Chapter 4

The Role of Two-Component Signal Transduction Systems in Bacterial Stress Responses

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Two-component signal transduction systems represent one of the primary means by which bacteria sense and respond to change in their environments, both intracellular and extracellular. These versatile signaling systems have been implicated in regulating a wide range of stress responses, from nutrient starvation to cell envelope stress and protein misfolding to antibiotics and many more. Two-component signaling proteins are found in nearly all bacteria, with most species encoding dozens and sometimes hundreds of these proteins. These molecules are crucial players in allowing bacteria to adapt and survive in the face of many different stresses and environments. The sensor histidine kinase can respond to intracellular or extracellular cues by catalyzing the phosphorylation of cognate response regulators, which are then capable of modifying gene expression or cellular physiology to help an organism cope with and survive changes in its environment. As two-component signaling proteins are conspicuously absent in animals and humans, they have begun to garner significant attention as possible antibiotic targets. Since their discovery more than 20 years ago, we have learned how specific pathways work at the cellular, molecular, and atomic levels. More recent work has also begun to reveal general design principles governing two-component pathways and the means by which cells coordinate the activity of many highly related pathways. This chapter summarizes the state of our understanding of two-component pathways on all levels, beginning with the initial input stimulus through the final output response. The goal, however, is not to comprehensively review all two-component pathways—which is infeasible here—but, instead, to demonstrate the key principles and paradigms for how these signaling pathways work by drawing on specific, illustrative examples.

The prototypical two-component signal transduction system involves a sensor histidine kinase and its cognate response regulator (Stock et al., 2000) (Fig. 1A). Receipt of an input signal by the histidine kinase stimulates its autophosphorylation whereby the gamma-phosphoryl group of an ATP molecule is transferred to a conserved histidine residue. Histidine kinases are often integral membrane proteins with a periplasmic domain that receives cues and then transduces this signal across the membrane to trigger a change in autophosphorylation activity. Some kinases are entirely cytoplasmic and use domains located upstream of the catalytic domains to sense input signals. An autophosphorylated kinase becomes a phosphodonor substrate for a cognate response regulator that catalyzes transfer of the phosphoryl group on the kinase to a conserved aspartic acid on itself. Phosphorylation of the response regulator typically induces a conformational change leading to activation of an output domain, frequently a DNA-binding domain. Two-component signaling pathways thus often culminate in gene expression changes, although, as discussed later, this is not always the case. When not stimulated to autophosphorylate, the histidine kinases can also act as phosphatases for their cognate response regulators. Or, to be precise, histidine kinases accelerate the intrinsic dephosphorylation rate of the aspartyl phosphate on their cognate response regulators, but, for simplicity, are referred to as phosphatases. Most histidine kinases are thus considered bifunctional, acting as both kinases and phosphatases.

Although many two-component signaling pathways follow this general paradigm, there are numerous variations. One common variant is the so-called phosphor relay (Fig. 1B), which initiates with the autophosphorylation of a histidine kinase that then phosphotransfers to a response regulator just like in a canonical pathway. The phosphoryl group is subsequently shuttled to a histidine phosphotransferase and then to a second, terminal response regulator that ultimately triggers an output or physiological response.

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response. In many cases, the first response regulator of a phosphorelay has only the phosphorylatable receiver domain and no output domain. And, frequently, this receiver domain is fused to the C-terminus of the initiating histidine kinase, forming a so-called hybrid histidine kinase, which phosphotransfers intramolecularly before serving as a phosphodonor for the histidine phosphotransferase. Unlike histidine kinases and response regulators, histidine phosphotransferases do not constitute a single paralogous protein family, but they are structurally similar to one another and to the domain that is autophosphorylated in histidine kinases (Kato et al., 1997; Varughese et al., 1998; Tomomori et al., 1999; Xu and West, 1999; Ulrich et al., 2005; Xu et al., 2009). It is not clear whether phosphorelays offer any advantage relative to canonical two-component pathways, but it has been suggested that phosphorelays offer additional points of control, enabling the integration of multiple signals. Phosphorelays are often associated with major cellular differentiation processes such as cell cycle transitions in Caulobacter crescentus (Biondi et al., 2006), the decision to sporulate in Bacillus subtilis (Burbuly et al., 1991), or the switch from individual to collective, or quorum, behavior in Vibrio harveyi (Freeman and Bassler, 1999a; Freeman and Bassler, 1999b).

Two-component signaling proteins are among the most prevalent signaling molecules in the bacterial kingdom and represent a primary means by which bacteria sense and respond to a range of stresses and environments. Most bacterial species encode dozens, if not hundreds, of histidine kinases and response regulators (Galperin, 2005), underscoring their importance.

These proteins are also found in some eukaryotes, such as plants, slime molds, and yeasts (Loomis et al., 1998), but they are conspicuously absent from animals, including humans, suggesting they may be valuable new antibiotic targets (Stephenson and Hoch, 2002; Watanabe et al., 2008). In fact, many pathogenic bacteria rely heavily on two-component signaling pathways to adapt to life inside a host and to upregulate genes critical to their virulence and the avoidance of host immune systems.

HISTORICAL BACKGROUND

Two-component signaling proteins were first characterized in diverse genetic screens for Escherichia coli mutants that could not properly chemotax, adapt to nitrogen starvation, or respond to cell envelope stress and in screens for B. subtilis mutants that could not sporulate. Each of these screens identified genes involved in executing a given response as well as two-component signaling genes that regulate that response. For instance, early studies of nitrogen starvation in E. coli identified ghnA, which encodes glutamine synthetase and is necessary for responding to changes in nitrogen availability, as well as glnG (ntrC) and glnI (ntrB), which comprise a prototypical two-component signaling pathway that regulates the transcription of ghnA and other genes required for adapting to nitrogen starvation (Ninfa and Magasanik, 1986; Keener and Kustu, 1988; Ninfa et al., 1988; Weiss and Magasanik, 1988). It was quickly recognized that the regulatory genes from these different genetic screens encoded
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Homologous proteins (Ferrari et al., 1985; Stock et al., 1985; Nixon et al., 1986). Subsequent biochemical studies revealed the general mechanisms underlying two-component signal transduction systems (Ninfa and Magasanik, 1986; Hess et al., 1987; Hess et al., 1988; Kecner and Kustu, 1988; Ninfa et al., 1988; Oosawa et al., 1988; Weiss and Magasanik, 1988; Igo et al., 1989a; Igo et al., 1989b) and, importantly, demonstrated that these proteins all utilize a common phosphotransfer mechanism, consistent with their being homologous proteins. For example, at high concentrations, the histidine kinase NtrB was shown, both in vivo and in vitro, to phosphorylate the chemotaxis regulator CheY as well as its cognate substrate NtrC (Ninfa et al., 1988).

Early studies of sporulation mutants in B. subtilis identified multiple two-component signaling proteins required for the initiation of sporulation. Elegant biochemical studies with these proteins led to the identification of the first phosphorelay. This four-step signaling pathway initiates with a histidine kinase, KinA, which then triggers a cascade of phosphotransfer events from KinA to the response regulator SpoOF to the histidine phosphotransferase SpoOB and, finally, to the master regulator of sporulation, SpoOA (Burbulys et al., 1991) (Fig. 2A).

These early studies laid the foundation for our understanding of two-component signaling pathways and, since then, additional histidine kinases and response regulators have been unearthed in countless genetic screens. We have also begun to identify and characterize numerous factors that influence the activity of these key signaling molecules, as discussed in this chapter. In addition, because two-component proteins share significant homology to one another, the sequencing of complete bacterial genomes has enabled their comprehensive cataloging in hundreds of organisms. These cataloging and related bioinformatic efforts have shed new light on both the diversity and commonalities of two-component pathways. They have also enabled global analyses that are starting to reveal how organisms simultaneously coordinate so many highly related proteins. This chapter discusses each aspect of two-component signaling pathways, working back from the myriad output responses to the flow of phosphoryl groups within pathways and finally to the initial sensing of signals and inputs.

OUTPUTS

Response regulators control an enormous array of physiological, metabolic, and morphological processes in bacteria, a testament to the versatility of two-component signaling proteins. These proteins typically contain two domains: a receiver domain that contains the conserved aspartate phosphorylation site and an output, or effector, domain (Fig. 3A). Although the most common output domain is a DNA-binding domain that allows response regulators to control gene expression, there are at least 10 additional types of output domains (Galperin, 2006). Whatever the output domain, structural and biochemical studies suggest a common mechanism by which phosphorylation activates all response regulators (Stock et al., 2000; West and Stock, 2001).

Receptor domains all adopt a conserved α/β fold in which a five-stranded β-sheet is surrounded by five amphipathic alpha helices. Phosphorylation of the conserved aspartate triggers conformational changes that propagate through a series of highly conserved residues stretching from the phosphorylation site to the α4-β5-α5 face of the molecule. Conformational changes in this region of the receiver domain then drive homodimerization or changes in protein-protein interactions, ultimately enabling response regulators to affect cellular physiology.

A systematic bioinformatic analysis of more than 4,500 response regulators classified effector domains into two broad categories: DNA-binding and those harboring enzymatic activities (Galperin, 2006; Gao et al., 2007). Although, as noted, at least 10 distinct effector domains have been identified. In addition, many response regulators, such as the chemotaxis regulator CheY, have only a receiver domain and use conformational changes in this domain alone to modulate interactions with target proteins (Jenal and Galperin, 2009).

More than 60% of all response regulators contain DNA-binding domains; two-component signaling pathways thus represent one of the primary means by which bacteria can couple changes in their environments or intracellular states to changes in gene expression. The DNA-binding effector domains can be further categorized based on sequence similarity into three major classes: OmpR-like, NarL-like, and NtrC-like (Fig. 3A). There are diverse mechanisms by which phosphorylation induces changes in activity of these DNA-binding response regulators, even within a group (Gao et al., 2007). In some cases, such as E. coli NarL and B. subtilis Spo0A, the receiver domain plays an autoinhibitory role such that its phosphorylation relieves inhibition and then permits DNA-binding (Ireton et al., 1993; Baikalov et al., 1996). In other cases, such as FixJ, phosphorylation of the receiver domain drives the dimerization of a response regulator, leading to tighter binding of target promoters (Da Re et al., 1999). And, finally, in other cases, such as PhoB and NtrC, receiver domain phosphorylation triggers rearrangement from an inactive dimer...
or multimer state to an alternative and active dimer or multimer state (Wyman et al., 1997; Bachhawat et al., 2005). Response regulators can either activate or repress transcription, usually depending on the promoter architecture and the location of the regulator's binding site relative to the transcriptional start site. Most response regulators that activate transcription are thought to do so by stimulating recruitment of RNA polymerase. The NtrC subfamily, however, contains an AAA+ domain (Fig. 3A) that has ATPase activity, enabling these regulators to catalyze closed to open complex formation in RNA polymerase that
is associated with the alternative σ factor σ54 (Kustu et al., 1991). The NtrC subfamily of regulators can also often bind at a distance and are often referred to as enhancer-binding proteins.

After DNA-binding domains, the most common effector domains are those involved in cyclic-di-GMP signaling (Fig. 3A). This includes so-called GGDEF domains, which are typically diguanyl cyclases that synthesize c-di-GMP, and EAL and HD-GYP domains, which function as phosphodiesterases for c-di-GMP (Jenal and Malone, 2006). In most bacteria, the production of c-di-GMP plays a critical role in the transition from a motile or planktonic growth phase to a sessile or biofilm state. Precisely how c-di-GMP influences cellular physiology is not yet entirely clear, but it likely involves the binding of this small molecule to specific target proteins that then control various metabolic, motility, or regulatory processes (Jenal and Malone, 2006). The decision to transition from a planktonic to a biofilm state is influenced by a wide range of signals, but is often coordinated by two-component signaling pathways that couple changes in environmental conditions to the production of c-di-GMP by phosphorylating response regulators with GGDEF output domains. For instance, in Pseudomonas aeruginosa, a chemotaxis-like system drives the phosphorylation of the response regulator WspR which, in turn, stimulates its diguanylate cyclase activity and the accumulation of intracellular c-di-GMP (Hickman et al., 2005). Similarly, in C. crescentus, the differentiation of a motile, swarm cell to a sessile, stalked cell requires a response regulator called PleD, which contains a GGDEF output domain (Paul et al., 2004; Hecht and Newton, 1995). The diguanylate cyclase domain of PleD functions as a dimer and phosphorylation of the receiver domain is thought to increase c-di-GMP production by driving protein dimerization (Wassmann et al., 2007). WspR and other GGDEF-containing response regulators likely work by a similar mechanism.

Finally, as noted, many response regulators contain only the receiver domain with no additional or discrete output domain (Fig. 3A). The prototypical member of this subfamily is the chemotaxis regulator CheY where phosphorylation induces a conformational change in the α4–β5–α5 face of the receiver domain that, in turn, modulates its ability to interact with the flagellum and control swimming behavior (Cho et al., 2000; Lee et al., 2001a; Lee et al., 2001b). The diverse roles for other single-domain response regulators are just now beginning to be dissected but they are likely to regulate a diverse range of processes beyond chemotaxis (Jenal and Galperin, 2009). In C. crescentus, two single-domain response regulators are critical to cell cycle progression. One is the single-domain response regulator DivK (Fig. 2C), which is essential for viability and for cell cycle progression (Hecht et al., 1995; Hung and Shapiro, 2002; Biondi et al., 2006). Phosphorylated DivK is required for the G1-S transition by downregulating the phosphorylation that initiates with the histidine kinase CckA.
and culminates in activation of the master regulator CtrA (Biondi et al., 2006) (Fig. 2C). DivK has also been shown to allosterically stimulate the autophosphorylation of its cognate kinase DivJ and to switch its cognate phosphatase PleC into the kinase state (Paul et al., 2008). These data suggest that DivK has two independent binding sites on DivJ and PleC, one for phosphotransfer and one for modulating autophosphorylation activity. Yeast two-hybrid screens indicate that DivK also binds to the histidine kinase DivL, an interaction that may be crucial to the role DivK plays in controlling CtrA (Ohba and Newton, 2003). Another important single-domain response regulator in Caulobacter, called CpdR, was first discovered in a systematic deletion study of two-component genes (Skerker et al., 2003). It was subsequently shown to control the localization and activity of the ClpXP protease which degrades the master cell cycle regulator CtrA at the G1-S transition (Biondi et al., 2006; Iniesta et al., 2006) (Fig. 2C). Precisely how CpdR influences ClpXP activity or localization remains unclear, but CpdR exemplifies the crucial and ever-expanding role of single-domain response regulators.

PHOSPHOTRANSFER

A critical step in two-component signaling is the transfer of a phosphoryl group from the histidine kinase to its cognate response regulator. In most cases, a histidine kinase will phosphorylate a single cognate response regulator, forming an exclusive one-to-one signaling pair. However, in other cases, cells exhibit many-to-one and one-to-many relationships, further enhancing the information-processing capabilities of bacteria.

A prime example of a many-to-one relationship is the multiple histidine kinases that phosphorylate Spo0F to drive the initiation of B. subtilis sporulation (Antoniewski et al., 1990; Trach and Hoch, 1993; Jiang et al., 2000). The kinases KinA and KinB are essential for proper sporulation and drive most of the phosphorylation of Spo0F under standard laboratory conditions. KinC, KinD, and KinE are formally dispensable, but each is capable of phosphorylating Spo0F (Fig. 2A). These three kinases may simply supplement the activity of KinA and KinB, or they may be essential for phosphorylating Spo0F in response to different environmental conditions. The convergence of multiple histidine kinases on Spo0F may enable different stress and starvation signals to drive the initiation of sporulation. Alternatively, such a topology may be allowing the integration of signals such that the subthreshold activation of multiple pathways triggers sporulation.

Another classic example of a many-to-one relationship in two-component signaling is the multiple hybrid histidine kinases in V. harveyi that converge on the histidine phosphotransferase LuxU to regulate the quorum sensing response of this organism (Fig. 2B). Under conditions of low cell density, the hybrid kinases LuxN, LuxQ, and CqsS can each autophosphorylate and drive the phosphorylation of LuxU, which in turn can phosphorylate the response regulator LuxO (Freeman and Bassler, 1999a; Freeman and Bassler, 1999b; Freeman et al., 2000; Henke and Bassler, 2004). Phosphorylated LuxO ultimately activates the expression of five small RNAs that inhibit translation of LuxR, a key transcription factor involved in quorum-dependent behavior. Under conditions of high cell density, three different autoinducers can accumulate and switch LuxN, LuxQ, and CqsS from their kinase to phosphatase states. Because phosphorelays are reversible, LuxO will backtransfer phosphoryl groups to LuxU, which then transfers to the receiver domain of a hybrid histidine kinase where the bifunctional kinase domain drives dephosphorylation. Hence, switching any of the quorum histidine kinases to the phosphatase state is likely sufficient to drive the complete dephosphorylation of LuxO, resulting in the synthesis of LuxR and its density-dependent genes.

There are also examples of one-to-many relationships and histidine kinases that have multiple, bona fide targets. The best such example is the chemotaxis kinase CheA that phosphorylates the single-domain regulator CheY to effect changes in flagellar rotation as well as the methylesterase CheB, which plays a critical role in signal adaptation by demethylating chemotaxis receptors such as Tar (Armitage, 1999).

The operation of two-component signaling pathways, whether branched or simply one-to-one, demands an exquisite specificity of information flow. Cells must somehow ensure that histidine kinases interact with and phosphotransfer to the "correct," or cognate, response regulator(s) while avoiding detrimental cross talk with noncognate regulators. This is a significant challenge for bacterial cells because histidine kinases and response regulators are often very similar to one another at the sequence and structural levels, and most organisms encode dozens, if not hundreds, of each type of protein.

The specificity of phosphotransfer is driven primarily by molecular recognition and an intrinsic specificity of histidine kinases for their cognate substrates (Skerker et al., 2005; Laub and Goulian, 2007). In principle, other mechanisms could contribute to or reinforce specificity. For instance, differential expression of signaling proteins could prevent the interaction of noncognate partners, or scaffold
proteins that simultaneously bind components of a signaling pathway and enforce spatial proximity of cognate proteins could help prevent cross talk between noncognate partners. However, there is a wealth of data that points to molecular recognition as the dominant force behind signaling fidelity. Early studies with the histidine kinase VanS from Enterococcus faecium demonstrated that it preferentially phosphorylated VanR relative to the noncognate substrate PhoB from E. coli (Fisher et al., 1996). Kinetic studies also demonstrated that KinA in B. subtilis can phosphorylate both Spo0F and Spo0A, but has a nearly 57,000-fold preference for phosphotransfer to Spo0F (Grimshaw et al., 1998). More recently, in E. coli and C. crescentus, histidine kinases were shown to exhibit a strong, global kinetic preference in vitro for their in vivo cognate substrates (Skerker et al., 2005). For example, autophosphorylated EnvZ was systematically examined for phosphotransfer in vitro to each of the 32 response regulators encoded in the E. coli genome. With extended incubation times, EnvZ phosphorylated several response regulators; but, with shorter incubations, EnvZ preferentially phosphorylated OmpR relative to all other E. coli response regulators. More detailed kinetic analyses revealed that EnvZ has an approximately 1,000-fold preference for phosphotransfer to OmpR relative to the next best substrate, CpxR, and hence even higher preferences relative to all other response regulators (Skerker et al., 2005). This preference corresponds with numerous in vivo studies indicating that EnvZ's primary target is OmpR.

Efforts to understand the specificity of molecular recognition in phosphotransfer have been hampered by the lack of structural data and, in particular, by the lack of a co-crystal structure of a histidine kinase in complex with a response regulator. There is a structure of the histidine phosphotransferase Spo0F from B. subtilis in complex with the response regulator Spo0F (Zapf et al., 2000) and, more recently, a structure has been solved of the Thermotoga maritima kinase TM0853 in complex with its cognate regulator TM0468 (Casino et al., 2009). However, these two static structures still do not fully reveal how cognate and noncognate substrates are distinguished from one another. To tackle this problem, alternative approaches have been pursued—most notably, analyses of amino acid coevolution in kinase-regulator pairs (White et al., 2007; Skerker et al. 2008). If the operation of a two-component signaling pathway depends on molecular recognition, then mutations in the histidine kinase may be accompanied by compensatory mutations in its cognate response regulator or vice versa. To identify patterns of amino acid coevolution, recent bioinformatic analyses have examined large sets of co- operonic histidine kinases and response regulators; such pairs typically interact in a one-to-one fashion and are thus expected to coevolve. Indeed, sequence analysis identified a relatively small set of amino acids that appear to coevolve in the two molecules. These putative specificity residues map to the molecular interface formed when a response regulator docks to a histidine kinase, but comprise only a subset of the interfacial residues (Skerker et al., 2008). Importantly, mutating these specificity residues in the histidine kinase EnvZ to match the corresponding residues found in other E. coli histidine kinases, such as RstB, was sufficient to reprogram the substrate selectivity of EnvZ (Skerker et al., 2008). These results have thus highlighted the key residues that dictate specificity. In addition, they open the door to the rational rewiring of two-component signaling pathways and the possibility of developing algorithms to predict kinase-regulator pairings in any bacterium.

Similar analyses of amino acid coevolution may also be possible to identify the residues that dictate the specificity of histidine kinase and response regulator homodimerization. A recent systematic analysis of Förster resonance energy transfer (FRET) between all possible pairs of response regulators in E. coli demonstrated that these molecules specifically homodimerize (Gao et al., 2008), suggesting that a set of residues exists to mediate homodimerization and to prevent all possible heterodimerizations.

Although molecular recognition is the dominant determinant of phosphotransfer specificity in two-component signaling pathways, other mechanisms do exist. One key mechanism involves the phosphatase activity of bifunctional histidine kinases. For example, the phosphorylation of OmpR by the noncognate histidine kinase CpxA is normally offset by EnvZ-dependent dephosphorylation of OmpR; similarly, phosphorylation of CpxR by EnvZ is offset by CpxA's dephosphorylation of CpxR (Siryaporn and Goulian, 2008). Hence, CpxA phosphorylates OmpR in a ΔenvZ mutant and EnvZ phosphorylates CpxR in a ΔcpxA mutant, but wild-type cells are effectively buffered against spurious cross talk.

Response regulator competition seems to further reinforce the fidelity of information flow in two-component pathways. For example, in E. coli, the relatively high abundance of the response regulator OmpR can prevent cross talk from EnvZ to CpxR and, conversely, CpxR can prevent cross talk from CpxA to OmpR (Siryaporn and Goulian, 2008). In each case, the cognate response regulator (which also has a higher affinity for its cognate kinase) competes for, and effectively occupies, its cognate histidine kinase, which is much lower in abundance. By occupying its
cognate kinase, a regulator prevents the kinase from phosphorylating a noncognate regulator. These two mechanisms—dephosphorylation by a cognate kinase and response regulator competition—have been characterized in numerous systems, not just with EnvZ-OmpR and CpxA-CpxR, suggesting they are general design principles of two-component signaling pathways (Laub and Goulian, 2007). These mechanisms, in combination with the intrinsic specificity of phosphotransfer, ensure the fidelity of two-component pathways and prevent significant cross talk at the level of phosphotransfer. Two-component signaling pathways are not, however, entirely insulated from one another because there are numerous cases of response regulators that regulate overlapping sets of genes (Laub and Goulian, 2007). Consequently, two distinct pathways can effect both common and pathway-specific transcription.

**AUXILIARY PROTEINS**

Given the importance of two-component signaling pathways to bacteria, it is perhaps not surprising that cells have evolved numerous ways of modulating the output of these signaling pathways, including cofactors, phosphatases, and protein inhibitors. These auxiliary components provide additional layers of regulation, in some cases fine-tuning the output of a signaling pathway, but in other cases significantly changing signaling dynamics.

**Regulators of Response Regulators**

In many cases, histidine kinases are bifunctional: acting as both kinases and phosphatases for their cognate substrates. However, many two-component signaling pathways are also regulated by exogenous, dedicated phosphatases. To date, all of the phosphatases characterized, with one possible exception (discussed later), target the aspartyl phosphates on response regulators. Unlike histidine kinases and response regulators, the aspartyl phosphatases do not constitute a single paralogous gene family and are often structurally dissimilar from one another. This fact has made their identification difficult and, hence, the repertoire of known phosphatases is still somewhat limited.

The aspartyl phosphate on most response regulators is often labile and hydrolyzes at an appreciable rate on its own. Nevertheless, many response regulators have cognate, dedicated phosphatases, or cognate proteins that accelerate the intrinsic rate of dephosphorylation. Even for response regulators with extremely short phosphoryl group half-lives there can be dedicated phosphatases, such as the phosphatase CheZ that specifically targets CheY. The chemotactic behavior of *E. coli* cells is highly sensitive to changes in CheZ concentration, such that either increasing or decreasing CheZ disrupts proper chemotaxis (Sanna and Simon, 1996), underscoring the critical role that phosphatases play in precisely controlling the activity of response regulators and the output of two-component signaling pathways.

Some response regulators have multiple phosphatases. In *B. subtilis*, several phosphatases have been identified that target the response regulators Spo0F and Spo0A to block the initiation of sporulation (Perego et al., 1996; Perego and Hoch, 1996a; Perego and Hoch, 1996b; Perego, 2001) (Fig. 2A). In some cases, these phosphatases are subject to further regulation themselves and hence can integrate additional signals into the decision to sporulate. For instance, the activity of the phosphatase RapA, which dephosphorylates Spo0F, is regulated by a gene immediately downstream of *rapA* called *phrA* (Perego and Hoch, 1996a). This gene encodes for a small protein that is secreted and subsequently processed to form a five amino acid peptide that binds to and antagonizes RapA. The accumulation of the *phrA*-derived peptide thus depends on cell population density—meaning, the RapA phosphatase effectively integrates a quorum signal into the decision to sporulate by modulating flow through the Spo0A phosphorelay. Intriguingly, the transcription of *rapA* is stimulated by another two-component pathway, ComP-ComA, which controls the cellular decision to become competent (Perego and Hoch, 1996a). The RapA phosphatase thus serves not only to integrate information on cell density, but may help coordinate two different pathways, helping to prevent their simultaneous activation. Another phosphatase, RapB, also targets Spo0F, but the signal that RapB responds to remains unclear (Perego et al., 1994). Several phosphatases appear to directly target Spo0A, including Spo0E and perhaps YnrD and YisI (Perego, 2001). The presence of numerous phosphatases dedicated to the sporulation phosphorelay indicates that *B. subtilis* cells integrate a wide range of disparate signals into the critical decision to sporulate or not (Fig. 2A).

In addition to phosphatases, there is a growing list of proteins that modulate response regulators by direct protein-protein interaction without driving dephosphorylation. These proteins act in diverse ways, but ultimately inhibit response regulator activity. Two of the Rap proteins from *B. subtilis*, RapC and RapG, interact with the response regulators ComA and DegU (Solomon et al., 1996; Ogura et al., 2003). Although RapC and RapG are homologous to the RapA and RapB phosphatases described previously,
they do not affect dephosphorylation of ComA and DegU, respectively, and instead block binding of these regulators to their target promoters. In *E. coli*, the protein TorI binds to the response regulator TorR and inhibits TorR-dependent gene expression (Ansaldi et al., 2004). However, in contrast to RapC and RapG, TorI does not prevent TorR from binding to DNA, suggesting it may instead disrupt activation of RNA polymerase. As a final, somewhat unconventional, example in *B. subtilis*, the protein Spx has been shown to inhibit transcriptional activation by multiple response regulators, including ResD and ComA (Nakano et al., 2003). However, Spx does not bind to the regulators themselves, but instead binds to a site on the alpha subunit of RNA polymerase that the regulators must normally interact with to drive gene expression.

Another emerging class of molecules that regulate response regulators is small inhibitory proteins, or peptides. In *E. coli*, the response regulator RssB controls the stability of RpoS, an alternative σ factor and master regulator of stationary phase (Muffler et al., 1996; Pratt and Silhavy, 1996; Zhou et al., 2001). During exponential phase, RpoS levels are kept low by RssB, which is a proteolytic targeting factor for RpoS, helping deliver the σ factor to its protease ClpXP. When cells enter stationary phase or are starved of key nutrients, such as phosphate, RpoS is stabilized allowing it to drive gene expression; in many cases this stabilization occurs by inhibiting the response regulator RssB. Recent work has identified a family of small proteins, termed anti-adapter, that are transcriptionally induced following starvation and that bind directly to RssB to inhibit it (Bougdour et al., 2006; Bougdour et al., 2008; Merrikh et al., 2009). The first anti-adapter characterized was IraP, which is induced following phosphate starvation, but others such as IraM and IraD, which respond to magnesium starvation and DNA damage, respectively, have since been identified and there are likely many more. Precisely how these small proteins inhibit RssB from delivering RpoS to the protease ClpXP or whether they bind similar regions of RssB is not yet clear. IraP, IraM, and IraD do not share significant similarity to one another suggesting they could bind RssB in different ways to inhibit its activity as a protease adaptor.

Proteins that bind and influence response regulators may not always function as negative regulators and can instead promote the activity of a response regulator. A case in point is the small protein PmrD in *Salmonella enterica* that binds to and stabilizes the aspartyl phosphate on the response regulator PmrA following phosphorylation by PmrB, its cognate histidine kinase (Kato and Grosman, 2004). PmrB responds to changes in extracellular iron concentrations by driving the phosphorylation of PmrA, which then triggers changes in gene expression. The presence of PmrD can thus reinforce these changes in transcription. The synthesis of PmrD itself is regulated by a different two-component signaling pathway, PhoQ-PhoP, which responds to changes in extracellular magnesium concentration. Consequently, activation of the PhoQ-PhoP system and the induction of PmrD can help stimulate PmrA-dependent gene expression, even after PmrB is no longer stimulated to phosphorylate PmrA.

**Regulators of Histidine Kinases**

To date, the only reported phosphatase targeting a histidyl phosphate is the protein SixA in *E. coli* that drives the dephosphorylation of the histidine phosphotransferase domain of ArcB in vitro (Garcia Vescovi et al., 1996). However, the role of SixA in modulating anaerobiosis in vivo, as ArcB does, remains unclear. Although histidyl phosphatases seem to be uncommon, many histidine kinases are modulated by small inhibitory proteins. In *B. subtilis*, the sporulation initiation kinases KinA and KinB are inhibited by Sda, a 46 amino acid protein that binds directly to a region near the conserved histidine within each kinase (Ogino et al., 1998; Burkholder et al., 2001; Bick et al., 2009) (Fig. 2A). Immediately prior to sporulating, *B. subtilis* cells must complete a final round of DNA replication to ensure that both the mother and daughter cells inherit a complete chromosome. Any disruption of DNA replication or replication stress, such as that caused by DNA damage, induces the expression of Sda, which then inhibits KinA, preventing both autophosphorylation and phosphotransfer to SpoOF (Cunningham and Burkholder, 2009). This Sda-based circuitry represents a checkpoint system, coupling DNA replication to sporulation, by modulating output from the sporulation phosphorelay as also described previously for the Rap phosphatases. The autophosphorylation of KinA (but not KinB) can also be inhibited by a small protein called Kipl, whose synthesis depends on the availability of fixed nitrogen (Fig. 2A); however, the precise signal sensed by Kipl and its role in sporulation remain unclear (Wang et al., 1997). Other examples of histidine kinase inhibitors include the PII protein GlnB in *E. coli* and FixT in *Sinorhizobium meliloti*. GlnB directly inhibits autophosphorylation of NtrB, a histidine kinase critical for responding to nitrogen starvation, and concomitantly enhances the phosphatase activity of NtrB on NtrC-~P (Jiang and Ninfa, 1999). FixT acts similarly, by inhibiting autophosphorylation of the oxygen-sensitive kinase FixL and promoting its phosphatase activity on FixJ-~P (Garnerone et al., 1999).
The examples mentioned include cytoplasmic regulators of histidine kinases; there are also examples of transmembrane and periplasmic proteins that regulate histidine kinases. Two examples are B1500, a small inner-membrane protein that directly stimulates the histidine kinase PhoQ (Eguchi et al., 2007), and CpxP, a periplasmic protein that inhibits the kinase CpxA (Fleischer et al., 2007). Finally, in P. aeruginosa, the histidine kinase GacS, which activates a response regulator called GacA that is crucial to the switch from acute to chronic infection, is directly inhibited by another membrane-bound histidine kinase called RetS (Goodman et al., 2009). This inhibition does not require the catalytic residues of RetS indicating that RetS inhibits via a protein-protein interaction that somehow disrupts autophosphorylation of GacS, although the domains involved are not yet known.

INPUTS

One of the least understood aspects of two-component signaling systems is the initial activation of histidine kinases. All histidine kinases have two conserved domains: the dimerization and phosphotransfer (DHp) domain and the catalytic and ATP-binding (CA) domain (Fig. 3B). The DHp domain is a four-helix bundle formed by the homodimerization of two histidine kinases and contains the conserved histidine in a solvent-exposed position within the first alpha helix. The CA domain binds ATP and catalyzes transfer of the gamma phosphoryl group to the DHp domain. Autophosphorylation activity is likely regulated by controlling the interaction between these two domains. Unlike the DHp and CA domains, the sensor domains of a histidine kinase are often structurally and phylogenetically unrelated, and in only a few cases are the precise ligands known (Bader et al., 2005; Cheung and Hendrickson, 2008; Cheung and Hendrickson, 2009; Kerff et al., 2003). Many of the known ligands are small molecules, metabolites, or ions that bind directly to the periplasmic domain of a histidine kinase. However, in some cases, adaptor proteins may bind ligands and then transduce this information to their partner kinase. For example, in V. harveyi, the histidine kinase LuxQ is found constitutively associated with a periplasmic binding protein called LuxP that reversibly binds the autoinducer Al-2 to regulate LuxQ kinase activity and quorum sensing (Neiditch et al., 2005; Neiditch et al., 2006).

It should also be noted that not all histidine kinases respond to periplasmic or extracellular cues and, in fact, many histidine kinases are entirely cytoplasmic. Signal recognition for these histidine kinases typically involves domains located immediately N-terminal to the DHp and CA domains; as discussed later, these adjacent domains are often PAS or HAMP domains.

Although a few direct signals are known, for the vast majority of histidine kinases, their stimuli have only been characterized phenomenologically. For example, envZ was initially implicated in responding to changes in osmolarity of E. coli growth media (Hall and Silhavy, 1981), but precisely how osmolarity is sensed by EnvZ remains a mystery. And some studies have reported that the small lipophilic molecule procaine is a more potent inducer of the EnvZ-OmpR system than changes in osmolarity (Batchelor and Goulian, 2006), leaving open the question of what EnvZ responds to in E. coli's natural setting and underscoring the gap in our understanding of the input signals for histidine kinases. As another example, the histidine kinase CpxA was initially implicated in responding to defects in F-pilus biogenesis (McFwan and Silverman, 1980) and then subsequently shown to respond to unfolded periplasmic proteins or the overexpression of periplasmic proteins such as NlpE (Danese et al., 1995). Even more recently it was reported that the CpxA-CpxR pathway is activated by exposure to aminoglycoside antibiotics such as gentamicin and kanamycin (Kohanski et al., 2008). Although these three inducers of CpxA are seemingly incongruous, they each likely lead to the misfolding of membrane proteins, providing a unifying notion for CpxA's sensory function. These studies of CpxA also underscore the central role that two-component signaling proteins play in coordinating diverse bacterial stress responses. However, it is still unclear whether the activation of CpxA in response to these diverse stressors proceeds via a common mechanism at the molecular level or whether there are different means by which CpxA can be activated.

Ultimately, periplasmic signals must be transduced across the membrane to induce changes in the autophosphorylation state of the histidine kinase. This critical step of the pathway remains mostly mysterious, but recent studies of nitrate-sensing by the histidine kinase NarX suggest that a piston-type displacement occurs between alpha helices in the periplasmic domain (Cheung and Hendrickson, 2009); similar movements have been suggested in the activation of the chemoreceptor Tar which ultimately activates the histidine kinase CheA (Chervitz and Falke, 1996; Ottemann et al., 1999). Precisely how such piston-like movements in the periplasm would be propagated to the cytoplasmic domains is unresolved. Studies of the histidine kinase LuxQ have also provided intriguing hints about the mechanism of signal transduction across a membrane by a histidine kinase (Neiditch et al., 2005; Neiditch et al., 2006). As noted previously, the periplasmic domain of LuxQ is constitutively...
associated with the protein LuxP, which can directly bind the quorum signal autoinducer AI-2 (Fig. 2B). Under conditions of low cell density when LuxP's binding site is unoccupied by ligand, the LuxP/LuxQ complex adopts a symmetric quaternary structure in which the LuxQ monomers are positioned in a manner that allows autophosphorylation. The binding of AI-2 to LuxP switches the periplasmic domains into an asymmetric configuration that is presumably propagated to the cytoplasmic domains and results in LuxQ transitioning from the kinase to phosphatase state. The studies of LuxQ and NazX have only scratched the surface and much remains to be understood about the mechanisms by which ligands and signals control their histidine kinases.

Translating periplasmic signals into changes in autophosphorylation activity probably also involves cytoplasmic domains that lie between the last transmembrane helix and the beginning of the DHp domain. A careful census of histidine kinase domain structure found that the two most common domains immediately N-terminal to the DHp domain are so-called HAMP and PAS domains (Anantharaman et al., 2006) (Fig. 3B). HAMP domains are coiled coils that have been suggested to relay receipt of a signal by adopting one of two conformations that are rotationally related (Hulko et al., 2006). PAS domains adopt a compact, globular fold and may relay signals by reversible docking and release of the last alpha helix in the domain (Harper et al., 2003; Taylor and Zhulin, 1999). Many PAS domains also participate directly in sensing various ligands or small molecules. For example, one subclass of PAS domains called LOV (light-oxygen-voltage) domains use flavin nucleotide cofactors to sense changes in redox state, oxygen tension, or available light.

Precisely how PAS and HAMP domains regulate autophosphorylation at a molecular and atomic level is unknown. However, HAMP and PAS domains are usually linked to the DHp domain by an S-helix, or signaling helix, which is thought to form a coiled coil linker (with each monomer contributing one helix) that connects the terminal alpha helices in the HAMP or PAS domain to the first alpha helix of the DHp domain. The S-helix likely plays a central role in converting ligand binding or signal recognition into changes in autophosphorylation activity of the kinase (Anantharaman et al., 2006). One recent study has made significant progress in characterizing how this linker controls a kinase's activity by rationally reprogramming FixL, an oxygen-sensing histidine kinase from *Bradyrhizobium japonicum* (Möglich et al., 2009). FixL normally contains a heme-binding PAS domain that allows it to sense changes in oxygen tension within the cell. This PAS domain was replaced with a structurally related LOV blue-light sensor from *B. subtilis* YtvA (not a histidine kinase) to make FixL sense and respond to light. This chimeric kinase, dubbed YF1, was light-regulatable; the default, or dark, state of YF1 was competent for autophosphorylation whereas exposure to light led to a more than 1,000-fold decrease in activity. A systematic mutational study of the linker connecting the LOV domain to the DHp domain demonstrated that only insertions of seven amino acids were tolerated and continued to yield a kinase that could be shut off by blue light. And, intriguingly, insertions of just two amino acids generated a protein whose default state was off, but that could now be activated by blue light. Together these observations provide strong evidence that input signals are transmitted to the DHp domain via a coiled coil linker that effectively acts as a rotary switch. Subsequent bioinformatic analysis revealed that the length of the linker between PAS and DHp domains in most histidine kinases is either a multiple of seven or a multiple of seven plus two. These studies are thus beginning to unveil general design principles of histidine kinases, while simultaneously enabling the rational rewiring of pathways. Such rewiring may provide new tools for probing regulatory systems that utilize two-component pathways and they may further enhance efforts in synthetic biology to design regulatory circuits de novo.

**OUTLOOK**

Most bacteria are faced with a constantly changing environment and a multitude of stressors that challenge their survival. Two-component signaling proteins are one of the predominant means by which bacteria sense and respond to such challenges. Since their initial discovery two decades ago, histidine kinases and response regulators have been implicated in countless stress responses. Early studies elucidated the general paradigms for how these signaling pathways function. Work since then has begun to reveal the complex regulatory networks that are built around these pathways and the many mechanisms controlling phosphate flux through these pathways, some of which are summarized in this chapter. Despite these advances, there is much that remains to be understood about two-component signaling. We still have a rudimentary understanding of precisely how signals are sensed and translated into changes in histidine kinase activity. We are also just beginning to understand how cells orchestrate the activities of so many highly related pathways that must function in parallel and in close proximity to one another. And, finally, it remains a major challenge to understand how cells evolve new signaling pathways to respond to new stressors.
REFERENCES


