JOURNAL OF BACTERIOLOGY, June 2010, p. 3055–3067 Vol. 192, No. 12
0021-9193/10/$12.00 doi:10.1128/JB.00213-10
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The Phosphoenolpyruvate Phosphotransferase System Regulates *Vibrio cholerae* Biofilm Formation through Multiple Independent Pathways[▽]

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Received 28 February 2010/Accepted 3 April 2010

The bacterial phosphoenolpyruvate phosphotransferase system (PTS) is a highly conserved phosphotransfer cascade that participates in the 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 EI^{Ntr}, the HPr homolog NPr, and the EIIA homolog $EIIA^{Ntr}$. 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 σ^{54}. 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 ($EIIA^{Glc}$) and two nitrogen-specific EIIA homologs, $EIIA^{Ntr1}$ and $EIIA^{Ntr2}$. 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 polyamines (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 PTS^{Ntr} (22, 49, 50). The components of this phosphotransfer cascade include EI^{Ntr}, NPr, and $EIIA^{Ntr}$. Unlike the carbohydrate-transporting PTS, PTS^{Ntr} does not include membrane-associated components and, therefore, does not partic-

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▽ Published ahead of print on 16 April 2010.

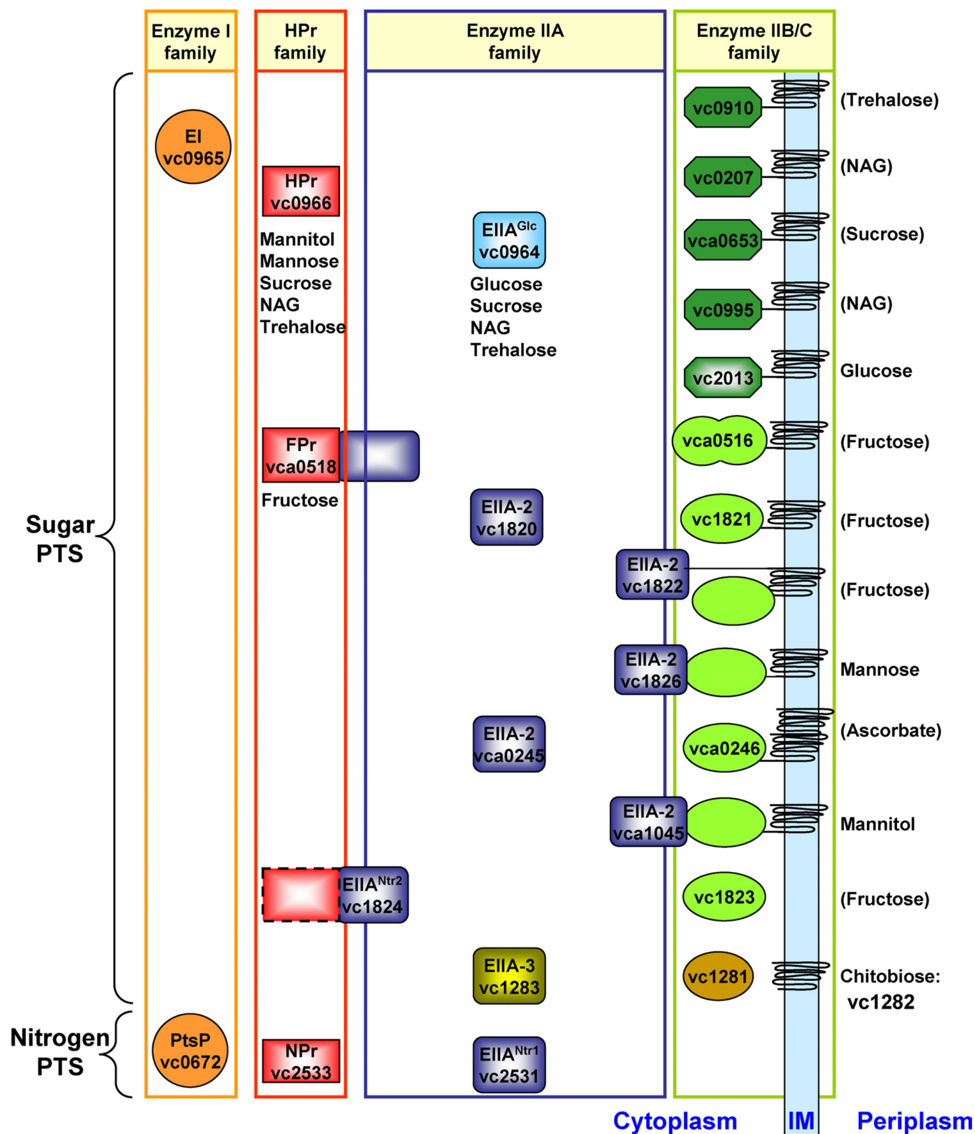

FIG. 1. Schematic diagram of the 25 *V. cholerae* PTS components. Homologs of the EI, HPr, and EII proteins are indicated by the same colors. Furthermore, proteins belonging to the same EIIA superfamily are color coded (blue, glucose superfamily; violet, mannitol-fructose superfamily; yellow, lactose superfamily). Sugar specificities are indicated for PTS components if they are known (16). Parentheses indicate presumptive specificities. The VC1824 HPr-like domain is surrounded by a dashed line to indicate its low level of similarity to HPr. The sugar specificity of VC1281 is based on previously described data (3). NAG, *N*-acetylglucosamine.

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 PTS^{Ntr} have not been defined, transfer of phosphate between the two PTS cascades may be one mechanism by which the PTS^{Ntr} 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).

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).

The *V. cholerae* genome encodes 25 PTS components, including two EI homologs, three HPr homologs, and nine EIIA homologs (Fig. 1). A role in carbohydrate transport has been established for a subset of these components (16). We recently

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or phenotype	Reference
E. coli SM10λpir	*thi thr leu tonA lacY supE recA*::RP4-2-Tc::Mul *pir* R6K; Km^r	38
V. cholerae strains		
PW357	MO10 *lacZ*::*vpsLp*→*lacZ*; Sm^r	15
PW751	MO10 *lacZ*::*vpsLp*→*lacZ* ΔPTS; Sm^r	17
PW961	MO10 *lacZ*::*vpsLp*→*lacZ* ΔEI; Sm^r	17
PW964	MO10 *lacZ*::*vpsLp*→*lacZ* ΔHPr; Sm^r	17
PW954	MO10 *lacZ*::*vpsLp*→*lacZ* ΔFPr; Sm^r	16
PW965	MO10 *lacZ*::*vpsLp*→*lacZ* ΔHPr ΔFPr; Sm^r	16
PW836	MO10 *lacZ*::*vpsLp*→*lacZ* ΔEIIA^{Glc}; Sm^r	17
PW989	MO10 *lacZ*::*vpsLp* ΔEIIBC^{Glc}; Sm^r	17
PW988	MO10 *lacZ*::*vpsLp* ΔEIIA^{Glc} ΔEIIBC^{Glc}; Sm^r	This study
PW996	MO10 *lacZ*::*vpsLp* ΔEIIBC^{Glc} ΔMlc; Sm^r	This study
PW994	MO10 *lacZ*::*vpsLp* ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc; Sm^r	This study
PW993	MO10 *lacZ*::*vpsLp* ΔMlc	This study
PW992	MO10 *lacZ*::*vpsLp*→*lacZ* ΔEIIA^{Ntr1}; Sm^r	16
PW991	MO10 *lacZ*::*vpsLp*→*lacZ* ΔEIIA^{Ntr2}; Sm^r	16
PW1002	MO10 *lacZ*::*vpsLp*→*lacZ* VC1283::pGP704; Sm^r Ap^r	16
PW1003	MO10 *lacZ*::*vpsLp*→*lacZ* VC1820::pGP704; Sm^r Ap^r	16
PW1004	MO10 *lacZ*::*vpsLp*→*lacZ* VC1822::pGP704; Sm^r Ap^r	16
PW1005	MO10 *lacZ*::*vpsLp*→*lacZ* VC1826::pGP704; Sm^r Ap^r	16
PW1006	MO10 *lacZ*::*vpsLp*→*lacZ* VCA0245::pGP704; Sm^r Ap^r	16
PW1007	MO10 *lacZ*::*vpsLp*→*lacZ* VCA1045::pGP704; Sm^r Ap^r	16
Plasmids used in rescue experiments		
pBAD-TOPO-*EI*	pBAD-TOPO carrying the coding sequence of VC0965	17
pBAD-TOPO-*EI*(*H189A*)	pBAD-TOPO carrying the coding sequence of VC0965 with an H-to-A mutation at position 189	17
pBAD-TOPO-*EIIA^{Glc}*	pBAD-TOPO carrying the coding sequence of VC0964	17
pBAD-TOPO-*EIIA^{Glc}*(*H91A*)	pBAD-TOPO carrying the coding sequence of VC0964 with an H-to-A mutation at position 91	This study
pBAD-TOPO-*EIIA^{Glc}*(*H91D*)	pBAD-TOPO carrying the coding sequence of VC0964 with an H-to-D mutation at position 91	This study
pBAD-TOPO-*mlc*	pBAD-TOPO carrying the coding sequence of VC2007	This study
pBAD-TOPO-*EIIA^{Ntr1}*	pBAD-TOPO carrying the coding sequence of VC2531	This study
pBAD-TOPO-*EIIA^{Ntr1}*(*H66A*)	pBAD-TOPO carrying the coding sequence of VC2531 with an H-to-A mutation at position 66	This study
pBAD-TOPO-*EIIA^{Ntr1}*(*H66D*)	pBAD-TOPO carrying the coding sequence of VC2531 with an H-to-D mutation at position 66	This study
pBAD-TOPO-*EIIA^{Ntr2}*	pBAD-TOPO carrying the coding sequence of VC1824	This study
pBAD-TOPO-*EIIA^{Ntr2}*(*H172A*)	pBAD-TOPO carrying the coding sequence of VC1824 with an H-to-A mutation at position 172	This study
pBAD-TOPO-*EIIA^{Ntr2}*(*H172D*)	pBAD-TOPO carrying the coding sequence of VC1824 with an H-to-D mutation at position 172	This study

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 indicated below, the medium was also supplemented with streptomycin (100 μg/ml), ampicillin (50 or 100 μg/ml), or L-arabinose (0.04% wt/vol). 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 Δ*mlc* mutant, pKEKΔ*mlc*, 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

TABLE 2. Primers used in this study

Protein	Primer sequences
Primers used for construction of expression plasmids	
Primers for wild-type alleles	
Mlc	TAAGAGGAATAATAAATGTACATGGCTCAA CCCGG, TCCTTCGACCACTTTCATTAA GAG
PtsN	CAATTGAGCGAAGTACTTTCATTGGACTG, TTGGTTAACCATGATGTTGTACAGC
VC1824	GCGTGACATCGCGTACATCAG, CATGAATTCCAAGTCACCTTCTTGG
Internal primers for construction of mutant alleles	
$EIIA^{Glc(H91A)}$	GCGTTGAGCTGTTTGTTGCCTTCGGTATCG, CGATACCGAAGGCAACAAACAGCTCAACGC
$EIIA^{Glc(H91D)}$	GCGTTGAGCTGTTTGTTGACTTCGGTATCG, CGATACCGAAGTCAACAAACAGCTCAACGC
$PtsN^{(H66A)}$	ATAGCCATCCCAGCCGCACGCATGT, ACATGCGTGCGGCTGGGATGGCTAT
$PtsN^{(H66D)}$	ATAGCCATCCCAGACGCACGCATGT, ACATGCGTGCGTCTGGGATGGCTAT
$VC1824^{(H172A)}$	GGGATTGCTATTCCCGCTGTGATGTTTGC, GCAAACATCACAGCGGGAATAGCAATCCC
$VC1824^{(H172D)}$	GGGATTGCTATTCCCGATGTGATGTTTGC, GCAAACATCACATCGGGAATAGCAATCCC
Primers used in quantitative RT-PCR	
EI	ACCGTTATCGAAGAGCAAGCCACT, TCTGCGCAGTTTCAGAAGGCGTTA
$EIIA^{Glc}$	GCGATCAAGCCTGCTGGTAACAAA, AAGCCTTCACCTTTCAGCTCAACC
HPr	TGTTCAAGCTGCAAACGCTAGGTC, GCAACTAGGTGCTCAACAGCTTCT
$EIIA^{Ntr1}$	GCTATTGCAGTGTGAAGAGCCGAT, CTGCGTTACGAAGCTGTTTGAGGA
$EIIA^{Ntr2}$	TCGGTATTGATGCTGAACTCGCCT, GGGCTTTGGCATAGTGCCATTTGA
ClpX	AGAGTTCATTGGTCGTCTGCCTGT, AACAACGCCGCATACTGTTTGGTC

introduced into *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 $EIIA^{Glc(H91A)}$ 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 (OD_{655}) 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 OD_{655} of the resulting cell suspension was measured. Each total growth value represented the sum of the OD_{655} recorded for the planktonic and surface-associated cell suspensions. All 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 *V. cholerae* was cultured for 18 h in LB broth or MM supplemented with pyruvate (0.5%, wt/vol). The cells were then pelleted by centrifugation, and total RNA was isolated using an RNeasy 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 used as the template for quantitative PCR using iTaq SYBR green Supermix with ROX (Bio-Rad) and 5 pmol of the relevant primers (Table 2) in a 20-μl reaction mixture. The level of the *clpX* (VC1921) transcript was used to normalize all measurements. Template-free reaction mixtures were included as controls to confirm the absence of contaminating DNA or RNA. The experiments were conducted with a StepOnePlus PCR system (Applied Biosystems) using the following steps: (i) 95°C for 10 min, (ii) 40 cycles of denaturation for 15 s at 95°C and annealing and extension for 1 min at 60°C, and (iii) dissociation curve analysis using temperatures from 60°C to 90°C. Data were analyzed using the StepOne V2.0 software (Applied Biosystems). Measurements were obtained in triplicate.

Measurement of *vpsL* transcription using a β-galactosidase reporter. Strains were inoculated into LB broth to obtain an initial OD_{655} of approximately 0.05. Cultures were incubated at 27°C for 4 h with shaking until the OD_{655} was approximately 0.5. The OD_{655} was determined and subsequently used for normalization of measurements of β-galactosidase activity. Fifty or 100 μl of each culture was moved to a white 96-well plate (Nunc). Three freeze-thaw cycles were performed, and an equal volume of the β-Glo luminescent substrate (Promega) 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 $\Delta EIIA^{Glc}$ 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 $\Delta EIIA^{Glc}$ mutant, and a ΔPTS mutant lacking the genes encoding EI, HPr, and $EIIA^{Glc}$ (Fig. 2). Similar to

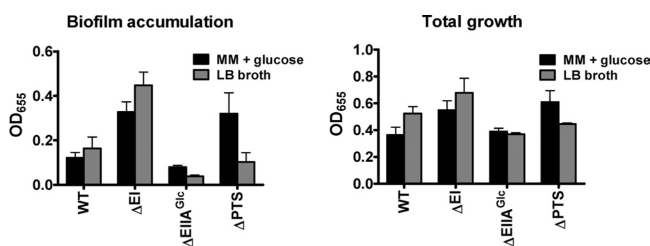

FIG. 2. PTS mutant displays distinct biofilm phenotypes in LB broth and minimal medium (MM) supplemented with glucose. Total growth and biofilm accumulation were determined for wild-type *V. cholerae* (WT), a ΔEI mutant, a $\Delta EIIA^{Glc}$ mutant, and a ΔPTS mutant in minimal medium supplemented with 0.5% (wt/vol) glucose (MM + glucose) and LB broth (LB). The values are the averages of four experimental replicates, and the error bars indicate standard deviations. There was a statistically significant difference ($P = 0.0062$)between biofilm formation by wild-type *V. cholerae* and biofilm formation by the ΔPTS mutant in minimal medium supplemented with glucose (indicated by an asterisk) but not in LB broth ($P = 0.116$).

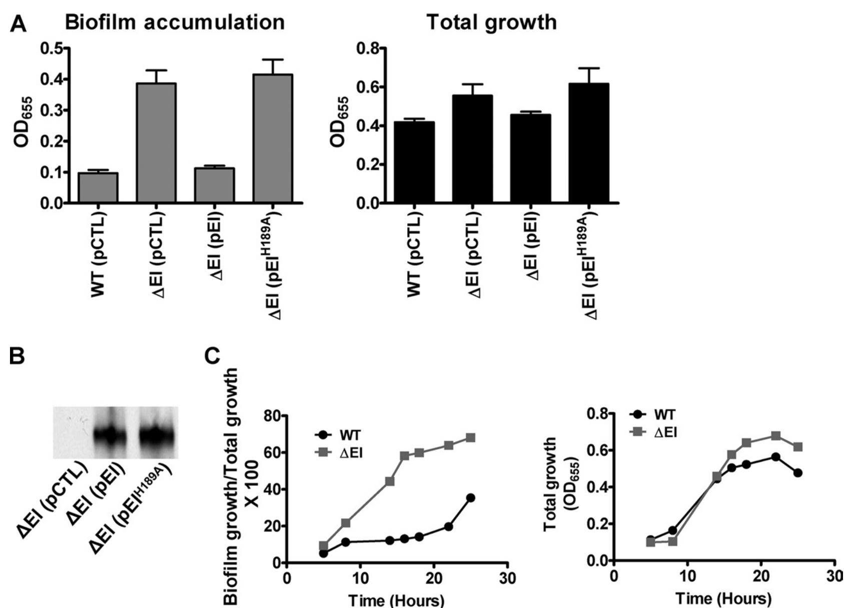

FIG. 3. EI-P represses biofilm formation and *vps* gene transcription in LB broth (biofilm regulatory pathway 1). (A) Total growth and biofilm accumulation in LB broth by wild-type *V. cholerae* (WT) or a ΔEI mutant rescued with either a control pBAD plasmid (pCTL), a pBAD plasmid expressing a wild-type 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.

what was observed in minimal medium, in LB broth a ΔEI mutant exhibited increased surface accumulation compared with wild-type *V. cholerae*, while a ΔEIIA^{Glc} mutant exhibited decreased surface accumulation. As previously noted (17), surface accumulation by a ΔPTS mutant lacking EI, HPr, and EIIA^{Glc} 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 supplemented with glucose, repression of biofilm accumulation by EI becomes apparent only during the stationary phase of growth. This corresponds to the time at which the glucose in the growth medium is depleted (17) and phosphorylated PTS intermediates begin to accumulate. Because PTS substrates are less abundant in LB broth (20), we predicted that during growth in LB broth, repression of biofilm accumulation by EI-P would be observed much earlier in the growth cycle. As predicted, the difference in biofilm accumulation between wild-type *V. cholerae* and the ΔEI mutant occurred during the exponential phase of growth and increased in early stationary phase (Fig. 3C).

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 accumulation by wild-type *V. cholerae* and biofilm accumulation by ΔHPr, ΔFPr, and ΔHPr ΔFPr mutants. As shown in Fig. 4A, biofilm accumulation 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-

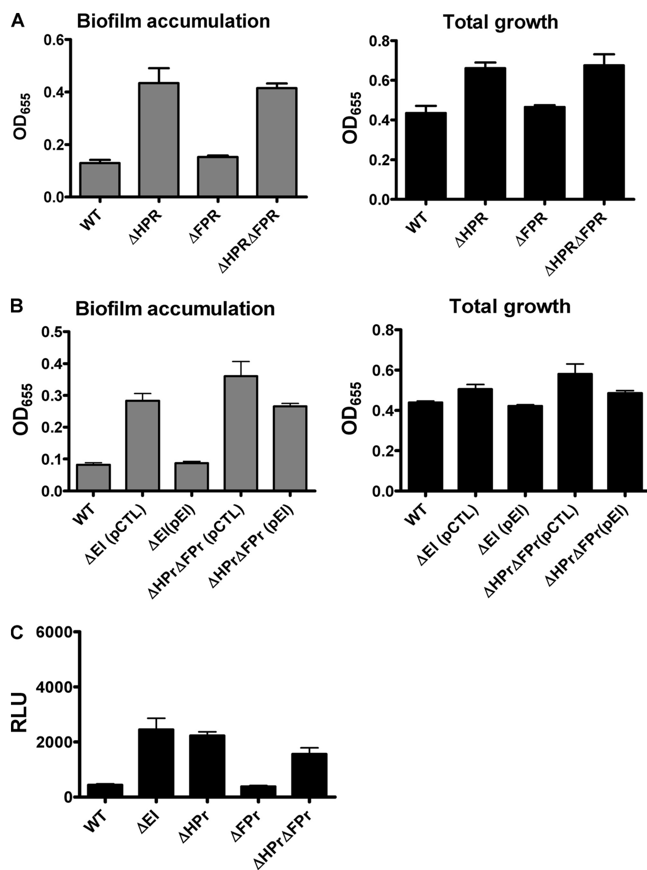

FIG. 4. EI and HPr are in the same biofilm regulatory pathway. (A) Total growth and biofilm accumulation by wild-type *V. cholerae* (WT), a ΔHPr mutant, a ΔFPr mutant, and a ΔHPr ΔFPr double mutant. Biofilm accumulation by a ΔHPr mutant is significantly different from that by wild-type *V. cholerae* ($P = 0.002$), while biofilm accumulation by a ΔHPr ΔFPr is not significantly different from that by ΔHPr ($P = 0.758$). (B) Total growth and biofilm accumulation by wild-type *V. cholerae* (WT), as well as ΔEI and ΔHPr ΔFPr mutants rescued either with a control pBAD plasmid (pCTL) or a pBAD plasmid expressing a wild-type EI allele (pEI). Protein expression was induced by addition of 0.04% L-arabinose. Deletion of HPr and FPr blocked the ability of EI to repress biofilm formation. (C) Measurement of *vps* gene transcription in wild-type *V. cholerae* and various PTS mutants harboring a chromosomal *vpsL-lacZ* fusion. *vpsL* transcription in the ΔEI ($P = 0.0002$), ΔHPr ($P < 0.0001$), and ΔHPr ΔFPr ($P = 0.0001$) mutants is significantly different from that in wild-type *V. cholerae*. The error bars indicate standard deviations. RLU, relative light units.

pression 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 the FPr gene, which encodes both EIIA and HPr domains, as well as in each of the nine genes in the *V. cholerae* genome encoding EIIA homologs (Fig. 1). As shown in Fig. 5, we identified three EIIA mutants with altered biofilm phenotypes compared with wild-type *V. cholerae*. First, approx-

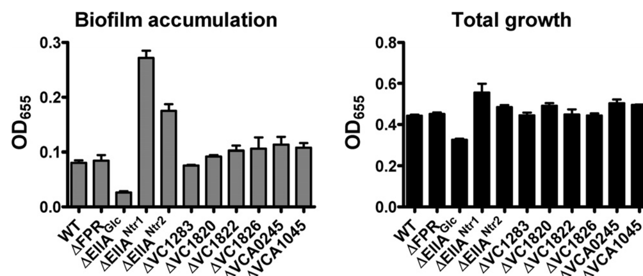

FIG. 5. EIIA homologs $EIIA^{Glc}$, $EIIA^{Ntr1}$, and $EIIA^{Ntr2}$ regulate biofilm formation: quantification of total growth and biofilm accumulation by wild-type *V. cholerae*, as well as strains having mutations in each of the 10 genes encoding EIIA domains. The biofilm accumulation by the $\Delta EIIA^{Glc}$ ($P < 0.0001$), $\Delta EIIA^{Ntr}$ ($P < 0.0001$), and ΔVC1824 ($P = 0.0004$) mutants was significantly different from that by wild-type *V. cholerae* (WT). The error bars indicate standard deviations.

imately 3-fold fewer $\Delta EIIA^{Glc}$ 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 ($EIIA^{Ntr1}$) and VC1824 ($EIIA^{Ntr2}$) both contain EIIA domains homologous to $EIIA^{Ntr}$, 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 $EIIA^{Glc}$, $EIIA^{Ntr1}$, and $EIIA^{Ntr2}$ 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, $EIIA^{Glc}$, $EIIA^{Ntr1}$, and $EIIA^{Ntr2}$ 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,

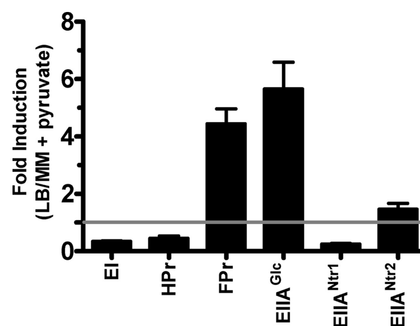

FIG. 6. Differential transcription of PTS components is not solely responsible for the patterns of biofilm regulation observed in LB broth compared with those observed in minimal medium. The EI, HPr, FPr, $EIIA^{Glc}$, $EIIA^{Ntr1}$, and $\Delta EIIA^{Ntr2}$ transcript levels in wild-type *V. cholerae* grown in LB broth were compared to the transcript levels in MM supplemented with pyruvate. The data were analyzed in triplicate using the $\Delta\Delta C_t$ method. *clpX* was used as a standard. The error bars indicate standard deviations.

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 EIIA^{Glc} was approximately 6-fold greater in LB broth, suggesting that activation of biofilm formation by EIIA^{Glc} in LB broth may be due at least in part to increased transcription of EIIA^{Glc}. Transcription of the PTS components EIIA^{Ntr1} and EIIA^{Ntr2} 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 EIIA^{Glc} 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

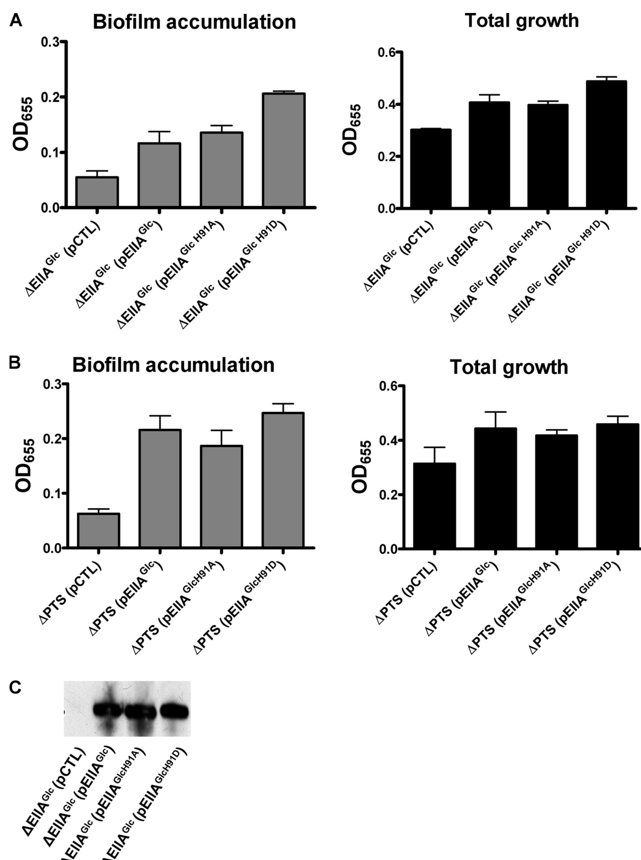

FIG. 7. Activation of biofilm formation by EIIA^{Glc} does not require phosphorylation and is independent of EI and HPr (biofilm regulatory pathway 2). (A) Quantification of total growth and biofilm accumulation by a ΔEIIA^{Glc} mutant rescued with a pBAD plasmid carrying a control sequence (pCTL) and pBAD plasmids with a wild-type EIIA^{Glc} allele (pEIIA^{Glc}) and a variety of mutant EIIA^{Glc} alleles. Protein expression was induced with 0.04% arabinose. Biofilm accumulation by the ΔEIIA^{Glc} mutant rescued with a wild-type EIIA^{Glc} glucose allele ($P = 0.0024$), an EIIA^{Glc} allele encoding a histidine-to-alanine substitution at position 191 (pEIIA^{GlcH91A}) ($P < 0.0001$), or an EIIA^{Glc} allele encoding a histidine-to-aspartate substitution at position 191 (pEIIA^{GlcH91D}) ($P < 0.0001$) was significantly increased compared with biofilm accumulation by the ΔEIIA^{Glc} mutant rescued with a control sequence. (B) Quantification of total growth and biofilm accumulation by a ΔPTS mutant (lacking EI, HPr, and EIIA^{Glc}) rescued with a pBAD plasmid carrying a control sequence (pCTL), a wild-type EIIA^{Glc} allele (pEIIA^{Glc}), an EIIA^{Glc} allele encoding a histidine-to-alanine substitution at position 191 (pEIIA^{GlcH91A}), or an EIIA^{Glc} allele encoding a histidine-to-aspartate substitution at position 191 (pEIIA^{GlcH91D}). Protein expression was induced with 0.04% arabinose. Biofilm accumulation by the ΔPTS mutant rescued with any of the EIIA^{Glc} alleles was significantly increased compared with biofilm accumulation by the ΔPTS mutant rescued with a control sequence ($P \leq 0.0002$ for all comparisons). The error bars indicate standard deviations. (C) Western blot demonstrating that the wild-type and mutant EIIA^{Glc} alleles used in these experiments are well expressed and produce full-length proteins.

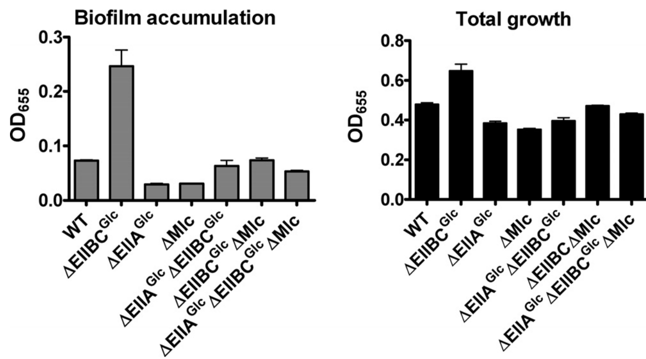

FIG. 8. Evidence for involvement of Mlc and EIIBC^{Glc} in pathway 2: quantification of total growth and biofilm accumulation by wild-type *V. cholerae* and various pathway 2 mutants. Biofilm accumulation by the ΔEI ($P = 0.0008$) or ΔEIIBC^{Glc} ($P = 0.0001$) mutant was significantly greater than that by wild-type *V. cholerae* (WT). Biofilm accumulation by the ΔEIIA^{Glc} or ΔMlc mutants was significantly less than that by wild-type *V.* cholerae ($P < 0.0001$). Biofilm accumulation by the ΔEIIA^{Glc} ΔEIIBC^{Glc} mutant ($P = 0.04$) or the ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutant ($P = 0.001$) was significantly greater than that by the ΔEIIA^{Glc} mutant. Biofilm accumulation by the ΔEIIBC^{Glc} ΔMlc mutant ($P = 0.0006$) or the ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutant ($P = 0.0005$) was significantly greater than that by the ΔMlc mutant. The error bars indicate standard deviations.

that the biofilm regulatory pathway shown in Fig. 9A is present. In this signal transduction pathway, EIIBC^{Glc} inhibits activation of EIIA^{Glc} by Mlc. EIIA^{Glc}, in turn, activates transcription of the *vps* genes by an as-yet-unidentified mechanism.

To further evaluate the presence of this signal transduction pathway, we measured *vpsL* transcription in wild-type *V. cholerae* and ΔEIIBC^{Glc}, ΔMlc, and ΔEIIA^{Glc} 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^{Glc} and is repressed by EIIBC^{Glc}. Furthermore, the level of *vps* transcription in a ΔEIIA^{Glc} ΔEIIBC^{Glc} mutant is higher than that in a ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutant, suggesting that Mlc activates *vps* transcription even in the absence of EIIA^{Glc}. Therefore, an additional pathway must be postulated, in which Mlc activates *vpsL* transcription independent of EIIA^{Glc}. 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 *vpsL* transcription. However, differences were found. First, there was no difference in biofilm accumulation between ΔEIIA^{Glc} and ΔMlc mutants. Second, biofilm formation by strains having mutations in EIIBC^{Glc} as well as in EIIA^{Glc} and/or Mlc all formed biofilms that were slightly but significantly and reproducibly larger than those formed by ΔEIIA^{Glc} and ΔMlc mutants. One possible explanation for these observations is that EIIBC^{Glc} also represses biofilm accumulation at the posttranscriptional level. The complex effect of EIIBC^{Glc} on biofilm formation is currently under investigation.

We hypothesized that if Mlc were partially dependent on EIIA^{Glc} for its effect on biofilm formation, the absence of

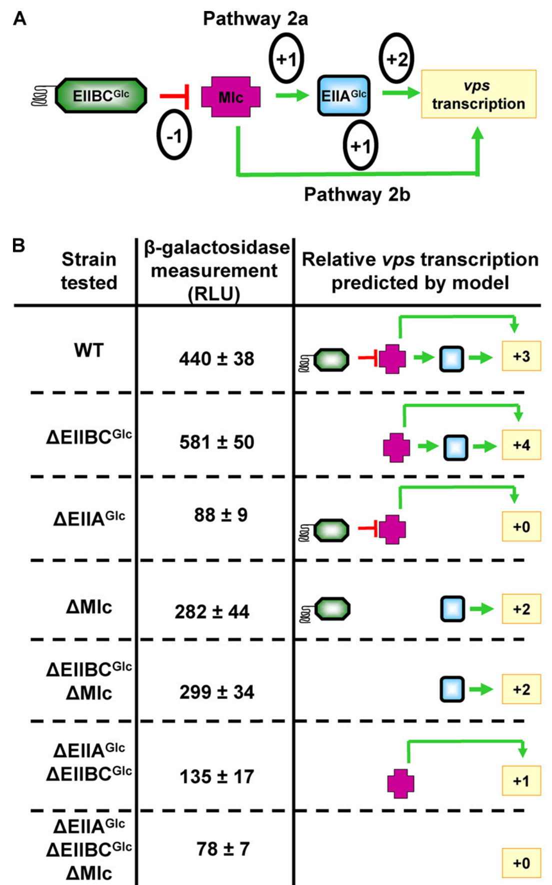

FIG. 9. Pathway 2 regulates biofilm accumulation at the transcriptional level. (A) Schematic diagram of pathways 2a and 2b. In this model, Mlc activates *vps* gene transcription through both EIIA^{Glc}-dependent and EIIA^{Glc}-independent pathways. EIIBC^{Glc} interferes with the action of Mlc. The numbers in circles indicate the predicted relative effect of each component in the regulatory cascade on *vps* gene transcription. (B) All the strains tested have *vpsL-lacZ* promoter fusions in the *V. cholerae lacZ* locus. The measurements of β-galactosidase activity reflect *vpsL* transcription. The measurements for the ΔEIIA^{Glc} ($P < 0.0001$), ΔEIIBC^{Glc} ($P = 0.04$), and ΔMlc ($P = 0.0154$) mutants were significantly different from that for wild-type *V. cholerae*. The *vpsL* transcription in the ΔEIIBC^{Glc} ΔMlc mutant was significantly different from that in the ΔEIIBC^{Glc} mutant ($P = 0.0003$) but not from that in the ΔMlc mutant ($P = 0.766$). The *vps* transcription in the ΔEIIA^{Glc} ΔEIIBC^{Glc} mutant was significantly different from that in the ΔEIIA^{Glc} mutant ($P = 0.032$) or the ΔEIIBC^{Glc} mutant ($P < 0.0001$). The *vps* transcription in the ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutant was significantly different both from that in the ΔEIIA^{Glc} ΔEIIBC^{Glc} mutant ($P = 0.0086$) or the ΔEIIBC^{Glc} ΔMlc mutant ($P < 0.0001$) but not from that in the ΔEIIA^{Glc} mutant ($P = 0.415$). The models on the right show the predicted impact of each genetic background on *vps* gene transcription. The relative contributions of the regulatory components to *vps* gene transcription as shown in panel A were added to obtain the predicted impact of the genetic background on *vps* gene transcription. WT, wild type; RLU, relative light units.

EIIA^{Glc} would compromise the ability of an *mlc* allele provided in *trans* to rescue an Mlc mutation. To test this hypothesis, the following epistasis experiment was performed. We introduced a wild-type allele of *mlc* into ΔMlc, ΔEIIBC^{Glc} ΔMlc, and ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutants. As shown in Fig. 10, while a control vector had no effect on biofilm accu-

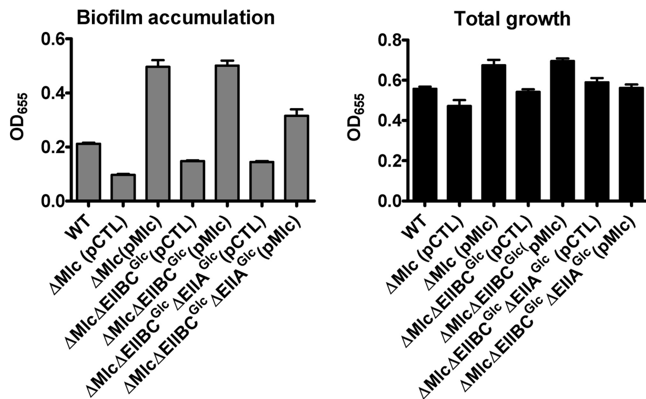

FIG. 10. Activation of biofilm accumulation by Mlc is partially dependent on EIIA^{Glc}: quantification of biofilm accumulation and total growth by wild-type *V. cholerae* (WT) and ΔMlc, ΔEIIBC^{Glc} ΔMlc, and ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutants rescued with either a control vector (pCTL) or a plasmid carrying a wild-type *mlc* allele (pMlc). Biofilm formation by ΔMlc ($P < 0.0001$), ΔEIIBC^{Glc} ΔMlc ($P < 0.0001$), and ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc ($P = 0.002$) mutants rescued with the plasmid carrying the wild-type *mlc* allele was significantly greater than that by mutants carrying a control vector. The error bars indicate standard deviations.

mulation by any of these mutants, introduction of the *mlc* allele into the ΔMlc and ΔEIIBC^{Glc} ΔMlc mutants increased biofilm accumulation approximately 4-fold. In contrast, introduction of the *mlc* allele into a ΔEIIA^{Glc} ΔEIIBC^{Glc} ΔMlc mutant increased biofilm accumulation only 2-fold. This result supports our model for pathway 2 in which Mlc activates biofilm accumulation through EIIA^{Glc}-dependent and -independent pathways.

EIIA^{Ntr1} and EIIA^{Ntr2} repress biofilm accumulation (regulatory pathway 3). The gene encoding EIIA^{Ntr}, which is located close to *rpoN*, has been found in many bacterial genomes (36, 48, 51). Mutation of the two *V. cholerae* EIIA^{Ntr} homologs, EIIA^{Ntr1} (60% identity and 75% similarity to *E. coli* EIIA^{Ntr}) and EIIA^{Ntr2} (26% identity and 42% similarity to *E. coli* EIIA^{Ntr}), resulted in increased biofilm accumulation (Fig. 5). The conserved histidine residues at position 66 of EIIA^{Ntr1} and position 172 of EIIA^{Ntr2} are predicted to be phosphorylated by phosphotransfer from the HPr homolog NPr, which in turn receives a phosphate from EI^{Ntr}. We first determined whether phosphorylation of the conserved histidine was required for the function of EIIA^{Ntr1} by comparing biofilm accumulation data for ΔEIIA^{Ntr1} mutants carrying either a plasmid encoding a control sequence, a wild-type allele of EIIA^{Ntr1}, 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 ΔEIIA^{Ntr1} mutant was observed with both mutant alleles, as well as with the wild-type allele of EIIA^{Ntr1} (Fig. 11A). Furthermore, all alleles of EIIA^{Ntr1} 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 EIIA^{Ntr1}. Based on this observation, we also predicted that the upstream components of the PTS^{Ntr} would not be required for repression of biofilm accumulation by EIIA^{Ntr1}. To test this hypothesis, we con-

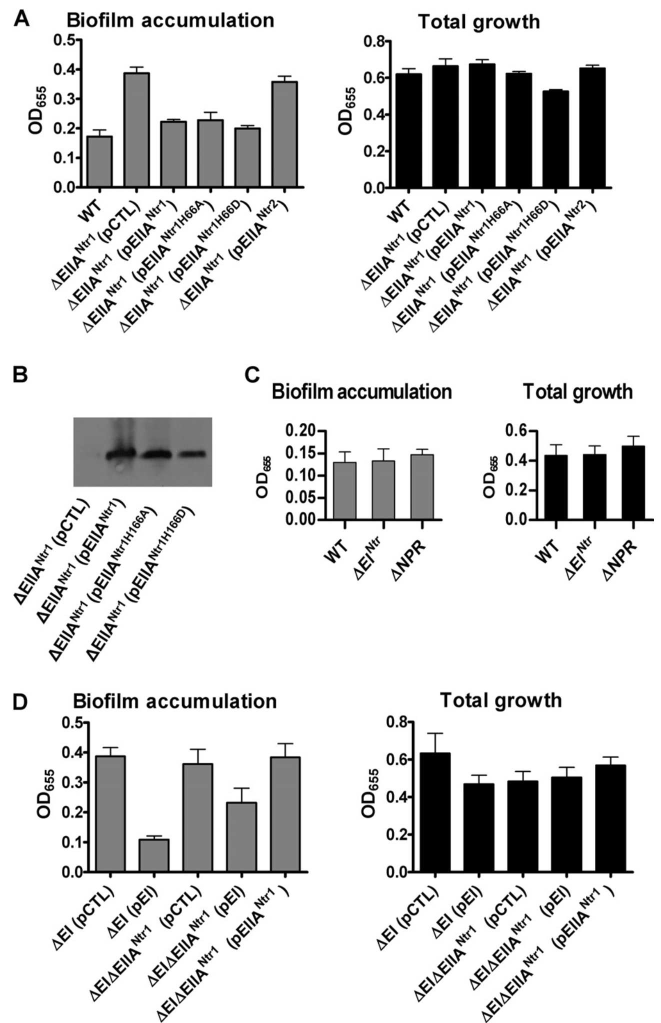

FIG. 11. Repression of biofilm formation by EIIA^{Ntr1} does not require phosphorylation of the conserved histidine at residue 66 and is independent of pathway 1 (biofilm regulatory pathway 3). (A) Quantification of total growth and biofilm accumulation by wild-type *V. cholerae* or a ΔEIIA^{Ntr1} mutant rescued with a control plasmid (pCTL), a plasmid carrying a wild-type EIIA^{Ntr1} allele (pEIIA^{Ntr1}), a plasmid carrying an EIIA^{Ntr1} allele resulting in an alanine substitution for the histidine at position 66 (pEIIA^{Ntr1H66A}), a plasmid carrying an EIIA^{Ntr1} allele resulting in an aspartate substitution for the histidine at position 66 (pEIIA^{Ntr1H66D}), and a plasmid carrying a wild-type EIIA^{Ntr2} allele (pEIIA^{Ntr2}). Only biofilm accumulation by the ΔEIIA^{Ntr1} (pCTL) ($P = 0.0003$) and ΔEIIA^{Ntr1} (pEIIA^{Ntr2}) ($P = 0.0004$) mutants was 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^{Ntr1} alleles used in these experiments are well expressed and produce full-length proteins. (C) Quantification of total growth and biofilm accumulation by wild-type *V. cholerae* and strains having mutations in the other two components of the PTS^{Ntr}, EI^{Ntr}, and NPr. Biofilm accumulation by the two mutants was not significantly different from that by wild-type *V. cholerae.* (D) Quantification of total growth and biofilm formation by a ΔEI mutant or a ΔEI ΔEIIA^{Ntr1} mutant rescued with a pBAD plasmid carrying a control sequence (pCTL), a wild-type EI allele (pEI), or a wild-type EIIA^{Ntr1} allele (pEIIA^{Ntr1}). Biofilm accumulation by the ΔEI ΔEIIA^{Ntr1} mutant rescued with a wild-type EI allele was significantly different from that by the ΔEI ΔEIIA^{Ntr1} mutant carrying a control sequence ($P = 0.0102$).

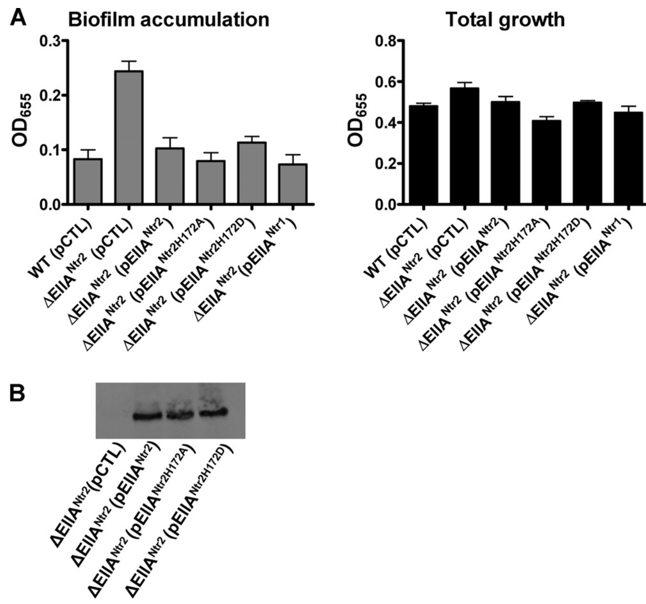

FIG. 12. Repression of biofilm formation by $EIIA^{Ntr2}$ does not require phosphorylation at histidine 172 and is rescued by $EIIA^{Ntr1}$ (biofilm regulatory pathway 3) (A) Quantification of total growth and biofilm accumulation by wild-type *V. cholerae* or a $\Delta EIIA^{Ntr2}$ mutant rescued with a pBAD plasmid carrying a control sequence (pCTL), a wild-type $\Delta EIIA^{Ntr2}$ allele ($pEIIA^{Ntr2}$), a $\Delta EIIA^{Ntr2}$ allele encoding an H-to-A point mutation at conserved residue 172 ($pEIIA^{Ntr2H172A}$), a $\Delta EIIA^{Ntr2}$ allele encoding an H-to-D point mutation at conserved residue 172 ($pEIIA^{Ntr2H172D}$), or a wild-type $EIIA^{Ntr1}$ allele ($pEIIA^{Ntr1}$). Protein expression was induced with 0.04% arabinose. Biofilm accumulation by a $\Delta EIIA^{Ntr2}$ mutant rescued with wild-type $EIIA^{Ntr2}$ ($P = 0.263$), $EIIA^{Ntr2H172A}$ ($P = 0.776$), $EIIA^{Ntr2H172D}$ ($P = 0.126$), or $EIIA^{Ntr1}$ ($P = 0.514$) 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.

structed strains having in-frame deletions in EI^{Ntr} and NPr and compared the biofilm accumulation by these strains to that by wild-type *V. cholerae.* As expected, we found that biofilm accumulation by ΔEI^{Ntr} and ΔNPr mutants was not significantly different from that by wild-type *V. cholerae* (Fig. 11C).

Because biofilm formation is also repressed by EI, we asked whether EI and $EIIA^{Ntr1}$ function independently. As shown in Fig. 11D, return of a wild-type EI allele to a ΔEI $\Delta EIIA^{Ntr1}$ 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 ΔEI $\Delta EIIA^{Ntr1}$ mutant. This suggests that the ΔEI mutation is dominant in the ΔEI $\Delta EIIA^{Ntr1}$ mutant. While we have not ruled out a model in which one function of EI is to act downstream of $EIIA^{Ntr1}$ in a common signaling pathway, we maintain that pathway 1 is independent of pathway 3 because it is present in minimal medium (16), while pathway 3 is not.

We then asked whether phosphorylation of the conserved histidine at position 172 was required for repression of biofilm accumulation by $EIIA^{Ntr2}$. Biofilm accumulation was measured for $\Delta EIIA^{Ntr2}$ mutants harboring plasmids containing either a control sequence, a wild-type allele of $EIIA^{Ntr2}$, an

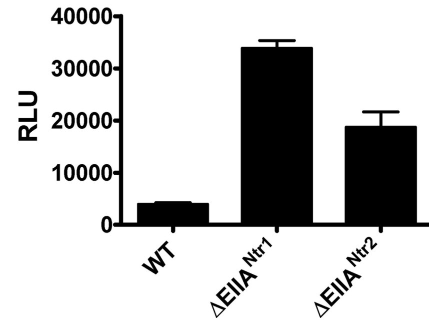

FIG. 13. $EIIA^{Ntr1}$ and $EIIA^{Ntr2}$ operate at the level of transcription: measurements of *vps* gene transcription in wild-type *V. cholerae*, *a* $\Delta EIIA^{Ntr1}$ mutant, and a $\Delta EIIA^{Ntr2}$ mutant harboring a chromosomal *vpsL-lacZ* fusion. The *vpsL* transcription in the $\Delta EIIA^{Ntr1}$ and $\Delta EIIA^{Ntr2}$ 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.

allele of $EIIA^{Ntr2}$ resulting in substitution of the conserved histidine for alanine at position 172, or an allele of $EIIA^{Ntr2}$ resulting in substitution of the conserved histidine for aspartate at position 172. As shown in Fig. 12A, the biofilm phenotype of a $\Delta EIIA^{Ntr2}$ mutant was rescued by the wild-type $EIIA^{Ntr2}$ allele, as well as by both nonphosphorylatable mutant alleles. Furthermore, all rescue constructs were expressed as full-length proteins (Fig. 12B).

In other systems, $EIIA^{Ntr}$ has been shown to operate at the transcriptional level (25). We used β-galactosidase assays to measure the effects of the $EIIA^{Ntr}$ homologs on *vpsL* gene transcription. As shown in Fig. 13, while both $EIIA^{Ntr1}$ and $EIIA^{Ntr2}$ repress biofilm formation at the transcriptional level, $EIIA^{Ntr1}$, which is more similar to the $EIIA^{Ntr}$ of *E.* coli, has a more pronounced effect.

Lastly, we examined whether $EIIA^{Ntr1}$ and $EIIA^{Ntr2}$ have redundant roles in repression of biofilm formation by attempting to rescue the biofilm phenotype of a $\Delta EIIA^{Ntr1}$ mutant with a wild-type $EIIA^{Ntr2}$ allele and *vice versa*. A wild-type $EIIA^{Ntr1}$ allele provided in *trans* did rescue the biofilm phenotype of the $\Delta EIIA^{Ntr2}$ mutant, supporting our conclusion that these two $EIIA^{Ntr}$ homologs function in the same biofilm regulatory pathway (Fig. 12A). $EIIA^{Ntr2}$ provided in *trans* was not able to rescue the $\Delta EIIA^{Ntr1}$ mutant phenotype (Fig. 11A). We attribute the inability of $EIIA^{Ntr2}$ to rescue the phenotype of the $\Delta EIIA^{Ntr1}$ mutant to its smaller effect on *vps* gene transcription and biofilm formation.

DISCUSSION

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 $EIIA^{Glc}$ and one of which (pathway 3) results in repression of biofilm formation by $EIIA^{Ntr}$ (Fig. 14). All three of these pathways regulate transcrip-

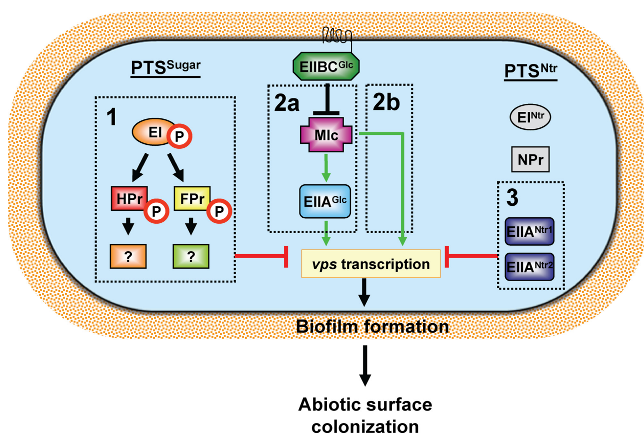

FIG. 14. Schematic diagram of three pathways in the PTS network that regulate biofilm formation. Pathway 1 requires phosphorylation of the PTS components EI and HPr or FPr to repress transcription of the *vps* genes. Pathway 2a is comprised of the components EIIBC^{Glc}, Mlc, and EIIA^{Glc}. EIIA^{Glc} activates *vps* transcription in a phosphorylation-independent manner. Mlc potentiates the action of EIIA^{Glc} but may also activate *vps* gene transcription through an EIIA^{Glc}-independent route (pathway 2b). EIIBC^{Glc} interferes with the action of Mlc. Pathway 3 includes EIIA^{Ntr1}and EIIA^{Ntr2}, whose functions display some redundancy in repression of *vps* transcription.

tion of the *vps* genes, which encode the biofilm matrix biosynthetic machinery. The regulatory cascade comprising pathway 2 suggests that there are differences from the homologous PTS-dependent regulatory pathway in *E. coli*, while pathway 3 defines a new role for EIIA^{Ntr} in regulation of bacterial biofilm formation.

In minimal medium, pathway 1 proceeds through EI-P to HPr-P and FPr-P, which have redundant functions. As a result, only a ΔHPr ΔFPr mutant forms a biofilm that is distinguishable from that formed by wild-type *V. cholerae*. In contrast, in LB broth, HPr is the dominant protein in the signal transduction cascade, resulting in increased biofilm accumulation by a ΔHPr mutant compared with wild-type *V. cholerae*. This is not due to increased transcription of HPr in LB broth compared with minimal medium (Fig. 6). Rather, we hypothesize that phosphotransfer through HPr is preferred in LB broth. Alternatively, if pathway 1 remains divergent downstream of HPr and FPr, subsequent components may be responsible for the differences observed in LB broth.

Signaling through pathway 2 results in activation of *vps* gene transcription. This activation requires EIIA^{Glc} and is observed in the absence of phosphorylation of the conserved histidine residue at position 91. In *E. coli*, EIIA^{Glc} has two important regulatory functions. EIIA^{Glc}-P potentiates the action of adenylate cyclase to increase intracellular concentrations of cyclic AMP (cAMP), a cofactor of the global regulator, the cAMP receptor protein (CRP) (56). The unphosphorylated form of EIIA^{Glc} directly blocks transport of non-PTS substrates (52). Here we discovered a phosphorylation-independent regulatory role for EIIA^{Glc} in activation of the *vps* genes and biofilm formation in *V. cholerae*. It is unlikely that the regulation is due to activation of adenylate cyclase as multiple studies have shown that cAMP represses rather than activates *vps* transcription and biofilm formation in *V. cholerae* (11, 17, 28).

Regulation of biofilm formation by EIIA^{Glc} does not require phosphorylation of the conserved histidine at position 91. Therefore, the mechanism by which this function of EIIA^{Glc} is controlled in response to environmental cues is unclear. Because transcription of *V. cholerae* EIIA^{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^{Glc} is regulated by its intracellular abundance.

The results of epistasis experiments and measurements of *vpsL* transcription are consistent with a model in which Mlc activates biofilm accumulation through EIIA^{Glc}-dependent and EIIA^{Glc}-independent pathways. EIIBC^{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^{Glc} activity either directly or indirectly. *E. coli* Mlc functions as a transcriptional repressor of many PTS components (47, 59). However, it does not regulate transcription of EIIA^{Glc}. *E. coli* EIIBC^{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^{Glc} may block activation of EIIA^{Glc} by Mlc (pathway 2a) (Fig. 14). Our data also suggest that a small portion of the observed activation of *vps* gene transcription by Mlc may proceed through an EIIA^{Glc}-independent pathway (pathway 2b) (Fig. 14).

We identified two EIIA^{Ntr} homologs that repress *V. cholerae* biofilm formation and *vps* gene transcription in LB broth but not in minimal medium (pathway 3) (Fig. 14). Transcription of the EIIA^{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^{Ntr1} or EIIA^{Ntr2} in a ΔEIIA^{Glc} background activates biofilm formation (data not shown), suggesting that EIIA^{Ntr1} and EIIA^{Ntr2} act independent of EIIA^{Glc}.

While EIIA^{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 *Pseudomonas putida* and repression of β-hydroxybutyrate synthesis in *Azotobacter vinelandii* by EIIA^{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 *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, *V. cholerae* EIIA^{Ntr} may have a function parallel to that of its homologs in *P. putida* and *A. vinelandii*.

The mechanism by which EIIA^{Ntr} represses polysaccharide synthesis in *P. putida* and *A. vinelandii* has not been discovered yet. However, the mechanism of action of EIIA^{Ntr} in transcriptional derepression of the *ilvBN* 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 $EIIA^{Ntr}$ inhibits the action of the K^+ transporter TrkA, which keeps intracellular K^+ levels low and preserves transcription of the *ilvBN* operon (24). In our experiments, phosphorylation of neither $EIIA^{Ntr1}$ nor $EIIA^{Ntr2}$ was required for activation of *vpsL* gene transcription. One possibility is that regulation of gene transcription by $EIIA^{Ntr}$ in *V. cholerae* is governed by intracellular K^+ levels in a phosphorylation state-independent manner. Alternatively, $EIIA^{Ntr}$ 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 the environmental signals to which pathways 2 and 3 respond, 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.

ACKNOWLEDGMENTS

We thank Joachim Reidl for generously providing plasmids for mutant construction.

This work was supported by NIH grant AI50082 to P.I.W.

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