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MEETING PRESENTATION

NtrC Adds a New Node to the Complex Regulatory Network of Biofilm Formation and *vps* Expression in *Vibrio cholerae*

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ABSTRACT The biofilm growth mode is important in both the intestinal and environmental phases of the *Vibrio cholerae* life cycle. Regulation of biofilm formation involves several transcriptional regulators and alternative sigma factors. One such factor is the alternative sigma factor RpoN, which positively regulates biofilm formation. RpoN requires bacterial enhancer-binding proteins (bEBPs) to initiate transcription. The *V. cholerae* genome encodes seven bEBPs (LuxO, VC1522, VC1926 [DctD-1], FlrC, NtrC, VCA0142 [DctD-2], and PgtA) that belong to the NtrC family of response regulators (RRs) of two-component regulatory systems. The contribution of these regulators to biofilm formation is not well understood. In this study, we analyzed biofilm formation and the regulation of *vpsL* expression by RpoN activators. Mutants lacking NtrC had increased biofilm formation and *vpsL* expression. NtrC negatively regulates the expression of core regulators of biofilm formation (*vpsR*, *vpsT*, and *hapR*). NtrC from *V. cholerae* supported growth and activated *glnA* expression when nitrogen availability was limited. However, the repressive activity of NtrC toward *vpsL* expression was not affected by the nitrogen sources present. This study unveils the role of NtrC as a regulator of *vps* expression and biofilm formation in *V. cholerae*.

IMPORTANCE Biofilms play an important role in the *Vibrio cholerae* life cycle, contributing to both environmental survival and transmission to a human host. Identifying key regulators of *V. cholerae* biofilm formation is necessary to fully understand how this important growth mode is modulated in response to various signals encountered in the environment and the host. In this study, we characterized the role of RRs that function as coactivators of RpoN in regulating biofilm formation and identified new components in the *V. cholerae* biofilm regulatory circuitry.

KEYWORDS *Vibrio cholerae*, VPS, NtrC, biofilm

Vibrio cholerae, the causative agent of the severe diarrheal disease cholera, can inhabit freshwater, estuaries, and human intestines. In its natural aquatic environment, *V. cholerae* can be found either as free-swimming planktonic cells or as biofilm-associated cells attached to surfaces (1). The ability of *V. cholerae* to form biofilms is critical for its survival in its natural habitats and transmission to the human host. The production of mature biofilms by *V. cholerae* requires extracellular matrix components. A major component of the *V. cholerae* biofilm matrix is the exopolysaccharide (EPS) *Vibrio* polysaccharide (VPS), which is required for the formation of three-dimensional biofilm structures (2, 3).

The regulatory network that controls biofilm formation is complex and involves several transcriptional regulators and alternative sigma factors. The primary components of this network consist of two positive transcriptional regulators, VpsT and VpsR, and three negative transcriptional regulators, HapR, cyclic AMP (cAMP) receptor protein (CRP), and histone-like nucleoid structuring protein (H-NS) (4–10). In addition to the main biofilm regulators, VpsR and VpsT, a number of other response regulators (RRs)

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have been identified to impact *V. cholerae* biofilm formation, including the positive regulators LuxO and VxrB and the negative regulators PhoB, CarR, VarA, and VieA (4, 5, 11–18). These RRs may affect biofilm formation through interactions with other key regulators or through the direct regulation of biofilm genes. The alternative sigma factors RpoS and RpoN also feed into the *V. cholerae* biofilm regulatory circuitry (6).

RpoN positively regulates biofilm formation and *vpsL* gene expression in *V. cholerae* (6). RpoN-dependent gene expression requires an activator, as RpoN is unable to initiate transcription by itself. Such regulators are classified as bacterial enhancer-binding proteins (bEBPs) (19). One group of bEBPs belongs to the NtrC family of RRs, named after its best-characterized representative, nitrogen regulatory protein C (NtrC) from *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (20–22). The contribution of the NtrC family regulators to the RpoN-mediated biofilm phenotype is not well characterized. NtrC family RRs have an N-terminal receiver (REC) domain, a central AAA^+ ATPase domain, an RpoN-binding domain, and a C-terminal DNA-binding domain. The *V. cholerae* genome is predicted to have seven genes encoding NtrC family RRs VC1021 (*luxO*), VC1522, VC1926 (*dctD*-1), VC2135 (*flrC*), VC2749 (*ntrC*), VCA0142 (*dctD*-2), and VCA0704 (*pgtA*) (see http://www.ncbi.nlm.nih.gov/Complete_Genomes/RRcensus.html and http://www.p2cs.org/).

In this study, we determined the regulatory role of the NtrC family RRs in biofilm formation and in the expression of *vps* genes. Consistent with data from previous studies, we found that LuxO positively regulates biofilm formation and that FlrC negatively regulates biofilm formation (23, 24). Additionally, we determined that NtrC negatively regulates *vpsL* gene expression and biofilm formation. NtrC from *V. cholerae* supports growth in poor nitrogen sources and is necessary to activate the expression of *glnA*, a gene that encodes glutamine synthetase, which is induced in response to nitrogen limitation. The expression of *vpsL* is downregulated by NtrC regardless of the nitrogen source available. Together, these results underscore the importance and complexity of the NtrC-dependent regulation of biofilm formation in *V. cholerae*.

RESULTS

NtrC family response regulators modulate biofilm formation and *vpsL* expression in *Vibrio cholerae*. We have previously shown that RpoN is a positive regulator of biofilm formation in *V. cholerae* (6). As RpoN requires bEBPs to activate transcription, we sought to understand the contribution of bEBPs to the RpoN-mediated regulation of biofilm formation. For this study, we focused on NtrC family RRs. To determine if the seven genes predicted to encode NtrC RRs in *V. cholerae* contain a conserved phosphorylation site and the GAFTGA motif necessary to interact with RpoN, we performed a multiple-sequence alignment (see Fig. S1 in the supplemental material). All these proteins have an aspartate residue that aligns with aspartate 56 from FlrC. This amino acid has been shown to be important for FlrC activity and is predicted to be the phosphorylation site (25). Furthermore, all proteins except for PgtA (VCA0704) had a conserved GAFTGA motif, which is required for the RpoN-dependent activation of target genes; for this reason, we excluded PgtA from further analysis. To evaluate the contribution of NtrC family regulators to the RpoN-mediated biofilm phenotype, we analyzed the biofilm formation capacities of strains lacking RpoN or the NtrC family RRs. After 48 h of incubation, the Δ*rpoN* and Δ*luxO* strains formed significantly less biofilm and lacked complete three-dimensional biofilm structures (Fig. 1A). COMSTAT analysis showed that the Δ*rpoN* and Δ*luxO* strains had ~65% less biomass and ~70% less average thickness than the wild type (WT) (Table 1). Other studies have shown that *rpoN*- and *luxO*-null mutants are deficient in biofilm formation; our results corroborated those data (6, 24, 26). Furthermore, we observed that the Δ*ntrC* and Δ*flrC* strains made thicker biofilms than the wild type (Fig. 1A). COMSTAT analysis showed that the Δ*ntrC* and Δ*flrC* strains made 32% and 27% more biomass, respectively, than the wild type and had 30% and 52% increases in average thickness, respectively, compared to the wild type (Table 1).

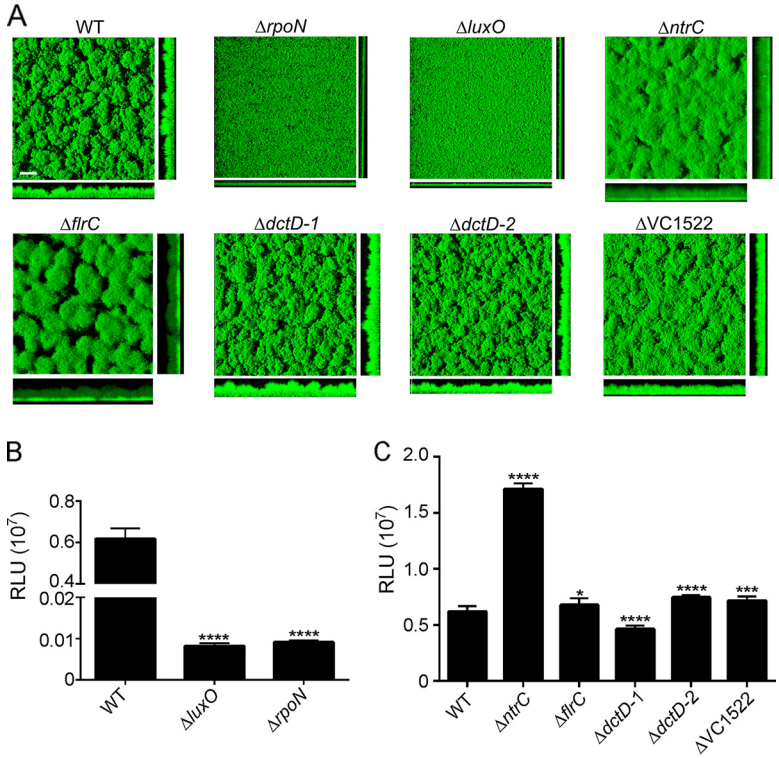

FIG 1 Analysis of biofilm formation and *vpsL* expression in NtrC family RR deletion mutants. (A) Three-dimensional biofilm structures of *V. cholerae* wild-type (WT), Δ*rpoN*, Δ*luxO*, Δ*ntrC*, Δ*flrC*, Δ*dctD*-1, Δ*dctD*-2, and ΔVC1522 strains after 48 h of incubation in flow cell chambers. Images of horizontal (*xy*) and vertical (*xz*) projections of biofilms are shown. The results shown are from one representative experiment of three independent experiments. Bar = 30 μm. (B) Expression of P*vpsL-luxCADBE* (pFY_0950) in the WT, Δ*luxO*, and Δ*rpoN* strains The graph represents the averages and standard deviations of relative light units (RLU) obtained from four technical replicates from two independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. One-way analysis of variance (ANOVA) followed by Dunnett's multiple-comparison test was used to compare expression levels between the WT and deletion mutants. ****, $P < 0.0001$. (C) Expression of P*vpsL-luxCADBE* (pFY_0950) in the WT, Δ*ntrC*, Δ*flrC*, Δ*dctD*-1, Δ*dctD*-2, and ΔVC1522 strains. The graph represents the averages and standard deviations of RLU obtained from four technical replicates from two independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. One-way ANOVA followed by Dunnett's multiple-comparison test was used to compare expression levels between the WT and deletion mutants. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$.

The most abundant component of the *V. cholerae* biofilm matrix is the exopolysaccharide VPS. The biosynthesis of VPS is encoded in two operons, *vps*-I and *vps*-II (2, 4). In addition to biofilm formation, we also analyzed the expression of *vps* genes using the P*vpsL-lux* transcriptional reporter, where the promoter of the *vps*-II operon (P*vpsL*) is cloned upstream of the luciferase reporter encoded by *luxCADBE* in plasmid pBBRlux (pFY-0950). We then analyzed *vpsL* transcription in the wild type and mutants lacking

TABLE 1 COMSTAT analysis of biofilms of NtrC family ΔRR strains at 48 h[a]

Strain	Mean biomass (μm^3) (SD)	Mean avg thickness (μm) (SD)	Mean maximum thickness (μm) (SD)
WT	17.1 (2.6)	19.0 (2.6)	22.1 (2.4)
Δ*rpoN* strain	6.4 (0.5)	5.8 (0.6)	7.2 (0.9)
Δ*luxO* strain	6.0 (0.2)	5.4 (0.6)	5.7 (0.6)
Δ*ntrC* strain	22.6 (0.6)	25.3 (1.3)	26.7 (2.5)
Δ*flrC* strain	21.8 (0.6)	29.0 (0.7)	31.0 (1.9)
Δ*dctD*-1 strain	13.5 (4.0)	16.0 (0.8)	23.9 (1.4)
Δ*dctD*-2 strain	13.9 (1.3)	17.0 (0.8)	22.7 (5.2)
ΔVC1522 strain	16.8 (1.9)	19.5 (0.2)	19.5 (0.2)

[a]The values are the means of data from six z-series image stacks.

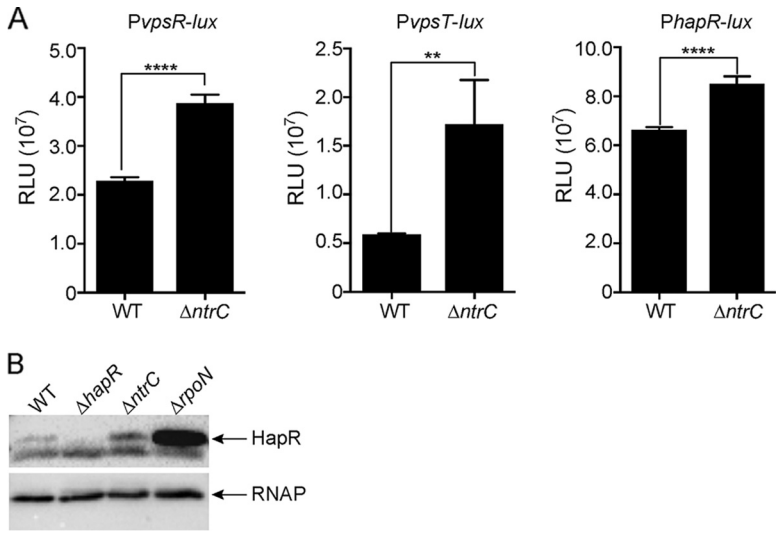

FIG 2 NtrC negatively regulates *vpsR*, *vpsT*, and *hapR* expression. (A) Expression of P*vpsR-luxCADBE*, P*vpsT-luxCADBE*, and P*hapR-luxCADBE* in the WT and Δ*ntrC* strains at the exponential growth phase. The graph represents the averages and standard deviations of RLU obtained from three technical replicates from two independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. A two-tailed unpaired *t* test with Welch's correction was used to compare expression levels between the WT and deletion mutants. **, $P < 0.01$; ****, $P < 0.0001$. (B) Western blot analysis of HapR production in the WT and Δ*ntrC* strains during the exponential growth phase (OD_{600} of 0.3). Equal amounts of total protein (determined by a BCA assay) were loaded onto an SDS–12% polyacrylamide gel. The abundance of RNA polymerase (RNAP) was used as a loading control.

RpoN or the NtrC family RRs. As previously reported, we found that *vpsL* transcription was abrogated in the Δ*rpoN* (6) and Δ*luxO* (27) strains (Fig. 1B). In agreement with its negative effect on biofilm formation, we observed that the absence of NtrC promotes a 3-fold upregulation of the expression of *vpsL* compared to the expression of this gene in the wild type. On the other hand, even though the absence of *flrC* showed a significant increase in biofilm formation, the absence of this regulator had only a modest effect on *vpsL* expression (~10% increase) (Fig. 1C). The expression of *vpsL* was slightly reduced in the Δ*dctD*-1 strain (~30% decrease) and modestly increased in the Δ*dctD*-2 (~21% increase) and ΔVC1522 (~16% increase) strains; however, none of these mutants showed an altered biofilm phenotype.

Collectively, these findings showed that LuxO is the only NtrC family RR that positively regulates both biofilm formation and *vpsL* expression; NtrC and FlrC negatively regulate biofilm formation, but NtrC has a much stronger effect on the downregulation of *vpsL* expression. We focused the rest of this study on the characterization of NtrC as a regulator of *vps* expression and biofilm formation in *V. cholerae*.

NtrC downregulates the core regulators of biofilm formation. VpsR and VpsT are positive transcriptional regulators of *vps* genes, while HapR negatively regulates *vps* genes. To determine if NtrC regulates *vpsR*, *vpsT*, and *hapR*, we used transcriptional fusions to the *luxCADBE* reporter and determined the expression levels of these constructs in the Δ*ntrC* strain compared to the wild type (Fig. 2A). The expression levels of *vpsR* and *vpsT* were increased 69% and 192%, respectively, in the Δ*ntrC* strain compared to the wild type. The expression of *hapR* showed a 28% increase in the Δ*ntrC* strain compared to the wild type. It is well known that *hapR* can be regulated at the posttranscriptional level through the quorum-sensing signaling pathway (17, 28). To evaluate the impact of NtrC on HapR production, we analyzed HapR levels. We found a small but reproducible increase in the HapR abundance in the Δ*ntrC* strain; as expected, HapR levels were markedly increased in the Δ*rpoN* strain compared to the wild type (Fig. 2B). Together, these results suggest that NtrC acts upstream as a negative regulator of the positive regulators of biofilms. The fact that NtrC negatively

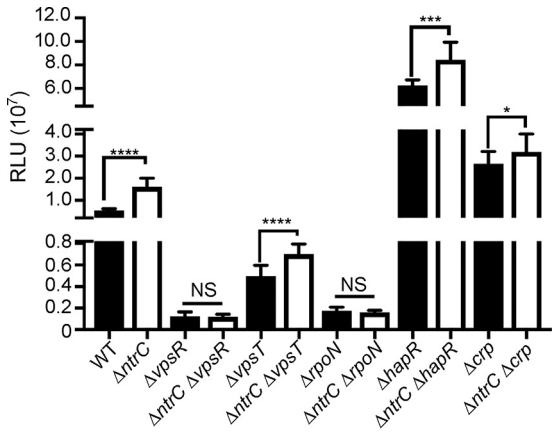

FIG 3 NtrC represses *vpsL* expression independently of regulators of biofilm formation, except for VpsR and RpoN. Shown are expression levels of P*vpsL-luxCADBE* (pFY_0950) in the WT, Δ*ntrC*, Δ*vpsR*, Δ*ntrC* Δ*vpsR*, Δ*vpsT*, Δ*ntrC* Δ*vpsT*, Δ*rpoN*, Δ*ntrC* Δ*rpoN*, Δ*hapR*, Δ*ntrC* Δ*hapR*, Δ*crp*, and Δ*ntrC* Δ*crp* strains. The graph represents the averages and standard deviations of RLU obtained from four technical replicates from at least three independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. Pairwise analysis was done by using a two-tailed unpaired *t* test with Welch's correction. *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$; NS, not significant.

regulates HapR was surprising due to the role of HapR as a negative regulator of biofilms.

NtrC represses *vpsL* expression independently of VpsT, HapR, and CRP. To determine the contribution of NtrC to the *vps* regulatory network, we characterized the genetic interaction between *ntrC*, *vpsR*, *vpsT*, *rpoN*, *hapR*, and *crp* (Fig. 3). As described above, the Δ*ntrC* strain exhibited increased *vpsL* expression. The deletion of *ntrC* in backgrounds that lack either *vpsR* or *rpoN* did not result in increased *vpsL* expression, indicating that the positive effect of these regulators is dominant over the NtrC-mediated repression of *vpsL*. The expression of *vpsL* in these genetic backgrounds is severely diminished. The expression of *vpsL* in the absence of VpsT was reduced ~91% compared to the wild type. The expression levels of *vpsL* in the Δ*ntrC* Δ*vpsT*, Δ*ntrC* Δ*hapR*, and Δ*ntrC* Δ*crp* strains showed ~40%, 35%, and 20% increases, respectively, compared to the levels in the individual mutants of these biofilm regulators (Δ*vpsT*, Δ*hapR*, and Δ*crp*) (Fig. 3). These findings suggest that NtrC can repress *vpsL* independently of VpsT, HapR, and CRP, but its negative effect is dependent on the presence of VpsR and RpoN.

RpoN contributes to *vpsL* expression in the absence of NtrC and HapR. To determine the contribution of RpoN to *vpsL* expression in the absence of HapR and NtrC, we characterized the genetic interaction between *rpoN*, *ntrC*, and *hapR* (Fig. 4). The expression levels of *vpsL-lux* in the Δ*rpoN* Δ*hapR* and Δ*hapR* strains were similar, indicating that the observed phenotype is likely due to increased HapR levels in the Δ*rpoN* strain. We found that the expression level of *vpsL* decreased significantly in the Δ*ntrC* Δ*hapR* Δ*rpoN* strain compared to the Δ*ntrC* Δ*hapR* strain, indicating that NtrC-dependent repression in the Δ*hapR* strain relies on the presence of *rpoN*.

The absence of NtrC has no effect on c-di-GMP levels. The expression of VPS biosynthetic genes and their regulators is controlled by the levels of the nucleotide-based second messenger cyclic dimeric GMP (c-di-GMP). High levels of c-di-GMP increase biofilm formation. We hypothesized that the effect of NtrC on *vpsL*, *vpsR*, and *vpsT* expression could be mediated through the modulation of the cellular c-di-GMP pool. We extracted nucleotides from exponentially grown wild-type and Δ*ntrC* strain cultures and measured cellular c-di-GMP levels. We found no significant difference in the abundance of c-di-GMP in the Δ*ntrC* strain compared to the abundance in the wild type, suggesting that the NtrC-mediated repression of *vps* expression is not due to changes in the cellular c-di-GMP levels under these conditions (see Fig. S2 in the supplemental material).

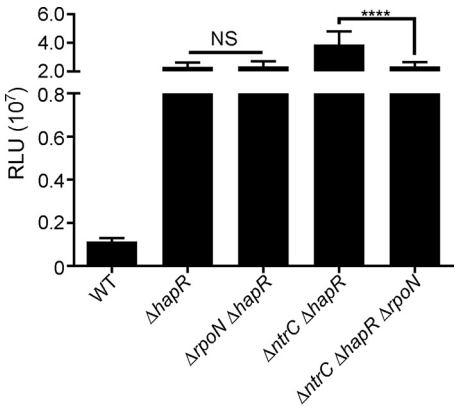

FIG 4 RpoN contributes to *vpsL* expression in the absence of NtrC and HapR. Shown are expression levels of P*vpsL-luxCADBE* (pFY_0950) in the WT, Δ*hapR*, Δ*rpoN* Δ*hapR*, Δ*ntrC* Δ*hapR*, and Δ*ntrC* Δ*hapR* Δ*rpoN* strains. The graph represents the averages and standard deviations of RLU obtained from four technical replicates from at least three independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. One-way ANOVA followed by Dunnett's multiple-comparison test was used to compare expression levels between the WT and deletion mutants. ****, $P < 0.0001$; NS, not significant.

***V. cholerae* NtrC supports growth under conditions of low nitrogen availability.** Amino acid sequence alignments of NtrC from *V. cholerae* to its counterparts in *E. coli* and *S.* Typhimurium showed that NtrC from *V. cholerae* is 73.12% and 72.9% identical to NtrC from *E. coli* and *S.* Typhimurium, respectively (see Fig. S3 in the supplemental material). To test if *V. cholerae* NtrC functions similarly to its counterpart in *E. coli*, we performed a complementation analysis using an *E. coli* strain lacking *ntrB* and *ntrC* (Δ*ntrBC*). For these studies, we expressed *V. cholerae ntrBC* from an arabinose-inducible promoter on a pBAD plasmid (Fig. 5A) and analyzed its ability to support growth on M9 agar supplemented with a poor nitrogen source (L-serine). Wild-type *E. coli* containing the empty vector (pBAD) grew on this medium; however, the Δ*ntrBC* strain containing the empty vector grew poorly, indicating that the Δ*ntrBC* strain has a decreased ability

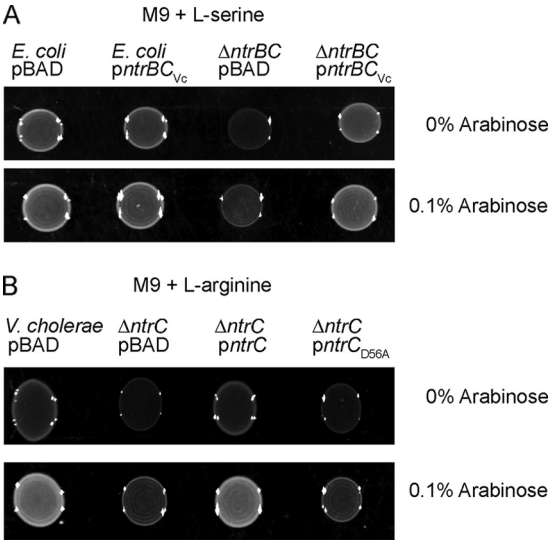

FIG 5 NtrC from *V. cholerae* allows growth under limiting nitrogen availability. (A) Images of spot colonies from *E. coli* and the Δ*ntrC* strain harboring an empty plasmid (pBAD) or a complementation plasmid expressing *ntrB-ntrC* or *ntrB-ntrC*$_{D56A}$ from *V. cholerae*. Cells were grown on M9 agar supplemented with 100 mM L-serine as a nitrogen source and 0 or 0.1% arabinose (for induction) for 24 h at 37°C. (B) Images of spot colonies from *V. cholerae* and the Δ*ntrC* strain harboring an empty plasmid (pBAD) or a complementation plasmid expressing *ntrC* or *ntrC*$_{D56A}$. Cells were grown on M9 agar supplemented with 100 mM L-arginine as a nitrogen source and 0 or 0.1% arabinose for 24 h at 37°C.

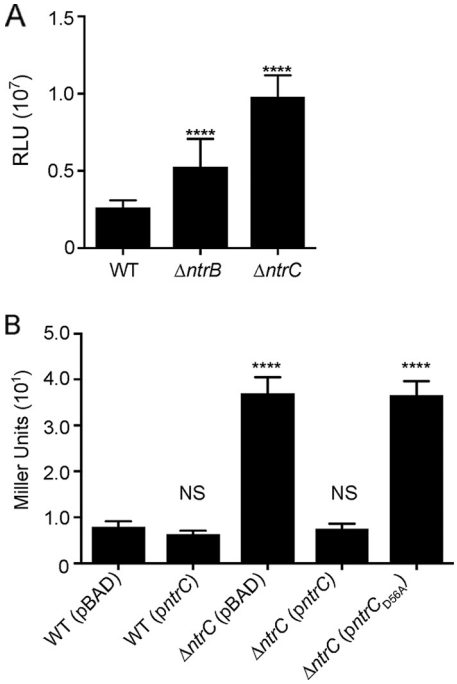

FIG 6 NtrB and aspartate 56 of NtrC contribute to *vpsL* repression. (A) Expression of P*vpsL-luxCADBE* (pFY_3406) in the WT, Δ*ntrB*, and Δ*ntrC* strains. The graph represents the averages and standard deviations of RLU obtained from at least three technical replicates from four independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. Expression levels of *vpsL* in the Δ*ntrB* and Δ*ntrC* strains were compared to that in the WT. (B) β-Galactosidase assay of P*vpsL-lacZ* reporter strains containing either the empty vector (pBAD) or a vector expressing *ntrC* (pBAD-*ntrC*) or *ntrC*$_{D56A}$ (pBAD-*ntrC*$_{D56A}$) under the control of an arabinose-inducible promoter. Cells were grown in LB medium supplemented with 0.1% arabinose to mid-exponential phase. The graph represents the averages and standard deviations of Miller units obtained from four technical replicates from two biological replicates. The expression levels of *vpsL* in all strains were compared to that in the WT (pBAD). ANOVA followed by Dunnett's test was performed for multiple-comparison analysis. ****, adjusted *P* value of <0.0001; NS, not significant.

to utilize L-serine as a nitrogen source (Fig. 5A). When *V. cholerae ntrBC* was introduced into the *E. coli* Δ*ntrBC* strain, growth on M9 agar with L-serine was restored (Fig. 5A). These findings suggest that *V. cholerae ntrC* and *ntrB* function similarly to their *E. coli* counterparts.

Using the same approach, we analyzed the contribution of NtrC to supporting the growth of *V. cholerae* in the presence of another poor nitrogen source, L-arginine. In the absence of NtrC, *V. cholerae* grew poorly on M9 broth agar supplemented with L-arginine as the sole nitrogen source. When expressed in *trans* from an arabinose-inducible promoter (pBAD-*ntrC*), NtrC was capable of supporting the growth of the Δ*ntrC* strain. Since the phosphorylation state of a RR likely determines its activity, we mutated the conserved aspartate residue at position 56 (D56) in the REC domain of NtrC to generate a potentially inactive RR (D56A). We found that the construct expressing *ntrC* with the point mutation D56A (pBAD-*ntrC*$_{D56A}$) was unable to complement the growth defect of the Δ*ntrC* strain (Fig. 5B). This finding suggests that the phosphorylation of NtrC is important for its activity as an effector of the nitrogen starvation response in *V. cholerae*.

NtrC phosphorylation contributes to *vpsL* repression. Since the phosphorylation state of response regulators generally dictates their activity, we examined if the absence of NtrC's cognate histidine kinase, NtrB, affects *vpsL* expression. The expression levels of P*vpsL-lux* were increased 100% in the Δ*ntrB* strain and 273% in the Δ*ntrC* strain compared to the wild type (Fig. 6A). We next analyzed the importance of the conserved D56 amino acid residue of NtrC for the negative regulation of *vpsL* expression. We utilized a reporter strain with P*vpsL* fused to *lacZ* on the chromosome (P*vpsL-lacZ*) and

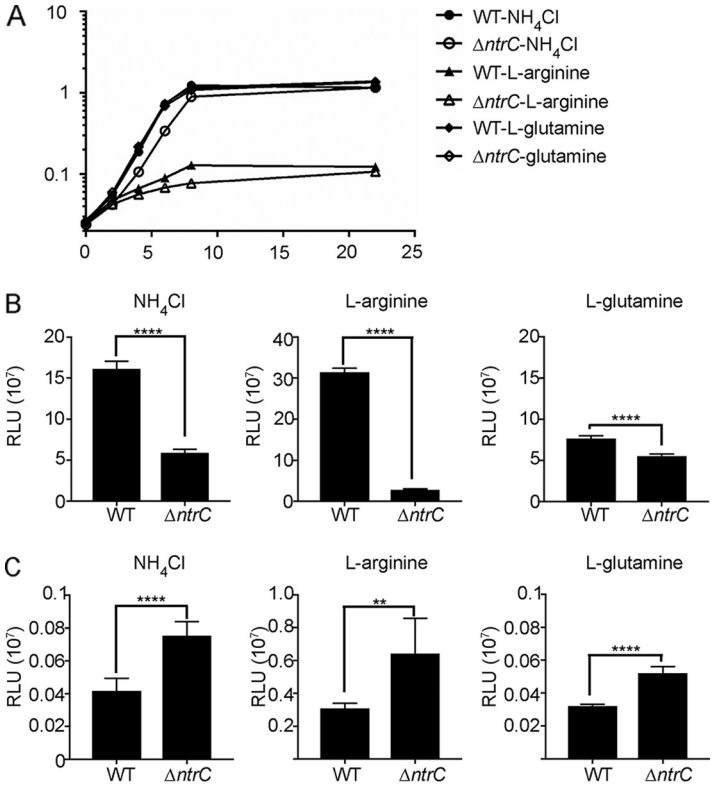

FIG 7 NtrC affects growth with different nitrogen sources and modulates *glnA* and *vpsL* expression in *V. cholerae*. (A) Growth curves of the wild-type and Δ*ntrC* strains grown in defined M9 medium supplemented with 0.1% NH_4Cl, 100 mM L-arginine, or 100 mM L-glutamine as the only nitrogen source. The graph represents the means and standard deviations of data for two technical replicates from two biological replicates. (B and C) Expression of P*glnA-luxCADBE* (B) and P*vpsL-luxCADBE* (C) in the WT and Δ*ntrC* strains grown for 4 h in the presence of 0.1% NH_4Cl, 100 mM L-arginine, or 100 mM L-glutamine as the sole nitrogen source. The graphs represent the averages and standard deviations of RLU obtained from four technical replicates from two independent biological samples. RLU are reported in luminescence counts per minute per milliliter per OD_{600}. Pairwise analysis was performed by using a two-tailed unpaired *t* test with Welch's correction. **, $P < 0.01$; ****, $P < 0.0001$.

analyzed *vpsL* expression using β-galactosidase production as a readout (Fig. 6B). When *ntrC* was overexpressed from a pBAD plasmid in the parental strain, we saw no significant changes in *vpsL* expression compared to the wild type carrying the empty vector (pBAD). A 363% increase in *vpsL* expression was observed in the Δ*ntrC* strain compared to the wild type when both strains carried the empty vector. The pBAD-*ntrC* construct restored *vpsL* expression in the Δ*ntrC* strain to wild-type levels. This complementation phenotype was lost when NtrC had a D56A point mutation.

Together, these results suggest that the phosphorylation of NtrC is important for repressing *vpsL* expression. The deletion of *ntrC* or the presence of an *ntrC* variant with a D56A point mutation has a more profound effect on *vpsL* expression than does the deletion of *ntrB*, which suggests that additional factors contribute to NtrC phosphorylation and/or activity under the conditions tested.

NtrC regulates the expression of *glnA* but not *vpsL* in a nitrogen source-dependent manner. The activity of canonical NtrC orthologues is modulated by nitrogen availability; hence, we hypothesized that *vpsL* expression would respond to the type of nitrogen source present in the growth medium. To test this possibility, we first monitored the growth of the wild-type and Δ*ntrC* strains in M9 minimal medium supplemented with either NH_4Cl (0.1%), L-arginine (100 mM), or L-glutamine (100 mM) as the sole nitrogen source (Fig. 7A). We observed that the levels of growth of the wild-type strain in the presence of NH_4Cl or L-glutamine were indistinguishable. However, the use of L-arginine as the sole nitrogen source markedly reduced the growth of

the wild type. The Δ*ntrC* strain showed a decrease in growth relative to the wild type when NH_4Cl or L-arginine was used as the sole nitrogen source.

As a proxy for NtrC activation, we analyzed the induction of *glnA*, encoding glutamine synthetase, in cells growing in the presence of the above-mentioned nitrogen sources. We first generated a transcriptional fusion of the regulatory region of *glnA* with the *lux* reporter (P*glnA-lux*) and then compared *glnA* expression levels between the wild-type and Δ*ntrC* strains grown in the presence of different nitrogen sources. The expression of P*glnA-lux* in the Δ*ntrC* strain decreased ~63% when NH_4Cl was used as the sole nitrogen source, ~91% when L-arginine was used as the sole nitrogen source, and ~28% when L-glutamine was used as the sole nitrogen source, compared to the wild type under the same conditions. These results suggest that NtrC is important for the activation of *glnA* expression, especially in cells growing with a poor nitrogen source (Fig. 7B).

We next evaluated *vpsL* expression (P*vpsL*-lux) in cells growing under the same conditions as those indicated above. The expression of *vpsL* in the Δ*ntrC* strain was increased ~80%, ~108%, and ~62% when NH_4Cl, L-arginine, and L-glutamine were used as the sole nitrogen sources, respectively, compared to the wild type under the same conditions. We hypothesized that the *vpsL* expression level would be lower with poor nitrogen sources due to NtrC activation. Surprisingly, we observed the opposite: *vpsL* expression in the presence of L-arginine showed a 637% increase compared to growth in NH_4Cl and an 859% increase compared to growth in the presence of L-glutamine as the sole nitrogen source. This result suggests that the regulation of *vpsL* by different nitrogen sources is more complex than anticipated and not dependent solely on NtrC activation.

DISCUSSION

In *V. cholerae*, the alternative sigma factor RpoN regulates the expression of genes involved in a variety of cellular processes, including, but not limited to, nitrogen assimilation, motility, quorum sensing, and the type VI secretion system (29–31). In this study, we determined the regulatory role of the NtrC family RRs in biofilm formation and the expression of *vps* genes. RpoN, together with LuxO, regulates the abundance of HapR in response to quorum-sensing signals. This regulation is indirect and involves the activation of the small regulatory RNAs *qrr1* to *qrr4* by LuxO. These small RNAs block the translation of *hapR* mRNA at a low cell density (28). The regulatory role of the quorum-sensing master regulator, HapR, in biofilm formation is well documented (6, 7, 32–34). We determined that HapR levels are higher in the Δ*rpoN* strain, suggesting that the decreased biofilm formation in the absence of RpoN and LuxO is due mainly to the inhibition of the transcription of *qrr1* to *qrr4* and, in turn, the increased accumulation of HapR. We found that the absence of FlrC and NtrC resulted in increased biofilm formation. Increased *vpsL* expression in the absence of NtrC depends on RpoN regardless of the presence of HapR (Fig. 8). From a systems biology perspective, RpoN is a highly interconnected node that can regulate a variety of processes through its interaction with specific activator partners. The abundance of RpoN and its specific coactivators can perhaps dictate the outcome of the response to environmental perturbations. Previous work has demonstrated that RpoN acts as a positive regulator of *vps*. Here we provided genetic evidence suggesting that RpoN can act as a positive or negative regulator of *vps* depending on the RpoN activator present. In this study, we focused only on the impact of the NtrC class of bEBPs on *vps* expression; it is possible that there is competition for RpoN availability and that the absence of *ntrC* results in an increased availability of RpoN, thereby allowing another RpoN-dependent regulator to positively impact levels of *vps*. Thus, further investigation of the role of sigma factor competition and the mechanism of NtrC-mediated *vps* regulation is warranted.

NtrC from *V. cholerae* is ~70% identical to NtrC from *E. coli*; this, together with data from an early study on RpoN-dependent regulators (30), suggests that in *V. cholerae*, NtrC is a regulator of nitrogen metabolism. Nitrogen acquisition is of great importance during the *V. cholerae* life cycle, and it has been reported that chitin, mucin, and

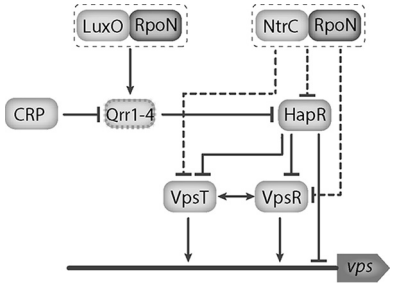

FIG 8 Model of RpoN-dependent regulation of *vps*. RpoN is a highly interconnected node that can regulate a variety of processes through its interaction with specific activator partners. In response to quorum-sensing signals, LuxO interacts with RpoN to positively regulate the small regulatory RNAs *qrr1-4*, which block the translation of *hapR* mRNA at a low cell density. HapR is the quorum-sensing master regulator and negatively regulates *vps* expression through both direct regulation and the repression of the master biofilm regulators VpsR and VpsT. We demonstrate that NtrC acts as a negative regulator of *vps*, likely through its repression of *vpsR* and *vpsT*, and this regulatory input is dependent on the presence of RpoN. Additionally, it appears that NtrC has a modest negative impact on HapR levels. Solid lines represent direct interactions, and dashed lines indicate indirect interactions.

nucleosides can be used as nitrogen sources by *V. cholerae* (35–37). Furthermore, nitrogen limitation leads to glycogen accumulation in *V. cholerae*, which was found to be important for persistence and transmission (38). Given the central role of NtrC in the regulation of nitrogen metabolism in other organisms, it is pertinent to better understand its involvement in different physiological processes where nitrogen availability dictates the fate of *V. cholerae*. Here we show that NtrC limits biofilm formation and downregulates the expression levels of a structural matrix gene, *vpsL*, as well as genes encoding biofilm regulators, such as *vpsR*, *vpsT*, and *hapR* (Fig. 1 and 2). None of these genes seem to have promoters with signals recognized by RpoN (data not shown), which suggests that regulation by NtrC is indirect. Our results revealed a negative effect of NtrC on HapR accumulation; it is unknown if this regulation occurs through the RpoN-LuxO-Qrr regulatory cascade or in a different pathway. HapR and Crp are the main negative regulators of biofilm formation in *V. cholerae*. We observed a 292% increase in *vpsL* expression in the Δ*ntrC* strain compared to the wild type but only 35% and 20% increases in the Δ*ntrC* Δ*hapR* and Δ*ntrC* Δ*crp* strains compared to the single Δ*hapR* and Δ*crp* mutants. Given the difference in the magnitudes of induction of *vpsL*, we cannot disregard the possibility of a genetic interaction between *ntrC*, *hapR*, and *crp*. For instance, an interaction between *hapR* and *crp* was proposed previously (8, 9), and here we report that the absence of NtrC has a modest effect on *hapR* expression. Undoubtedly, the balance between these regulators is crucial for controlled biofilm formation and may play a role in processes such as biofilm dispersal and transmission.

We observed the NtrC-repressive effect on biofilms in rich medium (lysogeny broth [LB]), suggesting that nitrogen limitation is not a prerequisite for NtrC activation and perhaps that basal levels of phosphorylation of NtrC by phosphodonors, such as acetyl phosphate, are sufficient to exert NtrC-dependent repression of biofilm formation. Our results suggest that growth with L-arginine as the sole nitrogen source results in nitrogen limitation. Under these conditions, NtrC is expected to be activated by NtrB-mediated phosphorylation; therefore, we predicted that the level of expression of *vpsL* would be lower than that under nitrogen-replete conditions. Interestingly, opposite of our prediction, we found that the expression of *vpsL* was induced under these conditions (Fig. 7). L-Arginine has been shown to promote c-di-GMP accumulation and cellulose production in *S*. Typhimurium (39), and it is possible that *V. cholerae* cells also respond to the presence of L-arginine by increasing c-di-GMP levels independently from NtrC and, as a consequence, *vpsL* expression. Our results suggest that nitrogen limitation may positively regulate *vpsL* expression independently from NtrC and that other *vps* regulators may play a role under these conditions.

The nitrogen-related phosphotransfer system (PTS^{Ntr}) has been shown to sense nitrogen limitation in *Escherichia coli* and *Caulobacter crescentus* (40, 41). For *V. cholerae*,

two nitrogen-specific enzyme IIA (EIIA^{Ntr}) homologs, but not the upstream members of the PTS^{Ntr}, were reported to repress *vpsL* expression (42). Interestingly, the effect of EIIA^{Ntr} proteins on biofilm formation occurs in cells grown in LB but not in M9 minimal medium (42). It is important to note that the role of the *V. cholerae* PTS^{Ntr} has not been studied in great depth under conditions of nitrogen limitation. Interestingly, in *Pseudomonas aeruginosa*, unphosphorylated EIIA^{Ntr} also acts as a repressor of biofilm formation (43). We observed that NtrC regulates biofilm formation in LB and *vpsL* expression both in LB and M9 minimal medium, regardless of the nitrogen source available. It would be informative to further characterize if there is a connection between the regulatory effects of NtrC and EIIA^{Ntr} on biofilm formation.

In *Vibrio vulnificus*, the NtrC orthologue has been shown to upregulate the expression of lipopolysaccharide (LPS)- and exopolysaccharide (EPS)-related genes (44, 45) when grown in minimal medium under nitrogen limitation. Additionally, NtrC in *Burkholderia cenocepacia* also appears to positively regulate EPS production (46). Further studies of the mechanism of biofilm regulation by NtrC in these organisms, as well as in *V. cholerae*, where NtrC has an opposite role, would allow us to better understand the factors that shape the evolution of related regulatory pathways and their outcomes in specific biological systems.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 2. *Escherichia coli* CC118λ*pir* strains were used for DNA manipulation, and *E. coli* S17-1λ*pir* strains were used for conjugation with *V. cholerae*. In-frame deletion mutants of *V. cholerae* were generated as described previously (32). All *V. cholerae* and *E. coli* strains were grown aerobically, at 30°C and 37°C, respectively, unless otherwise noted. Cells were grown in lysogeny broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl [pH 7.5]), unless otherwise stated. LB agar medium contains 1.5% (wt/vol) granulated agar (BD Difco, Franklin Lakes, NJ). Concentrations of antibiotics used were as follows: ampicillin at 100 μg/ml, rifampin at 100 μg/ml, and chloramphenicol at 5 μg/ml for *V. cholerae* and 20 μg/ml for *E. coli*. Cells were also grown in M9 minimal medium (0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.5% NaCl, 1 mM $MgSO_4$, 0.1 mM $CaCl_2$, 10 mM glucose) supplemented with 1× minimal essential medium vitamins (10 ml/liter; Gibco, Rockford, IL) and either 0.1% NH_4Cl or 100 mM the following L-amino acids: arginine and glutamine.

DNA manipulations and generation of in-frame deletion mutants and *gfp*-tagged strains. An overlapping PCR method was used to generate in-frame deletion constructs using previously described methods (32). The generation of deletions by double recombination was performed as described previously (47, 48). *V. cholerae* wild-type and mutant strains were tagged with the green fluorescent protein gene (*gfp*) as described previously (49). The *gfp*-tagged *V. cholerae* strains were verified by PCR and examined in flow cell experiments.

Flow cell experiments and confocal laser scanning microscopy. Flow cells were inoculated by normalizing cultures of *gfp*-tagged *V. cholerae* strains grown overnight to an optical density at 600 nm (OD_{600}) of 0.02 and injecting cells into an Ibidi m-Slide VI0.4 instrument (catalogue number 80601; Ibidi LLC, Verona, WI). After inoculation, the bacteria were allowed to adhere at room temperature for 1 h with no flow. Next, a flow of 2% (vol/vol) LB (0.2 g/liter tryptone, 0.1 g/liter yeast extract, 1% NaCl) was initiated at a rate of 7.5 ml/h. Confocal laser scanning microscopy (CLSM) images of the biofilms were captured with an LSM 5 Pascal system (Zeiss, Jena, Germany), using an excitation wavelength of 488 nm and an emission wavelength of 543 nm. Three-dimensional images of the biofilms were reconstructed by using Imaris software (Bitplane, Zurich, Switzerland) and quantified by using COMSTAT (50).

Luminescence assay. Cultures of *V. cholerae* cells grown overnight in LB were diluted 1:1,000 in LB containing chloramphenicol (5 μg/ml) or washed twice in M9 minimal medium without nitrogen and diluted to an OD_{600} of 0.02 in M9 minimal medium containing different nitrogen sources and chloramphenicol (2.5 μg/ml). Cultures grown in LB at 30°C were harvested at mid-exponential phase (OD_{600} of 0.3 to 0.4) for luminescence readings. Cultures grown in M9 minimal medium at 30°C were harvested for luminescence reading after reaching an OD_{600} of ~0.1 in the presence of NH_4Cl and L-glutamine or ~0.06 in the presence of L-arginine. Luminescence was measured by using a PerkinElmer Victor3 multilabel counter (PerkinElmer, Waltham, MA) and is reported as relative light units (RLU) in counts per minute per milliliter per OD_{600}. Assays were repeated with at least two biological replicates. Four technical replicates were measured for all assays.

Western blotting of HapR. Cells were grown to mid-exponential phase (OD_{600}), and 25 ml of the culture was then harvested and lysed in 5 ml of 2% sodium dodecyl sulfate (SDS) and 4 mg of DNase I (Sigma-Aldrich Corp., St. Louis, MO). The protein concentration was estimated with a bicinchoninic acid (BCA) assay (Thermo Fisher, Waltham, MA). Thirty micrograms of protein was loaded and resolved by electrophoresis on a 12% SDS-polyacrylamide gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a Trans-Blot Turbo transfer system (Bio-Rad, Hercules, CA). Immunoblotting was performed by using a polyclonal anti-HapR antibody (51) or purified anti-*E. coli* RNA polymerase α antibody (BioLegend, San Diego, CA). Proteins were detected by chemiluminescence using horseradish

TABLE 2 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant genotype or description	Reference or source(s)
Strains		
E. coli		
CC118λ*pir*	Δ(*ara-leu*) *araD* Δ*lacX74 galE galK phoA20 thi-1 rpsE rpoB argE*(Am) *recA1* λ*pir*	53
S17-1λ*pir*	Tp^r Sm^r *recA thi pro* (r_K^- m_K^+) RP4::2-Tc::Mu-Km Tn*7* λ*pir*	54
K-12 (BW28357)	*lacIp4000*(*lacI*^{q}) *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1*	55
K-12Δ*ntrBC* (BW30011)	*lacIp4000*(*lacI*^{q}) *rrnB3* Δ*lacZ4787 hsdR514* Δ(*araBAD*)*567* Δ(*rhaBAD*)*568 rph-1* Δ(*ntrB-ntrC*)*1318*	55
V. cholerae		
FY_VC_0001	*Vibrio cholerae* O1 El Tor A1552; wild type; Rif^r	6
FY_VC_0616	P*vpsL-lacZ*; Rif^r	27
FY_VC_2272	ΔVC0665 (*vpsR*)	4
FY_VC_0192	ΔVC1021 (*luxO*)	47
FY_VC_7998	ΔVC1522	47
FY_VC_8243	ΔVC1926 (*dctD*-1)	47
FY_VC_6286	ΔVC2135 (*flrC*)	47
FY_VC_6289	ΔVC2749 (*ntrC*)	47
FY_VC_8245	ΔVCA0142 (*dctD*-2)	47
FY_VC_2407	ΔVC0665 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_8068	ΔVC1021 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_8070	ΔVC1522 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_8319	ΔVC1926 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_8072	ΔVC2135 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_7061	ΔVC2749 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_8321	ΔVCA0142 mTn*7-gfp* Rif^r Gm^r	This study
FY_VC_7058	ΔVC2748 (*ntrB*)	This study
FY_VC_7057	Δ*ntrC* Δ*vpsR*	This study
FY_VC_0099	Δ*vpsT*	5
FY_VC_7051	Δ*ntrC* Δ*vpsT*	This study
FY_VC_7936	ΔVC2529 (*rpoN*)	This study
FY_VC_7304	Δ*ntrC* Δ*rpoN*	This study
FY_VC_0178	Δ*hapR*	This study
FY_VC_7054	Δ*ntrC* Δ*hapR*	This study
FY_VC_2326	Δ*crp*	9
FY_VC_7056	Δ*ntrC* Δ*crp*	This study
FY_VC_13619	Δ*rpoN* Δ*hapR*	This study
FY_VC_13621	Δ*rpoN* Δ*ntrC* Δ*hapR*	This study
Plasmids		
pGP704*sacB*28	pGP704 derivative; *mob-oriT sacB* Ap^r	G. Schoolnik
pFY-1422	pGP704-*sacB28*::ΔVC2748; Ap^r	This study
pBBRlux	*luxCDABE*-based promoter fusion vector; Cm^r	28
pFY-0950	pBBRlux *vpsL* promoter; Cm^r	56
pFY-1326	pBBRlux *glnA* promoter; Cm^r	This study
pFY-0989	pBBRlux *vpsR* promoter; Cm^r	This study
pFY-0988	pBBRlux *vpsT* promoter; Cm^r	This study
pFY-1049	pBBRlux *hapR* promoter; Cm^r	This study
pBAD/myc-His-B	Arabinose-inducible expression vector with C-terminal myc epitope and six-His tags	Invitrogen
pFY-1648	pBAD/myc-His-B::$ntrBC_{VC}$[a]; Ap^r	This study
pFY-1351	pBAD/myc-His-B::*ntrC*; Ap^r	This study
pFY-1349	pBAD/myc-His-B::$ntrC_{D56A}$; Ap^r	This study
pUX-BF13	oriR6K helper plasmid; *mob-oriT*; provides the Tn*7* transposition function in *trans*; Ap^r	57
pMCM11	pGP704::mTn*7-gfp*; Gm^r Ap^r	M. Miller and G. Schoolnik

[a]VC, *V. cholerae*.

peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) and SuperSignal West Pico Plus chemiluminescent substrate according to the instructions of the manufacturer (Thermo Fisher, Waltham, MA).

c-di-GMP measurements. Extraction of c-di-GMP was performed as described previously (52). Cultures were grown in LB to an OD_{600} of 0.3 to 0.4, and 40 ml was harvested for c-di-GMP quantification. The amount of c-di-GMP was calculated by interpolation using a standard curve generated from pure c-di-GMP suspended in 184 mM NaCl (Biolog Life Science Institute, Bremen, Germany). The c-di-GMP levels were expressed as picomoles per milligram of total protein. The protein concentration was determined from 4 ml of culture, cells were lysed in 1 ml of 2% SDS, and total protein was estimated with a BCA assay (Thermo Fisher, Waltham, MA). Each c-di-GMP quantification experiment was performed with

six biological replicates. Levels of c-di-GMP were compared between the samples by using a two-tailed Mann-Whitney test.

Growth on minimal medium. One milliliter of cultures grown overnight was harvested by centrifugation. The cell pellet was resuspended in 1× phosphate-buffered saline (PBS), and 5 μl of the resuspension mixture was spotted onto defined M9 agar containing either 100 mM L-serine or L-arginine as the only nitrogen source. The plates were incubated at 30°C for *V. cholerae* and at 37°C for *E. coli* for 24 h. Images were taken by using a Bio-Rad ChemiDoc MP system (Bio-Rad, Hercules, CA).

β-Galactosidase assay. β-Galactosidase assays were performed by using exponentially grown cultures. *V. cholerae* cells were grown overnight (18 to 20 h) aerobically in LB containing 100 μg/ml of ampicillin. Cultures grown overnight were diluted 1:200 in fresh LB medium with or without arabinose and grown aerobically to an OD_{600} of 0.3 to 0.4. The cells were then diluted again 1:200 in fresh LB with or without arabinose, grown aerobically to an OD_{600} of 0.3 to 0.4, and immediately harvested for assays. The β-galactosidase assays were carried out with MultiScreen 96-well microtiter plates fitted onto a MultiScreen filtration system (Millipore, Billerica, MA), using a previously described procedure (49).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/JB.00025-18.

SUPPLEMENTAL FILE 1, PDF file, 3.0 MB.

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