Glucose-Specific Enzyme IIA of the Phosphoenolpyruvate:Carbohydrate Phosphotransferase System Modulates Chitin Signaling Pathways in *Vibrio cholerae*

Shouji Yamamoto, Makoto Ohnishi

Department of Bacteriology I, National Institute of Infectious Diseases, Tokyo, Japan

ABSTRACT In *Vibrio cholerae*, the genes required for chitin utilization and natural competence are governed by the chitin-responsive two-component system (TCS) sensor kinase ChiS. In the classical TCS paradigm, a sensor kinase specifically phosphorylates a cognate response regulator to activate gene expression. However, our previous genetic study suggested that ChiS stimulates the non-TCS transcriptional regulator TfoS by using mechanisms distinct from classical phosphorylation reactions (S. Yamamoto, J. Mitobe, T. Ishikawa, S. N. Wai, M. Ohnishi, H. Watanabe, and H. Izumiya, *Mol Microbiol* 91:326–347, 2014, <https://doi.org/10.1111/mmi.12462>). TfoS specifically activates the transcription of *tfoR*, encoding a small regulatory RNA essential for competence gene expression. Whether ChiS and TfoS interact directly remains unknown. To determine if other factors mediate the communication between ChiS and TfoS, we isolated transposon mutants that turned off *tfoR::lacZ* expression but possessed intact *chiS* and *tfoS* genes. We demonstrated an unexpected association of chitin-induced signaling pathways with the glucose-specific enzyme IIA (EIIA^{glc}) of the phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) for carbohydrate uptake and catabolite control of gene expression. Genetic and physiological analyses revealed that dephosphorylated EIIA^{glc} inactivated natural competence and *tfoR* transcription. Chitin-induced expression of the *chb* operon, which is required for chitin transport and catabolism, was also repressed by dephosphorylated EIIA^{glc}. Furthermore, the regulation of *tfoR* and *chb* expression by EIIA^{glc} was dependent on ChiS and intracellular levels of ChiS were not affected by disruption of the gene encoding EIIA^{glc}. These results define a previously unknown connection between the PTS and chitin signaling pathways in *V. cholerae* and suggest a strategy whereby this bacterium can physiologically adapt to the existing nutrient status.

IMPORTANCE The EIIA^{glc} protein of the PTS coordinates a wide variety of physiological functions with carbon availability. In this report, we describe an unexpected association of chitin-activated signaling pathways in *V. cholerae* with EIIA^{glc}. The signaling pathways are governed by the chitin-responsive TCS sensor kinase ChiS and lead to the induction of chitin utilization and natural competence. We show that dephosphorylated EIIA^{glc} inhibits both signaling pathways in a ChiS-dependent manner. This inhibition is different from classical catabolite repression that is caused by lowered levels of cyclic AMP. This work represents a newly identified connection between the PTS and chitin signaling pathways in *V. cholerae* and suggests a strategy whereby this bacterium can physiologically adapt to the existing nutrient status.

KEYWORDS *Vibrio cholerae*, chitin, chitin utilization, natural competence, phosphoenolpyruvate:carbohydrate phosphotransferase system, two-component regulatory systems

Received 27 February 2017 Accepted 21 April 2017

Accepted manuscript posted online 1 May 2017

Citation Yamamoto S, Ohnishi M. 2017. Glucose-specific enzyme IIA of the phosphoenolpyruvate:carbohydrate phosphotransferase system modulates chitin signaling pathways in *Vibrio cholerae*. *J Bacteriol* 199:e00127-17. <https://doi.org/10.1128/JB.00127-17>.

Editor Igor B. Zhulin, University of Tennessee at Knoxville

Copyright © 2017 American Society for Microbiology. All Rights Reserved.

Address correspondence to Shouji Yamamoto, yshouji@nih.go.jp.

Bacteria can respond quickly and precisely to changes in environmental conditions such as stress levels, nutrient availability, and the extent of competition from other bacteria by employing signal transduction pathways. Two-component systems (TCSs) play important roles in transducing extracellular stimuli into the cell (1–5). A prototypical TCS is composed of a transmembrane sensor histidine kinase (HK) that regulates the activity of a single cytoplasmic response regulator (RR). In response to a specific signal, the HK itself autophosphorylates by transferring a phosphoryl group from ATP to the histidine residue of its conserved kinase domain. The phosphoryl group is then transferred to the aspartate residue in the conserved receiver domain of the cognate RR. Phosphorylation of the RR changes the activity of its output domain, which, in many cases, can control transcription through DNA binding (6). According to the prokaryotic TCS database (<http://www.p2cs.org/>), many bacteria possess multiple copies of TCS genes, ranging from a few to more than 250 copies (7), suggesting that the ability of bacteria to respond to environmental signals by TCSs varies in different species.

Another general signaling pathway in bacteria includes the phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (PTS) that represents the primary sugar transport system (8, 9). Two general cytoplasmic proteins in the PTS, enzyme I (EI) and the histidine phosphocarrier (Hpr) protein, mediate phosphoryl transfer from PEP to the various sugar-specific enzyme II (EII) transporters, which subsequently phosphorylate their sugar substrates during uptake. Thus, the phosphorylation state of the PTS proteins depends on both extracellular carbon availability and the intracellular metabolic state. In addition to sugar transport functions, PTS coordinates a wide variety of physiological functions that require carbon availability by modulating the activities of transporters, enzymes, and regulators of gene expression. In many cases, this regulation is mediated by glucose-specific enzyme IIA (EIIA^{glc}) (8, 9). EIIA^{glc} is the cytosolic component of the membrane-bound glucose transporter EIIBC. Dephosphorylated EIIA^{glc} causes inducer exclusion by binding to and inhibiting non-PTS carbohydrate transporters (10, 11). In contrast, phosphorylated EIIA^{glc} binds to and activates adenylate cyclase that produces cyclic AMP (cAMP). This small molecule acts as a secondary messenger and binds to the cAMP receptor protein (CRP), forming the cAMP-CRP complex, which has a profound impact on gene expression (12, 13). Therefore, catabolite repression of cAMP-CRP-dependent genes is caused by dephosphorylated EIIA^{glc}. With increasing numbers of novel EIIA^{glc} binding partners being revealed in various species, EIIA^{glc} has been found to be involved in multiple networks that regulate various bacterial functions and metabolic processes, including chemotaxis (14), respiration and fermentation (15), biofilm formation (16), and virulence (17, 18).

Vibrio cholerae, especially O1 and O139, causes the fatal diarrheal disease cholera. However, outside the human host, this bacterium is a natural inhabitant of aquatic environments, including rivers, estuaries, and coastal regions, where it is frequently attached to the chitinous exoskeletons of copepods and other crustaceans (19–21). Chitin, a polymer of *N*-acetylglucosamine (GlcNAc), is the most abundant polysaccharide in the aquatic biosphere and an important source of carbon and nitrogen for marine microorganisms. Association of *V. cholerae* with the chitin surface of copepods not only provides nutrients for growth (22, 23) but may also help the transmission of this pathogen from aquatic reservoirs to susceptible human hosts (24). Chitin pleiotropically affects physiological properties of *V. cholerae* and other *Vibrio* spp., including the development of colonization and biofilm formation, chemotaxis, survival during temperature and acid stresses (21), and the induction of natural competence for genetic transformation (25). This suggests that these bacteria have evolved to sense and adapt to the presence of chitin as a strategy for survival in natural environments.

By microarray gene expression analysis, Meibom et al. showed that chitin or its oligomers [(GlcNAc)_{n≥2}] induce the expression of 41 genes of *V. cholerae*, most of which are involved in chitin utilization and natural competence (25, 26). Chitin-induced natural transformation has only recently been recognized as an additional mechanism of horizontal gene transfer employed by *V. cholerae*. Natural competence is the ability of bacteria to actively take up DNA from the environment. The imported DNA is

subsequently recombined into the recipient chromosome, thereby undergoing transformation. Tight coupling of sensitivity of chitin availability with its utilization and DNA uptake might promote the niche-specific acquisition of genetic information. Li and Roseman proposed that all chitin-inducible genes are governed by the interactions among chitin, periplasmic chitin oligosaccharide-binding protein (CBP), and TCS HK ChiS (27). In the absence of chitin, the activity of ChiS is inhibited by CBP. In the presence of chitin, its major degradation product, (GlcNAc)₂, competes with ChiS to CBP and then, free ChiS activates an unidentified cytoplasmic RR called “ChiR” that interacts with target promoters. The ChiS regulon is divided into at least two distinct pathways; one is independent of the transcriptional regulator TfoX, and the other is TfoX dependent (25, 26, 28). TfoX (also called Sxy) is found in the bacterial families *Pasteurellaceae*, *Enterobacteriaceae*, and *Vibrionaceae* (29–31). It was first identified in *Haemophilus influenzae* (*Pasteurellaceae*) as the central regulator of the competence regulon (32–34), which activates the transcription of competence genes in collaboration with the cAMP-CRP complex (35, 36). In *V. cholerae*, TfoX is also dependent on cAMP-CRP for transcriptional regulation (37, 38), and it activates both competence genes (15 genes that encode a predicted type IV pilus, including *pilA*, *pilB*, and *pilQ*) and chitin utilization genes (two extracellular chitinase genes, *chiA-1* and *chiA-2*, and an outer membrane chitoporin gene, *chiP*). Furthermore, constitutive expression of TfoX led to the identification of new genes in the TfoX-dependent pathway, including two competence genes, *comEA* and *comEC*, and, surprisingly, three type 6 secretion system (T6SS) gene clusters that promote the acquisition of exogenous DNA by contact-dependent killing of neighboring bacteria (25, 37, 39–41). In contrast, the TfoX-independent pathway is now recognized to include the only (GlcNAc)₂ catabolic operon (*chb*) (25, 26, 28).

TfoX itself is transcriptionally and translationally activated by chitin and ChiS, with the translational impact being much stronger than the transcriptional one (28, 42, 43). Translation of *tfoX* is activated by the Hfq-dependent small RNA (sRNA) TfoR, which is also a member of the ChiS regulon (28, 43). Recently, we and another research group identified a novel transmembrane transcriptional regulator, termed “TfoS,” that is essential for *tfoR* transcription and natural competence (28, 44). Although the study by Dalia et al. suggested that TfoS itself acts as a chitin sensor (44), our results provided evidence that ChiS is required for the activity of TfoS (28). However, positive interactions between ChiS and TfoS have not been detected to date. Chitin-induced expression of *chb* depends on ChiS but does not involve TfoS. Thus, this operon should be controlled by an as-yet-unidentified regulator, ChiR (28). Because ChiS mutants that lacked the conserved phosphorylation residues were still able to activate the transcription of *tfoR* and *chb*, ChiS-initiated signaling is apparently mediated by mechanisms distinct from classical phosphorylation reactions (28).

In this study, we demonstrated that chitin signaling pathways in *V. cholerae* are modulated by the PTS. By genetic analyses of this signaling cascade, we unexpectedly found a novel association of EIIA^{glc} with the regulation of *tfoR* and *chb* expression. The results presented here provide new insights into the connectivity between the PTS and chitin signaling pathways in *V. cholerae* and suggest a strategy whereby this bacterium can physiologically adapt to the existing nutrient status.

RESULTS

Isolation and characterization of transposon mutants with defective *tfoR* expression. To determine which additional factors mediate the interplay of ChiS and TfoS, we screened transposon mutants of *V. cholerae* that turn off *tfoR* expression but possess intact *chiS* and *tfoS* genes. This approach is summarized in three steps illustrated in Fig. 1A. The first step involves efficient *in vitro* mutagenesis of chromosomal DNA by the *mariner* transposon (45–47). The second step was performed by natural transformation of the reporter strain (*tfoR::lacZ*) with mutagenized DNA. The third step selected mutants with decreased β -galactosidase activity. In this genetic screening system, however, insertion of *lacZ* into *tfoR* into the reporter strain inhibited transformation because *tfoR* is essential for the expression of TfoX, the central regulator of natural

FIG 1 Selection of transposon mutants with decreased *tfoR::lacZ* expression. (A) Schematic representation of the three steps of the strategy used for genetic screening. Chromosomal DNA and transformation-competent cells were prepared from SY0638S, a genetically modified *tfoR::lacZ* strain with *Com*⁺ and *Lac*⁺ phenotypes. (B) *Lac* phenotypes of transposon mutants grown on LA containing S-Gal. (C) Locations of transposon insertion sites in *vc0965* (*ptsI*). Numbering is according to the A base of the start codon (ATG) of *ptsI*.

competence (43). To convert this nontransformable strain into a highly transformable phenotype (*Com*⁺), we exchanged the *tfoX* promoter region with the *tac* promoter region, which resulted in constitutive expression of TfoX (42, 43). It should be noted that β -galactosidase activity from the *tfoR::lacZ* strain was too low to be reliably determined on a selective agar plate unless chitin was supplied in sufficient amounts. We therefore deleted *cbp*, which encodes a ChiS inhibitor, from the *Com*⁺ *tfoR::lacZ* strain to activate ChiS independently of the presence of chitin (27). The resulting strain, SY0638S, expressed *tfoR::lacZ* even in the absence of chitin, showing a *Lac*⁺ phenotype (black colonies) on Luria agar (LA) plates containing S-Gal (3,4-cyclohexenoesucletin- β -D-galactopyranoside) (Fig. 1B). (X-Gal [5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside] was not used in this study because it strongly inhibited *V. cholerae* growth [see Materials and Methods for details].) We screened ~40,000 transposon mutants of SY0638S on LA containing S-Gal and chloramphenicol (Cm) and observed three phenotypes of mutants in color (Fig. 1B). Whereas most of the mutants exhibited a *Lac*⁺ (black) phenotype, which is similar to that of parent strain SY0638S, some exhibited *Lac*^{+/-} (intermediate) and *Lac*⁻ (white) phenotypes. Sequencing analysis of the *Lac*⁻ mutants revealed transposon insertions in *lacZ* (data not shown). Therefore, we focused on *Lac*^{+/-} mutants in this study. Of 18 *Lac*^{+/-} mutants, 15 had mutations that were located in *tfoS* (data not shown), whereas the other three mutants had independent insertions in and near the *vc0965* (*ptsI*) gene, encoding EI of the PTS (Fig. 1C).

A *ptsI* deletion (Δ *ptsI*) mutant was constructed, and its phenotype was examined. The Δ *ptsI* mutation abolished chitin-induced expression of *tfoR* (Table 1). The defect of *tfoR* expression was complemented by the introduction of a low-copy-number plasmid carrying *ptsI* (pMW-SY*ptsI*) (Table 1). To determine whether phosphorylation is required for the ability of the EI protein to control *tfoR*, we created a plasmid encoding a

TABLE 1 Effects of PTS mutations on *tfoR* and *chb* expression

Mutation(s) ^a	Plasmid	β -Galactosidase enzyme activity ratio ^b	
		<i>tfoR::lacZ</i>	<i>chb::lacZ</i>
None		8.2	17.6
$\Delta ptsI$		0.9	3.6
$\Delta ptsI^c$		1.6	1.4
$\Delta ptsI$	pMW118	1.3	1.2
$\Delta ptsI$	pMW-SY $ptsI$	9.1	22.8
$\Delta ptsI$	pMW-SY $ptsI^{H189A}$	1.8	1.4
$\Delta ptsI \Delta ptsH \Delta crr$		11.6	19.5
$\Delta ptsI \Delta ptsH$		1.9	2.1
$\Delta ptsI \Delta crr$		9.5	13.6
$\Delta ptsI \Delta crr^c$		12.2	15.6
$\Delta ptsI \Delta crr$	pMW118	24	26.3
$\Delta ptsI \Delta crr$	pMW-SY crr	2.1	2.1
$\Delta ptsI \Delta crr$	pMW-SY crr^{H91Q}	1.8	2.1
Δcrr	pMW118	16.8	27
Δcrr	pMW-SY crr	9.3	18.6
Δcrr	pMW-SY crr^{H91Q}	2.5	3
$\Delta chiS$		1.2	1.1
$\Delta chiS \Delta ptsI$		0.7	1.1
$\Delta chiS \Delta ptsI \Delta crr$		0.9	1
$\Delta tfoS$		0.5	13.7
$\Delta tfoS \Delta ptsI$		1.0	1.2
$\Delta tfoS \Delta ptsI \Delta crr$		1.1	14.3
$chiS::3\times FLAG$		2.5	2.3

^aThe strains used were SY0627S (*tfoR::lacZ*), SY0639 (*tfoR::lacZ \Delta ptsI*), SY0640 (*tfoR::lacZ \Delta ptsI \Delta ptsH \Delta crr*), SY0641 (*tfoR::lacZ \Delta ptsI \Delta ptsH*), SY0642 (*tfoR::lacZ \Delta ptsI \Delta crr*), SY0643 (*tfoR::lacZ \Delta crr*), SY0627S30K (*tfoR::lacZ \Delta chiS*), SY0644 (*tfoR::lacZ \Delta chiS \Delta ptsI*), SY0645 (*tfoR::lacZ \Delta chiS \Delta ptsI \Delta crr*), SY0627S34 (*tfoR::lacZ \Delta tfoS*), SY0646 (*tfoR::lacZ \Delta tfoS \Delta ptsI*), SY0647 (*tfoR::lacZ \Delta tfoS \Delta ptsI \Delta crr*), SY0635S (*chb::lacZ*), SY0648 (*chb::lacZ \Delta ptsI*), SY0649 (*chb::lacZ \Delta ptsI \Delta ptsH \Delta crr*), SY0650 (*chb::lacZ \Delta ptsI \Delta ptsH*), SY0651 (*chb::lacZ \Delta ptsI \Delta crr*), SY0652 (*chb::lacZ \Delta crr*), SY0635S30K (*chb::lacZ \Delta chiS*), SY0653 (*chb::lacZ \Delta chiS \Delta ptsI*), SY0654 (*chb::lacZ \Delta chiS \Delta ptsI \Delta crr*), SY0635S34 (*chb::lacZ \Delta tfoS*), SY0655 (*chb::lacZ \Delta tfoS \Delta ptsI*), SY0656 (*chb::lacZ \Delta tfoS \Delta ptsI \Delta crr*), SY0673 (*tfoR::lacZ chiS::3\times FLAG*), and SY0674 (*chb::lacZ chiS::3\times FLAG*).

^b β -Galactosidase enzyme activity was assayed with cells grown in M9L medium (pH 7.6) in the presence or absence of 0.5 mM (GlcNAc)₄. Each value is the ratio of the β -galactosidase activity of cells cultured with (GlcNAc)₄ divided by the activity of cells cultured without (GlcNAc)₄. The actual values in units of β -galactosidase enzyme activity are shown in Table S6.

^cPlus 5 mM cAMP.

nonphosphorylatable mutant form of EI by replacing the conserved phosphorylated histidine residue with alanine (H189A, pMW-SY $ptsI^{H189A}$) (48–50). Introduction of the pMW-SY $ptsI^{H189A}$ plasmid into the $\Delta ptsI$ mutant failed to activate *tfoR* (Table 1). The $\Delta ptsI$ mutant was also unable to induce the competence genes *tfoX* and *pilA* (Table 2) and thus did not have detectable competence (Table 3). Previous studies suggested that the expression of the *chb* operon, which encodes proteins involved in the uptake and catabolism of chitin, is ChiS dependent (26, 27) but TfoS independent (28). Similar to the case with *tfoR*, activation of *chb* was dependent on EI and its intact H189 residue (Table 1). These results suggest that phosphorylated EI is required for the expression of *tfoR* and *chb*.

Negative regulation of *tfoR* and *chb* expression by EIIA^{91c} in the absence of EI.

Like the *Escherichia coli* genes that encode the general components of the PTS, their homologues in *V. cholerae* are located on the chromosome close to each other in the following order (Fig. 1C): *vc0966* (*ptsH* encoding Hpr), *ptsI*, and *vc0964* (*crr* encoding EIIA^{91c}). To examine whether EI is the PTS component solely responsible for the induction of *tfoR* and *chb*, we deleted the region spanning *ptsH*, *ptsI*, and *crr* ($\Delta ptsI \Delta ptsH \Delta crr$) and attempted to express only the EI protein from pMW-SY $ptsI$ in this triple mutant strain. However, in contrast to the observations in the $\Delta ptsI$ single mutant, the $\Delta ptsI \Delta ptsH \Delta crr$ triple mutant displayed full induction of *tfoR* and *chb* without exogenously expressing EI (Table 1). This result indicates that EI is not essential for *tfoR* and *chb* expression but rather regulates these genes in cooperation with other PTS components.

TABLE 2 Effects of PTS mutations on *tfoX* and *pilA* expression

<i>lacZ</i> fusion ^a and mutation(s)	Mean β -galactosidase activity ^b (Miller units) $\pm$ SD ^c		Fold activation ^d
	No supplement	0.5 mM (GlcNAc) ₄	
<i>tfoX</i> _{TC} :: <i>lacZ</i>			
None	118 $\pm$ 5	326 $\pm$ 13	2.8
$\Delta ptsI$	129 $\pm$ 6	146 $\pm$ 4	1.1
$\Delta ptsI^e$	191 $\pm$ 6	194 $\pm$ 8	1.0
$\Delta ptsI \Delta crr$	104 $\pm$ 8	113 $\pm$ 5	1.1
$\Delta ptsI \Delta crr^e$	141 $\pm$ 6	332 $\pm$ 8	2.4
<i>tfoX</i> _{TL} :: <i>lacZ</i>			
None	9 $\pm$ 1	381 $\pm$ 13	42
$\Delta ptsI$	13 $\pm$ 1	31 $\pm$ 2	2.4
$\Delta ptsI^e$	13 $\pm$ 1	17 $\pm$ 1	1.3
$\Delta ptsI \Delta crr$	16 $\pm$ 1	68 $\pm$ 6	4.3
$\Delta ptsI \Delta crr^e$	8 $\pm$ 1	165 $\pm$ 8	21
<i>pilA</i> :: <i>lacZ</i>			
None	12 $\pm$ 1	472 $\pm$ 14	39
$\Delta ptsI$	15 $\pm$ 2	50 $\pm$ 4	3.3
$\Delta ptsI^e$	17 $\pm$ 1	21 $\pm$ 1	1.2
$\Delta ptsI \Delta crr$	14 $\pm$ 1	27 $\pm$ 1	1.9
$\Delta ptsI \Delta crr^e$	15 $\pm$ 1	321 $\pm$ 13	21.4

^aThe strains used were SY06135 (*tfoX*_{TC}::*lacZ*; transcriptional fusion), SY0657 (*tfoX*_{TC}::*lacZ* Δ *ptsI*), SY0658 (*tfoX*_{TC}::*lacZ* Δ *ptsI* Δ *crr*), SY06165 (*tfoX*_{TL}::*lacZ*; translational fusion), SY0659 (*tfoX*_{TL}::*lacZ* Δ *ptsI*), SY0660 (*tfoX*_{TL}::*lacZ* Δ *ptsI* Δ *crr*), SY0623 (*pilA*::*lacZ*), SY0661 (*pilA*::*lacZ* Δ *ptsI*), and SY0662 (*pilA*::*lacZ* Δ *ptsI* Δ *crr*).

^bExperiments were performed in triplicate.

^cIn M9L medium (pH 7.6).

^dFold activation is indicated as the relative difference from the value observed in the culture in the absence of (GlcNAc)₄.

^ePlus 5 mM cAMP.

EI is the first component of the PTS phosphotransfer cascade. It is possible that the lack of EI prevents phosphorylation of the downstream PTS components Hpr and EII, leading to the repression of *tfoR* and *chb* by dephosphorylated forms of these PTS proteins. We constructed double mutants lacking *ptsI* and either *ptsH* or *crr* and examined the effect of the combined mutations on gene expression. As a result, deletion of *crr*, but not that of *ptsH*, on the Δ *ptsI* background rescued the deficiency of *tfoR* and *chb* expression (Table 1), indicating that in the absence of EI, EIIA^{91c} negatively regulated the transcription of *tfoR* and *chb*.

In contrast to the regulation of *tfoR* and *chb*, the repression of *tfoX* and *pilA* in the Δ *ptsI* mutant was not rescued by the additional deletion of *crr* (Table 2). Consistent with this, natural competence remained inactivated in this double mutant (Table 3). Classically, phosphorylated EIIA^{91c} is known to activate adenylate cyclase, the cAMP biosynthetic enzyme (8, 51). Synthesized cAMP, in turn, modulates CRP, a global transcrip-

TABLE 3 Effects of the PTS mutations on natural transformation

Recipient strain ^a	Transformation frequency (Cm ^r CFU/total CFU) ^b in DASWL medium supplemented with shrimp shell
WT	$2.8 \times 10^{-5} \pm 1.9 \times 10^{-5}$
Δ <i>chiS</i> mutant	<DL ^c
Δ <i>tfoS</i> mutant	<DL
Δ <i>ptsI</i> mutant	<DL
Δ <i>ptsI</i> mutant ^d	<DL
Δ <i>ptsI</i> Δ <i>crr</i> mutant	<DL
Δ <i>ptsI</i> Δ <i>crr</i> mutant ^d	$1.6 \times 10^{-5} \pm 1.1 \times 10^{-5}$

^aThe strains used were V060002 (WT), SY0630K (Δ *chiS*), SY0634S (Δ *tfoS*), SY0661 (Δ *ptsI*), and SY0664 (Δ *ptsI* Δ *crr*).

^bData from three independent experiments are presented as mean values $\pm$ standard deviations.

^c<DL, below detection limit ($\sim 3.7 \times 10^8$).

^dPlus 5 mM cAMP.

tional regulator of cellular physiology. When cells accumulate dephosphorylated EIIA^{91c} or lack this protein, catabolite repression occurs. Because the transcription of *tfoX* and *pilA* has been reported to be positively regulated by cAMP and CRP (37, 38, 52–54), we hypothesized that the lack of EIIA^{91c} would cause catabolite repression of these competence genes by promoting low intracellular cAMP levels. As expected, addition of cAMP to the culture medium recovered the phenotypes of the $\Delta ptsI \Delta crr$ mutant, including natural competence, as well as the induction of *tfoX* and *pilA* (Table 2 and 3). However, the deficiencies of *tfoR* and *chb* expression (also *tfoX* and *pilA*, genes governed by TfoR) and of natural competence in the $\Delta ptsI$ mutant were not rescued by exogenous cAMP (Tables 1 to 3). This suggests that the repression of *tfoR* and *chb* by dephosphorylated EIIA^{91c} is mechanistically distinct from classical catabolite repression. It should be noted that a *V. cholerae* mutant lacking adenylate cyclase or CRP did not grow with chitin or lactate as a sole carbon source, as described previously (38). Therefore, we could not test the effects of these proteins on gene expression under the same culture conditions.

Dephosphorylated EIIA^{91c} is important for negative regulation of *tfoR* and *chb*.

The results presented above suggested that dephosphorylated EIIA^{91c} downregulates gene expression in the ChiS regulon by two distinct mechanisms, (i) classical catabolite repression of *tfoX* and *pilA* and (ii) newly identified repression of *tfoR* and *chb*. We next determined the phosphorylation state of EIIA^{91c} in the wild-type (WT) and $\Delta ptsI$ mutant genetic backgrounds. To detect the EIIA^{91c} protein expressed by *V. cholerae* cells, a 3×FLAG sequence was chromosomally fused to the 3' end of the *crr* gene. Previous studies reported that the phosphorylated form of EIIA^{91c} has a slightly slower mobility in SDS-polyacrylamide gels than the dephosphorylated form (13, 55, 56). By combining this technique with phosphate-binding tag (Phos-tag) sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (57), we managed to detect these two different forms with better resolution. In WT cells, the EIIA^{91c}::3×FLAG protein was largely phosphorylated (slower form), whereas it became completely dephosphorylated (faster form) on the background of the $\Delta ptsI$ mutation (Fig. 2A).

To examine whether dephosphorylated EIIA^{91c} plays a crucial role in the repression of *tfoR* and *chb*, we constructed a plasmid encoding a nonphosphorylatable EIIA^{91c} mutant protein in which the conserved phosphorylated histidine residue was replaced with glutamine (H91Q, pMW-SYcrr^{H91Q}) (13, 58). This plasmid was introduced into the $\Delta ptsI \Delta crr$ and Δcrr mutants that represent genetic backgrounds lacking and containing El-to-Hpr phosphosignaling, respectively. In contrast to WT EIIA^{91c}, the EIIA^{91c}-H91Q mutant protein inhibited the expression of *tfoR* and *chb* in both genetic backgrounds (Table 1). These results suggested that dephosphorylated EIIA^{91c} is important for the negative regulation of *tfoR* and *chb*.

The phosphorylation state of EIIA^{91c} determines the expression of *tfoR* and *chb* in response to carbon availability. The phosphorylation state of EIIA^{91c} is determined by transported and catabolized carbon sources (59, 60). Preferred carbon sources such as glucose cause dephosphorylation of EIIA^{91c}, but unfavorable carbon sources or carbon depletion conditions lead to the accumulation of phosphorylated EIIA^{91c}. Intracellular EIIA^{91c} proteins were mainly phosphorylated under the conditions that favored *tfoR* and *chb* by using (GlcNAc)₄ (Fig. 2A). We expected that in the presence of glucose, chitin-induced expression of *tfoR* and *chb* would be downregulated and EIIA^{91c} would become dephosphorylated. Thus, we induced *tfoR* and *chb* expression by (GlcNAc)₄ and supplemented the culture medium with glucose at a concentration of 2.5 mM, i.e., five times that of (GlcNAc)₄. After further cultivation for 60 min, the expression levels of these genes were assayed. Glucose reduced *tfoR* and *chb* expression levels to 33 and 50% of those observed under control conditions, respectively, whereas the EIIA^{91c}-independent PTS sugar mannitol and the non-PTS sugar arabinose (61) had no significant effects on these genes (Table 4). Moreover, the reduction caused by glucose was suppressed by the Δcrr mutation (Table 4). Under the same culture conditions, the EIIA^{91c}::3×FLAG protein was dramatically changed into the dephosphorylated form by

FIG 2 Analysis of the phosphorylation state of EIIA^{glc}. (A) Phosphorylation state of the EIIA^{glc}::3×FLAG protein. Cells were cultured in M9L medium that contained 0.5 mM (GlcNAc)₄. Total protein samples were prepared and separated on a Phos-tag 15% polyacrylamide gel as described in Materials and Methods. The EIIA^{glc}::3×FLAG protein was detected by Western blotting with an anti-FLAG antibody. The strains used were WT V060002, SY0671S (*crr::3×FLAG*), and SY0672 (*crr::3×FLAG ΔptsI*). (B) Effects of various sugars on the phosphorylation state. The sugars indicated were added to cultures of SY0671S at a concentration of 2.5 mM, and the cultures were further incubated for 60 min. Total protein samples were subjected to Phos-tag SDS-PAGE and then analyzed as described above.

glucose but not by mannitol or arabinose (Fig. 2B). These results suggested that glucose availability regulates *tfoR* and *chb* through the phosphorylation state of EIIA^{glc}.

Like glucose, the chitin monomer GlcNAc is transported through the cytoplasmic membrane by the PTS driven by EIIA^{glc} and causes catabolite repression of natural competence (38, 61). Consistently, supplementation with GlcNAc combined with (GlcNAc)₄ converted the EIIA^{glc}::3×FLAG protein into the dephosphorylated form (Fig. 2B) and inhibited the expression of *tfoR* and *chb* in an EIIA^{glc}-dependent manner (Table 4). According to the model of the chitin catabolic cascade in *Vibrio* spp., GlcNAc is yielded by the periplasmic degradation of chitin oligosaccharides (27, 62). However, during growth in medium containing (GlcNAc)₄ as the sole sugar source, EIIA^{glc} was largely phosphorylated and expression of *tfoR* and *chb* was highly induced (Fig. 2 and Table 1). This may suggest some mechanism for which chitin oligosaccharides escape from degradation into GlcNAc in the periplasmic space and act as effective inducers, as has already been pointed out previously (63).

Negative regulation of *tfoR* and *chb* by dephosphorylated EIIA^{glc} involves ChiS.

To determine whether the repression of *tfoR* and *chb* by dephosphorylated EIIA^{glc} is dependent on ChiS, we introduced the *ΔptsI* and *ΔptsI Δcrr* mutations into the *tfoR::lacZ* and *chb::lacZ* strains with *chiS* deleted (*ΔchiS*). On the *ΔchiS* background, both *tfoR* and *chb* were not induced under any of the mutation combinations tested (Table 1). Furthermore, the expression of *chb*, which is independent of TfoS (28), was normally induced in the *ΔptsI Δcrr ΔtfoS* mutant (Table 1). These results suggested that negative regulation of *tfoR* and *chb* by dephosphorylated EIIA^{glc} is mediated through ChiS.

We examined the effects of the PTS genes on ChiS expression. The expression of the ChiS::3×FLAG protein was unaffected by the *ΔptsI* and *ΔptsI Δcrr* mutations (Fig. 3), raising the possibility that dephosphorylated EIIA^{glc} regulates ChiS at the posttranslational level. Similar result was obtained in the case of the TfoS::3×FLAG protein (Fig. 3).

TABLE 4 Effects of various sugars on *tfoR* and *chb* expression

<i>lacZ</i> fusion ^a and added sugar (2.5 mM)	β-Galactosidase activity ^b in M9L medium plus 0.5 mM (GlcNAc) ₄	Inhibition ^c
<i>tfoR::lacZ</i>		
None	183 ± 6	
Glucose	60 ± 2	0.33
GlcNAc	85 ± 5	0.46
Mannitol	162 ± 4	0.9
Arabinose	155 ± 11	0.85
<i>tfoR::lacZ Δcrr</i>		
None	248 ± 17	
Glucose	198 ± 6	0.8
GlcNAc	259 ± 13	1
Mannitol	225 ± 13	0.91
Arabinose	256 ± 3	1
<i>chb::lacZ</i>		
None	287 ± 7	
Glucose	143 ± 2	0.5
GlcNAc	149 ± 6	0.52
Mannitol	255 ± 11	0.89
Arabinose	335 ± 11	1.2
<i>chb::lacZ Δcrr</i>		
None	263 ± 11	
Glucose	232 ± 11	0.88
GlcNAc	288 ± 15	1.1
Mannitol	295 ± 17	1.1
Arabinose	312 ± 18	1.2

^aThe strains used were SY0627S (*tfoR::lacZ*), SY0643 (*tfoR::lacZ Δcrr*), SY0635S (*chb::lacZ*), and SY0652 (*chb::lacZ Δcrr*).

^bβ-Galactosidase activity (Miller units) is presented as mean values ± standard deviations. Experiments were performed in triplicate.

^cInhibition is presented as the ratio of the β-galactosidase activity of cells cultured with the added sugar divided by the activity of cells cultured without the added sugar.

DISCUSSION

Natural competence and chitin utilization in *V. cholerae* are catabolically repressed at multiple levels by dephosphorylated EIIA^{glc}. In many bacteria, the PTS is primarily known as a sugar transport system. The PTS also mediates the regulatory functions of a wide range of cellular activities in response to carbon availability, many of which are mediated by EIIA^{glc} (8, 9). The phosphorylation status of EIIA^{glc}, in general, determines the regulatory outputs and depends on the PTS transport activity and glycolysis flux. Utilization of unfavorable carbon sources generates a high PEP/pyruvate

FIG 3 Effects of the PTS genes on the expression of ChiS and TfoS. Western blotting analyses of the ChiS::3×FLAG and TfoS::3×FLAG proteins from cells grown in M9L medium containing 0.5 mM (GlcNAc)₄. Total proteins were separated on a 5 to 20% SDS-polyacrylamide gel and then analyzed by Western blotting with an anti-FLAG antibody (top) or an anti-RpoA antibody (bottom). Lanes: 1, VC060002; 2, SY0666S; 3, SY0667; 4, SY0668; 5, SY0636S; 6, SY0669; 7, SY0670.

ratio and leads to EIIA^{glc} phosphorylation. Furthermore, this ratio becomes low and EIIA^{glc} is predominantly dephosphorylated in the presence of preferred carbon sources (59, 60). In classical examples, inducer exclusion occurs because dephosphorylated EIIA^{glc} prevents the activity of non-PTS transporters (10, 11), whereas cAMP synthesis stimulated by the phosphorylated form of EIIA^{glc} leads to the activation of CRP-dependent genes (12, 13). A recent study of *E. coli* unveiled a novel connection between EIIA^{glc} and the carbon storage regulator (Csr) system, in which dephosphorylated EIIA^{glc} binds to the CsrD protein and promotes the decay of the CsrB and CsrC sRNA molecules by RNase E (64). EIIA^{glc} also interacts with the CsrD homologue MshH in *V. cholerae* to control biofilm formation (16). In some cases, EIIA^{glc} directly regulates virulence factors of pathogenic bacteria. The activity of the *Vibrio* insulin-degrading enzyme in *V. vulnificus* is stimulated by dephosphorylated EIIA^{glc} (18). EIIA^{glc} also binds to the ATPase specific for T3SS-2 of *Salmonella enterica*, but this binding occurs in a phosphorylation-independent manner (17).

The involvement of the PTS and cAMP-CRP in the induction of natural competence was previously described in *H. influenzae* (36, 65). Thus, phosphorylated EIIA^{glc} could also positively regulate competence genes through the activation of cAMP synthesis. In recent years, several lines of evidence have demonstrated that a set of *V. cholerae* and *E. coli* genes homologous to the competence genes in *H. influenzae*, including *tfoX* and *pilA*, are activated by cAMP-CRP (25, 29, 37, 38, 52–54, 66, 67). Consistent with these reports, the defects of competence, as well as of *tfoX* and *pilA* transcription, in a *V. cholerae* mutant lacking EIIA^{glc} were rescued by the exogenous addition of cAMP (Tables 2 and 3). In this study, we unexpectedly found that the dephosphorylated form of EIIA^{glc} is also involved in the regulation of natural competence. The activation of *tfoX* in *V. cholerae* requires an additional signaling cascade initiated by the chitin-responsive TCS HK ChiS (26). When activated by chitin, ChiS stimulates the transmembrane transcriptional regulator TfoS, which activates the transcription of *tfoR*, encoding the sRNA essential for *tfoX* translation (28, 43). Our results suggest that dephosphorylated EIIA^{glc} acts as a negative regulator of *tfoR* (Fig. 4). In the presence of only chitin, EIIA^{glc} is predominantly phosphorylated and thus *tfoR* is not repressed. Simultaneously, phosphorylated EIIA^{glc} increases the intracellular cAMP level to activate CRP, which, in turn, stimulates the transcription of *tfoX* and other cAMP-CRP-dependent competence genes. However, the coexistence of chitin with the competing PTS-dependent sugars, such as glucose and GlcNAc, dephosphorylates EIIA^{glc}, leading to the repression of *tfoR*. At the same time, smaller amounts of cAMP are produced and therefore, CRP-dependent genes are downregulated. ChiS is also required for the activation of the (GlcNAc)₂ catabolic operon *chb*, which is independent of TfoS (26–28). Similar to *tfoR*, *chb* is repressed by dephosphorylated EIIA^{glc} (Fig. 4). The regulation by EIIA^{glc} of the expression levels of both *tfoR* and *chb* was dependent on ChiS (Table 1), and EIIA^{glc} did not affect ChiS expression (Fig. 3). Therefore, it can be hypothesized that dephosphorylated EIIA^{glc} binds to ChiS to inhibit its activity, reminiscent of “inducer exclusion”; however, our attempts to detect interactions between EIIA^{glc} and ChiS by bacterial two-hybrid assays and chemical cross-linking experiments have not been successful (data not shown). Regarding chemical cross-linking, we fused a 3×FLAG peptide to ChiS to detect the protein. However, we found that the expression of this fusion protein decreases the induction levels of *tfoR* and *chb* (Table 1), raising the possibility that the incorporation of some foreign peptide or protein into ChiS inhibits its potential activities, including interactions with EIIA^{glc} and TfoS. We are now investigating peptides or proteins that do not affect ChiS functions. An alternative possibility is that EIIA^{glc} regulates ChiS via other factors, including cognate EIIBC transporters. According to the phosphotransfer cascade of the PTS, the lack of only EI and both EI and EIIA^{glc} would exhibit a common phosphorylation status of EIIBCs (all unphosphorylated forms). However, these mutations inversely affected *tfoR* and *chb* expression (Table 1). If EIIA^{glc}-specific EIIBCs were directly involved in the regulation of ChiS, factors beyond the phosphorylation state would determine the interactions of these transporters and ChiS. Recently, Pickering et al. have identified 13 binding partners of EIIA^{glc} in *V.*

FIG 4 Multiple-level control of the ChiS regulon by EIIA^{glc}. In the presence of chitin only, EIIA^{glc} is predominantly in the phosphorylated form, and thus, ChiS is active. Simultaneously, phosphorylated EIIA^{glc} increases the intracellular cAMP level and thereby modulates CRP, which, in turn, activates the transcription of *tfoX* and other competence genes. However, the combined presence of chitin and EIIA^{glc}-dependent PTS sugars such as GlcNAc and glucose promotes the dephosphorylation of EIIA^{glc} and, as a result, inhibition of ChiS. Under these conditions, cAMP-CRP-dependent competence genes are also downregulated.

cholerae by tandem affinity purification, but ChiS was not one of them (16). It is also possible that an additional protein(s), including the 13 proteins identified by Pickering et al., mediates the regulation of ChiS by EIIA^{glc}. At present, however, we cannot rule out a different possibility, that EIIA^{glc} acts in an unidentified pathway parallel to ChiS, because ChiS is essential for *tfoR* and *chb* expression, and therefore, the Δ *chiS* mutation could mask the effect of the Δ *crr* mutation.

In addition to the availability of chitin and other carbon sources, *V. cholerae* senses high cell density to stimulate natural competence. The quorum sensing regulator HapR, as well as TfoX, positively regulates not only two competence genes, *comEA* and *comEC*, but also three competence-promoting T6SS gene clusters (25, 37, 39–41). Because cAMP-CRP has also been shown to upregulate *hapR* expression by activating the synthesis of the autoinducer CAI-1 (68), EIIA^{glc} could indirectly modulate natural competence via quorum sensing. Moreover, like that of *pilA*, the transcription of two extracellular chitinase genes, *chiA-1* and *chiA-2*, as well as that of the chitoporin gene *chiP*, is dependent on both TfoX and cAMP-CRP (25, 37, 38) and thus is probably regulated by EIIA^{glc}. Considering the results presented here and other available information reported previously, all three signaling pathways (induced by chitin, cAMP, and CAI-1) for natural competence and chitin utilization could be catabolically repressed by dephosphorylated EIIA^{glc}.

Does EIIA^{glc} act as an interface between two lifestyles of *V. cholerae*? *V. cholerae* exhibits roughly two different lifestyles in nature, one in the aquatic environment, where it often attaches to the chitinous reservoirs of zooplankton, crabs, and shrimp (23), and the other in the human body, where it acts as the causative agent of cholera.

When infecting the human body, *V. cholerae* may encounter a wide range of carbohydrates, including PTS sugars, in the small intestine (69, 70) and therefore may catabolically repress natural competence and chitin utilization through EIIA^{glc}. In contrast, the latter two cellular functions are activated in the aquatic environment, where chitin is the most abundant carbon source available. *Vibrio* species are predominant bacterial species associated with zooplankton, e.g., copepods (71, 72), where *Vibrio* bacteria accumulate a pool of DNA from closely related species. In contrast, the human small intestine microbiota includes different bacterial genera, e.g., *Clostridium*, *Escherichia*, *Turicibacter*, *Streptococcus*, and *Veillonella* (73), so *Vibrio* species would have to coexist with distantly related bacterial species. In the human gut environment, therefore, *V. cholerae* should limit efficient DNA uptake and transformation, which can be detrimental, particularly in the case of interspecies genetic exchange with phylogenetically diverse species via illegitimate (nonhomologous) recombination. Multiple levels of control of natural competence and chitin utilization by EIIA^{glc} may help *V. cholerae* to quickly determine the availability of DNA and carbon sources according to the changes in the surrounding nutritional status, e.g., following the transfer of this bacterium from the environmental reservoirs to the human host. Interestingly, the expression of major virulence factors of *V. cholerae*, including cholera toxin and the toxin-coregulated pilus, was repressed by cAMP-CRP but activated by glucose and other PTS-dependent sugars (74, 75), suggesting that virulence and natural competence (or chitin utilization) may be inversely regulated by EIIA^{glc}. In addition, this protein positively regulates biofilm formation, which is associated with the stress resistance of *V. cholerae* in natural environments (16, 76). Therefore, it is possible that EIIA^{glc} acts as an interface for switching between two different lifestyles of *V. cholerae*, i.e., as an environmental inhabitant and as a human pathogen. Experiments are now in progress to test this possibility.

Links between PTS and TCS signal transduction cascades. In this study, we presented a genetic link between the PTS enzyme EIIA^{glc} and TCS HK ChiS. In recent years, auxiliary proteins, which recruit TCSs in response to distinct signals, have emerged as a novel class of regulatory proteins (77–79). Several auxiliary factors are known that modulate the activities of HKs and RRs through protein-protein interactions. Although very little is known about connections between the PTS and TCSs, a functional interaction of Hpr from enterococci with the RR of the CroRS TCS has been recently reported (80). In addition, the EIIA^{Ntr} protein, which is a component of a nitrogen-related PTS, paralogous to the canonical PTS, has been identified as another auxiliary protein that can modulate the activities of the HKs KdpD and PhoR in *E. coli* (81, 82). These findings suggest that more TCSs can be modulated by such nutrient-sensory cytoplasmic PTSs. Future studies of the regulation of chitin signaling pathways by EIIA^{glc} will improve our understanding of the connectivity between the PTS and TCSs.

MATERIALS AND METHODS

Bacterial strains, plasmids, media, and DNA manipulation. All of the strains and plasmids used in this study are listed in Table S1 in the supplemental material. *E. coli* strain HST16CR (TaKaRa) was used for DNA cloning. Bacteria were routinely grown in Luria broth (LB), on LA, and in super optimal broth medium with catabolite repression (2% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄, 20 mM glucose). The antibiotics used were 100 µg/ml ampicillin (Ap), 1 µg/ml Cm, and 30 µg/ml kanamycin. Arabinose was used at a concentration of 5 mg/ml. PCR amplification of DNA was performed with GeneAmp PCR system 9700 (Applied Biosystems) and Herculase II fusion polymerase (Stratagene). DNA sequencing was performed with a BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems). PCR products were purified with a High Pure PCR product purification kit (Roche). Customized oligonucleotide primers (Table S2) were purchased from Hokkaido System Science. Chromosomal and plasmid DNA molecules were extracted with a Wizard genomic DNA purification kit (Promega) and a High Pure plasmid isolation kit (Roche), respectively.

Protein analysis. SDS-PAGE and Western blotting analyses of proteins were performed as previously described (83).

Chromosomal engineering. Genetically modified *V. cholerae* strains were constructed with the λ Red-FLP recombination system (83–85) or natural transformation induced by shrimp shell (86). When an in-frame deletion was introduced into the *V. cholerae* chromosome, markerless gene replacement was

achieved with the *sacB*/sucrose counterselection system as described previously (28). The DNA molecules used for recombination are described in the supplemental material.

Plasmid constructions. See the supplemental material for details of plasmid construction.

Natural transformation. Natural transformation with shrimp shell was performed as previously described (28, 86). Cells were cultured in distilled artificial seawater medium containing 0.5% lactate (pH 7.6), termed DASWL medium. Lactate was added to support the growth of a *chiS* null mutant that cannot use (GlcNAc)_{n=2} as its sole carbon source (27). Chromosomal DNA from SY1003 (10 µg/ml), which harbors the Cm resistance gene (*cat*) (83), was used as a donor. If necessary, 5 mM cAMP (Nacalai Tesque) was added to the DASWL medium throughout cell culturing. After overnight incubation, bacterial cultures were plated onto LA with or without Cm. The transformation frequency was calculated as the number of Cm-resistant CFU divided by the total number of CFU.

Transposon mutagenesis, mutant selection, and insertion site mapping. Mutagenesis with *mariner* transposase was performed as reported previously (45, 87), with some modifications. MarC9 (a mutant protein with *Himar1* transposase) N-terminally fused to the maltose-binding protein (MBP::MarC9) was purified from *E. coli* BL21(DE3) (Promega) carrying pMAL-SYMarC9 as described previously (88). By using purified MBP::MarC9, the minitransposon *magellan2* (which is a derivative of the *Himar1* transposon, encoding Cm resistance) on pEMcat (45) was introduced into chromosomal DNA from SY0638S. Subsequently, single-strand gaps at each transposon-chromosome junction were repaired with T4 DNA polymerase (TaKaRa) and *E. coli* DNA ligase (New England BioLabs). Mutagenized DNA was introduced into SY0638S by natural transformation, and then the bacterial culture was directly plated onto LA containing 5-Gal (Sigma) and Cm to determine the Lac phenotypes of transformants. Note that X-Gal was not used as an indicator in this study because direct inoculation of a liquid culture of *V. cholerae* onto LA containing X-Gal caused severe growth inhibition. Ligation-mediated PCR was used to characterize transposon-*V. cholerae* DNA junctions. Briefly, chromosomal DNA from each strain harboring a *magellan2* insertion was digested with *Sau3A1* and then self-ligated. PCRs were performed on ligated templates with primers MarP1 and MarP2. Sequencing of the PCR products was performed with MarP1.

β-Galactosidase enzyme assay and bacterial culture. β-Galactosidase (LacZ) activity was measured as previously described (42), with *V. cholerae* cells assayed in triplicate. The cells were grown under the culture conditions described below. Unless otherwise stated, these conditions were also used for other experiments. Cells were grown in M9 minimal medium containing minimal essential medium vitamin solution (Gibco) and 0.5% lactate (pH 7.6) (termed M9L medium) in the presence or absence of 0.5 mM (GlcNAc)₄ (Tokyo Chemical Industry Co., Ltd.) until they reached an optical density at 600 nm of approximately 0.3. If necessary, Ap or 5 mM cAMP was also added to the medium.

Analysis of EIIA^{glc} phosphorylation state. SY0671S and its derivative strains that encoded the EIIA^{glc}::3×FLAG protein were cultured, and then total protein samples were prepared for determination of the phosphorylation state of EIIA^{glc} as described previously (55). To clearly distinguish the two forms of EIIA^{glc}, samples were separated on a Phos-tag 15% polyacrylamide gel (Wako) as described previously (57). Subsequently, the gel was washed twice with 1 mM EDTA for 15 min, followed by a third wash with Western transfer buffer. After transfer onto a polyvinylidene difluoride (PVDF) membrane, the EIIA^{glc}::3×FLAG protein was detected with a horseradish peroxidase-conjugated anti-FLAG M2 monoclonal antibody (Sigma).

Effects of various sugars on gene expression and phosphorylation state of EIIA^{glc}. Appropriate *V. cholerae* strains were cultured in M9L medium containing 0.5 mM (GlcNAc)₄, and then different sugars (GlcNAc, glucose, mannitol, and arabinose) were added to the culture medium at a concentration of 2.5 mM. After further incubation for 60 min, β-galactosidase activity and the EIIA^{glc} phosphorylation state were analyzed as described above.

Analysis of intracellular levels of the FLAG-tagged proteins. Proteins extracted from SY0636S, SY0666S, and their derivative strains that harbored a 3×FLAG sequence were separated by SDS-PAGE and then transferred onto a PVDF membrane. The FLAG-tagged proteins were detected with a horseradish peroxidase-conjugated anti-FLAG M2 monoclonal antibody. RpoA was also detected with anti-RpoA antiserum (Santa Cruz Biotechnology).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00127-17>.

SUPPLEMENTAL FILE 1, PDF file, 0.2 MB.

ACKNOWLEDGMENTS

This work was supported by the Japan Society for the Promotion of Sciences (JSPS KAKENHI, no. 16K08798).

We thank Brian J. Akerley and Jean-Pierre Claverys for providing plasmids pEMcat and pET-29b(+)/MarC9 and Hideyuki Takahashi for suggesting the method used to analyze the phosphorylation state of EIIA^{glc}.

REFERENCES

- Parkinson JS, Kofoed EC. 1992. Communication modules in bacterial signaling proteins. *Annu Rev Genet* 26:71–112. <https://doi.org/10.1146/annurev.ge.26.120192.000443>.
- Stock AM, Robinson VL, Goudreau PN. 2000. Two-component signal transduction. *Annu Rev Biochem* 69:183–215. <https://doi.org/10.1146/annurev.biochem.69.1.183>.
- Mascher T, Helmman JD, Unden G. 2006. Stimulus perception in bacterial signal-transducing histidine kinases. *Microbiol Mol Biol Rev* 70:910–938. <https://doi.org/10.1128/MMBR.00020-06>.
- Hoch JA, Silhavy TJ (ed). 1995. Two-component signal transduction. ASM Press, Washington, DC.
- Mizuno T. 1998. His-Asp phosphotransfer signal transduction. *J Biochem* 123:555–563. <https://doi.org/10.1093/oxfordjournals.jbchem.a021972>.
- Galperin MY. 2010. Diversity of structure and function of response regulator output domains. *Curr Opin Microbiol* 13:150–159. <https://doi.org/10.1016/j.mib.2010.01.005>.
- Barakat M, Ortet P, Jourlin-Castelli C, Ansaldi M, Mejean V, Whitworth DE. 2009. P2CS: a two-component system resource for prokaryotic signal transduction research. *BMC Genomics* 10:315. <https://doi.org/10.1186/1471-2164-10-315>.
- Deutscher J, Francke C, Postma PW. 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. *Microbiol Mol Biol Rev* 70:939–1031. <https://doi.org/10.1128/MMBR.00024-06>.
- Deutscher J, Aké FM, Derkaoui M, Zébré AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P. 2014. The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. *Microbiol Mol Biol Rev* 78:231–256. <https://doi.org/10.1128/MMBR.00001-14>.
- Osumi T, Saier MH, Jr. 1982. Regulation of lactose permease activity by the phosphoenolpyruvate:sugar phosphotransferase system: evidence for direct binding of the glucose-specific enzyme III to the lactose permease. *Proc Natl Acad Sci U S A* 79:1457–1461. <https://doi.org/10.1073/pnas.79.5.1457>.
- Saier MH, Jr, Roseman S. 1976. Sugar transport. The *crr* mutation: its effect on repression of enzyme synthesis. *J Biol Chem* 251:6598–6605.
- Feucht BU, Saier MH, Jr. 1980. Fine control of adenylate cyclase by the phosphoenolpyruvate:sugar phosphotransferase systems in *Escherichia coli* and *Salmonella typhimurium*. *J Bacteriol* 141:603–610.
- Dörschug M, Frank R, Kalbitzer HR, Hengstenberg W, Deutscher J. 1984. Phosphoenolpyruvate-dependent phosphorylation site in enzyme III^{glc} of the *Escherichia coli* phosphotransferase system. *Eur J Biochem* 144:113–119. <https://doi.org/10.1111/j.1432-1033.1984.tb08438.x>.
- Neumann S, Grosse K, Sourjik V. 2012. Chemotactic signaling via carbohydrate phosphotransferase systems in *Escherichia coli*. *Proc Natl Acad Sci U S A* 109:12159–12164. <https://doi.org/10.1073/pnas.1205307109>.
- Koo BM, Yoon MJ, Lee CR, Nam TW, Choe YJ, Jaffe H, Peterkofsky A, Seok YJ. 2004. A novel fermentation/respiration switch protein regulated by enzyme IIA^{glc} in *Escherichia coli*. *J Biol Chem* 279:31613–31621. <https://doi.org/10.1074/jbc.M405048200>.
- Pickering BS, Smith DR, Watnick PI. 2012. Glucose-specific enzyme IIA has unique binding partners in the *Vibrio cholerae* biofilm. *mBio* 3:e00228–e00212. <https://doi.org/10.1128/mBio.00228-12>.
- Mazé A, Glatte T, Bumann D. 2014. The central metabolism regulator EIIA^{glc} switches *Salmonella* from growth arrest to acute virulence through activation of virulence factor secretion. *Cell Rep* 7:1426–1433. <https://doi.org/10.1016/j.celrep.2014.04.022>.
- Kim YJ, Ryu Y, Koo BM, Lee NY, Chun SJ, Park SJ, Lee KH, Seok YJ. 2010. A mammalian insulysin homolog is regulated by enzyme IIA(Glc) of the glucose transport system in *Vibrio vulnificus*. *FEBS Lett* 584:4537–4544. <https://doi.org/10.1016/j.febslet.2010.10.035>.
- Colwell RR. 1996. Global climate and infectious disease: the cholera paradigm. *Science* 274:2025–2031. <https://doi.org/10.1126/science.274.5295.2025>.
- Lipp EK, Huq A, Colwell RR. 2002. Effects of global climate on infectious disease: the cholera model. *Clin Microbiol Rev* 15:757–770. <https://doi.org/10.1128/CMR.15.4.757-770.2002>.
- Pruzzo C, Vezzulli L, Colwell RR. 2008. Global impact of *Vibrio cholerae* interactions with chitin. *Environ Microbiol* 10:1400–1410. <https://doi.org/10.1111/j.1462-2920.2007.01559.x>.
- Heidelberg JF, Heidelberg KB, Colwell RR. 2002. Bacteria of the gamma-subclass *Proteobacteria* associated with zooplankton in Chesapeake Bay. *Appl Environ Microbiol* 68:5498–5507. <https://doi.org/10.1128/AEM.68.11.5498-5507.2002>.
- Nalin DR, Daya V, Reid A, Levine MM, Cisneros L. 1979. Adsorption and growth of *Vibrio cholerae* on chitin. *Infect Immun* 25:768–770.
- Colwell RR, Huq A, Islam MS, Aziz KM, Yunus M, Khan NH, Mahmud A, Sack RB, Nair GB, Chakraborty J, Sack DA, Russek-Cohen E. 2003. Reduction of cholera in Bangladeshi villages by simple filtration. *Proc Natl Acad Sci U S A* 100:1051–1055. <https://doi.org/10.1073/pnas.0237386100>.
- Meibom KL, Blokesch M, Dolganov NA, Wu CY, Schoolnik GK. 2005. Chitin induces natural competence in *Vibrio cholerae*. *Science* 310:1824–1827. <https://doi.org/10.1126/science.1120096>.
- Meibom KL, Li XB, Nielsen AT, Wu CY, Roseman S, Schoolnik GK. 2004. The *Vibrio cholerae* chitin utilization program. *Proc Natl Acad Sci U S A* 101:2524–2529. <https://doi.org/10.1073/pnas.0308707101>.
- Li X, Roseman S. 2004. The chitinolytic cascade in vibrios is regulated by chitin oligosaccharides and a two-component chitin catabolic sensor/kinase. *Proc Natl Acad Sci U S A* 101:627–631. <https://doi.org/10.1073/pnas.0307645100>.
- Yamamoto S, Mitobe J, Ishikawa T, Wai SN, Ohnishi M, Watanabe H, Izumiya H. 2014. Regulation of natural competence by the orphan two-component system sensor kinase ChiS involves a non-canonical transmembrane regulator in *Vibrio cholerae*. *Mol Microbiol* 91:326–347. <https://doi.org/10.1111/mmi.12462>.
- Cameron AD, Redfield RJ. 2006. Non-canonical CRP sites control competence regulons in *Escherichia coli* and many other gamma-proteobacteria. *Nucleic Acids Res* 34:6001–6014. <https://doi.org/10.1093/nar/gkl734>.
- Johnsborg O, Eldholm V, Havarstein LS. 2007. Natural genetic transformation: prevalence, mechanisms and function. *Res Microbiol* 158:767–778. <https://doi.org/10.1016/j.resmic.2007.09.004>.
- Johnston C, Martin B, Fichant G, Polard P, Claverys JP. 2014. Bacterial transformation: distribution, shared mechanisms and divergent control. *Nat Rev Microbiol* 12:181–196. <https://doi.org/10.1038/nrmicro3199>.
- Karudapuram S, Barcak GJ. 1997. The *Haemophilus influenzae* *dprABC* genes constitute a competence-inducible operon that requires the product of the *tfoX* (*sxy*) gene for transcriptional activation. *J Bacteriol* 179:4815–4820. <https://doi.org/10.1128/jb.179.15.4815-4820.1997>.
- Redfield RJ. 1991. *sxy-1*, a *Haemophilus influenzae* mutation causing greatly enhanced spontaneous competence. *J Bacteriol* 173:5612–5618. <https://doi.org/10.1128/jb.173.18.5612-5618.1991>.
- Williams PM, Bannister LA, Redfield RJ. 1994. The *Haemophilus influenzae* *sxy-1* mutation is in a newly identified gene essential for competence. *J Bacteriol* 176:6789–6794. <https://doi.org/10.1128/jb.176.22.6789-6794.1994>.
- Redfield RJ, Cameron AD, Qian Q, Hinds J, Ali TR, Kroll JS, Langford PR. 2005. A novel CRP-dependent regulon controls expression of competence genes in *Haemophilus influenzae*. *J Mol Biol* 347:735–747. <https://doi.org/10.1016/j.jmb.2005.01.012>.
- Macfadyen LP, Dorocicz IR, Reizer J, Saier MH, Jr, Redfield RJ. 1996. Regulation of competence development and sugar utilization in *Haemophilus influenzae* Rd by a phosphoenolpyruvate:fructose phosphotransferase system. *Mol Microbiol* 21:941–952. <https://doi.org/10.1046/j.1365-2958.1996.441420.x>.
- Antonova ES, Bernardy EE, Hammer BK. 2012. Natural competence in *Vibrio cholerae* is controlled by a nucleoside scavenging response that requires CytR-dependent anti-activation. *Mol Microbiol* 86:1215–1231. <https://doi.org/10.1111/mmi.12054>.
- Blokesch M. 2012. Chitin colonization, chitin degradation and chitin-induced natural competence of *Vibrio cholerae* are subject to catabolite repression. *Environ Microbiol* 14:1898–1912. <https://doi.org/10.1111/j.1462-2920.2011.02689.x>.
- Lo Scrudato M, Blokesch M. 2013. A transcriptional regulator linking quorum sensing and chitin induction to render *Vibrio cholerae* naturally transformable. *Nucleic Acids Res* 41:3644–3658. <https://doi.org/10.1093/nar/gkt041>.
- Wathe SS, Thomas J, Hammer BK. 2015. CytR is a global positive regulator of competence, type VI secretion, and chitinases in *Vibrio cholerae*. *PLoS One* 10:e0138834. <https://doi.org/10.1371/journal.pone.0138834>.

41. Borgeaud S, Metzger LC, Scrignari T, Blokesch M. 2015. The type VI secretion system of *Vibrio cholerae* fosters horizontal gene transfer. *Science* 347: 63–67. <https://doi.org/10.1126/science.1260064>.
42. Yamamoto S, Morita M, Izumiya H, Watanabe H. 2010. Chitin disaccharide (GlcNAc)₂ induces natural competence in *Vibrio cholerae* through transcriptional and translational activation of a positive regulatory gene *tfoX^{VC}*. *Gene* 457:42–49. <https://doi.org/10.1016/j.gene.2010.03.003>.
43. Yamamoto S, Izumiya H, Mitobe J, Morita M, Arakawa E, Ohnishi M, Watanabe H. 2011. Identification of a chitin-induced small RNA that regulates translation of the *tfoX* gene, encoding a positive regulator of natural competence in *Vibrio cholerae*. *J Bacteriol* 193:1953–1965. <https://doi.org/10.1128/JB.01340-10>.
44. Dalia AB, Lazinski DW, Camilli A. 2014. Identification of a membrane-bound transcriptional regulator that links chitin and natural competence in *Vibrio cholerae*. *mBio* 5:e01028–01013. <https://doi.org/10.1128/mBio.01028-13>.
45. Akerley BJ, Rubin EJ, Camilli A, Lampe DJ, Robertson HM, Mekalanos JJ. 1998. Systematic identification of essential genes by in vitro *mariner* mutagenesis. *Proc Natl Acad Sci U S A* 95:8927–8932. <https://doi.org/10.1073/pnas.95.15.8927>.
46. Martin B, Prudhomme M, Alloing G, Granadel C, Claverys JP. 2000. Cross-regulation of competence pheromone production and export in the early control of transformation in *Streptococcus pneumoniae*. *Mol Microbiol* 38: 867–878. <https://doi.org/10.1046/j.1365-2958.2000.02187.x>.
47. Lampe DJ, Akerley BJ, Rubin EJ, Mekalanos JJ, Robertson HM. 1999. Hyperactive transposase mutants of the *Himar1 mariner* transposon. *Proc Natl Acad Sci U S A* 96:11428–11433. <https://doi.org/10.1073/pnas.96.20.11428>.
48. Houot L, Watnick PI. 2008. A novel role for enzyme I of the *Vibrio cholerae* phosphoenolpyruvate phosphotransferase system in regulation of growth in a biofilm. *J Bacteriol* 190:311–320. <https://doi.org/10.1128/JB.01410-07>.
49. Meadow ND, Fox DK, Roseman S. 1990. The bacterial phosphoenolpyruvate: glucose phosphotransferase system. *Annu Rev Biochem* 59:497–542. <https://doi.org/10.1146/annurev.bi.59.070190.002433>.
50. Patel HV, Vyas KA, Savtchenko R, Roseman S. 2006. The monomer/dimer transition of enzyme I of the *Escherichia coli* phosphotransferase system. *J Biol Chem* 281:17570–17578. <https://doi.org/10.1074/jbc.M508965200>.
51. Postma PW, Lengeler JW, Jacobson GR. 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. *Microbiol Rev* 57:543–594.
52. Lo Scrudato M, Borgeaud S, Blokesch M. 2014. Regulatory elements involved in the expression of competence genes in naturally transformable *Vibrio cholerae*. *BMC Microbiol* 14:327. <https://doi.org/10.1186/s12866-014-0327-y>.
53. Lo Scrudato M, Blokesch M. 2012. The regulatory network of natural competence and transformation of *Vibrio cholerae*. *PLoS Genet* 8:e1002778. <https://doi.org/10.1371/journal.pgen.1002778>.
54. Wu R, Zhao M, Li J, Gao H, Kan B, Liang W. 2015. Direct regulation of the natural competence regulator gene *tfoX* by cyclic AMP (cAMP) and cAMP receptor protein (CRP) in vibrios. *Sci Rep* 5:14921. <https://doi.org/10.1038/srep14921>.
55. Takahashi H, Inada T, Postma P, Aiba H. 1998. CRP down-regulates adenylate cyclase activity by reducing the level of phosphorylated IIA^{Glc} (Glc), the glucose-specific phosphotransferase protein, in *Escherichia coli*. *Mol Genet* 259:317–326. <https://doi.org/10.1007/s004380050818>.
56. Nelson SO, Scholte BJ, Postma PW. 1982. Phosphoenolpyruvate:sugar phosphotransferase system-mediated regulation of carbohydrate metabolism in *Salmonella typhimurium*. *J Bacteriol* 150:604–615.
57. Kinoshita E, Kinoshita-Kikuta E, Takiyama K, Koike T. 2006. Phosphate-binding tag, a new tool to visualize phosphorylated proteins. *Mol Cell Proteomics* 5:749–757. <https://doi.org/10.1074/mcp.T500024-MCP200>.
58. Presper KA, Wong CY, Liu L, Meadow ND, Roseman S. 1989. Site-directed mutagenesis of the phosphocarrier protein. III^{Glc}, a major signal-transducing protein in *Escherichia coli*. *Proc Natl Acad Sci U S A* 86: 4052–4055.
59. Hogema BM, Arents JC, Bader R, Eijkemans K, Yoshida H, Takahashi H, Aiba H, Postma PW. 1998. Inducer exclusion in *Escherichia coli* by non-PTS substrates: the role of the PEP to pyruvate ratio in determining the phosphorylation state of enzyme IIA^{Glc}. *Mol Microbiol* 30:487–498. <https://doi.org/10.1046/j.1365-2958.1998.01053.x>.
60. Bettenbrock K, Sauter T, Jahreis K, Kremling A, Lengeler JW, Gilles ED. 2007. Correlation between growth rates, EIACrr phosphorylation, and intracellular cyclic AMP levels in *Escherichia coli* K-12. *J Bacteriol* 189: 6891–6900. <https://doi.org/10.1128/JB.00819-07>.
61. Houot L, Chang S, Absalon C, Watnick PI. 2010. *Vibrio cholerae* phosphoenolpyruvate phosphotransferase system control of carbohydrate transport, biofilm formation, and colonization of the germfree mouse intestine. *Infect Immun* 78:1482–1494. <https://doi.org/10.1128/IAI.01356-09>.
62. Keyhani NO, Roseman S. 1999. Physiological aspects of chitin catabolism in marine bacteria. *Biochim Biophys Acta* 1473:108–122. [https://doi.org/10.1016/S0304-4165\(99\)00172-5](https://doi.org/10.1016/S0304-4165(99)00172-5).
63. Keyhani NO, Roseman S. 1996. The chitin catabolic cascade in the marine bacterium *Vibrio furnissii*. Molecular cloning, isolation, and characterization of a periplasmic beta-N-acetylglucosaminidase. *J Biol Chem* 271: 33425–33432.
64. Leng Y, Vakulskas CA, Zere TR, Pickering BS, Watnick PI, Babsitzke P, Romeo T. 2016. Regulation of CsrB/C sRNA decay by EIIA(Glc) of the phosphoenolpyruvate: carbohydrate phosphotransferase system. *Mol Microbiol* 99:627–639. <https://doi.org/10.1111/mmi.13259>.
65. Dorocicz IR, Williams PM, Redfield RJ. 1993. The *Haemophilus influenzae* adenylate cyclase gene: cloning, sequence, and essential role in competence. *J Bacteriol* 175:7142–7149. <https://doi.org/10.1128/jb.175.22.7142-7149.1993>.
66. Cameron AD, Redfield RJ. 2008. CRP binding and transcription activation at CRP-S sites. *J Mol Biol* 383:313–323. <https://doi.org/10.1016/j.jmb.2008.08.027>.
67. Jaskólska M, Gerdes K. 2015. CRP-dependent positive autoregulation and proteolytic degradation regulate competence activator Sxy of *Escherichia coli*. *Mol Microbiol* 95:833–845. <https://doi.org/10.1111/mmi.12901>.
68. Liang W, Pascual-Montano A, Silva AJ, Benitez JA. 2007. The cyclic AMP receptor protein modulates quorum sensing, motility and multiple genes that affect intestinal colonization in *Vibrio cholerae*. *Microbiology* 153:2964–2975. <https://doi.org/10.1099/mic.0.2007/006668-0>.
69. Cockburn DW, Koropatkin NM. 2016. Polysaccharide degradation by the intestinal microbiota and its influence on human health and disease. *J Mol Biol* 428:3230–3252. <https://doi.org/10.1016/j.jmb.2016.06.021>.
70. Zoetendal EG, Raes J, van den Bogert B, Arumugam M, Booijink CC, Troost FJ, Bork P, Wels M, de Vos WM, Kleerebezem M. 2012. The human small intestinal microbiota is driven by rapid uptake and conversion of simple carbohydrates. *ISME J* 6:1415–1426. <https://doi.org/10.1038/ismej.2011.212>.
71. Sochard MR, Wilson DF, Austin B, Colwell RR. 1979. Bacteria associated with the surface and gut of marine copepods. *Appl Environ Microbiol* 37:750–759.
72. Simidu U, Ashino K, Kaneko E. 1971. Bacterial flora of phyto- and zooplankton in the inshore water of Japan. *Can J Microbiol* 17:1157–1160. <https://doi.org/10.1139/m71-184>.
73. Booijink CC, El-Aidy S, Rajilic-Stojanovic M, Heilig HG, Troost FJ, Smidt H, Kleerebezem M, De Vos WM, Zoetendal EG. 2010. High temporal and inter-individual variation detected in the human ileal microbiota. *Environ Microbiol* 12:3213–3227. <https://doi.org/10.1111/j.1462-2920.2010.02294.x>.
74. Kühn J, Finger F, Bertuzzo E, Borgeaud S, Gatto M, Rinaldo A, Blokesch M. 2014. Glucose- but not rice-based oral rehydration therapy enhances the production of virulence determinants in the human pathogen *Vibrio cholerae*. *PLoS Negl Trop Dis* 8:e3347. <https://doi.org/10.1371/journal.pntd.0003347>.
75. Skorupski K, Taylor RK. 1997. Cyclic AMP and its receptor protein negatively regulate the coordinate expression of cholera toxin and toxin-coregulated pilus in *Vibrio cholerae*. *Proc Natl Acad Sci U S A* 94: 265–270. <https://doi.org/10.1073/pnas.94.1.265>.
76. Houot L, Chang S, Pickering BS, Absalon C, Watnick PI. 2010. The phosphoenolpyruvate phosphotransferase system regulates *Vibrio cholerae* biofilm formation through multiple independent pathways. *J Bacteriol* 192:3055–3067. <https://doi.org/10.1128/JB.00213-10>.
77. Buelow DR, Raivio TL. 2010. Three (and more) component regulatory systems—auxiliary regulators of bacterial histidine kinases. *Mol Microbiol* 75:547–566. <https://doi.org/10.1111/j.1365-2958.2009.06982.x>.
78. Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in networks. *Curr Opin Microbiol* 15:118–124. <https://doi.org/10.1016/j.mib.2011.11.009>.
79. Mitrophanov AY, Groisman EA. 2008. Signal integration in bacterial two-component regulatory systems. *Genes Dev* 22:2601–2611. <https://doi.org/10.1101/gad.1700308>.
80. Snyder H, Kellogg SL, Skarda LM, Little JL, Kristich CJ. 2014. Nutritional

- control of antibiotic resistance via an interface between the phosphotransferase system and a two-component signaling system. *Antimicrob Agents Chemother* 58:957–965. <https://doi.org/10.1128/AAC.01919-13>.
81. Lüttmann D, Göpel Y, Görke B. 2012. The phosphotransferase protein EIIA(Ntr) modulates the phosphate starvation response through interaction with histidine kinase PhoR in *Escherichia coli*. *Mol Microbiol* 86: 96–110. <https://doi.org/10.1111/j.1365-2958.2012.08176.x>.
82. Lüttmann D, Heermann R, Zimmer B, Hillmann A, Rampp IS, Jung K, Görke B. 2009. Stimulation of the potassium sensor KdpD kinase activity by interaction with the phosphotransferase protein IIA(Ntr) in *Escherichia coli*. *Mol Microbiol* 72:978–994. <https://doi.org/10.1111/j.1365-2958.2009.06704.x>.
83. Yamamoto S, Izumiya H, Morita M, Arakawa E, Watanabe H. 2009. Application of lambda Red recombination system to *Vibrio cholerae* genetics: simple methods for inactivation and modification of chromosomal genes. *Gene* 438:57–64. <https://doi.org/10.1016/j.gene.2009.02.015>.
84. Datsenko KA, Wanner BL. 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci U S A* 97:6640–6645. <https://doi.org/10.1073/pnas.120163297>.
85. Uzzau S, Figueroa-Bossi N, Rubino S, Bossi L. 2001. Epitope tagging of chromosomal genes in *Salmonella*. *Proc Natl Acad Sci U S A* 98: 15264–15269. <https://doi.org/10.1073/pnas.261348198>.
86. Morita M, Yamamoto S, Hiyoshi H, Kodama T, Okura M, Arakawa E, Alam M, Ohnishi M, Izumiya H, Watanabe H. 2013. Horizontal gene transfer of a genetic island encoding a type III secretion system distributed in *Vibrio cholerae*. *Microbiol Immunol* 57:334–339. <https://doi.org/10.1111/1348-0421.12039>.
87. van Opijnen T, Lazinski DW, Camilli A. 2015. Genome-wide fitness and genetic interactions determined by Tn-seq, a high-throughput massively parallel sequencing method for microorganisms. *Curr Protoc Microbiol* 36:1E.3.1–1E.3.24. <https://doi.org/10.1002/9780471729259.mc01e03s36>.
88. Akerley BJ, Lampe DJ. 2002. Analysis of gene function in bacterial pathogens by GAMBIT. *Methods Enzymol* 358:100–108. [https://doi.org/10.1016/S0076-6879\(02\)58082-4](https://doi.org/10.1016/S0076-6879(02)58082-4).