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EnvZ/OmpR two-component signaling: an archetype system that can function non-canonically

Linda J. Kenney^{1,3}, Ganesh S. Anand²

¹Mechanobiology Institute, T-Lab, 5A Engineering Drive 1

²Department of Biological Sciences, National University of Singapore, Singapore

³Department of Biochemistry & Molecular Biology, University of Texas Medical Branch, Galveston, TX 77555 USA

Abstract

Two-component regulatory systems represent the major paradigm for signal transduction in prokaryotes. The simplest systems are composed of a sensor kinase and a response regulator. The sensor is often a membrane protein that senses a change in environmental conditions and is autophosphorylated by ATP on a histidine residue. The phosphoryl group is transferred onto an aspartate of the response regulator, which activates the regulator and alters its output, most often resulting in a change in gene expression. In this review, we present an historical view of the archetype EnvZ/OmpR two-component signaling system and then we provide a new view of signaling based on our recent experiments. EnvZ responds to cytoplasmic signals that arise from changes in the extracellular milieu (1), and OmpR acts canonically (requiring phosphorylation) to regulate the porin genes and non-canonically (without phosphorylation) to activate the acid stress response (2, 3). Herein we describe how insights gleaned from stimulus recognition and response in EnvZ are relevant to nearly all HK-RRs.

Keywords

EnvZ; OmpR; two-component regulatory system; phosphorylation; HDXMS

HISTORICAL ASPECTS OF ENVZ/OMPR SIGNALING

INTRODUCTION

Signal transduction in bacteria is largely achieved by two-component regulatory systems. The first component is a sensor kinase (HK), most often a membrane protein, which senses an environmental signal and is phosphorylated by ATP on a conserved histidine residue. The second component is a response regulator (RR), which catalyzes the transfer of the phosphoryl group from the histidine of the HK onto a conserved aspartic acid residue. Most RRs are two-domain proteins and phosphorylation of the N-terminal receiver domain alters the output of the C-terminal effector domain, which usually involves a stimulation of DNA

binding. In some systems, the HK alters the level of the phosphorylated RR (RR~P) by stimulating its dephosphorylation (referred to as phosphatase activity).

The HK EnvZ and its cognate RR OmpR are the products of the *ompB* operon and were historically identified as regulating the expression of outer membrane proteins OmpF and OmpC in response to changes in the osmolality of the growth medium (4, 5) (see Figure 1). The operon is organized with *ompR* upstream, and its stop signal overlaps the start of *envZ* (6), with the result that there is substantially greater OmpR protein compared to EnvZ (7). Approximately 50% of the two-component systems in *E. coli* are organized similarly. Porin levels are influenced by a wide variety of growth conditions, including pH (8–11), temperature (12), osmolality (13) and growth phase (14–16). In general, the total amount of OmpF and OmpC remains relatively constant, but their levels are reciprocally regulated by environmental stimuli (13). For example, at low osmolality, OmpF is the major porin in the outer membrane, and at high osmolality, *ompF* transcription is repressed and OmpC becomes the major porin. The two porins differ from one another by their pore size and flow rates, with OmpC exhibiting a slower flux and a smaller pore (17). It is believed that sensing and adjusting the porins in the outer membrane is one strategy whereby *E. coli* or *Salmonella* recognize whether or not they are in a host environment (high osmolality) compared to a dilute environment (low osmolality).

1.1 Domain Organization of EnvZ

EnvZ is composed of 450 amino acids and is located in the inner membrane. Two transmembrane domains (TM1 and TM2; residues 16–35 and 159–179) flank a 117 amino acid periplasmic domain (residues 36–158) (18). The cytoplasmic domain is divided into two functionally distinct subdomains (19, 20): a four-helix dimeric catalytic domain (amino acids 223–289) contains the histidine that is phosphorylated (His²⁴³), as well as the OmpR binding region (SINKDIEE, amino acids 269–276) (1, 3, 21). The ATP binding domain consists of amino acids 290–450. The catalytic domain is separated from TM2 by a HAMP linker (“histidine kinase, adenylyl cyclase, methyl-accepting chemotaxis protein and phosphatase”) of approximately 43 amino acids. The HAMP linker is structurally conserved among many sensor proteins and may play a role in signaling in some HKs (22).

The catalytic domain forms a stable homodimer in solution with an apparent molecular weight of 19 kDa (23). An NMR structure of the four-helix subdomain (20) provided the first structural insights of EnvZ at low osmolality (Figure 2). The homodimer consists of a four-helix bundle with 2-fold symmetry. Each monomer contains two alpha helices, α 1 (amino acids 235–255) and α 2 (amino acids 265–286) that are separated by a 9-amino acid loop. Within the dimer, the helices are packed such that α 1 is surrounded by an antiparallel α 2. The core is hydrophobic, and the structure is conserved among HKs. The phosphorylated histidine, His²⁴³ lies in α 1 and protrudes away from the helical bundle, where it is accessible to ATP from the ATP-binding subdomain. Within α 1 is a region of dynamic instability (20) that is exquisitely sensitive to intracellular signals including: acid pH (2, 3, 24) and high osmolality (1, 2). A soluble cytoplasmic construct of EnvZ (EnvZc; residues 180–450) lacking TMs 1 and 2, as well as the periplasmic domain, is fully capable of osmosensing and autophosphorylation *in vitro* and *in vivo* (1). More recently, others have

identified additional HKs that are capable of intracellular signaling, although most of these systems are still lacking in mechanistic detail (25–27).

The ATP binding domain is structurally similar to ATP binding proteins DNA gyrase B and Hsp90. It is composed of an $\alpha\beta$ sandwich composed of a five-stranded β -sheet (strand B: amino acids 319–323), D:356–362, E: 367–373, F:420–423 and G: 431–436) and three α -helices (α 1: 301–311, α 2:334–343, and α 3: 410–414). Between these two folds is a hydrophobic core that is highly conserved in HKs. Between α 3 and α 4, there is a “central loop”, which has no defined structure (Figure 2). Binding of ATP occurs at α 3 and the central loop and involves contacts with F and G β -strands. The central loop is near the ATP binding pocket formed by Asp-347, Asp-373, Ile-378 and Phe-387. The ATP triphosphate chain is exposed on the surface to allow transfer to His²⁴³ in the catalytic domain. Conserved glycines form G1 and G2 boxes located in the catalytic core and are essential for kinase activity.

1.2 Cis versus Trans Phosphorylation

Studies on the hybrid HK Taz, in which the extracellular aspartate receptor domain was fused with the EnvZ cytoplasmic domain (see section 1.3) explored whether EnvZ phosphorylation was intra-(cis) or intermolecular (trans). Autophosphorylation of Taz was stimulated by aspartate binding (28). Dimerization promotes formation of a four-helix bundle, putting the sites of phosphorylation in each monomer in equal proximity to bound ATP from either monomer (20), making it equally plausible for cis or trans autophosphorylation in HKs. Single mutants that either lacked phosphorylation (H243V) or were impaired for ATP binding (a deletion of 146 amino acids of the C-terminal domain) were unable to activate *ompC*, but a mixture of the two restored signaling (29), suggesting that EnvZ phosphorylation occurs in trans.

While trans phosphorylation was proposed for EnvZ, biochemical studies of the *Thermotoga maritima* HK HK853 reported cis phosphorylation (30). The ability for HKs to undergo both cis and trans autophosphorylation was corroborated in the crystallographic structure of an EnvZ chimera containing the four-helix bundle domain of HK853 fused to the ATP binding domain of EnvZ (31). The ability to undergo cis or trans phosphorylation was dependent upon linker length, as well as the handedness of the helix. Substituting the EnvZ linker between α 1 and α 2 with the linker from PhoR switched phosphorylation from trans to cis phosphorylation (30, 31). Thus, both cis and trans phosphorylation pathways are possible in EnvZ and its homologs.

Regardless of the mode of autophosphorylation, two functional groups impact His²⁴³ autophosphorylation (see “New View”, especially sections 4.2, 4.3). Asp²⁴⁴ functions as a base to abstract a proton from N δ and promotes nucleophilic attack on the γ -phosphate of ATP (31). Crystallographic analysis offers a snapshot of a fully ON (high osmolality) or OFF state (low osmolality), but amide hydrogen deuterium exchange mass spectrometry (HDXMS) provides direct insights into the ensemble behavior of EnvZ in solution, which is critical for function (1). In low osmolality, His²⁴³ is positioned by a coordinated relay of interactions from the backbone carbonyl of Ala²³⁹ (one helical turn above His²⁴³) through the imidazole side chain of His²⁴³ to the carboxylic acid side chain of Asp²⁴⁴. The interplay

of these two flanking moieties regulates His²⁴³ tautomerization, rotamerization and phosphorylation. The intrinsic disorder within the locus containing the His²⁴³ side chain affords a low basal level of autophosphorylation (32). High osmolality stabilizes the helical backbone, relieves the Ala²³⁹ carbonyl link to enable anchoring of the His²⁴³ imidazole by a H-bond with Asp²⁴⁴ at the N δ of His²⁴³, enabling phosphotransfer to the N ϵ of His²⁴³. Thus, the core of osmosensing is a His-Asp/Glu dyad positioned within a flexible helix.

1.3 The Role of EnvZ Transmembrane and Periplasmic Regions

It was assumed that because EnvZ was an inner membrane protein, that the TM and periplasmic domains were important for its function. HAMP domains have been shown to be important for relaying the effects of extracellular stimuli (22, 33, 34). The periplasmic domain shows the greatest diversity in length and sequence amongst EnvZ homologues from different bacterial species. These range from 6 residues in *Xenorhabdus* to 147 residues in *E. coli*. Chimeras of *E. coli* EnvZ with the periplasmic domain from *Xenorhabdus* were able to restore full functionality, offering evidence that the cytoplasmic domain was pivotal for osmosensing and the TMs were not essential (35). The integrative capacity of the cytoplasmic domain when coupled to alternative signaling domains was further inferred from functional chimeras of the N-terminal segments of the aspartate receptor (Tar) fused with the cytoplasmic domain of EnvZ (Taz1) (28). Because signaling occurred when the EnvZ TMs or periplasmic domain were absent or replaced to construct a chimera, these approaches ruled out an essential role for the EnvZ periplasmic domain and the TMs in EnvZ signaling. An N-terminal truncation of EnvZ (EnvZ115) that lacked TM1 and did not localize to the inner membrane, did not respond to osmolality shifts. It was therefore deduced that EnvZ required its transmembrane domains for signaling (36). However, EnvZ115 ended up in inclusion bodies (37), thus it was unable to respond to extracellular or intracellular signals. In contrast, the soluble, cytoplasmic construct EnvZc was fully capable of osmosensing by responding to signals that arose in the cytoplasm (1).

1.4 How Do Extracellular Signals Translate into Intracellular Signals?

In response to an increase in extracellular osmolality, a passive diffusion of water out of the cell occurs and cells reduce their volume as the intracellular water activity drops (38). This reduction in cell volume is evident in super-resolution images of *E. coli* at high osmolality (39). If it is available, extracellular K⁺ ions are taken up via the Trk channels (40), which are stimulated by the Kdp two-component system (41). Synthesis of organic osmolytes such as glycine betaine is stimulated and subsequently replaces the high levels of intracellular potassium (42). Measurements of intracellular osmolytes identified the large changes in osmolality that can be endured by Gram negative bacteria such as *E. coli* and *Salmonella* (up to 1800 mOsmKg⁻¹) (43). These results highlight the unique properties of bacteria compared to eukaryotes, which tightly regulate both osmolality and pH to be isosmotic with blood (290 mOsmKg⁻¹, pH 7.35-7.45). In response to extracellular osmotic stress, the bacterial cytoplasm becomes concentrated (43) and secondarily, a decrease in intracellular pH occurs (2, 3).

1.5 Freely Diffusible Osmolytes Do Not Stimulate EnvZ

Diffusing osmolytes such as glycerol do not stimulate EnvZ and now we understand the basis for this finding. Osmolytes are small organic compounds that exert a dramatic influence on the protein folding reaction, without making or breaking covalent bonds. Protective osmolytes push the folding equilibrium toward N, the native state, by raising the free energy of the unfolded state (U), favoring the folded population. Denaturing osmolytes push the equilibrium toward U, the unfolded state. The osmolyte effect operates predominantly on the peptide backbone, a component common to all amino acid residues in a protein (44, 45). The backbone transfer free energy (ΔG_{tr}) primarily determines the extent to which osmolytes stabilize ($\Delta G_{tr} > 0$) or destabilize ($\Delta G_{tr} < 0$) the protein relative to an equivalent aqueous solution. Interactions between the peptide backbone and osmolytes are dominated by the surface area and outer-group polarity of the osmolyte (46). Sucrose has a larger polar surface area compared to glycerol and a larger ΔG_{tr} (56 cal/mol vs 22 cal/mol). Glycerol is ranked the weakest of protecting osmolytes on the transfer energy scale (46). Thus, osmolytes such as sucrose and betaine for example, will exert much greater effects on peptide backbone stabilization of EnvZ, which then activate autophosphorylation (see Section 4.3).

1.6 Lipid Allostery Couples EnvZ Signaling and Activation, the effect of Procaine, Robustness

The observation that EnvZc was capable of osmosensing (1) raised the obvious question as to whether the TMs or the periplasmic domain or the lipid composition of the membrane contributed to EnvZ function. Earlier studies established that the four-helix bundle subdomain was sensitive to osmolytes and insensitive to nucleotide binding. In contrast, the ATP binding subdomain was sensitive to nucleotide binding, but insensitive to osmolytes (1). Membrane lipids serve to couple these two domains. A notable change in HDXMS of the full-length solubilized EnvZ incorporated into nanodiscs compared to EnvZc, was in the region of the Gly-rich motif in the ATP-binding site (32). In the presence of lipids, the rate of ATP hydrolysis in the nucleotide binding domain increased, evident as higher turnover. Thus, while EnvZc forms the core of the osmosensing locus, membrane anchoring of EnvZ enables an interaction of the ATP binding domain with the membrane that enhances autophosphorylation at His²⁴³. Thus, ATP hydrolysis is coupled to osmosensing. This effect of lipids likely forms the basis for how EnvZ/OmpR responds to procaine (47) and other membrane perturbants (48). These agents alter the lipids, which then affect interactions with the glycine-rich loop of the ATP binding domain, stimulating ATP turnover and subsequent phosphorylation at His²⁴³ (32). Stimulation of EnvZ autophosphorylation would lead to repression of *ompF* and activation of *ompC* (47–49). Regions of full-length EnvZ that showed increased deuterium exchange included the HAMP, kinase, TMs and periplasmic domains (32). Inside bacterial cells, over-expression of EnvZc resulted in a higher level of β -galactosidase activity of an *ompC-lacZ* fusion compared to the wildtype protein (1). This was not surprising, since HKs are typically expressed at very low levels and overexpression enhances activity (1). This finding implies that porin regulation by EnvZ/OmpR is not robust. An early proposal that OmpR~P levels were independent of the EnvZ concentration argued that the EnvZ/OmpR system was robust (50). Our findings conflict with claims of robustness because in one scenario, *ompC-lacZ* activity increased at elevated EnvZc

concentrations (1). In a separate experiment, higher levels of EnvZ over-expression inhibited *ompC* transcription, and the number of EnvZ molecules produced was verified by molecular counting using super-resolution imaging (21).

1.7 EnvZ Biochemical Activities Underlie Signaling

The kinase activity results from EnvZ binding ATP and its subsequent autophosphorylation of His²⁴³ (51). At high osmolality, autophosphorylation of His²⁴³ is enhanced (1). EnvZ then transfers the phosphoryl group to OmpR at its conserved aspartic acid (Asp⁵⁵) (52). This phosphorelay is at the heart of two-component signaling (Figure 3). EnvZ signals through OmpR, controlling the concentration of OmpR~P to control porin gene expression (5). The first step in the signaling cascade thus involves osmolyte stimulus recognition by EnvZ, leading to enhanced autophosphorylation of His²⁴³. At high EnvZ concentrations *in vitro*, EnvZ can stimulate the dephosphorylation of OmpR~P (referred to as a phosphatase activity). Thus, EnvZ can modulate the level of OmpR~P by altering any or all of these reactions.

1.8 EnvZ Mutants with Altered Biochemical Activities

Mutations have been isolated in *envZ* that alter its phosphorylation, phosphotransfer and dephosphorylation activities, although complete uncoupling of these activities has not effectively been demonstrated, apart from the requirement of His²⁴³ for autophosphorylation (51). The interpretation of the various results highlights the differences in approach and the “conflict” between geneticists and biochemists (53, 54). The biochemist complains that genetic experiments are indirect, while geneticists argue that *in vitro* experiments don’t necessarily recapitulate what happens *in vivo*. Localized mutagenesis of *envZ* identified two substitutions, one that was one helical turn away from the phosphorylated histidine, A239T, and a periplasmic substitution, P159S. These substitutions were described as kinase minus, phosphatase plus (K⁻P⁺) (56,57), but direct measurements revealed that the A239T substitution exhibited a 3-fold lower affinity for ATP and was also impaired in phosphotransfer to OmpR, indicating multiple effects on EnvZ activities from the single substitution (37).

1.9 EnvZ Dephosphorylation of OmpR~P

In many TCSs, dephosphorylation of the RR~P via the HK (the so-called “phosphatase activity”) serves to limit the level of the activated RR and reset the system. In some systems, it is clear that RR~P dephosphorylation occurs, or is stimulated by, accessory proteins. For example: PII-stimulated dephosphorylation of NtrC~P (58, 59) and CheY~P dephosphorylation by CheZ (60, 61). In the case of EnvZ, it is poised to increase autophosphorylation in response to signaling (i.e., OmpR~P levels are relatively low in the absence of *envZ*) (5), whereas its close homologue CpxA is poised to regulate CpxR~P turnover (i.e., CpxR~P basal levels are high in the absence of *cpxA*) (62, 63). Kinetic studies proposed that the HK phosphatase activity functions to limit cross-talk between highly homologous two-component systems (64), but more recent studies of co-varying residues (65) and our HDXMS experiments determined that what limits cross-talk is the sequence specificity of the RR binding domain of the HK (1, 32). It is important to note that concentrations of OmpR far exceed that of EnvZ in *E. coli*, and RRs in general compared to

HKs (7). Therefore, all of the EnvZ would be complexed with OmpR. The significance of this complex is that osmolyte stimulation of EnvZ phosphorylation and phosphotransfer from EnvZ to OmpR becomes a unidirectional process *in vivo* (see Figure 4). Experiments performed with kinase nulls strains alter the free concentration of the RR in the cell as it is no longer complexed with the HK. The observed OmpR~P 'phosphatase' activity of EnvZ is in part a limitation of *in vitro* biochemical experiments performed with a large excess of EnvZ (64, 66). When the assay was performed with EnvZ at concentrations similar to the *in vivo* ratios of EnvZ to OmpR, turnover was very slow (67), (see (68) for a review). While we do not discount the ability of EnvZ to dephosphorylate OmpR~P when it is in substantial excess, the relative concentrations of EnvZ and OmpR *in vivo* render the phosphorylation of OmpR as unidirectional from EnvZ to OmpR *in vivo*. A similar behavior is likely in many, but not all two-component systems. An important distinction may be those in which regulation is linked to central metabolism, as is the case with CpxA/R (63).

A demonstrated role for the phosphatase activity in signaling is based on events that occur in the absence of the HK, which as mentioned above, alters the RR free concentration in the cell. Acetyl phosphate (AcP) can phosphorylate RRs *in vitro* and *in vivo* (63, 69–72), although RRs vary in their ability to be phosphorylated by AcP. CpxR is a RR that is more readily phosphorylated by AcP compared to OmpR (our unpublished observations; (73, 74)). These differences are evident in the low activity of an *ompF-lacZ* reporter in an *envZ* null strain (56). In contrast, a *cpxA* null strain showed much higher activity of CpxR regulation of *degP*- and *cpxP-lacZ* transcriptional fusions (75), suggesting a higher concentration of CpxR~P produced by AcP. A gain of function mutant (Cpx*) (76, 77) located approximately one helical turn below the phosphorylated histidine was described as a K^+P^- mutant, but it clearly has severely reduced autophosphorylation and phosphotransfer activities as well (75). Growing cells in conditions that alter AcP levels (such as acetate or pyruvate), would also influence the cellular concentration of phosphorylated RRs (72). It is also worth noting that many of these measurements were made on protein fusions to maltose binding protein (MBP), which can dramatically effect protein:protein interactions, as well as phosphorylation and phosphotransfer. In particular, we have observed substantially lower levels of OmpR~P produced by MBP-EnvZ compared with other EnvZ constructs or from AcP phosphorylation. One interpretation of experimental observations is that the HK phosphatase activity is important for suppressing cross-talk with other two-component systems. An alternative interpretation is that the presence of the HK complexes the RR and reduces its availability as a substrate for phosphorylation by AcP. Thus, when the cognate HK is missing, the free RR concentration increases, and it can be a substrate for AcP (Figure 4). Single molecule localization microscopy experiments (SMLM) may help to identify such HK/RR complexes and determine whether they are simultaneously complexed with DNA *in vivo* and whether complex formation is influenced by environmental stress.

Strain differences are also likely to play a role in the observed behavior of EnvZ in response to environmental stresses, which also affects the interpretation of mutant behaviors. The majority of experiments on the EnvZ/OmpR system were conducted on the *E. coli* strain MC4100, which responds differently to extracellular acid or osmotic stress compared to the probiotic Nissle strain (78, 79) and to MG1655, a sequenced strain (80). It appears that the large deletions that occurred during the construction of MC4100 for making *lacZ*

transcriptional fusions may make it difficult to generalize results of physiological studies to other strains (81). For example, in response to acid or osmotic stress, MG1655 acidifies from an initial intracellular pH of 7.13 to 6.55 or 6.75, respectively. MC4100 was slightly acidified initially compared to MG1655 (pH 6.80 vs pH 7.13) (3), but MC4100 did not acidify further in response to acid stress (3). Instead, MC4100 maintained its cytoplasmic pH throughout the experiment (internal pH = 6.83). This was the case whether the external pH was 7.2, 5.6 or induced by the osmolyte sucrose. Addition of osmolytes actually increased the pH_i of MC4100 from 6.8 to 7.15, as reported (82). This was opposite to what we observed with *E. coli* MG1655 (3). Differences in intracellular pH resulting from environmental stress are likely to have significant effects on gene regulation, since histidyl phosphates are rapidly hydrolyzed under acidic conditions (3, 83), and acid pH alters the conformation of the HK (see section 4.2, 4.3).

2.0 What Activity is Regulated by Osmolality/Acid pH?

A long-standing question in the field was which activity of EnvZ was regulated by osmotic stress? A study by Jin and Inouye proposed that at high osmolality, OmpR~P levels increased as a result of a decrease in the phosphatase activity (84). Their hypothesis was based on experiments with the chimeric kinase Taz (28), which contains the periplasmic domain of the Tar aspartate chemoreceptor fused to the cytoplasmic domain of EnvZ. Taz activates *ompC* in the presence of aspartate. Although activating EnvZ using a known ligand was clever, the construct has several limitations that cast doubt on whether conclusions based on Taz are physiologically meaningful. The problem is that the domains derived from Tar behave differently from native Tar, so how does that impact the interpretation of the function of the EnvZ domains? For example, Taz requires 1-5 mM aspartate to activate *ompC* compared to Tar, which binds aspartate with a K_d of 1.2 μ M (85). Maltose also activates Tar, but it did not enhance *ompC* transcription, and finally, aspartate did not affect *ompF* transcription. More recent studies have shown that EnvZ phosphorylation is directly stimulated by osmolytes, including K^+ (86), Na^+ , and sucrose (1). Furthermore, HDXMS also demonstrated that EnvZc could sense osmolality without being in the membrane, and that the source of the signal was cytoplasmic (1) (see section 4.4). Thus, it is now established that the major effect of osmolality is to stimulate EnvZ kinase activity (see also section 3.1).

2.1 MzrA

MzrA was identified by a genetics approach as a modulator of the EnvZ/OmpR system (87). MzrA is a 127 amino acid periplasmic protein that interacts with EnvZ to modulate its kinase activity and elevate OmpR~P levels. Subsequent experiments determined that the periplasmic domain of MzrA, and not its single transmembrane domain, was the site of interaction with EnvZ (88). A construct that lacked the TM retained activity, indicating that TM localization was not essential. The role of MzrA appears to be important in envelope stress rather than osmotic stress. During envelope stress, the Cpx and σ^E pathways are activated, MzrA concentrations increase, and *ompF* is repressed via the action of OmpR~P (87, 88). Reducing OmpF levels appears to alleviate the ensuing envelope stress.

2.2 Cross-talk Between Noncognate HK/RR Pairs

Cross-talk between noncognate HK/RR pairs is suppressed by a high degree of sequence specificity encoded in the RR binding site on the HK (65). In EnvZ, this region is ²⁶⁷AESINKDIEECN²⁷⁸ (1). Labeling of Cys-277 with a fluorophore prevented OmpR binding, verifying the AESINKDIEECN peptide as a site of EnvZ/OmpR interaction (21). The closest homologue to OmpR is CpxR. In the absence of *envZ* and *cpxR*, CpxA can phosphorylate OmpR (73), increasing OmpR binding to *ompF* and *ompC* sites (89). This activity is entirely suppressed in a wildtype EnvZ strain. Why? We hypothesize that the presence of the cognate HK has higher affinity/specificity for its cognate RR, preventing a lower affinity interaction with CpxR. Now that new tools and super-resolution imaging in bacteria is possible (39, 90, 91), it will be worthwhile to determine the extent to which OmpR is bound to EnvZ using single particle tracking photoactivation localization microscopy, (spt-PALM) and compare with OmpR binding to CpxA in an *envZ* null strain. Presumably, an OmpR interaction with its cognate kinase EnvZ is favored, and we would expect to see a higher population of bound OmpR molecules in the EnvZ-labeled strain compared to the *envZ* null, CpxA-labelled strain.

2.3 Ensemble View of EnvZ Signaling

HDXMS has provided an overview of conformational dynamics of EnvZ in solution, where the four-helix bundle subdomain serves as a primary signaling module for changes in osmolality and pH (1). His²⁴³ is positioned within a highly disordered segment of a longer helix and flanked by a helix that is sensitive to OmpR binding (see Section 4.1). Together, these disordered helices adopt an ensemble of multiple conformations of EnvZ in solution. Osmolytes and environmental stimuli function to alter this ensemble to trigger non-discrete signaling outputs. The four-helix bundle is a point of convergence of multiple signaling inputs, including signals from membrane lipids (Section 1.6) and specific OmpR interactions (see Section 4.1). This provides EnvZ with a wide dynamic range for responding with high sensitivity to gradients of osmolytes as well as pH stimuli. Convergence of multiple signals from the TMs and the EnvZ periplasmic domain (including MzrA, section 2.1), as well as the membrane, explains the allostery observed with the Tar-EnvZ chimera Taz (28), the activating effects of a periplasmic mutant EnvZ P151A (57), and the lipid effects on the ATP binding domain (92).

3.0 The OmpR subfamily of response regulators

OmpR is a two-domain RR with an N-terminal receiver (phosphorylation domain) and a C-terminal DNA binding domain (93). The C-terminal effector domain is a winged helix-turn-helix motif that binds DNA (94–96). OmpR is an extensively studied member of a subfamily of RRs that includes PhoB, VirG, ResD and CpxR, among others. RRs are divided into subfamilies based on the structure of their DNA binding domains. The OmpR subfamily has 14 homologs in *E. coli* alone (97). The receiver domain is highly conserved among RRs and consists of five alpha helices surrounded by a central β -sheet of five parallel beta strands (see (98) and Figure 5A). Several critical invariant amino acids are in the loops between α helices and β strands: between β 4– α 4 is a threonine (Thr⁸³ in OmpR) (99), between β 5– α 5

is a tyrosine (Tyr¹⁰²) and the aspartic acid that is phosphorylated is in the loop between β 3– α 3 (Asp⁵⁵). Phosphorylation occurs at only this one site (69), Asp⁵⁵ (52).

OmpR binds as a dimer to multiple DNA sites at the *ompF* and *ompC* promoters between –100 and –60 upstream of the transcription start site (99, 100). Repression of *ompF* involves an additional upstream binding site between –384 and –354 (101). The crystal structure of the C-terminus of OmpR (OmpRc) was determined independently by two groups (95, 102). The structures revealed the presence of a winged helix-turn-helix motif (wHTH) in the DNA binding domain of OmpR (see Figure 5B). The recognition helix is unusually long and is the major determinant of DNA binding specificity. The turn of the HTH is also unusually long. Most canonical HTH proteins have turns of 3–4 amino acids, whereas OmpR subfamily members have turns of 10 amino acids (103). The turn is dynamic, and substitutions in the turn affect interactions with RNA polymerase (RNAP) (104–106). In the case of PhoB, there is strong evidence that the turn of the HTH interacts with the σ subunit of RNAP, but in the case of OmpR, substitutions in the turn affect DNA binding (107).

OmpR is somewhat unusual among response regulators in that the unphosphorylated protein has quite high affinity for DNA ($K_d \sim 150$ nM) (108), while the K_d of the phosphoprotein is 5 nM. These measurements were made possible by our ability to separate phosphorylated and unphosphorylated forms of the protein by HPLC (108), an approach that is now widely used with other RRs. High affinity binding by OmpR/OmpR~P is also observed in *in vitro* transcription studies (90, 109), as well as by atomic force microscopy (3) and super-resolution imaging (91). OmpR makes more phosphate backbone contacts and fewer specific base contacts (110) compared to RRs that recognize precise motifs such as PhoP, PhoB and others. This lack of specificity enables OmpR to contribute to acid and osmotic stress responses (2, 3, 24) (111) and to evolved virulence gene regulation in *Salmonella* (112, 113) (see “Virulence”). Until recently, it was believed that unphosphorylated OmpR did not play a role in gene expression (5), but it is now appreciated that OmpR can act non-canonically to regulate genes outside of the porin regulon that facilitate survival and growth in response to acid and osmotic stress (see “Non-canonical Activation”, section 4.6) (2, 3, 24).

One of the unique features of OmpR subfamily members is the presence of a four-stranded β -sheet at the N-terminus of the effector domain (see Figure 5B). A model whereby phosphorylation of the receiver domain results in subtle conformational changes that are transmitted to the β -sheet of the C-terminus was proposed from the co-crystal structure (114, 115). Cysteine-scanning mutagenesis through this region in OmpR suggested that the β -sheet may be involved in oligomeric interactions (116), and the role of the β -sheet may vary in function among OmpR subfamily members.

3.1 Activation of OmpR

Phosphorylation of EnvZ at His²⁴³ from ATP subsequently leads to the phosphorylation of OmpR at Asp⁵⁵ (52). Small molecule phosphodonors such as acetyl phosphate or phosphoramidate also phosphorylate OmpR at the same locus (69). Phosphorylation of OmpR in its N-terminal receiver domain increases the affinity of its C-terminal domain by at least 10-fold for the regulatory regions upstream of the *ompF* and *ompC* genes (108). The communication between OmpR domains is bidirectional; the presence of high affinity *ompF*

or *ompC* sites stimulates OmpR phosphorylation *in vitro*. These experiments led to the proposal that OmpR might be activated while bound to its target DNA. This would require the formation of a complex between the membrane bound EnvZ with OmpR, while it is physically bound to DNA. Genetic evidence for such an HK/RR/DNA complex exists, both in the EnvZ/OmpR system (117), and its close homologue CpxA/R (Champion & Silhavy, personal communication). In OmpR, two substitutions G94D and E96A eliminate *ompF* and *ompC* activation, and the G94D substitution is dominant (117, 118). These mutants are of interest because EnvZ was required for their dominance. At the time, the authors had no explanation for this surprising finding. Both mutants were unable to bind DNA, suggesting that oligomerization is an essential prerequisite to binding. These mutants lie on the face of OmpR that is opposite the phosphorylation site. Both were capable of phosphorylation, and it was proposed that the mutants were unable to oligomerize after phosphorylation. This interpretation absolutely fits with a recent observation that OmpR dimerization required EnvZ, but did not require phosphorylation (see section 4.6, below) (3).

A four-state model has been proposed in which OmpR exists as an equilibrium mixture between four distinct states (Figure 6). Free OmpR in the unphosphorylated form (A), OmpR~P (B), the unphosphorylated form bound to DNA (C) and OmpR~P bound to DNA (D). The reaction step that is most affected by DNA depends on the phosphodonor. In the presence of AcP, DNA binding stimulated OmpR phosphorylation dramatically (about 25-fold faster), without little or no effect on OmpR~P dephosphorylation (119). In Figure 6, this means that C to D is much faster than A to B. DNA binding also slowed dephosphorylation about 2-fold, i.e., D to C is slower than B to A. When phosphorylating with EnvZ, D to C was much slower than B to A. In either case, the overall effect of DNA was to increase the net rate of OmpR~P formation by about 50-fold. Qin and co-workers proposed that OmpR~P binding to DNA made it inaccessible to dephosphorylation by EnvZ (120). However, this does not fit with their proposal that the EnvZ phosphatase activity was the step that was regulated by high osmolality (84) (section 2.0). According to Qin et al., OmpR~P bound to DNA would not be able to be dephosphorylated, raising the question as to how discrimination between OmpR~P bound to *ompF* (presumably at low osmolality) and OmpR~P bound to *ompC* (at high osmolality), and differential regulation would occur.

These questions were addressed in studies using fluorescence anisotropy to measure EnvZ/OmpR interactions. OmpR was labeled at its N-terminus with fluorescein and EnvZc was added to the mixture. The K_d for EnvZ binding to unphosphorylated OmpR was 425 nM and binding was unaffected by the addition of *ompF* or *ompC* DNA (67), indicating that DNA binding did not affect the ability of OmpR to interact with EnvZ. Phosphorylation of OmpR reduced its affinity for EnvZ to unmeasurable levels, to at least ten-fold lower affinity (i.e. $>5 \mu\text{M}$). This finding is inconsistent with a proposed role for DNA in the OmpR/OmpR~P equilibrium in which DNA prevents EnvZ from dephosphorylating OmpR~P (120), because the affinity of EnvZ for OmpR~P is already too low (67). The binding measurements are consistent with measurements in the chemotaxis system in which phosphorylation of the RR CheY (an OmpR homologue) reduced its affinity for the CheA kinase (121). The reduced affinity of CheY~P for CheA would promote its binding to the switch proteins of the flagellar motor. Similarly, phosphorylation of OmpR by EnvZ~P while it is bound to DNA (C, Figure 6) would reduce its affinity for EnvZ and promote an interaction between

OmpR~P and RNA polymerase. Because the cellular concentration of EnvZ is so low, a $K_d > 5 \mu\text{M}$ for EnvZ/OmpR~P would mean that these two partners would hardly ever interact, implying that EnvZ cannot be the primary mechanism of OmpR~P dephosphorylation. Thus, we favor a view where OmpR~P turnover occurs rapidly enough *in vivo*, due to the intrinsic instability of aspartyl phosphate intermediates, eliminating the need for phosphatase activity in controlling signaling. Recent phosphorylation results clearly demonstrated the link between increasing osmolality and increasing levels of EnvZ~P (1), suggesting that the kinase activity was the step regulated by environmental signals. Of course, an ability to actually measure the level of phosphoproteins *in vivo* would be enormously helpful in providing a quantitative molecular picture of signaling events.

3.2 OmpR Binding Sites

OmpR binds to three tandem sites immediately upstream of the -35 element (see Figure 7) between approximately -100 and -40 from the transcriptional start site at both the *ompF* and *ompC* promoters (100, 122–124). At *ompF*, there is an additional upstream site F4 that is required for repression (125). A genetic approach was employed to determine which OmpR binding site(s) participates in the recruitment of RNAP (126). DNA fragments containing the binding sites were used in a gel retardation assay with purified OmpR, EnvZ and ATP. Only the DNA fragments containing the site between -100 and -80 of either promoter were shifted in the presence of protein and ATP (F1 and C1 sites; see Figure 7). The interpretation was that F1 and C1 each comprise high-affinity OmpR binding sites. The F1 and C1 fragments were cloned in either orientation upstream of the *ompC* -35 element. Both fragments were able to direct transcriptional activation when present in either direction, but, in both cases, the forward facing F1 and C1 fragments were more active. Insertion of a 4 bp spacer between the binding site and the -35 element abolished transcriptional activation. Insertion of a 10 bp fragment at either promoter reduced activation, but the level of transcription was still considerably higher than the control strain (126). The interaction between OmpR and RNAP must be flexible, provided that the proper helical phasing of each protein bound to DNA is maintained. Thus, OmpR bound immediately upstream of the -35 element or at one helical turn upstream is sufficient to activate transcription, indicating that at the *ompF* or *ompC* promoters occupancy of the F3 or C3 sites is required for activation (99). A prediction from these studies is that OmpR bound to DNA exists as a head-to-head or tail-to-tail dimer (116). This is intriguing, because the OmpR binding sites are asymmetric and protein dimers in head-to-head or tail-to-tail binding modes typically bind to inverted repeat elements. A previous study reported a head-to-tail mode of binding based on Cu-phenanthroline cleavage of OmpR bound to DNA (127). These conflicting results likely will be reconciled by the co-crystal structure of OmpR bound to *ompF/ompC* DNA. Although OmpR exists as a monomer in solution, dimerization occurs upon phosphorylation (128), or after interaction with EnvZ (3). Sequence requirements for binding to the downstream half site appear to be less stringent than for initial binding to the upstream half site, implying that at least some OmpR binding sites may actually contain a poorly defined inverted repeat.

3.3 Recruitment of RNA Polymerase to OmpR-dependent Promoters

OmpR-mediated transcription occurs through a recruitment mechanism whereby OmpR facilitates binding of RNAP at OmpR-dependent promoters. The *ompF* and *ompC* promoters contain –10 elements that deviate from the *E. coli* consensus sequence (see below). A genetic study examined the role of each –10 element in OmpR-dependent transcriptional activation.

Consensus TATAAT

ompF AAAGAT

ompC GAGAAT

When the first base of either –10 element was converted to a consensus T (labeled in red), the promoters were highly active in an *ompR* mutant background (129). In an *ompR* null background, the level of transcription was further enhanced. The non-consensus –10 promoter element necessitates the requirement for OmpR in transcriptional activation. The addition of OmpR and/or mutation towards the consensus sequence results in stimulation of transcription. These results are consistent with a recruitment mechanism for RNAP binding to promoter DNA. OmpR recruits RNAP to promoter DNA via a direct protein/protein interaction, or alternatively, OmpR binding could drive a structural change in the promoter that then facilitates RNAP binding. Since a single nucleotide change can stimulate OmpR-independent transcription, the former possibility seems more likely. A point mutation is more likely to result in the formation of a favorable nucleotide/amino acid interaction, rather than mimicking a structural change in the DNA due to binding of an upstream activator.

The location of the transcription factor binding site determines the requirement for α CTD at the promoters under examination. OmpR binds to sites upstream of the –35 region of the *ompC* promoter and requires the reconstituted RNAP containing the full-length α CTD to activate transcription, suggesting that a direct interaction occurs between OmpR and α CTD (130). In contrast, the OmpR family member PhoB binds to a site at the *pstS* promoter in the position normally occupied by the –35 element and does not require α CTD, it directly interacts with the $\sigma 70$ subunit of RNAP holoenzyme (104, 105). Thus, OmpR subfamily members can activate transcription via interactions with different subunits of RNAP and their mechanism of activation is not conserved throughout the subfamily.

Subsequent genetic studies identified substitutions in the α subunit that affected expression uniquely at the porin genes, but not at other test promoters (131, 132). However, it was not determined whether the substitutions affected the interaction between OmpR and α or the interaction between α and promoter DNA. It is well established that α stimulates transcription at certain promoters by directly binding to high-affinity sites termed UP elements (reviewed in (133)), but most activator-dependent promoters do not contain UP elements. It has been proposed that at some promoters that lack UP elements, non-specific or low-affinity interactions between α and DNA are stabilized by interactions with an activator and α or with $\sigma 70$ and α (134–140). Thus, it remains possible that mutations in a that

specifically affect transcription of OmpR-dependent promoters alter the interaction of α with DNA, and not with α and OmpR.

Evidence for an interaction between OmpR and α was provided by the identification of a suppressor mutation in *ipoA* (encoding the α subunit) of the OmpR mutant P179L (141). The suppressor resulted in a glycine substitution for valine at position 264 of α (141). Surprisingly, the co-crystal structure of a CAP/ α CTD/DNA complex identified residue 264 of α CTD as making direct contacts with the DNA backbone (134). It thus seems more likely that the V264G substitution enhances the affinity of α CTD for DNA, rather than suppressing the OmpR positive control defect. From further analysis of the OmpR P179L mutant, it is evident that the mutant is severely defective in binding to DNA. This defect can be overcome by increasing the concentration of the mutant in an *in vitro* transcription assay, leading to a stimulation of transcription (107). Thus, it is still unresolved whether the requirement for α at OmpR-dependent promoters is the result of an interaction with OmpR and α or the promoter DNA and α . Two studies reported inhibitory effects of over-expression of α on the *ompF* and *ompC* promoters, suggestive of a direct OmpR/ α interaction (132, 142). High concentrations of α inhibit OmpR-dependent transcription of *ompF*, presumably by sequestering OmpR from the promoter. However, it is possible that α bound to promoter DNA prevents RNAP holoenzyme from interacting with OmpR and/or the promoter DNA, a scenario that would not require a direct interaction between OmpR and α .

3.4 OmpR RNAP Interaction Surface

The precise interaction surfaces on OmpR and α remain undetermined. Genetic screens for positive control mutants, defined as mutants defective in their interaction with RNAP, but not with DNA, identified three potential surfaces on OmpR for interaction with RNAP (106, 143, 144). The criteria for positive control was narrowly defined (see below) and most mutants ended up having binding defects. This doesn't rule out a direct effect with α , but it does indicate that more experiments are needed to identify the interaction surface. These surfaces include the N-terminal receiver domain and two regions in the C-terminus, the α loop and the α_1 - α_2 loop (see Figure 5). However, the lack of a DNA binding defect was confirmed only with the high-affinity site C1 (106), and binding at other loci was not examined. In two of these cases, a partial defect in binding to C1 DNA was apparent using a direct *in vitro* approach (143, 144). Altered binding was shown to occur at the low-affinity sites without a simultaneous defect at the high-affinity C1 site (99, 105), suggesting that high-affinity binding is not a stringent enough requirement for a positive control mutant. This result raises the possibility that the positive control mutants previously identified could be defective in DNA binding at the low-affinity sites, rather than having an impaired interaction with RNAP. Because OmpR binding to C3 is required for activation of the wild-type *ompC* promoter, defects in binding to this region would confer a transcriptional activation defect (99, 105).

A subset of the positive control mutants was expressed and purified to examine their DNA binding and transcriptional activation properties. OmpR R42H is a substitution in the N-terminal domain (106); OmpR G191S and A196V are substitutions in the so-called α loop

(106), which is the turn of the HTH motif (95). OmpR P179L (106, 143) and S181P (144) are substitutions in the $\alpha 1$ – $\alpha 2$ loop, immediately upstream of the HTH motif. Four of the five mutants had DNA binding defects to varying degrees at both promoters (107). S181P was defective in binding to *ompC*, but protected *ompF* from DNase I cleavage similarly to wild-type OmpR. All of the mutants, with the exception of S181P, were able to significantly activate transcription *in vitro*. The results indicate that the substitutions in the N-terminus and the α loop are actually DNA binding mutants and not positive control mutants. When the DNA binding defect is overcome by increasing the concentration of the mutant proteins, they are able to activate transcription. Based on these results, it is proposed that a location in the N-terminus containing R42 ($\alpha 1$) and the α loop are potential candidates for regions that determine DNA binding specificity (107). The $\alpha 1$ – $\alpha 2$ loop remains a likely candidate as an RNAP interaction surface. The crystal structure of the OmpR C-terminus (OmpRc) reveals that residues in this region interact with residues within the recognition helix (95, 103, 145). Thus, it is not unexpected that substitutions in the $\alpha 1$ – $\alpha 2$ loop could also affect DNA binding. Clearly, additional studies are required to rigorously identify the regions of OmpR required for an interaction with RNAP.

3.5 Affinity Model of Porin Gene Regulation

A model that explained the differential regulation of *ompF* and *ompC* was referred to as the affinity hypothesis. The model was based on genetic analysis of a series of mutants that indicated that (i) OmpR~P was the form that regulated the porin genes and OmpR did not play a role (4) and (ii) EnvZ controls the concentration of OmpR~P via its biochemical activities (described in Section 1.7) (57). We now appreciate that OmpR plays a surprising, non-canonical role in regulating many genes (see section 3.6 and “New View”) (2, 3, 24). The hypothesis is outlined briefly as follows: at low osmolality, there is a low level of OmpR~P present, either due to a low activity of the EnvZ kinase or a high activity of the EnvZ phosphatase. OmpR~P binds to high-affinity sites on *ompF* that activate its expression. At high osmolality, the kinase activity of EnvZ increases, or the phosphatase activity decreases, increasing the OmpR~P concentration. OmpR~P binds to low-affinity sites on *ompF* that repress OmpF expression and it also binds to low-affinity sites on *ompC* that activate expression of OmpC. The hypothesis had two basic predictions: that phosphorylation of OmpR increases its affinity for the porin regulatory regions and that the upstream sites have different affinities for OmpR~P. The *ompF* activating sites should be high affinity, the *ompF* repressing site should be low affinity; and the *ompC* activating sites should be low affinity. Thus, at low OmpR~P (low osmolality), the high-affinity site on *ompF* would be occupied, leading to OmpF production. At high OmpR~P (high osmolality), the low-affinity sites at *ompF* would be bound, repressing *ompF* transcription and the low-affinity sites at *ompC* would also be occupied, activating transcription of *ompC*. It was proposed that the differences in affinity would need to be on the order of 20-fold in order to account for the observed differences in OmpF and OmpC expression levels (57).

Testing the Affinity Model—A quantitative analysis of the binding affinities of OmpR and OmpR~P for individual OmpR binding sites and the composite *ompF* and *ompC* promoters was performed using fluorescence anisotropy (108). OmpR was phosphorylated by AcP and OmpR~P concentrations were measured on a C4 column using reverse-phase

HPLC. The binding assays revealed that OmpR~P has similar affinities for both the isolated F1 and C1 sites. This result was surprising, as the affinity hypothesis had predicted that the F1 site should be considerably higher affinity than the C1 site. When OmpR~P binding to the composite F1-F2-F3 and C1-C2-C3 sites was examined, the affinity for *ompF* DNA ($K_d = 15 \mu\text{M}$) was only 2-fold higher than for *ompC* DNA ($K_d = 31 \mu\text{M}$). The binding assays indicated that the affinity of OmpR~P for each promoter was not sufficiently dissimilar to account for osmoregulation of porin gene expression on the basis of differences in binding site affinity. More interestingly, the binding data indicated that the affinity of unphosphorylated OmpR was greater for *ompC* ($K_d = 87 \mu\text{M}$) than for *ompF* ($K_d = 194 \mu\text{M}$). Thus, even at low OmpR~P concentrations, it is then predicted that occupancy of *ompC* and *ompF* by either OmpR or OmpR~P would be quite similar. Considering that the affinities of OmpR~P for *ompF* and *ompC* were quite similar, the simplest explanation for the lack of *ompC* activation at low osmolality is a type of gene silencing mechanism. Such a mechanism would prevent OmpR and/or RNAP binding to negatively regulate steps downstream of RNAP recruitment (146). Occlusion of OmpR binding to *ompC* at low osmolality by another bound factor or by an altered DNA structure that requires high concentrations of OmpR~P to activate *ompC* is in agreement with one aspect of the affinity hypothesis: that differences in the porin levels in response to changes in medium osmolality require higher concentrations of OmpR~P.

Differential Regulation of the Porin Genes is Driven by Conformational

Changes in OmpR—The affinity hypothesis was elegant and simple, but if the hypothesis is no longer valid, how can differential osmoregulation of the porin genes be explained?

Recent studies of the OmpR mutant T83I led to an alternate model for differential porin gene expression (99). A threonine near the phosphorylation site is a highly conserved residue among RRs (position 83 in OmpR). It participates in a phosphorylation-dependent conformational change in the N-terminal receiver domain and transmits it to the C-terminal DNA binding domain of OmpR. Upon phosphorylation at Asp⁵⁵, the side-chain hydroxyl of T83 hydrogen bonds to the phosphoryl group. This creates a pocket that enables Y102 to rotate from an outward, solvent-exposed to an inward, buried position (114, 147–150). This “aromatic switch” is likely a general mechanism to transduce the phosphorylation event into a conformational change in the protein. Such a conformational change would then modulate protein function, for example, by increasing affinity for DNA. When over-expressed *in vivo*, OmpR T83I was unable to activate transcription of either *ompF* or *ompC*. This defect was not due to a defect in phosphorylation by EnvZ. DNase I footprinting assays indicated that the OmpRT83I mutant was able to bind to *ompF* identically as wild-type OmpR, yet displayed altered binding at *ompC*. Thus, OmpR must adopt different conformations when bound to *ompF* versus *ompC*. In the case of T83I, the mutant appears to be locked into a low osmolality conformation that enables normal binding at *ompF*, but not at *ompC*. It is especially intriguing that this substitution is in the phosphorylation site, not in the DNA binding domain of OmpR. Binding at *ompC* would require that OmpR adopt a different conformation, and this switch is prevented by the substitution. The mechanism that facilitates the conformational change of OmpR when bound to one promoter versus the other remains elusive but will hopefully be revealed when co-crystal structures are available. It is possible that OmpR changes selectivity in response to intracellular changes in osmolality or

pH (3). Another possibility is that the different conformation stabilizing DNA binding at *ompC* occurs post initial DNA recognition (119). In other words, once OmpR is bound to *ompC* DNA, a conformational change might occur to facilitate oligomerization, RNAP interaction or some other event required for transcriptional activation. A shift to high osmolality may directly or indirectly alter the structure of the *ompC* promoter that facilitates DNA binding. For example, a previous report demonstrated that integration host factor (IHF) protects approximately 40 bp upstream of the OmpR binding sites at the *ompC* promoter (151). If IHF binding at *ompC* depends on environmental osmolality, binding or release at high osmolality could lead to a structural change in the downstream DNA that favors OmpR binding. In agreement with this prediction, a study demonstrated that IHF binding to an upstream activating sequence of the *ilvPG* promoter mediates duplex destabilization in the -10 element that favors RNAP binding (152). In other words, a structural change in the DNA transmitted by distally bound IHF favors RNAP binding in the absence of any direct interaction between the two proteins. The characterization of OmpR T83I revealed an additional property of OmpR-dependent transcriptional activation. T83I binds to *ompF* similarly to wild-type OmpR, yet it is unable to activate transcription. Since EnvZ can phosphorylate T83I *in vitro*, the substitution likely confers a defect in signaling that is subsequent to phosphorylation and is required for transcriptional activation (99). Likely possibilities are OmpR oligomerization or a conformational change that is required for a productive interaction with RNAP.

The OmpR mutant V203M constitutively expresses *ompF* but is defective at activating *ompC*. V203M has a reduced affinity for *ompC* DNA when compared to wild-type OmpR (153). In a *himA* null background (encoding one of the two IHF subunits), the *ompC* expression defect was suppressed (151). These results suggest that IHF inhibits OmpR binding at the *ompC* promoter, although this possibility has not been tested directly. It is unclear whether or not IHF levels are coordinated to changes in medium osmolality and under what conditions IHF binds at *ompF* and *ompC* (i. e. at low versus high osmolality or both). Thus, the precise contributions made by IHF to differential porin gene expression and the signals that feed into this pathway remain elusive. A further complication is that IHF has also been shown to negatively regulate transcription of the *ompR-envZ* locus in both *in vitro* and *in vivo* assays (154). An intriguing question is whether or not changes in IHF levels and/or function in response to changes in medium osmolality are important for differential regulation of the three loci. However, IHF is a pleiotropic, global regulatory protein, and any models concerning IHF-dependent regulation will likely be difficult to test *in vivo*.

3.6 OmpR is a Global Regulator

OmpR is well known for its role in modulating the ratio of OmpF to OmpC in the cell in response to changes in osmolality, but it is now evident that OmpR plays a much wider role in regulating gene expression. Earlier transcriptome analysis reported that an *ompR/envZ* deletion resulted in changes in the expression of 125 different genes (155). The *ompR/envZ* response was second only to a deletion of the *arcA/B* two-component system in terms of the number of genes affected. Although some of these changes may be the result of defects in porin synthesis, they include a diverse array of genes, such as those necessary for flagellar-mediated motility, amino acid biosynthesis (cysteine and leucine) and enterochelin synthesis.

The lack of both EnvZ and OmpR results in a variety of phenotypic changes, including increased hexose utilization (allose, fructose, mannitol, N-acetyl-d-glucosamine and glucose), increased resistance to antibiotics (cephalosporins, β -lactams, topoisomerase inhibitors and folate antagonists) and a decreased resistance to some metal ions (cobalt, selenite, chromate, lithium and dichromate), but, interestingly, little change in sensitivity to osmolality (156). More recently, a deletion in *ompR* was shown to increase expression of a variety of drug exporters (157). Previous work from many groups has shown that OmpR plays a role in the regulation of a wide variety of genes, including the flagellar master regulon *flhDC* in *E. coli*, but not in *Salmonella* (158), expression of the *csgDEFG* operon and curv fimbriae production (159–161), expression of cryptic porins OmpS1 and OmpS2 in *Salmonella* Typhi (162, 163), the stationary phase acid tolerance response in *S. Typhimurium* (164, 165), and expression of many virulence genes (112, 113, 166–169).

In *E. coli* MG1655, whole-genome expression profiling identified acid-responsive genes including: chaperones, regulators and genes involved in metabolism (e.g., glutamine decarboxylase) and some genes associated with the cell envelope (170). In another study, the *E. coli* response to mild and strong acidic conditions was compared, revealing a complex transcriptional program that was dependent on OmpR and the switch between aerobic and anaerobic growth (111). OmpR was connected to genes involved in pyruvate metabolism and glycolysis, signal transduction and transport and some components of the glutamate decarboxylation (GAD) system. Direct OmpR targets were not identified, most likely because OmpR regulation of acid stress pathways occurs most commonly via repression of transcription, which has less precise recognition requirements (3, 24). Identifying OmpR binding sites by sequence gazing is difficult, because OmpR has a high capacity for non-specific binding to DNA, in part because it makes more phosphate backbone contacts and fewer DNA base contacts (96). Thus, it is now evident that OmpR drives a major reprogramming in response to acid and osmotic stress. What remains to be determined is how many of these genes are directly regulated by OmpR.

OmpR also regulates two small RNAs, *omrA* and *omrB* which negatively regulate iron transport genes *cirA*, *fecA*, *fepA* and the outer membrane protease *ompT* (171). DNaseI footprinting determined that *omrA/B* binding sites were exquisitely sensitive to OmpR~P (binding at nM concentrations) and unphosphorylated OmpR did not bind to the promoter DNA (107). This observation was validated by *in vitro* transcription assays that were also completely dependent on OmpR~P (and not OmpR), although the precise concentration of OmpR~P in the reaction was not determined (109).

A larger role for OmpR in regulating gene expression is particularly evident in the response to acid and osmotic stress in *S. Typhimurium* and *E. coli* (2, 3, 24, 111, 172). Based on an observed intracellular pH threshold of ~6.5, OmpR represses distinct sets of genes that neutralize the bacterial cytoplasm, thus enabling acidification (3). During acid stress in *S. Typhimurium* and *E. coli*, OmpR contributes to cytoplasmic acidification by repressing the *cadC/BA* operon (3, 24). CadC is in the OmpR subfamily of response regulators and normally it activates transcription of *cadBA*. CadA is a lysine decarboxylase, which consumes a proton during decarboxylation. The product, cadaverine, is then transported out of the cell by the CadB antiporter. Repression of *cadC/BA* by OmpR thus prevents

neutralization. Because the pH optima of the CAD system is 6.1–6.5 (173), it is the most important acid stress system at vacuolar pH (24) and shutting it off enables the bacterial cytoplasm to acidify in the vacuole.

OmpR also promotes cytoplasmic acidification in response to osmotic stress, but different pathways are involved. In *S. Typhimurium*, OmpR represses the alternative stationary phase sigma factor, *rpoS*, relieving RpoS repression of *yghA*. YghA is a putative oxidoreductase that is predicted to produce protons (3). In *E. coli*, the intracellular pH was less acidic and OmpR regulated different pathways. OmpR repressed *speF*, the arginine decarboxylation system, which has a higher pH optimum of 7.0 (174). Normally, ornithine decarboxylase decarboxylates arginine, consumes protons and produces putrescine, which allows for recovery from acidification. Repression of *speF* by OmpR prevents recovery at high osmolality (3).

A recent microarray analysis compared the OmpR-dependent response to acid stress in *E. coli* and reported that about six times as many genes were involved compared to *S. Typhimurium* (2). The involvement of 1360 genes in the OmpR-dependent *E. coli* MG1655 acid stress response (and 1538 genes in the overall OmpR-independent acid stress response) was similar to a study of *E. coli* BW25113, in which 1871 genes were differentially expressed after a 15 minute acid exposure to pH 5.5 (111). These two studies differ from a ChIP-on-chip study using the *E. coli* K-12 strain CSH-50 (172). In that study, only 144 OmpR-regulated acid stress genes were identified in CSH-50, which was only ~10% compared to the 1360 genes identified in *E. coli* MG1655. In contrast, the magnitude of the response of *S. Typhimurium* was similar: 240 vs. 212 acid stress genes identified (2, 172). Fifteen OmpR-regulated genes were common to *E. coli* CSH-50 and *S. Typhimurium* (172), whereas in *E. coli* MG1655 and *S. Typhimurium* strain 14028, 25 genes were common (2). Surprisingly, there was very little overlap between the genes involved in both acid and osmotic stress pathways of *E. coli* and *S. Typhimurium* (2, 172). The genes that were in common were known OmpR targets: *ompC*, *ompF* and the tripartite permease, *tpxB*. These same targets appear in the common genes of the acid and osmotic stress responses of *S. Typhimurium* and *E. coli* (2). Extensive genetic differences between *E. coli* K-12 CSH-50 (175, 176) and MG1655 likely explains the poor overlap between genes identified in these studies.

Flagellar Biosynthesis—The flagellar master regulator FlhDC is encoded by the *flhDC* operon and is responsible for activating the expression of motility and chemotaxis genes, including the flagellar components, the motor apparatus, the chemoreceptors and *che* genes (for a review, see (177, 178)). Several studies report that expression of the *flhDC* operon was partially affected by AcP concentration (179–181). Shin and Park demonstrated that OmpR~P binds to the promoter and represses *flhDC* (158). Mutations in phosphotransacetylase (*pta*) or *ompR* increase *flhDC* expression, whereas mutations in acetate kinase (*ackA*), *envZ11* or increasing osmolality decrease *flhDC* expression. On soft-agar motility plates, (*ompR-envZ*) mutations have a stronger swarm phenotype than the wild-type (155). Therefore, AcP may regulate flagellar biosynthesis through phosphorylation of OmpR, which binds to the *flhDC* promoter region and inhibits expression of the FlhDC master regulator. Electrophoretic mobility shift assays and DNase I

footprinting identify two binding sites in the *flhDC* promoter region (158). Phosphorylation of OmpR leads to a 10-fold increase in DNA binding affinity, as also observed for binding at *ompF* and *ompC* (108) and results in protection at regions -179 to -116 and -17 to +49 from the *flhDC* transcriptional start site. An IHF-binding site is located between the two OmpR binding sites at *flhDC*. A repression loop, similar to that predicted at *ompF* may also occur between the two binding sites at *flhDC* to inhibit transcription of this operon.

Curli Fimbriae Production—Many bacteria form biofilms on surfaces and life in a biofilm is a prevalent lifestyle for microbes in the environment. Biofilms are characterized as a community of microorganisms suspended in a polysaccharide matrix (182). Bacteria in these environments are extremely resistant to most antimicrobial agents and display different patterns of gene expression and morphology distinct from their free-swimming counterparts. A mutation in *ompR* (Leu-43 to Arg substitution; *ompR234* allele) causes a dramatic increase in bacterial surface binding and biofilm formation compared to the wild-type (161). This phenotype is caused by an overproduction of curli, which are long, fimbrial structures composed of the products of the *csgBA* operon. In either wild-type or *ompR* null backgrounds, curli production is substantially reduced. Expression of *csgBA* is stimulated by the transcriptional activator CsgD, whose expression is dependent upon OmpR (160). Although OmpR is proposed to act as a positive regulator at *csgD*, curli formation is repressed at high osmolality. *S. Typhimurium* contains six OmpR binding sites upstream of the *csgD* transcriptional start site, one of which is reported to have an inhibitory effect on *csgD* expression (159). In *Salmonella*, OmpR also regulates the SsrA/B two-component system (112, 113) and the RR SsrB non-canonically activates *csgD* in the absence of phosphorylation by derepressing H-NS (183–185).

Virulence—OmpR modulates the virulence properties of several pathogens and OmpR mutants have a reduced ability to infect hosts. In *Shigella flexneri*, OmpR alters expression of the *vir* genes necessary for invasion of epithelial cells (166). Expression of the *vir* genes is reduced in an *envZ* null mutant but can be restored by increasing medium osmolality. In an *ompR-envZ* double mutant, osmolality changes are uncoupled from *vir* expression and the mutants are attenuated in the invasion of HeLa cells. In *Yersinia enterocolitica*, an *ompR* null mutant is essentially avirulent and is unable to infect orally challenged BALB/c mice beyond 21 days post-inoculation (168). The *ompR* null strain exhibits decreased survival when exposed to high osmolality (1.5 M NaCl) or high temperature (55°C) compared to the wildtype. It remains to be determined whether the avirulent phenotype of *Y. enterocolitica ompR* null strains is caused by this increased susceptibility to environmental stress or, more likely, whether OmpR directly interacts with the regulatory regions of a subset of virulence genes necessary to sustain infection.

In *S. Typhimurium*, *ompR* and *envZ* deletions are attenuated for virulence and are unable to kill phagocytes and spread to other macrophages. The avirulent phenotype is not a result of reduced expression of *ompF*, *ompC* or *tppB* (169). OmpR activates expression of SsrAB, a two-component regulatory system located on *Salmonella* pathogenicity island 2 (112, 113). SsrA/B subsequently regulates a type three secretion system that is required for *Salmonella* to replicate inside macrophages. This is one of only a few examples where a response

regulator of one two-component system affects the transcription of another two-component system. Footprinting analysis identified several OmpR binding sites upstream of *ssrA* and both upstream and within the *ssrB* coding sequence (112, 113). The expression of the kinase SsrA is uncoupled from the expression of the response regulator SsrB (90, 186). This is unusual compared to most two-component regulatory systems in which the ratio of kinase to response regulator is tightly controlled. In *S. Typhi*, OmpR also directly binds and regulates the Vi capsule regulator *IviA* (187).

3.7 A caution regarding C-terminal fusions to OmpR

Recent ChIP-on-chip results with OmpR reported OmpR binding at the *mgtC* promoter (172). In contrast, microarray and qRT-PCR analysis of OmpR-regulated genes at acid pH did not identify *mgtC* as a target of OmpR regulation, nor did *mgtC* contribute to intracellular acidification (24). A likely explanation for this discrepancy is that OmpR containing a 3XFLAG tag was employed in the ChIP-on-chip study, which also contained a D55E substitution (172). C-terminal tags to OmpR affect its DNA binding ability (187) and also alter its specificity (188). For example, the D55E-3XFLAG-tagged OmpR required concentrations $>1 \mu\text{M}$ to bind in electrophoretic mobility shift assays (189), even though the affinity of wildtype, unphosphorylated OmpR for the porin genes *ompF* and *ompC* is $\sim 150 \text{ nM}$ (108). OmpR targets identified by SELEX (188), were not able to be validated (190), and different OmpR targets were identified depending on whether a C-terminal or N-terminal tag was employed (188). Thus, extreme caution should be used when interpreting results using OmpR C-terminal fusions.

A NEW VIEW OF EnvZ/OmpR BASED ON NON-CANONICAL SIGNALING

The old view of two-component signaling was based on a phosphorelay in which the membrane-embedded HK was activated by an external signal (Figure 3). An environmental signal results in a conformational change that drives ATP binding and subsequent autophosphorylation at the conserved histidine (His²⁴³ of EnvZ). The RR then engages the HK~P and phosphotransfer to the RR occurs. The RR-P is then released from the HK to find its target DNA binding sites, allowing activation or repression of transcription. In some systems, the HK plays an additional role as a phosphatase to turn over the RR~P, but in others, this step is controversial (see above) (68).

Our new view of signaling is based on our recent observations of non-canonical signaling by EnvZ/OmpR in response to acid pH (2, 3, 24), and the HDXMS readout of EnvZ conformational changes in response to osmolytes (32). During non-canonical signaling, EnvZ phosphorylation is very low (for example, at acid pH (3)), but OmpR still binds to EnvZ and dimerizes, due to an effect of acid pH on the OmpR binding site of EnvZ (the SINKDIEE peptide). OmpR dimerization mimics an activated state that then binds to DNA and activates or represses transcription (see (183) for a review). This signaling is non-canonical in that the reaction does not involve OmpR phosphorylation but requires an interaction of OmpR with EnvZ (3).

The prominent changes in EnvZ conformation as determined by HDXMS after exposure to osmolytes involved two loci: the histidine-containing peptide (HDXMS peptide amino acids

238-254) and the OmpR binding site (HDXMS peptide amino acids 269-276: SINKDIEE). Note that the actual region might be larger than the peptides identified by HDXMS, for example C²⁷⁷ is C-terminal to the SINKDIEE peptide, and fluorescent labeling of C²⁷⁷ eliminates OmpR interaction with EnvZ (21). In the His²⁴³ locus, the central positioning role of the flanking carbonyl and acidic side chains also make the His-Asp/Glu dyad sensitive to acid pH sensing, although only osmolyte-induced conformational changes enhance autophosphorylation. Lower pH, on the other hand, increases protonation of Asp²⁴⁴, and coupled with stronger helical main chain bonding would alter conformation of the His²⁴³ locus without phosphorylation.

Analysis of the side chain interactions during EnvZ activation provides us with a view in which the signal (intracellular acid or osmotic stress in the case of EnvZ) modifies in parallel, both the RR binding site and the histidine-containing peptide of the HK. This change drives a switch between a non-functional interaction of the RR with the HK to a functional interaction, promoting autophosphorylation and subsequent stimulation of phosphotransfer to the RR. In this scenario, autophosphorylation and phosphotransfer are essentially coupled because the RR is already complexed to the HK. The phosphorylated RR~P dimer (or the unphosphorylated RR dimer in the case of non-canonical signaling), binds DNA, or may already be bound to DNA (119), and activates or represses transcription. The revised series of events are illustrated in Figure 4.

4.1 “An Asp for an Asp” Drives Phosphotransfer

In the resting state, OmpR is bound to the region spanning the EnvZ SINKDIEE locus via interactions in the α 1 helix of the OmpR N-terminal receiver domain (65). In step 1, a signal activates ATP binding **AND** stimulates the acidic cluster of the SINKDIEE locus, loosening its interaction with OmpR at its α 1 helix. The His²⁴³-Asp²⁴⁴ dyad of EnvZ is destabilized via osmolytes or acid pH (32,192), reducing the brake that keeps the histidine-spanning peptide disordered. The helix containing His²⁴³ forms, allowing changes in the histidine side chain rotamer, which promote phosphorylation of His²⁴³ (1). Subsequently, Asp⁵⁵ and residues in the β 3- α 3 and β 4- α 4 loops of the OmpR N-terminus engage the His~P of EnvZ, and phosphotransfer to OmpR occurs. As a consequence, the inhibitory His-anchoring role of Asp²⁴⁴ in EnvZ is now replaced by Asp⁵⁵ of OmpR. Thus, an Asp²⁴⁴(EnvZ)-Asp⁵⁵(OmpR) switch drives phosphotransfer.

EnvZ and OmpR thus function as a composite signaling entity, wherein changes due to osmolality are detected in a continuum by modular units in both proteins, leading to autophosphorylation and phosphotransfer. This new model incorporates inherent specificity that is built into the unique interface mediated by the HK bound to its RR (SINKDIEE of EnvZ). This programs each HK-RR cognate complex to respond with exquisite specificity to precise environmental stimuli, while retaining modular elements of ATP binding, autophosphorylation and phosphotransfer chemistry, which are conserved across nearly all two-component signaling systems. This modularity also effectively allows for non-canonical signaling inputs that can modulate the HK-RR interaction interface to drive additional signaling responses. For example, an altered conformation of OmpR involves a switch from α 1 engagement with EnvZ in the resting state to engagement via β 3- α 3 and β 4- α 4 loops as

the EnvZ SINKDIEE peptide changes conformation in acidic pH. This generates an altered output response, e.g., activation of the acid stress response (2, 3, 24) and explains how the HK-RR composite signaling unit can be specifically driven by phosphorylation-independent non-canonical signaling outputs.

HOW DO SIGNALS ALTER ENVZ/OMPR CONFORMATION?

4.2 A Combination of Side Chain and Peptide Backbone Effects Drive Signaling

Structures of diverse HKs indicate that the autophosphorylated His locus is always positioned within α -helices. In addition, directly C-terminal to the His residue is an acidic Asp/Glu residue that is strikingly conserved in almost all HKs, except PhoQ (32). A series of interactions relay multiple signaling inputs that converge onto the His-Asp/Glu dyad to alter the signaling state of the His and drive autophosphorylation. Parallel side chain interactions in tandem with helical backbone contacts mediate a coordinated response to osmolytes or acid pH in the case of EnvZ.

4.3 Side Chain Effects from the His-Asp Dyad

Specific side chain interactions at the His-Asp dyad directly impact the stability of the helical backbone holding the His side chain. These side chain interactions then modulate the stability of the helical backbone (Figure 8). The positioning of His and Asp residues and their effects on the stability of α -helices has been extensively examined by Huyghues-Despointes and Baldwin (191). The occurrence of His and Asp in helices is not stochastic. Positioning of an Asp residue C-terminal to a His residue enhances α -helix stability only when the Asp is in the $i, i+3$ and $i, i+4$ positions. In EnvZ, the Asp is in the $i, i+1$ position. This makes the His-Asp dyad in EnvZ intrinsically de-stabilizing, and it works to maintain the histidine locus in a locally disordered state (32). Because the Asp is at an $i, i+1$ position, generation of more favorable side chain interactions necessitates puckering of the backbone helix at His²⁴³. This forms the core of the signaling unit: stabilization of the helical backbone occurs in EnvZ in the presence of osmolytes, and negatively impacts the His-Asp side chain contacts. Thus, osmolytes shield the His-Asp side chain contacts, which promotes the ordering of the helical backbone and repositions the His²⁴³ rotamer. These events stimulate autophosphorylation. Interestingly, osmolyte-mediated stabilization has been observed only in the His-Asp orientation and not in the Asp-His orientation of synthetic peptides (191).

Peptide Backbone Stabilization—Examination of the dynamics of the cytoplasmic domain of EnvZ by HDXMS revealed that the four-helix bundle formed the primary signaling locus for osmosensing (1). Residues flanking His²⁴³ were positioned in an island of greater disorder, relative to the rest of the protein (20). Osmolytes facilitated stabilization of this locus, resulting in enhanced autophosphorylation through integration of helical main chain H-bonding and side chain interactions (32). A recent structure of EnvZ_c solved by X-ray crystallography at high osmolality further corroborated these results (192)(Figure 8). The “side chain-main chain swap” model we developed (1, 32) emerged from the use of strategic inhibitory and activating mutants in EnvZ that were located one turn above and one

turn below the phosphorylated His²⁴³ that had opposing effects on autophosphorylation (51, 193, 194).

4.4 The Effect of Osmolytes or Acid pH on EnvZ: a two-way street

Osmolytes function to couple helical backbone stabilization (1) and relieve the His-Asp dyad side chain interactions. Stabilization occurs when the H-bond between the carbonyl C=O of Ala²³⁹ and the imidazole nitrogen of His²⁴³ switches to that between the carbonyl C=O of Ala and the main chain amide N-H of His²⁴³ (32), freeing the Ne for phosphorylation. Osmolytes also shield His²⁴³-Asp²⁴⁴ side-chain interactions, releasing the brake and promoting stabilization of the helical backbone. These two effects of osmolytes are coupled: altering the His-Asp side chain interactions promotes helical stabilization of the His²⁴³ backbone, and helical backbone stabilization at the His²⁴³ locus alters the positioning of the His-Asp dyad. Together, these parallel effects of osmolytes on helical backbone and side chain interactions constitute the basis of osmosensing, and enhanced His²⁴³ phosphorylation.

Substitution of Asp²⁴⁴ with Ala in EnvZc eliminated autophosphorylation of His²⁴³ (195), by enhancing ATP turnover (32). The Asp²⁴⁴ side chain serves both as a brake on His²⁴³ autophosphorylation at low osmolality but repositioning of Asp²⁴⁴ in the activated conformation also stabilizes phospho-His²⁴³. Thus, in the phosphorylation experiments (195), no His~P was detected, because its turnover was enhanced. Both of these results point to a critical role of the Asp/Glu residue of the His-Asp dyad in modulating both His autophosphorylation as well as stability of the phospho-histidine in HKs.

The His-Asp dyad also forms the integrative node for relaying the effects of RR interactions. In EnvZ/OmpR, OmpR contact with EnvZ through the OmpR α helix is relayed to the acidic SINKDIEE cluster in EnvZ through a network of charged residues that modulate the conformation of the His-Asp dyad. The composite EnvZ/OmpR interface forms the basis for specificity of two-component signaling responses, while retaining the conserved chemistries of His-Asp phosphorelays. The His-Asp dyad is highly sensitive to protonation states and pH, providing an alternate stimulus for mediating a phosphorylation-independent response in HK-RRs (2, 3).

IF NEARLY ALL HKs POSSESS THE HIS-ASP DYAD, WHAT PROVIDES SPECIFICITY IN THE SIGNALING RESPONSE?

The answer lies in the RR binding domain of the HK (SINKDIEE in EnvZ), which is unique for each HK/RR cognate pair. In an analysis of co-varying residues between HKs and their cognate RRs, two apparent clusters of specificity determinants were identified in each HK and RR (65). In the HK, one cluster was at the base of the four helix bundle and was implicated in NMR studies as a site of RR interaction (20); in EnvZ, this site was confirmed to be a site of interaction with OmpR by native SDS-PAGE and fluorescence cross-correlation spectroscopy (3, 21, 32). Osmotic stimuli affect both the His-Asp dyad, by releasing the brake and activating the helix surrounding His²⁴³ and also by driving the acidic SINKDIEE locus into an active configuration. The change in the SINKDIEE locus, which is

coupled to activation by environmental stimuli, shifts the interface of OmpR interaction with EnvZ from the $\alpha 1$ helix of the OmpR receiver domain to the $\beta 3$ - $\alpha 3$ and $\beta 4$ - $\alpha 4$ loops of OmpR where Asp⁵⁵ resides. Thus, OmpR binds to EnvZ using a different interface in its inactive and active signaling states, and this difference explains why OmpR~P has a lower affinity for EnvZ than OmpR (67), because it has a different interface. This may also explain why AcP phosphorylation of RRs *in vivo* is reduced in the presence of the cognate HK, because the free RR concentration is reduced. Signal fidelity then resides in how signals affect the RR binding site of the HK, for example, non-cognate HKs will not experience a change in their RR binding site in response to “EnvZ signals”. With advances in super-resolution imaging, we are optimistic that even low abundant complexes such as EnvZ/OmpR can soon be visualized in single bacterial cells to examine how the complex is affected by environmental stimuli (see section 4.6).

4.5 Cytoplasmic Signaling

Despite evidence for the modular nature of the cytoplasmic domain of EnvZ, a stand-alone role for EnvZc was unequivocally demonstrated only recently (1). In this study, EnvZ lacking the N-terminal 179 residues, or EnvZc, fully restored osmosensing in an *E. coli envZ* null mutant, as well as osmolyte stimulation of His²⁴³ autophosphorylation. This result also clearly demonstrated that the TM and periplasmic domains were not obligatory for EnvZ function.

4.6 Non-canonical Activation of OmpR

Although unphosphorylated OmpR does not play a role in porin gene regulation (4), it plays an important role in regulating many genes in response to acid and osmotic stress. Earlier studies of the acid stress response reported a central role for OmpR (111), although no direct targets were identified. It was subsequently determined that the cytoplasm of *E. coli* and *Salmonella* became acidified in an OmpR-dependent manner in response to acid or osmotic stress (3, 24). During acid stress, OmpR repressed *cadC/BA*, preventing a return to neutral pH, by preventing expression of the CAD lysine decarboxylation system. Normally, in response to acid stress, the OmpR family member CadC activates *cadB/A*. CadA is a lysine decarboxylase, which consumes a proton during decarboxylation. The product of decarboxylation, cadaverine, is transported out of the cell by CadB. Repression of the CAD system by OmpR maintained an acidic cytoplasm (3, 24). Mechanistically, how does this work? From our unpublished studies, OmpR phosphorylation by AcP was lowest at pH 6.0 and highest at pH 8.0. EnvZ phosphorylation was also substantially reduced at acid pH to 28% of the level at pH 7.5 (3). The histidine nitrogen that is phosphorylated in HKs needs to be unprotonated to act as a nucleophile toward ATP (196). An aspartic acid mutant of OmpR behaved similarly to the wildtype OmpR in supporting acidification, thus phosphorylation was not required. Surprisingly, OmpR still required the EnvZ kinase (3), because an EnvZ mutant that was unable to interact with OmpR (21) was unable to support OmpR-dependent acidification (3). Thus, OmpR can act non-canonically to drive acid and osmotic stress responses. The observation that OmpR required an interaction with EnvZ (but not phosphorylation) highlights the importance of revising our thinking to consider HK/RR complexes in signaling, even though phosphorylation might not result from the interaction.

In fact, recent evidence of non-canonical regulation by other RRs has been reported, including ArcA, RcsB, and others (see (183) for a recent review).

4.7 Analysis of OmpR in Single Cells

Due to the small size of bacterial cells and the diffraction limit of light, conventional fluorescence microscopy is of limited use (197). Super-resolution microscopy allows visualization of biological molecules far below the light diffraction limit (198). In order to image and count OmpR molecules in single cells, we constructed a photoactivatable fusion protein that was chromosomally expressed under its native promoter and it was capable of activating *ompF* and *ompC* (21). We applied single molecule localization microscopy (SMLM) to visualize OmpR molecules. The bacterial nucleoid was labeled with EdU followed by click chemistry fluorescent labeling and then applying a sequential workflow for imaging (39, 91, 199). In SMLM, the fluorescence signal is separated in time and only a subset of fluorophores is detected, and their position can be precisely determined. Photoactivatable localization microscopy (PALM) can then provide copy numbers of OmpR in single cells (39, 91, 200) and single particle tracking (Spt-PALM) can determine the fraction of OmpR molecules that are immobilized (39, 90). At high osmolality, OmpR molecules were localized close to the edge of the nucleoid (Figure 9) and the chromosome was not decorated by OmpR (39). At low osmolality, the nucleoid was more condensed compared to high osmolality. In acid conditions, OmpR localized near the plasma membrane and appeared to be more clustered, consistent with a separate study in which OmpR dimerization and EnvZ interaction was acid-induced (3). Again, the nucleoids were more condensed (39, 90, 200). This finding is at odds with a report that argued the nucleoids were more relaxed at acid pH, allowing OmpR to bind to more target genes (189). Single cells in acid were also ~ 2 μm smaller than in LB (39).

Early attempts to localize OmpR in cells employed a fusion protein in which the C-terminus of OmpR was directly fused to GFP, and the spatial distribution of OmpR-GFP was examined (197). Although the fusion was described as functional, the activation of the target gene *ompC* was only 20% compared to wildtype OmpR. This level was similar to an early photoactivatable mCherry (PAmCherry) fusion that we constructed (39). Addition of a linker between the C-terminus of OmpR and the fluorescent protein increased activation to >70%. Fluorescent OmpR-GFP foci were visualized at what appeared to be near the membrane. The number of foci increased when EnvZ was over-expressed, and the foci were eliminated by overexpression of native OmpR; these foci were therefore interpreted as being complexes of OmpR-GFP with EnvZ (197). We were able to visualize EnvZ using PALM super-resolution imaging, although it required over-expression of EnvZ (21). We are optimistic that new advances in fluorophores and labeling (201) will now enable visualization of EnvZ/OmpR complexes *in vivo*, and these experiments are currently in progress in our laboratory.

When plasmids containing clusters of OmpR binding sites were imaged, additional OmpR-GFP foci were observed, indicative of OmpR binding to DNA (197). When OmpR was bound to these multiple DNA sites, OmpR-GFP foci near the membrane (suggestive of EnvZ/OmpR complexes) were not observed (197). The results from this study are supportive of a model in which EnvZ is first complexed with OmpR (although whether EnvZ is first

phosphorylated or EnvZ is phosphorylated while complexed with OmpR is not known), OmpR is then phosphorylated and released to bind to DNA (see Figure 3). An improvement on this approach employed arrays of *tetO* and *lacO* binding sites near the *ompF* and *ompC* promoters, respectively (202). OmpR-YFP colocalized with TetR-mCherry or CFP-LacI, and OmpR-YFP foci were absent in the absence of *envZ*. The authors interpreted this to mean that OmpR was not phosphorylated and thus did not bind to DNA. However, the K_d of unphosphorylated OmpR binding to *ompF* and *ompC* is quite low, ~100–150 nM (108). Based on our recent studies of non-canonical effects of OmpR, we offer a different interpretation of this result. We observed that OmpR was required to physically interact with EnvZ in order to activate the acid stress pathway (3), although OmpR phosphorylation was not required (reviewed in (2)). In the presence of an *envZ* mutant, OmpR fails to dimerize and no OmpR transcriptional effects would be observed. A similar effect might better explain the absence of OmpR binding to DNA in the *envZ* null strain (202). Advances in imaging should enable visualization of EnvZ/OmpR at higher resolution and may distinguish between EnvZ/OmpR complexes and OmpR/DNA complexes.

SUMMARY

Our recent work establishes the following events in EnvZ/OmpR signaling: OmpR is complexed with EnvZ *in vivo*. Osmolytes destabilize a negative effect of the His²⁴³-Asp²⁴⁴ dyad on the His²⁴³ locus, this stabilizes the helix to better position the histidine side chain for enhanced phosphorylation. In concert, osmolytes also alter the conformation of the SINKDIEE peptide (OmpR binding site), shifting the OmpR/EnvZ interface from the α 1 interface of OmpR to the β 3- α 3 and β 4- α 4 loops, driving phosphotransfer to Asp⁵⁵ of OmpR. Even though the features of the His locus and the His-Asp dyad are conserved in TCSs, specificity to environmental signals resides in how the signals alter the RR binding site. Future work will focus on imaging these complexes *in vivo* using enhanced super-resolution techniques.

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Abbreviations:

HDXMS	amide hydrogen/deuterium exchange mass spectrometry
TM	transmembrane
HK	histidine kinase
RR	response regulator
spt-PALM	single particle tracking photoactivation localization microscopy
SMLM	single molecule localization microscopy

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Figure 1:

A classical view of EnvZ/OmpR regulation of outer membrane porins in response to osmotic or acid shifts. At low osmolality and neutral pH, OmpF is the major porin in the outer membrane. At high osmolality, *ompF* transcription is repressed and OmpC becomes the predominant porin. EnvZ autophosphorylation is stimulated by increasing osmolality, driving phosphotransfer to OmpR, dimerization, and high affinity binding to DNA.

Figure 2:

EnvZ domain structure. The periplasmic domain of an EnvZ dimer protrudes above the membrane, which is shown as a space-filling model. The transmembrane domains (TMs) connect to the four-helix bundle formed from a dimer of two monomers, the His²⁴³ sidechain (phosphorylation site) is highlighted in red. The ATP binding domains flank His²⁴³.

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Figure 3:

Reaction scheme of EnvZ-OmpR two-component signaling. EnvZ binds ATP and is autophosphorylated at His²⁴³. Phosphorylated EnvZ transfers the phosphoryl group to OmpR. OmpR~phosphate binds with higher affinity to the porin promoters and activates transcription. At high concentrations, EnvZ can catalyze OmpR~P dephosphorylation.

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Figure 4:

A modified reaction scheme of two-component signaling. Because the RR is in great excess compared to the HK, all of the HK would be complexed in vivo. ATP binds to the HK and autophosphorylates, driving phosphoryl transfer and dimerization of the complexed RR. The RR~P is then stimulated to activate its downstream pathway, usually through enhanced DNA binding. The phosphorylated RR dimer can undergo dephosphorylation to then rebind to the HK.

Figure 5:

(A) A highly conserved, receiver domain (phosphorylation site) structure. Key residues that contribute to phosphorylation are highlighted. (B) Ribbon diagram of the NMR structure of the C-terminal domain of OmpR in one orientation (left) and rotated 90° (right)(reprinted with permission from (98)).

Figure 6:

A reaction scheme of events that drive OmpR phosphorylation and DNA binding. OmpR is a two-domain protein, the N-terminal phosphorylation or receiver domain is joined to the C-terminal DNA binding effector domain via a flexible linker. (A) The protein is shown in the uncomplexed state (B) phosphorylated, (C) bound to DNA in the unphosphorylated state and (D) bound to DNA while phosphorylated. The arrows depict transitions between these four states. Further complicating the scheme is the dimerization of OmpR (not depicted for simplicity). OmpR is a monomer in solution and phosphorylation or interaction with EnvZ drives dimerization. In B, C and D, the conformation of the linker is altered by phosphorylation, DNA binding or both events.

Figure 7:

The porin promoters. OmpR~P binds as a dimer to sites located between –100 and –40 upstream of the transcription start site. The site between –100 and –80 (F1 and C1) are the highest affinity for OmpR and OmpR~P. Phosphorylation is required for occupancy of the lower affinity sites F2-F3 and C2-C3. An additional site at *ompF* is required for repression between –380 and –350, and repression is predicted to occur via loop formation assisted by the DNA bending protein IHF. Presumably, the loop then occludes and prevents RNA polymerase binding and subsequent activation of transcription, leading to *ompF* repression.

Figure 8: Model of EnvZ osmosensing and autophosphorylation. High resolution structures of the His²⁴³ neighborhood under conditions of low osmolality (by NMR PDB ID: 1JOY) **A**) and high osmolality (by X-ray crystallography PDB ID: 4KP4). **B**) The osmolality-dependent conformational change is associated with helix stabilization across the four helix bundle subdomain. **C**) An inset shows the local disorder within the His²⁴³-containing helix. The imidazole ring nitrogens (blue) are anchored by competing H-bonds with the Ala²³⁹ backbone carbonyl group and Asp²⁴⁴ side chain (red dashed lines) to maintain low basal levels of His²⁴³ phosphorylation. **D**) Osmolality-induced helical backbone stabilization strengthens the backbone H-bond between the Ala²³⁹ carbonyl and aids in positioning the Asp²⁴⁴ side chain for enhanced His²⁴³ phosphorylation at Ne.

Figure 9:

Sequential super-resolution imaging of OmpR-PAmCherry (red), bacterial cell membrane (green) and DNA (cyan) in various growth media. LB (A), 100 mM Tris pH 7.2 (B), 100 mM MES pH 5.6 (C) and 0.5 x M9 buffer (D). Evidence for DNA compaction is evident in panels in the third row, where the green outlines the nucleoid edges. OmpR was distributed around the nucleoid edges (outlined in green) in LB, whereas it was more uniformly distributed in Tris buffer. In acidic (MES, pH 5.6) and hypotonic (0.5x M9) conditions, OmpR was recruited to the plasma membrane (scale bar = 1 μ m). In the bottom row, images show the averaged distribution of the bacterial membrane (green) and OmpR (red). These average images indicate a localization of OmpR near the membrane in acid and low osmolality. The recruitment of OmpR to DNA in LB is averaged out because of the nucleoid heterogeneity and asymmetric segregation. Cells grown in Tris and 0.5x M9 medium exhibited a smaller cell diameter ($\sim 0.8 \mu$ m), resulting in projection effects for the membrane average image. The number of images used for averaging was: 19, cell length 3.75-4.25 μ m (A), 20 cell length 2.0-2.5 μ m (B), 15 cell length 1.75-2.25 μ m (C) and 13 cell length 1.5-2.0 μ m (D). Reprinted with permission from (40).