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

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## 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 Mlc, and glucose induction of the Mlc regulon genes is achieved by its interaction with glucose-specific enzyme II ($EII^{Glc}$). In this study, we revealed that, in *Vibrio* species, which are phylogenetically older than *Enterobacteriaceae*, the membrane sequestration of Mlc and thereby the induction of its regulon genes is mediated by *N*-acetylglucosamine (NAG)-specific EII. While *Vibrio* Mlc interacts only with the EIIB domain of $EII^{Nag}$, *E. coli* Mlc interacts with the EIIB domain of both $EII^{Glc}$ and $EII^{Nag}$. The present data suggest that $EII^{Nag}$ may be the primordial regulator of Mlc, and $EII^{Glc}$ has evolved to interact with Mlc since an EIIA domain was fused to $EII^{Nag}$ 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.**

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## 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 (Faruque *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 β-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 β-*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 non-sugar-specific cytoplasmic proteins, enzyme I (EI, encoded by *ptsI*) and histidine phosphocarrier protein (HPr, encoded by *ptsH*), and several sugar-specific proteins collectively known as enzymes II (EIIs) (Postma *et al*., 1993). EIIs 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 ($EIICBA^{Nag}$ encoded by *nagE*), or encoded separately in two or more ORFs, as in the case of the glucose-specific EII ($EIICB^{Glc}$ and $EIIA^{Glc}$ encoded by *ptsG* and *crr*, respectively), in *Escherichia coli*. EI and HPr mediate the phosphoryl

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 NAG-specific PTS transporter NagE (hereafter vEII$^{Nag}$) and showed that in *Vibrio* species, dephosphorylated vEIIB$^{Nag}$ 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$^{Glc}$) 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$^{Glc}$, EIICB$^{Nag}$ and some other EIIs in *Vibrio* species, whereas it is specific for EIICB$^{Glc}$, and therefore named EIIA$^{Glc}$ 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$^{Glc}$ (Ferenci, 1996; Hayes *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$^{Nag}$ 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$^{Nag}$, 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™ metal affinity resin (Takara Bio) and vEIIB$^{Nag}$ with an N-terminal $His_6$ tag (His-vEIIB$^{Nag}$, residues from 425 to 524) or His-vEIIB$^{Glc}$ (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

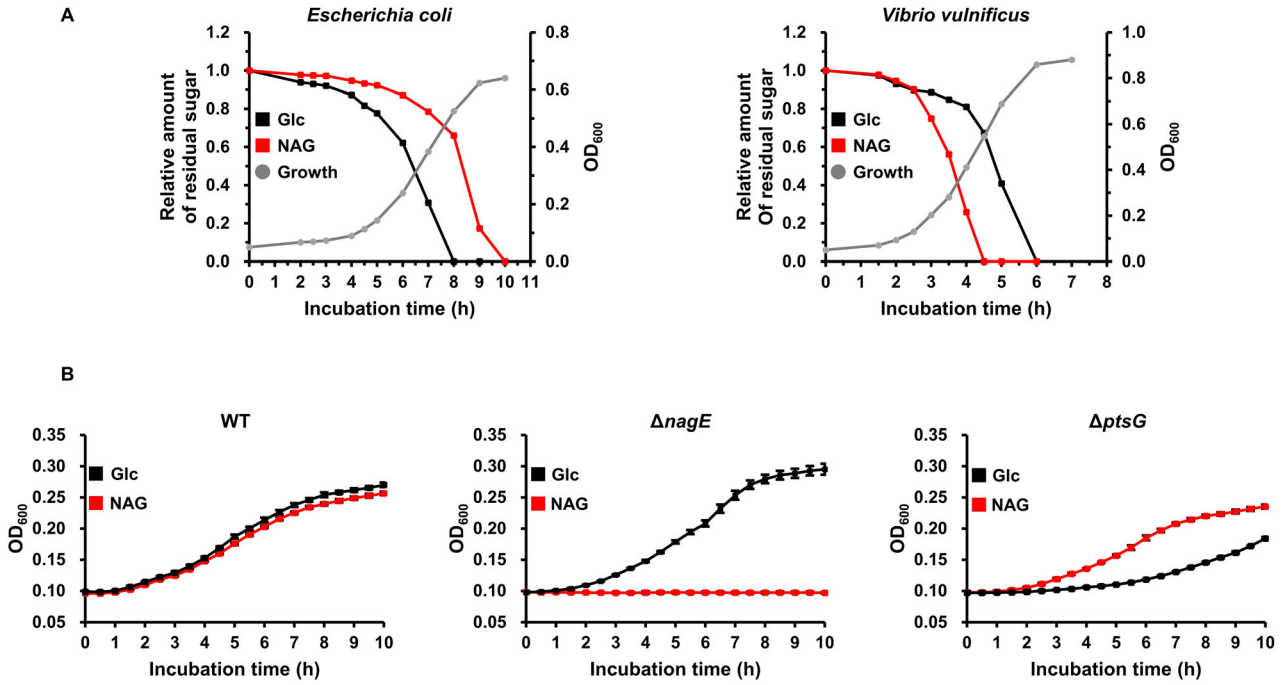


**Fig 1.** Distinct carbohydrate preference between *Escherichia coli* and *Vibrio vulnificus*.
A. *E. coli* MG1655 and *V. vulnificus* MO6-24/O were grown in M9-based minimal medium supplemented with 0.04% glucose and 0.04% NAG. Growth rates (optical density at 600 nm) and the concentration of sugars 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® 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$^{Glc}$ regardless of its phosphorylation state, we found a protein band migrating at ~50 kDa co-eluting only with dephosphorylated vEIIB$^{Nag}$ 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 *ptsHIcrr* operon and of a number of genes encoding transporters for glucose and related sugars such as EII$^{Glc}$, EII$^{Man}$ 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 eMlc is disabled by binding to the dephosphorylated eEIIB domain of eEII$^{Glc}$ in the membrane (Lee *et al*., 2000; Tanaka *et al*., 2000; Nam *et al*., 2001).

To verify the specific interaction between vEIIB$^{Nag}$ and vMlc and to confirm that the interaction is affected by the phosphorylation state of vEIIB$^{Nag}$, we first performed pull-down assays with purified vMlc in the presence of vEI, vHPr and vCrr with and without PEP. Direct interaction of vMlc with vEIIB$^{Nag}$ was observed in the absence but not in the presence of PEP, indicating that only the dephosphorylated form of vEIIB$^{Nag}$ can interact with vMlc (Fig. 2B). We then tested whether the interaction between vMlc and vEIIB$^{Nag}$ 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$^{Nag}$ (residues from 427 to 524) and vEIIB$^{Glc}$ (residues from 387 to 477) we found a significant interaction between vMlc and vEIIB$^{Nag}$ in both reciprocal combinations (Fig. 2C), but no interaction between vMlc and vEIIB$^{Glc}$ (Supporting Information Fig. S1B). Interestingly, we observed much higher levels of β-galactosidase activity only when the gene encoding vEIIB$^{Nag}$ in the fusion plasmids was mutated to a non-phosphorylatable form (vEIIB$^{Nag}$(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$^{Nag}$ interaction is specific and dependent on the phosphorylation state of vEIIB$^{Nag}$ *in vivo* as well as *in vitro*.

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

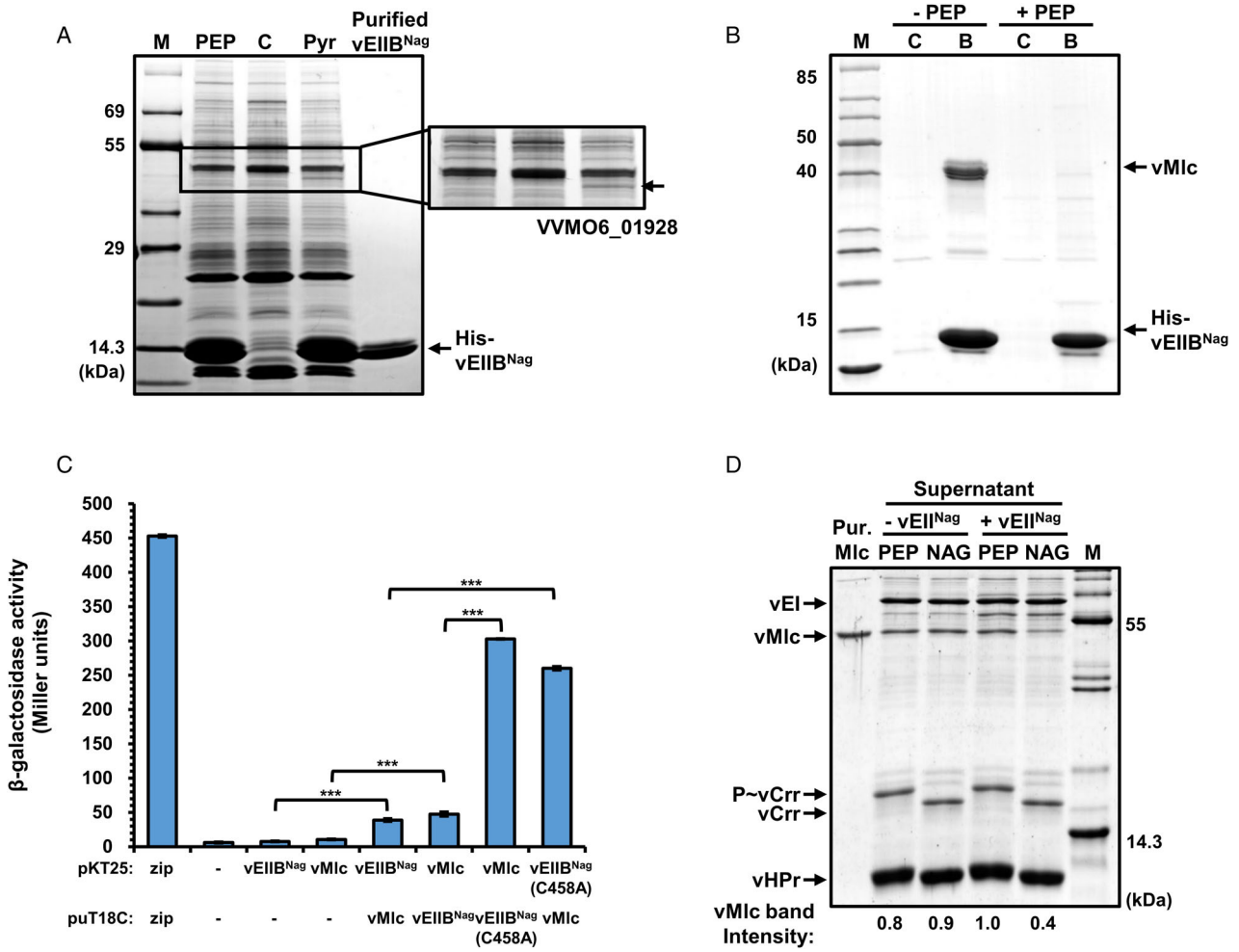


**Fig 2.** Specific interaction between dephospho-vEIIB$^{Nag}$ and vMlc in *Vibrio vulnificus*.

A. Ligand fishing experiment was carried out to search for protein(s) interacting with the vEIIB$^{Nag}$ 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 His-**v**EIIB$^{Nag}$. The mixture containing His-vEIIB$^{Nag}$ was supplemented with either 2 mM PEP to phosphorylate vEIIB$^{Nag}$ (lane PEP) or 2 mM pyruvate to dephosphorylate vEIIB$^{Nag}$ (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. vMlc (90 μg) was mixed with vEI (2 μg), vHPr (6 μg) and vCrr (4 μg), and buffer A (lane C) or His-vEIIB$^{Nag}$ (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$^{Nag}$ 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$^{Nag}$ domain (residues 427–524). Zip, the leucine zipper domain of *Saccharomyces cerevisiae* GCN4, served as a positive control. After growth in LB medium overnight, β-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$^{Nag}$ and vMlc. Membrane vesicles in which vEII$^{Nag}$ is enriched (lane vEII$^{Nag}$) or lacking (lane −vEII$^{Nag}$) 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$^{Glc}$ (Lee *et al*., 2000; Tanaka *et al*., 2000; Nam *et al*., 2001). vEII$^{Nag}$-depleted and -enriched membrane vesicles were prepared from *E. coli* ER2566 transformed with pET and pET-vEII$^{Nag}$ plasmids, respectively, as described previously, and we explored the interaction between vMlc and vEII$^{Nag}$ 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 $vEII^{Nag}$. However, whereas the presence of $vEII^{Nag}$ did not affect the amount of vMlc remaining in the supernatants in the reaction mixtures containing PEP, the presence of $vEII^{Nag}$ significantly decreased the amount of free vMlc in the reaction mixtures containing NAG (Fig. 2D), indicating that only dephosphorylated $vEII^{Nag}$ sequesters vMlc to the membrane. In addition, we prepared $vEII^{Glc}$-enriched membrane vesicles and performed an interaction test with vMlc (Supporting Information Fig. S1C). The presence of $vEII^{Glc}$ did not affect the amount of vMlc remaining in the supernatants regardless of its phosphorylation state. Thus, we confirmed that there is no direct interaction between $vEII^{Glc}$ and vMlc in *V. vulnificus*. The dissociation constant ($K_D$) for the vMlc–$vEIIB^{Nag}$ 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 $K_D$ for the eMlc–$eEIIB^{Glc}$ 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 eMlc. Since it was reported that eMlc 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 vMlc 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 vMlc-binding 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, +14 of 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 Δ*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 vMlc 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 $eEII^{Nag}$ 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

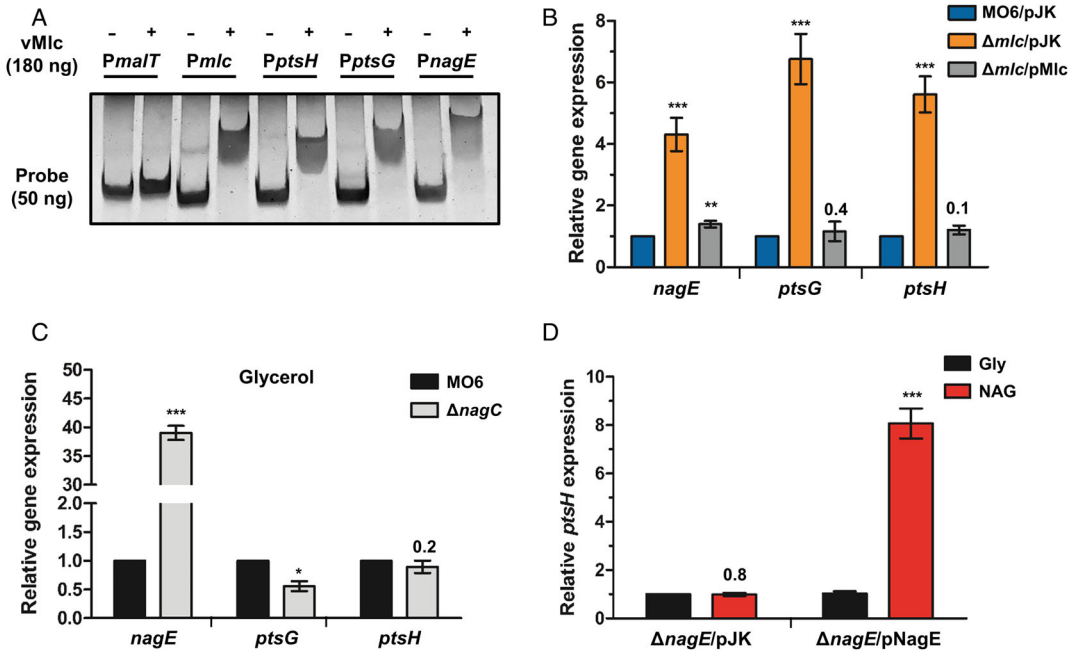


**Fig 3.** NAG induces repression of vMlc regulon expression in *Vibrio vulnificus*.
A. vMlc binding to the promoter regions of PTS-related genes was examined with DNA fragments (approximately −280 to 0 bp relative to the translation start codon) amplified by PCR using gene-specific primer pairs (Supporting 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 $EII^{Nag}$, 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, Δ*nagC* and Δ*nagC* Δ*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 $vEII^{Nag}$ antagonizes the repression of the vMlc regulon depending on its phosphorylation state in response to the availability of NAG in *V. vulnificus*.

### *$vEII^{Nag}$ displaces vMlc from its target promoters to induce expression of its regulon*

To determine whether dephosphorylated $vEII^{Nag}$ 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 $vEII^{Nag}$ 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$^{Nag}$ to the reaction mixture inhibited the binding of vMlc to all the three promoters in EMSA assays (Fig. 4A). Displacement of vMlc by vEII$^{Nag}$ 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$^{Nag}$ 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$^{Nag}$ 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 Mlc 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 Mlc is different between the two groups of bacteria. Unlike *E. coli* EII$^{Nag}$ (eEII$^{Nag}$), which consists of C, B and A domains (thus eEIICBA$^{Nag}$), *V. vulnificus* EII$^{Nag}$ (vEII$^{Nag}$) is devoid of the

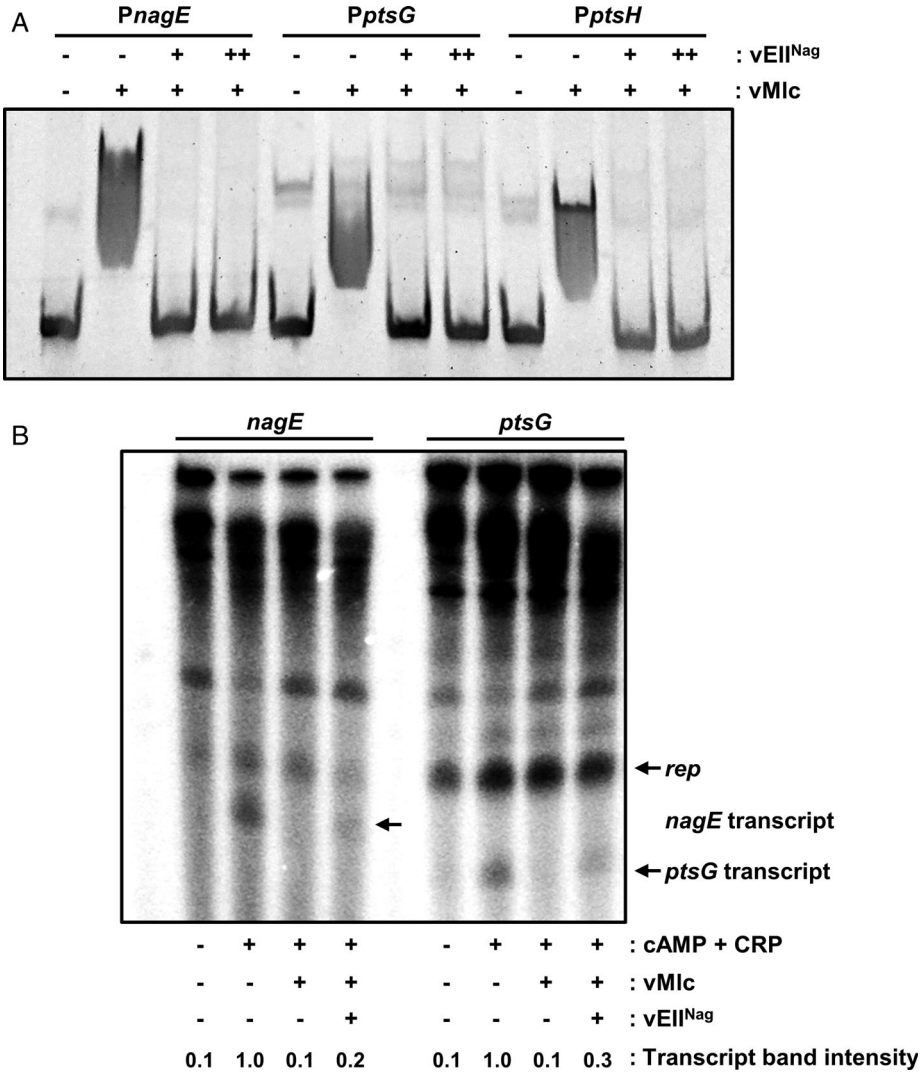


**Fig 4.** Displacement of vMlc from its target DNA by vEII$^{Nag}$ to induce transcription of target genes.
A. Inhibition of vMlc binding to its target DNA by vEII$^{Nag}$. Promoter DNA fragments (the same as in Fig. 3a; 100 ng each) were mixed with 400 ng of vMlc and varying amounts of vEII$^{Nag}$ (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 vMlc was approximately 1:16.
B. Induction of *nagE* and *ptsG* transcription by vEII$^{Nag}$ *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 a 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).

A domain (thus vEIICB$^{Nag}$), whereas both eEII$^{Glc}$ and vEII$^{Glc}$ consist of C and B domains. Therefore, vEII$^{Glc}$ and vEII$^{Nag}$ share vCrr in all *Vibrio* species including *V. vulnificus* (Houot *et al*., 2010). Since it has been established that eEII$^{Glc}$ regulates the activity of eMlc in *Enterobacteriaceae* and BLAST searches revealed that *V. vulnificus* vMlc and the vEIIB$^{Glc}$ 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$^{Glc}$ and vMlc. Pull-down assays using a cell-free extract of *E. coli* expressing vMlc and purified His-vEIIB$^{Nag}$ and His-vEIIB$^{Glc}$ revealed that vMlc specifically interacts with vEIIB$^{Nag}$ but not with vEIIB$^{Glc}$ (Fig. 5A). We then wondered whether vMlc interacts with the B domains of eEII$^{Nag}$ (residues from 373 to 468) and eEII$^{Glc}$ (residues from 391 to 480) since *V. vulnificus* vEIIB$^{Nag}$ and vEIIB$^{Glc}$ domains have amino acid sequence identities of 50% and 77%, respectively, with their *E. coli* orthologs. Interestingly, pull-down assays revealed that vMlc interacts with eEIIB$^{Nag}$, but not eEIIB$^{Glc}$. Since Mlc and PTS components are highly conserved among vibrios, we assumed that the specific interaction of vMlc with vEII$^{Nag}$ could be conserved in other *Vibrio* species. Pull-down assays with a cell-free extract of *E. coli* expressing *V. cholerae* Mlc (vcMlc) using purified His-vcEIIB$^{Nag}$ (residues from 428 to 524) and His-vcEIIB$^{Glc}$ (residues from 412 to 501) as bait revealed that vcMlc also interacts with vcEII$^{Nag}$, but not with vcEII$^{Glc}$ (Supporting Information Fig. S5), indicating that the interaction between Mlc and EII$^{Nag}$ is widespread among *Vibrionaceae* species.

Since *Vibrionaceae* is phylogenetically older than *Enterobacteriaceae* (Williams *et al*., 2010), we postulated that the interaction between vMlc and vEIIB$^{Nag}$ could have a longer evolutionary history than that between eMlc and eEII$^{Glc}$. Therefore, the question arose as to whether this trait remains in *Enterobacteriaceae*. To this

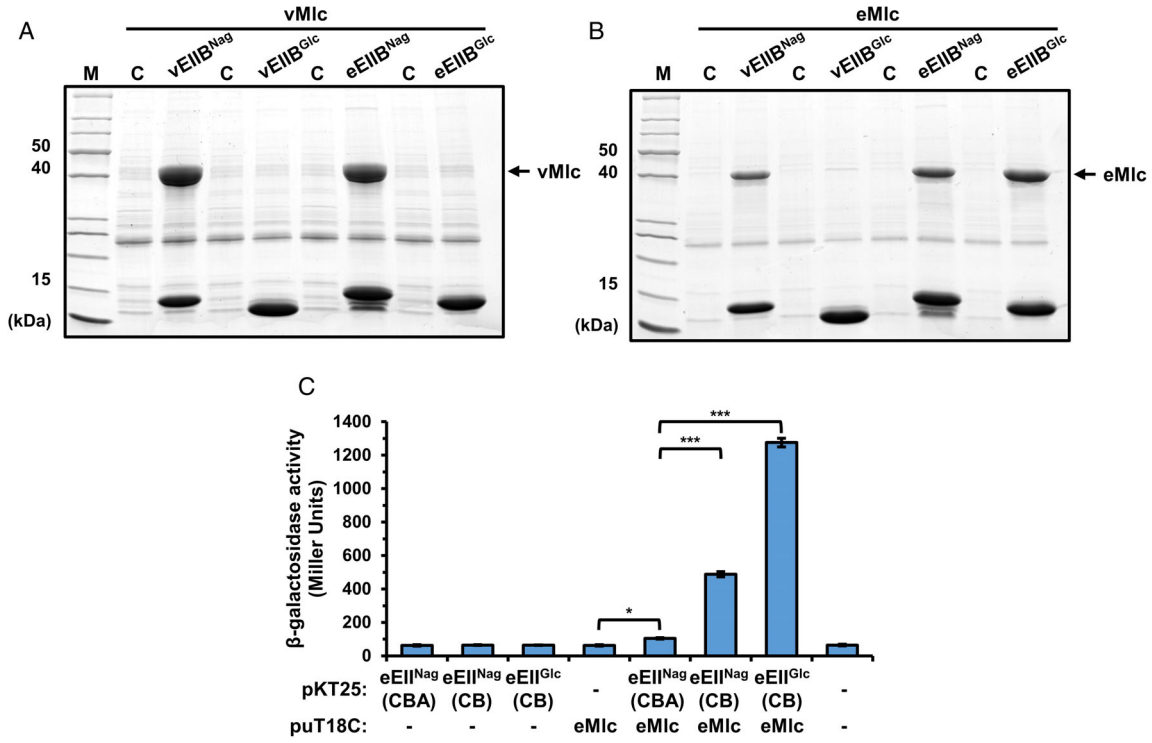


**Fig 5.** Analysis of the cross-species interaction between Mlcs and EIIB domains from *E. coli* and *Vibrio vulnificus*.
A,B. A cell-free extract of *E. coli* expressing vMlc (A) or eMlc (B) was mixed with buffer A (lane C) or the His-tagged form of the indicated EIIB domain from *E. coli* (eEIIB$^{Nag}$ domain, residues 373–468); and eEIIB$^{Glc}$ domain, residues 391–480) or *V. vulnificus* (**v**EIIB$^{Glc}$ 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 eMlc with the full-length eEIICBA$^{Nag}$ and the eEIICB$^{Nag}$ 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 eMlc and either of the two forms of eEII$^{Nag}$ respectively. The pair of T18-eMlc and T25-eEII$^{Glc}$ fusions served as a positive control. After growth in LB medium overnight, β-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 eMlc interacts with the $EIIB^{Nag}$ domain from *E. coli* and *V. vulnificus*. Notably, eMlc interacted with $vEIIB^{Nag}$ as well as $eEIIB^{Glc}$ (Fig. 5B). Since the $eEIIB^{Nag}$ domain exists between the C and A domains in $eEII^{Nag}$, we therefore constructed an expression vector for the His-tagged form of the B domain (residues from 372 to 468) of $eEII^{Nag}$ to test its interaction with eMlc. Interestingly, eMlc interacted with $eEIIB^{Nag}$ as well as $vEIIB^{Nag}$ (Fig. 5B). Therefore, eMlc interacts with both $EIIB^{Glc}$ and $EIIB^{Nag}$ domains, whereas vMlc interacts only with the $EIIB^{Nag}$ domain. The possibility of the interaction between $eEII^{Nag}$ 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-Baey *et al*., 2015). The binding of eMlc to $eEIIB^{Nag}$ led us to ask whether *E. coli* evolved to change the cognate Mlc regulator from $EII^{Nag}$ to $EII^{Glc}$ since the A domain was fused to the C-terminal end of $EII^{Nag}$. Therefore, we examined whether the presence of the A domain hinders the interaction between eMlc and $eEII^{Nag}$ in *E. coli*. If this is true, $eEII^{Nag}$ might recover the capability to interact with eMlc when the A domain is removed. To test this hypothesis, we examined the interaction of full-length $eEII^{Nag}$ with eMlc *in vivo* by the BACTH assay. We found a slight but statistically significant increase in the β-galactosidase activity in the strain carrying the plasmid pair of the full-length $eEII^{Nag}$ and eMlc compared to the cell expressing eMlc alone. However, the strain carrying the plasmid pair of the A domain-truncated $eEII^{Nag}$ (residues from 1 to 468) and eMlc gave a much higher β-galactosidase activity than the strain carrying the plasmid pair of the full-length $eEII^{Nag}$ and eMlc (Fig. 5C), suggesting that the vMlc–$vEII^{Nag}$ interaction may have a longer evolutionary history than the eMlc–$eEII^{Glc}$ interaction.

Finally, although the data in Fig. 1B shows that $vEII^{Nag}$ is the only transporter for NAG, a question remains whether $vEII^{Nag}$ could transport other sugars such as glucose in addition to NAG, since some EIIs 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 $vEII^{Nag}$. When $vEII^{Nag}$ 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 $vEII^{Nag}$. Altogether, our data suggest that the global repressor Mlc 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 Mlc and the regulatory mechanism of Mlc are highly conserved, the cognate PTS transporters regulating Mlc are differentially evolved between the orders *Vibrionales* and *Enterobacterales*. We then wondered how the regulators of Mlc were differentially evolved in the two closely related groups of bacteria. Several lines of evidence led us to postulate that the primordial regulator of Mlc could be $EII^{Nag}$ (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 $eEII^{Nag}$ and eMlc was very weak, the isolated $eEIIB^{Nag}$ domain and the EIIA domain-deleted form of $eEII^{Nag}$ (i.e., $eEIICB^{Nag}$) showed a high affinity interaction with eMlc (Fig. 5). These data suggest that $eEII^{Nag}$ might have lost the ability to interact with eMlc since $eEII^{Nag}$ was fused with the eEIIA domain in *E. coli* (Fig. 6). Because eMlc interacts with both $eEIIB^{Nag}$ and $eEIIB^{Glc}$, while vMlc interacted only with $vEIIB^{Nag}$ (Fig. 5), it is likely that eMlc acquired the capability to interact with $eEIIB^{Glc}$ as well as $eEIIB^{Nag}$ through accumulated mutations in *E. coli* (Fig. 6).

Phylogenetic analysis also supports our assumption. Mlc is mostly conserved in the class *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 $EII^{Nag}$ in all genome-announced genera of the orders *Aeromonadales*, *Enterobacterales*, *Pasteurellales* and *Vibrionales* of the class *Gammaproteobacteria* shows that an EIIA domain is fused to $EII^{Nag}$ only in the order *Enterobacterales* (Supporting Information Table S3). Therefore, it can be

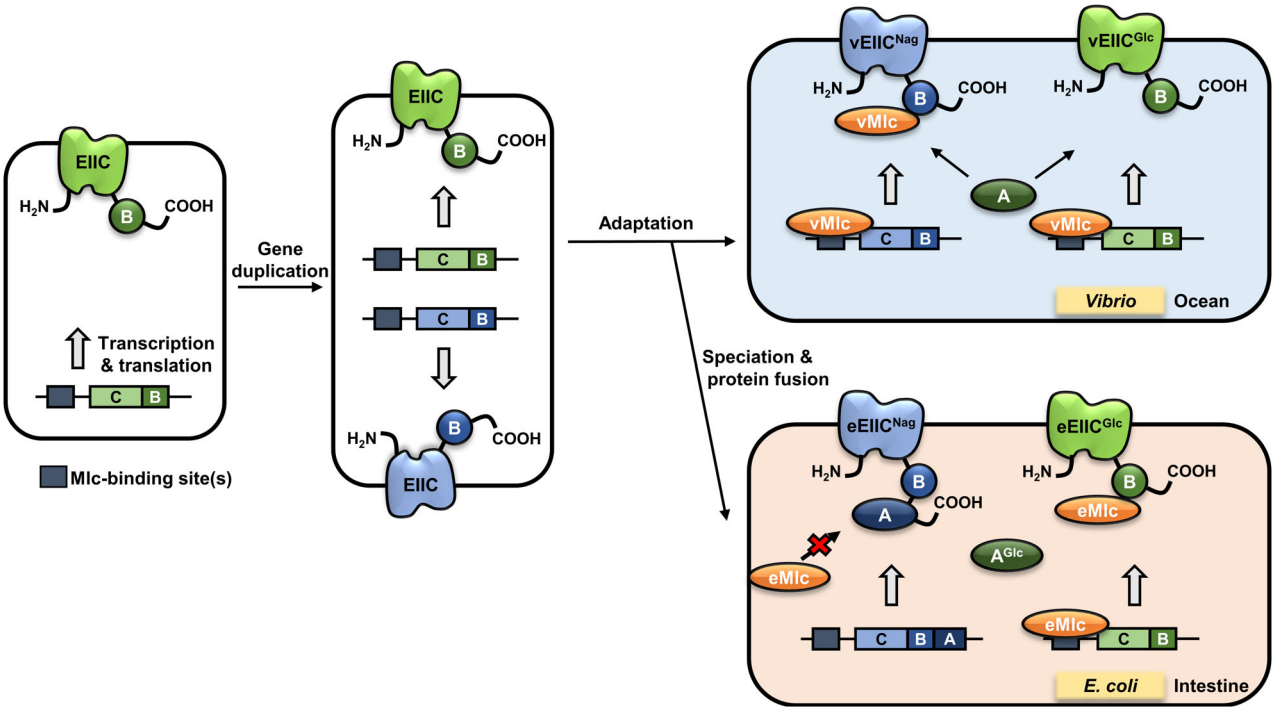


**Fig 6.** A hypothesis for the reciprocal evolution of Mlc and its cognate PTS sugar transporter. It has been proposed that the eubacterial PTS evolved from a much simpler genetic system (Saier, 1977). Exposure of an evolving bacterium to increasing numbers of carbohydrates might have acted as selective 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 Mlc and its cognate PTS sugar transporter. We hypothesize that vEII$^{Nag}$ is the primordial Mlc regulator as the case in *Vibrionaceae* which dwell in the ocean where NAG has long been the most abundant carbohydrate. However, eMlc and eEII$^{Glc}$ have evolved to interact with each other in *Enterobacteriaceae* since the Crr-encoding gene was duplicated and added to the C-terminus of eEII$^{Nag}$. Therefore, eEII$^{Nag}$ lost the ability to interact with eMlc. 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 C-terminus of EII$^{Nag}$ after *Enterobacterales* diverged from the order *Pasteurellales*. A comparative analysis of amino acid sequences of Mlc, EIIB$^{Glc}$ and EIIB$^{Nag}$ domains in representative species for each order shows that EIIB$^{Glc}$ 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$^{Glc}$ in the order *Enterobacterales*. Therefore, all lines of evidence suggest the reciprocal evolution of EII$^{Nag}$ and EII$^{Glc}$ 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 μg $mL^{-1}$ for *V. vulnificus* and 50 μg $mL^{-1}$ for *E. coli*; ampicillin, 20 μg $mL^{-1}$ for *V. vulnificus* and 100 μg $mL^{-1}$ for *E. coli*; chloramphenicol, 2 μg $mL^{-1}$ for *V. vulnificus* and 10 μg $mL^{-1}$ for *E. 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 × *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 β-mercaptoethanol and 5% glycerol].

Untagged proteins were purified using MonoQ™ 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 β-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°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 100 000*g* 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 10 000*g* 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 $H_2SO_4$ 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 10 000*g* at 4°C for 20 min, the supernatant was mixed with either buffer A as a control or purified His-vEIIB$^{Nag}$ or His-vEIIB$^{Glc}$ as bait. His-vEIIB$^{Nag}$ and His-vEIIB$^{Glc}$ 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× 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$^{Nag}$ was excised from the gel, and tryptic in-gel digestion and peptide mass fingerprinting 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 assay as described previously (Karimova *et al*., 1998). The *cya*-deficient *E. coli* BTH101 strain was co-transformed with pKT25 and pUT18C derivatives encoding Mlc and PTS components fused to T25 and T18 fragments of *B. pertussis* adenylate cyclase singly 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 $Na_2HPO_4$, 40 mM $NaH_2PO_4$, 10 mM KCl, 1 mM $MgSO_4$ and 50 mM β-mercaptoethanol) and lysed with chloroform and 0.1% SDS. After incubation at 30°C for 10 min, *O*-nitrophenyl-β-galactoside was added to the mixture and the reaction was stopped by the addition of $Na_2CO_3$. β-Galactosidase activity was measured as described by Miller (1972).

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

vEII$^{Nag}$- or vEII$^{Glc}$-enriched membrane vesicles were prepared from *E. coli* ER2566 transformed with the pET-*nagE* 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 10 000*g* 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 10 000*g* at 4°C for 5 min. The pellet was dissolved in buffer A containing 5 M urea. After centrifugation at 100 000*g* 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 vEI, 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 100 000*g* at 4°C for 30 min. The supernatants containing 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 vMlc in 20 μl of binding buffer B [10 mM Tris–HCl (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 Taq 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 μl 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 $vEII^{Nag}$ 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).

## Acknowledgements

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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.