The phosphoenolpyruvate phosphotransferase system (PTS) mediates transport and phosphorylation of selected carbohydrates and modulates many cellular functions in response to carbohydrate availability. It plays a role in the virulence of many bacterial pathogens. Components of the carbohydrate-specific PTS include the general cytoplasmic components enzyme I (EI) and histidine protein (HPr), the sugar-specific cytoplasmic components enzymes IIA (EIIA) and IIB (EIIB), and the sugar-specific membrane-associated multisubunit components enzymes IIC (EIIC) and IID (EIID). Many bacterial genomes also encode a parallel PTS pathway that includes the EI homolog EINtr, the HPr homolog NPr, and the EIIA homolog EIIANtr. This pathway is thought to be nitrogen specific because of the proximity of the genes encoding this pathway to the genes encoding the nitrogen-specific σ factor σ54. We previously reported that phosphorylation of HPr and FPr by EI represses Vibrio cholerae biofilm formation in minimal medium supplemented with glucose or pyruvate. Here we report two additional PTS-based biofilm regulatory pathways that are active in LB broth but not in minimal medium. These pathways involve the glucose-specific enzyme EIIA (EIIAGlc) and two nitrogen-specific EIIA homologs, EIIANtr1 and EIIANtr2. The presence of multiple, independent biofilm regulatory circuits in the PTS supports the hypothesis that the PTS and PTS-dependent substrates have a central role in sensing environments suitable for a surface-associated existence.

Attachment of a free-swimming bacterium to a surface, which is termed biofilm formation, is the result of a complex decision tree that occurs when a bacterium encounters a surface (19). Environmental signals dictate the decisions made at each branch point. The advantages afforded to a bacterium by surface attachment depend on the environmental niche of the bacterium being studied, and the environmental signals that induce biofilm formation reflect this. Formation of a multilayer bacterial biofilm often requires synthesis of an extracellular matrix composed of biological polymers that enhance interbacterium attachment. These extracellular polymers may be proteins, polysaccharides, and/or DNA (7). Synthesis of the biofilm matrix is often tightly regulated. The environmental activators of many signaling pathways that modulate multilayer biofilm accumulation have been identified. These activators include specific carbohydrates, quorum-sensing molecules, nucleic acids and their precursors, and polyanimes (1, 6, 9, 13, 15, 17, 18, 26, 31, 33, 35, 41, 45, 53). However, there are many known biofilm regulatory pathways for which no environmental activator has been identified yet.

The phosphoenolpyruvate phosphotransferase system (PTS) is a multicomponent phosphotransfer cascade that mediates transport and phosphorylation of selected sugars, such as glucose, sucrose, mannose, and N-acetylglucosamine (10). In addition, it has been implicated in the formation of biofilms by diverse organisms (1, 2, 17, 31, 42). Phosphate enters the PTS through transfer from phosphoenolpyruvate to the first PTS component, the phosphoenolpyruvate-protein phosphotransferase or enzyme I (EI). EI in turn transfers the phosphate group to another component of the PTS, histidine protein (HPr). Many bacterial genomes also encode a protein homologous to HPr termed FPr, which is preferred for transport of fructose through the PTS. HPr and FPr transfer phosphate to a number of enzymes II, which are multisubunit, membrane-associated complexes that carry out transport and phosphorylation of specific PTS substrates. Because transport of PTS substrates rapidly depletes the PTS of phosphorylated intermediates, the phosphorylation states of PTS components serve as cytoplasmic reporters of environmental nutrient availability. These reporters then modulate cellular functions such as chemotaxis (60), uptake and catabolism of PTS-independent carbohydrates (1, 12, 39), and glycogen breakdown (54, 55).

The genomes of many Gram-negative organisms contain genes encoding another phosphotransfer cascade that is homologous to the carbohydrate-transporting PTS. Because these genes are close to rpoN, which encodes the sigma factor involved in transcription of many genes required for nitrogen assimilation, this phosphotransfer cascade is termed the nitrogen-related PTS or PTSNtr (22, 49, 50). The components of this phosphotransfer cascade include EINtr, NPr, and EIIANtr. Unlike the carbohydrate-transporting PTS, PTSNtr does not include membrane-associated components and, therefore, does not partic-
ipate directly in transport of nutrients into the cell. Rather, its primary function in the cell is thought to be regulatory (22, 24, 25, 32, 44). While the breadth and overarching goal of the PTSNtr have not been defined, transfer of phosphate between the two PTS cascades may be one mechanism by which the PTSNtr influences cellular function (46).

*Vibrio cholerae* is a Gram-negative bacterium whose natural habitat includes environments with low and intermediate salinities, such as ponds and estuaries (8). There is some evidence that *V. cholerae* forms a multilayer, exopolysaccharide-based biofilm in freshwater environments (20). The exopolysaccharide is referred to as VPS (*Vibrio* polysaccharide), and the region of the *V. cholerae* genome containing many of the genes required to make the biofilm matrix is known as the *vps* island (64).

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When pathogenic *V. cholerae* is ingested by humans in contaminated food or water, the diarrheal disease cholera results. While many studies of human and murine infection have suggested that proteins required for biofilm matrix synthesis are expressed *in vivo* (14, 30), no study has found that these proteins are essential for colonization of the mammalian intestine (16, 23, 63).

*Vibrio cholerae* is a Gram-negative bacterium whose natural habitat includes environments with low and intermediate salinities, such as ponds and estuaries (8). There is some evidence that *V. cholerae* forms a multilayer, exopolysaccharide-based biofilm in freshwater environments (20). The exopolysaccharide is referred to as VPS (*Vibrio* polysaccharide), and the region of the *V. cholerae* genome containing

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**FIG. 1.** Schematic diagram of the 25 *V. cholerae* PTS components. Homologs of the EI, HPt, and EII proteins are indicated by the same colors. Furthermore, proteins belonging to the same EIIA superfamily are color coded (blue, glucose superfamily; violet, mannitol-fructose superfamily; yellow, lactose superfamily). Sugar specificities are indicated for PTS components if they are known (16). Parentheses indicate presumptive specificities. The VC1824 HPt-like domain is surrounded by a dashed line to indicate its low level of similarity to HPt. The sugar specificity of VC1281 is based on previously described data (3). NAG, N-acetylglucosamine.
discovered that supplementation of minimal medium (MM) with PTS sugars activates transcription of the vps genes and formation of a multilayer biofilm (40). Furthermore, we found that phosphorylated form of EI represses *V. cholerae* biofilm formation in minimal medium (17). Subsequent biofilm assays conducted with Luria-Bertani (LB) broth suggested that there are additional PTS-based regulatory pathways. Here we define three PTS-based biofilm regulatory pathways that are present when this medium is used. These studies illustrate the complexity of the regulation of *V. cholerae* biofilm accumulation by PTS components and underscore the importance of carbohydrates as signals in the decision of *V. cholerae* cells to join a biofilm.

### MATERIALS AND METHODS

**Bacterial strains, plasmids, and media.** The bacterial strains and plasmids used in this study are listed in Table 1. *V. cholerae* strain PW357 was used for biofilm assays. Bacteria were cultivated in Luria-Bertani broth or minimal medium supplemented with 0.5% (wt/vol) glucose or pyruvate (Sigma) (17). Where specified below, a 0.1 M phosphate-buffered saline solution (PBS) (pH 7.0) was used.

**Construction of mutants.** The plasmid used to make the ΔvpsL mutant, pKEK::ΔvpsLp, was generously provided by J. Reidl (3). All other plasmids used in the construction of insertion and in-frame deletion mutants were available in our laboratory (16, 17). Suicide plasmids were used to generate insertion and in-frame deletion mutants by single and double homologous recombination, respectively.

**Construction of rescue plasmids.** Plasmids used for rescue experiments, which are listed in Table 1, were constructed as previously described (17, 61) using the primers listed in Table 2. Briefly, the native sequence of the targeted gene was amplified by PCR and cloned into pBAD-TOPO (Invitrogen) using the manufacturer’s protocol. Point mutation sequences were generated by amplification of two gene fragments using internal primers with overlapping sequences containing the desired mutation. The two fragments were joined using the splicing by overlap extension (SOE) technique and cloned into pBAD-TOPO (Invitrogen) using the manufacturer’s protocol. The sequence of the inserted fragment was confirmed by amplification and sequence analysis. The rescue plasmids were then

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introduced into \emph{V. cholerae} strains by electroporation, and transformants were selected on LB agar supplemented with ampicillin. A pBAD-TOPO vector containing an antisense fragment of the coding sequence for EIIAGlc(H91A) was used as a control plasmid for rescue experiments. Expression of the cloned protein was evaluated by Western blot analysis as previously described (16).

**Quantitative analysis of total growth and biofilm formation.** Quantification of surface association was performed as described previously (20). Briefly, the strains were grown overnight on LB agar plates at 27°C. The following morning, the resulting colonies were used to inoculate borosilicate tubes filled with 300 μl of LB broth or MM supplemented with glucose or pyruvate. After incubation for 18 to 24 h at 27°C, each planktonic cell suspension was collected, and the planktonic cell density was estimated by measuring the optical density at 655 nm (OD655) using a Benchmark Plus microplate spectrophotometer (Bio-Rad). To quantify the surface-attached cells, 300 μl of PBS and a small number of 1-mm glass beads were added to the surface-attached cells remaining in the borosilicate tube, and the cells were dispersed by vortexing. The OD655 of the resulting cell suspension was measured. Each total growth value represented the sum of the planktonic and biofilm-associated cell densities. The values are the means of at least three experimental replicates, and standard deviations were determined. Statistical significance was calculated using a two-tailed \( t \)-test.

**Quantification of PTS component transcripts.** Wild-type \emph{V. cholerae} was cultered for 18 h in LB broth or MM supplemented with pyruvate (0.5%, wt/vol). The cells were then pelletted by centrifugation, and total RNA was isolated using an RNaseasy kit (Qiagen), followed by RNase-free DNase I treatment to remove contaminating DNA. Reverse transcription-PCR (RT-PCR) was performed using 1 to 2 ng of total RNA with a SuperScriptIII first-strand kit (Invitrogen). Subsequently, 15 ng of the resulting cDNA was added to each well. After incubation in the dark at room temperature for 30 min, luminescence was measured with an Infinite 200 spectrophotometer (Tecan). Three experimental replicates were included each time that the experiment was performed, the experiment was repeated three times, and similar results were obtained in the three experiments. Values for a representative experiment are reported below and are the means of three experimental replicates; standard deviations were also determined, and statistical significance was calculated using a two-tailed \( t \)-test.

**RESULTS**

The biofilm phenotypes of \( ΔEi \) and \( ΔEiΔGlc \) mutants in LB broth are additive. We first compared biofilm formation in LB broth and minimal medium supplemented with glucose by a \( ΔEi \) mutant, a \( ΔEiΔGlc \) mutant, and a \( ΔPTS \) mutant lacking the genes encoding EI, HPr, and EIIA Glc (Fig. 2). Similar to

![Biofilm accumulation](attachment:biofilm.png)  
**FIG. 2.** PTS mutant displays distinct biofilm phenotypes in LB broth and minimal medium (MM) supplemented with glucose. Total growth and biofilm accumulation were determined for wild-type \emph{V. cholerae} (WT), a \( ΔEi \) mutant, a \( ΔEiΔGlc \) mutant, and a \( ΔPTS \) mutant lacking the genes encoding EI, HPr, and EIIA Glc (Fig. 2). Similar to

\[ \text{Biofilm accumulation} \]

\[ \text{Total growth} \]

\[ \text{MM + glucose} \]

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what was observed in minimal medium, in LB broth a ΔEI mutant exhibited increased surface accumulation compared with wild-type *V. cholerae*, while a ΔEIIAGlc mutant exhibited decreased surface accumulation. As previously noted (17), surface accumulation by a ΔPTS mutant lacking EI, HPr, and EIIAGlc was increased in minimal medium supplemented with glucose. In contrast, the surface accumulation by the ΔPTS mutant was similar to that by wild-type *V. cholerae* in LB broth. This suggested that the biofilm phenotype of the ΔPTS mutant in LB broth might reflect the additive effects of two opposing regulatory pathways.

**Evidence for repression of biofilm accumulation by EI-P and HPr-P in LB broth (regulatory pathway 1).** In both minimal medium and LB broth, EI represses biofilm formation (17). To determine if the phosphorylated form of EI is required for this repression, we measured biofilm formation by a ΔEI mutant rescued with a control plasmid, with a plasmid carrying a wild-type EI allele, or with a plasmid carrying a mutant EI allele in which phosphorylated histidine 189 had been changed to alanine. As shown in Fig. 3A, while the biofilm phenotype of a ΔEI mutant was rescued by a wild-type EI allele provided in trans, the EI^H189A allele was unable to rescue the biofilm phenotype despite adequate expression of the protein (Fig. 3B). This suggests that, similar to what was observed in minimal medium (17), EI-P represses *V. cholerae* biofilm formation in LB broth. We previously reported that in minimal medium (17), EI-P represses biofilm formation by a ΔEI mutant rescued with a control pBAD plasmid (pCTL), a pBAD plasmid expressing a wild-type EI allele (pEI), or a pBAD plasmid expressing an EI allele encoding an H-to-A mutation at position 189 (pEI^H189A). Protein expression was induced by addition of 0.04% L-arabinose. The values are the averages of four experimental replicates, and the error bars indicate standard deviations. Biofilm formation by the ΔEI mutant rescued with a wild-type EI allele was not statistically different from biofilm formation by wild-type *V. cholerae* (P = 0.290), while biofilm formation by a ΔEI mutant rescued with the EI^H189A allele was statistically different (P = 0.0007). (B) Western blot analysis demonstrating that the wild-type EI and EI^H189A alleles are well expressed in the ΔEI genetic background. (C) Quantification of total growth and biofilm formation over time for wild-type *V. cholerae* and a ΔEI mutant. The values are the means of two experimental replicates. The ΔEI mutant demonstrated more biofilm accumulation than wild-type *V. cholerae* throughout the course of the experiment.

In minimal medium, repression of biofilm formation by EI requires phosphotransfer to either HPr or FPr (16). Furthermore, this repression occurs at the level of transcription. To determine if phosphotransfer from EI to HPr or FPr contributed to repression of biofilm accumulation in LB broth, we compared biofilm formation by wild-type *V. cholerae* and biofilm formation by ΔHPr, ΔFPr, and ΔHPr ΔFPr mutants. As shown in Fig. 4A, biofilm formation by a ΔHPr mutant was approximately 2-fold greater than that by wild-type *V. cholerae*, while biofilm formation by a ΔFPr mutant was not significantly different. To demonstrate that EI is upstream of HPr/FPr in the signaling pathway, we documented that a wild-type EI allele provided in trans repressed biofilm formation by a ΔEI mutant but was unable to repress biofilm formation in the absence of HPr and FPr (Fig. 4B). Lastly, we measured activation of the *vps* genes in wild-type *V. cholerae* and ΔEI, ΔHPr, ΔFPr, and ΔHPr ΔFPr mutants using a chromosomal lacZ reporter fusion to the *vpsL* promoter. As shown in Fig. 4C, deletion of EI and HPr but not FPr increased transcription of the *vps* genes. Thus, the regulatory pathway leading to re-
expression of biofilm formation and vps gene transcription through phosphorylation of EI and HPr is present in both minimal medium and LB broth. This pathway is referred to in this paper as pathway 1 (see Fig. 14).

Three EIIA homologs regulate biofilm accumulation. To further define regulation of biofilm formation by the PTS, we compared biofilm accumulation in LB broth by wild-type _V. cholerae_ and biofilm accumulation in LB broth by strains having mutations in each of the 10 genes encoding EIIA domains. The biofilm accumulation by the ∆EIIAGlc (P < 0.0001), ∆EIIANtr1 (P < 0.0001), and ∆VC1824 (P = 0.0004) mutants was significantly different from that by wild-type _V. cholerae_ (WT). The error bars indicate standard deviations.

Because independent control of biofilm formation by an EIIA component was not observed in minimal medium (16), we hypothesized that transcription of EIIAGlc, EIIANtr1, and EIIANtr2 might be activated in LB broth compared to their transcription in minimal medium. To test this hypothesis, we used quantitative RT-PCR to compare the transcript levels of EI, HPr, FPr, EIIAGlc, EIIANtr1, and EIIANtr2 in LB broth and minimal medium supplemented with pyruvate (Fig. 6). Pathway 1 was present in both minimal medium and LB broth. However, the transcript levels of the pathway 1 components EI and HPr were lower in LB broth. The transcription of FPr, imately 3-fold fewer ∆EIIAGlc mutant cells accumulated in a biofilm than wild-type _V. cholerae_ cells. Second, mutations in VC2531 and VC1824 led to increases in biofilm accumulation. VC2531 (EIIANtr1) and VC1824 (EIIANtr2) both contain EIIA domains homologous to EIIANtr, and VC1824 also includes an N-terminal domain which is homologous to HPr but is not as closely related as the other _V. cholerae_ HPr homologs (Fig. 1). Our observations suggested that at least two independent PTS pathways control biofilm formation at the level of EIIA in LB broth.

Because independent control of biofilm formation by an EIIA component was not observed in minimal medium (16), we hypothesized that transcription of EIIAGlc, EIIANtr1, and EIIANtr2 might be activated in LB broth compared to their transcription in minimal medium. To test this hypothesis, we used quantitative RT-PCR to compare the transcript levels of EI, HPr, FPr, EIIAGlc, EIIANtr1, and EIIANtr2 in LB broth and minimal medium supplemented with pyruvate (Fig. 6). Pathway 1 was present in both minimal medium and LB broth. However, the transcript levels of the pathway 1 components EI and HPr were lower in LB broth. The transcription of FPr,
which does not appear to play a role in regulation of biofilm accumulation in LB broth, was elevated in LB broth compared with minimal medium. The transcription of EIIAGlc was approximately 6-fold greater in LB broth, suggesting that activation of biofilm formation by EIIAGlc in LB broth may be due at least in part to increased transcription of EIIAGlc. Transcription of the PTS components EIINA and EIINIC was not increased in LB broth. Therefore, differential transcription of these components does not underlie the observation that they regulate biofilm formation in LB broth but not in minimal medium.

Activation of biofilm formation by EIIAGlc does not require phosphorylation of the conserved histidine at position 91 (regulatory pathway 2). Phosphotransfer through EI and HPr is predicted to lead to EIIA Glc phosphorylation at histidine 91. To determine whether regulation of biofilm formation by EIIA Glc requires phosphorylation of this residue, we studied biofilm accumulation by a ΔEIIA Glc mutant containing an expression plasmid carrying either a control sequence, a wild-type EIIA Glc allele, an EIIA Glc allele with a histidine-to-alanine mutation at position 91, or an EIIA Glc allele with a histidine-to-aspartate mutation at position 91. As shown in Fig. 7A, while these EIIA Glc alleles did not affect the total growth, all of them activated biofilm accumulation in the ΔEIIA Glc mutant background. To further establish that this rescue was not dependent on either EI or HPr and, therefore, was not part of regulatory pathway 1, we measured activation of biofilm accumulation by the same EIIA Glc alleles in a ΔPTS mutant background, which lacks EI, HPr, and EIIA Glc (Fig. 7B). If EIIA Glc were dependent on EI or HPr for activation of biofilm formation, an EIIA Glc allele would be unable to activate biofilm accumulation in the absence of EI or HPr. However, we observed that rescue of biofilm accumulation by the ΔPTS mutant was possible with each of the three EIIA Glc alleles but not with the control plasmid. Lastly, we demonstrated by Western analysis that all rescue constructs resulted in expression of a full-length protein in the ΔEIIA Glc mutant background (Fig. 7C). Therefore, we concluded that activation of biofilm accumulation by EIIA Glc does not require phosphorylation of the conserved histidine at position 91 and that it represents a second, independent PTS-based biofilm regulatory pathway (regulatory pathway 2).

Evidence that EIIBC Glc and the transcription factor Mlc participate in regulatory pathway 2. Several proteins are known to interact with EIIA Glc. Two of these proteins are EIIBC Glc and Mlc. EIIBC Glc is downstream of EIIA Glc in the glucose-specific PTS phosphotransfer cascade. Furthermore, in Escherichia coli, the DNA-binding protein Mlc has been shown to repress transcription of EIIBC Glc when PTS substrates are scarce (21). Unphosphorylated EIIBC Glc interacts directly with Mlc to relieve this repression (27, 43). The V. cholerae Mlc and EIIBC Glc proteins are very similar to the corresponding proteins of E. coli. We therefore asked whether EIIBC Glc and Mlc might play a role in regulation of V. cholerae biofilm formation by EIIA Glc. We first measured biofilm accumulation by wild-type V. cholerae, a ΔEIIBC Glc mutant, a ΔMlc mutant, and a ΔEIIA Glc mutant, as well as by strains with combinations of the mutations. As shown in Fig. 8, the total levels of growth were comparable for all of the strains studied. However, biofilm formation by the ΔMlc and ΔEIIA Glc mutants was significantly decreased, while biofilm accumulation by the ΔEIIBC Glc mutant was increased compared with wild-type V. cholerae biofilm accumulation. Furthermore, deletion of either Mlc or EIIA Glc in a ΔEIIBC Glc mutant background was sufficient to decrease the biofilm accumulation almost to the levels observed for the ΔEIIA Glc mutant. This led us to hypothesize
that the biofilm regulatory pathway shown in Fig. 9A is present. In this signal transduction pathway, EIIBC<sup>Glc</sup> activates transcription of the vps genes by an as-yet-unidentified mechanism.

To further evaluate the presence of this signal transduction pathway, we measured vps<sub>L</sub> transcription in wild-type V. cholerae and ΔEIIA<sup>Glc</sup> and ΔEIIBC<sup>Glc</sup> mutants, as well as in strains with combinations of these mutations. The results of this experiment are shown in Fig. 9B along with the components of the signal transduction pathway present in each genetic background and the relative amount of vps transcription expected based on functional pathway components. The data support a model in which Mlc activates ΔEIIA<sup>ΔGlc</sup> and is repressed by EIIBC<sup>ΔGlc</sup>. Furthermore, the level of vps transcription in a ΔEIIA<sup>Glc</sup>ΔEIIBC<sup>Glc</sup> mutant is higher than that in a ΔEIIA<sup>Glc</sup>ΔEIIBC<sup>Glc</sup>ΔMlc mutant, suggesting that Mlc activates vps<sub>L</sub> transcription even in the absence of EIIA<sup>Glc</sup>. Therefore, an additional pathway must be postulated, in which Mlc activates vps<sub>L</sub> transcription independent of EIIA<sup>Glc</sup>. These pathways are referred to in Fig. 9A as pathways 2a and 2b, respectively.

In general, the measurements of biofilm accumulation correlated well with measurements of vps<sub>L</sub> transcription. However, differences were found. First, there was no difference in vps<sub>L</sub> transcription by the ΔEIIA<sup>Glc</sup> ΔEIIBC<sup>Glc</sup> mutant as well as in ΔEIIA<sup>Glc</sup>ΔMlc and ΔEIIA<sup>Glc</sup>ΔMlc mutants, as shown in Fig. 10, while a control vector had no effect on biofilm accumulation by wild-type V. cholerae lacZ-locus. The measurements of β-galactosidase activity reflect vps<sub>L</sub> transcription. The measurements for the ΔEIIA<sup>Glc</sup>ΔEIIBC<sup>Glc</sup>ΔMlc mutant was significantly different from that for wild-type V. cholerae. The vps<sub>L</sub> transcription in the ΔEIIA<sup>Glc</sup>ΔEIIBC<sup>Glc</sup>ΔMlc mutant was significantly different from that in the ΔEIIBC<sup>Glc</sup>ΔMlc mutant (P = 0.0003) but not from that in the ΔMlc mutant (P = 0.766). The relative contributions of the regulatory components to vps<sub>L</sub> gene transcription as shown in panel A were added to obtain the predicted impact of each genetic background on vps<sub>L</sub> gene transcription. The relative contributions of the regulatory components to vps<sub>L</sub> gene transcription as shown in panel A were added to obtain the predicted impact of each genetic background on vps<sub>L</sub> gene transcription. The relative contributions of the regulatory components to vps<sub>L</sub> gene transcription as shown in panel A were added to obtain the predicted impact of each genetic background on vps<sub>L</sub> gene transcription. The relative contributions of the regulatory components to vps<sub>L</sub> gene transcription as shown in panel A were added to obtain the predicted impact of each genetic background on vps<sub>L</sub> gene transcription.
mulation by any of these mutants, introduction of the mlc allele into the ΔMlc and ΔEIIBC\textsuperscript{Glc} ΔMlc mutants increased biofilm accumulation approximately 4-fold. In contrast, introduction of the mlc allele into a ΔEIIBC\textsuperscript{Glc} ΔMlc mutant increased biofilm accumulation only 2-fold. This result supports our model for pathway 2 in which Mlc activates biofilm accumulation through EIIBC\textsuperscript{Glc}-dependent and -independent pathways.

\textbf{EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} and EI\textsuperscript{A\text{Ntr}}\textsuperscript{2} repress biofilm accumulation (regulatory pathway 3).} The gene encoding EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}, which is located close to \textit{rpoN}, has been found in many bacterial genomes (36, 48, 51). Mutation of the two \textit{V. cholerae} EI\textsuperscript{A\text{Ntr}} homologs, EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} (60\% identity and 75\% similarity to \textit{E. coli} EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}) and EI\textsuperscript{A\text{Ntr}}\textsuperscript{2} (26\% identity and 42\% similarity to \textit{E. coli} EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}), resulted in increased biofilm accumulation (Fig. 5). The conserved histidine residues at position 66 of EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} and position 172 of EI\textsuperscript{A\text{Ntr}}\textsuperscript{2} are predicted to be phosphorylated by phosphotransfer from the HPr homolog NPr, which in turn receives a phosphate from EI\textsuperscript{B\text{CGlc}}. We first determined whether phosphorylation of the conserved histidine was required for the function of EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} by comparing biofilm accumulation data for ΔEI\textsuperscript{A\text{Ntr}}\textsuperscript{1} mutants carrying either a plasmid encoding a control sequence, a wild-type allele of EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}, a mutant allele having a histidine-to-alanine substitution at residue 66, or a mutant allele having a histidine-to-aspartate substitution at residue 66. Rescue of the biofilm phenotype of a ΔEI\textsuperscript{A\text{Ntr}}\textsuperscript{1} mutant was observed with both mutant alleles, as well as with the wild-type allele of EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} (Fig. 11A). Furthermore, all alleles of EI\textsuperscript{A\text{Ntr}}\textsuperscript{1} were well expressed as full-length transcripts (Fig. 11B). This result suggests that phosphorylation of the conserved histidine at position 66 is not required for repression of biofilm accumulation by EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}. Based on this observation, we also predicted that the upstream components of the PTS\textsuperscript{Ntr} would not be required for repression of biofilm accumulation by EI\textsuperscript{A\text{Ntr}}\textsuperscript{1}. To test this hypothesis, we con-
The function of EI is to act downstream of EIIA Ntr1 in a common target. While we have not ruled out a model in which one whether EI and EIIA Ntr1 function independently. As shown in Fig. 11D, return of a wild-type EI allele to a mutant partially rescued the increased biofilm phenotype, suggesting that EI regulates biofilm formation independent of EIIA Ntr1. Return of a wild-type EIIA Ntr1 allele did not reduce biofilm formation by a wild-type V. cholerae (biofilm regulatory pathway 3) (A) Quantification of total growth and biofilm accumulation by wild-type V. cholerae or a ΔEIIA Ntr2 mutant rescued with a pBAD plasmid carrying a control sequence (pCtL), a wild-type ΔEIIA Ntr2 allele (pEIIA Ntr2), a ΔEIIA Ntr2 allele encoding an H-to-A point mutation at conserved residue 172 (pEIIA Ntr2H172A), a ΔEIIA Ntr2 allele encoding an H-to-D point mutation at conserved residue 172 (pEIIA Ntr2H172D), or a wild-type EIIA Ntr2 allele (pEIIA Ntr2). Protein expression was induced with 0.04% arabinose. As expected, we found that biofilm accumulation by wild-type V. cholerae was not significantly different from that by wild-type V. cholerae (WT). The error bars indicate standard deviations. RLU, relative light units.

Because biofilm formation is also repressed by EI, we asked whether phosphorylation of the conserved histidine at position 172 is required for repression of biofilm formation by EI. We previously described a PTS-dependent biofilm regulatory pathway present in minimal medium. This pathway requires phosphotransfer from EI to HPr or FPr for action (pathway 1) (16, 17). Here, we examined regulation of biofilm formation by the PTS in LB broth, a complex medium. Our studies established the action of pathway 1 in V. cholerae biofilms formed in LB broth and also identified two novel PTS-dependent pathways, one of which (pathway 2) leads to activation of biofilm formation by EIIA Ntr1 and one of which (pathway 3) results in repression of biofilm formation by EIIA Ntr2 (Fig. 14). All three of these pathways regulate transcription of vps gene transcription in wild-type V. cholerae, a ΔEIIA Ntr2 mutant, and a ΔEIIA Ntr2 mutant harboring a chromosomal vpsL-lacZ fusion. The vpsL transcription in the ΔEIIA Ntr1 and ΔEIIA Ntr2 mutants was significantly different from that in wild-type V. cholerae (WT) (P < 0.0001). The error bars indicate standard deviations.

FIG. 13. EIIA Ntr1 and EIIA Ntr2 operate at the level of transcription: measurements of vps gene transcription in wild-type V. cholerae, a ΔEIIA Ntr2 mutant, and a ΔEIIA Ntr2 mutant harboring a chromosomal vpsL-lacZ fusion. The vpsL transcription in the ΔEIIA Ntr1 and ΔEIIA Ntr2 mutants was significantly different from that in wild-type V. cholerae (WT) (P < 0.0001). The error bars indicate standard deviations. RLU, relative light units.

FIG. 12. Repression of biofilm formation by EIIA Ntr2 does not require phosphorylation at histidine 172 and is rescued by EIIA Ntr1 (biofilm regulatory pathway 3) (A) Quantification of total growth and biofilm accumulation by wild-type V. cholerae or a ΔEIIA Ntr2 mutant rescued with a pBAD plasmid carrying a control sequence (pCtL), a wild-type ΔEIIA Ntr2 allele (pEIIA Ntr2), a ΔEIIA Ntr2 allele encoding an H-to-A point mutation at conserved residue 172 (pEIIA Ntr2H172A), a ΔEIIA Ntr2 allele encoding an H-to-D point mutation at conserved residue 172 (pEIIA Ntr2H172D), or a wild-type EIIA Ntr2 allele (pEIIA Ntr2). Protein expression was induced with 0.04% arabinose. As expected, we found that biofilm accumulation by wild-type V. cholerae was not significantly different from that by wild-type V. cholerae (WT). The error bars indicate standard deviations. (B) Western blot demonstrating that the wild-type and mutant EIIA Ntr2 alleles used in these experiments are well expressed and produce full-length proteins.
Regulation of biofilm formation by EIIA\text{Glc} does not require phosphorylation of the conserved histidine at position 91. Therefore, the mechanism by which this function of EIIA\text{Glc} is controlled in response to environmental cues is unclear. Because transcription of \textit{V. cholerae} EIIA\text{Glc} is 6-fold greater in LB broth than in minimal medium and 10-fold greater in response to addition of a PTS substrate to minimal medium (17), one possibility is that the activity of EIIA\text{Glc} is regulated by its intracellular abundance.

The results of epistasis experiments and measurements of \textit{vpsL} transcription are consistent with a model in which Mlc activates biofilm accumulation through EIIA\text{Glc}-dependent and EIIA\text{Glc}-independent pathways. EIIBC\text{Glc}, in turn, inhibits the action of Mlc. Because Mlc is a transcription factor, we hypothesize that it acts at the transcriptional level to increase EIIA\text{Glc} activity either directly or indirectly. \textit{E. coli} Mlc functions as a transcriptional repressor of many PTS components (47, 59). However, it does not regulate transcription of EIIA\text{Glc} \textit{E. coli} EIIBC\text{Glc}, an integral membrane protein, blocks the action of Mlc by sequestering it at the inner membrane (27, 58). We hypothesize that a similar interaction between Mlc and EIIBC\text{Glc} may block activation of EIIA\text{Glc} by Mlc (pathway 2a) (Fig. 14). Our data also suggest that a small portion of the observed activation of \textit{vps} gene transcription by Mlc may proceed through an EIIA\text{Glc}-independent pathway (pathway 2b) (Fig. 14).

We identified two EIIA\text{Ntr} homologs that repress \textit{V. cholerae} biofilm formation and \textit{vps} gene transcription in LB broth but not in minimal medium (pathway 3) (Fig. 14). Transcription of the EIIA\text{Ntr} homologs in these two media is similar. Therefore, this regulatory pathway is not controlled at the transcriptional level. The mechanism by which this pathway is activated remains to be identified.

We have not ruled out the possibility that there is an interaction between pathways 2 and 3. However, mutation of EIIA\text{Ntr1} or EIIA\text{Ntr2} in a \textit{ΔEIIA\text{Glc}} background activates biofilm formation (data not shown), suggesting that EIIA\text{Ntr1} and EIIA\text{Ntr2} act independent of EIIA\text{Glc}.

While EIIA\text{Ntr} homologs have not been associated with biofilm formation in other organisms, they have been implicated in regulation of diverse metabolic functions, some of which have a clear role in regulating the balance of carbon and nitrogen in the cell. Two examples of this are repression of polyhydroxyalkanoate synthesis in \textit{Pseudomonas putida} and repression of \(\beta\)-hydroxybutyrate synthesis in \textit{Azobacter vinelandii} by EIIA\text{Ntr} homologs (44, 62). Both of these carbohydrate polymers are intracellular carbon storage compounds that are accumulated when the environmental carbohydrate supply exceeds the bacterial requirement. VPS, the extracellular polysaccharide which is a component of the \textit{V. cholerae} biofilm matrix, may also play a role in carbohydrate balance. VPS synthesis is activated when PTS substrates are abundant and is repressed when PTS substrates become scarce (17). Thus, in repressing VPS synthesis, \textit{V. cholerae} EIIA\text{Ntr} may have a function parallel to that of its homologs in \textit{P. putida} and \textit{A. vinelandii}.

The mechanism by which EIIA\text{Ntr} represses polysaccharide synthesis in \textit{P. putida} and \textit{A. vinelandii} has not been discovered yet. However, the mechanism of action of EIIA\text{Ntr} in transcriptional derepression of the \textit{ihB} operon, which encodes aceto-
hydroxy acid synthase I, a common enzyme in biosynthesis of branched-chain amino acids, has been established (25). By an unknown mechanism, transcription of this operon is inhibited by high intracellular levels of K⁺. The unphosphorylated form of EIIANtr inhibits the action of the K⁺ transporter TrkA, which keeps intracellular K⁺ levels low and preserves transcription of the ib/vn operon (24). In our experiments, phosphorylation of neither EIIANtr1 nor EIIANtr2 was required for activation of vpsL gene transcription. One possibility is that regulation of gene transcription by EIIANtr in V. cholerae is governed by intracellular K⁺ levels in a phosphorylation state-independent manner. Alternatively, EIIANtr may be involved in a regulatory interaction with a different protein.

There are several examples of V. cholerae functions that are governed by complex regulatory networks with multiple inputs. These functions include production of virulence factors (34), quorum sensing (37), chemotaxis, and regulation of biofilm formation by cyclic-di-GMP (4, 5, 29, 57). In each case, the mechanism by which various environmental signals are integrated and result in the observed phenotype is unknown. Regulation of biofilm formation in V. cholerae by the PTS also appears to be quite complex. Immediate goals include identification of downstream components of these pathways, and integration of the three pathways in regulation of biofilm formation by the PTS. In particular, it seems likely that these pathways eventually interface with members of the large family of V. cholerae proteins that modulate intracellular levels of c-di-GMP and whose activators remain largely unidentified.

The V. cholerae PTS participates in colonization of both environmental surfaces and the mammalian intestine (16). We previously demonstrated that the influence of the PTS on VPS-dependent biofilm formation does not play a role in colonization of the mammalian intestine. The regulatory pathways outlined here are hypothesized to participate in colonization of the environment. In particular, a close environmental relationship between V. cholerae and zooplankton has been demonstrated. V. cholerae is thought to degrade the chitinaceous exoskeletons of these organisms to N-acetylglucosamine, a sugar transported exclusively by the PTS (16). Therefore, accumulation of V. cholerae on the exoskeletons of zooplankton is likely to be regulated by these PTS-dependent pathways. We envision that after formation of a monolayer on a surface, augmentation of the biofilm through cell division coupled with VPS synthesis is finely tuned to the nutritive potential of the surface itself and the environment in which the surface occurs. The signaling pathways outlined here contribute to bacterial sensing of surfaces and their environments and highlight the importance of PTS substrates in this process.

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