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# Evidence for reciprocal evolution of the global repressor MIc and its cognate phosphotransferase system sugar transporter

# Ji-Hee Yoon,<sup>1</sup> Min-Seung Jeon,<sup>2</sup> Seong-il Eyun<sup>2</sup> and Yeong-Jae Seok <sup>(1),3\*</sup>

<sup>1</sup>Department of Biophysics and Chemical Biology, Seoul National University, Seoul, South Korea.

<sup>2</sup>Department of Life Science, Chung-Ang University, Seoul, South Korea.

<sup>3</sup>School of Biological Sciences and Institute of Microbiology, Seoul National University, Seoul, South Korea.

#### Summary

Because the bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system (PTS) is involved in the regulation of various physiological processes in addition to carbohydrate transport, its expression is precisely regulated in response to the availability of PTS sugars. The PTS consists of enzyme I and histidine phosphocarrier protein, and several sugar-specific enzymes II. In Escherichia coli, genes for enzymes II specific for glucose and related sugars are co-regulated by the global repressor MIc, and glucose induction of the MIc regulon genes is achieved by its interaction with glucose-specific enzyme II (Ell<sup>Gic</sup>). In this study, we revealed that, in Vibrio species, which are phylogenetically older than Enterobacteriaceae, the membrane sequestration of MIc and thereby the induction of its regulon genes is mediated by N-acetylglucosamine (NAG)-specific Ell. While Vibrio MIc interacts only with the EIIB domain of EII<sup>Nag</sup>, E. coli MIc interacts with the EIIB domain of both EIIGIC and Ell<sup>Nag</sup>. The present data suggest that Ell<sup>Nag</sup> may be the primordial regulator of MIc, and Ell<sup>Gic</sup> has evolved to interact with MIc since an EIIA domain was fused to EII<sup>Nag</sup> in Enterobacteriaceae. Our findings provide insight into the coevolutionary dynamics between a transcription factor and its cognate regulator according to long-term resource availability in the bacterial natural habitat.

#### Introduction

Several members of the bacterial genus Vibrio are associated with food-borne diseases in humans. Vibrio cholerae is best known as the causative agent of cholera, a severe diarrheal disease resulting from poor water supplies and sanitation (Farugue et al., 1998). Vibrio vulnificus is an opportunistic human pathogen that causes primary septicemia after the ingestion of contaminated seafood or direct wound infection with a mortality rate higher than 50% in immunocompromised patients (Bross et al., 2007). The ingestion of raw or undercooked seafood is also the predominant cause of acute gastroenteritis caused by Vibrio parahaemolyticus (Chowdhury et al., 2000). Vibrios are typically found in salt water such as estuarine and marine environments, where chitin, a polymer of  $\beta$ -1,4-linked *N*-acetylglucosamine (NAG), is the most abundant and important source of nutrients and energy (Yu et al., 1991; Thompson et al., 2011).

All members of the genus Vibrio are chitinivorous (Yu et al., 1993). To efficiently utilize chitin, vibrios secrete chitinase into the extracellular space (Hirano et al., 2019). Chitin oligosaccharides are then degraded by chitinodextrinase and  $\beta$ -N-acetylglucosaminidase to produce chitobiose and NAG monomers (Hunt et al., 2008). Although chitobiose is transported through an ABC transporter, the monomer NAG is transported into the cytoplasm via the phosphoenolpyruvate (PEP) : carbohydrate phosphotransferase system (PTS) (Yu et al., 1991). The PTS is composed of two nonsugar-specific cytoplasmic proteins, enzyme I (EI, encoded by ptsl) and histidine phosphocarrier protein (HPr, encoded by ptsH), and several sugar-specific proteins collectively known as enzymes II (EIIs) (Postma et al., 1993). Ells usually have two cytosolic domains (A and B) and one transmembrane domain (C) harbouring the sugar-binding site, and these domains may be encoded in a single open-reading frame (ORF), as in the case of the NAG-specific EII (EIICBA<sup>Nag</sup> encoded by nagE), or encoded separately in two or more ORFs, as in the case of the glucose-specific EII (EIICBGIc and EIIA<sup>Gic</sup> encoded by *ptsG* and *crr*, respectively), in Escherichia coli. El and HPr mediate the phosphoryl

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transfer from PEP to EIIA and EIIB, which leads to the concomitant phosphorylation of a PTS sugar during its cellular influx through EIIC (Postma *et al.*, 1993).

In addition to sugar transport, the PTS regulates various physiological processes both in Gram-negative and Gram-positive bacteria (Deutscher *et al.*, 2014). In *V. vulnificus* and *V. cholerae*, Crr has been shown to regulate the activity of insulin-degrading enzymes (Kim *et al.*, 2010), fermentation-respiration switch protein FrsA (Lee *et al.*, 2011), flagellar biosynthesis protein FapA (Park *et al.*, 2016; 2019) and c-di-GMP phosphodiesterase PdeS (Heo *et al.*, 2019), whereas HPr was shown to regulate the activity of pyruvate kinase A (Kim *et al.*, 2015; 2018) (Supporting Information Table S1). These regulatory functions of the PTS components are exerted by protein–protein interactions depending on their phosphorylation state, which varies depending on the availability of PTS substrates.

Here, we explored the regulatory function of the NAGspecific PTS transporter NagE (hereafter vEII<sup>Nag</sup>) and showed that in *Vibrio* species, dephosphorylated vEIIB<sup>Nag</sup> interacts with and inactivates the global repressor vMlc. Since it is well established that eMlc interacts with the glucose-specific PTS transporter PtsG (hereafter eEII<sup>Glc</sup>) in *Enterobacteriaceae*, we discuss how the PTS sugar transporter/Mlc pairs could have reciprocally evolved in these two groups of bacteria.

#### Results

## N-acetylglucosamine is consumed faster than glucose in V. vulnificus

Since both Vibrionaceae and Enterobacteriaceae belong to the Gammaproteobacteria class, the PTS components of vibrios have very high amino acid sequence identities with their E. coli orthologs (see Supporting Information -Table S1). One of the most prominent differences in the PTS between Enterobacteriaceae and Vibrio species is that the crr gene product EIIA is shared among EIICB<sup>GIC</sup>, EIICB<sup>Nag</sup> and some other EIIs in Vibrio species, whereas it is specific for EIICB<sup>Glc</sup>, and therefore named EIIA<sup>Glc</sup> in Enterobacteriaceae. While the natural habitat of the members of Enterobacteriaceae is the gastrointestinal tract of warm-blooded animals, where glucose is usually available, that of Vibrio species is known to be the aquatic environment, where NAG is abundant but glucose is scarce. Since Crr is known to be the major player in the hierarchical use of sugars in Gram-negative bacteria (Gorke and Stulke, 2008), we wondered which sugar is the primary substrate for Crr in Vibrio species and whether the primary substrate is related to the most available sugar in the natural habitat of a bacterial species. When E. coli MG1655 and V. vulnificus MO6-24/O strains were incubated in M9-based minimal medium supplemented with 0.04% glucose and 0.04% NAG, *E. coli* consumed glucose faster than NAG, as expected, whereas *V. vulnificus* consumed NAG faster than glucose (Fig. 1A).

In both E, coli and V, cholerae, glucose can be transported by other transporters in addition to the major glucose transporter, EII<sup>Gic</sup> (Ferenci. 1996: Haves et al., 2017). However, NAG is transported only through NagE in V. cholerae, while it is transported through NagE and the mannose PTS (ManXYZ) in E. coli (Jones-Mortimer and Kornberg, 1980; Hayes et al., 2017). Therefore, we examined growth of V, vulnificus nagE and ptsG mutants in M9-based minimal medium supplemented with 0.2% NAG or glucose as the sole carbon source to examine the substrate specificity of PtsG and NagE in V. vulnificus. While the ptsG deletion mutant showed a significantly reduced growth on glucose compared to the wild-type strain, the nagE deletion strain showed a complete growth defect on NAG (Fig. 1B). These data suggest that, while PtsG is the major glucose transporter. NagE is the only transporter of NAG in V. vulnificus, as in V. cholerae, and that the favoured sugar, which has a faster consumption rate, could have been selected depending on the most abundant carbon source in the natural environment of a bacterial species over a long period.

#### vMlc specifically interacts with vEIIB<sup>Nag</sup> in V. vulnificus

NAG has been shown to influence a variety of cellular processes, including regulation of genes required for degradation, and utilization of chitin, energy metabolism, competence, biofilm formation and host colonization of vibrios (Meibom et al., 2004; Ghosh et al., 2011; Neiman et al., 2011; Thompson et al., 2011). These lines of evidence led us to reason that the NAG-specific PTS transporter could be directly implicated in some of these physiological role(s) in addition to NAG transport. Unlike E. coli NagE, which carries its own eEIIA domain at the C-terminus of eEIICB<sup>Nag</sup>, the NagE of vibrios has only the EIIB domain at the C-terminus of the vEIIC domain, like PtsG. In E. coli, the EIIB domain of PtsG was shown to be sufficient to interact with Mlc (Nam et al., 2001). Therefore, we decided to search for proteins interacting with the vEIIB domain of NagE in V. vulnificus by employing the ligand-fishing approach (Kim et al., 2015; Park et al., 2016; Heo et al., 2019). The crude extract of MO6-24/O cells was mixed with Talon<sup>™</sup> metal affinity resin (Takara Bio) and vEIIB<sup>Nag</sup> with an N-terminal His<sub>6</sub> tag (His-vEIIB<sup>Nag</sup>, residues from 425 to 524) or HisvEIIB<sup>Gic</sup> (residues from 387 to 477) as a control in the presence of PEP to phosphorylate or pyruvate to dephosphorylate EIIB before being subjected to a pull-down



**Fig 1.** Distinct carbohydrate preference between *Escherichia coli* and *Vibrio vulnificus*. A. *E. coli* MG1655 and *V. vulnificus* MO6-24/O were grown in M9-based minimal medium supplemented with 0.04% glucose and 0.04% NAG. Growth rates (optical density at 600 nm) and the concentration of sugars remaining in the medium were measured as a function of incubation time as described in 'Experimental Procedures' section.

B. Wild-type and indicated mutant strains of V. vulnificus MO6-24/O were inoculated into a 96-well plate containing M9S medium supplemented with 0.2% glucose or NAG and cultivated at 30°C using a Spark<sup>®</sup> multimode microplate reader (TECAN Group Ltd). Error bars represent the standard error of the mean (SEM) of three independent experiments.

assay. While we could not detect any protein interacting with vEIIB<sup>Gic</sup> regardless of its phosphorylation state, we found a protein band migrating at  $\sim$ 50 kDa co-eluting only with dephosphorylated vEIIB<sup>Nag</sup> but not with its phosphorylated form (Figs. 2A S1A). After in-gel trypsin digestion and peptide mass fingerprinting, the protein band was identified as VVMO6 01928 (hereafter vMlc), which is an ortholog of E. coli Mlc (eMlc). vMlc shares 47% amino acid sequence identity with eMlc. In E. coli, eMlc represses not only its own expression, but also that of the ptsHlcrr operon and of a number of genes encoding transporters for glucose and related sugars such as Ell<sup>Gic</sup>, Ell<sup>Man</sup> and MalT (Decker et al., 1998; Kimata et al., 1998; Plumbridge, 1998a; 1998b; 1999; Kim et al., 1999; Tanaka et al., 1999). The repressor activity of eMIc is disabled by binding to the dephosphorvlated eEIIB domain of eEII<sup>Gic</sup> in the membrane (Lee et al., 2000; Tanaka et al., 2000; Nam et al., 2001).

To verify the specific interaction between vEIIB<sup>Nag</sup> and vMlc and to confirm that the interaction is affected by the phosphorylation state of vEIIB<sup>Nag</sup>, we first performed pulldown assays with purified vMlc in the presence of vEI, vHPr and vCrr with and without PEP. Direct interaction of vMlc with vEIIB<sup>Nag</sup> was observed in the absence but not in the presence of PEP, indicating that only the dephosphorylated form of vEIIB<sup>Nag</sup> can interact with vMlc (Fig. 2B). We then tested whether the interaction between vMIc and vEIIB<sup>Nag</sup> occurs in vivo by the bacterial two-hybrid (BACTH) assay (Karimova et al., 1998). When protein-protein interactions were measured in the E. coli BTH101 strain co-transformed with both plasmids carrying T18 and T25 fusions to vMlc, vEIIB<sup>Nag</sup> (residues from 427 to 524) and vEIIB<sup>GIc</sup> (residues from 387 to 477) we found a significant interaction between vMIc and vEIIB<sup>Nag</sup> in both reciprocal combinations (Fig. 2C), but no interaction between vMlc and vEIIB<sup>Glc</sup> (Supporting Information Fig. S1B). Interestingly, we observed much higher levels of  $\beta$ -galactosidase activity only when the gene encoding vEIIB<sup>Nag</sup> in the fusion plasmids was mutated to non-phosphorylatable form а (vEIIB<sup>Nag</sup>(C458A)) (Meins et al., 1993) in the BACTH assay. Because PTS components are known to exist predominantly in the phosphorylated form in cells growing in LB medium used for the BACTH assay (Park et al., 2013; 2016), our data indicate that the vMlc-vEIIB<sup>Nag</sup> interaction is specific and dependent on the phosphorylation state of vEIIB<sup>Nag</sup> in vivo as well as in vitro.

We then examined whether full-length  $vEII^{Nag}$  interacts with and sequesters vMIc to the membrane depending on



Fig 2. Specific interaction between dephospho-vEIIB<sup>Nag</sup> and vMIc in Vibrio vulnificus.

A. Ligand fishing experiment was carried out to search for protein(s) interacting with the vEIIB<sup>Nag</sup> domain (residues 425–524) as described in 'Experimental Procedures' section. A cell-free extract of *V. vulnificus* MO6-24/O was mixed with buffer A (lane C) or 400 μg of purified HisvEIIB<sup>Nag</sup>. The mixture containing His-vEIIB<sup>Nag</sup> was supplemented with either 2 mM PEP to phosphorylate vEIIB<sup>Nag</sup> (lane PEP) or 2 mM pyruvate to dephosphorylate vEIIB<sup>Nag</sup> (lane Pyr). Each mixture was subjected to TALON metal affinity chromatography, and proteins bound to the column were analysed by SDS-PAGE using a 4% to 20% gradient gel (KOMA Biotech) and staining with Coomassie brilliant blue. EzWay Protein Blue MW Marker (KOMA Biotech) was used as the molecular mass marker (lane M).

B. vMIc (90 μg) was mixed with vEI (2 μg), vHPr (6 μg) and vCrr (4 μg), and buffer A (lane C) or His-vEIIB<sup>Nag</sup> (150 μg, lane B) was added in the presence and absence of 2 mM PEP. The mixtures were then subjected to TALON metal affinity chromatography as in panel A. PageRulerTM unstained protein ladder (Therimo Scientific) was used as the molecular marker (lane M).

C. BACTH assays to measure the phosphorylation state-dependent interaction of vEIIB<sup>Nag</sup> with vMlc in vivo. *E. coli* BTH101 was transformed with a pair of plasmids encoding T25 and T18 fragments of *Bordetella pertussis* adenylate cyclase fused to *V. vulnificus* vMlc or the vEIIB<sup>Nag</sup> domain (residues 427–524). Zip, the leucine zipper domain of *Saccharomyces cerevisiae* GCN4, served as a positive control. After growth in LB medium overnight,  $\beta$ -galactosidase activities were measured as described in 'Experimental Procedures' section. Error bars represent the SEM of three independent experiments. Statistical significance was determined using Student's *t* test (\*\*\**p* < 0.0005). D. Direct interaction between the dephospho-form of full-length vEII<sup>Nag</sup> and vMlc. Membrane vesicles in which vEII<sup>Nag</sup> is enriched (lane vEII<sup>Nag</sup>)

D. Direct interaction between the dephospho-form of full-length vEII<sup>Nag</sup> and vMlc. Membrane vesicles in which vEII<sup>Nag</sup> is enriched (lane vEII<sup>Nag</sup>) or lacking (lane –vEII<sup>Nag</sup>) were mixed with vMlc and soluble PTS proteins (vEI, vHPr and vCrr) in the presence of 2 mM PEP or 2 mM NAG as indicated. After incubation for 10 min, membrane vesicles and bound proteins were precipitated by centrifugation at 100,000*g* for 30 min and the supernatants were analysed by SDS-PAGE and staining with Coomassie brilliant blue. EzWay Protein Blue MW Marker (KOMA Biotech) was used as the molecular mass marker (lane M). vMlc band intensity is indicated in relative values at the bottom of each lane.

its phosphorylation state, as does eEII<sup>Gic</sup> (Lee *et al.*, 2000; Tanaka *et al.*, 2000; Nam *et al.*, 2001). vEII<sup>Nag</sup>-depleted and -enriched membrane vesicles were prepared from *E. coli* ER2566 transformed with pET and pET-vEII<sup>Nag</sup> plasmids, respectively, as described

previously, and we explored the interaction between vMlc and vEII<sup>Nag</sup> in the form of membrane vesicles in the presence of vEI, vHPr and vCrr with and without PEP (Nam *et al.*, 2001). After membrane vesicles and bound proteins were sedimented, proteins remaining in the

supernatants were analysed (Fig. 2D). As expected, the amounts of the three soluble PTS components in the supernatants were not changed regardless of their phosphorylation state and the presence of vEII<sup>Nag</sup>. However, whereas the presence of vEII<sup>Nag</sup> did not affect the amount of vMIc remaining in the supernatants in the reaction mixtures containing PEP, the presence of vEII<sup>Nag</sup> significantly decreased the amount of free vMlc in the reaction mixtures containing NAG (Fig. 2D), indicating that only dephosphorylated vEII<sup>Nag</sup> sequesters vMIc to the membrane. In addition, we prepared vEII<sup>Gic</sup>-enriched membrane vesicles and performed an interaction test with vMIc (Supporting Information Fig. S1C). The presence of vEll<sup>Gic</sup> did not affect the amount of vMlc remaining in the supernatants regardless of its phosphorvlation state. Thus, we confirmed that there is no direct interaction between vEII<sup>GIc</sup> and vMIc in V. vulnificus. The dissociation constant ( $K_D$ ) for the vMIc-vEIIB<sup>Nag</sup> interaction was determined to be approximately  $4.48 \times 10^{-8}$  M (Supporting Information Fig. S2), assuming a 1:1 interaction by employing surface plasmon resonance technology (Seok et al., 1997), which is approximately 10-fold higher than the  $K_{\rm D}$  for the eMlc-eEIIB<sup>Glc</sup> interaction in *E. coli* (Nam et al., 2008).

#### vMlc binds to specific sequences to repress expression of its regulon in the absence of NAG

Because the DNA binding domain (residues 1-81) of vMlc has 71% amino acid sequence identity with that of eMlc, we assumed that vMlc may recognize similar binding sequences and control the transcription of similar genes with eMIc. Since it was reported that eMIc binds to promoter regions of ptsG, ptsH, manX, malT and mlc genes in E. coli (Decker et al., 1998; Kimata et al., 1998; Plumbridge, 1998a; 1998b; 1999; Kim et al., 1999; Tanaka et al., 1999), we attempted to confirm the binding of vMlc to these genes and nagE, but excluding manX which is absent in V. vulnificus, by electrophoretic mobility shift assay (EMSA). Our data show that vMIc binds to the promoters of mlc, ptsH, ptsG and nagE, but not to the promoter of malT, in V. vulnificus (Fig. 3A). We then searched for putative binding sites of vMlc in nagE, ptsG, ptsH and mlc promoters using the consensus binding sequence of eMlc (Decker et al., 1998; Kimata et al., 1998; Plumbridge 1998a; 1998b; 1999; Kim et al., 1999; Tanaka et al., 1999). As in E. coli, we found two putative vMlc-binding sequences in the ptsG promoter and one each in the ptsH and mlc promoters in V. vulnificus. The V. vulnificus nagE promoter also appeared to have two vMlc-binding sites (Supporting Information Fig. S3A). To determine the precise vMlcbinding sites, we performed DNase I footprinting experiments with 6-carboxyfluorescein (6-FAM)-labelled nagE,

ptsG and ptsH promoter probes. As expected from in silico analyses, the DNA sequences corresponding exactly to the predicted vMlc-binding sites were protected by vMlc from DNase I digestion in these promoter DNA probes (Supporting Information Fig. S3B). When we aligned all vMlc-binding sequences, a consensus sequence could be deduced (Supporting Information Fig. S3C), which is similar to the consensus binding sequence of eMlc in E. coli (Decker et al., 1998; Kimata et al., 1998; Plumbridge, 1998a; 1998b; 1999; Kim et al., 1999; Tanaka et al., 1999). Because the regulatory effect of a transcription factor is usually determined by the location of its binding site(s) relative to the transcriptional start site (TSS), TSSs were identified by 5'-rapid amplification of cDNA ends (5'-RACE) experiments. The centres of the vMlc-binding sites were located at -121 and -28 of the nagE promoter, -164 and -2 of the *ptsG* promoter, +14of the *ptsH* promoter and -4 of the *mlc* promoter, relative to each TSS (Supporting Information Fig. S3A). Therefore, we assumed that binding of vMlc near the TSS might decrease transcription, as is usually observed in E. coli (Collado-Vides et al., 1991). To confirm whether vMlc inhibits the transcription of genes, we compared the relative expression levels of the vMlc regulon genes in the wild-type and  $\Delta mlc$  strains harbouring either the empty plasmid pJK1113 (Lim et al., 2014) or the vMlc expression vector pJK-vMlc (Supporting Information -Table S2). As expected, the mlc mutant carrying pJK1113 showed four to eight times increased expression levels of ptsG, ptsH and nagE genes compared to the wild-type strain, whereas the same strain carrying pJK-vMlc showed similar transcript levels to the wild-type strain (Fig. 3B). These data indicate that vMIc represses the expression of its regulon by binding to specific target sites, as does eMlc. In E. coli, transcription of nagE is repressed by eNagC and the specific inducer of eNagC is NAG-6-phosphate, which is produced from NAG during translocation through nagE-encoded eEII<sup>Nag</sup> in the membrane (Plumbridge, 1991). Despite the fact that eNagC also binds to ptsG and ptsH promoters in vitro, a nagC mutation had no effect on the expression of ptsG or ptsH in E. coli (Plumbridge, 1999). Therefore, we examined the effect of *nagC* deletion on the expression of *nagE*, ptsG and ptsH, before we investigate the inducibility of the vMlc regulon by NAG in V. vulnificus. We compared the expression levels of nagE, ptsG and ptsH in the wild type with those in the nagC mutant grown on glycerol (Fig. 3C). It should be noted that the PTS components exist mostly in the dephosphorylated forms in the presence of a favoured PTS carbohydrate such as NAG, whereas they are mostly phosphorylated in the presence of a gluconeogenic carbon source such as glycerol (Hogema et al., 1998; Nam et al., 2005; Park et al., 2016). The expression of nagE was dramatically



Fig 3. NAG induces repression of vMlc regulon expression in Vibrio vulnificus.

A. vMlc binding to the promoter regions of PTS-related genes was examined with DNA fragments (approximately -280 to 0 bp relative to the translation start codon) amplified by PCR using gene-specific primer pairs (Supporting Information Table S4). After DNA fragments (50 ng each) were incubated with 180 ng of vMlc in binding buffer B, EMSA was performed as described in 'Experimental Procedures' section. The molar ratio between DNA and vMlc was approximately 1:15.

B. Total RNAs were isolated from the wild-type and *mlc* deletion mutant strains harbouring either pJK1113 (pJK) or pJK1113·vMlc (pMlc) grown to mid-exponential phase in LBS medium, and relative expression levels of the vMlc regulon genes were determined by qRT-PCR.

C. Total RNAs were isolated from the wild-type (black bars) and *nagC* deletion mutant (grey bars) strains grown to mid-exponential phase in M9S medium containing 0.4% glycerol and the expression levels of *nagE*, *ptsG* and *ptsH* were quantified by qRT-PCR.

D. Total RNAs were isolated from the *nagE* deletion mutant harbouring pJK1113 or pJK1113-vNagE (pNagE) grown to mid-exponential phase in M9S medium containing 0.4% glycerol (black bars) or 0.2% NAG (red bars) and the transcript level of *ptsH* was determined by qRT-PCR. Error bars denote standard deviations from three measurements. Statistical significance was assessed using Student's *t*-test (\*\*\*p < 0.0005, \*\*p < 0.005 and \*p < 0.05, and p values higher than 0.05 were presented).

increased, whereas that of *ptsG* was significantly decreased, in the nagC mutant compared to the wild-type strain. However, the effect of nagC deletion on ptsH expression was not statistically significant. As in E. coli (Plumbridge, 1998b, 2001; Shin et al., 2003; Jeong et al., 2004; Zheng et al., 2004; Rungrassamee et al., 2008), expression of ptsG appears to be regulated by several transcription factors in addition to NagC and Mlc in V. vulnificus. Therefore, we chose to examine whether NAG can induce ptsH expression solely by dephosphorylating Ell<sup>Nag</sup>, independently of vNagC. To this end, we examined the expression level of ptsH in a nagE mutant of V. vulnificus harbouring the empty vector pJK1113, or the vector carrying wild-type nagE (pNagE) grown on glycerol and NAG. ptsH expression was remarkably increased in response to NAG in the nagE mutant harbouring pNagE, whereas it was not induced by NAG in the same strain carrying pJK1113 (Fig. 3D). In addition, the relative expression levels of ptsH were determined in the wild-type,  $\Delta nagC$  and  $\Delta nagC$   $\Delta nagE$ mutant strains grown on either 0.4% glycerol or 0.2% NAG (Supporting Information Fig. S4). The expression of *ptsH* was found to increase in response to NAG in both the wild-type and *nagC*-deletion strains. When *nagE* was deleted in the *nagC*-deficient strain, we could not observe the expression of *ptsH*, even in the presence of NAG. These data suggest that vMlc represses the expression of its regulon in the absence of NAG but vEII<sup>Nag</sup> antagonizes the repression of the vMlc regulon depending on its phosphorylation state in response to the availability of NAG in *V. vulnificus*.

# vEll<sup>Nag</sup> displaces vMlc from its target promoters to induce expression of its regulon

To determine whether dephosphorylated vEII<sup>Nag</sup> induces the vMlc regulon by interacting with and displacing vMlc from its target DNA in *V. vulnificus* as does  $eEII^{Glc}$  in *E. coli* (Lee *et al.*, 2000; Tanaka *et al.*, 2000; Nam *et al.*, 2001), the effect of vEII<sup>Nag</sup> on the binding of vMlc to its target sites on the *nagE*, *ptsG* and *ptsH* promoters

was examined by EMSA and in vitro transcription assays. The addition of purified vEII<sup>Nag</sup> to the reaction mixture inhibited the binding of vMlc to all the three promoters in EMSA assays (Fig. 4A). Displacement of vMlc by vEII<sup>Nag</sup> from its target site was also verified by in vitro transcription assays using a supercoiled plasmid containing the nagE or ptsG promoter of V. vulnificus as template (Fig. 4B). We observed that both the ptsG and nagE transcripts are expressed only in the presence of cAMP/ CRP, which is in accordance with previous reports showing that *ptsG* expression is absolutely dependent on cAMP/CRP in E. coli (Kimata et al., 1997; Jeong et al., 2004). As expected, vMlc decreased the cAMP/ CRP-induced transcription of both nagE and ptsG but did not affect transcription from the plasmid origin of replication (rep), indicating the specificity of the repression by vMlc. Interestingly, vEII<sup>Nag</sup> relieved (at least partially) vMlc-mediated repression of transcription from the nagE and *ptsG* promoters. Taken together, these data indicate that the interaction of vEII<sup>Nag</sup> with vMlc results in sequestration of the repressor from its binding sites on the target promoters, thereby inducing expression of its regulon genes in response to the availability of the PTS substrate, NAG.

### Evolutionary implications of the interaction between Mlc and its cognate sugar transporter

Our data show that the regulatory mechanism of MIc is highly conserved between *Vibrionaceae* and *Enterobacteriaceae* except for the cognate PTS sugar transporter regulating its activity. Therefore, we next sought to understand why the regulator of MIc is different between the two groups of bacteria. Unlike *E. coli* EII<sup>Nag</sup> (eEII<sup>Nag</sup>), which consists of C, B and A domains (thus eEIICBA<sup>Nag</sup>), *V. vulnificus* EII<sup>Nag</sup> (vEII<sup>Nag</sup>) is devoid of the

Fig 4. Displacement of vMlc from its target DNA by vEll^{Nag} to induce transcription of target genes.

A. Inhibition of vMic binding to its target DNA by vEII<sup>Nag</sup>. Promoter DNA fragments (the same as in Fig. 3a; 100 ng each) were mixed with 400 ng of vMic and varying amounts of vEII<sup>Nag</sup> (0, 540 and 1600 ng) in the binding buffer B, and EMSA was performed as described in 'Experimental Procedures' section. The molar ratio between DNA and vMic was approximately 1:16.

B. Induction of *nagE* and *ptsG* transcription by vEII<sup>Nag</sup> in vitro. The *nagE* and ptsG promoters on the supercoiled plasmids (Supporting Information Table S2) were used for in vitro transcription assays as describes in 'Experimental Procedures' section. An 110-nucleotide transcript from nagE and а 75-nucleotide transcript from ptsG promoters are indicated by arrows. An 150-nucleotide rep transcript from the plasmid origin of replication provided an internal control. The transcript intensity was quantified by densitometric analysis using ImageJ software (http://rsb.info.nih.gov/ij/ download.html).



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A domain (thus vEIICB<sup>Nag</sup>), whereas both eEII<sup>GIc</sup> and vEII<sup>Gic</sup> consist of C and B domains. Therefore, vEII<sup>Gic</sup> and vEll<sup>Nag</sup> share vCrr in all Vibrio species including V. vulnificus (Houot et al., 2010). Since it has been established that eEII<sup>Gic</sup> regulates the activity of eMIc in Enterobacteriaceae and BLAST searches revealed that V. vulnificus vMlc and the vEIIB<sup>Glc</sup> domain have overall amino acid sequence identities of 47% and 77%, respectively, with their E. coli orthologs, we investigated the possibility of an interaction between vEIIB<sup>Glc</sup> and vMlc. Pull-down assays using a cell-free extract of E. coli expressing vMIc and purified His-vEIIB<sup>Nag</sup> and HisvEIIB<sup>Gic</sup> revealed that vMic specifically interacts with vEIIB<sup>Nag</sup> but not with vEIIB<sup>GIc</sup> (Fig. 5A). We then wondered whether vMlc interacts with the B domains of eEII<sup>Nag</sup> (residues from 373 to 468) and eEII<sup>Gic</sup> (residues from 391 to 480) since V. vulnificus vEIIB<sup>Nag</sup> and vEIIB<sup>Glc</sup> domains have amino acid sequence identities of 50% and 77%, respectively, with their E. coli orthologs.

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Interestingly, pull-down assays revealed that vMIc interacts with eEIIB<sup>Nag</sup>, but not eEIIB<sup>Glc</sup>. Since MIc and PTS components are highly conserved among vibrios, we assumed that the specific interaction of vMIc with vEII<sup>Nag</sup> could be conserved in other Vibrio species. Pull-down assavs with a cell-free extract of E. coli expressing V. cholerae Mlc (vcMlc) using purified His-vcEIIB<sup>Nag</sup> (residues from 428 to 524) and His-vcEIIB<sup>Gic</sup> (residues from 412 to 501) as bait revealed that vcMlc also interacts with vcEll<sup>Nag</sup>. vcEll<sup>Glc</sup> but not with (Supporting Information Fig. S5), indicating that the interaction between MIc and EII<sup>Nag</sup> is widespread among Vibrionaceae species.

Since *Vibrionaceae* is phylogenetically older than *Enterobacteriaceae* (Williams *et al.*, 2010), we postulated that the interaction between vMIc and vEIIB<sup>Nag</sup> could have a longer evolutionary history than that between eMIc and eEII<sup>Gic</sup>. Therefore, the question arose as to whether this trait remains in *Enterobacteriaceae*. To this



Fig 5. Analysis of the cross-species interaction between MIcs and EIIB domains from E. coli and Vibrio vulnificus.

A,B. A cell-free extract of *E. coli* expressing vMic (A) or eMic (B) was mixed with buffer A (lane C) or the His-tagged form of the indicated EIIB domain from *E. coli* (eEIIB<sup>Nag</sup> domain, residues 373–468); and eEIIB<sup>Gic</sup> domain, residues 391–480) or *V. vulnificus* (vEIIB<sup>Gic</sup> domain, residues 387–477) (100 μg each) and subjected to TALON metal affinity chromatography. Proteins bound to each column were analysed by 4% to 20% gradient SDS-PAGE and staining with Coomassie brilliant blue. PageRulerTM unstained protein ladder (Thermo Scientific) was used as the molecular marker (lane M).

C. BACTH assays to test the interaction of eMIc with the full-length eEIICBA<sup>Nag</sup> and the eEIICB<sup>Nag</sup> mutant (residues 1–468) *in vivo. E. coli* BTH101 was transformed with a pair of plasmids encoding T18 and T25 fragments of *Bordetella pertussis* adenylate cyclase fused to eMIc and either of the two forms of eEII<sup>Nag</sup> respectively. The pair of T18-eMIc and T25-eEII<sup>Gic</sup> fusions served as a positive control. After growth in LB medium overnight,  $\beta$ -galactosidase activities were measured as described in 'Experimental Procedures' section. The mean and standard deviation of five independent measurements are shown. Statistical significance was determined using Student's *t*-test (\*\*\**p* < 0.0005, \*\**p* < 0.005 and \**p* < 0.05).

end, we examined whether eMIc interacts with the EIIB<sup>Nag</sup> domain from *E. coli* and *V. vulnificus*. Notably, eMlc interacted with vEIIB<sup>Nag</sup> as well as eEIIB<sup>Glc</sup> (Fig. 5B). Since the eEIIB<sup>Nag</sup> domain exists between the C and A domains in eEII<sup>Nag</sup>, we therefore constructed an expression vector for the His-tagged form of the B domain (residues from 372 to 468) of eEll<sup>Nag</sup> to test its interaction with eMlc. Interestingly, eMlc interacted with eEIIB<sup>Nag</sup> as well as vEIIB<sup>Nag</sup> (Fig. 5B). Therefore, eMIc interacts with both EIIB<sup>Glc</sup> and EIIB<sup>Nag</sup> domains, whereas vMIc interacts only with the EIIB<sup>Nag</sup> domain. The possibility of the interaction between eEII<sup>Nag</sup> and vcMlc in E. coli was previously raised by the observation that when vcMlc was expressed in an E. coli mlc nagC double mutant, repression of ptsG by vcMlc was partially relieved by growth on NAG (Brechemier-Baev et al., 2015). The binding of eMIc to eEIIB<sup>Nag</sup> led us to ask whether E. coli evolved to change the cognate MIc regulator from EII<sup>Nag</sup> to Ell<sup>Gic</sup> since the A domain was fused to the C-terminal end of EII<sup>Nag</sup>. Therefore, we examined whether the presence of the A domain hinders the interaction between eMIc and eEII<sup>Nag</sup> in *E. coli*. If this is true, eEII<sup>Nag</sup> might recover the capability to interact with eMIc when the A domain is removed. To test this hypothesis, we examined the interaction of full-length eEll<sup>Nag</sup> with eMlc in vivo by the BACTH assay. We found a slight but statistically significant increase in the  $\beta$ -galactosidase activity in the strain carrying the plasmid pair of the full-length eEll<sup>Nag</sup> and eMIc compared to the cell expressing eMIc alone. However, the strain carrying the plasmid pair of the A domain-truncated eEII<sup>Nag</sup> (residues from 1 to 468) and eMlc gave a much higher  $\beta$ -galactosidase activity than the strain carrying the plasmid pair of the full-length eEll<sup>Nag</sup> and eMlc (Fig. 5C), suggesting that the vMlcvEII<sup>Nag</sup> interaction may have a longer evolutionary history than the eMIc-eEII<sup>GIc</sup> interaction.

Finally, although the data in Fig. 1B shows that vEII<sup>Nag</sup> is the only transporter for NAG, a question remains whether vEII<sup>Nag</sup> could transport other sugars such as glucose in addition to NAG, since some Ells show broad substrate specificity. For example, the mannose PTS is the only effective transport system for mannose, but it can also transport NAG, glucosamine, glucose and fructose in enteric bacteria (Stock et al., 1982; Postma et al., 1993). It was previously shown that the mobility of Crr in an SDS-PAGE gel decreases when it is phosphorylated at its active site histidine (Hogema et al., 1998; Lee et al., 2019). Therefore, we employed a phosphorylation-dependent mobility shift assay to assess the substrate specificity of vEII<sup>Nag</sup>. When vEII<sup>Nag</sup> was incubated with vEI, vHPr, vCrr, PEP and various carbohydrates, vCrr showed increased mobility only in the mixture containing NAG (Supporting Information Fig. S6), indicating that NAG is the only substrate of vEII<sup>Nag</sup>.

Altogether, our data suggest that the global repressor MIc and its cognate PTS sugar transporter could have reciprocally evolved to make optimum use of accessible nutrients in *Vibrionaceae* and *Enterobacteriaceae*.

#### Discussion

The bacterial PTS, one of the signal transduction systems responding to nutritional changes in the environment, is a structurally and functionally complex system with a surprising evolutionary history (Saier et al., 2005). In this study, we show that, although the amino acid sequences of PTS components and MIc and the regulatory mechanism of MIc are highly conserved, the cognate PTS transporters regulating MIc are differentially evolved between the orders Vibrionales and Enterobacterales. We then wondered how the regulators of MIc were differentially evolved in the two closely related groups of bacteria. Several lines of evidence led us to postulate that the primordial regulator of MIc could be EII<sup>Nag</sup> (Fig. 6). First, ocean-dwelling Vibrionales are phylogenetically older than Enterobacterales dwelling in the mammalian gut (Williams et al., 2010). Second, slow-evolving organisms tend to retain a primordial signalling system, whereas fast-evolving organisms may modify the system extensively (Saier, 1977). The ocean has been a stable environment since the Earth's infancy, so not much selective pressure for evolutionary change would have been exerted on ocean-dwelling bacteria. Finally, more direct evidence comes from the biochemical data in this study. Although the interaction between full-length eEII<sup>Nag</sup> and eMIc was very weak, the isolated eEIIB<sup>Nag</sup> domain and the EIIA domain-deleted form of eEII<sup>Nag</sup> (i.e., eEIICB<sup>Nag</sup>) showed a high affinity interaction with eMlc (Fig. 5). These data suggest that eEII<sup>Nag</sup> might have lost the ability to interact with eMIc since eEII<sup>Nag</sup> was fused with the eEIIA domain in E. coli (Fig. 6). Because eMIc interacts with both eEIIB<sup>Nag</sup> and eEIIB<sup>GIc</sup>, while vMlc interacted only with vEIIB<sup>Nag</sup> (Fig. 5), it is likely that eMIc acquired the capability to interact with eEIIB<sup>GIc</sup> as well as eEIIB<sup>Nag</sup> through accumulated mutations in E. coli (Fig. 6).

Phylogenetic analysis also supports our assumption. mostly conserved in the class MIc is Gammaproteobacteria. In Gammaproteobacteria, the orders Enterobacterales and Pasteurellales diverged from each other after their common ancestor had diverged from the order Vibrionales. An analysis of amino acid sequences of Ell<sup>Nag</sup> in all genome-announced genera of the orders Aeromonadales, Enterobacterales, Vibrionales Pasteurellales and of the class Gammaproteobacteria shows that an EIIA domain is fused to Ell<sup>Nag</sup> only in the order Enterobacterales (Supporting Information Table S3). Therefore, it can be



**Fig 6.** A hypothesis for the reciprocal evolution of MIc and its cognate PTS sugar transporter. It has been proposed that the eubacterial PTS evolved from a much simpler genetic system (Saier, 1977). Exposure of an evolving bacterium to increasing numbers of carbohydrates might have acted as selective pressure to increase the number of sugar-specific EIIs by gene duplication. In the meanwhile, the habitat shift from the ocean to the terrestrial environment could have led to speciation. Furthermore, switching from NAG to glucose as the most available carbohydrate might have led to the reciprocal evolution of MIc and its cognate PTS sugar transporter. We hypothesize that vEII<sup>Nag</sup> is the primordial MIc regulator as the case in *Vibrionaceae* which dwell in the ocean where NAG has long been the most abundant carbohydrate. However, eMIc and eEII<sup>GIc</sup> have evolved to interact with each other in *Enterobacteriaceae* since the Crreencoding gene was duplicated and added to the C-terminus of eEII<sup>Nag</sup>. Therefore, eEII<sup>Nag</sup> lost the ability to interact with eMIc. This evolution event could be related to the change in the most frequently encountered carbohydrate from NAG to glucose since *Enterobacteriaceae* species started colonizing the intestines of warm-blooded animals.

assumed that the EIIA domain was fused to the Cterminus of EII<sup>Nag</sup> after *Enterobacterales* diverged from the order *Pasteurellales*. A comparative analysis of amino acid sequences of Mlc, EIIB<sup>Glc</sup> and EIIB<sup>Nag</sup> domains in representative species for each order shows that EIIB<sup>Glc</sup> is most conserved whereas Mlc is least conserved (Supporting Information Fig. S7), suggesting that Mlc has undergone the most evolution out of the three proteins among species in the three orders. We postulate that the fusion of an EIIA domain to the Nag-specific EII might have decreased the binding affinity of Mlc and therefore Mlc might have been evolved to interact with EII<sup>Glc</sup> in the order *Enterobacterales*. Therefore, all lines of evidence suggest the reciprocal evolution of EII<sup>Nag</sup> and EII<sup>Glc</sup> and Mlc in *Enterobacteriaceae*.

Although glucose is the most preferred carbohydrate for most organisms (Gorke and Stulke, 2008), our data show different sugar consumption rate between two closely related groups of bacteria. We then wondered why the favoured sugar became to serve as a signal to regulate the expression of genes encoding transporters for other related sugars. It has been proposed that the PTS evolved from a much simpler genetic system consisting of soluble components and a membrane-bound sugar-specific component (Saier, 1977). Exposure of an evolving bacterium to increasing numbers of carbohydrates might have acted as selective pressure for an increased spectrum of transport capabilities. Therefore, the number of genes for sugar-specific components carrying the same operator should have increased through gene duplication and divergence, thus expanding the substrate repertoire (Fig. 6).

We postulate that Crr could have been a general PTS component for several reasons. First, it is encoded in an operon together with EI and HPr in diverse groups of bacteria. Second, Crr is not homologous to EIIA domains for other PTS sugars and has an entirely different fold than that observed for other EIIs (Saier *et al.*, 2005). Finally, while Crr is regarded as a glucose-specific IIA in *Enterobacteriaceae*, it is shared among EIIs for several sugars as well as glucose and NAG in *Vibrio* species (Houot *et al.*, 2010). We found that *crr* deletion caused a severe growth defect on NAG, glucose and trehalose, whereas this strain showed no significant growth defect

on other PTS sugars, fructose and mannose, in *V. vulnificus* (Supporting Information Fig. S8). Taken together, our findings in this study on the co-evolutionary dynamics between the PTS and a transcription regulator provide insight into how the long-term resource availability in the bacterial natural habitat affects evolution of solute transporters and their transcriptional regulators.

#### **Experimental procedures**

#### Bacterial strains, plasmids and culture conditions

Details of the strains, plasmids and oligonucleotides used in this study are listed in Supporting Information Tables S2 and S3. V. vulnificus strains were grown in Luria-Bertani medium supplemented with 2.5% NaCl (LBS) at 30°C, and E. coli strains were cultured in LB medium at 37°C, unless otherwise indicated. The following supplements were added if necessary: kanamycin, 200  $\mu$ g mL<sup>-1</sup> for V. vulnificus and 50  $\mu$ g mL<sup>-1</sup> for *E. coli*; ampicillin, 20  $\mu$ g mL<sup>-1</sup> for V. vulnificus and 100  $\mu$ g mL<sup>-1</sup> for E. coli; chloramphenicol, 2  $\mu$ g mL<sup>-1</sup> for V. vulnificus and 10  $\mu g m L^{-1}$ for Ε. coli; isopropyl-b-D-1-thiogalactopyranoside (IPTG), 1 mM. mlc, nagE and ptsG deletion mutants of V. vulnificus were constructed by allelic exchange using the plasmid pDM4 as described previously (Kim et al., 2015; Park et al., 2016; Heo et al., 2019).

#### Purification of proteins

Proteins were expressed from pET vectors in *E. coli* BL21 (DE3) pLysS or ER2566. Harvested cells were disrupted by three passages through a French pressure cell at 8000 psi and centrifuged at 10,000  $\times$  *g* at 4°C for 10 min. His-tagged proteins were purified by immobilized metal affinity chromatography using TALON metal affinity resin (Takara Bio) according to the manufacturer's instructions. Resin-bound proteins were eluted with 200 mM imidazole. To remove imidazole and increase purity, proteins were chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE Healthcare Life Science) equilibrated with buffer A [50 mM Hepes (pH 7.5), 100 mM NaCl, 5 mM  $\beta$ -mercaptoethanol and 5% glycerol].

Untagged proteins were purified using MonoQ<sup>TM</sup> 10/100 GL and HiLoad 16/60 Superdex 75 prepgrade columns (GE Healthcare Life Science). The cell lysate was applied to a MonoQ 10/100 GL column equilibrated with 20 mM Tris–HCl (pH 8.0), containing 50 mM NaCl, 5 mM  $\beta$ -mercaptoethanol and 5% glycerol. The fractions containing the desired protein were concentrated and chromatographed on a HiLoad 16/60 Superdex

75 prepgrade column equilibrated with buffer A. The purified proteins were stored at  $-80^{\circ}$ C until use.

His-tagged full-length NagE was purified from the E. coli cell membrane after solubilization with 1% n-dodecyl-β-D-maltopyranoside (DDM). After removal of cell debris from the lysate, the membrane was pelleted at 100 000g at 4°C for 60 min. The pellet was resuspended in buffer A containing 1% DDM, and NagE was purified using TALON metal affinity resin (Takara Bio) according to the manufacturer's instructions. The eluted protein was further chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (GE Healthcare Life Science) equilibrated with buffer A containing 0.05% DDM. Purified proteins were concentrated using Amicon Ultracel-3K centrifugal filters (Merck Millipore), and the purified proteins in buffer A containing 0.05% DDM were stored at -80°C until use. Total protein concentration was determined by the Bradford protein assay using bovine serum albumin as the standard.

#### Determination of the amounts of sugars in medium

Overnight-grown E. coli and V. vulnificus cells were harvested and, after a brief wash, incubated in M9 medium and M9 medium containing 2.5% NaCl (M9S) supplemented with 0.04% glucose and 0.04% NAG respectively. One millilitre aliquots were withdrawn at various time points to determine concentrations of residual sugars in the medium as a function of cell growth. After centrifugation at 10 000g for 3 min, the sugars remaining in the culture supernatant were analysed using a Sugar-Pak H column connected to a Dionex Ultimate 3000 high-performance liquid chromatography system equipped with a refractive index detector (Thermo Fisher Scientific). The mobile phase used was 0.01 N H<sub>2</sub>SO<sub>4</sub> and the column was maintained at 60°C during the experiment.

## Ligand fishing experiments using metal affinity chromatography

Overnight-grown V. vulnificus MO6-24/O cells were harvested, resuspended in buffer A, and disrupted by three passages through a French pressure cell at 8000 psi. After centrifugation at 10 000g at 4°C for 20 min, the supernatant was mixed with either buffer A as a control or purified His-vEIIB<sup>Nag</sup> or His-vEIIB<sup>Glc</sup> as His-vEIIB<sup>Nag</sup> His-vEIIB<sup>Glc</sup> bait. and were dephosphorylated by adding 2 mM pyruvate or phosphorylated by adding 2 mM PEP to the mixture. Each mixture was then incubated with TALON metal affinity resin at 4°C for 15 min. After brief washes with buffer A, proteins bound to the column were eluted with  $2 \times$  SDS sample buffer. The eluted proteins were analysed by SDS-PAGE

using a 4% to 20% gradient gel (KOMA biotech) and staining with Coomassie brilliant blue. The protein band specifically bound to His-vEIIB<sup>Nag</sup> was excised from the gel, and tryptic in-gel digestion and peptide mass finger-printing were performed as previously described (Kim *et al.*, 2015; Park *et al.*, 2016).

#### BACTH assays

To detect protein interactions in E. coli cells, we performed a BACTH assav as described previously (Karimova et al., 1998). The cya-deficient E. coli BTH101 strain was co-transformed with pKT25 and pUT18C derivatives encoding MIc and PTS components fused to T25 and T18 fragments of B. pertussis adenylate cyclase sinalv or in pairwise combinations. To determine the level of β-galactosidase activity, co-transformants were grown in LB medium at 30°C overnight. Cultured cells were diluted 10-fold with Z-buffer (60 mM Na<sub>2</sub>HPO<sub>4</sub>, 40 mM NaH<sub>2</sub>PO<sub>4</sub>, 10 mM KCl, 1 mM MgSO<sub>4</sub> and 50 mM  $\beta$ -mercaptoethanol) and lysed with chloroform and 0.1% SDS. After incubation at 30°C for 10 min, O-nitrophenylβ-galactoside was added to the mixture and the reaction was stopped by the addition of Na<sub>2</sub>CO<sub>3</sub>. β-Galactosidase activity was measured as described by Miller (1972).

#### Membrane vesicle preparation and Mlc-binding assay

vEII<sup>Nag</sup>- or vEII<sup>Glc</sup>-enriched membrane vesicles were prepared from E. coli ER2566 transformed with the pETnagE or pET-ptsG plasmid (Supporting Information -Table S2), respectively, after induction by adding 1 mM IPTG. Membrane vesicles lacking EII were prepared from E. coli ER2566 transformed with pET plasmid. The cells were harvested by centrifugation and resuspended in buffer A containing 10 mM dithiothreitol (DTT). Cells were disrupted by three passages through a French pressure cell at 8000 psi, and centrifuged at 10 000g at 4°C for 5 min. Ammonium sulfate was added to the supernatant to yield a final concentration of 42% saturation and the precipitate was collected by centrifugation at 10 000g at 4°C for 5 min. The pellet was dissolved in buffer A containing 5 M urea. After centrifugation at 100 000g at 4°C for 60 min, the pellet was rinsed twice with buffer A and resuspended in the same buffer. The resulting membrane vesicles were used for the Mlc-binding assay. Incubation mixtures contained buffer A with 10 mM DTT, three soluble PTS proteins (12 µg of vEl, 18 µg of vHPr, 12 µg of vCrr), and 4 µg of vMlc with the membrane vesicles in a total volume of 150 µl. The reaction mixtures were incubated at room temperature for 10 min in the presence of 2 mM NAG or 2 mM PEP. The membrane vesicles with bound proteins were separated by centrifugation at 100 000g at 4°C for 30 min. The supernatants containing

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unbound vMlc (15 µl) were examined by SDS-PAGE using a 4% to 20% gradient gel (KOMA biotech) and staining with Coomassie brilliant blue. The vMlc band intensity was quantified by densitometric analysis using ImageJ software (http://rsb.info.nih.gov/ij/download.html).

#### Electrophoretic mobility shift assay

The DNA fragments were amplified by PCR using the *V. vulnificus* MO6/24-O chromosome as a template with the primers listed in Supporting Information Table S4. Binding reactions were performed with DNA fragments and purified vMIc in 20  $\mu$ I of binding buffer B [10 mM Tris–HCI (pH 8.0), 5% glycerol, 0.1 mM ethylenediaminetetraacetic acid (EDTA) and 1 mM DTT]. Following incubation at 37°C for 10 min, the binding mixture was subjected to electrophoresis on a 6% polyacrylamide gel in TBE (89 mM Tris-borate and 2 mM EDTA [pH 8.0]). After electrophoresis, the gel was stained with ethidium bromide and visualized under UV light and photographed using a gel documentation system (GDS-200C, KBT).

#### RNA isolation and qRT-PCR

Vibrio vulnificus was grown in LBS or M9S medium supplemented with 0.4% glycerol or 0.2% NAG and fixed with methanol for 1 h at -20°C. Total RNA was prepared using the TaKaRa MiniBEST Universal RNA Extraction Kit (Takara Bio) according to the manufacturer's instructions. Total RNA (2500 ng) from each sample was converted into cDNA using the RNA to cDNA EcoDry Premix (Clontech Laboratories). The cDNA was subjected to real-time PCR amplification with specific primers listed in Supporting Information Table S4 using SYBR Premix Ex Tag II (Takara Bio) in the CFX96 Real-Time System (Bio-Rad). To normalize the transcript level, the 16S rRNA expression level was used as a reference in this study. The relative expression level was calculated as the difference between the threshold cycle (Ct) of the target gene and the Ct of the reference gene for each template.

#### DNase I footprinting assays

DNase I footprinting assays were performed as described previously (Choi *et al.*, 2017). The promoter regions of *nagE*, *ptsG* and *ptsH* were amplified by PCR using 5'-6-FAM-labelled forward primers and unlabelled reverse primers (Supporting Information Table S4). Binding reactions were performed as in EMSA, except using 200 ng probe DNA in a total volume of 40  $\mu$ l reaction mixture. After 10 min incubation at 37°C, DNA was digested with 0.01 U DNase I (New England Biolabs) at 37°C for 1 min.

DNase I reaction was terminated by adding the same volume of stop solution [200 mM NaCl, 1% NaCl and 30 mM EDTA (pH 8.0)], followed by phenol extraction and ethanol precipitation. Samples were analysed by capillary electrophoresis in an ABI 3730xl DNA Analyser (Applied Biosystems) with Peak Scanner software v1.0 (Applied Biosystems).

#### 5' Rapid amplification of cDNA ends

The TSSs of *nagE*, *ptsG* and *mlc* were determined using the SMARTer RACE 5'/3' Kit (Clontech Lab) following the manufacturer's instructions. The cDNA specific for the target gene was synthesized using the primers listed in Supporting Information Table S4 and a poly(C) tail was added to the 3'-end of the cDNA. The 5'-RACE product was amplified by PCR from the poly(C)-tailed cDNA using the Universal Primer Short included in the kit and the primer specific for the gene of interest. The TSS of the target gene was identified by sequencing.

#### In vitro transcription assay

In vitro transcription experiments were performed as described previously (Park *et al.*, 2013), with slight modifications. Transcription reactions were carried out using *E. coli* RNA polymerase core enzyme (2 U) and  $\sigma^{70}$  from *V. cholerae* in the presence and absence of vMlc, cAMP, CRP or vEII<sup>Nag</sup> in a total volume of 20 µl at 37°C for 10 min. The transcription reaction was initiated by adding nucleotides and, after incubation at 37°C for 1 h, the reaction was terminated by adding 20 µl of formamide loading buffer containing 5 mM EDTA. The synthesized transcripts were analysed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea. The gel was dried and exposed to a phosphor screen (BAS MP 2040) and analysed using a Phosphorimager BAS-2500 (Fuji, Tokyo).

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#### Author contributions

J.Y. generated data. J.Y. and Y.-J.S. designed the study, analysed the results, and drafted the manuscript. M.-S.J.

and S.-I.E. carried out bioinformatic analyses and contributed to discussion.

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#### Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting Information.