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RESEARCH ARTICLE

Altered Regulation of the Diguanylate Cyclase YaiC Reduces Production of Type 1 Fimbriae in a Pst Mutant of Uropathogenic *Escherichia coli* CFT073

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ABSTRACT The *pst* gene cluster encodes the phosphate-specific transport (Pst) system. Inactivation of the Pst system constitutively activates the two-component regulatory system PhoBR and attenuates the virulence of pathogenic bacteria. In uropathogenic *Escherichia coli* strain CFT073, attenuation by inactivation of *pst* is predominantly attributed to the decreased expression of type 1 fimbriae. However, the molecular mechanisms connecting the Pst system and type 1 fimbriae are unknown. To address this, a transposon library was constructed in the *pst* mutant, and clones were tested for a regain in type 1 fimbrial production. Among them, the diguanylate cyclase encoded by *yaiC* (*adrA* in *Salmonella*) was identified to connect the Pst system and type 1 fimbrial expression. In the *pst* mutant, the decreased expression of type 1 fimbriae is connected by the induction of *yaiC*. This is predominantly due to altered expression of the FimBE-like recombinase genes *ipuA* and *ipbA*, affecting at the same time the inversion of the *fim* promoter switch (*fimS*). In the *pst* mutant, inactivation of *yaiC* restored *fim*-dependent adhesion to bladder cells and virulence. Interestingly, the expression of *yaiC* was activated by PhoB, since transcription of *yaiC* was linked to the PhoB-dependent *phoA-psiF* operon. As YaiC is involved in cyclic di-GMP (c-di-GMP) biosynthesis, an increased accumulation of c-di-GMP was observed in the *pst* mutant. Hence, the results suggest that one mechanism by which deletion of the Pst system reduces the expression of type 1 fimbriae is through PhoBR-mediated activation of *yaiC*, which in turn increases the accumulation of c-di-GMP, represses the *fim* operon, and, consequently, attenuates virulence in the mouse urinary tract infection model.

IMPORTANCE Urinary tract infections (UTIs) are common bacterial infections in humans. They are mainly caused by uropathogenic *Escherichia coli* (UPEC). We previously showed that interference with phosphate homeostasis decreases the expression of type 1 fimbriae and attenuates UPEC virulence. Herein, we identified that alteration of the phosphate metabolism increases production of the signaling molecule c-di-GMP, which in turn decreases the expression of type 1 fimbriae. We also determine the regulatory cascade leading to the accumulation of c-di-GMP and identify the Pho regulon as new players in c-di-GMP-mediated cell signaling. By understanding the molecular mechanisms leading to the expression of virulence factors, we will be in a better position to develop new therapeutics.

KEYWORDS *Escherichia coli*, Pho regulon, type 1 fimbriae, UPEC, c-di-GMP, phosphate, *pst*, urinary tract infection

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An important aspect of bacterial physiology and virulence is the capacity to sense environmental signals. Sensing the environmental changes leads to adaptation, since genes responding to these changes will be specifically and coordinately regulated. Two-component signal transduction systems (TCSs) are one of the mechanisms by which bacteria respond to environmental signals. TCSs typically comprise an inner membrane histidine kinase sensor protein and a cytoplasmic response regulator (1).

The TCS PhoBR comprises PhoR, which is the sensor histidine kinase, and PhoB, the response regulator, and responds to phosphate limitation, i.e., when the extracellular phosphate concentration falls below 4 μM (2, 3). Thereby, PhoBR regulates genes belonging to the Pho regulon, such as those mediating phosphate transport and metabolism. Genes belonging to the Pho regulon possess PhoB-binding specific DNA sequences, known as Pho boxes, located within their promoter regions (2–5). During phosphate limitation, the TCS PhoBR is activated, and PhoB binds to Pho boxes to induce or repress gene expression (2, 3). The periplasmic alkaline phosphatase (PhoA), which catalyzes the hydrolysis and phosphorylation of a wide variety of phosphate monoesters, and the phosphate-specific transport (Pst) system, an ATP-binding cassette (ABC) transporter specific for inorganic phosphate (P_i), are among members of the Pho regulon (2, 3). In addition to being involved in the transport of P_i, the Pst system negatively regulates the activity of PhoBR, as the disruption of Pst constitutively activates PhoBR regardless of environmental phosphate availability (2, 3). Thus, inactivation of the *pst* system mimics phosphate-limiting conditions. Moreover, the Pst system is also linked with pathogenesis, as its deletion attenuates the virulence of pathogenic strains (6–9). In uropathogenic *Escherichia coli* (UPEC), we showed that virulence attenuation of the *pst* mutant is mainly attributed to the decreased expression of type 1 fimbriae (10).

In UPEC strains, type 1 fimbriae are a key virulence factor and are required to establish infection (11–14). Type 1 fimbriae are expressed in the bladder, and in addition to promoting adhesion to bladder cells and its colonization, they are involved in the invasion of bladder cells (15–18). Type 1 fimbriae are encoded by the *fimAICDFGH* operon (*fim*), where *fimA* encodes the major subunit and *fimH* encodes the mannose-specific adhesin (19). Expression of the *fim* operon depends on a promoter located on an invertible element (*fimS*) (20). The expression of type 1 fimbriae is therefore subjected to phase variation. The expression of type 1 fimbriae is mediated by the switching of *fimS* between the on and off orientations. The orientation of *fimS* is mainly controlled by the FimB and FimE recombinases (21). FimB mediates switching in both directions, from phase off to phase on and phase on to phase off, where the on orientation is favored, while FimE promotes switching to the off orientation, i.e., from phase on to phase off (21, 22). In addition to FimB and FimE, UPEC strain CFT073 encodes FimBE-like recombinases IpuA and IpbA (*fimX* product in UPEC strain UTI89) (23–25). IpuA promotes switching, like FimB, whereas IpbA only promotes the switching to the on position. Furthermore, IpuA and IpbA are sufficient for switching *fimS* and influencing type 1 fimbria expression *in vitro* and *in vivo* (23–25).

Cyclic di-GMP (c-di-GMP) is a bacterial second messenger that controls various processes, such as flagellar motility, biofilm formation, the cell cycle, and virulence of pathogenic bacteria (26, 27). c-di-GMP is synthesized by diguanylate cyclase proteins, which contain GGDEF domains, and is degraded by phosphodiesterase proteins, which contain EAL or HD-GYP domains. c-di-GMP acts via a variety of receptors containing PilZ or I-site domains (28) and acts by influencing transcriptional, translational, and post-translational regulation (26, 27). Its role in cellular processes is well studied in *Salmonella*, *Vibrio*, *Yersinia*, and *Pseudomonas* species. In *Salmonella*, AdrA (homolog of YaiC, also named DgcC [29] in *Escherichia coli*) is one of the major diguanylate cyclases. By activating the biosynthesis of c-di-GMP, AdrA induces the formation of biofilm by increasing the production of cellulose through the induction of the *bcs* (bacterial cellulose synthesis) operon (30–33). The expression of *adrA* is activated by the curli biosynthesis regulator CsgD (30, 34, 35). Cellulose production, along with curli, forms an

extracellular matrix favorable to surface adhesion, cellular aggregation, persistence in the environment, and biofilm formation (36).

As mentioned above, deletion of the *pst* system attenuated virulence of the UPEC CFT073 strain by decreasing the expression of type 1 fimbriae (10). An *in silico* analysis showed that genes involved in regulation of or in biosynthesis of type 1 fimbriae do not possess Pho boxes in their promoter regions (5). Given that the specific mechanisms by which induction of the Pho regulon (PhoBR) inhibits the expression of type 1 fimbriae are unknown and that the Pho regulon seems to act indirectly, possible mechanisms linking the Pho regulon and type 1 fimbriae were investigated herein by the construction of a transposon library in the *pst* mutant. In this study, we show that YaiC is one of the important mediators in the *pst* mutant that contribute to decreased expression of type 1 fimbriae in strain CFT073. Indeed, *yaiC* is induced in the *pst* mutant and represses type 1 fimbriae. In the *pst* mutant strain, *yaiC* is activated by PhoB due to increased transcription of *yaiC* from the adjacent *phoA-psiF* operon, and the increased transcription of *yaiC* required the PhoB-dependent promoter of the *phoA-psiF* operon. An accumulation of c-di-GMP was observed in the *pst* mutant and was concomitant with the increased expression of *yaiC*. Taken together, our results demonstrate that the induction of *yaiC* in the *pst* mutant alters the expression of type 1 fimbriae and contributes to the fitness defect of the *pst* mutant in the murine model of UTI.

RESULTS

Screening for genes involved in repression of type 1 fimbriae in the *pst* mutant. We previously demonstrated that constitutive activation of PhoBR, by disrupting the Pst system, decreased expression and production of type 1 fimbriae and attenuated virulence (10, 37, 38). However, the molecular mechanisms connecting the Pho regulon and regulation of type 1 fimbriae have not yet been determined. Furthermore, an *in silico* search revealed that the *fimB*, *fimE*, and *fimA* genes do not possess a Pho box(es) in their promoter region (5), which suggests that PhoBR may indirectly affect type 1 fimbrial expression (10). To identify genes that could connect the Pho regulon (Pst system and PhoBR) to the expression of type 1 fimbriae, a transposon library was constructed in the *pst* mutant. Transposon mutants were screened for an increase in the production of type 1 fimbriae. Inactivation of the *pst* system constitutively activates PhoBR, which can be monitored by the detection of alkaline phosphatase PhoA activity on lysogeny broth (LB) agar plates supplemented with 5-bromo-4-chloro-3-indolyl phosphate disodium (BCIP). Indeed, the *pst* mutant appears blue on these plates, whereas the wild-type (WT) strain remains white. Furthermore, as the deletion of *phoB* in the *pst* mutant abrogated the activation of the Pho regulon induced by inactivating the *pst* system (38) and the *pst phoB* double mutant regained the production of type 1 fimbriae to WT levels (10), we tested our approach by first screening white colonies grown on BCIP-containing plates. Surprisingly, one of the mutants identified in this screen, which demonstrated a regain in type 1 fimbrial expression, did not have a mutation in the *phoBR* regulatory genes but was found to have a disrupted *phoA* gene (Fig. 1A). Since *phoA* encodes a periplasmic enzyme not known to have a regulatory function, we sought to explain the molecular mechanisms linking PhoA and type 1 fimbrial expression in the CFT073 *pst* mutant.

The *yaiC* gene encoding a GGDEF domain protein contributes to the repression of type 1 fimbriae in the *pst* mutant. PhoA is a periplasmic enzyme catalyzing the hydrolysis of a wide variety of phosphomonoesters. Given that the transposon was inserted in the opposite orientation of the *phoA-psiF* operon (Fig. 1A), we hypothesized that the transposon had a polar effect on a gene(s) downstream of *phoA* by blocking the transcription of *psiF* and *yaiC*. This hypothesis is also based on the fact that PhoA does not possess known regulatory domains or functions. To test this possibility, we introduced a nonpolar mutation in both WT and the Δ*pst* mutant strain in *phoA*, as well as in downstream genes *psiF* and *yaiC*. The production of type 1 fimbriae was then evaluated by yeast agglutination on strains cultured to mid-log phase in LB. As expected, mutations within *phoA*, *psiF*, or *yaiC* in the WT strain had no effect on the

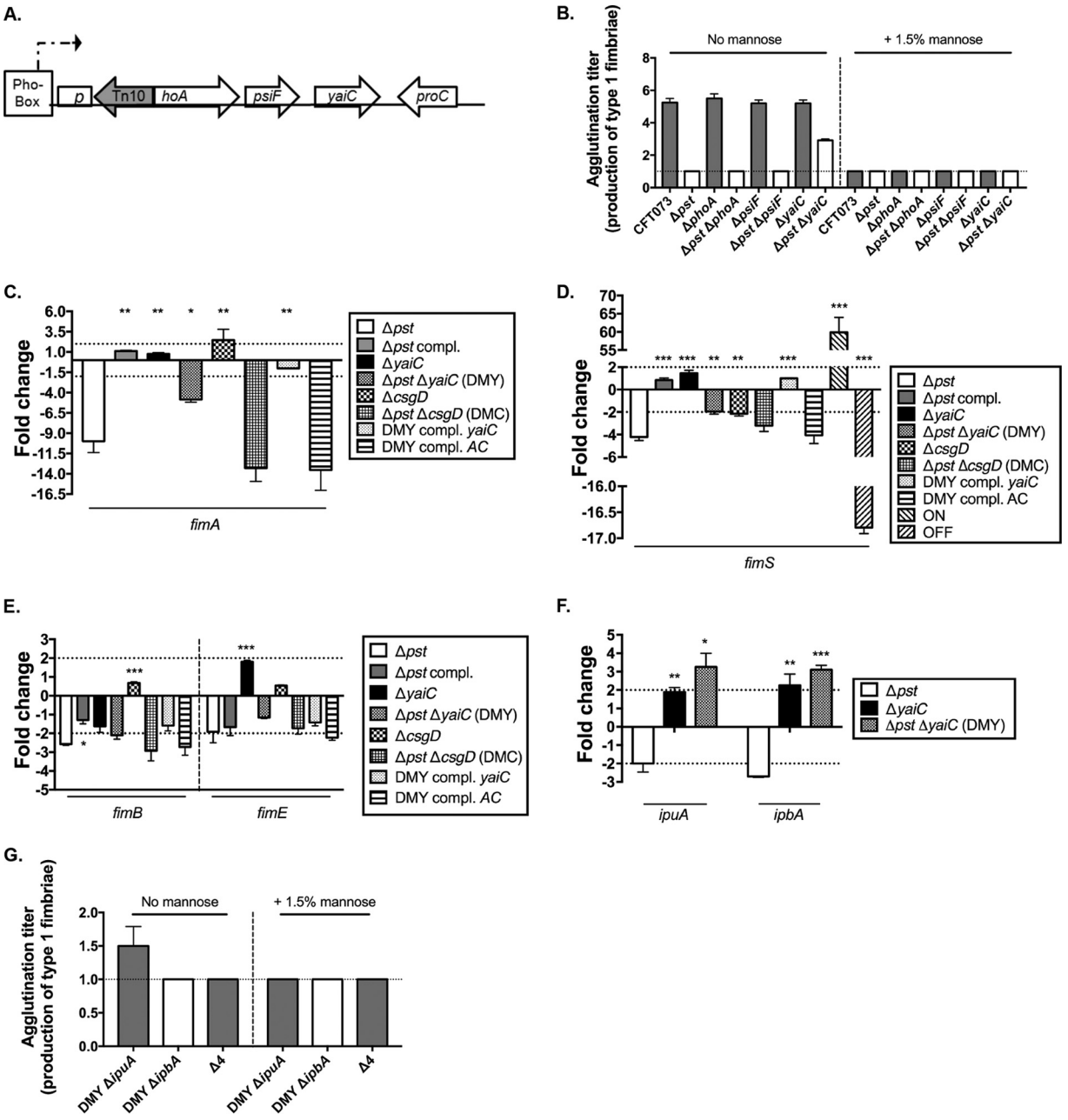

FIG 1 The *yaiC* gene alters expression of type 1 fimbriae. (A) Schematic representation of a transposon insertion which caused a regain in expression of type 1 fimbriae. (B) Quantification of type 1 fimbriae by yeast agglutination assay in nonpolar mutants with and without 1.5% mannopyranose. The CFT073 Δ*fim* and Δ*pst* mutant strains were used as negative controls, since they did not agglutinate yeast. The *y* axis represents the well in which we observed agglutination. Each well corresponds to a 2-fold dilution. For example, an agglutination titer of 2 corresponds to a 2-fold dilution, while a titer of 4 corresponds to an 8-fold dilution. (C) Transcription of *fimA* in mutant strains compared to the WT strain. (D) On orientation of *fimS* in the mutant strains compared to the WT strain. (E and F) Expression of *fimB* and *fimE* (E) and *ipuA* and *ipbA* (F) in mutant strains compared to the WT strain. (G) Quantification of type 1 fimbriae by yeast agglutination assay in mutants with deleted recombinase genes *ipuA* and/or *ipbA* with and without mannose. The dotted lines in panels B and G represent the limit of detection that corresponds to the nondiluted sample. The dotted lines in panels C, E, and F correspond to the cutoff of a significant difference in expression, while in panel D, they correspond to the level of on orientation. All results shown are the mean values and standard deviations of the results from four biological experiments. Statistical significance was calculated by the Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

production of type 1 fimbriae (Fig. 1B), as the Pho regulon is not induced in LB. In contrast, the production of type 1 fimbriae was restored in the *pst yaiC* double mutant (DMY) (Fig. 1B). The *yaiC* gene, an ortholog of the diguanylate cyclase gene *adrA* in *Salmonella enterica*, encodes a GGDEF domain, is immediately adjacent to the *phoA-psiF* operon, and is not annotated as being part of this operon (Fig. 1A). To confirm that

yeast agglutination was mediated by type 1 fimbriae, the assay was also performed in the presence of 1.5% mannopyranose, which blocks the interaction of the type 1 fimbrial adhesin and yeast and inhibits agglutination. As expected, no yeast agglutination was observed when 1.5% mannopyranose was added to the bacteria (Fig. 1B). To corroborate the yeast agglutination assay, the expression of *fimA* was quantified by reverse transcription-quantitative PCR (qRT-PCR). Compared to the Δ*pst* mutant strain, the expression of *fimA* was increased 2-fold in the DMY strain (Δ*pst* Δ*yaiC*) (Fig. 1C). These results suggest that induction of the Pho regulon in the *pst* mutant mediates the repression of type 1 fimbriae through the deregulation of *yaiC* expression, but the *phoA* gene product itself does not play a role in this process.

The expression of the genes encoding type 1 fimbriae is dependent on an invertible element (*fimS*), which contains the *fim* promoter (20). This invertible element alternates between the on and off orientations, which leads to activation and repression of type 1 fimbrial expression, respectively. To correlate increased *fim* expression in the DMY strain with the orientation of *fimS*, its orientation was determined by quantitative PCR (qPCR). In a comparison of the on position of strains cultured to mid-log phase in LB, the on orientation is 2.0-fold higher than for the *pst* mutant (Fig. 1D).

In vitro, we previously demonstrated that repression of *fimA* in the *pst* mutant is due to a bias toward the off orientation of *fimS*, which is mainly linked to the repression of *fimB* and, possibly, to the downregulation of the Fim-like recombinase genes *ipuA* and *ipbA* (10). To correlate the restoration of increased type 1 fimbrial production and *fimS* orientation with the expression levels of the recombinases, the expression of *fimB*, *fimE*, *ipuA*, and *ipbA* was quantified by qRT-PCR. Thereby, the deletion of *yaiC* in the Δ*pst* mutant background had no effect on *fimB* and *fimE* expression (Fig. 1E). However, the expression of *ipuA* and *ipbA* was restored in the DMY mutant strain, as they were induced 3.9- and 2.9-fold, respectively (Fig. 1E). Since IpuA and IpbA promote the orientation of *fimS* in the on position, restoration of type 1 fimbrial expression could be attributed to the increase expression of these two Fim-like recombinases. To confirm the contribution of *ipuA* and *ipbA* in the regain in the production of type 1 fimbriae in the DMY mutant, we inactivated either *ipuA* (DMY Δ*ipuA* mutant) or *ipbA* (DMY Δ*ipbA* mutant) or both genes (Δ4 mutant) in the DMY background. We then determined the production of type 1 fimbriae in these strains by yeast agglutination in the presence and absence of mannopyranose. As expected, the production of type 1 fimbriae was inhibited in all three mutants and restored production to the *pst* mutant (Fig. 1G). The addition of 1.5% mannopyranose did not affect yeast agglutination, since the production of type 1 fimbriae was inhibited in the mutant strains (Fig. 1G). These data confirm the contribution of *ipuA* and *ipbA* in the restoration in the expression of the type 1 fimbriae in the Δ*pst* Δ*yaiC* double mutant.

Taken together, these results show that *yaiC* plays a role in decreasing the expression of type 1 fimbriae in the *pst* mutant. Indeed, its deletion restored the expression of *fimA* and increased the expression of the *ipuA* and *ipbA* recombinases, which in turn promoted orientation of the invertible promoter toward the on position and increased expression of the type 1 fimbriae.

The *yaiC* gene is induced under phosphate-limiting conditions. As the deletion of *yaiC* in the Δ*pst* mutant strain restores the expression/production of type 1 fimbriae, this suggests that *yaiC* is induced in the *pst* mutant. To test this possibility, the expression of *yaiC* was evaluated by qRT-PCR. As shown in Fig. 2A, compared to the WT strain, *yaiC* was induced 4.4-fold in the *pst* mutant. Since constitutive induction of the Pho regulon through inactivation of the Pst system induced the expression of *yaiC*, we tested if physiological activation of the Pho regulon had the same effect. To do so, bacteria were grown in morpholinepropanesulfonic acid (MOPS) low-phosphate (LP) and high-phosphate (HP) broth, and the expression of *yaiC* was analyzed by qRT-PCR. When grown in LP broth, compared to HP broth, the expression of *yaiC* was 4.1- and 3.3-fold induced in the WT and Δ*pst* complemented (compl.) mutant strains (Fig. 2B). Since disruption of the *pst* system constitutively activates the Pho regulon regardless of

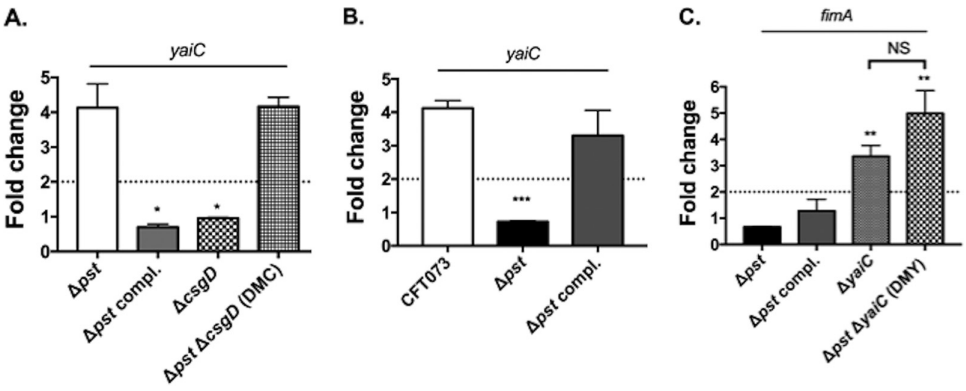

FIG 2 Expression of *yaiC* and *fimA* under phosphate-limiting conditions. (A) Expression of *yaiC* in strains grown in LB compared to WT strain. (B) Expression of *yaiC* in strains grown under low-phosphate (LP) conditions. Expression in LP broth was compared to expression of the corresponding strains grown under high-phosphate (HP) conditions. (C) Expression of *fimA* in strains grown in LP broth. The expression was compared with those of the WT strain grown in LP broth. The dotted lines correspond to the cutoff of a significant difference in expression. All results shown are the mean values and standard deviations of the results from four biological experiments. Statistical significance was calculated by the Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$; NS, nonsignificant.

environmental phosphate availability (3), it was not surprising to observe no difference in *yaiC* expression between the LP and HP media in the *pst* mutant.

We previously showed that growth under phosphate-limiting conditions decreased the expression of type 1 fimbriae (Fig. 2C) (10). This was reflected by similar expression patterns of *fimA* in the CFT073 and Δ*pst* mutant strains grown in LP broth. As the deletion of *yaiC* in the *pst* mutant restored the transcription of *fimA* in LB, we investigated whether the expression of *fimA* was restored in the Δ*yaiC* and the DMY mutants grown in LP broth. Indeed, compared with the WT strain grown in LP broth, *fimA* was induced 3.4- and 4.0-fold in the CFT073 Δ*yaiC* and DMY mutants, respectively (Fig. 2C).

These results indicate that induction of the Pho regulon, physiologically or by inactivation of *pst*, increases the expression of *yaiC* and consequently represses the expression of type 1 fimbriae. Thereby, these results suggest that *yaiC* is a repressor of type 1 fimbriae.

Deletion of *yaiC* restores type 1 fimbria-dependent adhesion of the Pst mutant. *In vitro*, we previously demonstrated that the *pst* mutant adheres to bladder cells as well as the WT strain. However, unlike the WT parent, adhesion of the *pst* mutant was *fim* independent and implied that adherence was mediated by other adhesins (10). Nevertheless, the production of other adhesins by the *pst* mutant was not sufficient to allow efficient colonization of the bladder, as the *pst* mutant is attenuated in the UTI mouse model (10). Since the deletion of *yaiC* in the *pst* mutant restored the expression of type 1 fimbriae, we wondered whether adhesion of the *pst yaiC* double (DMY) mutant to bladder cells was dependent on type 1 fimbriae. To do so, adhesion to 5637 human bladder epithelial cells was tested in the presence or absence of 1.5% α-D-mannopyranose.

In the absence of mannopyranose, the WT strain adhered to the bladder cells at 26.8% (Fig. 3A). Similarly, the *pst* and the DMY strains adhered to bladder cells at 21.0 and 22.0%, respectively (Fig. 3A). As previously observed (10), the addition of mannopyranose to the culture medium decreased adherence of the WT strain significantly to 5.8%, whereas it had no significant effect on adhesion of the Δ*pst* mutant strain, which adhered at 17.4% (Fig. 3A). As expected, the deletion of *yaiC* in the Δ*pst* background restored the type 1 fimbria-dependent adherence to bladder cells, since the presence of mannopyranose decreased the adherence of the DMY strain to 10.7%, which is similar to what is observed in the WT strain (Fig. 3A). Thereby, these results confirm that *yaiC* is involved in the repression of type 1 fimbriae.

Deletion of *yaiC* restored the colonization of the urinary tract in the Pst mutant. Since YaiC represses the expression of type 1 fimbriae and these fimbriae are

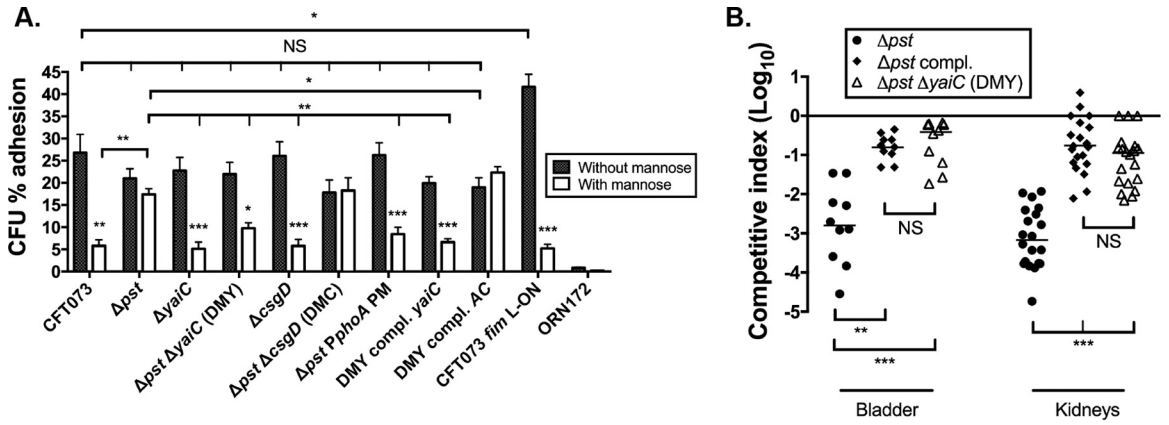

FIG 3 Adhesion and virulence of *yaiC* derivative strains. (A) Adherence of strain CFT073 and its derivatives to human 5637 bladder epithelial cells in the presence and absence of 1.5% mannopyranose (mannose). The CFT073 *fim*-locked-on strain and the *E. coli* K-12 *fim*-negative strain ORN172 were used as positive and negative controls, respectively. All results shown are the mean values and standard deviations of the results from four biological experiments. Statistical significance was calculated by the Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$; NS, nonsignificant. (B) CBA/J mice were coinfected with a 1:1 ratio of CFT073 Δ*lac* and either the Δ*pst* mutant or the Δ*pst* Δ*yaiC* (DMY) mutant strain. Results are presented as the $\log_{10}$ CFU · g^{-1}. Each data point represents a sample from an individual mouse, and the horizontal bars indicate the medians. Each kidney was sampled separately. A Wilcoxon signed-rank test (two-tailed) was used to determine statistical significance; **, $P < 0.005$; ***, $P < 0.0001$; NS, nonsignificant.

important for infection, we tested whether inactivation of *yaiC* in the *pst* mutant restores its urinary tract colonization defect. To do so, CBA/J mice were coinfected with a 1:1 ratio of CFT073 Δ*lac* and the Δ*pst* mutant or CFT073 Δ*lac* and the DMY mutant strain. As expected, and as we previously observed (10), at 48 h postinfection (p.i.), the *pst* mutant was outcompeted 618- and 1,323-fold in the bladder and kidneys, respectively (Fig. 3B). On the other hand, the DMY mutant was only outcompeted 5- and 13-fold in the bladder and kidneys, respectively (Fig. 3B). Although the DMY mutant strain did not colonize the urinary tract at the WT level, it had the same colonization profile as the Δ*pst* compl. mutant strain (Fig. 3B). Thereby, we can conclude that the deletion of *yaiC* significantly restores the virulence of the *pst* mutant.

In the *pst* mutant, expression of *yaiC* is independent of CsgD. The transcription of *yaiC* has been shown to be dependent on CsgD (39). As *csgD* is regulated by more than 10 transcription factors and several environmental conditions (36, 40, 41), we tested whether induction of the Pho regulon influenced its transcription and, subsequently, *yaiC*. To do so, qRT-PCR was performed on strains cultured to mid-log phase of growth at 37°C in LB. Interestingly, *csgD* was not differentially expressed between the WT, the Δ*pst* mutant, and Δ*pst* compl. mutant strains (data not shown). In order to eliminate any potential role of CsgD in *yaiC* regulation, a nonpolar mutation in *csgD* was introduced in the *pst* mutant, and the expression of *yaiC* was monitored by qRT-PCR. As shown in Fig. 2A, deletion of *csgD* in the Δ*pst* mutant did not affect *yaiC* transcription, as it was expressed at the same level as the single Δ*pst* mutant. Furthermore, we tested whether *csgD* had any effects on type 1 fimbrial expression. As shown in Fig. 1C to E, deletion of *csgD* in the *pst* mutant (DMC mutant) had no effect on *fimA*, *fimB*, or *fimE*, or on the orientation of the *fimS* switch, since their expression and orientation are similar to those of the single *pst* mutant. These results are also in agreement with the adhesion capacity of the DMC mutant to bladder epithelial cells. Indeed, this mutant adhered to bladder cells in a *fim*-independent manner, similarly to the single *pst* mutant (Fig. 3A). Taken together, these results suggest that under conditions in which the Pho regulon is induced, CsgD is not involved in the regulation of type 1 fimbriae. In addition, these results demonstrate that the transcription of *yaiC* in the *pst* mutant is independent of CsgD.

In the *pst* mutant, *yaiC* is transcribed as part of the *phoA-psiF* operon and is dependent on the *phoA* promoter. As the expression of *yaiC* is independent of CsgD in the *pst* mutant, and as *yaiC* does not possess a Pho box in its promoter region (5),

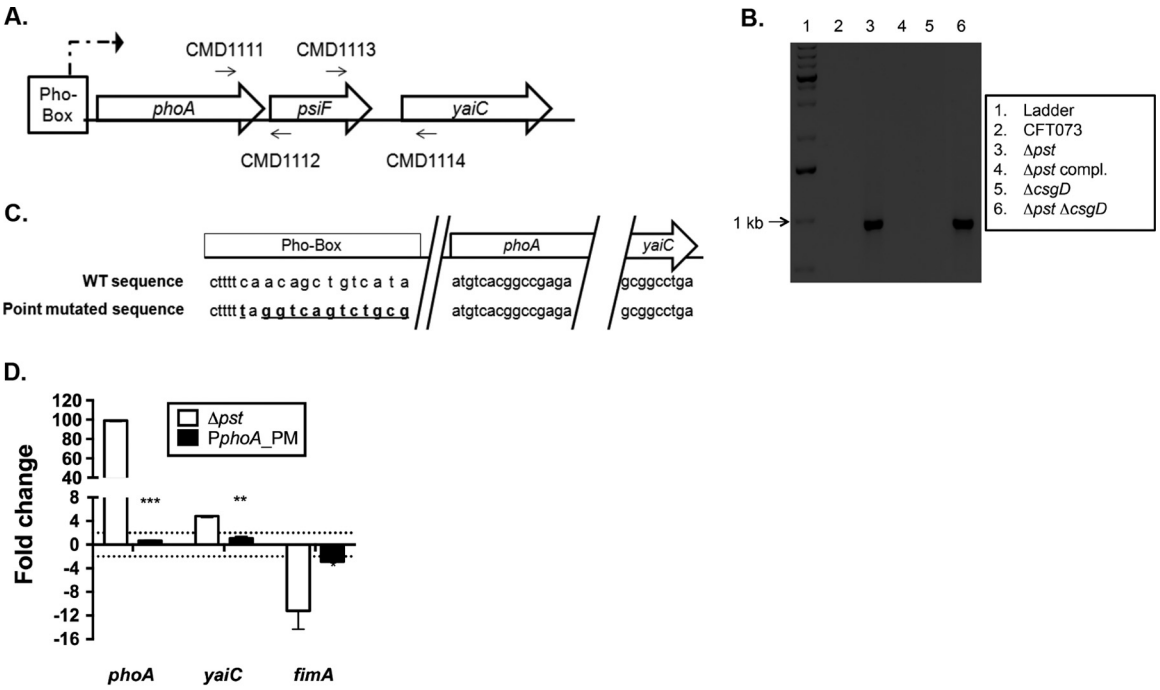

FIG 4 *yaiC* is transcribed as part of the *phoA-psiF* operon. (A) Schematic representation of how the RT-PCR experiments were performed. The *phoA-psiF* region was retrotranscribed and amplified with primers CMD1111 and CMD1112, the *psiF-yaiC* region with primers CMD1113 and CMD1114, and the *phoA-yaiC* region with primers CMD1111 and CMD1114. (B) Gel electrophoresis of RT-PCR products from amplification of the *phoA-psiF* region (primers CMD1111 and CMD1114). (C) Schematic representation of the WT and mutated promoter (Pho box) of *phoA*. The mutated nucleotides are represented by the bold and underlined nucleotides. (D) Expression of *phoA*, *yaiC*, and *fimA* in the *pst* mutant and its derivative *PphoA*_PM mutant. The expression was compared to that of the WT strain. The dotted line corresponds to the cutoff of a significant difference in expression. The calculated C_T of *phoA*, *yaiC*, and *fimA* was normalized to the C_T of the *tus* gene amplified from the corresponding sample and compared with the WT strain. All results shown are the mean values and standard deviations of the results from four biological experiments. Statistical significance was calculated by the Student *t* test; *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0001$.

its induction in the *pst* mutant and under phosphate starvation conditions seems to be indirectly activated by the Pho regulon. We hypothesized that in the *pst* mutant, *yaiC* is cotranscribed as part of the *phoA-psiF* operon (Fig. 1A). We reasoned this since *phoA* is positively regulated by PhoB and is strongly induced in a *pst* mutant (3, 37). Furthermore, an *in silico* analysis showed that the intergenic region between the *phoA-psiF* operon and *yaiC* is only 100 nucleotides. To validate this hypothesis, reverse transcription-PCR (RT-PCR) was performed on the *phoA-psiF* and *yaiC* regions from strains cultured to mid-log phase of growth in LB (Fig. 4A and B). The *phoA-psiF-yaiC* region was reverse transcribed with primers CMD1111 and CMD1114. As expected, no band was observed for the WT, Δ*pst* compl. mutant, or Δ*csgD* mutant strains, since the Pho regulon was not induced in these strains under these conditions. However, a 1-kb band, corresponding to the expected product size, was observed for the Δ*pst* and DMC (Δ*pst* Δ*csgD*) mutant strains (Fig. 4B), supporting the hypothesis that *yaiC* is cotranscribed with *phoA-psiF*.

As *yaiC* was cotranscribed with *phoA-psiF* in the *pst* mutant, we tested whether its transcription was dependent on the promoter of *phoA*, which is positively regulated by PhoB. In order to disrupt the expression of *phoA-psiF* and therefore *yaiC*, we chromosomally changed 13 nucleotides (see Materials and Methods) (Fig. 4C) in the Pho box of *phoA* in the *pst* mutant (P*phoA*_PM strain). To confirm that the introduced point mutations inhibit *phoA* expression, qRT-PCR was performed on RNA sampled from the WT, the Δ*pst* mutant, and the P*phoA*_PM mutant strains cultured to mid-log phase of growth in LB. In the *pst* mutant, the transcription of *phoA* was induced 99.0-fold (Fig. 4D). On the other hand, the expression of *phoA* was abolished in the P*phoA*_PM mutant strain, as *phoA* was not differentially expressed compared to the WT strain. Similarly, the expression of *yaiC* was inhibited in the P*phoA*_PM mutant strain, whereas it is induced

4.80-fold in the *pst* single mutant (Fig. 4D). These results strongly suggest that *yaiC* forms an operon with *phoA-psiF* and that its transcription is dependent on PhoB.

In addition, the P*phoA*_PM mutant strain also demonstrated a regain in the expression of type 1 fimbriae. Compared to the WT parent strain, *fimA* expression is decreased 2.9-fold (Fig. 4D), which is similar to what was observed in the DMY (Δ*pst* Δ*yaiC*) mutant strain, where the expression of type 1 fimbriae was decreased 4.8-fold (Fig. 1C). Furthermore, adhesion of the P*phoA*_PM mutant to bladder cells is type 1 fimbria dependent (Fig. 3A). Indeed, adherence of the P*phoA*_PM mutant strain dropped from 25% to 12.0% in the presence of mannopyranose, which was similar to the type 1 fimbria-dependent adherence of the DMY mutant, which is decreased from 24% to 12.2% upon the addition of mannopyranose (Fig. 3A).

To further demonstrate the role of P*phoA*-dependent induction of *yaiC* on expression of type 1 fimbriae in the *pst* mutant, we introduced either the *yaiC* gene alone, with its proximal *csgD*-dependent promoter, or the *phoA-psiF-yaiC* region, including the P*phoA* promoter and its Pho box, to the *att*Tn*7* site of the DMY mutant strain. As the expression of *yaiC* in the *pst* mutant depends on the *phoA* promoter, the introduction of *yaiC* alone, from its native promoter, should behave similarly to the DMY (Δ*pst* Δ*yaiC*) mutant strain. On the other hand, the introduction of *phoA-psiF-yaiC* in the DMY mutant should resemble the single *pst* mutant phenotype. To test this hypothesis, the expression of *fimA* and adhesion properties of these two complemented strains were determined. As expected, the introduction of *yaiC* in the DMY mutant strain (DMY compl. *yaiC*) did not affect the expression of *fimA*, since no difference was observed with the DMY mutant strain (Fig. 1C). However, when the *phoA-psiF-yaiC* region was introduced in the DMY mutant strain (DMY compl. *AC* mutant), the expression of *fimA* was decreased by 13.5-fold, which is similar to the level in the Δ*pst* single mutant (Fig. 1C). The adhesion properties of these two complemented strains to bladder epithelial cells were also distinct. Indeed, the DMY compl. *yaiC* mutant strain demonstrated type 1 fimbria-dependent adhesion, whereas the DMY compl. *AC* mutant strain demonstrated type 1 fimbria-dependent adhesion to bladder epithelial cells (Fig. 3A). Thus, adhesion of the DMY compl. *AC* mutant strain was similar to the adhesion of the single *pst* mutant.

Taken together, these results demonstrate that under conditions in which the Pho regulon is induced, *yaiC* is cotranscribed as part of an extended transcriptional unit, which includes *phoA-psiF* (the *phoA-psiF-yaiC* operon). Thus, in a *pst* mutant, *yaiC* expression is under the control of the *phoA* promoter and is consequently positively regulated by PhoB.

c-di-GMP influences the expression of type 1 fimbriae. It has been observed that elevated concentrations of c-di-GMP decrease adhesion and invasion and lower the expression of type 1 fimbriae in the adherent-invasive *E. coli* strain LF82 (42). Since the *yaiC* gene is involved in biosynthesis of the second messenger c-di-GMP (30, 32) and is induced in the *pst* mutant, we asked whether the *pst* mutant produces higher levels of c-di-GMP than the WT strain. To test this hypothesis, we quantified the production of c-di-GMP in different strain backgrounds. As shown in Fig. 5A, the *pst* mutant produced 91.2 ng · ml^{-1} c-di-GMP, whereas the WT strain produced 65.4 ng · ml^{-1}. Although the difference was not statistically significant, the *pst* mutant produced 1.4-fold more c-di-GMP than the WT strain, corresponding to an increase of 25.8 ng · ml^{-1} of c-di-GMP.

To confirm that c-di-GMP influences the expression of type 1 fimbriae, the *ydaM* (GGDEF) and *yhjH* (EAL) genes were cloned into the inducible pTRC99a vector. Thereby, the production of c-di-GMP by YdaM should decrease the expression of type 1 fimbriae in the WT strain similarly to what is observed in the *pst* mutant, where the induction of *yaiC* increased c-di-GMP concentration. On the other hand, the induction of *yhjH* in the *pst* mutant will degrade the c-di-GMP and should restore the production of type 1 fimbriae similarly to the WT strain. These plasmid-carried genes were induced in the WT and the Δ*pst*, Δ*yaiC*, and DMY mutants, and the production of type 1 fimbriae was

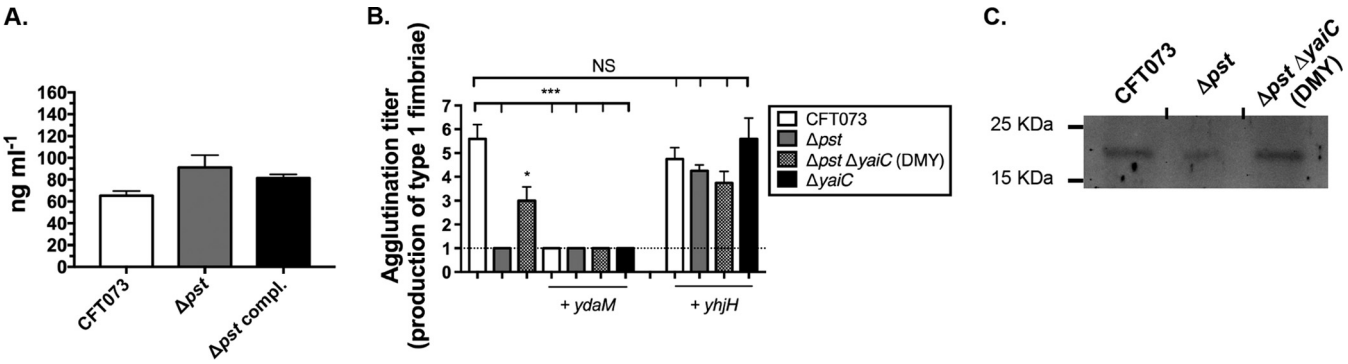

FIG 5 The c-di-GMP pathway influences the expression of type 1 fimbriae. (A) Production of c-di-GMP in the WT, Δ*pst* mutant, and the Δ*pst* compl. mutant strains. (B) Production of type 1 fimbriae by yeast agglutination assay in strains producing *ydaM*, from pTRC::*ydaM*, or *yhjH*, Each well corresponds to a 2-fold dilution. For example, an agglutination titer of 2 corresponds to a 2-fold dilution, while a titer of 4 corresponds to 8-fold dilution. The dotted line represents the limit of detection that corresponds to the nondiluted sample. (C) Western blot of fimbrial extracts using an anti-FimA serum. All results shown are the mean values and standard deviations of the results from three biological experiments. Statistical significance was calculated by the Student *t* test; *, $P < 0.05$; ***, $P < 0.0001$; NS, nonsignificant.

quantified by a yeast agglutination assay. Thereby, the expression of *ydaM* in either the WT strain or DMY mutant inhibited the expression of type 1 fimbriae to levels similar to those in the single *pst* mutant (Fig. 5B). As the *pst* mutant did not agglutinate yeast, it is not surprising to note that the induction of *ydaM* had no effect. On the other hand, expression of the *yhjH* gene in the *pst* mutant restored the production of type 1 fimbriae to the WT level, while, not surprisingly, it had no effect in the WT and Δ*yaiC* and Δ*pst yaiC* mutant strains (Fig. 5B).

Since the accumulation of c-di-GMP inhibits the production of type 1 fimbriae, Western blot analysis was performed on the WT and the *pst* and DMY mutants using an anti-FimA serum. As shown in Fig. 5C, the accumulation of c-di-GMP, through inactivation of *pst*, inhibits the production of type 1 fimbriae, while the inactivation of *yaiC*, in the Δ*pst* mutant background, restored the production of type 1 fimbriae to the WT level.

Taken together, these results show that although the increase in c-di-GMP concentration in the *pst* mutant was not statistically different from that in the WT strain, increased biosynthesis of c-di-GMP through the activation of *yaiC* in the *pst* mutant might explain the diminished expression of type 1 fimbriae which results in a decreased capacity to colonize the urinary tract.

DISCUSSION

Inactivation of the Pst system not only constitutively activates the Pho regulon, it also attenuates virulence of pathogenic strains (7, 8). Likewise, in extraintestinal pathogenic *E. coli* from swine or poultry, attenuation seems to be mainly attributed to alterations in membrane integrity (37, 43–45). In enteropathogenic *E. coli*, inactivation of the Pst system impairs adhesion to epithelial cells (46, 47). In *Vibrio cholerae*, the induction of PhoB represses virulence gene expression and induces genes involved in c-di-GMP metabolism (48, 49). In UPEC, we showed that inactivation of Pst attenuates virulence by decreasing the expression of type 1 fimbriae (10). In the current study, we investigated the molecular mechanisms by which activation of the Pho regulon represses the expression of type 1 fimbriae. Herein, we focused on the role of the *yaiC*-mediated pathway in reducing the expression of type 1 fimbriae.

In the *pst* mutant, we observed a decrease in the expression of type 1 fimbriae, which is linked to the upregulation of the GGDEF domain-containing gene product of *yaiC*. By knocking out *yaiC* in the *pst* mutant, the expression of type 1 fimbriae was restored. The regain in the expression of type 1 fimbriae in the DMY mutant is demonstrated by the increased type 1 fimbria-dependent adhesion to bladder cells, which was similar to the WT strain. The loss of *yaiC* in the *pst* mutant also resulted in a regain in virulence in the UTI mouse model (Fig. 3B). Although the DMY mutant is

somewhat outcompeted by the WT strain in the bladder and kidneys, it behaves similarly to the Δ*pst* compl. mutant strain. In the *pst* mutant, we previously demonstrated that the repression of type 1 fimbriae is linked to the downregulation of *fimB*, *ipuA*, and *ipbA* and to the upregulation of *fimE*. This differential expression promotes inversion of the *fim* promoter to the off position (10). As *fimB* and *fimE* were not differentially expressed in the DMY mutant strain, the restoration of type 1 fimbrial expression could solely be attributed to the upregulation of *ipuA* and *ipbA* (Fig. 1F and G), since these two genes are sufficient for switching the *fim* promoter and influencing type 1 fimbrial expression (23–25). Since *ipuA* and/or *ipbA* are not present in all UPEC or other *E. coli* strains, regulation of type 1 fimbriae through *pst*, or through other regulators, may vary among other strains. However, since *yaiC* is induced in the *pst* mutant and its deletion restores the expression of type 1 fimbriae, YaiC could be considered a repressor of these fimbriae.

As *yaiC* does not possess a Pho box in its promoter region and CsgD positively regulates it, we first hypothesized that the Pho regulon may affect *yaiC* through CsgD. However, we found that under conditions that activate the Pho regulon, the expression of *yaiC* was dependent on PhoB instead of CsgD. Indeed, the expression of *yaiC* was dependent on the Pho-regulated promoter of the *phoA-psiF* operon. Differential regulatory transcription from promoters that are active under distinct conditions has also been observed elsewhere. For instance, in *Borrelia burgdorferi*, *rpoS* possesses a short and a long transcript. The short transcript depends on RpoN, whereas the long transcript is dependent on a promoter found 1.5 kb upstream of the *rpoS* gene (50). The transcription of *rpoS* from this promoter is via a read-through and includes, in order, the *flgI*, *flgJ*, and *rpoS* genes. Furthermore, in *V. cholerae*, the *acgAB* operon is regulated via a read-through transcription from the upstream operon *alsDSO* (51).

As *yaiC* is involved in the biosynthesis of c-di-GMP, phosphate starvation seems to be an activating signal of c-di-GMP metabolism. Indeed, cultivation under phosphate-limiting conditions induced the expression of *yaiC*. This induction was also observed in the *pst* mutant when cultured in LB. These results are in agreement with what was observed in *V. cholerae*. Indeed, induction of the Pho regulon activates the transcription of *acgAB* encoding GGDEF and EAL proteins (49). The expression of *acgAB* is dependent on PhoB. In the UPEC CFT073 *pst* mutant, the induction of *yaiC* increased the intracellular concentration of c-di-GMP (Fig. 5A). Further, the role of c-di-GMP in regulation of type 1 fimbriae was confirmed by expressing the *ydaM* (GGDEF) or *yhjH* (EAL) gene in the WT strain and the *pst* mutant (Fig. 5B). In line with the role of c-di-GMP in the downregulation of type 1 fimbriae, their production was abolished by overexpressing *ydaM* (GGDEF) in the WT strain; conversely, type 1 fimbriae were restored in the *pst* mutant by overexpressing *yhjH* (EAL).

Many groups have reported that increased levels of c-di-GMP attenuate the virulence of pathogenic strains. For example, in *Yersinia pestis*, inactivation of the EAL gene *hmsP* increased the production of extracellular polysaccharide (EPS) and decreased virulence (52). In *V. cholerae*, elevated c-di-GMP levels reduced colonization of the mouse small intestine and decreased expression of the major virulence gene transcriptional activator *toxT* (53). Furthermore, the phosphodiesterase VieA is necessary for virulence in the mouse infant model and positively regulated the expression of *toxT* and the cholera toxin *ctxAB* (54). In *E. coli*, however, the role of c-di-GMP in virulence is not well studied. In the adherent-invasive *E. coli* strain LF82, which is associated with Crohn's disease, it has been observed that the c-di-GMP pathway decreased adhesion and invasion of intestinal epithelial cells by repressing the expression of type 1 fimbriae (42). Furthermore, in a clinical UPEC strain, it has been observed that the production of cellulose decreased adhesion to bladder cells and decreased kidney colonization (55). Accordingly, Raterman et al. (56) showed that deregulation of the YfiN diguanylate cyclase in UPEC strain CFT073 can induce the synthesis of curli and cellulose production, which decreased competitive colonization of the murine urinary tract. In our current report, we demonstrated, for the first time, that the regulation of type 1 fimbriae by the Pho regulon implicates the metabolism of c-di-GMP, and that the

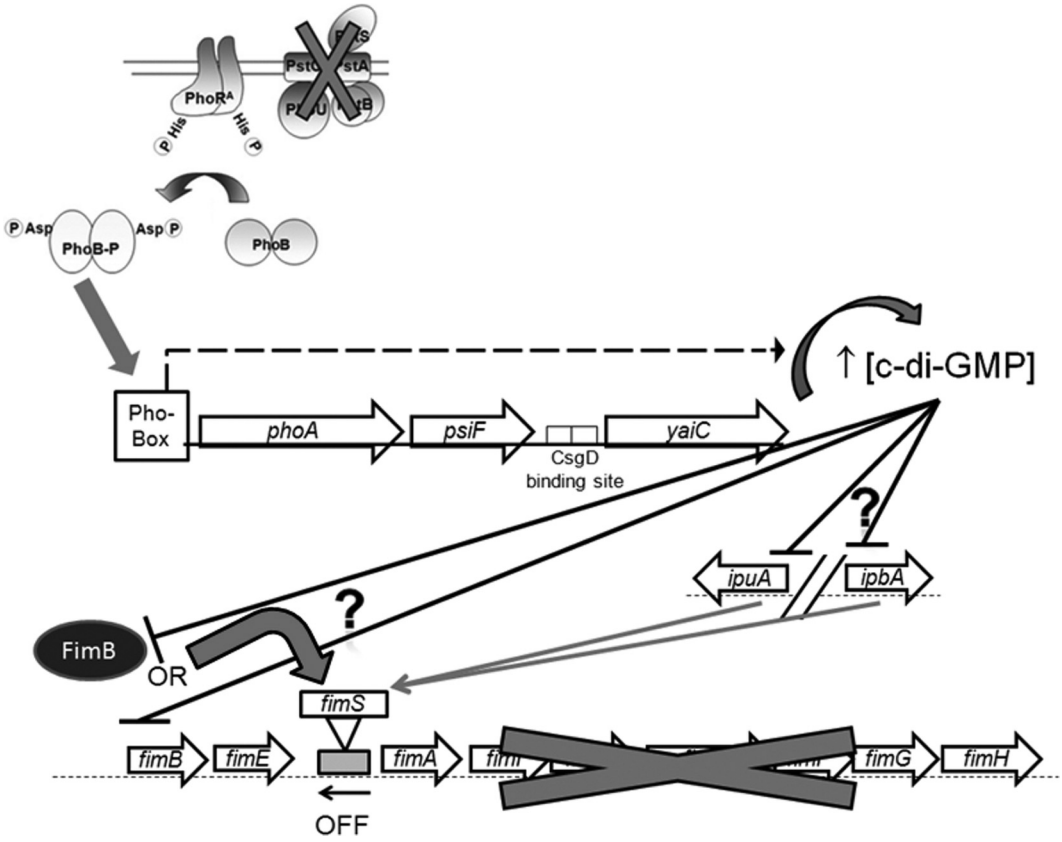

FIG 6 Model illustrating the interactions between the Pho regulon, *yaiC*, and type 1 fimbriae. Arrows represent an activation/promotion, while ⊥ represents an inhibition. The induction of PhoBR leads to transcription of *yaiC* from the promoter of *phoA*, which leads to the biosynthesis of c-di-GMP, repression of *ipuA* and *ipbA*, and then reduced expression of the *fim* operon by promoting the off orientation of *fimS*. It is not known (question mark on the right) whether c-di-GMP directly or indirectly inhibits the expression of *ipuA* and *ipbA*. Furthermore, c-di-GMP could inhibit the recombinase activity instead of its transcription. As for *ipuA* and *ipbA*, it is not known (question mark on the left) whether c-di-GMP directly or indirectly affects FimB or *fimB*.

repression of type 1 fimbriae through c-di-GMP production underlies one of the mechanisms leading to virulence attenuation of the *pst* mutant in the UTI mouse model (Fig. 3B) (10).

According to the results shown in this study, under conditions in which the Pho regulon is activated, PhoB binds to the Pho box promoter of *phoA*, leading to the transcription of the *phoA-psiF* operon. Transcription from this promoter extends through to *yaiC*. Increased expression of *yaiC* leads to an elevated intracellular concentration of c-di-GMP. The accumulation of c-di-GMP represses transcription of the *fimB*, *ipuA*, and *ipbA* Fim recombinase genes, leading to increased orientation of the *fim* promoter in the off position, repressed expression of type 1 fimbriae (Fig. 6), and consequently, decreased urinary tract colonization. As *yaiC* is transcribed from the *phoA* promoter and is positively regulated by PhoB, *yaiC* could be considered a new member of the Pho regulon and a regulator of type 1 fimbriae. It remains to be determined how *yaiC* (c-di-GMP gene) specifically influences expression of the recombinase genes *fimB*, *ipuA*, and *ipbA*, the *fim* promoter switch, and type 1 fimbriae (Fig. 6).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The *E. coli* strains and plasmids used in this study are listed in Table 1. Bacteria were grown in lysogeny broth (LB) at 37°C. Bacteria were also grown in MOPS minimal medium (Teknova) supplemented with 0.4% glucose, 0.2% $(NH_4)_2SO_4$, 1.32 mM K_2HPO_4, and 1 μg/ml thiamine (high phosphate). MOPS low-phosphate medium contained 1 μM K_2HPO_4 (57). 5637 human bladder cells (ATCC HTB-9) were grown in RPMI 1640 medium (Wisent Bioproducts) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/liter glucose,

TABLE 1 Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics[a]	Source or reference(s)
Strains		
CFT073	UPEC wild-type pyelonephritis strain	70, 71
MGN-617	*thi thr leu tonA lacY glnV supE* DΔ*asdA4 recA*::RP4 2-Tc::Mu [*pir*]; Km^r	72
ORN172	*thr-1 leuB thi-1* Δ*(argF-lac)U169 xyl-7 ara-13 mtl-2 gal-6 rpsL tonA2 supE44* Δ*(fimBEACDFGH)*::*kan pilG1*	73
CFT073 Δ*lac* (QT1081)	CFT073 Δ*lacZYA*::FRT	66
Δ*pst* mutant (QT1911)	CFT073 Δ*pstSCA*::FRT	10
Δ*pst* compl. mutant (QT2117)	QT1911::Tn*7*T-Gm::*pstSCA*; Gm^r	10
CFT073 Δ*fim* (QT2138)	CFT073 Δ*fimAICDFGH*::km; Km^r	10
CFT073 *fim* L-ON (QT2285)	CFT073 *fimS* phase locked-on; Cm^r	10
Δ*phoA* mutant (QT2334)	CFT073 Δ*phoA*::FRT	This study
Δ*psiF* mutant (QT2335)	CFT073 Δ*psiF*::FRT	This study
Δ*pst* Δ*phoA* mutant (QT2336)	QT1911 Δ*phoA*::FRT	This study
Δ*pst* Δ*psiF* mutant (QT2337)	QT1911 Δ*psiF*::FRT	This study
Δ*pst* Δ*yaiC* mutant (DMY; QT2065)	QT1911 Δ*yaiC*::FRT	This study
Δ*yaiC* mutant (QT2100)	CFT073 Δ*yaiC*::FRT	This study
DMY compl. *yaiC* mutant (QT2222)	QT1911::Tn*7*T-Gm::*yaiC*; Gm^r	This study
DMY compl. *AC* mutant (QT2244)	QT1911::Tn*7*T-Gm::*phoA-psiF-yaiC*; Gm^r	This study
P*phoA*_PM mutant (QT2729)	QT1911:: P*phoA*_PM (mutations in promoter of *phoA*	This study
DMC mutant (QT2099)	QT1911 Δ*csgD*::FRT	This study
Δ*csgD* mutant (QT2141)	CFT073 Δ*csgD*::FRT	This study
QT2209	MGN-617/pIJ297; Ap^r Gm^r	This study
QT2208	MGN-617/pIJ296; Ap^r Gm^r	This study
QT2087	MGN-617/pLOF/Km; Ap^r Km^r	This study
QT2893	CFT073/pTRC::*ydaM*; Ap^r	This study
Δ*pst* mutant/pTRC::*ydaM* (QT2895)	QT1911/pTRC::*ydaM*; Ap^r	This study
QT2894	CFT073/pTRC::*yhjH*; Ap^r	This study
Δ*pst* mutant/pTRC::*yhjH* (QT2896)	QT1911/pTRC::*yhjH*; Ap^r	This study
QT2059	CFT073/pIJ280; Ap^r	This study
QT2067	QT1911/pIJ280; Ap^r	This study
DMY Δ*ipuA* mutant (QT3015)	QT2065 Δ*ipuA*::Cm; Cm^r	This study
DMY Δ*ipbA* mutant (QT3016)	QT2065 Δ*ipbA*::Km; Km^r	This study
Δ4 mutant (QT3031)	QT3015 Δ*ipbA*::Km; Cm^r Km^r	This study
Plasmids		
pCP20	FLP helper plasmid Ts replicon; Ap^r Cm^r	60
pSTNSK	pST76-K::*tnsABCD*; Km^r	62
pKD13	Template plasmid for amplification of the km cassette bordered by FRT sites; Ap^r Km^r	60
pKD46	λ-Red recombinase plasmid Ts replicon; Ap^r	60
pGP-Tn7-Gm	pGP704::Tn*7*T-Gm; Ap^r Gm^r	62
pIJ280	pBAD24::*yaiC*; Ap^r	
pIJ297	pGP-Tn7-Gm::*yaiC*; Ap^r Gm^r	This study
pIJ296	pGP-Tn7-Gm::*phoA-psiF-yaiC* (*AC*); Ap^r Gm^r	This study
pGEM-T	TA cloning of PCR product; Ap^r	Promega
pSG76C	*ori*R6K suicide vector possessing an I-SceI cleavage site; Cm^r	64
pGEM::P*phoA*	pGEM-T:: P*phoA-phoA* (*phoA* with its promoter)	This study
pGEM::P*phoA*_PM	pGEM-T::P-*pho*APM-*phoA*; introduction of mutations (PM) into the promoter of *phoA*;	This study
pSG76C:: P*phoA*_PM	pSG76C::P*pho*APM-*phoA*	This study
pPIRK	Helper plasmid carrying the *pir* gene and the pSC101^{ts} origin; Km^r	64
pST76-ASceP	PSC101^{ts} origin suicide vector expression the I-SceI meganuclease; Ap^r	63
pLOF-Km	Tn*10*-based transposon vector delivery plasmid; Ap^r Km^r	74
pTRC::*ydaM*	pTRC99a::*ydaM*; Ap^r	This study
pTRC::*yhjH*	pTRC99a::*yhjH*; Ap^r	This study

[a]Km^r, kanamycin resistance; Gm^r, gentamicin resistance; Cm^r, chloramphenicol resistance; Ap^r, ampicillin resistance; Ts, temperature sensitive.

and 1.5 g/liter sodium bicarbonate. Antibiotics and reagents were added as required at the following concentrations: kanamycin, 40 μg/ml; ampicillin, 100 to 200 μg/ml; chloramphenicol, 30 μg/ml; gentamicin, 15 μg/ml; diaminopimelic acid (DAP), 50 μg/ml; 5-bromo-4-chloro-3 indolylphosphate disodium (BCIP), 40 μg/ml; and isopropyl-β-D-1-thiogalactopyranoside (IPTG), 500 μM.

Transposon mutagenesis. Transposon mutagenesis was performed as described by Simms and Mobley (58). Briefly, the MGN-617/pLOF-Km) donor strain and recipient strain CFT073 Δ*pst* were cultured overnight (O/N) at 37°C in LB with appropriate antibiotics and supplements. Cultures were gently mixed at a 1:4 donor-to-recipient ratio, placed onto LB agar plates supplemented with DAP and IPTG, and

incubated for 5 h at 37°C. Following incubation, the bacterial lawn was suspended in 1 ml of phosphate-buffered saline (PBS), washed twice in PBS, serially diluted, and spread onto LB agar plates supplemented with kanamycin and incubated O/N at 37°C to select the recovery of kanamycin-resistant transposon mutants of the CFT073 Δ*pst* recipient strain. To confirm the loss of the pLOF-Km vector, transconjugants were screened for their susceptibility to ampicillin (100 μg/ml).

Evaluation of type 1 fimbrial production. The production of type 1 fimbriae by transposon mutants was quantified by a yeast agglutination assay (10, 37). The transposon mutants were cultured in 96-well microtiter plates to mid-log phase of growth. Following centrifugation, the pellet was suspended in 40 μl of PBS and transferred to other microtiter wells containing equal volumes of a 3% commercial yeast suspension in PBS. After 30 min of incubation on ice, yeast aggregation was monitored visually, and the agglutination titer was recorded as the most diluted bacterial sample giving a positive agglutination reaction. To inhibit type 1 fimbria-dependent agglutination, a final concentration of 1.5% α-D-mannopyranose was added to the samples.

Preparation of fimbrial extracts and Western blotting. Preparation of fimbrial extracts and Western blotting were performed, as previously described (37), with anti-FimA serum from *E. coli* strain B_{AM}.

Site-specific integration of Tn*10*. Transposon site-specific integrations were identified as described by Nichols et al. (59) and as shown in Fig. 1. Chromosomal DNA isolated from the transposon mutants was digested with RsaI. The fragmented DNA was self-ligated with T4 DNA ligase (Fermentas), and a first round of inverted PCR, with primers Pri1 and Pri2 (see Table S1 in the supplemental material), was performed onto the circularized DNA. The PCR product was diluted 1:10, and 1 μl was used as the template for a second round of inverted PCR using Pri3 and Pri4. PCR products from this second round were gel electrophoresed, and fragments were sequenced (Génome Québec Innovation Centre, McGill University). BLASTN was used to identify the site-specific integration site in the genome of the CFT073 Δ*pst* strain.

Construction of nonpolar mutants and complemented strains. All mutants were generated by the procedure described by Datsenko and Wanner using plasmid pKD3 as the kanamycin resistance cassette (60). The primers used are listed in Table S1 in the supplemental material. Antibiotic cassettes flanked by FLIP recombination target (FRT) sequences were removed by transforming the mutant strains with pCP20 expressing the FLP recombinase (61).

The Δ*pstSCA* Δ*yaiC* (DMY) mutant strain was complemented as described by Crépin et al. (62) by inserting the *yaiC* gene or *phoA-psiF yaiC* genes with their respective promoters into the chromosomal *att*Tn*7* site, resulting in DMY compl. (*att*Tn*7*::*yaiC*) and DMY compl. *AC* (*att*Tn*7*::*phoA-psiF-yaiC*) mutant strains, respectively. Briefly, *yaiC* and its native promoter were amplified with primers CMD1156 and CMD1157 (Table S1) and cloned into the XmaI and XhoI sites of pGP-Tn7-Gm, creating vector pGP-Tn7-Gm-*yaiC*. The *phoA-psiF-yaiC* region, with the promoter of *phoA*, were amplified with primers CMD1260 and CMD1261 and cloned into the XmaI and XhoI sites of pGP-Tn7-Gm, creating vector pGP-Tn7-Gm-*AC*. The DMY mutant strain (pSTNSK) was conjugated overnight with strain MGN-617 (pGP-Tn7-Gm-*yaiC* or pGP-Tn7-Gm-*AC*) at 30°C on LB agar plates supplemented with DAP. After incubation, the bacterial lawn was suspended in 1 ml of PBS, washed twice in PBS, serially diluted, spread on LB agar supplemented with gentamicin, and incubated at 37°C. Colonies were verified for sensitivity to kanamycin and ampicillin, the expected phenotype for integration at *att*Tn*7*, and loss of the transposase-containing plasmid pSTNSK. Tn*7* insertion into the *att*Tn*7* site was verified by PCR using primers CMD1070 and CMD1072 (Table S1).

The introduction of point mutations into the promoter (Pho box) of *phoA* was performed as described by Pósfai et al. (63, 64). The *phoA* gene and its upstream promoter (Pho box) were amplified with primers CMD1245 and CMD1336 and were cloned in pGEM-T vector (Promega), creating vector pGEM::P*phoA*. The introduction of 13 point mutations into the Pho box of *phoA* was performed according to the QuikChange site-directed mutagenesis kit (Stratagene) with primers CMD1334 and CMD1335. The point mutations were confirmed by sequencing the vector with M13-specific primers. The resulting plasmid (pGEM::P*phoA*_PM) was digested with SacI and SphI and cloned into the respective sites of pSG76-C, creating vector pSG76C::P*phoA*_PM. Plasmid pSG76C::P*phoA*_PM was introduced in strain CFT073 Δ*pst*, containing the p-PIRK vector, and grown for 5 h at 30°C. This step allowed the replication of the pSG76C::P*phoA*_PM vector as the π protein is provided by p-PIRK. Following growth at 30°C, the bacterial suspension was spread on LB with chloramphenicol (LB-Cm) plates and grown 5 h at 42°C and O/N at 37°C. This step was done to inhibit the replication of pSG76C::P*phoA*_PM while selecting for integration by Cm selection, as the helper plasmid is unable to replicate at these temperatures. In this manner, pSG76C::P*phoA*_PM integrates by single-crossover recombination at the homologous site, i.e., the *phoA* gene and its promoter (Pho box) into the CFT073 Δ*pst* mutant strain. The replacement of the native pPhoA promoter with a modified promoter was achieved by introducing the pST76-ASceP vector in the resulting strain. The expression of SceP from pST76-ASceP can introduce a double strand break at the I-SceI homing endonuclease site on pSG76C::P*phoA*_PM, which can be avoided through homologous recombination between the mutant and the WT allele (64), generating the markerless exchange of the pPhoA promoter. Loss of pST76-ASceP temperature-sensitive plasmid is then achieved by growing the bacteria at 37°C or 42°C. The resulting strain was named P*phoA*_PM.

Experimental UTI in CBA/J mice. Experimental infections were carried out using coinfection models as described by Hagberg et al. (65) and Sabri et al. (66). Prior to inoculation, strains were grown for 16 h at 37°C with shaking (250 rpm) in 55 ml of LB medium. Cultures were then centrifuged, and pellets of the WT and derivative strains were mixed 1:1. Six-week-old CBA/J female mice were transurethrally inoculated with 20 μl of the 1:1 mixture containing 5×10^8 CFU of UPEC CFT073 Δ*lacZYA* strain and 5 ×

10^8 CFU of either CFT073 Δ*pstSCA* strain or CFT073 Δ*pstSCA* Δ*yaiC* (DMY). The CFT073 Δ*lac* strain is as virulent as the CFT073 wild-type parent and presented no statistical differences from the WT strain (66). Furthermore, the Δ*lac* strain provided a differential Lac-negative phenotype on MacConkey agar plates. At 48 h p.i., the mice were euthanized; bladders and kidneys were aseptically removed, homogenized, diluted, and plated onto MacConkey agar to determine bacterial counts.

RNA extraction and quantification of gene expression. RNAs from bacterial cultures grown to mid-log phase in LB or MOPS minimal medium to mid-log phase were extracted using TRIzol reagent (Invitrogen), according to the manufacturer's recommendations, with the exception that DNase I treatment was performed twice. The iScript cDNA synthesis kit and the SsoFast Evagreen Supermix kit (Bio-Rad) were used for qRT-PCR, according to the manufacturer's instructions. The *tus* gene was used as a housekeeping control (37). Each qRT-PCR run was done in quadruplicate, and for each reaction, the calculated threshold cycle (C_T) was normalized to the C_T of the *tus* gene amplified from the corresponding sample. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (67). Genes with a fold change above or below the defined threshold of 2 were considered to be differentially expressed.

Quantification of the on/off state of the *fimS* region. Quantification of the orientation of the *fimS* switch was performed by quantitative PCR (qPCR) with iQ SYBR Green Supermix (Bio-Rad), according to Crépin et al. (10). The qPCR was performed on 10 ng of genomic DNA (gDNA) extracted from bacteria grown to mid-log phase of growth in LB. Primers CMD1246 and CMD1248 were used to amplify the on orientation, while the CMD1247 and CMD1248 primers amplified the off orientation. The threshold cycle (C_T) of the on and off orientations was normalized to the C_T of the *vat* gene (amplified with primers CMD96 and CMD97), an uninvertible element. The fold change was calculated using the $2^{-\Delta\Delta CT}$ method (67). A fold change above or below the defined threshold of 2 was considered to be differentially oriented.

Adhesion assay. The human bladder epithelial cell line 5637 (ATCC HTB-9) was grown to confluence in 24-well plates in RPMI 1640. UPEC CFT073 and its derivative strains were grown in LB medium at 37°C to mid-log phase of growth (optical density [OD], 0.6). The bacterial cells were centrifuged, washed twice with PBS, resuspended at a 10^6 CFU · ml^{-1} suspension in RPMI 1640 medium (Wisent Bio Products, St-Bruno, Canada), supplemented with 10% fetal bovine serum, and added to each well. Bacterium-host cell contact was enhanced by a 5-min centrifugation at 600 × *g*. At 2 h postadhesion, cells were washed three times and lysed with PBS–0.1% sodium deoxycholate (DOC), serially diluted, and plated onto LB agar plates. Quantification of cell-associated bacteria was performed as previously described (16, 68). To block adherence mediated by type 1 fimbriae, 1.5% α-D-mannopyranose was added to the culture medium.

Determination of intracellular c-di-GMP levels. c-di-GMP was quantified as described by Waters et al. (69). Bacteria were grown in LB medium to mid-log phase of growth. After cell lysis, supernatants were collected and analyzed by liquid chromatography-tandem mass spectrometry on a Finnigan TSQ Quantum Discovery MAX on a Quattro-Micro triple quadrupole mass spectrometer (Waters Corporation, Milford, MA), coupled with an Alliance high-performance liquid chromatography (HPLC) system (Waters Corporation). The MassLynx software from Waters was used for instrument control, data acquisition, and data processing. c-di-GMP was detected by using selected reaction monitoring (SRM) in negative ionization mode at *m/z* 689.0/150.1. SRM was used for simultaneous tracking of *m/z* 6,893,344 at 32 eV and *m/z* 6,893,150 at 45 eV, which gave a signal ratio of 1:0.4. The mass spectrometry parameters were as follows: cone energy, 45 eV; collision energy, 42 eV; capillary, 3,300 V; desolvation gas, 500 liters/h; and cone gas, 0 liters/h. The temperature source was 120°C and the temperature desolvation was 450°C. The interscan delay was 0.01 s. Purified c-di-GMP (Biolog, Germany) was used to generate the standard curve. Data are given as the c-di-GMP concentration in nanograms per milliliter per unit of optical density at 600 nm and are the means of the results from three independent experiments. Each sample was quantified in duplicate and showed less than 10% variation between duplicates.

Expression of GGDEF and EAL genes from an inducible promoter. The *yaiC* gene was amplified with primers CMD1099 and CMD1100. The amplified product was then cloned into pBAD24 at the XbaI and HindIII sites, creating plasmid pIJ280. The induction of *yaiC* from the pBAD promoter was induced with 0.05% L-arabinose. The gene *ydaM* (GGDEF) was amplified with primers CMD1461 and CMD1462 and *yhjH* (EAL) with primers CMD1463 and CMD1464. The respective genes were cloned into the NcoI and PstI sites of pTRC99a (Pharmacia Biotech). Gene expression was induced by adding 50 μM IPTG to the culture medium.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00168-17.

SUPPLEMENTAL FILE 1, PDF file, 0.1 MB.

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