $P < 0.01$; ****, $P < 0.0001$.

domonas homologs are also involved in a cellular pathway(s) affecting motility, though in the opposite direction.

Among the Gram-negative enteric bacteria in the class of *Gammaproteobacteria*, motile bacteria such as *Serratia marcescens* and *Yersinia enterocolitica* do not possess homologs of the *Salmonella* STM14_1829 (and its paralogs), while a nonmotile *Shigella* sp. does (see Fig. S7A). The class of *Betaproteobacteria* includes motile bacteria such as *Burkholderia pseudomallei* that has polar flagella (60) and nonflagellated bacteria such as *Neisseria gonorrhoeae*; however, no bacterial species in this class was retrieved in our BLAST search. Interestingly, several Gram-positive *Streptomyces* species that do not produce flagella were also found to have a protein with high homology to STM14_1829 (Fig. S7B). These results reveal that orthologs of *Salmonella* STM14_1829 are conserved in a wide range of flagellated and nonflagellated bacteria. The fact that there is no correlation between the presence or absence of STM14_1829 orthologs and motile or nonmotile bacteria suggests that these proteins may play roles in a cellular pathway(s) conserved in both motile and nonmotile bacteria.

DISCUSSION

In this study, we identified three paralogous small proteins with previously unknown connections to flagellum-dependent motility in *Salmonella*. One of them (STM14_1829) appears to be involved in indirect regulation of the expression of *flhDC*, and the other two (YciG and YmdF) are only required for wild-type motility under certain growth conditions. In *Salmonella*, many small proteins (60 aa or fewer) have recently been characterized to have various functions: PmrR inhibits the LpxT enzyme (61), MgtR promotes the degradation of the MgtA and MgtC inner membrane proteins by the FtsH protease (62, 63), KdpF stabilizes the Kdp potassium transporter complex, and MgrB

FIG 8 Conservation of STM14_1829 orthologs in other Gram-negative bacteria. (A) Amino acid sequences of orthologs of STM14_1829 (1829_{St}) in *E. coli* MG1655 (YmdF_{Ec} and YciG_{Ec}) and in *P. aeruginosa* PAO1 (PA2146 and PA2190). A repeated motif is marked with arrowed line and conserved amino acid residues in the repeat of each small protein are in bold. (B) Swimming motility in ΔyciG_{Ec} (HL2328) and ΔymdF_{Ec} (HL2326) single and ΔyciG ΔymdF_{Ec} (HL2338) double mutants of *E. coli* MG1655 (HL657). Shown are the means ± SDs from three independent experiments performed in duplicate. Statistical significance was determined by one-way ANOVA followed by a *post hoc* Dunnett's test (versus WT). *, *P* < 0.05. Error bars indicate SDs. (C) Swarming surface motility in WT_{Pa} (HL2340), PA2146 (HL2341), and PA2190 (HL2342) strains determined as described in Materials and Methods. Shown is representative of two independent experiments.

inhibits the kinase activity of PhoQ (64). These small proteins are all α -helical hydrophobic membrane proteins. In contrast, YciG, STM14_1829, and YmdF are all predicted and confirmed to be IDPs (Fig. 2B), and their functions are likely distinct from those of other small proteins previously characterized in *Salmonella*.

The expression of all three proteins (YciG, STM14_1829, and YmdF) was recently shown to be RpoS (σ^S)-dependent in *Salmonella* cells grown in LB medium (65). A *Salmonella* rpoS mutant is defective in swimming and swarming motility (44), and on the basis of our results in this study, the motility defect in the rpoS mutant is likely due, at least in part, to the lack of STM14_1829 expression. Despite this common requirement of RpoS for expression, the transcriptional regulation of each of these genes appears to be distinct. The σ^S -dependent yciGFE-katN operon was previously shown to be repressed by the xenogeneic silencer H-NS (66–69), which can be antagonized by the DNA-binding transcription regulator YncC (69). It was proposed that yciFE comprises horizontally transferred genes in both *E. coli* and *Salmonella*, while yciG is not (69). YncC upregulates transcription of the yciGFE-katN operon by directly binding to the operon's upstream region (called the YncC box) in a σ^S -dependent manner (69). However, the STM14_1829 and ymdF genes do not appear to contain YncC boxes in their upstream regions (data not shown); H-NS does not seem to bind neighboring or coding regions of the ymdF gene, although it is shown to bind the upstream region of STM14_1829 (67, 68). Moreover, the upstream nucleotide sequences of the yciG, STM14_1829, and ymdF genes are significantly different (data not shown), and the yciG gene, but not ymdF and STM14_1829, has been shown to be regulated by ArcA (70).

The three paralogs (YciG, STM14_1829, and YmdF) have tandem repeats that contain the "KGG" motif (Fig. 2A). Though no bacterial protein with the KGG motif has been functionally characterized, this motif has been suggested to be present in

stress-inducible proteins. This is somewhat consistent with the fact that the expression of the three paralogs depends on RpoS, the sigma factor that activates the expression of many stress-resistance genes (71). Interestingly, a *Salmonella yciG* deletion mutant was previously reported to have several altered phenotypes compared to its parent strain *S. Typhimurium* SB300 (72): a lower survival under both acidic conditions (pH 3.1) and inside macrophages, impaired adhesion and invasion into epithelial cells, and reduced swimming motility. However, we did not observe any swimming motility defect in the $\Delta yciG$ mutant of strain 14028s under our test conditions (Fig. 1A; see also Fig. S2 in the supplemental material). Subtle genetic differences between strains SB300 and 14028s might account for this discrepant phenotype, and it would be interesting to examine the phenotypes of $\Delta STM14_1829$ and $\Delta ymdF$ mutants in the genetic background of strain SB300.

Of the three paralogous proteins identified in strain 14028s, STM14_1829 appears to play a key role (directly or indirectly) in controlling flagellum-dependent motility, because the inactivation of either *YciG* or *YmdF*, or even both, does not worsen the swimming motility defect in the $\Delta STM14_1829$ single mutant (Fig. 1B). Unlike *YciG* and *YmdF* that are conserved in the closely related nonpathogenic *E. coli* MG1655, STM14_1829 is unique to *Salmonella*. Note that the $\Delta yciG \Delta ymdF$ double mutants in both *S. Typhimurium* 14028s and *E. coli* MG1655 exhibit only minor motility defects (see Fig. 1A versus Fig. 8B). The unique presence of STM14_1829 in *Salmonella* may indicate that its involvement in motility control may have evolved in coordination with *Salmonella* pathogenesis and/or *Salmonella*-specific adaptation to environments. Moreover, the majority of the STM14_1829 orthologs in other bacterial species only differ in their six to ten C-terminal residues (data not shown) (see Fig. S7), suggesting that these varied C-terminal regions may provide species-specifically adapted roles.

Our results indicate that the motility defect in the $\Delta STM14_1829$ mutant (Fig. 1B; also see Fig. S2) is unlikely due to an increase in intracellular c-di-GMP levels (Fig. 4D). These results, however, do not completely rule out the possibility of localized or temporal changes and effects of intracellular c-di-GMP in relation to the role of STM14_1829 in motility control. Whether c-di-GMP is involved in the function of STM14_1829 and the other two paralogs remains to be determined.

The genetic evidence provided in this study for higher PhoP activity in the $\Delta STM14_1829$ mutant (Fig. 6) raises interesting ideas about the function of STM14_1829. While it is not well understood how PhoP controls *flhDC* expression (20, 29), it is tempting to speculate that STM14_1829 might function upstream of PhoP in a regulatory hierarchy, as a *phoP* deletion restores the motility of the $\Delta STM14_1829$ mutant to the wild-type level (Fig. 7A). STM14_1829 might interact with the sensor kinase/phosphatase PhoQ that activates PhoP (73, 74), either inhibiting the kinase activity or stimulating the phosphatase activity of PhoQ. In either case, the deletion of STM14_1829 may result in an increase in the abundance of PhoP~P, leading to a downregulation of flagellum-dependent motility. Considering this, our observation that the inactivation of STM14_1829 homologs in *P. aeruginosa* results in hypermotility is intriguing (Fig. 8C). Similar to that in *Salmonella*, the PhoP-PhoQ two-component regulatory system in *Pseudomonas* is activated under low extracellular Mg^{2+} conditions (75) and also regulates flagellum-dependent motility (76). However, unlike in *Salmonella*, it is proposed that the *Pseudomonas* PhoQ acts mainly as a phosphatase and downregulates PhoP activity, while a yet to be identified protein phosphorylates and activates the *Pseudomonas* PhoP (75, 77). Despite this difference, *Pseudomonas* homologs of STM14_1829 may still function in the same regulatory pathway.

STM14_1829 (and its homologs in other bacteria) likely has an alternative and/or additional role. The large number of differentially expressed genes (>1,600 genes) in the $\Delta STM14_1829$ mutant relative to that in the wild type is somewhat surprising (see Table S3), as this cannot be explained simply by the hyperactivation of PhoP, which is known to directly and indirectly regulate the expression of ~400 genes in *Salmonella* (78). The question remains as to how a deletion of a 60-aa small protein leads to such dramatic changes in gene expression. The fact that STM14_1829 (and its two paralogs)

is an IDP (Fig. 2B) may offer a possible explanation. Some IDPs and intrinsically disordered regions (IDRs) of larger proteins are known to be involved in signal transduction pathways through protein-protein interactions with their partner proteins, and an IDP (or IDR) may bind to multiple partners (79). Therefore, it is possible that STM14_1829 interacts with more than one partner protein, such as multiple transcriptional regulators. Following this notion, we are currently working to identify potential partner proteins of STM14_1829.

In summary, our study reports the identification of a novel family of small intrinsically disordered proteins that play a role in the regulation of flagellum-dependent motility in *Salmonella*, as well as in *E. coli* and *P. aeruginosa*. Given that their homologs are conserved in a wide range of bacteria, including nonflagellated bacteria, their primary functions may involve another cellular pathway(s) (that affects motility in the case of *Salmonella*) and remain to be uncovered.

MATERIALS AND METHODS

Bacterial strains, transductions, and growth conditions. All strains used in this study are listed in Table S1 in the supplemental material. *Salmonella enterica* serovar Typhimurium 14028s (80) was used as the wild-type strain, and all knockout mutants were constructed by the one-step inactivation method (81); P1 and P22 transductions were conducted as described previously (82). All strains were typically grown in Luria-Bertani (LB) medium in 50-ml conical tubes or Erlenmeyer flasks at 37°C with shaking at 200 rpm. When necessary, appropriate antibiotics were added to the medium at the following concentrations for both *E. coli* and *S. enterica*: ampicillin, 100 µg/ml; kanamycin, 40 µg/ml; and chloramphenicol, 20 µg/ml. *E. coli* DH5α was used as the host strain for the cloning and preparation of plasmids.

Construction of mutants. All mutants were constructed using the one-step inactivation method as described by Datsenko and Wanner (81) (see Tables S1 and S2 for primers and strain information). Mutant strains constructed in-house or obtained from BEI Resources were transduced into a fresh wild-type background and verified by PCR.

Motility assay. Cells of the strains grown overnight in LB medium were normalized to an optical density at 600 nm (OD₆₀₀) of 4 in their own supernatants of overnight cultures, and 1 to 2 µl was inoculated on swim agar (0.5% tryptone, 0.5% NaCl, and 0.3% Bacto agar) or swarm agar (0.8% nutrient broth, 0.5% glucose, and 0.5% Bacto agar). Swim diameters and photos were captured after incubation at 37°C for 4 to 4.5 h.

Construction of complementation plasmid. To construct a complementation plasmid carrying a wild-type copy of *STM14_1829*, the *STM14_1829* gene with its native promoter was PCR amplified using the genomic DNA of strain 14028s as the template and primers HP1326 and HP1515 (Table S2). After digesting with HindIII and BamHI, the DNA fragment was cloned into pUC19 digested with the same restriction enzymes, followed by transformation into chemically competent *E. coli* DH5α cells. The resultant plasmid was verified by Sanger sequencing and used for complementation of the Δ *STM14_1829* mutant.

Construction of SUMO fusion protein overexpression plasmids. Plasmids pET-SUMO-YciG, pET-SUMO-STM14_1829, and pET-SUMO-YmdF were constructed as follows. A DNA fragment containing *yciG*, *STM14_1829*, or *ymdF* was amplified by PCR with wild-type strain 14028s chromosomal DNA as the template. The primers used in PCRs for each gene are listed in Table S2. The amplified PCR fragments were purified and cloned into linearized TA-cloning plasmid pET-SUMO (Champion pET SUMO expression system; Thermo Fisher Scientific). The resulting plasmids (pET-SUMO-YciG, pET-SUMO-STM14_1829, and pET-SUMO-YmdF) were verified by Sanger sequencing and transformed into a host strain, *E. coli* BL21(DE3). In each of the overexpression plasmids, all proteins were overexpressed with a His₆-SUMO (H-SUMO) tag fused to their N termini.

Purification of YciG, STM14_1829, and YmdF proteins. *E. coli* strains carrying the overexpression plasmids were grown in LB medium. Overnight cultures were diluted 100-fold into 4 liters of LB medium supplemented with kanamycin (50 µg/ml). After growing the cultures to an OD₆₀₀ of ~0.8 at 37°C, IPTG (isopropyl-β-D-thiogalactopyranoside) was added at a final concentration of 1 mM, and growth was continued overnight at 20°C. Cells were harvested by centrifugation, and the cell pellets were stored at -80°C until use for protein purification. For purification, the cell pellets were thawed on ice and resuspended in 40 ml of cold lysis buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 25 mM imidazole) and lysed using an Emulsiflex-C5; cell debris was removed by centrifugation at 30,000 × *g* for 20 min at 4°C. The cleared lysate was passed over a nickel-Sepharose column to bind the His₆-SUMO protein chimeras. After binding, the column was washed with 50× volume lysis buffer, followed by the elution of the His-tagged protein with 3× column volume elution buffer (50 mM Tris [pH 7.5], 500 mM NaCl, 500 mM imidazole). After elution, His₆-tagged SUMO protease (H-SP) was added to the sample at a ratio of 1/50 and allowed to digest overnight at 4°C while simultaneously dialyzing against 2 liters of 50 mM Tris (pH 7.5) containing 250 mM NaCl. After dialysis, the protein was passed over nickel-nitrilotriacetic acid (Ni-NTA) resin three times to remove free His₆-SUMO and H-SP, yielding a pure preparation of the desired protein without added nonnative amino acid residues. After purification, the proteins were dialyzed overnight at 4°C in 20 mM Tris (pH 7.5) containing 150 mM NaCl. Finally, the purified proteins were confirmed by mass spectrometry, with custom-synthesized YciG, STM14_1829, and YmdF (from NeoBiolab) as the controls.

Circular dichroism measurements. Purified proteins were diluted to a final concentration of 0.05 mg/ml in 20 mM KH_2PO_4 , 100 mM NaF at pH 7.5 and placed in a 0.1-cm-path-length cuvette. The spectra were collected using a Jasco-815 from a wavelength of 260 nm to 190 nm. The background spectrum from the buffer alone was subtracted, and the data were plotted as mean residue ellipticity.

Evaluation of flagellin expression on the cell surface. Cell-bound flagellin samples were prepared as described by Le Guyon et al. (53) with a slight modification. Overnight cultures in LB medium were diluted 100-fold in 5 ml of fresh LB medium and grown at 37°C for 3 h with shaking at 200 rpm. The cells were harvested by centrifugation, resuspended in phosphate-buffered saline, and adjusted to an OD_{600} of 1. Flagella were sheared from the cell surface by passing the cell suspension 10 times through a 25-gauge 1.5 needle (BD Biosciences). The final suspension (1 ml) was centrifuged at $18,000 \times g$ at 4°C for 10 min, and to precipitate sheared flagella, supernatants (900 μl) were mixed with trichloroacetic acid (at a final concentration of 10%) and incubated on ice for 1 h. Precipitated proteins were pelleted by centrifugation at $18,000 \times g$ at 4°C for 10 min, and the pellets were washed 3 times with 1 ml ice-cold acetone and dried in a SpeedVac. The pellets were resuspended in SDS sample loading buffer, boiled at 95°C for 10 min, run on a 12% SDS-PAGE gel, and visualized by Coomassie brilliant blue staining.

c-di-GMP measurement. Samples were prepared as described by Simm et al. (83), and c-di-GMP levels were determined as described by Irie and Parsek (84), with minor modifications. Overnight LB cultures of the strains were diluted 100-fold in 100 ml of fresh LB medium and grown to an OD_{600} of ~0.6 at 37°C with shaking at 200 rpm. Ten milliliters of the culture was added to a swarming agar plate, and after 40 to 60 s, the extra liquid was removed. The swarming agar plates were then dried for 10 min and incubated at 37°C for 2.5 h, at which time, the cells were harvested and resuspended in ice-cold 0.19% formaldehyde. After incubating on ice for 10 min, the cells were harvested by centrifugation at $5,000 \times g$ for 15 min. The supernatants were discarded, and the cell pellet was resuspended in 330 μl high-performance liquid chromatography (HPLC)-grade water. A portion (30 μl) of the cell suspension was saved for the determination of protein concentrations using the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Fisher Scientific). The rest was heated at 95°C for 10 min, followed by the addition of 700 μl of ethanol to extract the nucleotides. After adding 10 μl of a 10 μM solution of 2-Cl-5'-AMP (Axxora) as the internal standard (84) and incubating at room temperature for 15 min with intermittent vortexing, the samples were centrifuged at 12,000 rpm for 10 min. The supernatant was saved and cell debris was resuspended in 1 ml of 70% ethanol for the second extraction. Nucleotide extracts were pooled and dried in a SpeedVac. Dried samples were reconstituted in 300 μl of HPLC-grade water, vortexed for 30 s, and centrifuged at $16,100 \times g$ for 20 min at 4°C. The supernatant was used for liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis. Separation was performed on an Agilent 1200 series LC using Atlantis T3 (100 Å, 3 μm , 3 mm by 100 mm) column with an injection volume of 40 μl . The following mobile phases were used: 10 mM ammonium acetate and 0.1% acetic acid in water (mobile phase A) and acetonitrile (mobile phase B) in gradient elution (0 min, 0% B; 1.5 min, 10% B; 5 min, 30% B; 8 to 8.5 min, 0% B; and 15 min, 0% B) at a flow rate of 0.4 ml/min. A QTRAP 5500 MS/MS system (Sciex) with an electrospray ionization (ESI) source in positive ion mode was used for the MS analysis. The multiple reaction monitoring (MRM) transitions used for the quantification of c-di-GMP and 2-Cl-5'-AMP were 691.0/152.1 and 382.1/170.0, respectively. A standard curve was generated using a series of c-di-GMP solutions (90 to 900 nM) containing 100 nM 2-Cl-5'-AMP. Data acquisition and analysis were performed using Analyst 1.6.2 software. The c-di-GMP levels obtained were normalized to the protein concentrations.

Transcriptomic analysis using RNA sequencing. Wild-type and $\Delta\text{STM14_1829}$ cells grown overnight in LB medium were diluted 100-fold in 10 ml of fresh LB medium and grown at 37°C for 3 h with shaking at 200 rpm, at which time two volumes of RNeasy Protect (Qiagen) were added to the cultures with incubation at room temperature for 10 min. The cells were harvested by centrifugation at 4,000 rpm at 4°C for 15 min, and total RNA was isolated using the RiboPure microbial RNA isolation kit (Ambion) according to the manufacturer's instructions. The isolated RNA was then treated with RNase-free DNase I supplied with the RiboPure RNA isolation kit for 30 min at 37°C, purified by phenol-chloroform extraction and ethanol-Na-acetate (pH 5.2) precipitation, and resuspended in 20 μl of nuclease-free water. Three independent RNA samples of wild-type and $\Delta\text{STM14_1829}$ strains were prepared and sent to the sequencing facility at the University of Illinois at Chicago for the preparation of RNA sequencing libraries with Illumina's ScriptSeq kit with bacterial ribosomal RNA depletion. Sequencing was performed on the Illumina HiSeq 4000 in paired-end mode (2×75 bp).

The raw sequencing reads were preprocessed to remove adapter sequences and bases with low sequencing quality, and the preprocessed reads were mapped to the genome of *Salmonella enterica* Typhimurium 14028s (CP001363.1) using bowtie 2 (v. 2.3.3) with default parameters (85). On average, 81% of the sequencing reads were uniquely mapped to the reference genome. Using featureCounts from subread (v. 1.5.1) (86), the expression levels of the annotated genes (CP001363.gff3) were quantified with uniquely mapped reads. To identify the genes that were differentially expressed, DESeq2 package (version 1.10.1) in R was used (87). Genes with adjusted *P* values of ≤ 0.001 and with fold changes ≥ 2 were considered statistically significant and differentially expressed.

Quantitative real-time PCR. Total RNAs of the wild-type *Salmonella* and $\Delta\text{STM14_1829}$ strains were prepared as described in "Transcriptomic analysis using RNA sequencing" above and used as the template (~5 μg) for cDNA synthesis with random hexamers and SuperScript IV reverse transcriptase (Invitrogen) according to manufacturer's instructions. Upon the completion of reaction, cDNA was purified using a Wizard SV gel and PCR cleanup kit (Promega), and the concentrations were quantified with a NanoDrop. qRT-PCR was performed in a CFX Connect system (Bio-Rad). Each 10- μl reaction mixture contained ~5 ng of total cDNA, 1 μM (each) forward and reverse primers for a target gene, and

5 μ l SensiFast SYBR Low-ROX MasterMix (Bioline). The primers used in qRT-PCR are listed in Table S2. Three biological replicates of each strain were performed with 3 technical replicates per sample. The program cycle was as follows: denaturation at 95°C for 1 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 60°C for 15 s, and extension at 72°C for 10 s, with autothreshold. The *rpoD* gene was used as a control for normalization. The fold change in gene expression was calculated using the comparative C_T ($2^{-\Delta\Delta C_T}$) method (88).

β -Galactosidase and luciferase assays. β -Galactosidase activities were determined as described previously (89). Luciferase activity was determined on a SpectraMax plate reader (Molecular Devices) as described by Mouslim et al. (54).

Statistics. Statistical significance was determined using GraphPad Prism (v. 7).

Accession number(s). RNA-seq data are available at GEO with the accession number GSE113988.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00415-18>.

SUPPLEMENTAL FILE 1, PDF file, 1 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.7 MB.

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