The global regulators ArcA and CytR collaboratively modulate Vibrio cholerae motility

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Abstract

Background: Vibrio cholerae, a Gram-negative bacterium, is highly motile owing to the presence of a single polar flagellum. The global anaerobiosis response regulator, ArcA regulates the expression of virulence factors and enhances biofilm formation in V. cholerae. However, the function of ArcA for the motility of V. cholerae is yet to be elucidated. CytR, which represses nucleoside uptake and catabolism, is known to play a chief role in V. cholerae pathogenesis and flagellar synthesis but the mechanism that CytR influences motility is unclear.

Results: In this study, we found that the ΔarcA mutant strain exhibited higher motility than the WT strain due to ArcA directly repressed flrA expression. We further discovered that CytR directly enhanced fliK expression, which explained why the ΔcytR mutant strain was retarded in motility. On the other hand, cytR was a direct ArcA target and cytR expression was directly repressed by ArcA. As expected, cytR expression was down-regulated.

Conclusions: Overall, ArcA plays a critical role in V. cholerae motility by regulating flrA expression directly and fliK indirectly in the manner of cytR.

Keywords: Vibrio cholerae, Motility, ArcA, CytR, Flagellum

Background

V. cholerae is ubiquitous in aquatic environments and intestines of host [1]. The flagellum of V. cholerae is a complex self-assembling organelle that is attached to the cell surface and allows bacterial cells to move in their environment [2]; moreover, it plays a pivotal role in substrates adhesion, biofilm formation, and virulence [3–6]. Elucidating the mechanisms underlying the regulation of flagellum should enhance our understanding of the lifecycle of V. cholerae both in the intestinal and aquatic phases. V. cholerae flagellar genes are expressed within a four-tiered transcriptional hierarchy [4]. The sole Class I gene encodes the σ54-dependent transcriptional activator FlrA, which is the master regulator of the flagellar hierarchy because without it no flagellar genes are expressed [7]. FliK is in the Class III level of the flagellar hierarchy. The assembly of a flagellum occurs in a number of stages, and FliK is the “checkpoint control” protein. When the flagellar hook substructure has reached its optimal length, FliK then terminates hook export and assembly and transmits a signal to begin filament export, in the final stage of the flagellar biosynthesis [8].

The ArcA/ArcB two-component system evidently has a key role in the response to oxygen. The membrane sensor protein ArcB is phosphorylated, resulting in the transfer of its phosphoryl group to ArcA. Phosphorylated ArcA is subsequently activated as a transcription factor, resulting in the up-or downregulation of several downstream genes [9, 10]. In Escherichia coli, approximately 50% genes whose expression levels are affected due to aerobic to anaerobic transitions are also affected by ArcA; in total 1139 genes in the E. coli genome are in fact either directly or indirectly regulated by ArcA [11]. In Salmonella Typhimurium, ArcA has been shown to affect...
metabolic processes, stress response, and surface adherence [12]. A study reported that the ΔarcA mutant strain lacked flagella in S. enterica sv. Typhimurium and was thus non-motile [13]. In E. coli, ArcA enhances motility by increasing flmA expression [14]. Further ArcA was found to directly repress the expression of motA, motB, and cheA in avian pathogenic E. coli, but it did not affect the expression of flagella genes [15]. Considering that the function of ArcA in V. cholerae motility remains unexplored, in this study, we aimed to determine the relationship between ArcA and V. cholerae motility.

The CytR repressor belongs to the LacI family, and possesses, like the other members, an N-terminal helix–turn–helix (HTH) DNA-binding motif [16]. However, unlike a typical bacterial repressor, the CytR repressor and the cAMP receptor protein (CRP) bind cooperatively to several promoters in E. coli to repress transcription initiation [17]. In addition, researches had shown that the CRP protein and the CytR regulator can act either to repress or to activate transcription depending on the context [18]. CytR negatively regulates the genes that are involved in nucleosides uptake and catabolism [19]. In uropathogenic E. coli, CytR is a modulator of flagellar expression activated by CRP. The ΔcytR mutant strain was observed to show higher motility and flagellar expression [20]; further, CytR bound to the upstream region of flhD, which encodes the master regulator for flagellar expression. In V. cholerae, CytR positively regulates competence genes, type VI secretion operons, and chitinases [21]. Moreover, it plays an important role in V. cholerae pathogenesis and flagella synthesis [22]. In V. cholerae, the ΔcytR mutant strain was found to show downregulated expression levels of the class II flagellar genes flrB and flrC, and several class III flagellar genes [22]. Nevertheless, further studies are warranted to comprehensively understand this process. In the current study, we identified that low oxygen levels enhanced arcA expression, but repressed cytR expression. Furthermore, ArcA reduces V. cholerae motility not only in the manner of directly regulating flrA, but also in the manner of indirectly regulating fliK via directly binding and regulating cytR. Collectively, our findings enhance our understanding of how ArcA and CytR collaboratively modulate V. cholerae motility.

Results
ArcA repressed V. cholerae motility
In E. coli and S. enterica sv. Typhimurium, ArcA acts as the positive regulator of motility [13, 14]. To evaluate the role of ArcA in V. cholerae motility, we investigated the surface motility of ΔarcA mutant strain on soft agar plates in aerobic conditions. The motility zones of the ΔarcA mutant strain (diameter, 2.25 ± 0.15 cm) were larger than those of the WT strain (diameter, 1.27 ± 0.17 cm) (Figs. 1a and 1b). Moreover, we constructed the arcA complemented strain ΔarcA::ParcA containing a functional copy of the arcA sequence, using the plasmid pBAD33; and found the complementation strain (diameter, 1.51 ± 0.11 cm) restore the motility to the WT level (p = 0.0913) (Figs. 1a and 1b). These data indicated that ArcA functions as a negative regulator of V. cholerae motility.

ArcA directly repressed flrA expression
The increase in the motility of the ΔarcA mutant strain could be attributed to lack of flagella (fla phenotype), loss of motility (mot phenotype), or loss of chemotaxis (che phenotype) [14, 23]. As previous reports, ArcA is proposed to bind to a conservative sequence (5’TGTAT-3’) in E. coli [24]. Then, A search for the conservative ArcA binding sites in E. coli at flrA promoter region was performed using Virtual Footprint 3.0. We found a similar sequence (TGTTC-AAAACGTGCAAACAAAC T-TCTTA) with a 19 base-spacing at positions -14 to -42 on the upstream region of flrA that ArcA probably binds (Fig. S10). To determine the precise cause, the EMSA screening was performed to assess whether ArcA directly binds to the promoter region of the 16 different core regulons in the flagellar hierarchy, and found that the phosphorylated ArcA directly bound to the promoter region of flrA (Fig. 2a), not with the other 15 ones. The following qRT-PCR indicated that flrA expression was increased by approximately 3.4-fold in the ΔarcA mutant strain compared with that in the WT strain in aerobic conditions (Fig. 2b). Moreover, we constructed the arcA complemented strain ΔarcA::ParcA containing a functional copy of the arcA gene, using the plasmid pBAD33; and found flrA expression in the complementation strain was restored to the WT level (p = 0.1320) (Fig. 2b). Furthermore, the promoter-lux fusion reporter plasmid was constructed with the promoter of flrA inserted into pMS402, and found that the activity of flrA promoter-lux was approximately up-regulation by 1.5-fold in the ΔarcA mutant strain compared to that in the WT (Fig. 2c). These data indicated that ArcA functions as a negative regulator of V. cholerae motility by directly repressing the expression of flrA.

CytR enhanced the fliK expression and activated by CRP
Previous studies reported that the ΔcytR mutant strain shows reduced motility and the expression of flagellar-synthesis regulatory genes flrBC and class III flagellar-synthesis genes were reduced in the ΔcytR mutant strain [22]. We hypothesized that CytR modulates flagellar regulatory genes by directly binding to their promoter regions. CytR is proposed to bind to octameric inverted
Fig. 1  ArcA represses V. cholerae motility.  

a. Plate showing the motility zones of the WT strain, ΔarcA mutant strain, and the complementary strain ΔarcA::ParcA in aerobic condition.  
b. The diameter of motility zones (mean ± SEM) for nine independent biological replicates (See figure on next page.)

Fig. 2  ArcA directly represses flrA expression.  

a. The EMSA between phosphorylated ArcA protein and the flrA promoter. The concentration of phosphorylated ArcA protein increased gradually (0 to 2.4 μM), and the amount of promoter DNA used in each reaction was 50 ng. 4.5S RNA served as the negative control.  
b. mRNA levels of flrA in the WT, ΔarcA mutant strain and arcA complementary strain ΔarcA::ParcA in aerobic conditions. *, p < 0.05; **, p < 0.01; ***, p < 0.001, NS, no significance, p > 0.05.  
c. Expression of flrA-lux in WT strain and ΔarcA mutant strain. CPS (counts per second) values represent relative promoter-lux activities. All experiments were independently repeated at least three times. Values represent means ± SEM.
Fig. 2  (See legend on previous page.)
or direct repeats containing the consensus half sites (5’-TGCAA-3’) with variable spacing in E. coli [16]. We found a similar sequence (TGCAA-TAACCTTGCTTG) with a 24 base-spacing at positions -15 to -48 on the upstream region of fliK that CytR probably binds (Fig S11). This site overlays 18 bases of CRP-binding site (TG GAT -GCA ATA AAA CCT -TCACT ) located at position -31 to -52 (Fig. S2) [24].

The EMSA was performed to assess whether CytR directly binds to the promoter region of the 16 different core regulons in the flagellar heirarchy, and found that the CytR protein directly bound to the promoter region of fliK (Fig. 3a), not with the other 15 ones. Studies of gene regulation have revealed that the CRP protein and the CytR regulator can act either to repress or to activate transcription depending on the context [18]. When CytR binds to the promoter region of target genes, it forms a complex with CRP and RNA polymerase [25]. To determine if the transcriptional regulation of fliK by CytR occurs in the same manner as that of the above cases, we also observed CRP protein only (Fig. 3b) and co-binding of the CytR and CRP proteins by EMSA assay (Fig. 3c).

qRT-PCR revealed that fliK expression was approximately down-regulated by 2.4-fold in the ΔcytR mutant strain compared with that in the WT strain in aerobic conditions (Fig. 3d). Moreover, we constructed the cytR complemented strain ΔcytR::PcytR containing a functional copy of the cytR gene, using the plasmid pBAD33; and found fliK expression in the complementation strain was restored to the WT level (p = 0.1126) (Fig. 3d). Furthermore, the promoter-lux fusion reporter plasmid was constructed with the promoter of fliK inserted into pMS402 and found that the activity of fliK promoter-lux was approximately down-regulation by 1.5-fold in the ΔcytR mutant strain compared to that in the WT (Fig. 3e). As a whole, CytR and CRP
cooperatively bind to the upstream region of fliK, then CytR enhance fliK expression in the presence of CRP as a modulator.

**ArcA directly repressed cytR expression**

In addition to ArcA and CytR being related to the *V. cholerae* motility, we also found that CytR is a new downstream regulatory gene of ArcA. As previous reports, ArcA is proposed to bind to a conservative sequence (5'-TGTTA-3') in *E. coli* [24]. Then, A search for the conservative ArcA binding sites in *E. coli* at cytR promoter region was performed using Virtual Footprint 3.0. We found a similar sequence (TGTTA-ATT TTG TCA GCA AAT TAA TGC-TTATTA) with a 21 base-spacing at positions -11 to -42 on the upstream region of cytR that ArcA probably binds (Fig. S12). Then EMSA showed that the phosphorylated ArcA directly binds to the promoter of cytR (Fig. 4a). qRT-PCR was performed in both aerobic and anaerobic conditions. The results showed that the cytR expression was increased by 2.1-fold in aerobic conditions, and 5.0-fold in anaerobic condition in the ΔarcA mutant strain compared to that in the WT strain (Fig. 4b and 4c). Moreover, we constructed the arcA complemented strain ΔarcA::ParcA containing a functional copy of the arcA sequence, using the plasmid pBAD33; and found cytR expression in the complementation strain restore to the WT level in aerobic conditions (p = 0.6134) and anaerobic conditions (p = 0.3347) (Fig. 4b and 4c). Furthermore, the promoter-lux fusion reporter plasmid was constructed with the promoter of cytR inserted into pMS402 and found that the activity of cytR promoter-lux was approximately up-regulation by 1.3-fold in the ΔarcA mutant strain compared to that in the WT (Fig. 4d). These data indicated that ArcA repressed cytR expression by directly binding to its promoter region.
Discussion
ArcA and CytR involved in multiple regulation in *V. cholerae*. ArcA, being a regulator, its function in *V. cholerae* motility is still not known. Here, we for the first time aimed to understand the role of this multifunctional transcription factor in *V. cholerae* motility. Our results also suggest an expanded role of ArcA in *V. cholerae* in the manner of CytR and further evidence the relationship between CytR and *V. cholerae* motility.

More than 50 genes are involved in flagella synthesis and regulation in *V. cholerae* [7]. The flagella-synthesis genes in *V. cholerae* are categorized into a four transcriptional hierarchy [26]. The σ54-dependent transcriptional activator FlrA is the only class I gene in this hierarchy [27]. FlrA is the master regulator of the *V. cholerae* flagellar transcription hierarchy because it is important for the expression of all other flagellar genes. The *fliK* operon is transcribed from a class III promoter [28]. Flagellar assembly occurs in a number of stages, and in this process, the “checkpoint control” protein FliK functions in detecting when the flagellar hook substructure has reached its optimal length. FliK then terminates hook export and assembly and transmits a signal to begin filament export [8, 29].

In *E. coli*, the ΔarcA mutant strain has been reported to show loss of motility, with ArcA being necessary for the expression of *fliA*, but not for that of the master regulators *flhDC* [30]. In avian pathogenic *E. coli*, ArcA directly regulates the expression of *motA*, *motB*, and *cheA* [15], whereas in *S. enterica* *avium*, it activates class II and III flagellar genes and seems to slightly repress *flhDC* [31]. In contrast, in *V. cholerae*, we found that ArcA reduced motility by a directly way of repressing the expression of the class I flagellar regulatory gene *flrA*, and an indirectly way of repressing the expression of the class III flagellar regulatory gene *fliK* via CytR (Fig. 5). The expression of *cyrR* was up-regulated by 2.1-fold in ΔarcA stain compared to the WT under the aerobic condition (Fig. 4b); likewise, up-regulated by 5.0-fold under the anaerobic condition (Fig. 4c). So the further qRT-PCR was performed on the *cyrR* in ΔarcA stain under both the aerobic and anaerobic conditions, and the data showed that the expression of *cyrR* between these two conditions was not significantly different. In other word, the

![Fig. 5](image-url)  
**Fig. 5** Schematic representation of *V. cholerae* motility regulation network by ArcA regulating the expression of *flrA* directly and *fliK* indirectly in the manner of CytR.
repression of ArcA on cytR was consistent, no matter under the aerobic or anaerobic conditions.

In uropathogenic E. coli, CytR evidently represses motility and flagellar expression by directly binding to the upstream region of flhD, which encodes the master regulator for flagellar expression [20]. In the ΔcytR mutant strain of V. cholerae, qRT-PCR showed that the expression of the class II flagellar genes flrB and flrC, and that of several class III flagellar genes was downregulated [32]. We further investigated the target binding site of CytR and found that CytR bound to the promoter region of flik and acts as a positive regulator of flik (Fig. 5).

Our results suggest an extended role of ArcA in V. cholerae motility, and found its new downstream regulatory gene, cytR, which is also a global regulator and influences the motility by directly increasing flik expression.

Conclusions
In this work, we report that the global regulators ArcA and CytR collaboratively modulate V. cholerae motility. Here, we provide evidence that ArcA plays a fundamental role in V. cholerae motility by regulating the expression of flrA directly and flik indirectly in the manner of cytR.

Materials and methods

Bacterial strains, plasmids, and growth conditions
All strains and plasmids used in this study are shown in Table 1. In aerobic condition, all strains were grown overnight at 37 °C in lysogeny broth/agar. In anaerobic condition, bacterial cultures in an anaerobic incubator were grown in the presence of 1 g/l cysteine and 1 mg/l resazurin. Antibiotics were added, as required 40 μg/ml, polymyxin B or 25 μg/ml, chloramphenicol. All chemicals were purchased from Sigma (St. Louis, MO, USA).

Construction of the deletion mutant of ArcA and its complementation
The ArcA isogenic deletion mutant was constructed using the suicide plasmid pRE112 method, as previously described [33]. Briefly, 1) the recombinant plasmid pRE112-ΔarcA-V.cholerae was constructed and

Table 1 Bacterial strains and plasmids used in this study

| Strain or plasmid | Characteristics* | Reference or source |
|-------------------|------------------|---------------------|
| **Vibrio cholerae** |                  |                     |
| EI2382            | Virulent strain, O1 El Tor type, PmBl | Shanghai Municipal Center for Disease Control & Prevention |
| ΔarcA             | EI2382, deletion of arcA, PmBl | This study |
| ΔarcA::ParcA      | ΔarcA containing pBAD33 carrying arcA ORF with its own promoter, PmBl | This study |
| ΔcytR             | EI2382, deletion of cytR, PmBl | This study |
| ΔcytR::PcytR      | ΔcytR containing pBAD33 carrying cytR ORF with its own promoter, PmBl | This study |
| **Escherichia coli** |                  |                     |
| S17-1 (DE3)       | ṭpr Smr recA thi pro Rk- mkl- RP4-2-Tc-MuKm Tn7 λ pir (thi pro hsdR hsdM + recA RP4-2-Tc: Mu-Km-Tn7) | [1] |
| BL21(DE3)         | Host strain for protein expression | This study |
| BL21/pET28a:arcA  | BL21(DE3) with pET28a carrying the arcA ORF, Km' | This study |
| BL21/pET28a:cytR  | BL21(DE3) with pET28a carrying the cytR ORF, Km' | This study |
| BL21/pET28a:crp   | BL21(DE3) with pET28a carrying the crp ORF, Km' | This study |
| **Plasmids**      |                  |                     |
| pRE112            | pGP704 suicide plasmid, pir dependent, oriT, oriV, sacB, Km' | [2] |
| pBAD33            | arabinose inducible promoter, Km' | [3] |
| pET28a            | Expression vector, Km' | This study |
| pET28a:arcA       | pET28a carrying the arcA ORF, Km' | This study |
| pET28a:cytR       | pET28a carrying the cytR ORF, Km' | This study |
| pET28a:crp        | pET28a carrying the crp ORF, Km' | This study |
| pMS402            | For construct promoter-luxCDABE reporter fusion; Km' | This study |
| cytR-lux          | pMS402 carrying the cytR promoter region, Km' | This study |
| flrA-lux          | pMS402 carrying the flrA promoter region, Km' | This study |
| flik-lux          | pMS402 carrying the flik promoter region, Km' | This study |

* r resistant. Cm, chloramphenicol, PmB, polymyxinB, Km, kanamycin
### Table 2 Primers used in this study

| Name | Sequence (5′–3′) | Amplified fragment |
|------|------------------|--------------------|
| **Primers for construction of mutants** | | |
| ∆arcA-S-F | GCTCTAGACGATCAAGCATTGCTGTA | ∆arcA-S (500) |
| ∆arcA-S-R | AAAGAAAGGCTAGTTACCACTAACTTGA | |
| ∆arcA-X-F | GTAAACGGCCTACCTGTTTTATATTAG | ∆arcA-X (500) |
| ∆arcA-X-R | CCGGACCTACATCAGCCGGTGA | ∆arcA-SX (1000) |
| arcA-F | ATGCAACCCGGCAAGATCT | arcA (717) |
| arcA-R | TTAATCCTCTCAAATCACCCAG | |
| ∆cytR-S-F | CGGGGTACTTCCGGAGGACACGATAC | ∆cytR-S (512) |
| ∆cytR-S-R | GGGAAATCACCACAATCTGGAACGG | |
| ∆cytR-X-F | CCAAGTGGTGTTTATACCCCTTCTCTCCTCG | ∆cytR-X (534) |
| ∆cytR-X-R | CGAGCTTTCCAGCTGAAGCCAATC | ∆cytR-SX (1046) |
| cytR-F | ATGGCGACAATGAAGGATG | cytR (1015) |
| cytR-R | AAGTGGTGTACCTTCCTCTTG | |
| **Primers for identification of plasmid** | | |
| pRE112-U-F | CACGTGTCGTCATCCATTCCG | pRE112-UD (567) |
| pRE112-D-R | TTTGTCCTCAGCAATCCCT | pRE112-U-arcA-D (1284) |
| pBAD33-U-F | AACAAAGCCGGACAAAG | |
| pBAD33-D-R | AGAGGTTCACCGACAAA7 | pBAD33-UD (529) |
| pET28a-U-F | TAATACGACTCACTATAGGG | pET28a-UD (318) |
| pET28a-D-R | GCTATTTGCTCATCGGG | pET28a-U-arcA-D (1395) |
| pET28a-D-D | GCTATTTGCTCATCGGG | pET28a-U-cytR-D (918) |
| **Primers for construction of complemented strain** | | |
| ∆arcA::ParcA-F | CGAGCTTAATCAAACAAAGTGATGGA | ∆arcA::ParcA (732) |
| ∆arcA::ParcA-R | GGGAAATCACCACAATCTGGAACGG | |
| ∆cytR::PcytR-F | CGGGGTACTTCCGGAGGACACGATAC | ∆cytR::PcytR (1031) |
| ∆cytR::PcytR-R | GGGAAATCACCACAATCTGGAACGG | |
| **Primers for protein cloning** | | |
| pET28a-arcA-F | CGGAGATCCATGCAAAAGCAGATACCT | pET28a-arcA (734) |
| pET28a-cytR-F | CCGCCTACTTTAACTCCTAATACCAC | |
| pET28a-cytR-R | CCGCCTACTTTAACTCCTAATACCAC | pET28a-cytR (1095) |
| pET28a-crp-F | CCGGATCATCTTTCTTCTTATTGAGGATG | |
| pET28a-crp-R | CCGGATCATCTTTCTTCTTATTGAGGATG | pET28a-crp (617) |
| **Primers for bioluminescent reporter assays** | | |
| pMS402-cytR-F | CCGGATCCCATGCAAAAGCAGATACCT | pMS402-cytR (1032) |
| pMS402-cyrR-R | CCGCCTACTTTAACTCCTAATACCAC | |
| pMS402-flrA-F | CCGGATCCCATGCAAAAGCAGATACCT | pMS402-flrA (1484) |
| pMS402-flrA-R | CCGCCTACTTTAACTCCTAATACCAC | pMS402-flrA (1484) |
| pMS402-fliK-F | CCGGATCCCATGCAAAAGCAGATACCT | pMS402-fliK (2042) |
| pMS402-fliK-R | CCGGATCCCATGCAAAAGCAGATACCT | |
| **Primers for qRT-PCR** | | |
| 165S rRNA-F | GTGACCGGTGAATGCGTGA | 275 bp |
| 165S rRNA-R | GCGGATCCCATGCAAAAGCAGATACCT | |
| qRT-cytR-F | ATTCGCGGATTAGGATG | |
| qRT-cytR-R | AAGCGGAATTCCTCCGTCAG | 189 bp |
transformed into *E. coli* λpir; 2) intergeneric conjugation between *E. coli* and *V. cholerae*. DNA sequencing was performed to confirm the sequences of the constructed deletion plasmids. The Δ*arcA* mutant strain were complemented with the plasmid cloned into the vector pBAD33. The complemented strain was constructed using a previously reported procedure [34]. Table 2 lists the primers used in this study.

Soft agar motility assay
*V. cholerae* strains were grown in LB broth for overnight and inoculated (1 μl) into freshly poured 0.3% agar plates, followed by incubation and grown at 30 °C for 24 h. The diameters of motility zones at least six independent colonies were averaged [35, 36].

RNA isolation and quantitative real time PCR (qRT-PCR)
Bacterial cultures were grown in LB medium aerobically or anaerobically at 37 °C to the mid-logarithmic phase (OD₆₀₀ approximately 0.6). Total RNA was extracted using TRIzol (Invitrogen, Waltham, MA, USA, #15,596–018), as per manufacturer instructions. cDNA was synthesized using a Prime Script RT Reagent Kit with gDNA Eraser (Takara, Shiga, Japan). qRT-PCR was performed on an Applied Biosystems 7500 sequence detection system with SYBR green fluorescence dye. The 16 s rRNA gene was used as the reference control for sample normalization [37]. Table 2 lists the primers used in this study. The relative expression levels of target transcripts were calculated according to the 2⁻^ΔΔCT method [38]. Each experiment was performed in triplicate. Expression changes of > twofold with *p* < 0.05 were considered statistically significant.

Electrophoretic mobility shift assay (EMSA)
A sequence encoding a ArcA/CytR/CRP-His₆ fusion protein was cloned into vector pET-28a, expressed in *E. coli* BL21 (DE3), and purified using an Ni–NTA-Sefinose Column in accordance with the protocol provided by the manufacturer [37, 39]. EMSA was performed by adding increasing amounts of purified phosphorylated ArcA protein (0, 1.5, 3.0, 4.5, and 6.0 μM or 0, 0.6, 1.2, 1.8 and 2.4 μM) to cytR or *flrA* DNA fragments (50 ng) in a binding buffer [10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM DTT, 50 mM KCl, 50 μg/mL BSA, 10% glycerol] supplemented with 20 nM acetyl phosphate [39], followed by incubation for 40 min at room temperature. Similarly, *fliK* DNA fragments (50 ng) were incubated with increasing amounts of 6× His-tagged CytR or CRP protein (0, 1.2, 2.4, and 3.6 μM or 0, 2, 3, and 4 μM) in a binding buffer [20 mM Tris–HCl (pH 7.5), 50 mM KCl, 1 mM dithiothreitol, 200 μM cAMP, and 10% glycerol] [20], followed by incubation for 40 min at room temperature. The concentration of cAMP used in each reaction was 0.1 mM. 4.5 s RNA served as the negative control. The reaction mixtures were then electrophoresed on a 6% native polyacrylamide gel. Protein was visualized using a Typhoon phosphorimager (GE Healthcare, Chicago, IL, USA).

Bioluminescent reporter assays
The procedures of the lux bioluminescent reporter assay were described in previous study [40]. Briefly, bacterial cultures were grown in LB medium at 37 °C to the mid-logarithmic phase (OD₆₀₀ approximately 0.6). The cultures were transferred into a black 96-well plate with a transparent bottom. Promoter activities were measured and bacterial growth was measured by OD₆₀₀ in a Synergy 2 plate reader (BioTek) at the same time.

Table 2 (continued)

| Name           | Sequence (5′–3′)                     | Amplified fragment |
|----------------|-------------------------------------|--------------------|
| qRT-*flrA*-F   | CCTGAAGGGGTTGAATCTCAA                | 157 bp             |
| qRT-*flrA*-R   | GCATGGTGTATTGGCGGATC                | 235 bp             |
| qRT-*fliK*-F   | CTCAAAGCGTACGGTCAAAT                | 300 bp             |
| qRT-*fliK*-R   | TGACCAGTTGGACTAGC                  |                    |
| Primers for EMSA |                                    |                    |
| EMSA-cytR-F  | ATCCGGTTTTATAACCGTGAT                | 200 bp             |
| EMSA-cytR-R  | CTAGAAAACTATGGCCATAACCA              |                    |
| EMSA-*flrA*-F | ATAAAGTCAGCTTGGAGCTCAA              | 300 bp             |
| EMSA-*flrA*-R | AGGTGAGATTATTTGCTTTATAT             |                    |
| EMSA-*fliK*-F | GTCAAAAACGGAAATCTCATCA              |                    |
| EMSA-*fliK*-R | AGTGGAAAAAAGATCCTGAGCAT             |                    |

Underlined letters show Xba I, Sac I, Kpn I, BamHI, or Xhol restriction site
F/R: upstream and downstream primers of gene, S/X-F/R: The upstream and downstream primers for the upstream and downstream gene fragments of arcA in the E12382 genome, U/D-F/R: Upstream and downstream sequencing primers of plasmid
Statistical analyses
All data are expressed as means ± standard deviation (SD). Differences between two groups were evaluated using independent-samples t-test or Mann–Whitney U test. Values of \( p \leq 0.05 \), 0.01, or 0.001 were considered to be statistically significant (*), highly significant (**), or extremely significant (***) respectively.

Abbreviations
ArcA: Anoxic redox control cognate response regulator; CRP: Cyclic-AMP receptor protein; CytR: Cytidine repressor; WT: Wild-type; ΔarcA:: ArcA isogenic deletion mutant strain; ΔcyaA: CyaA complementation strain of arcA; LB: Luria–Bertani; EMSA: Electrophoretic mobility shift.

Supplementary Information
The online version contains supplementary material available at https://doi.org/10.1186/s12866-022-02435-y.

Additional file 1.

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Availability of data and materials
All data generated or analyzed during this study are included in this published article.

Declarations
Ethics approval and consent to participate
Not applicable

Consent for publication
Not applicable

Competing interests
The authors declare that they have no competing interests

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