RESEARCH ARTICLE

Glucose-Specific Enzyme IIA Has Unique Binding Partners in The *Vibrio cholerae* Biofilm

Bradley S. Pickering, Daniel R. Smith, and Paula I. Watnick

Division of Infectious Diseases, Boston Children's Hospital, Boston, Massachusetts, USA

ABSTRACT **Glucose-specific enzyme IIA (EIIA^{Glc}) is a central regulator of bacterial metabolism and an intermediate in the phosphoenolpyruvate phosphotransferase system (PTS), a conserved phosphotransfer cascade that controls carbohydrate transport. We previously reported that EIIA^{Glc} activates transcription of the genes required for *Vibrio cholerae* biofilm formation. While EIIA^{Glc} modulates the function of many proteins through a direct interaction, none of the known regulatory binding partners of EIIA^{Glc} activates biofilm formation. Therefore, we used tandem affinity purification (TAP) to compare binding partners of EIIA^{Glc} in both planktonic and biofilm cells. A surprising number of novel EIIA^{Glc} binding partners were identified predominantly under one condition or the other. Studies of planktonic cells revealed established partners of EIIA^{Glc}, such as adenylate cyclase and glycerol kinase. In biofilms, MshH, a homolog of *Escherichia coli* CsrD, was found to be a dominant binding partner of EIIA^{Glc}. Further studies revealed that MshH inhibits biofilm formation. This function was independent of the Carbon storage regulator (Csr) pathway and dependent on EIIA^{Glc}. To explore the existence of multiprotein complexes centered on EIIA^{Glc}, we also affinity purified the binding partners of adenylate cyclase from biofilm cells. In addition to EIIA^{Glc}, this analysis yielded many of the same proteins that copurified with EIIA^{Glc}. We hypothesize that EIIA^{Glc} serves as a hub for multiprotein complexes and furthermore that these complexes may provide a mechanism for competitive and cooperative interactions between binding partners.**

IMPORTANCE **EIIA^{Glc} is a global regulator of microbial physiology that acts through direct interactions with other proteins. This work represents the first demonstration that the protein partners of EIIA^{Glc} are distinct in the microbial biofilm. Furthermore, it provides the first evidence that EIIA^{Glc} may exist in multiprotein complexes with its partners, setting the stage for an investigation of how the multiple partners of EIIA^{Glc} influence one another. Last, it provides a connection between the phosphoenolpyruvate phosphotransferase (PTS) and Csr regulatory systems. This work increases our understanding of the complexity of regulation by EIIA^{Glc} and provides a link between the PTS and Csr networks, two global regulatory cascades that influence microbial physiology.**

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Address correspondence to Paula I. Watnick, paula.watnick@childrens.harvard.edu.

Vibrio cholerae is an intestinal pathogen and a natural inhabitant of aquatic environments (1). In these environments, *V. cholerae* is thought to exist in a surface-attached or biofilm state in or on zooplankton and insects, leading some to suggest that arthropods may serve as reservoirs or vectors of disease (2–8). Our laboratory has shown that *V. cholerae* forms a dense biofilm in the rectum of the model arthropod *Drosophila melanogaster* (9). Formation of this biofilm requires elaboration of a matrix comprised of the VPS exopolysaccharide as well as several structural proteins (10–16).

Transcription of the *vps* genes, which encode proteins required for biosynthesis of the *V. cholerae* biofilm matrix, is controlled by a complex regulatory network that integrates multiple environmental signals, including bacterial autoinducers, polyamines, nucleosides, indole, and carbohydrates transported by the phosphoenolpyruvate phosphotransferase system (PTS), a multicomponent phosphotransfer cascade (14, 17–22).

The PTS consists of four regulatory intermediates and a terminal apparatus that both transports and phosphorylates specific sugars (23). In order of phosphotransfer, the regulatory intermediates include enzyme I (EI), which accepts a phosphate from phosphoenolpyruvate (PEP), histidine protein (HPr), and enzymes IIA and IIB (EIIA and EIIB). Enzymes IIC, which are not part of the phosphotransfer cascade, form the transport apparatuses. While EI and HPr are considered to be general PTS components, each EII component responds to and transports a specific group of sugars. For this reason, the *V. cholerae* genome encodes 19 EIIA, -B, and -C homologs, each with distinct substrate specificities (24).

The phosphorylation state of PTS intermediates depends upon the intracellular pool of PEP and the abundance of PTS sugars in the environment. A high concentration of intracellular PEP increases the proportion of phosphorylated PTS intermediates, while transport of sugars through the PTS depletes the phosphate

TABLE 1 EIIA^{Glc} and AC interaction partners

Locus	Gene product	No. of specific peptides[b]			
		EIIA^{Glc} mid-log	EIIA^{Glc} biofilm	EIIA^{GlcH91A} biofilm	AC biofilm
VC0122[a]	Adenylate cyclase	77	2	1	(117)
VC0398	MshH	2	22	3	1
VC0900	GGDEF family protein	6	ND	ND	ND
VC0964	EIIA^{Glc}	(37)	(64)	(81)	17
VC1087	Response regulator	ND	1	ND	ND
VC1291	Hypothetical protein	1	10	2	5
VC1655	Mg transporter	ND	11	3	ND
VC1721	PurR	7	ND	ND	2
VC1817	Fis family transcriptional regulator	ND	1	ND	ND
VC2013[a]	EIIBC^{Glc}	ND	1	ND	ND
VC2614	CRP	ND	2	ND	6
VC2689	6-Phosphofructokinase (PFK)	4	2	ND	1
VC2738	Phosphoenolpyruvate carboxykinase (PCK)	ND	8	10	1
VCA0744[a]	Glycerol kinase (GlpK)	100	2	6	4

[a] Previously documented interaction with EIIA^{Glc}.

[b] Numbers indicate the total number of peptides identified that were specific to the indicated protein. "ND" indicates that peptides associated with this protein were not detected. Parentheses indicate identified peptides corresponding to the TAP-tagged protein.

stored within the PTS. Bacteria are equipped with signal transduction pathways that monitor the phosphorylation state of PTS components and adjust their cellular physiology accordingly (25).

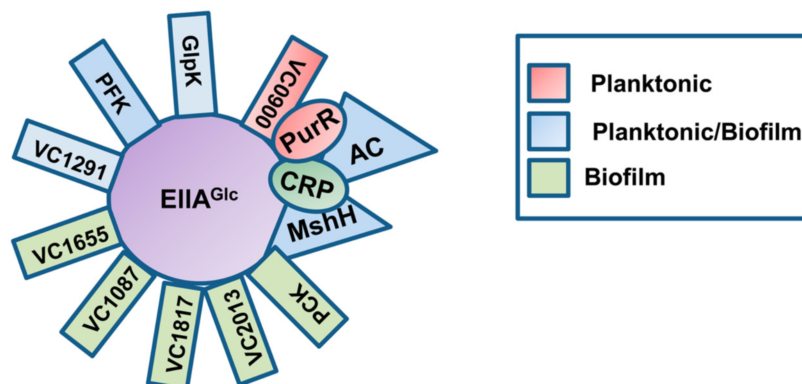
Our laboratory recently uncovered multiple independent pathways within the *V. cholerae* PTS that regulate synthesis of the biofilm matrix at the transcriptional level (26). One of these involves activation of biofilm formation by the glucose-specific EIIA component (EIIA^{Glc}). The phosphorylated form of EIIA^{Glc} modulates the action of the cAMP receptor protein (CRP), a global regulator of cellular physiology, by activating synthesis of cAMP through a direct interaction with adenylate cyclase (AC) (25). Because both CRP and cAMP have been reported to repress *vps* transcription and biofilm formation in *V. cholerae* (19, 27, 28), we hypothesized that EIIA^{Glc} must interact with additional protein partners that activate biofilm formation. To test this, we isolated interaction partners of EIIA^{Glc} in both planktonic and biofilm cells by tandem affinity purification (TAP) and then identified these proteins by mass spectrometry (29). Distinct EIIA^{Glc} binding partners were identified in planktonic and biofilm cells. In particular, AC and glycerol kinase (GlpK), both well-established binding partners of EIIA^{Glc}, were the most abundant interaction partners in planktonic cells, while MshH was the most abundant partner in the biofilm. We confirmed and further elucidated these interactions using a bacterial two-hybrid system. Both TAP and bacterial two-hybrid analyses suggested that factors in addition to the phosphorylation state of EIIA^{Glc} contribute to the differential abundance of these protein-protein interactions in planktonic and biofilm cells.

MshH is a homolog of *Escherichia coli* CsrD, a component of an unusual and complex posttranscriptional regulatory cascade known as Carbon storage regulator (Csr) that has been uncovered by Romeo and colleagues in a series of elegant experiments (30–32). CsrD enhances the RNase E-dependent degradation of the small *csr* RNAs that inhibit binding of the regulatory protein CsrA to mRNA. Here we find that MshH represses *V. cholerae* biofilm formation only when EIIA^{Glc} is present. While both MshH and EIIA^{Glc} modulate the levels of the *csr* RNAs, this function does not play a role in regulation of biofilm formation in this *V. cholerae* strain. We conclude that MshH is a repressor of biofilm formation that may function upstream of EII^{Glc}. Furthermore, we hypothesize that EIIA^{Glc} provides a platform for cooperative and competitive interactions between its many binding partners.

RESULTS

Tandem affinity purification reveals distinct EIIA^{Glc} interaction partners in the planktonic and biofilm states. EIIA^{Glc} regulates cellular physiology through interactions with other proteins. We used TAP followed by liquid chromatography-mass spectrometry (LC-MS) to identify interaction partners of EIIA^{Glc} in planktonic and biofilm cells cultured in LB broth (Table 1 and Fig. 1A; see also Tables S1 and S2 in the supplemental material). Some proteins copurified with EIIA^{Glc} in planktonic or biofilm cells specifically, while others copurified with EIIA^{Glc} under both conditions (Fig. 1A; Table 1). Proving the validity of our analysis, glycerol kinase (GlpK) and AC copurified with EIIA^{Glc} quite abundantly in planktonic cells. GlpK converts glycerol to glycerol-3-phosphate as it enters the cell. Unphosphorylated EIIA^{Glc} is an established allosteric inhibitor of GlpK that blocks glycerol utilization when PTS substrates are present (33, 34). EIIA^{Glc}-P has long been known to potentiate the activity of AC. However, direct contact between these two proteins has only recently been demonstrated (35). In this work, the investigators created an *E. coli* strain expressing native AC and another expressing AC tethered to an integral membrane protein. They found that EIIA^{Glc} associated with the cell membrane only in cells expressing tethered AC. Our results build on these by showing that EIIA^{Glc} interacts directly with the native form of AC.

Two proteins, PurR and the GGDEF domain-containing protein VC0900, copurified with EIIA^{Glc} only in the planktonic state (Fig. 1A). Proteins copurifying with EIIA^{Glc} in the biofilm alone included the putative response regulator VC1087, VC1655, a distant homolog of the cyclic nucleotide binding domain-containing magnesium transporter MgtE, VC1817, a GAF domain-containing σ^{54}-dependent transcriptional regulator, EIIBC^{Glc}, CRP, and PEP carboxykinase (PCK), which converts oxaloacetate to PEP. Proteins copurifying with EIIA^{Glc} in both states included



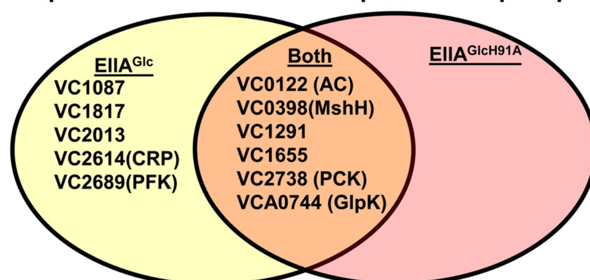


FIG 1 Analysis of the binding partners of EIIA^{Glc} and adenylate cyclase in the biofilm state. (A) Diagram illustrating the binding partners of EIIA^{Glc} found in planktonic cells, biofilm cells, or both. As proposed in the text, CRP and PurR are illustrated as binding to AC. (B) Venn diagram illustrating the overlap between the binding partners of EIIA^{GlcH91A} and those of the wild-type EIIA^{Glc} allele in biofilm cells.

AC, GlpK, MshH, a predicted regulatory protein containing at least one cyclic nucleotide binding domain at locus VC1291, and 6-phosphofructokinase (PFK). PFK is an allosterically regulated enzyme in the glycolytic pathway that generates fructose 1,6-biphosphate. While AC and GlpK showed a strong preference for association with EIIA^{Glc} in the planktonic state, MshH, VC1291, and PCK showed a strong preference for association in the biofilm state. Only PFK copurified equally well with EIIA^{Glc} in both the planktonic and biofilm states.

Dependence of protein interactions in the biofilm on phosphorylation of EIIA^{Glc}. To investigate the dependence of biofilm-specific EIIA^{Glc} binding partners on the phosphorylation state of EIIA^{Glc}, we performed a biofilm TAP analysis using EIIA^{GlcH91A}, a point mutant in which an alanine is substituted for the phosphorylated histidine. These results are listed in Table 1 (see also Table S3 in the supplemental material) and illustrated in Fig. 1B. Previous findings for *E. coli* suggest that GlpK interacts with the unphosphorylated form of EIIA^{Glc} (36). However, in biofilm cells, it copurified only slightly better with EIIA^{GlcH91A} than with native EIIA^{Glc}. Of the proteins that interacted with EIIA^{Glc} preferentially in the biofilm, some were able to interact with EIIA^{GlcH91A}, while others were not. These data suggest that factors in addition to the phosphorylation state of EIIA^{Glc} determine the specificity of these interactions for the biofilm state. However, it is also possible that the point mutation itself interferes with binding of some but not all of the interaction partners of EIIA^{Glc}.

TAP analysis of AC binding partners suggests that EIIA^{Glc} forms multiprotein complexes. We were curious to know if EIIA^{Glc} is able to interact concurrently with more than one binding partner in biofilm cells. To explore this further, we performed TAP analysis with AC and MshH, the principal binding partners of EIIA^{Glc} in planktonic and biofilm cells, respectively. TAP analysis with MshH was not successful. The binding partners of AC revealed by TAP are shown in Table 1 and also in Table S4 in the supplemental material. EIIA^{Glc} was the most abundant binding partner of AC in the biofilm. Furthermore, every other protein identified as a binding partner of AC had previously been identified as a binding partner of EIIA^{Glc}. Some binding partners of EIIA^{Glc}, including MshH, VC1655, and PCK, were poorly represented in the AC TAP analysis, while others, such as the transcription factors PurR and CRP, were better represented in the AC TAP analysis than in that with EIIA^{Glc}. Our data suggest the hypotheses that (i) EIIA^{Glc} and AC participate in multiprotein complexes with the other interaction partners identified here, (ii) PurR and CRP interact with AC, whereas the other partners interact with EIIA^{Glc}, and (iii) the interactions of EIIA^{Glc} with some protein partners are inhibited when AC is bound (Fig. 1A).

Bacterial two-hybrid assays further elucidate the interaction of EIIA^{Glc} with AC and MshH. While our TAP analyses gave rise to many hypotheses, we chose to focus on AC and MshH, the dominant binding partners of EIIA^{Glc} in planktonic and biofilm cells, respectively. To further examine the interaction of EIIA^{Glc} with AC and MshH, bacterial two-hybrid assays were performed as follows. We constructed a plasmid encoding EIIA^{Glc} fused to the N terminus of a zinc finger DNA-binding protein known as Zif and a plasmid encoding the putative EIIA^{Glc} binding partner fused to the N terminus of the ω subunit of RNA polymerase. These plasmids were transformed into an *E. coli* strain carrying a Zif

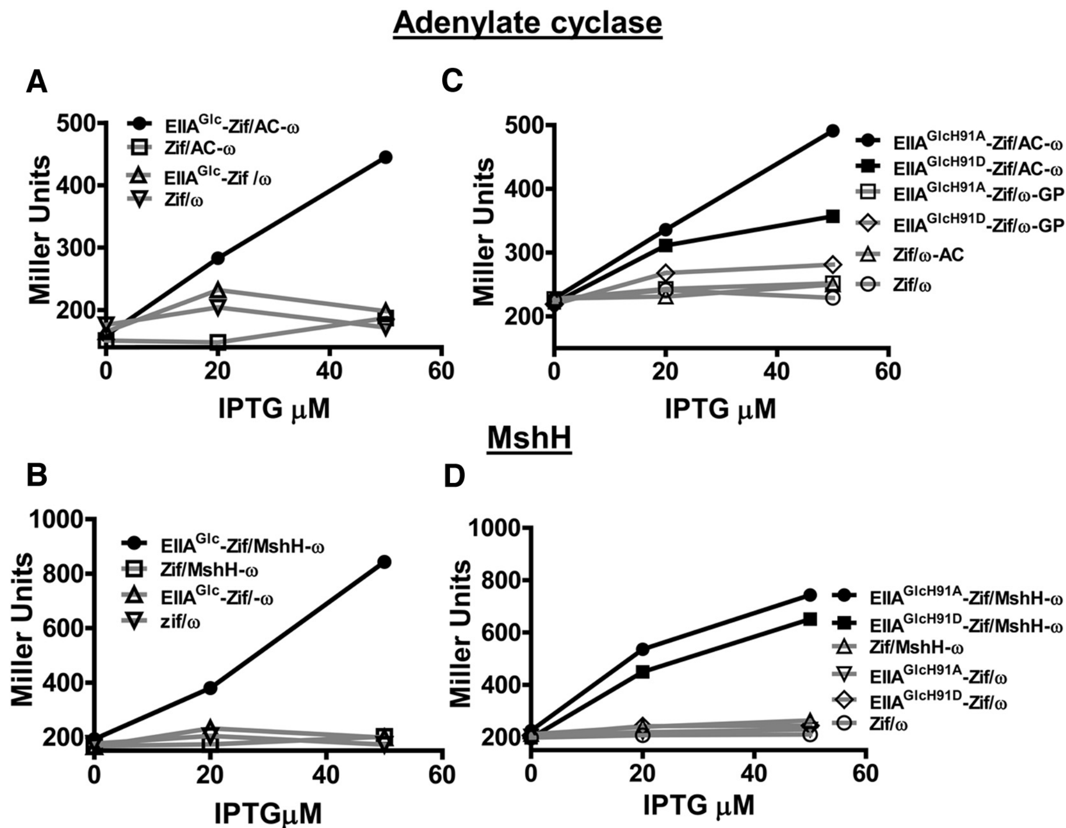


FIG 2 Bacterial two-hybrid assays confirm the interaction of adenylate cyclase and MshH with native $EIIA^{Glc}$ and $EIIA^{Glc}$ point mutants mimicking the phosphorylated and unphosphorylated states. (A and B) Interaction of $EIIA^{Glc}$ fused to the N terminus of the DNA-binding protein Zif ($EIIA^{Glc}$-Zif) with adenylate cyclase (AC-ω) (A) or MshH (MshH-ω) (B) fused to the ω subunit of RNA polymerase in an *E. coli* strain encoding the *lacZ* gene preceded by a Zif binding site. (C and D) A similar experiment was performed to assess the interactions of adenylate cyclase (AC-ω) (C) or MshH (MshH-ω) (D) with $EIIA^{Glc}$ point mutants in which an alanine ($EIIA^{GlcH91A}$-Zif) or an aspartate ($EIIA^{GlcH91D}$-Zif) was substituted for histidine 91 to mimic the unphosphorylated and phosphorylated states of $EIIA^{Glc}$, respectively. Controls, which are traced in gray for each experiment, include (i) a vector encoding Zif alone (Zif) combined with a vector encoding the protein of interest fused to ω, (ii) a vector encoding native $EIIA^{Glc}$ or point mutants fused to Zif combined with a vector encoding ω alone (ω), and (iii) vectors encoding Zif and ω alone. Two experimental replicates were included in each trial, and several trials were performed. A representative trial is shown here.

binding site upstream of the *lacZ* gene. In this strain, the level of β-galactosidase activity reflects the ability of $EIIA^{Glc}$ to recruit RNA polymerase to the *lacZ* promoter by interacting with its binding partner (37). Full-length adenylate cyclase was expressed for use in these assays. MshH has an N-terminal extracytoplasmic domain, a transmembrane domain, and C-terminal, cytoplasmic GGDEF and EAL domains. For these experiments, we expressed only the cytoplasmic portion of MshH, which consists of the terminal 578 amino acids of the protein. As shown in Fig. 2A and 2B, bacterial two-hybrid assays confirmed an interaction between $EIIA^{Glc}$ and both of these proteins. To examine the requirement for phosphorylation of $EIIA^{Glc}$ at position 91, we also performed two-hybrid assays with $EIIA^{GlcH91A}$ and $EIIA^{GlcH91D}$, which mimic the unphosphorylated and phosphorylated forms of $EIIA^{Glc}$, respectively. As shown in Fig. 2C and 2D, adenylate cyclase and MshH interacted well with both forms of $EIIA^{Glc}$. Based on these findings and those of our TAP analyses, we hypothesize that factors in addition to the phosphorylation state of $EIIA^{Glc}$ determine the differential affinity of adenylate cyclase and MshH for $EIIA^{Glc}$ in planktonic and biofilm cells. For MshH, it is also possible that the extracellular or transmembrane portions of the protein, which were eliminated for these experiments, restrict its interaction with $EIIA^{Glc}$ to a particular phosphorylation state.

MshH modulates biofilm formation in a Csr-independent manner. $EIIA^{Glc}$ is an activator of biofilm formation (26). Because MshH appeared to be the primary binding partner of $EIIA^{Glc}$ in the biofilm, we focused our subsequent studies on the role of MshH in regulation of biofilm formation. We first compared the biofilm-forming propensity of $\Delta EIIA^{Glc}$ and Δ*mshH* mutants with that of wild-type *V. cholerae*. As shown in Fig. 3A, deletion of $EIIA^{Glc}$ decreased biofilm formation, while deletion of *mshH* increased biofilm formation. Because our findings proved that MshH was not the downstream factor responsible for activation of biofilm formation by $EIIA^{Glc}$, we reasoned that MshH might interfere with potentiation of this downstream factor through its interaction with $EIIA^{Glc}$. If this were the case, we predicted that deletion of *mshH* should have a biofilm phenotype only in the presence of $EIIA^{Glc}$. To test this, we constructed a Δ*mshH* $\Delta EIIA^{Glc}$ double mutant and measured its ability to form a biofilm. As predicted, MshH did not impact biofilm formation in the absence of $EIIA^{Glc}$.

To further confirm a role for MshH in regulation of biofilm

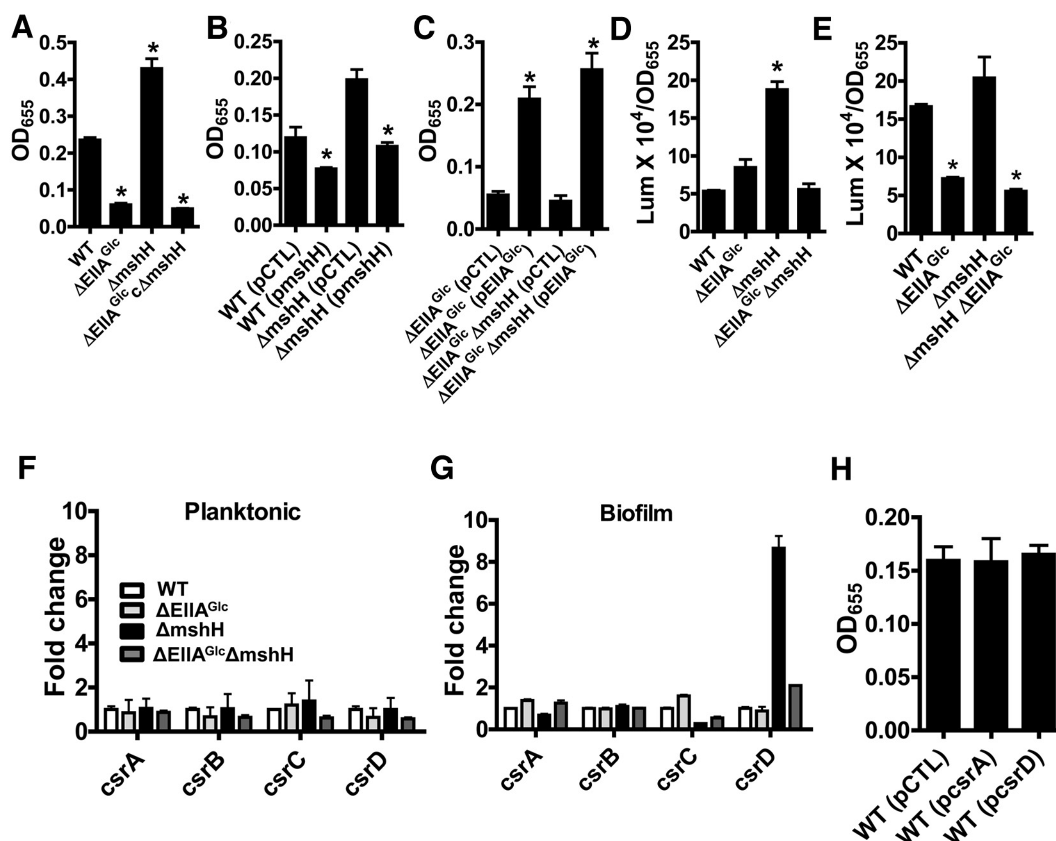


FIG 3 Evidence that MshH inhibits activation of biofilm formation by EIIA^{Glc}. (A) Biofilms formed by wild-type *V. cholerae* MO10 (WT) or ΔEIIA^{Glc}, Δ*mshH*, and ΔEIIA^{Glc} Δ*mshH* mutants. The biofilms formed by the ΔEIIA^{Glc} ($P < 0.0001$), Δ*mshH* ($P = 0.002$), and ΔEIIA^{Glc} Δ*mshH* ($P < 0.0001$) mutants are significantly different from that formed by wild-type *V. cholerae*. (B) Biofilm formation by a Δ*mshH* mutant carrying an empty vector (pCTL) or a plasmid encoding a wild-type allele of *mshH* (pmshH). The biofilms formed by the wild-type strain ($P = 0.04$) and the Δ*mshH* mutant ($P = 0.003$) carrying the plasmid expressing MshH are significantly different from those formed by the corresponding strains carrying a control plasmid. (C) Biofilm formed by ΔEIIA^{Glc} or ΔEIIA^{Glc} Δ*mshH* mutant carrying either an empty vector (pCTL) or a wild-type EIIA^{Glc} allele (pEIIA^{Glc}). The biofilm formed by the ΔEIIA^{Glc} (pEIIA^{Glc}) mutant is significantly different from that formed by the ΔEIIA^{Glc} (pCTL) mutant ($P = 0.0002$). The biofilm formed by the ΔEIIA ^{Glc} Δ*mshH* (pEIIA^{Glc}) mutant is significantly different from that formed by the ΔEIIA ^{Glc} Δ*mshH* (pCTL) mutant ($P = 0.0002$). (D and E) β-Galactosidase measurements reflecting planktonic (D) or biofilm (E) *vpsL* transcription in strains with chromosomal *vpsL-lacZ* reporter fusions. In planktonic cells, *vpsL* transcription in the Δ*mshH* mutant is significantly greater than that in wild-type cells ($P = 0.0002$). In the biofilm, *vpsL* transcription in the ΔEIIA^{Glc} and ΔEIIA^{Glc} Δ*mshH* strains is significantly different from that of wild-type *V. cholerae* ($P < 0.0001$). (F and G) qRT-PCR quantification of *csrA*, *csrB*, *csrC*, and *csrD* transcripts in planktonic and biofilm cultures. Transcript levels were measured in wild-type strains (WT) as well as ΔEIIA^{Glc}, Δ*mshH*, and ΔEIIA^{Glc} Δ*mshH* mutants. (H) Biofilm formation by wild-type *V. cholerae* with a control plasmid (pCTL), a plasmid expressing CsrA (pcsrA), or a plasmid carrying a *csrD* transcript (pcsrD).

formation, we cloned the wild-type *mshH* allele into an inducible vector and tested the effect of MshH overexpression on biofilm formation by both wild-type *V. cholerae* and a Δ*mshH* mutant. As shown in Fig. 3B, overexpression of MshH reduced biofilm formation in both wild-type and mutant cells. We further predicted that provision of EIIA^{Glc} in *trans* would increase biofilm formation by the Δ*mshH* ΔEIIA^{Glc} double mutant to levels equal to or greater than those observed for rescue of a ΔEIIA^{Glc} mutant. As shown in Fig. 3C, this was in fact the case. Last, to examine the effect of these mutations on transcription of the *vpsL* genes, we performed β-galactosidase assays in planktonic (Fig. 3D) and biofilm (Fig. 3E) cells with our control *V. cholerae* strain as well as the Δ*mshH*, ΔEIIA^{Glc}, and Δ*mshH* ΔEIIA^{Glc} double mutants, which all have a *vpsL-lacZ* transcriptional fusion cloned into a neutral site on the chromosome. In planktonic cells, deletion of EIIA^{Glc} had little effect on β-galactosidase activity, but deletion of *mshH* increased β-galactosidase activity 3-fold. When EIIA^{Glc} was deleted in a Δ*mshH* mutant background, β-galactosidase activity decreased to that of the control strain. In biofilms, a different pattern was observed. Deletion of EIIA^{Glc} decreased β-galactosidase activity, but deletion of MshH had only a small effect on β-galactosidase activity. Last, deletion of EIIA^{Glc} in a Δ*mshH* mutant background reduced β-galactosidase activity to that of the ΔEIIA^{Glc} mutant. These results suggest that both MshH and EIIA^{Glc} regulate biofilm formation at the transcriptional level. MshH has its greatest effect on transcription of the *vps* genes in planktonic cells, while EIIA^{Glc} has its greatest effect in biofilm cells. Because the effect of MshH on *vps* gene transcription in planktonic cells is dependent on EIIA^{Glc}, we hypothesize that MshH interferes with activation of *vps* gene transcription by EIIA^{Glc} in planktonic cells.

MshH is a homolog of *E. coli* CsrD, showing 33% identity and 53% similarity. CsrD accelerates degradation of the small RNAs *csrB* and *csrC* (32). These small RNAs, in turn, inhibit the action of CsrA, an mRNA-binding protein that regulates many aspects of cellular physiology at the posttranscriptional level (38). *V. cholerae*

has three small RNAs that are similar to *csrB* and *csrC*. The third *V. cholerae* small RNA has also been called *csrD*. Therefore, to avoid confusion, we refer to the *V. cholerae* homolog of the CsrD protein as MshH.

We hypothesized that MshH and $EIIA^{Glc}$ might modulate the levels of the *csr* RNAs in *V. cholerae*. To measure the effect of MshH and $EIIA^{Glc}$ on levels of the *csr* RNAs, we quantified *csrA*, *csrB*, *csrC*, and *csrD* transcripts in both planktonic and biofilm cells by quantitative reverse transcriptase-PCR (qRT-PCR). As shown in Fig. 3F, deletion of either *mshH* or the $EIIA^{Glc}$ gene did not alter the levels of the *csr* RNAs in planktonic cells. In contrast, in biofilm cells, deletion of *mshH* increased levels of the *csrD* transcript approximately 8-fold (Fig. 3G). While deletion of $EIIA^{Glc}$ in a wild-type background had no effect on *csr* transcript levels, deletion of $EIIA^{Glc}$ in a Δ*mshH* mutant background decreased *csrD* levels in the biofilm. We hypothesize that while MshH modulates activation of the *vps* genes by $EIIA^{Glc}$ in planktonic cells, $EIIA^{Glc}$ modulates the role of MshH in degradation of *csrD* in the biofilm.

We predicted that if increased *csrD* transcript contributed to the biofilm phenotype of the Δ*mshH* mutant, overexpression of *csrD* should increase the biofilm formed by wild-type *V. cholerae*, and overexpression of *csrA* should decrease biofilm formation. As shown in Fig. 3H, neither of these interventions altered biofilm formation by wild-type *V. cholerae.* Therefore, we conclude that *csrA* and *csrD* do not modulate biofilm formation by this *V. cholerae* strain.

DISCUSSION

Previous experiments showed that $EIIA^{Glc}$ activates *V. cholerae* biofilm formation at the transcriptional level (26). We hypothesized that this occurred through an interaction with a novel signaling or regulatory protein. Therefore, we employed a proteomic approach to identify binding partners of $EIIA^{Glc}$ in planktonic and biofilm cells that might participate in regulation of biofilm formation. Our results suggested that the binding partners of $EIIA^{Glc}$ in planktonic and biofilm cells are distinct. In planktonic cells, we isolated known binding partners of $EIIA^{Glc}$, such as adenylate cyclase and glycerol kinase. In addition, novel binding partners of $EIIA^{Glc}$ were identified in both planktonic and biofilm cells. These include a putative Mg^{2+} transporter, two additional metabolic enzymes, and several putative signaling proteins and transcription factors, including the *V. cholerae* CsrD homolog MshH. Through bacterial two-hybrid assays, we confirmed a direct interaction of MshH with $EIIA^{Glc}$. Our data suggest that MshH inhibits *V. cholerae* biofilm formation by interfering with the ability of $EIIA^{Glc}$ to activate biofilm formation. Furthermore, although both MshH and $EIIA^{Glc}$ modulate cellular levels of the *csr* RNAs, these do not appear to be involved in regulation of biofilm formation. We propose a novel function for MshH as a regulatory link between the PTS and Csr global signaling pathways.

To our knowledge, this study represents the first comparison of the binding partners of $EIIA^{Glc}$ in planktonic and biofilm cells. In the past, the planktonic state has been used to study the binding partners of $EIIA^{Glc}$. Therefore, it is not surprising that the known interaction partners of $EIIA^{Glc}$, GlpK and AC, copurified most abundantly with $EIIA^{Glc}$ in the planktonic phase. In contrast, most of the biofilm-specific binding partners of $EIIA^{Glc}$ are novel. A common theme among these binding partners is the presence of conserved cyclic nucleotide binding domains, which are known to serve a regulatory function.

Several pieces of evidence suggest that the phosphorylation state of $EIIA^{Glc}$ alone does not account for the different interaction partners observed in planktonic and biofilm cells. First of all, both AC, which is thought to bind to phosphorylated $EIIA^{Glc}$, and GlpK, which is thought to bind to unphosphorylated $EIIA^{Glc}$, are major interaction partners in planktonic cells but not the biofilm. Second, only a subset of the proteins that interact with $EIIA^{Glc}$ in biofilm cells are able to interact with $EIIA^{GlcH91A}$, which mimics the unphosphorylated form. Last, our bacterial two-hybrid studies conducted in *E. coli* suggest that MshH and AC interact well with point mutants that mimic both the unphosphorylated and phosphorylated forms of $EIIA^{Glc}$. Therefore, we predict the existence of factors beyond phosphorylation state that determine the interactions of $EIIA^{Glc}$ with its protein partners in the biofilm and planktonic states.

We also studied the interaction partners of AC in biofilm cells. Based on these results, we hypothesize the existence of multiprotein complexes in biofilm cells that include $EIIA^{Glc}$ and AC. Because $EIIA^{Glc}$ is a small protein, binding partners that interact with it under similar conditions are likely to influence each other's binding in either a cooperative or competitive manner.

mshH is the first gene in an operon encoding synthesis of the mannose-sensitive hemagglutinin pilus (MSHA), a structure that promotes attachment of single cells to surfaces but inhibits colonization of the mammalian intestine (39, 40). Previous research has shown that MshH is dispensable for pilus elaboration (41). The transcription factor ToxT coordinates repression of *mshA* and *mshH* with activation of the major virulence factors of *V. cholerae*, the toxin-coregulated pilus and cholera toxin (42). However, the significance of *mshH* regulation by ToxT requires further investigation.

MshH is comprised of an N-terminal periplasmic domain flanked by two predicted transmembrane domains. The cytoplasmic portion of MshH consists of a HAMP-like linker domain followed by GGDEF and EAL domains that are poorly conserved (43). In *E. coli*, these last two domains are not thought to play a direct role in modulating intracellular levels of c-di-GMP (32). Rather, they are thought to bind the *csr* small RNAs, which inhibit the Csr system, thus targeting them for degradation by RNase E. We found that mutation of MshH increases the *csrD* transcript in biofilm cells only. Interestingly, while mutation of $EIIA^{Glc}$ in a wild-type genetic background had no effect on *csr* transcript levels, deletion of $EIIA^{Glc}$ in a Δ*mshH* background returned the *csrD* transcript level almost to wild-type levels. One possible mechanism for this is that the $EIIA^{Glc}$-MshH complex, which is found principally in the biofilm, is responsible for spatial colocalization of *csrD* and RNase E. In the absence of MshH, binding of *csrD* to $EIIA^{Glc}$ may protect it from degradation. However, it is also possible that MshH and $EIIA^{Glc}$ independently regulate *csr* RNAs at the transcriptional level. A direct measurement of RNA stability in the presence and absence of Msh and $EIIA^{Glc}$ would be required to distinguish between these two possibilities.

In *V. cholerae*, the CSR system inhibits the quorum-sensing master regulator HapR, resulting in activation of the *vps* genes and biofilm formation (44). In such strains, we predict that MshH would activate biofilm formation. However, disruption of the quorum-sensing cascade is frequently found in environmental and clinical strains of *V. cholerae* (45), and this is the case for the clinical strain used in the studies presented here. Therefore, our

data are consistent with a model in which the *csr* RNAs do not affect biofilm formation when quorum sensing is disabled.

Many decades of investigation have established EIIA^{Glc} as a regulator of nutrient uptake and utilization in planktonic bacteria (35, 36, 46, 47). Here we have shown that in *V. cholerae*, the biofilm-specific interactions of EIIA^{Glc} are distinct from those in planktonic cells, suggesting that it participates in a different physiologic response. Furthermore, our findings suggest a novel paradigm for EIIA^{Glc} as a hub for multiprotein complexes. Such complexes could provide a mechanism for interaction and competition among the binding partners of EIIA^{Glc}.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Bacterial strains and plasmids used in this study are listed in Table S5 in the supplemental material. Bacteria were grown in LB. When required, the following antibiotics were added to the growth medium: streptomycin (100 μg/ml), ampicillin (50 or 100 μg/ml, as noted), carbenicillin (100 μg/ml), kanamycin (30 μg/ml), and tetracycline (10 μg/ml). For rescue experiments, protein expression was induced with 0, 0.01, 0.1, or 1 mM isopropyl β-d-1-thiogalactopyranoside (IPTG). For bacterial two-hybrid assays, concentrations of 0, 20, and 50 μM IPTG were used for induction.

Tandem affinity purification and LC/MS analysis. A bacterial strain encoding EIIA^{Glc} with a C-terminal TAP tag at its native chromosomal location was generated as follows. A fragment encoding protein A linked by a tobacco etch virus (TEV) protease (TEVP) cleavage site to calmodulin binding protein was inserted into the suicide plasmid pGP704 between the SphI and SmaI restriction sites to create the plasmid pGP704-TAP (48). The terminal 382 bp of the EIIA^{Glc} gene excluding the stop codon were amplified and ligated into pGP704-TAP in frame with the TAP sequence using SalI and SphI restriction sites. The plasmid was then inserted in the chromosome by homologous recombination. Correct insertion was verified by amplification of the inserted fragment from the chromosome and by Western analysis.

For purification of EIIA^{Glc} interaction partners from mid-log, planktonic cells, *V. cholerae* strains were inoculated into 1-liter flasks containing LB supplemented with ampicillin and grown to an optical density at 655 nm (OD_{655}) of 0.5. The planktonic cells were then pelleted, washed, resuspended in lysis buffer (20 mM K-HEPES [pH 7.9], 50 mM KCl, 0.5 mM dithiothreitol [DTT], and 10% glycerol) with protease inhibitors, and sonicated to disrupt cells. To isolate proteins interacting within the biofilm, static cultures of *V. cholerae* were grown for 60 h in 47-mm petri plates containing 40 ml of LB supplemented with ampicillin. The planktonic cells were aspirated, and the biofilm was washed three times with phosphate-buffered saline (PBS). The biofilm was then scraped off the plate into a 50-ml conical tube and washed in lysis buffer.

Particulates were removed from Trizol lysates by centrifugation, and clarified lysates were applied to a column containing either 400 μl (planktonic lysate) or 800 μl (biofilm lysate) of IgG Sepharose 6 Fast Flow resin (GE Healthcare) and incubated at 4°C for 2 h. The column was then washed three times with IPP150 buffer (10 mM Tris-HCl, 150 mM NaCl, and 0.1% NP-40) and equilibrated with TEVP cleavage buffer (IPP150 including 0.5 mM EDTA, 1 mM DTT, and 100 U TEV protease). This suspension was incubated overnight. After removal of the IgG resin, the supernatant was applied to a 400-μl volume of calmodulin affinity resin (Agilent Technologies). This was incubated for 1 h at 4°C, washed three times with IPP150 calmodulin binding buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM magnesium acetate [MgOAc], 1 mM imidazole, 2 mM $CaCl_2$, and 0.1% NP-40) and eluted in elution buffer (10 mM β-mercaptoethanol, 10 mM Tris-HCl, pH 8, 150 mM NaCl, 1 mM MgOAc, 1 mM imidazole, 2 mM EGTA, and 0.1% NP-40). Fractions containing the eluted proteins were concentrated using an Amicon Ultra-4 spin column and run into a 12% SDS-PAGE gel. Gel fragments above and below the tagged protein were removed and submitted for LC-MS analysis.

Generation of deletion mutants. Gene deletions were engineered as previously described using the splice overlap extension (SOE) method and double homologous recombination (18, 49). The primers used for making deletions are listed in Table S6 in the supplemental material.

Construction of plasmids used for protein expression. Rescue experiments were performed using pFLAG-CTC (Invitrogen). The pFLAG-MshH, pFLAG-csrD, and pFLAG-CsrA constructs were generated by PCR amplification of *mshH*, *csrD*, or *csrA*, respectively, using the primers listed in Table S6 in the supplemental material. These fragments were cloned into pFLAG–CTC.

Biofilm assays. *Vibrio cholerae* strains to be tested were grown overnight and then diluted to an OD_{655} of 0.05 in fresh LB. 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 separated from the biofilm, and planktonic growth was assessed by measuring the OD_{655}. Three hundred microliters of PBS solution was added to the biofilm 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 the number of cells associated with the biofilm. Error bars represent the standard deviation, and statistical significance was calculated using a Student *t* test.

Western blot analysis. For detection of TAP-tagged proteins in elution fractions, 12 μl of each eluant fraction was added to 3 μl of 5× Laemmli buffer, boiled for 5 min, and centrifuged briefly. Protein components were separated on a 12% gel (Pierce) by SDS-PAGE. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane using a semidry transfer method. The membrane was then incubated for 1 h at room temperature in a blocking solution consisting of PBS with 0.1% Tween 20 (PBS-T) and 5% skim milk. Blocking solution was removed and replaced with fresh blocking solution supplemented with an horseradish peroxidase (HRP)-conjugated anti-calmodulin binding antibody (1: 5,000) (Immunology Consultants Laboratory, Inc.). After incubation for 1 h, the membrane was washed three times with PBS-T and detected with ECL Plus Western blotting detection reagent (GE Healthcare).

Bacterial two-hybrid analysis. For bacterial two-hybrid analysis, plasmids were constructed as follows. For construction of the pACTR-EIIA^{Glc}-Zif plasmid, the gene encoding EIIA^{Glc} was amplified and ligated into the plasmid pACTR-AP-Zif between NotI and BamHI restriction sites (37). Plasmids encoding the point mutants EIIA^{GlcH91A} and EIIA^{GlcH91D} were constructed similarly except that SOE was used to engineer point mutations prior to amplification. The plasmids pBRω-*mshH* and pBRω-*cya* were constructed by amplifying the genes encoding these proteins and cloning the resulting PCR products into the plasmid pBRGPω. Bacterial two-hybrid assays were performed as previously described (37). In brief, both vectors were electroporated into KDZif1ΔZ cells. Transformants containing both vectors were selected on LB agar plates supplemented with kanamycin, carbenicillin, and tetracycline, inoculated into LB supplemented with the same antibiotics as well as either 0, 20, or 50 μM IPTG, and incubated overnight at 37°C with shaking. The following day, cultures were diluted in fresh LB supplemented with antibiotics and the indicated IPTG concentrations and grown to mid-log phase. Cells were harvested and used in β-galactosidase assays performed according to standard procedures. Two experimental replicates were performed in each trial, and several trials were performed.

***β*-Galactosidase assays.** Strains were grown overnight at 27°C on LB agar plates. Several colonies were resuspended in 1 ml of LB broth supplemented with streptomycin to yield the desired OD_{655}. For mid-log assays, 5-ml cell suspensions of an OD_{655} of 0.004 to 0.005 were placed in 50-ml Falcon tubes and grown with agitation at 27°C for 3 h. Final OD_{655}s, which were between 0.2 and 0.3, were recorded. These cells were pelleted, washed with 500 μl of Z buffer, and then resuspended in 250 μl of Z buffer. For biofilm assays, 300 μl of a 0.05-OD_{655} culture was grown statically for 18 to 20 h in 10- by 75-mm glass tubes (Fisher) at 27°C (50). The OD_{655} of the final culture was measured. A 150-μl volume of cells was pelleted and

resuspended in 200 μl of Z buffer. For both planktonic and biofilm assays, cells were lysed by three freeze-thaw cycles, and 10 μl of the lysate was added to 70 μl of β-galactosidase Chemiluminescent Substrate Plus (Michigan Diagnostics, LLC) in a white 96-well plate and incubated at room temperature for 30 min. Triggering reagent (70 μl) was added to each well, and luminescence was read after 5 min. Three experimental replicates were included in each assay, and the assay was repeated multiple times. β-Galactosidase activity was reported as the luminescence measurement divided by the OD_{655} of the final culture.

Quantitative reverse transcription-PCR. RNA was harvested either from mid-log cultures grown with aeration (planktonic cells) or from cultures grown statically at 27°C (biofilm cells). For planktonic cultures, cells were harvested at an OD_{655} of 0.5 and resuspended in 1 ml of Trizol (Invitrogen). For biofilm cultures, a mid-log culture of *V. cholerae* was inoculated into 90-mm petri dishes to yield a starting OD_{655} of 0.05, incubated for 19 h at 28°C, harvested into 50-ml conical tubes, resuspended in 2.5 ml of Trizol, and diluted 1:1 with fresh Trizol. RNA was isolated from 1 ml of the Trizol suspension as directed by the manufacturer. cDNA was synthesized using the SuperScript III kit (Invitrogen) as directed. Transcripts were quantified by qRT-PCR using the iTaq SYBR green kit (Bio-Rad) along with the Step One Plus real-time PCR system (Applied Biosystems). Transcript levels of *csrA*, *csrB*, *csrC*, and *csrD* were normalized to that of *clpX*. Fold expression was determined using the $\Delta\Delta CT$ method. Three experimental replicates were performed in each trial, and multiple trials were performed.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00228-12/-/DCSupplemental.

Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.
Table S4, DOCX file, 0.1 MB.
Table S5, DOCX file, 0.1 MB.
Table S6, DOCX file, 0.1 MB.

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