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The Transcription Factor Mlc Promotes *Vibrio cholerae* Biofilm Formation through Repression of Phosphotransferase System Components

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The phosphoenol phosphotransferase system (PTS) is a multicomponent signal transduction cascade that regulates diverse aspects of bacterial cellular physiology in response to the availability of high-energy sugars in the environment. Many PTS components are repressed at the transcriptional level when the substrates they transport are not available. In *Escherichia* coli, the transcription factor Mlc (for *m*akes *l*arge *c*olonies) represses transcription of the genes encoding enzyme I (EI), histidine protein (HPr), and the glucose-specific enzyme IIBC (EIIBC^{Glc}) in defined media that lack PTS substrates. When glucose is present, the unphosphorylated form of EIIBC^{Glc} sequesters Mlc to the cell membrane, preventing its interaction with DNA. Very little is known about *Vibrio cholerae* Mlc. We found that *V. cholerae* Mlc activates biofilm formation in LB broth but not in defined medium supplemented with either pyruvate or glucose. Therefore, we questioned whether *V. cholerae* Mlc functions differently than *E. coli* Mlc. Here we have shown that, like *E. coli* Mlc, *V. cholerae* Mlc represses transcription of PTS components in both defined medium and LB broth and that *E. coli* Mlc is able to rescue the biofilm defect of a *V. cholerae* Δ*mlc* mutant. Furthermore, we provide evidence that Mlc indirectly activates transcription of the *vps* genes by repressing expression of EI. Because activation of the *vps* genes by Mlc occurs under only a subset of the conditions in which repression of PTS components is observed, we conclude that additional inputs present in LB broth are required for activation of *vps* gene transcription by Mlc.

To thrive, a bacterium must sense and respond to carbohydrates in its environment. The phosphoenolpyruvate phosphotransferase system (PTS) is a central and far-reaching multicomponent signal transduction cascade that regulates many aspects of the bacterial response to carbohydrates, from chemotaxis and transport to storage and catabolism (1).

The PTS cascade is initiated by phosphotransfer from phosphoenolpyruvate (PEP) to enzyme I (EI) (Fig. 1). From here, phosphate is transferred sequentially to histidine protein (HPr) or one of its homologs and then to the multicomponent, sugar-specific enzymes II. There are more than 10 enzymes II encoded in the *Vibrio cholerae* genome, and the sugar specificities of only a few are known (2). While the enzymes IIA (EIIA) and IIB (EIIB) accept phosphate in turn, the enzyme IIC (EIIC) subunit, which spans the cytoplasmic membrane, transports PTS-dependent sugars into the cell, allowing them to be phosphorylated by EIIB. Thus, phosphate is channeled from PEP through PTS components to incoming PTS-dependent sugars.

The phosphorylation state of PTS components depends on the intracellular stores of PEP and the abundance of PTS-dependent sugars in the environment. For example, when PTS-dependent sugars are scarce and PEP is available as a phosphodonor, phosphate accumulates on PTS components. In contrast, when PTS sugars are abundant in the environment, sugar transport through the PTS removes phosphate from the general PTS components EI and HPr. These in turn receive phosphate from expressed sugar-specific PTS components that do not participate directly in transport of the available sugar, and these become dephosphorylated as well. Therefore, PTS components are ideal reporters of the overall nutritional status of the cell. In addition to facilitating the transport of high-energy carbohydrates, they coordinate the bacterial response to carbohydrate availability through direct interactions with other proteins (3, 4).

Transcription of many PTS components is repressed when they are not required for transport. For example, the *Escherichia coli* ROK (*r*epressor *O*rf *k*inase) family transcription factor Mlc represses general as well as selected sugar-specific PTS components, such as the glucose-specific EIIBC (EIIBC^{Glc}), when glucose is scarce (Fig. 1) (5–10). When glucose is abundant, Mlc is sequestered to the inner membrane through a direct interaction with the unphosphorylated form of the EIIB subunit of EIIBC^{Glc}. This prevents its interaction with DNA (11–14).

Vibrio cholerae uses an exopolysaccharide and protein-based extracellular matrix to attach to surfaces. Synthesis of the *Vibrio* polysaccharide (VPS) biofilm exopolysaccharide is encoded by the *vps* genes (15). In addition, three proteins, RbmA, Bap1, and RbmC, are required for formation of contacts with other cells and with the surface (16). Synthesis of this extracellular matrix is tightly regulated. We recently determined that sugars transported by the PTS activate transcription of the *vps* genes and biofilm formation (17–20). Furthermore, even in the absence of PTS substrates, the network of *Vibrio cholerae* PTS components forms a complex web that regulates *vps* gene transcription and biofilm formation through multiple independent pathways (2, 20–23).

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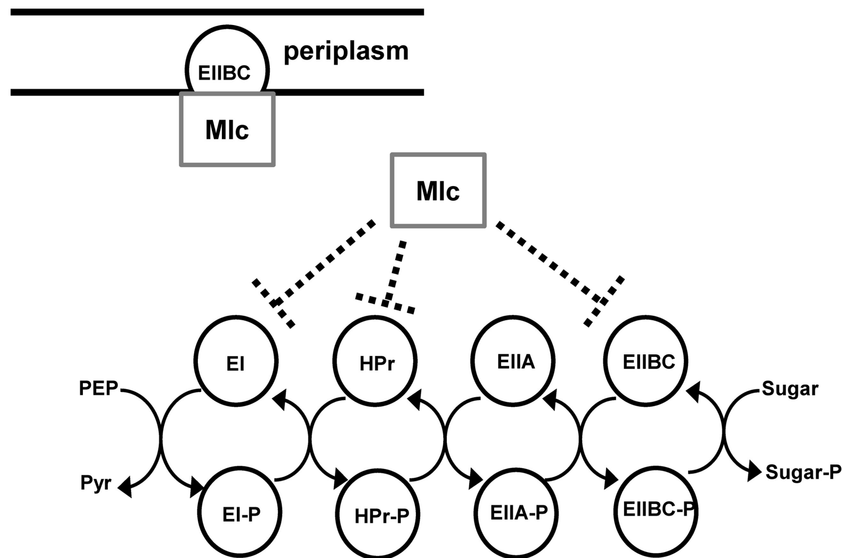

FIG 1 Mlc interacts with the PTS. A schematic representation of the PTS, including the various ways in which *E. coli* Mlc is known to interact with PTS components.

We observed that Mlc activated *V. cholerae* biofilm formation in LB broth but not in a defined medium supplemented either with glucose, which is transported by the PTS, or pyruvate, which is PTS independent. Because *E. coli* Mlc was reported to regulate transcription of PTS components in defined medium, we questioned whether Mlc might function differently in *V. cholerae*. Here we have shown that *V. cholerae* Mlc represses transcription of the EI, HPr, and $EIIBC^{Glc}$ genes in both defined medium and LB broth and furthermore that *E. coli* Mlc rescues the biofilm defect of a *V. cholerae* Δ*mlc* mutant. EI and $EIIBC^{Glc}$ indirectly repress *V. cholerae* biofilm formation at the transcriptional level (21). We have shown that repression of EI gene transcription by Mlc is responsible for activation of *vps* gene transcription and biofilm formation, while $EIIBC^{Glc}$ likely functions upstream of Mlc. Because activation of *vps* gene transcription occurs under only a subset of the growth conditions in which repression of EI and $EIIBC^{Glc}$ gene transcription is observed, we hypothesize that a second regulatory circuit present only in LB broth is required for activation of the *vps* genes. We previously showed that $EIIA^{Glc}$ activates *V. cholerae* biofilm formation in LB broth (20, 21). Here we provide evidence that $EIIA^{Glc}$ may be a component of the second regulatory circuit required for full activation of *V. cholerae* biofilm formation in LB broth.

MATERIALS AND METHODS

Bacteria strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table 1. Bacteria were grown in Luria-Bertani broth (LB) or a previously described defined medium supplemented with 0.5% (wt/vol) of the appropriate carbohydrate (20). When required, the following antibiotics were added to the growth medium: streptomycin (100 μg/ml), ampicillin (50 or 100 μg/ml as appropriate), carbenicillin (100 μg/ml), kanamycin (30 μg/ml), and tetracycline (10 μg/ml). For rescue experiments, protein expression was induced by adding arabinose to yield a final concentration of 0.02%. pBAD plasmids used as controls in rescue experiments included either the *lacZ* gene or the $EIIA^{Glc}$ gene in reverse coding orientation following the pBAD promoter as noted in Table 1. These plasmids were used because the empty pBAD vector was found to decrease bacterial growth. Control vectors were used with arabinose supplementation. Neither the control vectors nor arabinose supplementation affected biofilm formation.

Generation of deletion mutants. Gene deletions were engineered as described previously using the splice overlap extension (SOE) method and double homologous recombination (25, 26). These primers and all others used in this study are available upon request.

Construction of pBADTOPO-*mlc* for biofilm complementation. Constructs used for rescue experiments were pBAD-TOPO-$mlc^{V.\ cholerae}$ and pBAD-TOPO-$mlc^{E.\ coli}$. pBADTOPO-$mlc^{V.\ cholerae}$ was generated by PCR amplification of *V. cholerae mlc*, excluding the native stop codon, and cloning of this PCR product into the pBAD-TOPO vector (Invitrogen) according to the manufacturer's instructions. Construction of pBAD-TOPO-$mlc^{E.\ coli}$ was generated by PCR amplification of *mlc* from the *E. coli* strain K-12, excluding the native stop codon. This was also cloned into the pBAD-TOPO vector.

Biofilm assays. *Vibrio cholerae* strains to be tested were cultured overnight in LB broth and then diluted to an optical density at 655 nm (OD_{655}) of 0.05 in the appropriate medium. Three hundred microliters of this cell suspension was aliquoted into three borosilicate tubes and incubated at 27°C for 21 to 24 h. Planktonic cells were removed, and planktonic growth was assessed by measuring the OD_{655}. Three hundred microliters of 0.1 M phosphate-buffered saline (PBS) solution was added to the remaining adherent biofilm pellicle along with 1-mm diameter glass beads (30% [vol/vol]) (BioSpec Products, Inc.). Tubes were vortexed to disperse biofilm-associated cells, and the OD_{655} of the resultant cell suspension was measured to quantify biofilm formation. Total growth was calculated by adding the planktonic and biofilm measurements. Three independent replicates were included in each experiment, and experiments were repeated at least three times. Error bars represent the standard deviations, and a Student *t* test was used to assess statistical significance. *P* values of less than 0.05 were taken to indicate a statistically significant difference.

qRT-PCR analyses. For measurements of transcription in mid-log phase, cells were harvested at an OD_{655} of 0.5 and resuspended in 1 ml of TRIzol (Invitrogen). For measurements of transcription in stationary-phase cultures, a mid-log culture of *V. cholerae* was used to prepare a suspension with an OD_{655} of 0.05 in 90-mm petri dishes. This was incubated for 19 h at 28°C. All cells were then removed to 50-ml conical tubes, pelleted, resuspended in 2.5 ml of TRIzol, and diluted 1:1 with fresh TRIzol. Total RNA was prepared from 1 ml of the TRIzol suspension as

TABLE 1 Bacterial strains and plasmids

Bacterial strains	Genotype and/or description	Reference or source
E. coli		
SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mu λ*pirR6K*; Km^r	24
V. cholerae		
PW357	MO10 Δ*lacZ*::*vpsL*→ *lacZ*; Sm^r	25
PW988	PW357 ΔEIIA^{Glc}	20
PW961	PW357 ΔEI	20
PW1573	PW357 ΔEI ΔEIIA^{Glc}	This study
PW993	PW357 Δ*mlc*	21
PW1557	PW357 ΔEI Δ*mlc*	This study
PW1558	PW357 ΔEI ΔEIIBC^{Glc}	This study
PW1559	PW357 ΔEl ΔEIIBC^{Glc} Δ*mlc*	This study
PW996	PW357 ΔEIIBC^{Glc} Δ*mlc*	21
Plasmids		
pBAD-TOPO	Arabinose-inducible expression vector used to generate His6/V5 epitope-tagged proteins	Life Technologies
pBAD-TOPO-*lacZ*	pBAD-TOPO containing the *lacZ* gene; provided as a control	Life Technologies
pBAD-TOPO-*EIIA^{Glc} rev*	pBAD-TOPO containing the EIIA^{Glc} gene in reverse, noncoding orientation	20
pBAD-TOPO- Mlc$^{V. cholerae}$	pBAD-TOPO containing full-length *V. cholerae mlc*::His6/V5	This study
pBAD-TOPO-Mlc$^{E. coli}$	pBAD-TOPO containing full-length *E. coli mlc*::His6/V5	This study

directed by the manufacturer. cDNA was synthesized using the SuperScript III kit (Invitrogen) as directed. Transcripts were quantified by quantitative reverse transcription-PCR (qRT-PCR) using the iTaq SYBR green kit (Bio-Rad) along with the Step One Plus real-time PCR system (Applied Biosystems). Transcript levels were normalized to *clpX*. Fold expression was determined using the $\Delta\Delta C_T$ method. Three experimental replicates were performed in each trial, and multiple trials were performed. A Student *t* test was used to calculate statistical significance. *P* values of less than 0.05 were taken to be statistically significant.

RESULTS AND DISCUSSION

***V. cholerae* Mlc promotes biofilm formation in LB broth only.** We previously showed that Mlc activates biofilm formation in LB broth, which has relatively small amounts of PTS sugars (17, 21). To study its role more precisely, we repeated these experiments, including chemically defined medium containing glucose, a PTS substrate, or pyruvate, a PTS-independent substrate. As shown in Fig. 2A, deletion of *mlc* decreased biofilm formation in LB broth but not in defined medium supplemented with either glucose or pyruvate. Overexpression of Mlc from an arabinose-inducible promoter rescued the biofilm defect of a Δ*mlc* mutant but had no effect on biofilm formation in defined media (Fig. 2A). Overexpression of Mlc did decrease total cell growth in defined medium containing glucose, consistent with repression of PTS components by Mlc and hence a reduction in glucose transport under these conditions (Fig. 2B).

A change in *V. cholerae* biofilm formation is often correlated with a similar change in transcription of the *vpsA-vpsQ* genes, which encode the synthesis of the VPS biofilm exopolysaccharide,

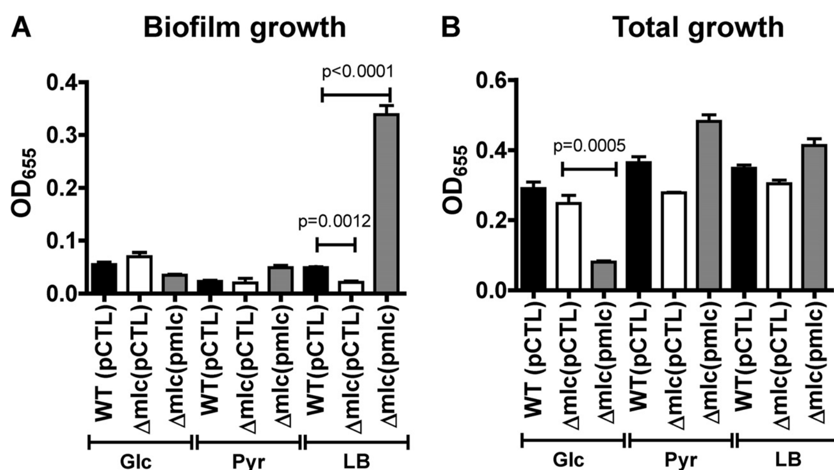

FIG 2 Mlc activates *V. cholerae* biofilm formation in LB broth only: biofilm-associated growth (A) or total growth (B) was measured for wild-type *V. cholerae* (WT) or a Δ*mlc* mutant carrying either the pBAD-*lacZ* control vector (pCTL) or the same plasmid expressing Mlc (pmlc). Static biofilms were formed in LB broth and defined medium supplemented with glucose (Glc) or pyruvate (Pyr).

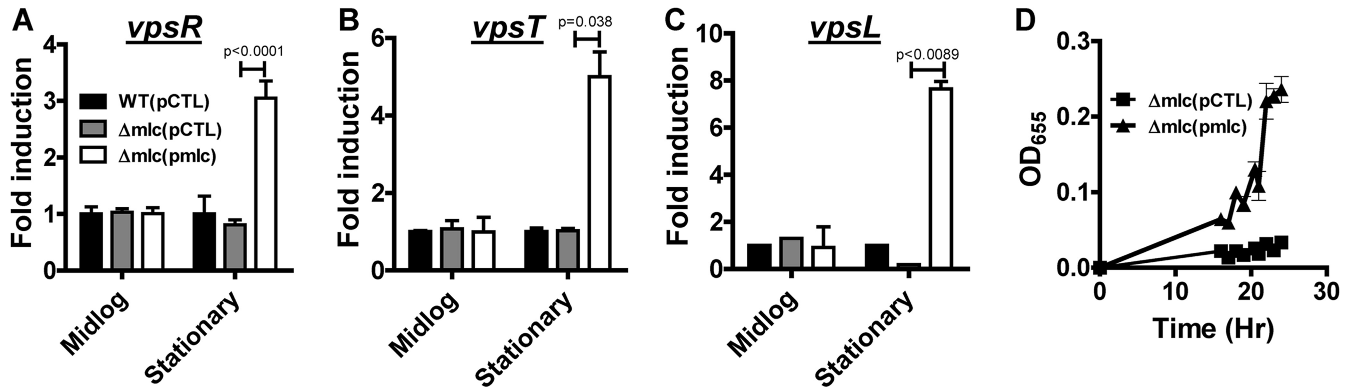

FIG 3 Mlc activates *vps* gene transcription and biofilm formation in later stages of growth in LB broth. qRT-PCR analysis of transcription of *vpsR* (A), *vpsT* (B), or *vpsL* (C) in wild-type *V. cholerae* carrying the pBAD-*lacZ* control or a Δ*mlc* mutant carrying the pBAD-*lacZ* control vector (pCTL) or the same plasmid expressing Mlc (pmlc). Cells were harvested from LB broth in either the mid-log or stationary phase of growth. Values are normalized to wild-type measurements, which were set to 1. (D) Quantification of biofilm formation over time by a Δ*mlc* mutant carrying either a pBAD-*EIIA^Glc^ rev* control vector (pCTL) or a vector encoding Mlc.

as well as the two master regulators of these genes, *vpsR* and *vpsT* (15, 27, 28). To determine if Mlc regulated transcription of these genes, we measured mRNA levels by qRT-PCR for wild-type *V. cholerae*, a Δ*mlc* mutant, or a Δ*mlc* mutant rescued with wild-type *mlc* in cells harvested from an LB culture in mid-log phase. Surprisingly, there was no evidence of regulation of the *vps* genes by Mlc in exponentially growing cells (Fig. 3A to C). We then performed a similar experiment using cells harvested from stationary-phase cultures. In this case, transcriptional activation of *vps* genes by Mlc was observed. This suggested to us that Mlc might have its largest effect on biofilm formation in later stages of growth. To test this, we performed a time course comparing biofilm formation by a Δ*mlc* mutant and that by a Δ*mlc* mutant overexpressing wild-type *mlc*. As shown in Fig. 3D, although Mlc expression was induced at the initiation of the experiment, biofilm formation did not increase until stationary phase was reached. Deletion or overexpression of Mlc had no effect on total growth (data not shown).

***V. cholerae* Mlc represses transcription of PTS components in cells cultured in defined media and LB broth.** Because multiple studies of *E. coli* Mlc have demonstrated repression of PTS components in cells cultured in defined medium and because it was recently suggested that *V. cholerae* Mlc activates transcription of *chsB*, which encodes a chitobiose-specific EII component (29), we questioned whether Mlc might have a fundamentally different function in *V. cholerae* than in *E. coli* (6, 9, 30). To investigate this, we used qRT-PCR to measure transcription of the EI, HPr, EIIA^{Glc}, and EIIBC^{Glc} genes and *chsB* in cells cultured in LB broth or in defined medium supplemented with glucose or pyruvate. Measurements were made in wild-type cells and a Δ*mlc* mutant carrying either a control plasmid or a plasmid encoding Mlc and normalized to the wild-type value. Therefore, values greater than 1

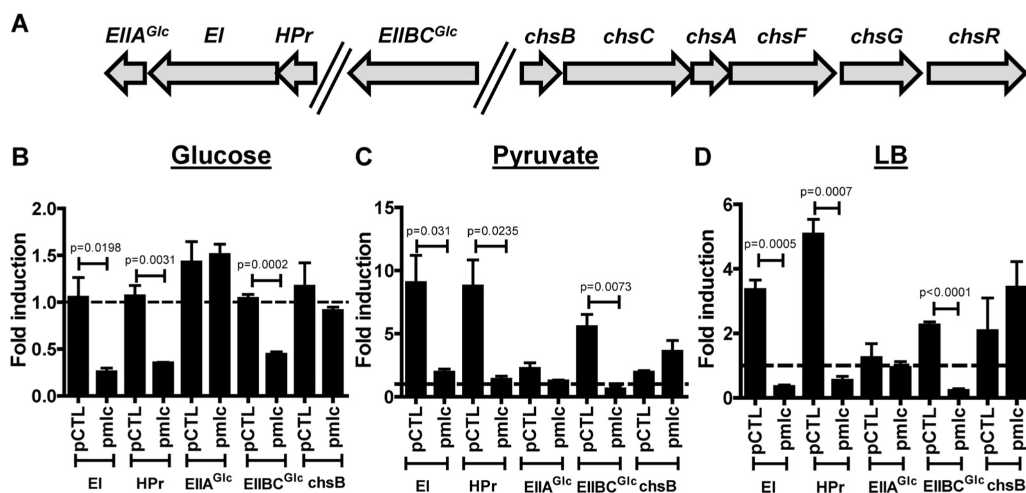

FIG 4 In the presence of non-PTS substrates, native levels of *V. cholerae* Mlc repress transcription of the genes encoding selected PTS components; when overexpressed, Mlc represses transcription under all conditions. (A) Operon structure corresponding to the transcripts measured. (B) qRT-PCR analysis comparing transcription of the genes encoding the specified PTS components in a Δ*mlc* mutant carrying either the control vector pBAD-*lacZ* (pCTL) or a similar plasmid encoding Mlc. Strains were cultured in minimal medium supplemented with glucose (B), minimal medium supplemented with pyruvate (C), or LB broth (D). Transcript levels were normalized to measurements for wild-type *V. cholerae* carrying the same control vector and cultured in the same medium. The broken line indicates the wild-type transcription level, which was set to 1. Transcription was measured in cells harvested in the mid-log phase of growth.

FIG 5 *V. cholerae* and *E. coli* Mlc and $EIIB^{Glc}$ are highly similar. (A) An alignment of the *V. cholerae* (Vc) and *E. coli* (Ec) Mlc proteins, which are 46% identical and 69% similar. Zn^{2+}-binding residues, which are shown in blue, are conserved. Residues predicted to be involved in sugar binding, which are shown in green, are conserved in *V. cholerae* Mlc. In *E. coli* Mlc, residue 194 is not conserved. The helix-turn-helix motifs of each protein are shown in red. (B) An alignment of the cytoplasmic portions of *V. cholerae* (Vc) and *E. coli* (Ec) $EIIBC^{Glc}$, which are 74% identical and 84% similar. In both panels A and B, the residues boxed in pink are known to be required for the interaction between $EIIBC^{Glc}$ and Mlc.

indicate that transcription is increased relative to the wild-type measurement, whereas values less than 1 indicate decreased transcription. As shown in Fig. 4A, the HPr, EI, and $EIIA^{Glc}$ genes form a putative operon, while the $EIIBC^{Glc}$ gene and *chsB* are in different chromosomal locations. In defined medium supplemented with glucose, deletion of *mlc* had very little effect on gene transcription (Fig. 4B). In defined medium supplemented with pyruvate or in LB broth, deletion of *mlc* greatly increased transcription of the EI, HPr, and $EIIBC^{Glc}$ genes but had no effect on the $EIIA^{Glc}$ gene or *chsB* (Fig. 4C and D). This suggests that Mlc represses transcription of the EI, HPr, and $EIIBC^{Glc}$ genes but not *chsB* or the $EIIA^{Glc}$ gene when *V. cholerae* is cultured in LB broth or defined medium supplemented with pyruvate, both of which contain a low level of or no PTS substrates.

To determine whether Mlc was inactive in the presence of glucose due to a change in the cofactor concentration or due to inadequate amounts of Mlc available for binding to DNA, we overexpressed Mlc from a plasmid using an inducible promoter. In all the media tested, overexpression of Mlc in a Δ*mlc* mutant background repressed transcription of the EI, HPr, and $EIIBC^{Glc}$ genes (Fig. 4B to D). These data suggest that when Mlc is available, it represses gene transcription in the presence of both a PTS-dependent substrate, such as glucose, and a PTS-independent substrate, such as pyruvate. From these observations, we conclude that transcriptional regulation of PTS components by Mlc is similar in *V. cholerae* and *E. coli*. Specifically, Mlc represses transcription of the general PTS components in LB broth and defined medium supplemented with pyruvate, both of which contain a low level of or no PTS substrates. Repression of PTS components by Mlc in defined medium supplemented with the PTS substrate glucose is dependent on Mlc availability. We hypothesize that $EIIBC^{Glc}$, which is predicted to be predominantly in its unphosphorylated form when *V. cholerae* is cultured in medium containing glucose, sequesters *V. cholerae* Mlc to the inner membrane, preventing it from binding to DNA. Thus, regulation by Mlc is observed only when excess Mlc is provided by overexpression. Furthermore, similar to what is observed in *E. coli*, transcription of $EIIA^{Glc}$ is independent of Mlc. Although the *E. coli* HPr, EI, and $EIIA^{Glc}$ genes are also in an operon in *E. coli*, an additional promoter just upstream of the $EIIA^{Glc}$ gene is responsible for its different pattern of transcription (31). This is consistent with the key role of $EIIA^{Glc}$ in directing the cell to utilize non-PTS carbohydrates, which demands its presence even when PTS substrates are scarce.

***V. cholerae* and *E. coli* $EIIBC^{Glc}$ and Mlc are highly similar.** Our data suggest that *V. cholerae* and *E. coli* Mlc similarly regulate gene transcription. To explore this further, we compared the amino acid sequences of *V. cholerae* and *E. coli* Mlc. These proteins are 46% identical and 69% similar (Fig. 5A). Furthermore, the amino acids that form the helix-turn-helix motif and that participate in Zn^{2+} binding are conserved. The action of ROK domain regulators, such as NagC, is often modulated by a carbohydrate cofactor (32). However, there is no evidence that *E. coli* Mlc binds

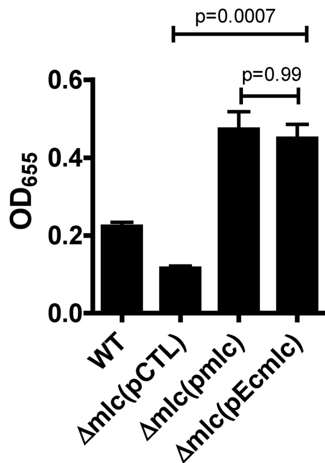

FIG 6 *E. coli* Mlc rescues the biofilm formation defect of a *V. cholerae* Δ*mlc* mutant. Quantification of biofilm formation by wild-type *V. cholerae* (WT) and a *V. cholerae* Δ*mlc* mutant carrying the control vector pBAD-*lacZ* (pCTL), pBAD-TOPO-Mlc$^{V.\ cholerae}$ (pmlc), or pBAD-TOPO-Mlc$^{E.\ coli}$ (pEcmlc). All cultures were supplemented with arabinose.

to or coregulates with a carbohydrate. Some have attributed this to the conservation of only 4 out of the 5 sugar binding residues of ROK domain proteins (33). Interestingly, all 5 sugar-binding residues are conserved in *V. cholerae* Mlc.

When expressed at native levels, we did not observe transcriptional regulation by Mlc in defined medium supplemented with the PTS substrate glucose (Fig. 4B). We hypothesized that, similar to *E. coli* Mlc, *V. cholerae* Mlc might interact with the cytoplasmic portion of EIIBC^{Glc}, resulting in sequestration to the membrane. As shown in Fig. 5B, the cytoplasmic domain of *V. cholerae* EIIBC^{Glc} is 74% identical and 84% similar to that of *E. coli*. Cys421, Arg424, Val449, Ala451, and Gln456 of EIIBC^{Glc}, as well as Phe136 of Mlc, are essential for the interaction of *E. coli* EIIB with Mlc (34, 35). As shown in Fig. 5, with the exception of Ala451, these residues are conserved in *V. cholerae* EIIBC^{Glc}. Because *E. coli* and *V. cholerae* EIIBC^{Glc} and Mlc are highly similar, it seems likely that these two proteins also interact in *V. cholerae*.

***E. coli mlc* can rescue a *V. cholerae* Δ*mlc* mutant.** Because of the similarity of *E. coli* and *V. cholerae* Mlc, we questioned whether *E. coli* Mlc expressed from a plasmid could rescue the biofilm defect of a *V. cholerae* Δ*mlc* mutant. As shown in Fig. 6, both *E. coli* and *V. cholerae* Mlc rescued the biofilm formation defect of a *V. cholerae* Δ*mlc* mutant. This demonstrates that the role played by *V. cholerae* Mlc in activation of biofilm formation can also be fulfilled by *E. coli* Mlc.

Mlc activates biofilm formation by repression of EI. Transcription of the genes encoding EI and EIIBC^{Glc} is repressed by Mlc. We previously showed that EI and EIIBC^{Glc} repress *vps* gene transcription and biofilm formation by cells cultured in LB broth (21). We hypothesized that one or both of these proteins might be downstream of Mlc in a regulatory pathway. As shown in Fig. 7A, deletion of EIIBC^{Glc} increased biofilm formation. Mutation of Mlc in this background, however, decreased biofilm formation to wild-type levels. This result is consistent with a model in which the primary function of EIIBC^{Glc} in regulation of biofilm formation is to sequester Mlc to the membrane. Furthermore, this suggests that Mlc must act on another protein to activate biofilm formation. In agreement with this model, overexpression of Mlc in a Δ*mlc* ΔEIIBC^{Glc} mutant background increased biofilm formation (Fig. 7B). This demonstrates that activation of biofilm formation by Mlc is not dependent on EIIBC^{Glc}.

As shown in Fig. 7A and C, deletion or overexpression of *mlc* had no effect on biofilm formation by a ΔEI mutant. Because we have demonstrated that Mlc represses transcription of the EI gene, EI represses biofilm formation, and Mlc has no effect on biofilm formation in the absence of EI, we hypothesize that Mlc activates *V. cholerae* biofilm formation indirectly by repressing expression of EI.

Evidence that EIIA^{Glc} is a component of a second regulatory pathway required in LB broth for derepression of biofilm formation in the absence of EI. Although Mlc repressed EI when *V. cholerae* was cultured in either LB broth or minimal medium supplemented with pyruvate (Fig. 4C and D), biofilm formation was activated by Mlc in LB broth only. Therefore, we hypothesized that repression of EI gene transcription by Mlc was necessary but not sufficient for activation of *vps* gene transcription and *V. cholerae* biofilm formation. We previously reported that EIIA^{Glc} activates biofilm formation in LB broth and that overexpression of Mlc only partially rescues biofilm formation in a Δ*mlc* ΔEIIA^{Glc} mutant background (20, 21). We suggested that expression of EIIA^{Glc} might be regulated by Mlc (21). However, our current findings show that this is not the case (Fig. 4). Another possibility is that EIIA^{Glc} is a component of a second regulatory pathway

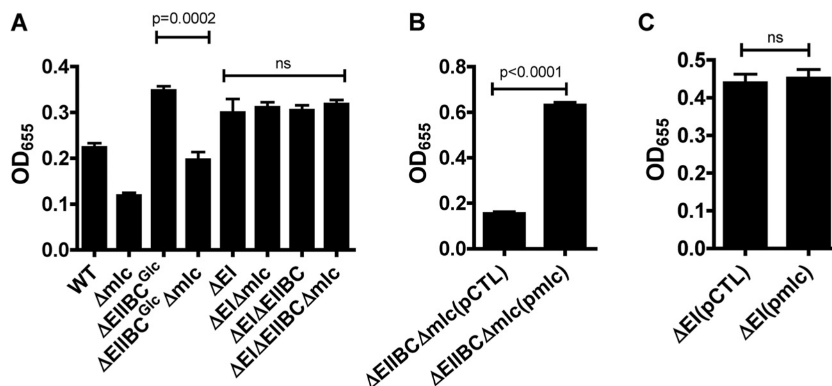

FIG 7 Evidence that Mlc activates biofilm formation through repression of EI. Quantification of biofilms formed by wild-type *V. cholerae* and Δ*mlc*, ΔEI, and ΔEI Δ*mlc* mutants (A), a ΔEIIBC^{Glc} Δ*mlc* mutant carrying either a control plasmid (pCTL) or a plasmid encoding Mlc (pmlc) (B), or wild-type *V. cholerae* and a ΔEI mutant carrying either a control plasmid (pCTL) or a plasmid encoding Mlc (pmlc) (C). *P* values are shown above. ns, not significant.

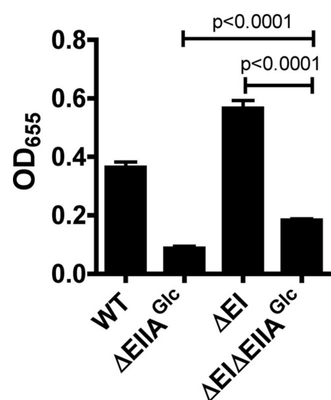

FIG 8 Derepression of biofilm formation in the absence of EI requires $EIIA^{Glc}$. Quantification of biofilm formation by ΔEI, $\Delta EIIA^{Glc}$, and ΔEI $\Delta EIIA^{Glc}$ mutants in LB broth.

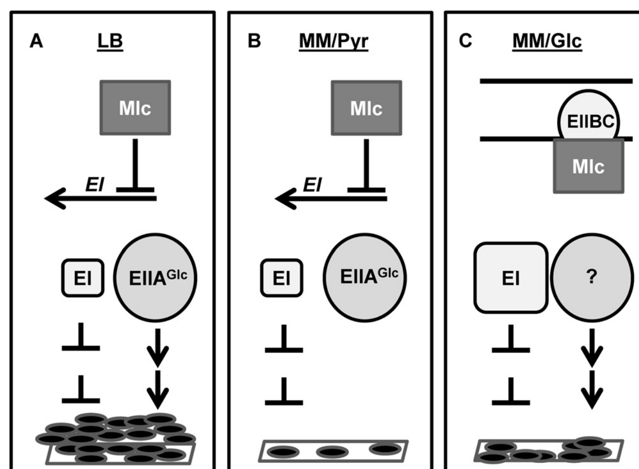

FIG 9 A model for regulation of *V. cholerae* biofilm formation by the transcription factor Mlc. (A) LB broth (LB): biofilm formation reflects decreased expression of the biofilm repressor EI due to repression by Mlc and activation of biofilm formation by a signal transduction pathway involving $EIIA^{Glc}$. (B) Minimal medium supplemented with pyruvate (MM/Pyr): Mlc partially represses *EI* transcription. However, the small amount of EI in the cell maximally represses biofilm formation because the biofilm-activating pathway involving $EIIA^{Glc}$ is not present. (C) Minimal medium supplemented with glucose (MM/Glc): Mlc is inactive due to sequestration to the membrane by the unphosphorylated form of $EIIBC^{Glc}$. This prevents repression of *EI* transcription by Mlc. In spite of repression by EI, a biofilm does form. This may indicate the presence of a second regulatory pathway that activates biofilm formation.

required for activation of biofilm formation by Mlc. Because Mlc activates biofilm formation through repression of EI, we evaluated the effect of a $\Delta EIIA^{Glc}$ gene deletion on biofilm formation by a ΔEI mutant. As shown in Fig. 8, deletion of the $EIIA^{Glc}$ gene in a ΔEI mutant background greatly reduced biofilm formation. However, repression of biofilm formation by EI was significant even in the $\Delta EIIA^{Glc}$ mutant background. These findings support a model in which the EI and $EIIA^{Glc}$ proteins function in independent regulatory pathways.

Our previous results show that $EIIA^{Glc}$ does not activate biofilm formation in minimal medium supplemented with pyruvate (20). The role of $EIIA^{Glc}$ in a particular growth condition is governed by its interactions with other proteins (22). We hypothesize that activation of biofilm formation by $EIIA^{Glc}$ in LB broth reflects its interaction with an as yet unexplored partner.

Model for regulation of biofilm formation by Mlc. Based on our observations, we present the following model for activation of *V. cholerae* biofilm formation by the transcription factor Mlc (Fig. 9). In media that do not contain PTS substrates, such as LB broth and minimal medium supplemented with pyruvate, Mlc decreases but does not eliminate transcription of PTS components. Thus, there is residual repression of biofilm formation by EI, and deletion of EI increases biofilm formation in these media (2, 21). In LB broth, a second, independent regulatory pathway that includes $EIIA^{Glc}$ activates biofilm formation, resulting in formation of a biofilm (Fig. 9A). In this scenario, deletion of either *mlc* or the $EIIA^{Glc}$ gene decreases biofilm formation. $EIIA^{Glc}$ does not activate biofilm formation in minimal medium supplemented with pyruvate. Therefore, biofilm formation by wild-type *V. cholerae* is completely repressed by the small amount of EI that is made (Fig. 9B). Further repression of biofilm formation by deletion of Mlc and the concomitant derepression of EI is not possible. In minimal medium supplemented with the PTS substrate glucose, Mlc is sequestered to the membrane by the unphosphorylated form of $EIIBC^{Glc}$ (Fig. 9C). Because Mlc is inactive, EI is maximally expressed, and deletion of *mlc* does not further alter biofilm formation. Surface accumulation is observed under these growth conditions. Therefore, a second, biofilm-activating pathway may be operative.

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