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The Functionally Active Mistic-Fused Histidine Kinase Receptor, EnvZ†

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ABSTRACT: Mistic is a small *Bacillus subtilis* protein which is of current interest to the field of structural biology and biochemistry because of its unique ability to increase integral membrane protein yields in *Escherichia coli* expression. Using the osmosensing histidine kinase receptor, EnvZ, an *E. coli* two-component system, and its cytoplasmic cognate response regulator, OmpR, we provide the first evidence that a Mistic-fused integral membrane protein maintains functionality both *in vitro* and *in vivo*. When the purified and detergent-solubilized receptor EnvZ is fused to Mistic, it maintains the ability to autophosphorylate on residue His_{243} and phosphotransfers to residue Asp_{55} located on OmpR. Functionality was also observed *in vivo* by means of a β-galactosidase assay in which RU1012 [Φ(*ompC-lacZ*)*10-15*, Δ*envZ*::Km^r] cells transformed with Mistic-fused EnvZ led to an increase in downstream signal transduction events detected by the activation of *ompC* gene expression. These findings illustrate that Mistic preserves the functionality of the Mistic-fused cargo protein and thus provides a beneficial alternate approach to study integral membrane proteins not only by improving expression levels but also for direct use in functional characterization.

Accounting for approximately 30% of all proteins in both prokaryotic and eukaryotic organisms are the integral membrane proteins. They are required for major cellular functions and are thus important pharmaceutical targets (*1*−*4*). Unfortunately, structural and biochemical studies of integral membrane proteins are hampered in part by low levels of expression. Therefore, a heterologous expression system is often employed to overcome this setback. Mistic is a 13 kDa, 110 amino acid *Bacillus subtilis* protein that has unique structural and functional properties. The NMR[1] structure of Mistic has illustrated that it consists of a four α-helical bundle with a hydrophilic surface (*5*). Mistic differs from other membrane-integrated proteins in that it appears to interact with the lipid bilayer and can bypass the traditional cellular translocon machinery for membrane integration. Previous studies illustrate that both prokaryotic and eukaryotic membrane protein expression levels were boosted when target proteins are fused to Mistic (*6*, *7*). Despite the utility of this Mistic-fusion system in improving expression levels of membrane proteins, the critical question still remains whether the overexpressed cargo protein remains functional as a fusion partner to Mistic. In this study, we chose to analyze the prokaryotic two-component signal transduction system EnvZ-OmpR to address this question.

Prokaryotic organisms utilize two-component signal transduction systems as their principal mode for adapting to various environmental stresses (*8*). One of the most widely studied and best characterized two-component systems involves the interaction between the osmosensing histidine kinase receptor, EnvZ, and its cytoplasmic cognate response regulator, OmpR (*8*−*10*). EnvZ is a 450 amino acid inner membrane protein consisting of an NH_2-terminal cytoplasmic tail, periplasmic sensor domain, two transmembrane domains, and a COOH-terminal cytoplasmic domain. The cytoplasmic domain is further divided into a HAMP linker (histidine kinases, adenylyl cyclases, methyl-accepting chemotaxis proteins, and phosphatases), DHp (dimerization and histidine phosphotransfer) domain, and the CA (catalytic and ATP-binding) domain (*11*−*14*) (Figure 1A). Upon activation EnvZ will autophosphorylate on His_{243} (*15*) and phophotransfer the phosphoryl group to residue Asp_{55} on OmpR. Phosphorylated OmpR, OmpR-P, functions as a transcription factor and subsequently controls the expression of the genes for the two major outer membrane porins, OmpF and OmpC (*16*−*18*). Like the majority of other histidine kinases, EnvZ has two major functions, possessing not only kinase activity but also the ability to act as a phosphatase when complexed with OmpR (*16*, *18*), where it dephosphorylates OmpR-P and in turn regulates the concentration of OmpR-P in the cytoplasm (*19*).

In addition to its use in studies involving two-component phosphorelays, EnvZ has also been exploited for various protein engineering purposes (*20*). In the past, EnvZ has been utilized to create many different chimeras which involve domain swapping with different chemoreceptors such as Tar (*21*, *22*) and Trg (*23*) in order to study the signaling mechanisms behind two-component systems. More recently, EnvZ has been fused to the cyanobacterium light sensing phytochrome, Cph1, to create a chimera which functions in a unique system with an image processing role, thus permitting bacteria to exhibit properties like that of film (*24*). In this study we have used EnvZ to test if the Mistic fusion affects its catalytic and signaling capabilities (Figure 1B). Here we provide the first report which illustrates that the *Escherichia coli* histidine

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[1]Abbreviations: NMR, nuclear magnetic resonance; IPTG, isopropyl β-D-1-thiogalactopyranoside; EDTA, ethylenediaminetetraacetic acid; PMSF, phenylmethanesulfonyl fluoride; β-Me, β-mercaptoethanol; FC-12, FOS-choline-12; FPLC, fast protein liquid chromatography; DTT, dithiothreitol; MWCO, molecular weight cutoff; SDS, sodium dodecyl sulfate; ONPG, *o*-nitrophenyl β-D-galactopyranoside; β-gal, β-galactosidase.

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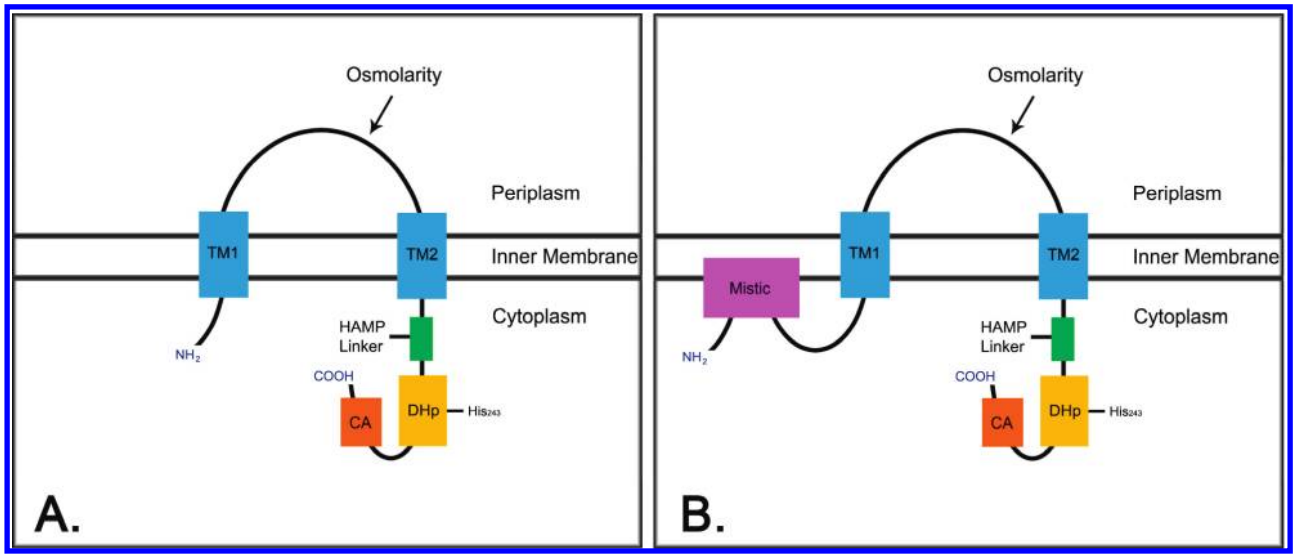

FIGURE 1: Schematic representation of EnvZ's domain organization. EnvZ is composed of two transmembrane domains (blue), a periplasmic sensor domain, and a cytoplasmic domain composed of a HAMP linker (green), DHp (gold), and CA domain (orange) (A). EnvZ domain organization when Mistic is NH_2-terminally fused (B).

kinase receptor, EnvZ, maintains its transmembrane signaling abilities when fused to Mistic, based on the data from both *in vitro* assays, through autophosphorylation and phosphotransfer to OmpR, and *in vivo* assays through activation of *ompC-lacZ* gene expression.

MATERIALS AND METHODS

Strains and Plasmids. All vector construction was Gateway (Invitrogen) adapted. For the [γ-^{32}P]ATP kinase assay, Mistic was fused to the NH_2 terminus of all targets (referred to as "misticated"), following an NH_2-terminal octahistidine tag in the Gateway-adapted vector, pMis3.0E (*5*). The genes that were not misticated were placed in-frame in a Gateway-adapted NH_2-terminal nonylhistidine-tagged vector modified from pET28 (referred to as "pHis9", nonmisticated). A thrombin cleavage site is present between the histidine tag and the target protein on each construct. The Gateway vector pDEST17 (Invitrogen) was used for expression of nonmisticated targets in the β-galactosidase assay to keep the antibiotic resistance (Amp) consistent between misticated and nonmisticated samples.

E. coli BL21 (DE3) cells (Invitrogen) were used for expression of all samples. Experimental *E. coli* RU1012 [Φ(*ompC-lacZ*)*10-15*, Δ*envZ*::Km^r] cells (courtesy of Dr. Masayori Inouye) and control *E. coli* MC4100 cells (courtesy of Dr. Kit Pogliano) were used for the β-galactosidase assay.

Expression and Membrane Isolation. Recombinant vectors were used to transform *E. coli* BL21 (DE3) cells (Invitrogen). A 5 mL overnight culture was used to inoculate 1 L of Terrific broth (EMD) at a 1:1000 ratio. Cells were grown at 37 °C to OD_{600} = 1. Temperature was decreased to 18 °C and 0.5 mM IPTG was added to induce expression of EnvZ constructs. For the soluble protein OmpR and EnvZ cytoplasmic domain, cells were grown at 37 °C to OD_{600} = 0.4. One millimolar IPTG was added to induce expression, the temperature was kept at 37 °C, and cells were harvested 3 h later.

The cell pellet was weighed, and the lysis buffer was added at 4× the weight of the cell pellet (20 mM Tris, pH 8.0, 200 mM NaCl, 10 mM EDTA, 5 mM PMSF). The pellet was resuspended, then 5 mM β-Me and 1 mg/mL lysozyme was added, and the sample was stirred at 4 °C for 30 min. Cells were further lysed by sonication 3× on ice for a total of 1 min, pulses at 1 s on and 2 s off, in volumes of no more than 40 mL at a time. The sample was then centrifuged at 100000*g* for 2 h. The pellet was resuspended in lysis buffer and centrifuged at 10000*g* for 20 m. The supernatant was collected and centrifuged at 100000*g* for 2 h. The membrane pellets were then resuspended in cold salt wash buffer (20 mM Tris, pH 8.0, 500 mM NaCl, 5 mM β-Me, 5 mM PMSF, 10 mM EDTA) and stirred overnight at 4 °C. The next day, the salt-washed membranes were centrifuged at 100000*g* for 2 h, and the pellet was then resuspended in cold storage buffer (20 mM Tris, pH 8.0, 0.1 M NaCl, 5 mM β-Me, 20% (v/v) glycerol and protease inhibitor cocktail tablets (Roche)). The homogeneous membrane mixture was then aliquoted and frozen at −80 °C.

Purification. (*A*) *EnvZ Purification*. Membranes were solubilized in solubilization buffer (20 mM Tris, pH 8.0, 20 mM FC-12, 0.3 M NaCl, 1 mM $MgCl_2$, 5 mM β-Me) and stirred overnight at 4 °C. Solubilized membranes were centrifuged at 100000*g* for 2 h. The protein was purified on a Ni-NTA column (Qiagen), and the detergent was exchanged by washing with wash buffer (20 mM Tris, pH 8.0, 0.2 M NaCl, 4 mM FC-12, 10 mM imidazole, 3 mM β-Me). The protein was eluted with elution buffer (20 mM Tris, pH 8.0, 0.2 M NaCl, 4 mM FC-12, 0.3 M imidazole, 3 mM β-Me) and concentrated to 2 mL using a Vivaspin concentrator and injected on a S200 16/60 size exclusion column (Pharmacia) with FPLC buffer (20 mM Tris, pH 8.0, 0.2 M NaCl, 1 mM DTT, 1 mM EDTA, 2 mM FC-12). To digest with thrombin, a 1:200 molar ratio of thrombin:protein was added, and the sample was dialyzed overnight at 4 °C, post Ni-NTA purification. Following cleavage on the next day, the thrombin and uncleaved protein were removed by purification on a benzamidine and Ni-NTA column, and the protein was concentrated to 2 mL and purified via size exclusion chromatography as stated above.

(*B*) *OmpR and EnvZ Cytoplasmic Domain Purification*. Cells were lysed as stated above using lysis buffer (20 mM Tris, pH 8.8, 0.3 M NaCl, 1 mM imidazole, 5 mM PMSF, 5 mM β-Me). Protein was purified on a Ni-NTA column (Qiagen), washed with wash buffer (20 mM Tris, pH 8.8, 0.3 M NaCl, 20 mM imdazole), and eluted with elution buffer (20 mM Tris, pH 8.8, 0.3 M NaCl, 0.25 M imidazole, 2 mM $CaCl_2$). Thrombin (Sigma) was added with a 1:2000 dilution and dialyzed overnight at 4 °C, 3500 MWCO tubing in dialysis buffer (20 mM

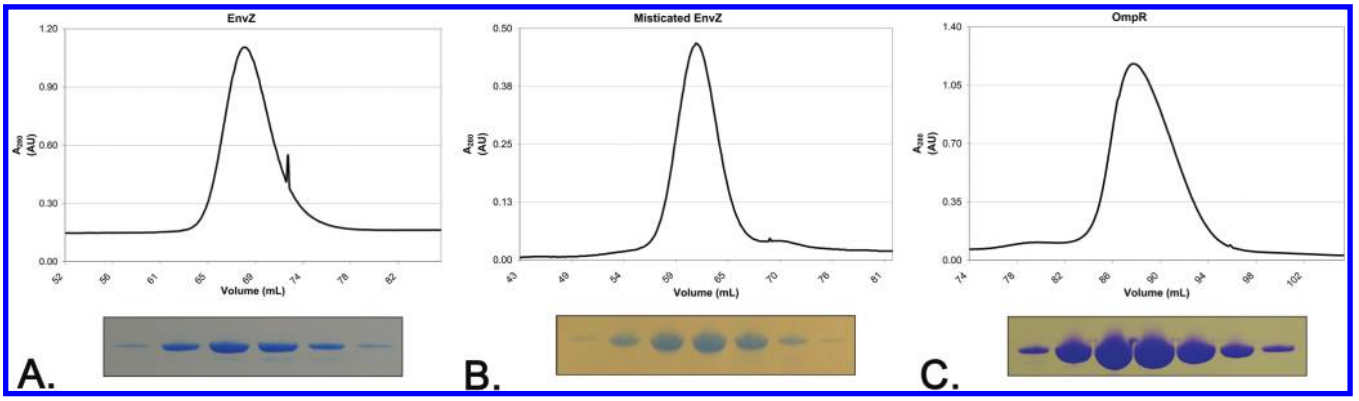

FIGURE 2: Purification of EnvZ, misticated-EnvZ, and OmpR. Size exclusion chromatogram profiles of EnvZ (A), misticated EnvZ (B), and OmpR (C) from a FPLC run on a S200 16/60 column, post Ni-NTA affinity chromatography. Coomassie-stained SDS−PAGE gels of the peak fractions are shown below each chromatogram.

Tris, pH 8.8, 0.3 M NaCl, 2.5 mM $CaCl_2$). After thrombin cleavage, the protein was purified on Ni-NTA and benzamidine resin to remove uncleaved protein and thrombin. The sample was concentrated in a Vivaspin concentrator and injected on a S200 16/60 size exclusion column (Pharmacia) with FPLC buffer (20 mM Tris, pH 8.8, 0.2 M NaCl, 5 mM DTT, 1 mM EDTA).

(*C*) [γ-^{32}*P*]*ATP Kinase Assay*. One micromolar protein was added to the reaction mixture (100 mM Tris, pH 8.0, 100 mM KCl, 10 mM $MgCl_2$, 10% glycerol) along with 20 μM cold ATP and 10 μCi of [γ-^{32}P]ATP in a total volume of 20 μL and incubated at room temperature for 15 min. For EnvZ/OmpR phosphotransfer, the complex was incubated at room temperature for 10 min before and after addition of ATP. Ten microliters of 2× SDS−PAGE sample buffer was added to stop the reaction. Samples were heated in a 95 °C water bath for 2 min and loaded on a 10% SDS−polyacrylamide gel along with the Bio-Rad precision plus prestained molecular weight marker. The gel was incubated with Amberlite cation/anion-exchange resin (Polysciences, Inc., polylite MB-3) to absorb free [γ-^{32}P]ATP, dried, and then exposed to Kodak BioMax XAR film for analysis.

(*D*) *β-Galactosidase Assay*. RU1012 cells or MC4100 cells were electroporated with the recombinant plasmid of choice and spread. The next day colonies were picked, and a 5 mL overnight culture was made. Cells were diluted 1:100 into 150 mL Luria broth (EMD). Cells were then grown to mid log phase and induced with 0.5 mM IPTG. Ten milliliter aliquots were taken out before induction and 0.5, 1, 1.5, 2, 2.5, 3, and 20 h post induction, harvested, and frozen in −80 °C.

Cells were thawed and resuspended in chilled Z buffer (0.06 M $Na_2PO_4 \cdot 7H_2O$, 0.04 M $NaH_2PO_4 \cdot H_2O$, 0.01 M KCl, 0.001 M $MgSO_4$, 0.05 M β-Me, pH 7.0) and normalized to 0.2 OD_{600}. A fraction of cells were taken out and diluted to about 1:2 with Z buffer for a total of 1 mL; if this ratio did not yield a sufficient yellow color, the ratio was changed by the addition of more cells (or less cells if the sample turned yellow too fast). The cells were permeabilized by the addition of 100 μL of chloroform and 50 μL of 0.1% SDS, then vortexed, and equilibrated for 5 min in a 28 °C water bath. The reaction was started by the addition of 0.2 mL of 4 mg/mL ONPG, followed by incubation at 28 °C. The reaction was terminated by the addition of 0.5 mL of 1 M Na_2CO_3 once a sufficient yellow color developed. Cells were centrifuged at 17000*g* for 5 min to remove chloroform and cell debris. OD_{420} and OD_{550} was recorded for all samples to calculate the units of activity (*25*).

RESULTS

Expression and Purification of EnvZ, Misticated EnvZ, and OmpR. One major challenge of studying membrane proteins is the ability to overexpress and isolate a pure homogeneous sample. With the use of a Mistic fusion (Figure 1B), approximately 19 mg of pure homogeneous misticated EnvZ was obtained by Ni-NTA affinity and size exclusion chromatography from 1 L of cultured media. In contrast, EnvZ expressed without Mistic fusion yielded approximately 7 mg of pure homogeneous protein/L of culture. The soluble response regulator OmpR also expressed to large quantities as well, yielding approximately 7 mg of pure homogeneous protein by Ni-NTA affinity and size exclusion chromatography (Figure 2).

Autophosphorylation and Phosphotransfer of EnvZ and Misticated EnvZ in Vitro. [γ-^{32}P]ATP kinase assay was used to test EnvZ and misticated EnvZ's ability to autophosphorylate *in vitro*. Here purified EnvZ and misticated EnvZ samples were incubated in the presence of Mg^{2+} and [γ-^{32}P]ATP. Autoradiography was performed after running samples on a 10% acrylamide gel. The soluble cytoplasmic domain demonstrated the ability to autophosphorylate in the absence of the sensor and transmembrane domains and confirmed the location of autophosphorylation as previously shown (*17*, *26*) (Figure 3, lane 1).

Lanes 3 and 9 of Figure 3 illustrate that both purified full-length EnvZ and misticated EnvZ, when solubilized in FC-12, are also able to autophosphorylate. To demonstrate that this autophosphorylation event occurs on the predicted site of phosphorylation, His_{243}, the point mutant H243V was created which has been previously shown to knock out kinase activity (*17*). Figure 3 (lanes 6 and 12) shows that no autophosphorylation takes place for EnvZ H243V, indicating that the autophosphorylation is dependent on residue His_{243}.

EnvZ's ability to phosphotransfer to its cognate response regulator OmpR can also be detected using this method. When solubilized in FC-12, both EnvZ and misticated EnvZ were incubated in the presence of OmpR and [γ-^{32}P]ATP and exhibited the ability to autophosphorylate and phosphotransfer to OmpR (Figure 3, lanes 3, 4, 9, 10). When EnvZ H243V and misticated EnvZ H243V were incubated in the presence of OmpR, no phosphotransfer (Figure 3, lanes 7 and 13) took place, demonstrating the dependence of these events on the initial autophosphorylation of EnvZ His_{243}. To determine if phosphorylation of OmpR is dependent on EnvZ, we repeated the assay in the absence of EnvZ and misticated EnvZ. No phosphorylation was detected, thus illustrating its dependence on the histidine

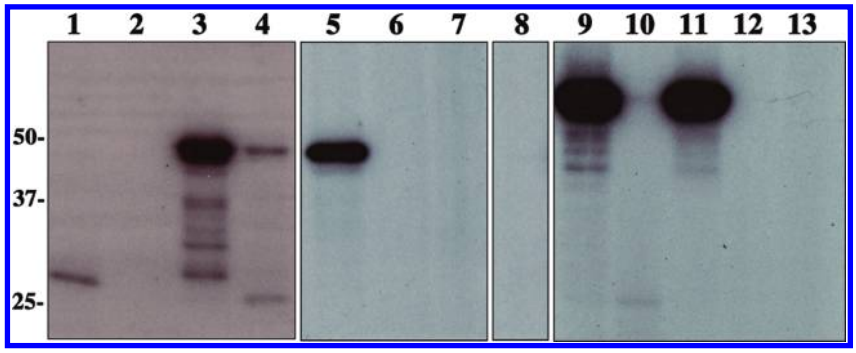

FIGURE 3: [γ-^{32}P]ATP kinase assay detecting autophosphorylation and phosphotransfer of EnvZ and misticated EnvZ. Autoradiogram of samples which were incubated in the presence of [γ-^{32}P]ATP as described previously. Lane 1, EnvZ cytoplasmic domain; lane 2, OmpR; lane 3, EnvZ; lane 4, EnvZ (upper band) and OmpR (lower band); lane 5, EnvZ and OmpR D55Q; lane 6, EnvZ H243V; lane 7, EnvZ H243V and OmpR; lane 8, OmpR D55Q; lane 9, misticated EnvZ; lane 10, misticated EnvZ (upper band) and OmpR (lower band); lane 11, misticated EnvZ and OmpR D55Q; lane 12, misticated EnvZ H243V; lane 13, misticated EnvZ H243V and OmpR.

kinase (Figure 3, lane 2). To confirm the phosphorylation site, Asp_{55} of OmpR, we created the point mutant D55Q which was previously described to knock out phosphorylation (*27*). Figure 3 (lanes 5 and 11) shows that EnvZ and misticated EnvZ are not able to phosphotransfer to OmpR D55Q, confirming its residue specificity for residue Asp_{55}. The reaction mixture alone, in the absence of EnvZ, does not phosphorylate OmpR D55Q non-specifically (Figure 3, lane 8).

β-Galactosidase Assay Illustrates EnvZ and Misticated EnvZ Signaling in Vivo. The ability of EnvZ and misticated EnvZ to autophosphorylate and phosphotransfer *in vitro* suggests that the CA domain and DHp domain are properly oriented, allowing for such activities to take place. However, this does not give insight into the functionality of the periplasmic sensor domain, transmembrane domains, or the activity of all domains of EnvZ and misticated EnvZ as a whole. In order to analyze the activity of all the domains of EnvZ and to look at EnvZ's ability to signal downstream to the level of inducing porin expression, an *in vivo* β-galactosidase assay was performed. Various EnvZ constructs were transformed into two different *E. coli* strains: RU1012 [Φ(*ompC-lacZ*)*10-15*, Δ*envZ*::Km^r] (*21*) and MC4100 (*lac*$^-$). In this assay we electroporated cells with full-length EnvZ, misticated EnvZ, EnvZ H243V, misticated EnvZ H243V, and misticated KvPae, a voltage-gated K^+ channel-like protein from *Pseudomonas aeruginosa*, as a negative control. In addition, cells were tested in the absence of vector, as an additional negative control. Cells were grown to mid log phase at 37 °C. Ten milliliter aliquots were harvested before the cells reached mid log phase and 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 20 h post mid log phase. The samples were centrifuged and frozen, and the Miller assay was completed on all samples.

The MC4100 (*lac*$^-$) *E. coli* cell strain was used as a control to ensure that any activity seen in the Miller assay was due to the production of β-gal. When the Miller assay was completed, there was no significant β-gal activity, measured in Miller units, from any of the six samples (Figure 4A). The use of the RU1012 [Φ(*ompC-lacZ*)*10-15*, Δ*envZ*::Km^r] (*21*) strain allows for the measurement of *ompC* gene expression as the consequence of the downstream signal transduction events of EnvZ and misticated EnvZ. EnvZ- and misticated EnvZ-transformed cells showed activity before mid log phase with a rise in activity up to 3 h post mid log phase and continuing up to 20 h post mid log phase. The negative control which lacked a transformed vector exhibited a low level of β-gal activity starting at 2 h post mid log phase in comparison to EnvZ and misticated EnvZ samples. Null mutants EnvZ H243V and misticated EnvZ H243V exhibited the lowest amount of activity than any other samples (Figure 4B).

DISCUSSION

In this study, we provide direct evidence that Mistic-fused full-length EnvZ is active both *in vitro* and *in vivo*. By means of the *in vitro* [γ-^{32}P]ATP kinase assay, we illustrate that EnvZ and misticated EnvZ autophosphorylate residue His_{243}, the conserved site of phosphorylation. In addition, we show that EnvZ and misticated EnvZ are both able to phosphotransfer the phosphoryl group from EnvZ His_{243} to OmpR Asp_{55} in a site-specific manner. Results from these assays demonstrate that both full-length constructs solubilized in the presence of the detergent FC-12 have a properly folded and functional cytoplasmic domain.

To further address the functionality of the whole receptor as a transmembrane signaling molecule, we performed an *in vivo* β-galactosidase assay using RU1012 *E. coli* cells, where EnvZ and misticated EnvZ were tested to determine the activation of *ompC-lacZ* gene expression through the binding of OmpR to the *ompC* promoter. The results from this experiment illustrate that both EnvZ and misticated EnvZ are active in their natural cell environment and are able to transduce a downstream signal such that the *ompC* promoter becomes activated. The lowest β-gal activity was found in the H243V samples and might be caused by the physical presence of the null EnvZ receptor. The expression of the nonfunctional EnvZ could possibly interfere or turn off alternative pathways subsequently inhibiting the otherwise recoverable *ompC* gene expression. When the negative control was tested in the absence of vector, a small rise in activity was seen 2 h post mid log phase, which could be explained by the complexity of the gene regulation system of the major *E. coli* outer membrane porins.

Since OmpC is one of the major *E. coli* outer membrane porins under complex gene regulation (*28*), the slight rise in β-gal activity seen in our negative control of the *in vivo* β-gal assay could be due to the interference of other pathways attempting to compensate for the absence of EnvZ. Both major *E. coli* outer membrane porins, OmpC and OmpF, are under the control of a very intricate regulatory system comprised of many components within the cell including sRNAs such as MicF (*29*−*32*), MicC (*33*), RseX (*34*, *35*), RybB (*36*, *37*), and Ipex (*38*, *39*) which function by forming base pairs with their target mRNAs in the translation start site

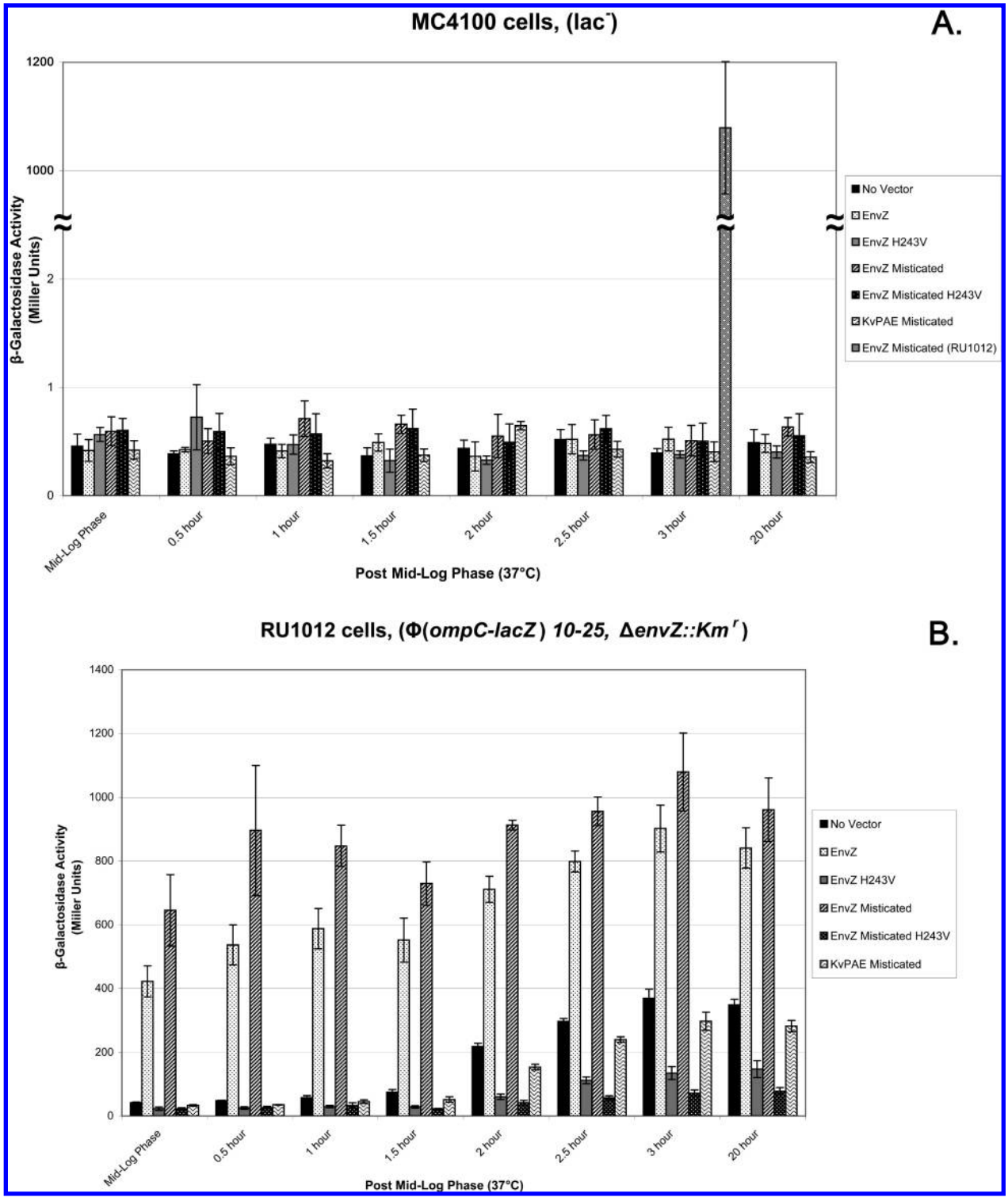

FIGURE 4: β-Galactosidase assay using the MC4100 and RU1012 *E. coli* strains illustrating EnvZ and misticated EnvZ activity. (A) The MC4100 *E. coli* strain illustrating that any β-galactosidase activity (in Miller units) is a result of the production of β-galactosidase produced by the lac-Z reporter gene. (B) The experimental RU1012 [Φ(*ompC-lacZ*)*10-15*, Δ*envZ*::Km^r] *E. coli* strain was tested to measure downstream signaling of EnvZ and misticated-EnvZ as a result of β-galactosidase activity (in Miller units). The following samples were tested: no vector control, EnvZ, EnvZ H243V mutant, misticated EnvZ, misticated EnvZ H243V, and misticated KvPAE. Ten milliliter aliquots of cells were harvested and frozen 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, and 20 h post mid log phase. The Miller assay was completed when all aliquots were collected.

region and thus prevent translation. There are also numerous indirect/direct protein regulators, some of which include Rob, SoxS, MarA, CpxR, Lrp, HU, IHF, and H-NS (Figure 5). Belonging to the AraC/XylS family of transcriptional regulators are Rob, SoxS, and MarA, and they function by repressing OmpF expression through activation of *micF* transcription (*31*, *40*−*49*). The histidine kinase receptor CpxA responds to different stimuli, some of which include misfolded proteins and alkaline pH, and functions in conjunction with the response regulator CpxR to positively and negatively regulate *ompC* and *ompF*, respectively (*50*−*52*). The activity of the regulator Lrp increases when the cell is exposed to conditions of limited accessibility to nutrients such as that of minimal medium, where this protein negatively regulates *ompC* and positively regulates *ompF* through repression of

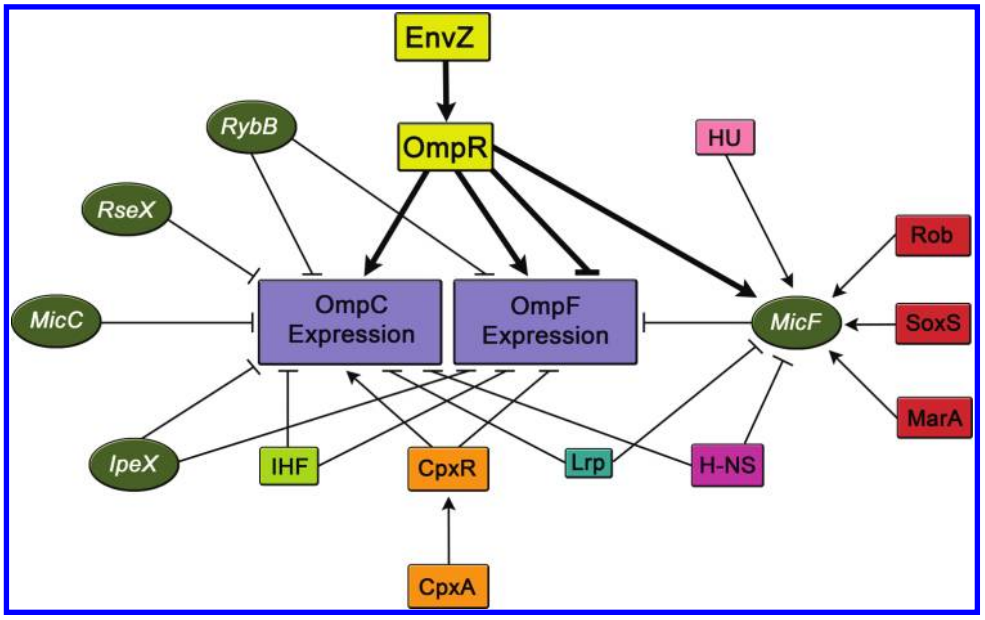

FIGURE 5: The complexity of OmpC and OmpF porin expression regulation. These are a number of the several different factors within the cell which are involved in the regulation of OmpC and OmpF porin expression. Various constituents illustrated include regulatory sRNAs (green circles) and indirect and direct protein regulators (multicolored boxes). The → symbol represents gene activation and −| represents gene repression.

micF (*53*, *54*). Histone-like proteins such as HU, IHF, and H-NS are also involved in the regulation of OmpC and OmpF. The nucleoid protein HU partakes in porin regulation through its involvement in a pathway which decreases OmpF levels through regulation of *micF* expression (*55*). IHF is a DNA-binding protein that functions not only by negatively regulating the *ompR-envZ* operon but also by negatively regulating both *ompC* and *ompF* by binding near their promoter region (*56*−*59*). The histone-like protein H-NS plays a role through repression of *ompC* and affects OmpF expression through the regulation of *micF* (*60*, *61*). Due to the complexity of porin regulation it is possible that one of these other pathways may take over the regulation of the *ompC* gene when EnvZ is absent.

Since there is an increasing demand to overcome the difficulties facing the structural studies of integral membrane proteins, biochemists and structural biologists have looked into alternative modes not only to increase the expression level of integral membrane proteins but also to ensure that these proteins are functionally active. We describe in this study that the Mistic-fusion system provided one such alternative, by not only increasing the expression level of EnvZ but also preserving its functional activity both *in vitro* and *in vivo*.

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