MlrA, a MerR family regulator in *Vibrio cholerae*, senses the anaerobic signal in the small intestine of the host to promote bacterial intestinal colonization

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**ABSTRACT**

*Vibrio cholerae* (*V.* cholerae*), one of the most important bacterial pathogens in history, is a gram-negative motile bacterium that causes fatal pandemic disease in humans via oral ingestion of contaminated water or food. This process involves the coordinated actions of numerous regulatory factors. The MerR family regulators, which are widespread in prokaryotes, have been reported to be associated with pathogenicity. However, the role of the MerR family regulators in *V. cholerae* virulence remains unknown. Our study systematically investigated the influence of MerR family regulators on intestinal colonization of *V. cholerae* within the host. Among the five MerR family regulators, MlrA was found to significantly promote the colonization capacity of *V. cholerae* in infant mice. Furthermore, we revealed that MlrA increases bacterial intestinal colonization by directly enhancing the expression of tcpA, which encodes one of the most important virulence factors in *V. cholerae*, by binding to its promoter region. In addition, we revealed that during infection, *mlrA* is activated by anaerobic signals in the small intestine of the host through *Fnr*. In summary, our findings reveal a MlrA-mediated virulence regulation pathway that enables *V. cholerae* to sense environmental signals at the infection site to precisely activate virulence gene expression, thus providing useful insights into the pathogenic mechanisms of *V. cholerae*.

**Introduction**

*Vibrio cholerae* (*V.* cholerae*), a gram-negative bacterium, is the etiological agent of cholera, which affects millions of individuals leading to approximately 100,000 deaths per year.\(^1\) Although the development of oral rehydration therapy has dramatically reduced the fatality rate of treated cases, cholera continues to present a severe global health and economic challenge.\(^2\) Thus far, over 200 serogroups of *V. cholerae* have been identified. The serogroup O1 El Tor biotype, which appears to have emerged in 1961 in Indonesia, is currently the predominant cause of cholera globally.\(^3\)

*V. cholerae* has a complex life cycle involving transitions between various aquatic environments, such as surface seawater, and the human small intestine.\(^4\) *V. cholerae* can survive in aquatic environments year-round.\(^5\) In the host, *V. cholerae* preferentially colonizes the epithelium of the distal small intestine.\(^6\) Once it enters the small intestine, *V. cholerae* mainly produces two major virulence factors: the cholera toxin (CT) encoded by *ctxAB* on the lysogenic CTXΦ bacteriophage, which directly causes diarrhea,\(^7\) and the toxin-coregulated pilus (TCP), which is required for bacterial attachment to enterocytes and intestinal colonization.\(^8\) TCP belongs to the type-4 Pilli family,\(^9\) and has been identified as a critical colonization factor for *V. cholerae* in both animal models and humans.\(^10\) TCP is a polymer of repeating subunits of the major pilin protein, TcpA,\(^11\) which is encoded by *tcpA*.\(^12\) In *V. cholerae*, the regulation of TCP biosynthesis is complex and orchestrated, forming an elaborate regulatory network. The TCP operon is mainly activated by the AraC/XylS-family transcriptional regulator, ToxT,\(^13\) which is regulated by TcpP and...
ToxR. Recently, TCP was also found to be regulated by other regulators, including Fur, HapR, AhpAB, CRP, and CarR. However, the regulatory mechanisms underlying TCP have not been fully elucidated.

Upon transition into the gut, V. cholerae undergoes a carefully orchestrated set of gene expression changes to adapt to host-specific environmental stresses, such as temperature, pH, oxygen concentration, and bile salts. Among these intestinal signals, oxygen concentration is a key environmental factor that governs the physiology of V. cholerae. Within the small intestine, oxygen concentration decreases below 3% and is further lowered to a nearly anoxic environment by the action of the commensal microbiota and host metabolism. It has been shown that V. cholerae promotes its colonization capacity in response to anaerobic environment. For example, TCP induction was detected when V. cholerae cultures were subjected to oxygen deprivation. To adapt to the low oxygen concentration, trimethylamine-N-oxide (TMAO) can substitute oxygen as the final electron acceptor, and stimulate CT production in V. cholerae.

Members of the MerR family of regulators are widespread in bacteria and play important roles in adaptation to environmental challenges. In general, MerR family regulators consist of an N-terminal helix-turn-helix DNA binding region and a C-terminal effector binding region, which respond to environmental stimuli including metal ions. Binding of the metal ion at the C-terminal repressor-binding site of MerR family regulators provokes an allosteric change at the N-terminal DNA binding region of the protein, which in turn transduces changes in the promoter structure, resulting in transcription activation.

MerR family regulators have been proven to affect virulence in many bacteria, such as ZntR in Brucella strains, VarN in Salmonella enterica serovar Typhimurium (Salmonella Typhimurium); CueR in Pseudomonas aeruginosa, and SoxR in Vibrio vulnificus. However, whether MerR family regulators are involved in the virulence regulation of V. cholerae has not been well characterized.

In this study, we aimed to systematically investigate the effects of all five annotated MerR family regulators on V. cholerae pathogenicity. Among these regulators, VCA0056 (MlrA) was shown to be essential for the efficient intestinal colonization of bacteria. Moreover, MlrA was activated by Fnr in response to the anaerobic signal in small intestine, and then it activated the TCP operon by directly binding to the promoter of tcpA. Overall, this study characterizes a novel MlrA-mediated regulatory pathway to enhance the virulence and colonization capacity of V. cholerae.

Results

Multiple MerR family regulators impact intestinal colonization in V. cholerae

Homologs of MerR family regulators were screened for in the genome of V. cholerae EL2382, the 7th pandemic strain. Five genes were annotated as MerR family regulators: vca0056 (mlrA), vca0084 (soxR), vca0264 (merR1), vco077 (zntR), and vco974 (cueR). Domain structure analysis of these regulators revealed that all five proteins contain an N-terminal DNA-binding helix-turn-helix (HTH) motif (Figure 1a). C-terminal domain structure analysis showed that VCO077 and VCO974 contain a metal-binding site, VCA0084 contains a [2Fe-2S] cluster-binding site, and VCA0264 contains a methyltransferase domain. No predicted domains were found in VCA0056 (MlrA). This suggests that these five MerR family regulators may have different functions and perform different roles in V. cholerae virulence.

In-frame deletion mutants of MerR family regulators were generated to evaluate their effect on bacterial intestinal colonization. The competitive assay in infant mice showed that the competitive index (CI) values of ΔmlrA, ΔsoxR, ΔmerR1, ΔzntR, and ΔcueR strains versus wild type (WT) were 0.058, 0.439, 0.788, 1.132, and 4.161 (Figure 1b), respectively. Consistently, the competitive infection assay results exhibited trends similar to those of a previous Tn-Seq analysis in infant rabbit. These data indicate that MlrA may play an important role in the intestinal colonization of V. cholerae.

Inducing the expression of mlrA in small intestine promotes the colonization of V. cholerae

Next, we compared the expression of mlrA by qRT-PCR in V. cholerae-infected infant mouse intestine
and Luria-Bertani broth (LB) media. The results revealed that *mlrA* expression was approximately 5.25-fold higher in the small intestine of mice than in vitro (Figure 2a). This indicated that *mlrA* may have a positive effect on the colonization of *V. cholerae* in vivo. We further analyzed the colonization ability of *ΔmlrA* and the complementary strain (*ΔmlrA+*) using a competitive assay. Consistent with the previous results, the competitive assay showed that the colonization ability of *ΔmlrA* was restored to the WT level upon complementation (Figure 2b). These results confirmed that a mutation in *mlrA* attenuated the colonization ability of *V. cholerae* in mice. As the mouse intestine is an anaerobic environment, we examined the growth curves of WT, *ΔmlrA*, and *ΔmlrA+* under anaerobic conditions. The growth curve showed that no significant growth defect was identified among WT, *ΔmlrA*, and *ΔmlrA+* in vitro (Figure 2c), indicating that the influence of MlrA on the intestinal colonization of *V. cholerae* was not due to different growth rates.

*MlrA increases bacterial virulence by regulating tcpA expression by directly binding to its promoter region*

To analyze the mechanism by which *mlrA* regulates the colonization of *V. cholerae*, RNA-seq of WT and *ΔmlrA* grown in AKI medium was performed. A total of 153 genes showed differential expression in *ΔmlrA* compared to WT.
between WT and ΔmlrA, when considering p-values <0.05, and |fold-change|≥2. Overall, 46 downregulated and 107 upregulated genes were identified in the ΔmlrA strain (Table S1). Among these genes, the TCP operon genes were significantly downregulated in ΔmlrA. qRT-PCR analysis of the expression of major virulence genes in *V. cholerae*, including tcpA, toxR, toxT, ctxA, and tcpP, showed that only the expression of tcpA, which encodes the basic protein of TCP, was significantly decreased in ΔmlrA compared with WT, and was restored to wild-type levels in ΔmlrA+ (Figure 3a). However, the expression of tcpP, toxR, toxT, and ctxA was not significantly different among the WT, ΔmlrA, and ΔmlrA+ strains (Fig. S1A), which is consistent with the RNA-seq results (Table S1). Furthermore, western blotting revealed that the production of TcpA was reduced in ΔmlrA compared with that in WT, which was restored to WT levels in ΔmlrA+ (Figure 3b). In contrast, no difference in the production of cholera toxin was detected among WT, ΔmlrA, and ΔmlrA+ (Fig. S1B). These results indicate that MlrA positively regulates the expression of the TCP operon but not other virulence genes.

Next, we investigated whether MlrA directly regulated tcpA expression by binding to its promoter. An electrophoretic mobility shift (EMSA) assay

![Figure 3](image_url) MlrA directly promotes the expression of tcpA. (a) qRT-PCR expression level of tcpA in WT, ΔmlrA, and ΔmlrA+ strains at the logarithmic phase in AKI medium. Data are represented as the mean ± SD (n = 3). (b) Representative western blotting image and quantitative analysis of TcpA in WT, ΔmlrA and ΔmlrA+ strains in AKI medium. RNA polymerase (RNAP) was used as a loading control. Data are represented as the mean ± SD (n = 3). (c) The fold enrichment of the promoters for toxT and the negative control (rpoS) in the chromatin immunoprecipitation assay. (d) EMSA of the specific binding of purified MlrA to the promoter region of tcpA and kana (negative control). (e) EMSA of the specific binding of purified MlrA to the promoter region of tcpA without the −10 and −35 elements (PtcpA−1), and the promoter region of tcpA without the binding site (PtcpA−2). (f) MlrA binds to a motif in the tcpA promoter region. The protected region shows a significantly reduced peak intensities (blue) pattern compared to the intensities seen in the control (red). The identified MlrA-binding motif is shown in a box at the bottom of the figure. Two-way ANOVA (a, b) and two-tailed unpaired Student’s t-test (C) were used to calculate P values. * P < .05, ** P < .01, *** P < .001; n.s., not significant.
was performed using purified MlrA-His6. As shown in Figure 3d, with increasing concentrations of MlrA protein; migrating bands were observed for the promoter of tcpA. MlrA could not bind to the promoter region of tcpP, toxR, toxT ctxA, or kana (negative control) under the same experimental conditions (Fig. S2A and Figure 3d). Consistent with this, chromatin immunoprecipitation-qPCR (ChIP-qPCR) results showed that the promoter of tcpA was enriched 2.76-fold in MlrA-ChIP samples compared with mock ChIP samples (Figure 3c), which confirmed the results of EMSA. In contrast, fold enrichment of rpoS and the promoters of tcpP, toxR, toxT, and ctxA showed no significant difference between the MlrA-ChIP and mock ChIP samples (Figure 3c and S2B). These results indicated that MlrA specifically binds to the tcpA promoter region both in vitro and in vivo.

Typically, the binding site of classical MerR family regulators is always located in the spacer between the −10 and −35 elements of the promoters of downstream genes. When the C-terminal effector-recognition domain binds to its cognate ligand, the transcription factor untwists and shortens the DNA, realigning the −10 and −35 elements to allow the RNA Polymerase (RNAP) holoenzyme to bind and activate transcription. To verify whether MlrA specifically binds to the −10 and −35 elements of the tcpA promoter, an EMSA of PtcpA−1(−10 and −35 elements deleted in the tcpA promoter) was performed (Figure 3e). The results showed that MlrA could still bind to the tcpA promoter without the −10 and −35 elements. This indicated that the binding site of MlrA was not located between the −10 and −35 elements of the tcpA promoter region. To identify the precise binding site for MlrA in the tcpA promoter region, a dye-based DNase I footprinting assay was performed. The results revealed a specific MlrA-bound sequence containing an 18-base pair motif (5’-GAATTGAATAAGTTAGTA-3’, −234 to −217 bp from the tcpA translational start site) in the tcpA promoter region (figure 3f). To further confirm that this motif is necessary for the binding of MlrA, EMSA of PtcpA-2 (the binding site identified by the dye-based DNase I footprinting assay deleted in the tcpA promoter) was performed (Figure 3e). The results showed that PtcpA-2 cannot bind to MlrA, confirming that the 18 base pair motif we identified is required for the binding ability of MlrA to PtcpA.

**Activation of tcpA by MlrA is not mediated by metal ions**

Members of the MerR family regulators are commonly found in different bacteria as metal sensing regulators. Therefore, we speculated that the activation of tcpA by MlrA also depends on metal ions. The growth curve was plotted for ΔmlrA and WT in LB media supplemented with ZnCl2, CuCl2, and HgSO4, which always interact with the MerR family regulators. The results showed that there were no differences in growth between ΔmlrA and WT samples under these conditions (Figure 4a-c). To investigate whether MlrA-mediated activation of tcpA expression is influenced by metal ions, the expression of tcpA in WT and ΔmlrA in AKI media with or without metal ions was analyzed by qRT-PCR. The results showed that the presence of metal ions did not influence the expression of tcpA in ΔmlrA or the WT (Figure 4d, e). Furthermore, EMSA confirmed that the binding of MlrA to the tcpA promoter was not influenced by metal ions (figure 4f). These results suggest that the regulation of the tcpA by MlrA is not regulated by metal ions.

**mlrA is activated by Fnr in an anaerobic environment**

As mlrA does not respond to metal ion signals, it exhibits significant upregulation in the small intestine of mice. We speculated that MlrA might respond to some unique signals in vivo. Temperature, pH, oxygen level, and bile salt presence are the major differing factors between the aquatic environment and the infant mouse intestine. The expression of mlrA under these four different conditions was compared using qRT-PCR. The results showed that the expression of mlrA was enhanced under anaerobic condition, but was not influenced by temperature, pH, or bile salts (Figure 5a-d). This indicates that mlrA expression is upregulated in an anaerobic environment.

Fnr and ArcA are the two major global regulators in response to anaerobic signals in *V. cholerae*, and
qRT-PCR assays were performed to investigate whether mlrA expression is regulated by Fnr and/or ArcA under anaerobic conditions. The qRT-PCR results showed that the expression of mlrA exhibited a 3.80-fold decrease in Δfmr compared to WT under anaerobic conditions (Figure 5e). However, mlrA expression was not significantly different between ΔarcA and WT under the same conditions (Figure 5e). Furthermore, mlrA expression in infant mouse intestines was analyzed in WT, Δfmr, and ΔarcA variants. The results showed that the expression of mlrA was downregulated in Δfmr compared to WT in vivo, while there was no significant difference in mlrA expression between WT and ΔarcA in vivo (figure 5f). These results suggest that mlrA expression is regulated by anaerobic signaling via Fnr in vitro and in vivo.

Taken together, these data indicate the expression of mlrA is activated by Fnr in response to the anaerobic signal in the small intestine of host, which leads to the increased pathogenicity of V. cholerae by directly enhancing the expression of tcpA.

Discussion

The MerR family transcriptional regulators are widespread in bacteria and activate the transcription of genes to perform multiple functions. However, the roles of the MerR family regulators in V. cholerae remain unclear. In this study, a novel MerR family regulator-mediated virulence regulation mechanism was identified in V. cholerae. We found that the expression of mlrA is upregulated in the small intestine of the host, and thus promotes the colonization of bacteria by directly upregulating the expression of TCP. MlrA in E. coli, which shares 50% sequence similarity with MlrA in V. cholerae, regulates biofilm formation through the major matrix regulator CsgD. However, the
RNA-seq results did not show differences between the WT and ΔmlrA in the expression of genes related to biofilm formation, such as biofilm formation master regulator vpsR and Vibrio polysaccharide (VPS) operons, indicating that the function of MlrA in V. cholerae differs from its homolog in E. coli. By analyzing the genome of V. cholerae EL2382, we found that there were homologous sequences of identified MlrA-binding sites in the promoter region of two genes (vca0219 and vc0910). The RNA-seq data showed that the expression of vca0219 and vc0910 are downregulated 3.12-fold and upregulated 2.10-fold, respectively, in ΔmlrA, suggesting that MlrA may directly regulate the expression of these two genes. Vca0219 encodes the hemolysin (HlyA), which is an important virulence factor in V. cholerae that belongs to the pore-forming toxin family. Vc0910 encodes the EIIB/EIIC proteins of the carbohydrate phosphotransferase system (PTS). Previous research has shown that cooperative regulation of PTS is important for bacterial survival and virulence gene expression. Thus, we speculate that MlrA may also contribute to the virulence and/or intestinal survival through regulating hemolysin synthesis and carbon source utilization in V. cholerae, and plan to investigate this in future studies.

Unlike most MerR family regulators, a unique binding site for MlrA was identified by us from −234 to −217 bp from the translational start site of tcpA. We also showed that, unlike most MerR family regulators, MlrA cannot bind to the −35 and −10 elements of the promoter region of the gene (tcpA) that it regulates. To date, very few
MerR family regulators have been shown to bind to parts of the DNA region other than the –35 and –10 elements. For example, MlrA in *E. coli* directly binds to –146 to –133 bp segment from the csgD translational start site with a palindromic sequence of AAAATTGTACA(12 N) TGTACAATTTT. In addition, MerR-like protein BldC in *Streptomyces venezuelae* binds to the –50 to –80 bp segment from the translational start site of *whiI*, which plays a critical role in *Streptomyces* differentiation. Although these two binding sites do not share obvious sequence homology with that of MlrA in *V. cholerae*, all three binding sites contain a conserved AATT motif. It is likely that this AATT motif act as an essential domain for DNA-protein interactions of MerR family regulators.

The C-terminal effector-binding domains of the MerR family transcriptional regulators are highly divergent and can sense a variety of cellular signals. Several signals sensed by MerR family regulators have been described, including metal ions, oxidative stress, carbonyl and nitrosative stress, and diverse drug-like compounds. In this study, five potential MerR family regulators were detected in *V. cholerae*. Domain-structure analysis showed that ligand-binding domains were present in the C-terminal effector-binding domain of SoxR, MerR1, ZntR and CueR, but not MlrA. Previous studies have shown that the homolog of ZntR and CueR are metal-sensing transcription regulators that play important roles in the virulence regulation of *Yersinia pseudotuberculosis* and *Pseudomonas aeruginosa*, respectively. The homolog of SoxR is an oxidative stress-sensing transcriptional activator associated with the virulence of *P. aeruginosa* and *Salmonella Typhimurium*. Comparative genomics analysis showed that zntR and cueR are present in all in *V. cholerae* strains, while soxR and mlrA are present in all pandemic strains, but absent from some non-pandemic strains (Fig. S3). However, competition assays showed that these regulators had no obvious association with intestinal colonization by *V. cholerae* in infant mice. It is likely that these regulators may contribute to the virulence of *V. cholerae* via mechanisms that cannot be detected in our animal model, or play a role in the environmental survival of bacteria. Unlike SoxR, MerR1, ZntR, and CueR, MlrA does not contain a predicted ligand-binding domain. Furthermore, we showed that *mlrA* expression is activated by Fnr under anaerobic conditions. To the best of our knowledge, this study provides the first demonstration that an anaerobic environment acts as a signal to activate a MerR family regulator.

In conclusion, we describe a novel signaling pathway with detailed mechanisms that links MlrA in *V. cholerae* to anaerobic signals in the small intestine of hosts. These findings provide a paradigm of *V. cholerae* signal perception and virulence regulation in the small intestine, which can be employed to investigate other pathogens in the human gastrointestinal tract.

**Materials and Methods**

**Ethics statement**

All animal experiments were performed according to the standards set forth by the Guide for the Care and Use of Laboratory Animals. The experimental protocols were approved by the Institutional Animal Care Committee of Nankai University.

**Bacterial strains, plasmids, and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table S2 and S3. Briefly *V. cholerae* O1 El Tor strain EL2382, isolated in 1994, was provided by Shanghai Municipal Centers for Disease Control and Prevention. *Escherichia coli* BL21-DE3 cells were used as recombinant protein expression hosts. The *E. coli* S17/λpir strain was used for conjugation. The bacterial strains were grown in Luria-Bertani (LB) broth or AKI medium (1.5% Bacto Peptone, 0.4% yeast extract, 0.5% NaCl, and 0.3% NaHCO₃). For aerobic condition, bacteria were grown at 37°C with shaking at 180 rpm. For anaerobic condition, bacteria were grown at 37°C in an anaerobic incubator (YQX-II, Shanghai, China) and oxygen-free nitrogen was used as the carrier gas. Antibiotics were used as following concentrations: polymyxin B, 40 μg/mL; ampicillin, 50 μg/mL; chloramphenicol, 25 μg/mL.
**Mutant construction and complementation**

All primers used in this study are listed in Table S4. Construction of the mutants was performed using the suicide vector pRE112, according to a previously described procedure. For complementation, genes with native promoters were amplified by PCR and cloned into the pBAD33 vector. For ChIP-qPCR, mlrA was amplified along with its promoter regions and cloned into pBAD33 in frame with a C-terminal 3× FLAG-tag. For MlrA purification, mlrA was amplified and cloned into the pET28a vector.

**RNA isolation and qRT-PCR**

To detect the gene expression in vivo, the samples were harvested from the small intestinal tissue of mice. To analyze the expression of virulence genes in vitro, the samples were collected from AKI medium under aerobic condition. To analyze the expression of mlrA in response to the anaerobic signal, we collected the samples incubated in LB medium under aerobic or anaerobic condition. When comparing the expression of mlrA in WT, Δfin, and ΔarcA, the samples were collected under anaerobic condition. Total RNA was isolated using TRizol Reagent (15596026; Invitrogen, Waltham, MA, USA), according to the manufacturer’s protocol. Next, total RNA content was determined using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). Three independent experiments were performed. cDNA was synthesized using a PrimeScript™ RT Reagent Kit with gDNA Eraser (Takara, Kusatsu, Japan) according to the manufacturer’s instructions.

The qRT-PCR analysis was conducted with Applied Biosystems ABI 7500 (Applied Biosystems, Waltham, MA, USA) using SYBR green fluorescence dye. In order to normalize samples, the rrsA gene was used as a reference control, and relative expression levels were calculated as fold-change values using the 2^{-ΔΔCT} method. Each experiment was performed in triplicate.

**RNA-seq**

WT and ΔmlrA strains were grown overnight in LB broth and diluted at 1:100 in a fresh AKI medium. After the cultures were grown for 4 h, the bacteria were collected by centrifugation. Total RNA was isolated using TRizol Reagent (Invitrogen) according to the manufacturer’s protocol. Three independent experiments were performed. Samples were analyzed by Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

**Growth curve**

To determine the growth curve of each strain, overnight cultures were diluted to 10^6 /mL in LB broth in a flask containing 20 mL of LB broth with or without metal ions (1 mM ZnCl₂, 50 μM CuCl₂, and 1 μM HgSO₄). For the aerobic condition, a 200 μL aliquot was added to a 96-well microplate and incubated for 24 hours at 37°C with shaking. The absorbance was recorded at 600 nm. For the anaerobic condition: a 100 μL aliquot was removed from the tube and suitable dilutions were plated on LB agar plates in anaerobic incubator. The absorbance was recorded at 600 nm. The experiments were independently performed three times.

**Electrophoretic mobility shift assay (EMSA)**

The 6× His-tagged MlrA protein was expressed and purified in *Escherichia coli* BL21-DE3 cells. Target DNA fragments were amplified and purified using a SPARKeasy Gel DNA Extraction Kit (AE0101 Spark jade, Jinan, China). Purified DNA fragments (40 ng) were incubated at 30°C for 30 min with 6× His-tagged MlrA protein at concentrations ranging from 0–2 μM, in 20 μL solutions containing binding buffer (10 mM Tris-HCl [pH 7.5], 0.2 mM dithiothreitol, 5 mM MgCl₂, 10 mM KCl, and 10% glycerol). The protein-DNA fragments were electrophoretically separated on a native polyacrylamide gel at 4°C and 90 V/cm. UV transillumination was used to visualize the protein bands on the gel after 10 minutes of staining in 0.1% GelRed. During effector screening, the purified 6× His-tagged MlrA protein was incubated at 25°C for 10 min with 1 mM ZnCl₂, 50 μM CuCl₂, or 1 μM HgSO₄ before being added to the binding buffer.
**Chromatin immunoprecipitation (ChIP) and ChIP qPCR**

Bacteria were grown at 37°C to mid-exponential phase and induced L-arabinose. After centrifugation, 1% formaldehyde was added and incubated at approximately 25°C for 25 min. Then, 0.5 M Glycine was added and mixed. The bacteria were incubated for an additional 5 min to quench the cross-linking reaction. Cross-linked bacteria were harvested and washed three times with ice-cold Tris-buffered saline. The cross-linked bacteria were resuspended in 500 mL lysis buffer (10 mM Tris [pH 7.5], 1 mM EDTA, 100 mM NaCl, 1 mM protease inhibitor cocktail, 1 mg/mL lysozyme, 0.1 mg/mL RNase A) and incubated at 37°C for 30 min. Then, immunoprecipitation (IP) buffer (100 mM Tris-HCl [pH 7.5], 200 mM NaCl, 1 mM EDTA, 2% v/v Triton X-100, 1 mM phenylmethylene sulfonyl fluoride) was incubated, and the lysates were further sonicated to generate DNA fragments of approximately 300 bp length. After centrifugation at 12,000 g for 10 minutes, supernatants were incubated with anti-3× FLAG antibody. (#F1804; Sigma-Aldrich, St. Louis, MO, USA) and protein A magnetic beads (Invitrogen; #10002D). DNA samples were subsequently purified using a PCR purification kit (#28104; Qiagen; #28104) and eluted in 15 mL distilled water. The samples were analyzed by MAP Biotech Co., Ltd. (Shanghai, China). The results were analyzed using a peak scanner (Applied Biosystems).

**Western blotting**

The *V. cholerae* strains were grown overnight and diluted 1:100 in a fresh AKI medium. After being grown anaerobically for 4 h and reaching an optical density of 0.2, the cultures were shaken for 2–2.5 h to reach an optical density of 1.0. Bacterial cells were harvested, washed, and resuspended in phosphate-buffered saline (PBS) at 4°C, sonicated for 15 cycles of 30 s on/off at 95% power. The cell debris was removed by centrifugation at 12,000 × g for 10 min at 4°C. To quantify the supernatants, we used BSA method and separated equal amounts of the total protein using SDS-PAGE with a 4–12% gel and transferred onto PVDF membranes (Bio-Rad) by electroblotting. Blots for RNA polymerase (RNAP), cholera toxin, and TcpA were incubated with anti-RNA polymerase beta (ab191598), anti-cholera toxin (ab123129), and anti-TcpA monoclonal antibodies (Willget Biotech Co., Ltd., Shanghai, China), respectively, at a dilution of 1:2000. To detect proteins, the blots were incubated with goat anti-rabbit IgG secondary antibodies (1:5000 dilution; Sparkjade, EF0002) with horse-radish peroxidase. Detection was performed using a Sparkjade ECL Plus (ED0016; Sparkjade) detection system. Images were acquired using an Amersham Imager 600 system (General Electric).

**Dye primer-based DNase I footprinting assay**

DNase I footprinting procedures were modified from published procedures. Approximately 200-bp fragments of the tcpA promoter regions were generated by PCR with 6-FAM primers. Various amounts of MlrA protein were added to 40 nanograms of 6-FAM-labeled tcpA promoter in a binding buffer (10 mM Tris-HCl [pH 7.5], 0.2 mM dithiothreitol, 5 mM MgCl2, 10 mM KCl, and 10% glycerol) from 0 to 1 mM. 0.05 U DNase I (Sigma; AMPD1) was added to a 20-μL solution for 10 min at 37°C. The reaction was stopped by heating at 85°C for 10 min in the presence of 250 mM ethylenediaminetetraacetic acid (EDTA). DNA fragments were purified using the QIAquick PCR Purification Kit (Qiagen; #28104) and eluted in 15 μL distilled water. The samples were analyzed by MAP Biotech Co., Ltd. (Shanghai, China). The results were analyzed using a peak scanner (Applied Biosystems).

**Intestinal colonization assay**

Five-day-old CD-1 mice were purchased from Beijing Vital River Laboratory Animal Technology (Beijing, China) and placed in
incubators at 30°C. An in vivo competition assay for intestinal colonization was performed as previously described with minor modification. Briefly, the V. cholerae lacZ+ strains (wild-type and mutants) and lacZ− strains (ΔlacZ) were grown overnight at 37°C with aeration in LB broth. Approximately 10^5 lacZ+ strains were mixed with an equal number of lacZ− cells and the mixtures were intragastrically administered to groups of eight anesthetized mice. In order to enumerate the recovered bacteria and obtain output ratios, the small intestine was removed, weighed, homogenized, and plated on LB agar plates containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal). The competitive index (CI) was determined as the output ratio of lacZ+ to lacZ− cells, divided by the input ratio of lacZ+ to lacZ− cells.

Statistical analyses

Data were analyzed using a t-test, two-way ANOVA or Mann–Whitney U test, and differences were evaluated using independent samples t-tests. Values of p < .05, 0.01, or 0.001 were considered statistically significant (*), highly significant (**), or extremely significant (***) respectively, and n.s. represents no significance. Figures were drawn using Origin 8.5 (Origin Lab Corporation).

Disclosure statement

The authors report there are no competing interests to declare.

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Author contributions

BL and DH designed the study; JLW, YTL, FL, WDL, RYL, HS, JLQ, and XHF conducted the experiments and data analyses. BL, JLW, and YTL wrote and edited the manuscript.

Data availability statement

RNA sequencing data generated in this study are available in the NCBI SRA database. Accession to cite SRA data: PRJNA870313 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA870313.

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