***In vivo* cross-linking and transmembrane helix dynamics support a bidirectional non-piston model of signaling within *E. coli* EnvZ**

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Abstract

In Gram-negative bacteria, porins span the outer membrane and control the influx of several prominent groups of antibiotics. Thus, it should not be surprising that expression of these porins is often altered in clinical isolates exhibiting multidrug resistance (MDR). The major regulator of porin expression in *Escherichia coli* is EnvZ, a canonical sensor histidine kinase (SHK). It allosterically processes periplasmic interactions with MzcA and cytoplasmic osmosensing into a single unified change in the ratio of its kinase and phosphatase activities. Unfortunately, the role of the EnvZ transmembrane domain (TMD) in bidirectional communication of these signals remains not well understood. Here, we employed *in vivo* sulfhydryl-reactivity to probe the dynamics of the TM2 helices and demonstrate that upon stimulus perception, only the region proximal to the periplasm undergoes conformational rearrangement. Furthermore, *in silico* coarse-grained molecular dynamics (CG-MD) simulations with aromatically tuned variants of EnvZ TM2 demonstrate the existence of both tilting and azimuthal rotational components to transmembrane communication while ruling out piston-type repositioning of TM2. Finally, in contrast to a similar analysis of TM1, we identified position-specific mutants possessing a “flipped” phenotype by dual-color fluorescent reporter analysis suggesting that both the periplasmic and cytoplasmic ends of TM2 are critical for maintenance of EnvZ signal output. Taken together, these data strongly support that EnvZ employs a non-piston-type mechanism during transmembrane communication. We conclude by discussing these results within the context of allosteric processing by EnvZ and propose that these results can be used to predict and classify transmembrane communication by various SHKs.

Importance

The EnvZ sensor histidine kinase serves as the major regulator of porin expression within *Escherichia coli*. A long-standing question is how stimulus perception by a bacterial receptor on one side of a biological membrane is transmitted to the opposite side of the membrane. To address this question, we monitored the dynamics of the transmembrane domain of EnvZ *in vivo* and coupled these results with *in silico* simulations of membrane-embedded EnvZ transmembrane domains. Taken together, these results demonstrate that detection of osmotic stress by the cytoplasmic domain of EnvZ results in non-piston communication across the inner membrane of *E. coli*. Thus, in addition to understanding how

92 EnvZ regulates porin balance and antibiotic influx, these results contribute to answering the long-
93 standing question of how transmembrane communication is performed by bacterial receptors. Our work
94 concludes with a framework that correlates receptor domain composition and signal transduction
95 mechanisms that could be employed by other research groups on their particular receptors of interest.
96

97   **Keywords**

98   porin balance / transmembrane communication / coarse-grained molecular dynamics / sulfhydryl-

99   reactivity / transmembrane helix dynamics

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Introduction

Most porins involved in antibiotic transport by Gram-negative bacteria belong to the classical OmpF and OmpC families (1). Transcription of these porins is governed by the intracellular concentration of phospho-OmpR (OmpR-P), which is controlled by EnvZ in response to changes in periplasmic interactions with MzcA and environmental osmolarity (2-7) (Fig. 1A). At low intracellular levels of OmpR-P, transcription of *ompF* is upregulated, whereas at higher levels of OmpR-P, transcription of *ompF* is repressed and transcription of *ompC* is activated. This results in a predominance of OmpF at low osmolarity and OmpC at higher osmolarities or in the presence of MzcA (8-10) (Fig. 1B). Dramatic modification of porin balance, which has been observed within clinical isolates from patients undergoing antibiotic treatment, strongly supports further characterization of the underlying mechanisms of porin regulation by EnvZ (11-17). In addition, it was recently shown that mutations in EnvZ within a porin-deficient (*ompC ompF*) *E. coli* strain resulted in increased carbapenem resistance (18). Thus, in addition to maintenance of porin balance, EnvZ also plays another not well-understood role in mediating antibacterial resistance that warrants further elucidation.

A substantial body of evidence suggests that EnvZ participates in bidirectional allosteric processing. A cytoplasmic fragment of EnvZ (EnvZ_c), consisting of residues Arg-180 through Gly-450, has been shown to mediate physiologically appropriate responses to increasing NaCl and sucrose concentrations *in vitro* and to increasing sucrose *in vivo* (19). These results led the suggestion that the periplasmic and transmembrane domains of EnvZ play a negligible role in osmosensing with the exception of anchoring EnvZ within the membrane and thereby reducing a three-dimensional search by OmpR to a two-dimensional search (19). In addition, it was initially suggested membrane anchoring might reduce the conformational dynamics of EnvZ to physiologically relevant levels, as EnvZ_c was found to have greater specific activity than full-length membrane-bound EnvZ (19, 20). Further studies demonstrated that peripheral interactions between the cytoplasmic domains and the lipid membrane were essential in increasing the kinase activity of the cytoplasmic domain of EnvZ, including the observation of a roughly 25-fold in EnvZ_c signal output while maintaining regulation, *i.e.* a 2-fold increase in autophosphorylation, in response to sucrose *in vitro* (21). In summary, these results confirm that molecular dynamics within the cytoplasmic domain of EnvZ govern response to osmolarity and that lipid-receptor interactions play a modulating the signal output of EnvZ.

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134 Alternatively, MzrA, modulator of EnvZ and OmpR protein A, was identified as a suppressor of a
135 $\Delta bamB \Delta degP$ double mutation. Upon further analysis, it was shown that MzrA is an upstream
136 regulator of EnvZ signal output that is independent of signal output modulation due to osmolarity, pH
137 and procaine (6). MzrA was subsequently shown to localize to the inner membrane and interact with
138 EnvZ within the periplasmic space (7) to serve as a connector between the CpxA/CpxR and
139 EnvZ/OmpR signaling circuits (6). It is also known as a connector of which only a few examples have
140 been found (22-24).

141

142 When taken together, these results demonstrate that the TMD of EnvZ is responsible for allosterically
143 coupling sensory input from the attached periplasmic and cytoplasmic domains. By extension,
144 bidirectional communication through the TMD is required in order to properly modulate porin
145 expression. Thus, we were interested in how cytoplasmic osmosensing could modulate the
146 conformational dynamics of the TMD and subsequently that of the periplasmic domain. Understanding
147 how EnvZ transduces signal across the biological membrane would be a significant step toward direct
148 manipulation of porin balance in bacterial cells exhibiting MDR.

149

150 To address these lines of enquiry, we have created a single-Cys-containing library of EnvZ mutants
151 across the second TM helix (TM2) that physically connects the periplasmic and the cytoplasmic
152 domains of EnvZ. *In vivo* analysis of this library revealed three regions within the TM domain, of
153 which the periplasmic region undergoes a non-piston-type conformational change. CG-MD simulations
154 of the aromatically tuned and wild-type EnvZ TM2 variants supported these results. We conclude by
155 discussing these results in the context of governance of porin balance and that mechanisms of
156 transmembrane communication can be classified based on the periplasmic domains that the bacterial
157 receptor possesses.

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159

160 **Results**

161

162 *Creating a single-Cys-containing library within TM2 of EnvZ*

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We previously created a Cys-less version of EnvZ from *E. coli* that had its sole Cys residue changed to an Ala residue (C277A). The Cys-less variant is expressed from pRD400, which results in the addition of a seven-residue linker (GGSSAAG) and a C-terminal V5 epitope (GKPIPPLLGLDST). We previously found that the Cys-less version of EnvZ had similar steady-state signal output and response to environmental osmolarity as the wild-type version of EnvZ making it suitable for comparisons of *in vivo* sulfhydryl-reactivity and signal output analysis. We initially determined that no major rearrangements occur along the TM1-TM1' interface upon stimulus perception (25). As minimal change was observed along this helical interface in response to osmolarity, we continued by examining the TM2-TM2' interface. We began by determining which residues comprise TM2 by subjecting the full EnvZ sequence to DGPred (26) and TMHMM v2.0 (27), which suggested that Leu-160 to Ile-181 and Leu-160 to Ile-179 comprise TM2 respectively. Based on these analyses, we employed site-directed mutagenesis using the Cys-less variant as a template to create a library of single-Cys-containing EnvZ proteins that spanned from positions 156 to 184 (Fig. 2). We observed that nearly the entire library was expressed within EPB30/pRD400 cells grown under the low- or high-osmolarity regime. Variants possessing a Cys at position 156 showed low levels of expression when grown under the low-osmolarity (0% sucrose) regime. However, when grown under the high-osmolarity regime, no variants showed reduced expression level (Fig. S1). These results indicate that the library was suitable for further *in vivo* experimentation.

Mapping TM2 surfaces important for maintenance of EnvZ signal output

We began by expressing each of the single-Cys-containing variants in EPB30/pRD400 cells, which allowed us to measure CFP fluorescence, YFP fluorescence, and to calculate the CFP/YFP ratio that estimates steady-state EnvZ signal output (Fig. 1B). Cells expressing the Cys-less C277A were used as a baseline comparison (Fig. S2). When EPB30/pRD400 cells are grown under the low-osmolarity regime, a shift in signalling output toward the “on” or kinase-dominant state results in increased CFP fluorescence, reduced YFP fluorescence and an increase in the overall CFP/YFP ratio, while a shift toward the “off” or phosphatase-dominant state appears as decreased CFP, increased YFP and a decrease in CFP/YFP ratio (Fig. 3 and S3).

Several trends were observed during analysis of the library of Cys-containing EnvZ receptors. When

195 EPB30/pRD400 cells were grown under the low-osmolarity regime, EnvZ was less tolerant of Cys
 196 substitutions at the N- and C-terminal regions of the library. At the N-terminus, signal output from
 197 receptors containing a Cys at positions 156, 162 and 163 were very elevated, exhibiting greater than a
 198 5-fold increase in CFP/YFP, while receptors possessing a Cys in the C-terminus at positions 179, 181,
 199 182 and 184 were elevated, possessing over a 2-fold increase in CFP/YFP. These boundary regions
 200 appear to flank a core of alternating increases and decreases in EnvZ signal output, as observed
 201 between residue positions 165 and 180, suggesting that multiple tightly packed EnvZ helices exist
 202 within the hydrophobic core of the inner membrane (Fig. 3A and 3B). When grown under the high-
 203 osmolarity regime, a pattern appeared where Cys substitutions resulted in significant decreases in
 204 signal output (Fig. 3C). Of the 29 mutants analysed, 13 supported less than 75% of the normal wild-
 205 type signal output.

206
 207 Most striking is the inverse effect on EnvZ signal output of the Cys substitutions that flank the
 208 hydrophobic core of TM2. For these residues, when grown under the low-osmolarity regime, the
 209 presence of a Cys resulted in an increase in signal output of more than 25%, i.e. shifted toward the on
 210 or kinase-dominant state (red dots in Fig. 3A) and a reduction in signal output of more than 25%, i.e.
 211 shifted toward the off or phosphate-dominant state, when grown under the high-osmolarity regime
 212 (blue dots in Fig. 3C). These flipped positions reside at the N- and C-terminal ends of the region we
 213 examined and outside of the proposed hydrophobic TMD core (Fig. 3). Within the hydrophobic core,
 214 Cys substitutions show similar changes when cells are grown under the low- and high-osmolality
 215 regimes. These results suggest that the flanking regions are not simply rigid structural conduits for
 216 signal transduction but may have higher-order roles in signal transduction, such as MzrA interaction or
 217 functioning as a control cable at the N- and C-terminal regions respectively (6, 7, 28-31).

218 219 *Identifying surfaces involved in TM2-TM2' interactions*

220
 221 Sulfhydryl-reactivity experimentation is well-characterised and has been employed on many soluble
 222 and membrane-spanning proteins and higher-order complexes (32). The *in vivo* nature of this assay
 223 facilitated mapping of the TM2-TM2' interface under different osmotic conditions, which is an
 224 important first step toward understanding how EnvZ processes allosteric inputs from periplasmic MzrA
 225 binding and cytoplasmic osmosensing into a single uniform modulation of bacterial porin balance (Fig.

1). In a similar manner to mapping TM1-TM1' interactions (25), Cys-containing EnvZ variants were expressed in EPB30/pRD400 cells and upon entering the early exponential phase ($OD_{600nm} \approx 0.25$) they were subjected to 250 μ M molecular iodine for 10 minutes analysed by non-reducing SDS-PAGE and immunoblotting (Fig. S4).

We observed three distinct regions within TM2. The N-terminal region (region I in Figure 3), comprised of residues 156 to 163, exhibited significant cross-linking under the low-osmolarity regime (0% sucrose) and almost no crosslinking under the high-osmolarity (15% sucrose) regime. The second region (II) consisting of positions 164 to 179, demonstrated altering low and high levels of disulphide-formation consistent the crossing of TM2 and TM2' within the hydrophobic core of the TMD. The final region (III), from residues 180 to 184, shows no crosslinking (Fig. 4). It should be noted that this significant difference at the periplasmic end of the TMD between cells grown under the low- and high-osmolarity regime was not observed during similar analyses of TM1 (25).

Molecular simulations of the wild-type and aromatically tuned variants of EnvZ TM2

The piston-type model of transmembrane communication is founded upon the central tenant that the vertical position of TM2 relative to the lipid bilayer changes upon stimulus perception. Previous work with TM2 of Tar demonstrates that repositioning the aromatic residues at the cytoplasmic end of TM2, known as aromatic tuning (33), repositions the helix within a biological membrane (34) and that this repositioning causes an incremental change in signal output (33). These results served as an experimental framework to optimise SIDEKICK software capable of high-throughput parallelised coarse-grained molecular dynamics (CG-MD) simulations, which demonstrated that aromatic tuning repositioned the TM2 helix *in silico* in a manner consistent with both the *in vivo* results and a piston-type mechanism of transmembrane communication (35).

Based on these extensive results and the recent suggestion that mechanisms employed by SHKs during TM communication correlate with the presence of a membrane-adjacent HAMP domain (36), we performed analogous *in silico* experimentation with EnvZ, which possesses a membrane-adjacent HAMP domain but substitutes a periplasmic PDC/CACHE domain for the four-helix bundle present in Tar and NarQ (36-39). We previously performed aromatic tuning with TM2 of EnvZ and found that

signal output was not correlated with the absolute vertical position of the aromatic residues as was shown with Tar TM2, which suggested that EnvZ does not transduce signal output across the membrane by a piston-type displacement (40).

Interpretation and assessment of the CG-MD results

To interpret the results of our GC-MD analysis, we categorised the signal output of these aromatically tuned EnvZ variants (40). For several two-component signalling circuits, including the EnvZ/OmpR, PhoQ/PhoP and CpxA/CpxR circuits, the steady-state output of the signalling circuits has been shown to be independent of the level of SHK present (40-43). However, in circuits containing the tuned variants of EnvZ, a different relationship between steady-state signal output and receptor level was observed, suggesting that the ratio of kinase to phosphatase activities was different within each receptor and always different than wild-type EnvZ (40). Based on this analysis, we found that the WLF-1 variant possessed the highest signal output while the WLF-5, WLF-4 and WLF-3 variants possessed slightly higher activity than wild-type EnvZ, which maintained receptor-concentration dependent robustness unlike the tuned variants. WLF-2 and WLF+1 were found to possess reduced signal output, while WLF+2 possessed the lowest overall signal output. These differences were previously quantified by analysing the slope of the change in CFP/YFP against the amount of receptor present and are visually represented in Figure S5 (40). These classifications are employed during interpretation of the CG-MD results in Figure 5.

Based on these previous results and the absence of asymmetric TM2 displacement observed within the *in vivo* sulfhydryl-reactivity assay (Fig. 4), we assessed whether molecular simulations would lend credibility to a non-piston type of transmembrane communication employed by EnvZ. We began by designing tuned TM2 sequences to subject to GC-MD simulations that matched those previously used during the aromatic tuning experimentation (Fig. 5A). Snapshots of individual frames and a movie from the CG-MD analysis with the wild-type EnvZ TM2 segment can be found in the Supporting Information (Fig. S6 and Movie S1). Unlike analogous experimentation with TM2 of Tar, no trend in helix displacement was observed (Fig. 5B, Table S1). This indicates that the mutations do not in isolation move the helix up and down in the membrane and would appear to rule out a pure piston motion as previously observed in Tar (35).

288

289 An alternative mechanism for transmembrane communication is to induce a tilt in the helix relative to
 290 the bilayer normal. This was originally proposed in Tar on the basis of crystallographic analysis of the
 291 receptor domain (44), and led to the proposal of a “swinging piston” where both displacement and tilt
 292 contributed to signal transduction. In addition, this would be consistent with a scissor-type model of
 293 TM communication proposed to be utilised by PhoQ (45, 46), which possesses a periplasmic
 294 PDC/CACHE domain (47). In testing the tuned EnvZ TM2 helices, a change in the extent of tilting was
 295 observed during the CG-MD simulations (Fig.5C, Table S2). It was found that a reduction in tilt, to a
 296 mean tilt of between 22 and 24 degrees, compared to the wild-type TM2 sequence with a tilt of
 297 approximately 26 degrees correlated with a decreased signal output (phosphatase-biased, dark blue).
 298 Interestingly, an increase in tilt to more than 29 degrees correlated with extremely biased signal output,
 299 *i.e.* kinase-dominant (WLF-1, bright red) and phosphatase-dominant (WLF+2, bright blue). Taken
 300 together, these results demonstrate that tilt, rather than vertical displacement, does correlate with
 301 modulated signal output from EnvZ.

302

303 Finally, a rotational/gearbox model of TMD-HAMP communication has also been proposed for EnvZ
 304 (48, 49). Central to this model is that interconversion between the kinase-dominant and phosphatase-
 305 dominant signalling states can be accomplished by rotation of individual HAMP helices by 26 degrees.
 306 With our simulations, we observed some consistency with this gearbox model of HAMP signalling,
 307 especially when the WLF-1 helix possessed a mean azimuthal rotation that is 25 degrees different than
 308 wild-type EnvZ (bright red in Figure 5D). In the full-length EnvZ WLF-1 receptor, this variant
 309 possessed the highest signal output. Further rotation of an additional 15 degrees for a total of 40
 310 degrees from wild-type correlated with the most phosphatase-dominant receptor, WLF+2. For the other
 311 simulated helices, it was not possible to differentiate them into separate classes.

312

313 Discussion

314

315 *Non-piston transmembrane communication by EnvZ*

316

317 EnvZ has been shown to allosterically process cytoplasmic changes in osmolarity and upon interaction
 318 with MzrA within the periplasmic. Here, our *in vivo* analysis demonstrated that only the periplasmic

end of EnvZ TM2 undergoes a conformational transition upon cytoplasmic stimulus perception and suggests that the asymmetric piston-type displacement employed by Tar is not used by EnvZ. To our knowledge, this is the first example of a periplasmic end of TM2 being affected by a cytoplasmic stimulus observed within EnvZ. Various experimentation has also been performed with the aromatically tuned variants of TM2 from both Tar and EnvZ. Previously, a linear correlation was observed between the position of the aromatic residue in Tar TM2, the position of the helices *in vitro* and *in silico* and the signal output from each Tar receptor (33, 34, 40, 50). Here, *in silico* analysis of EnvZ TM2 demonstrates that such a linear correlation is absent and that EnvZ functions by a non-piston mechanism in which both tilting and azimuthal rotation play a substantial role in modulation of signal output (Figure 5).

Correlations between domain composition and transmembrane communication

Comparisons of recently published *apo* and *holo* high-resolution (~1.9 Å) crystal structures of the *E. coli* nitrate sensor NarQ that contain the periplasmic, TM and HAMP domains reveal extensive structural rearrangements involving a piston-like motion of TM1 relative to TM2 of approximately 2.5 Å. These displacements result in a lever-like rotation of individual HAMP domains upon binding of cognate ligand (36). Based on these results, the authors posit that receptors containing a membrane-adjacent HAMP domain function by a piston-type displacement of TM helices while those that lack such domains transduce signal by rotation of TM helices. We previously postulated a related yet different categorization of signaling mechanisms also based on the domain structure of bacterial receptors (51). We proposed that receptors containing a periplasmic four-helix bundle transduce signal across the membrane by piston-type displacements and that the attached membrane-adjacent HAMP domains might possess one of a multitude of signaling mechanisms including a gearbox-type rotation (48), a dynamic bundle (28) or potentially other mechanisms (51). Differentiating between these classification systems will provide a theoretical framework for understanding domain-based intra-protein allosteric communication by bacterial receptors. A recent authoritative structural-based review of transmembrane communication by bacterial receptors addresses these different suggestions (52).

This results presented here also examines whether SHKs that possess membrane-adjacent HAMP domains function solely by piston-type displacements or whether other signaling mechanisms might be

employed. The results here with EnvZ should be compared with previous findings from the aspartate chemoreceptor (Tar) and the recent NarQ structures (36, 53-55) as these three are ideal candidates for comparison because they all possess a membrane-adjacent HAMP domain, however, while Tar and NarQ possess a periplasmic four-helix bundle, EnvZ possesses a periplasmic PDC/CACHE domain (37, 38). The authors of the recent NarQ structures posit that the presence or absence of the membrane-adjacent HAMP domain may be the difference between receptors employing piston-type mechanisms of transmembrane communication as compared to other mechanisms (36). However, differences employed during transmembrane communication by the Tar and EnvZ TMDs observed here and previously strongly suggest that Tar and EnvZ possess different mechanism of TM communication even though both possess a membrane-adjacent HAMP domain. Our previous work analyzed AS1 helices from *E. coli* NarX, *E. coli* Tar, *E. coli* EnvZ and Af1503, the HAMP domain resulting in the initial high-resolution structure (48), and found that the Tar and NarX AS1 helices possess similar properties, which the AS1 helices from both EnvZ and Af1503 fail to possess. Recent comparisons of the *apo* and ligand-bound structures of the combined periplasmic-TM-HAMP domain from *E. coli* NarQ demonstrate that binding of ligand results in symmetrical displacements of TM1 relative to TM2 of approximately 2.5 Å (36). These results are similar to Tar, which functions by asymmetrical TM2 displacement also possesses a periplasmic four-helix-bundle (39, 53-55).

Conclusion

Based on these results, we conclude that *E. coli* EnvZ functions by a non-piston mechanism of transmembrane communication that is different than Tar, NarX and NarQ, which communicate across the membrane by piston-type displacements. Furthermore, we propose that TM signaling mechanisms can be predicted and assigned based upon the domain(s) present in the periplasmic region of a bacterial membrane-spanning receptor.

Materials and Methods

Bacterial strains and plasmids

380 *E. coli* strains DH10B (New England Biolabs) or MC1061 (56) were used for DNA manipulations,
381 while strain K-12 MG1655 (57) served as a non-fluorescent strain that was used to control for light
382 scattering and cellular autofluorescence. *E. coli* strains MDG147 [MG1655 $\Phi(ompF^+-yfp^+)$ $\Phi(ompC^+-$
383 $cfp^+)$] (58) and EPB30 (MDG147 *envZ::kan*) (43) were employed for analysis of EnvZ signal output.
384 As the C-terminus of bacterial receptors can be sensitive to the presence of an epitope tag, we
385 previously ensured that the addition of a V5-epitope tag did not alter the signaling properties of EnvZ
386 (40, 59). Plasmid pEB5 was employed as an empty control vector (41). Plasmid pRD400 (40) retains
387 the IPTG-based induction of EnvZ from plasmid pEnvZ (60) while adding a seven-residue linker
388 (GGSSAAG) (61) and a C-terminal V5 epitope tag (GKIPNPLLGLDST) (62). Plasmid pEB5 was
389 employed as an empty control vector.

390

391 *Selection of residues comprising TM2 of EnvZ*

392

393 The primary sequence of EnvZ from *E. coli* K-12 MG1655 was subjected to DGPred using a minimal
394 window of 9 residues and a maximal window of 40 residues (26). Alternatively, a software package
395 that identifies TM helices with a Markov model (TMHMM v2.0) (27) was also employed. These
396 software packages suggested that Leu-160 to Ile-181 and Leu-160 to Ile-179 comprise TM2
397 respectively. Based on these results and to maximize the probability of including all residues within
398 TM2, we targeted all residues between positions 156 to 184 for the creation of a library of single-Cys-
399 containing EnvZ receptors.

400

401 *Analysis of EnvZ signal output in vivo*

402

403 Bacterial cultures were grown as described previously (40) with minor modification. MDG147 or
404 EPB30 cells were transformed with pRD400 expressing one of the single-Cys-containing EnvZ
405 receptors or pEB5 (empty). Fresh colonies were used to inoculate 2-ml overnight cultures of minimal
406 medium A (63) supplemented with 0.2% glucose. Ampicillin, sucrose and IPTG were added as
407 appropriate. Cells were grown overnight at 37 °C and diluted at least 1:1000 into 7 ml of fresh medium.
408 Upon reaching an $OD_{600nm} \approx 0.3$, chloramphenicol was added to a final concentration of 170 μ g/ml.
409 Fluorescent analysis was immediately conducted with 2 ml of culture and a Varian Cary Eclipse (Palo
410 Alto, CA). CFP fluorescence was measured using an excitation wavelength of 434 nm and an emission

wavelength of 477 nm, while YFP fluorescence was measured using an excitation wavelength of 505 nm and an emission wavelength of 527 nm. These values were corrected for cell density and for light scattering/cellular autofluorescence by subtracting the CFP and YFP fluorescence intensities determined for MG1655/pEB5 cells.

Analysis of sulfhydryl-reactivity in vivo

Cells were grown as described above with minor changes. Upon reaching an $OD_{600nm} \sim 0.3$, cells were subjected to between 250 μ M molecular iodine for 10 min while incubating at 37 °C. The reaction was terminated with 8 mM N-ethylmaleimide (NEM) and 10 mM EDTA. Cells were harvested by centrifugation and resuspended in standard 6X non-reducing SDS-PAGE buffer supplemented with 12.5 mM NEM. Cell pellets were analysed on 10% SDS/acrylamide gels. Standard buffers and conditions were used for electrophoresis, immunoblotting and detection with enhanced chemiluminescence (64). Anti-V5 (Invitrogen) was the primary antibody and peroxidase-conjugated anti-mouse IgG (Sigma) was the secondary antibody. Digitized images were acquired with a ChemiDoc MP workstation (Bio-RAD), analysed with ImageJ v1.49 (65) and quantified with QtiPlot v0.9.8.10.

CG-MD simulations with SIDEKICK

As previously described (35), coarse-grained molecular dynamics (CG-MD) simulations were performed using the MARTINI forcefield with an approximately 4:1 mapping of non-H atoms to CG particles. Lennard-Jones interactions between 4 classes of particles: polar (P), charged (Q), mixed polar/apolar (N) and hydrophobic apolar (C) were used to treat interparticle interactions. Within MARTINI, P and C particle types were subdivided to reflect varying degrees of polarity. Short range electrostatic interactions were treated Coulombically, shifted to zero between 0 and 12 Å. Lennard-Jones interactions were shifted to zero between 9 and 12 Å. α -helix integrity was maintained via dihedral restraints. Peptide termini were treated as uncharged. Simulations were performed using Gromacs 3 (66). Temperature was coupled using a Berendsen thermostat at 323 K ($\tau_T = 1$ ps), and pressure was coupled semi-isotropically (across XY/Z) at 1 bar (compressibility $= 3 \times 10^{-5}$ bar⁻¹, $\tau_P = 10$ ps). The initial simulation timestep was 20 fs. Initial models of the TM α -helices were generated as ideal, atomistically detailed α -helices using standard backbone angles and side-chain

conformers. These were then converted to coarse-grained as described previously(35). Around 128 DPPC molecules were used in each simulation along with around 3000 CG water particles, giving a final system size of $\square 65\times 65\times 13 \text{ \AA}$.

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Figure legends

Figure 1. Monitoring modulation of EnvZ signal output upon stimulus perception. (A) EnvZ signal output controls porin expression. It is a bifunctional sensor histidine kinase with both kinase and phosphatase activities. The ratio of these activities is modulated by the presence of extracellular osmolarity and the absence/presence of MzrA (blue). (B) The intracellular level of phosphorylated OmpR (OmpR-P) is controlled by EnvZ signal output and OmpR-P levels govern transcription of *ompF* and *ompC*. Strains MDG147 and EPB30 ($\Delta envZ$) contain transcriptional fusions of *yfp* to *ompF* (yellow) and *cfp* to *ompC* (cyan), which facilitates easy monitoring of intracellular OmpR-P levels by measuring the CFP/YFP ratio. The dashed line indicates an estimation of the baseline OmpR-P levels from EPB30/pRD400 cells grown under the low-osmolarity regime (0% sucrose).

Figure 2. EnvZ functions as a homodimer with a cytoplasmic N-terminus, the first transmembrane helix (TM1, white), a large periplasmic domain (sensor, white), the second transmembrane helix (TM2, red), a membrane-adjacent HAMP domain (grey) and the cytoplasmic domains responsible for dimerization and histidylphosphotransfer (DHp, black) and catalytic ATPase activity (CA, black). The position of the original Cys-277 residue that was mutated to Ala to produce the Cys-less EnvZ is provided. The residues subjected to Cys substitution and their position in the primary sequence is provided. Signal output from each single-Cys-containing variant is compared to the Cys-less (C277A) variant: less than

473 50% of Cys-less (light blue), between 50% and 75% of Cys-less (dark blue), between 75% and 125%
474 of Cys-less (grey), between 125% and 200% (dark red) and greater than 200% (light red). Residue
475 positions exhibited flipped signal output are indicated with a plus. The extent of sulphhydryl-reactivity is
476 also presented in five categories based on dimer-to-monomer ratio: no dimer present (white), less than
477 0.05 (light grey), between 0.05 and 0.2 (medium grey), between 0.2 and 0.5 (dark grey) and greater
478 than 0.5 (red). Positions that exhibit a significant change in cross-linking between the low- and high-
479 osmolarity regimes are indicated with a plus.

480

481 Figure 3. Signal output from the library of single-Cys EnvZ variants. (A) CFP/YFP from
482 EPB30/pRD400 cells expressing one of single-Cys variants grown under the low-osmolarity (0%
483 sucrose) regime. On the right axis, these CFP/YFP ratios are compared to the Cys-less (C277A)
484 variant. (B) Magnified version of panel A in order to emphasise the region up to a 2-fold increase in
485 CFP/YFP. (C) CFP/YFP from EPB30/pRD400 cells expressing one of the single-Cys variants grown
486 under the high-osmolarity (15% sucrose) regime. On the right axis, these CFP/YFP ratios are compared
487 to the Cys-less (C277A) variant. The flipped mutants are highlighted with a red dot in panel A
488 (increased signal output) and a blue dot in panel C (decreased signal output). The shaded areas
489 represent the mean signal output from the Cys-less variant of EnvZ with a range of one standard error
490 of mean. These values are provided to aid in comparison. Error bars represent standard deviation of the
491 mean with a sample size of $n \geq 3$.

492 Figure 4. Extent of sulphhydryl-reactivity for each single-Cys-containing EnvZ variant. EPB30/pRD400
493 cells were grown under the low- (empty circles, 0% sucrose) or high-osmolarity (filled circles, 15%
494 sucrose) regimes and subjected to 250 μ M molecular iodine for 10 minutes when their OD_{600nm} reached
495 approximately 0.25. As shown in Figure S5, this allowed us to determine the dimer/monomer ratio
496 represented on the Y-axis. Three distinct regions, denoted I, II and III were observed and are described
497 in the text. Error bars represent the standard error of the mean with a sample size of $n \geq 3$.

498

499 Figure 5. Aromatic tuning and signal output from EnvZ. (A) When aromatic tuning was performed in
500 EnvZ, a Trp-Leu-Phe triplet (red) was repositioned within the C-terminal region of TM2. (B) Helix
501 displacements, (C) tilt distributions and (D) azimuthal rotational distributions of the aromatically tuned
502 EnvZ TM2 helices. Histograms are shown for all time points of all membranes of each ensemble.
503 Aromatically tuned mutants have been classified based on their signal output as shown in Fig. S6 into

five categories: highly kinase biased (WLF-1; pink), moderately kinase biased (WLF-5, WLF-4, WLF-3; red), balanced (wild-type; black), moderately phosphatase biased (WLF-2, WLF+1, dark blue) and highly phosphatase biased (WLF+2, bright blue).

References

1. Nikaido H. 2003. Molecular basis of bacterial outer membrane permeability revisited. *Microbiol Mol Biol Rev* 67:593-656.
2. Egger LA, Inouye M. 1997. Purification and characterization of the periplasmic domain of EnvZ osmosensor in *Escherichia coli*. *Biochem Biophys Res Commun* 231:68-72.
3. Forst SA, Roberts DL. 1994. Signal transduction by the EnvZ-OmpR phosphotransfer system in bacteria. *Res Microbiol* 145:363-73.
4. Mizuno T. 1998. His-Asp phosphotransfer signal transduction. *J Biochem* 123:555-63.
5. Hoch JA, Silhavy TJ (ed). 1995. Two-Component Signal Transduction. American Society of Microbiology Press, Washington, DC, USA.
6. Gerken H, Charlson ES, Cicirelli EM, Kenney LJ, Misra R. 2009. MzrA: a novel modulator of the EnvZ/OmpR two-component regulon. *Mol Microbiol* 72:1408-22.
7. Gerken H, Misra R. 2010. MzrA-EnvZ interactions in the periplasm influence the EnvZ/OmpR two-component regulon. *J Bacteriol* 192:6271-8.
8. Forst SA, Delgado J, Inouye M. 1989. DNA-binding properties of the transcription activator (OmpR) for the upstream sequences of *ompF* in *Escherichia coli* are altered by *envZ* mutations and medium osmolarity. *J Bacteriol* 171:2949-55.
9. Lan CY, Igo MM. 1998. Differential expression of the OmpF and OmpC porin proteins in *Escherichia coli* K-12 depends upon the level of active OmpR. *J Bacteriol* 180:171-4.
10. Russo FD, Silhavy TJ. 1991. EnvZ controls the concentration of phosphorylated OmpR to mediate osmoregulation of the porin genes. *J Mol Biol* 222:567-80.
11. Elliott E, Brink AJ, van Greune J, Els Z, Woodford N, Turton J, Warner M, Livermore DM. 2006. In vivo development of ertapenem resistance in a patient with pneumonia caused by *Klebsiella pneumoniae* with an extended-spectrum beta-lactamase. *Clin Infect Dis* 42:e95-8.
12. Hernandez-Alles S, Conejo M, Pascual A, Tomas JM, Benedi VJ, Martinez-Martinez L. 2000. Relationship between outer membrane alterations and susceptibility to antimicrobial agents in isogenic strains of *Klebsiella pneumoniae*. *J Antimicrob Chemother* 46:273-7.

535 13. Jacoby GA, Mills DM, Chow N. 2004. Role of beta-lactamases and porins in resistance to
536 ertapenem and other beta-lactams in *Klebsiella pneumoniae*. *Antimicrob Agents Chemother*
537 48:3203-6.

538 14. Kaczmarek FM, Dib-Hajj F, Shang W, Gootz TD. 2006. High-level carbapenem resistance in a
539 *Klebsiella pneumoniae* clinical isolate is due to the combination of bla(ACT-1) beta-lactamase
540 production, porin OmpK35/36 insertional inactivation, and down-regulation of the phosphate
541 transport porin phoe. *Antimicrob Agents Chemother* 50:3396-406.

542 15. Loli A, Tzouvelekis LS, Tzelepi E, Carattoli A, Vatopoulos AC, Tassios PT, Miriagou V. 2006.
543 Sources of diversity of carbapenem resistance levels in *Klebsiella pneumoniae* carrying
544 blaVIM-1. *J Antimicrob Chemother* 58:669-72.

545 16. Martinez-Martinez L, Conejo MC, Pascual A, Hernandez-Alles S, Ballesta S, Ramirez De
546 Arellano-Ramos E, Benedi VJ, Perea EJ. 2000. Activities of imipenem and cephalosporins
547 against clonally related strains of *Escherichia coli* hyperproducing chromosomal beta-lactamase
548 and showing altered porin profiles. *Antimicrob Agents Chemother* 44:2534-6.

549 17. Mena A, Plasencia V, Garcia L, Hidalgo O, Ayestaran JI, Alberti S, Borrell N, Perez JL, Oliver
550 A. 2006. Characterization of a large outbreak by CTX-M-1-producing *Klebsiella pneumoniae*
551 and mechanisms leading to in vivo carbapenem resistance development. *J Clin Microbiol*
552 44:2831-7.

553 18. Adler M, Anjum M, Andersson DI, Sandegren L. 2016. Combinations of mutations in envZ,
554 ftsI, mrdA, acrB and acrR can cause high-level carbapenem resistance in *Escherichia coli*. *J*
555 *Antimicrob Chemother* 71:1188-98.

556 19. Wang LC, Morgan LK, Godakumbura P, Kenney LJ, Anand GS. 2012. The inner membrane
557 histidine kinase EnvZ senses osmolality via helix-coil transitions in the cytoplasm. *EMBO J*
558 31:2648-59.

559 20. Foo YH, Gao Y, Zhang H, Kenney LJ. 2015. Cytoplasmic sensing by the inner membrane
560 histidine kinase EnvZ. *Prog Biophys Mol Biol* 118:119-129.

561 21. Ghosh M, Wang LC, Ramesh R, Morgan LK, Kenney LJ, Anand GS. 2017. Lipid-Mediated
562 Regulation of Embedded Receptor Kinases via Parallel Allosteric Relays. *Biophys J* 112:643-
563 654.

564 22. Goulian M. 2010. Two-component signaling circuit structure and properties. *Curr Opin*
565 *Microbiol* 13:184-9.

566 23. Jung K, Fried L, Behr S, Heermann R. 2012. Histidine kinases and response regulators in
567 networks. *Curr Opin Microbiol* 15:118-24.

568 24. Misra R. 2016. Modulators of the bacterial two-component systems involved in envelope stress,
569 transport, and virulence. *In* de Bruijn FJ (ed), *Stress and Environmental Regulation of Gene*
570 *Expression and Adaptation in Bacteria*. Wiley-Blackwell, Hoboken, New Jersey, USA.

571 25. Heininger A, Yusuf R, Lawrence R, Draheim RR. 2016. Identification of transmembrane helix
572 1 (TM1) surfaces important for EnvZ dimerisation and signal output. *Biochim Biophys Acta*
573 1858:1868-1875.

574 26. Hessa T, Meindl-Beinker NM, Bernsel A, Kim H, Sato Y, Lerch-Bader M, Nilsson I, White
575 SH, von Heijne G. 2007. Molecular code for transmembrane-helix recognition by the Sec61
576 translocon. *Nature* 450:1026-30.

577 27. Krogh A, Larsson B, von Heijne G, Sonnhammer EL. 2001. Predicting transmembrane protein
578 topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305:567-
579 80.

580 28. Zhou Q, Ames P, Parkinson JS. 2009. Mutational analyses of HAMP helices suggest a dynamic
581 bundle model of input-output signalling in chemoreceptors. *Mol Microbiol* 73:801-14.

582 29. Wright GA, Crowder RL, Draheim RR, Manson MD. 2011. Mutational analysis of the
583 transmembrane helix 2-HAMP domain connection in the *Escherichia coli* aspartate
584 chemoreceptor tar. *J Bacteriol* 193:82-90.

585 30. Adase CA, Draheim RR, Manson MD. 2012. The residue composition of the aromatic anchor of
586 the second transmembrane helix determines the signaling properties of the aspartate/maltose
587 chemoreceptor Tar of *Escherichia coli*. *Biochemistry (Mosc)* 51:1925-32.

588 31. Adase CA, Draheim RR, Rueda G, Desai R, Manson MD. 2013. Residues at the cytoplasmic
589 end of transmembrane helix 2 determine the signal output of the TarEc chemoreceptor.
590 *Biochemistry (Mosc)* 52:2729-38.

591 32. Bass RB, Butler SL, Chervitz SA, Gloor SL, Falke JJ. 2007. Use of site-directed cysteine and
592 disulfide chemistry to probe protein structure and dynamics: applications to soluble and
593 transmembrane receptors of bacterial chemotaxis. *Methods Enzymol* 423:25-51.

594 33. Draheim RR, Bormans AF, Lai RZ, Manson MD. 2006. Tuning a bacterial chemoreceptor with
595 protein-membrane interactions. *Biochemistry (Mosc)* 45:14655-64.

596 34. Botelho SC, Enquist K, von Heijne G, Draheim RR. 2015. Differential repositioning of the
597 second transmembrane helices from E. coli Tar and EnvZ upon moving the flanking aromatic
598 residues. *Biochim Biophys Acta* 1848:615-21.

599 35. Hall BA, Armitage JP, Sansom MS. 2011. Transmembrane helix dynamics of bacterial
600 chemoreceptors supports a piston model of signalling. *PLoS Comput Biol* 7:e1002204.

601 36. Gushchin I, Melnikov I, Polovinkin V, Ishchenko A, Yuzhakova A, Buslaev P, Bourenkov G,
602 Grudinin S, Round E, Balandin T, Borshchevskiy V, Willbold D, Leonard G, Buldt G, Popov
603 A, Gordeliy V. 2017. Mechanism of transmembrane signaling by sensor histidine kinases.
604 *Science* doi:10.1126/science.aah6345.

605 37. Hwang E, Cheong HK, Kim SY, Kwon O, Blain KY, Choe S, Yeo KJ, Jung YW, Jeon YH,
606 Cheong C. 2017. Crystal structure of the EnvZ periplasmic domain with CHAPS. *FEBS Lett*
607 591:1419-1428.

608 38. Upadhyay AA, Fleetwood AD, Adebali O, Finn RD, Zhulin IB. 2016. Cache Domains That are
609 Homologous to, but Different from PAS Domains Comprise the Largest Superfamily of
610 Extracellular Sensors in Prokaryotes. *PLoS Comput Biol* 12:e1004862.

611 39. Mise T. 2016. Structural Analysis of the Ligand-Binding Domain of the Aspartate Receptor Tar
612 from *Escherichia coli*. *Biochemistry (Mosc)* 55:3708-13.

613 40. Norholm MH, von Heijne G, Draheim RR. 2015. Forcing the Issue: Aromatic Tuning
614 Facilitates Stimulus-Independent Modulation of a Two-Component Signaling Circuit. *ACS*
615 *Synth Biol* 4:474-81.

616 41. Batchelor E, Goulian M. 2003. Robustness and the cycle of phosphorylation and
617 dephosphorylation in a two-component regulatory system. *Proc Natl Acad Sci U S A* 100:691-
618 6.

619 42. Miyashiro T, Goulian M. 2008. High stimulus unmasks positive feedback in an autoregulated
620 bacterial signaling circuit. *Proc Natl Acad Sci U S A* 105:17457-62.

621 43. Siryaporn A, Goulian M. 2008. Cross-talk suppression between the CpxA-CpxR and EnvZ-
622 OmpR two-component systems in *E. coli*. *Mol Microbiol* 70:494-506.

623 44. Chervitz SA, Falke JJ. 1996. Molecular mechanism of transmembrane signaling by the
624 aspartate receptor: a model. *Proc Natl Acad Sci U S A* 93:2545-50.

625 45. Falke JJ. 2014. Piston versus Scissors: Chemotaxis Receptors versus Sensor His-Kinase
626 Receptors in Two-Component Signaling Pathways. *Structure* 22:1219-20.

627 46. Molnar KS, Bonomi M, Pellarin R, Clinthorne GD, Gonzalez G, Goldberg SD, Goulian M, Sali
628 A, DeGrado WF. 2014. Cys-Scanning Disulfide Crosslinking and Bayesian Modeling Probe the
629 Transmembrane Signaling Mechanism of the Histidine Kinase, PhoQ. *Structure* 22:1239-51.

630 47. Cheung J, Bingman CA, Reyngold M, Hendrickson WA, Waldburger CD. 2008. Crystal
631 structure of a functional dimer of the PhoQ sensor domain. *J Biol Chem* 283:13762-70.

632 48. Hulko M, Berndt F, Gruber M, Linder JU, Truffault V, Schultz A, Martin J, Schultz JE, Lupas
633 AN, Coles M. 2006. The HAMP domain structure implies helix rotation in transmembrane
634 signaling. *Cell* 126:929-40.

635 49. Inouye M. 2006. Signaling by transmembrane proteins shifts gears. *Cell* 126:829-31.

636 50. Yusuf R, Draheim RR. 2015. Employing aromatic tuning to modulate output from two-
637 component signaling circuits. *J Biol Eng* 9:7.

638 51. Unnerstale S, Maler L, Draheim RR. 2011. Structural characterization of AS1-membrane
639 interactions from a subset of HAMP domains. *Biochim Biophys Acta* 1808:2403-12.

640 52. Gushchin I, Gordeliy V. 2018. Transmembrane Signal Transduction in Two-Component
641 Systems: Piston, Scissoring, or Helical Rotation? *Bioessays* 40.

642 53. Falke JJ, Hazelbauer GL. 2001. Transmembrane signaling in bacterial chemoreceptors. *Trends*
643 *Biochem Sci* 26:257-65.

644 54. Hazelbauer GL, Falke JJ, Parkinson JS. 2008. Bacterial chemoreceptors: high-performance
645 signaling in networked arrays. *Trends Biochem Sci* 33:9-19.

646 55. Parkinson JS, Hazelbauer GL, Falke JJ. 2015. Signaling and sensory adaptation in *Escherichia*
647 *coli* chemoreceptors: 2015 update. *Trends Microbiol* 23:257-66.

648 56. Casadaban MJ, Cohen SN. 1980. Analysis of gene control signals by DNA fusion and cloning
649 in *Escherichia coli*. *J Mol Biol* 138:179-207.

650 57. Guyer MS, Reed RR, Steitz JA, Low KB. 1981. Identification of a sex-factor-affinity site in *E.*
651 *coli* as gamma delta. *Cold Spring Harb Symp Quant Biol* 45 Pt 1:135-40.

652 58. Batchelor E, Silhavy TJ, Goulian M. 2004. Continuous control in bacterial regulatory circuits. *J*
653 *Bacteriol* 186:7618-25.

654 59. Lai RZ, Bormans AF, Draheim RR, Wright GA, Manson MD. 2008. The region preceding the
655 C-terminal NWETF pentapeptide modulates baseline activity and aspartate inhibition of
656 *Escherichia coli* Tar. *Biochemistry (Mosc)* 47:13287-95.

657 60. Hsing W, Silhavy TJ. 1997. Function of conserved histidine-243 in phosphatase activity of
658 EnvZ, the sensor for porin osmoregulation in Escherichia coli. J Bacteriol 179:3729-35.

659 61. Cantwell BJ, Draheim RR, Weart RB, Nguyen C, Stewart RC, Manson MD. 2003. CheZ
660 phosphatase localizes to chemoreceptor patches via CheA-short. J Bacteriol 185:2354-61.

661 62. Southern JA, Young DF, Heaney F, Baumgartner WK, Randall RE. 1991. Identification of an
662 epitope on the P and V proteins of simian virus 5 that distinguishes between two isolates with
663 different biological characteristics. J Gen Virol 72 (Pt 7):1551-7.

664 63. Miller JH. 1992. A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for
665 Escherichia coli and Related Bacteria. Cold Spring Harbor Laboratory Press, Plainview, NY.

666 64. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K. 1998.
667 Current Protocols in Molecular Biology. Wiley, New York, NY.

668 65. Schneider CA, Rasband WS, Eliceiri KW. 2012. NIH Image to ImageJ: 25 years of image
669 analysis. Nat Methods 9:671-5.

670 66. Van Der Spoel D, Lindahl E, Hess B, Groenhof G, Mark AE, Berendsen HJ. 2005.
671 GROMACS: fast, flexible, and free. J Comput Chem 26:1701-18.

672

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Supplemental Information

Figure S1. Steady-state expression of EnvZ variants containing a single Cys residue within TM2. EPB30/pRD400 cells expressing one of the single-Cys-containing variants were grown under the low- (0% sucrose) or high-osmolarity (15% sucrose) regimes. Under the low-osmolarity regime, reduced steady-state levels of the D156C variant were observed. In addition, disulfide formation was observed for the F157C and L160C variants in the absence of any additional oxidizing agent. When EPB30/pRD400 cells were grown under the high-osmolarity regime, the F157C, L160C and F161C variants exhibited disulfide formation in the absence of any oxidizing agent.

Figure S2. Steady-state signal output from the Cys-variant of EnvZ. (A) CFP and YFP fluorescence from MDG147/pEB5 (filled) and EPB30/pRD400 C277A (Cys-less; empty) cells grown under the low- (0% sucrose) and high-osmolarity (15% sucrose) regimes. (B) The CFP/YFP ratio from MDG147/pEB5 (filled) and EPB30/pRD400 C277A (Cys-less; empty) cells grown under the low- and high-osmolarity regimes estimates EnvZ signal output. Error bars represent standard error of the mean with a sample size of $n \geq 3$. Reprinted from BBA Biomembranes, volume 1858, Annika Heininger, Rahmi Yusuf, Robert J. Lawrence and Roger R. Draheim, Identification of transmembrane helix 1 (TM1) surfaces important for EnvZ dimerisation and signal output, pages 1868-1875, Copyright 2016, with permission from Elsevier.

Figure S3. Signal output from the single-Cys-containing EnvZ variants. CFP (A) and YFP (B) fluorescence from EPB30/pRD400 cells expressing one of the receptors from the library grown under the low-osmolarity (0% sucrose) regime. CFP (C) and YFP (D) fluorescence from EPB30/pRD400 cells expressing one of the single-Cys-mutants from the library grown under the high-osmolarity (15% sucrose) regime. On the right axes, the ratio of signal output compared to EPB30/pRD400 cells expressing the Cys-less mutant is also presented to aid in comparison. In all panels, the shaded area represents the mean with a range of one standard deviation of the mean from EPB30/pRD400 cells expressing the Cys-less variant of EnvZ (Figure S2). Error bars represent standard error of the mean with an $n \geq 3$.

704 Figure S4. Immunoblotting analysis of the sulfhydryl-reactivity experimentation. EPB30/pRD400 cells
705 expressing one of the single-Cys-containing EnvZ receptors were grown under the low- (0%) or high-
706 (15%) regimes until an OD_{600nm} of approximately 0.25 was reached. Cultures were then subjected to
707 250 μM molecular iodine. Particular Cys-containing EnvZ receptors resulted in the presence of dimeric
708 EnvZ moieties that migrated at a slower rate than the monomeric species. A minimum of three
709 immunoblots were used for each of the data points present in Figure 3.

710

711 Figure S5. Comparison of signal output from osmosensing circuits containing the various aromatically
712 tuned EnvZ receptors. Steady-state signal output from circuits containing the WLF series of
713 aromatically tuned variants expressed in EPB30/pRD400 cells grown under the low (upper panel) or
714 high (lower panel) osmolarity regime is shown. The intracellular levels of phospho-OmpR are
715 estimated through use of the antisymmetrical reporter system presented in Figure S1B. Signal output at
716 low (open circles), medium (gray circles), and high levels (filled circles) of EnvZ-V5 expression (0.2,
717 0.5, and 0.8, respectively) is presented for comparison. The extent of sensitivity to changes in the
718 amount of EnvZ present is also summarized as robustness. In this column, N/A represents not
719 applicable, as in there is no reasonable amount of signal output, whereas REV indicates reversed,
720 where a decrease in activity is observed as the level of EnvZ-V5 increases. Reprinted from ACS
721 Synthetic Biology, volume 4, Morten Nøholm, Gunnar von Heijne and Roger R. Draheim, Forcing the
722 Issue: Aromatic Tuning Facilitates Stimulus-Independent Modulation of a Two-Component Signaling
723 Circuit, pages 474-481, Copyright 2015, with permission from the American Chemical Society.

724

725 Figure S6. A randomly selected (number 63) simulation of the wild-type EnvZ TM2 was selected for
726 demonstration. The blue and cyan dots represent water molecules, while the bronze spheres represent
727 the phosphate in DPPC. The backbone carbon atoms of the peptide are shown pink, while the yellow
728 spheres represent side chains.

729

730 Table S1: Mean displacement of the EnvZ TM2 segments in the membrane bilayer.

731

732 Table S2: Mean tilt of the EnvZ TM2 segments in the membrane bilayer.

733

734 Table S3: Mean azimuthal rotation of the EnvZ TM2 segments in the membrane bilayer.

735

736   Movie S1. A movie composed of frames from the simulation used for Figure S6. To compose the
737   movie, 500 frames spaced 200 ns apart were selected. The blue and cyan dots represent water
738   molecules, while the bronze spheres represent the phosphate in DPPC. The backbone carbon atoms of
739   the peptide are shown pink, while the yellow spheres represent side chains.

740

A **B**

Sensor

TM

HAMP

CA

DHp

A **B** **C**

A

EnvZ WLF-5: **I****M****L****L****W****L****F****A****I****G****G****A****I****R****I****Q****N****R**

EnvZ WLF-4: **I****M****L****L****A****W****L****F****I****G****G****A****I****R****I****Q****N****R**

EnvZ WLF-3: **I****M****L****L****A****I****W****L****F****G****G****A****I****R****I****Q****N****R**

EnvZ WLF-2: **I****M****L****L****A****I****G****W****L****F****G****A****I****R****I****Q****N****R**

EnvZ WLF-1: **I****M****L****L****A****I****G****G****W****L****F****A****I****R****I****Q****N****R**

EnvZ WLF 0: **I****M****L****L****A****I****G****G****A****W****L****F****I****R****I****Q****N****R**

EnvZ WLF+1: **I****M****L****L****A****I****G****G****A****G****W****L****F****R****I****Q****N****R**

EnvZ WLF+2: **I****M****L****L****A****I****G****G****A****G****G****W****L****F****I****Q****N****R**

B **C** **D**