Two-Component Regulatory Systems of Mycobacteria

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ABSTRACT

Two-component regulatory systems (2CRSs) are widely used by bacteria to sense and respond to environmental stimuli with coordinated changes in gene expression. Systems are normally comprised of a sensory kinase protein that activates a transcriptional regulator by phosphorylation. Mycobacteria have few 2CRSs, but they are of key importance for bacterial survival and play important roles in pathogenicity. Mycobacterium tuberculosis has 12 paired two-component regulatory systems (which include a system with two regulators and one sensor, and a split sensor system), as well as four orphan regulators. Several systems are involved in virulence, and disruption of different systems leads to attenuation or hypervirulence. PhoPR plays a major role in regulating cell wall composition, and its inactivation results in sufficient attenuation of M. tuberculosis that deletion strains are live vaccine candidates. MprAB controls the stress response and is required for persistent infections. SenX3-RegX3 is required for control of aerobic respiration and phosphate uptake, and PrrAB is required for adaptation to intracellular infection. MtrAB is an essential system that controls DNA replication and cell division. The remaining systems (KdpDE, NarL, TrcRS, TcrXY, TcrA, PdtARS, and four orphan regulators) are less well understood. The structure and binding motifs for several regulators have been characterized, revealing variations in function and operation. The sensors are less well characterized, and stimuli for many remain to be confirmed. This chapter reviews our current understanding of the role of two-component systems in mycobacteria, in particular M. tuberculosis.

INTRODUCTION

Two-component regulatory systems (2CRSs) are key players in bacterial responses to changing environments (1, 2). These systems act to integrate multiple stimuli into coordinated changes in global gene expression. Bacteria normally possess many 2CRSs which respond to specific signals and allow adaptive responses (2–7).

Two-component systems are composed of a sensor histidine kinase (HK) and a response regulator (RR) (1, 2, 4, 7). Changes in the external environment result in activation of the HK, which autophosphorylates on a conserved histidine residue; the HK then mediates phosphotransfer to a conserved aspartate residue on the RR (Fig. 1). Phosphorylation of the RR normally activates this protein, leading to DNA binding and promotion of transcription for a set of genes, termed the regulon.

Sensor HKs

HKs are usually membrane proteins, composed of a receiver domain, which senses the specific signal, and the transmitter domain, with kinase activity (2, 8). The specificity of the system lies in the receiver domain, which senses changes in the environment; HKs can respond to a number of stimuli including phosphate concentration, temperature, light, oxygen availability, pH, and redox states. The kinase domain is largely conserved and provides both the site of autophosphorylation and the interaction domain with the RR. HKs function as dimers in which one monomer catalyzes phosphorylation of the histidine residue in the other monomers. The C-terminal transfer domain of HKs is generally composed of a dimerization and phosphotransfer (DHp)
domain and the catalytic/ATP binding domain. DHp domains are the interaction sites between two monomers of the HK and the interface with the RR; thus, they control the specificity of the HK and RR interaction. A small number of HKs also have phosphatase activity and can mediate both phosphorylation and dephosphorylation of their cognate RRs.

**RRs**

RRs are the cytoplasmic partners responsible for receiving the signal via phosphorelay from the HK and for effecting a change in gene expression (1, 2, 4, 5). RRs preserve a two-domain structure comprising a receiver domain containing the conserved aspartate residue and an effector domain containing a DNA binding motif. The effector domains are used to group RRs into subfamilies depending on the DNA binding domain; for example, the OmpR family contains a winged helix-turn-helix motif, whereas the NarL family has a four-helix domain and the LuxR family has a tetrahelical helix-turn-helix domain. RRs are normally phosphorylated by their cognate HK partner, but some can also actively catalyze phosphotransfer from acetyl phosphate in vitro, although the biological significance of this is far from clear. In addition, most RRs have autophosphatase activity.

**Mycobacterial 2CRSs**

Mycobacteria have relatively few 2CRSs compared to other bacterial species (3, 6). Most studies have focused on their role in the pathogenic species Mycobacterium tuberculosis, although some data are available for the vaccine strain Mycobacterium bovis BCG and the saprophyte Mycobacterium smegmatis. This chapter will largely focus on the 2CRSs of M. tuberculosis, where most studies have been conducted.

*M. tuberculosis* has 12 complete 2CRSs, comprising an HK and an RR (Table 1). PhoPR, SenX3/RegX3,
PrrAB, and MprAB are all involved in virulence. Among their key roles, PhoP regulates cell wall composition and SenX3-RegX3 controls phosphate acquisition. MprAB is part of a complex regulatory cascade involved in the stress response and maintaining cell envelope integrity. MprAB is the only essential system that controls DNA replication and cell division. KdpDE may control potassium uptake. NarL may be responsive to hypoxia and is induced in starvation. The DosRST system incorporates two RRs that interact with a single HK. The function of TrcRS remains unclear. There are also four “orphan” RRs for which no cognate sensory proteins have been identified and about which little is known of their function. The stimuli for the sensors are largely uncharacterized; PhoPR may respond to pH, KdpDE may control potassium concentration, and SenX3 is indirectly stimulated by phosphate, although accessory proteins may be involved in actually sensing these parameters.

Although most HKs and RRs function as pairs, cross-talk between systems can occur where an HK phosphorylates a noncognate RR. Several such interactions have been demonstrated between M. tuberculosis recombinant proteins (10), although it is not clear what significance these interactions have, nor whether they truly occur in the mycobacterial cell. For example, PhoR and DosT can interact with NarL and PrrA, and PdtaS can interact with TcrX (10). PhoP appears to control the hypoxic response via interaction with DosR (11).

### SPECIFIC SYSTEMS

A number of two-component systems have been studied in detail, in particular the DosRST, PhoPR, MprAB, and SenX3-RegX3 2CRSs.

### PhoPR

The PhoPR system is one of the most studied of the mycobacterial 2CRSs and plays a key role in virulence. The structure of the PhoP DNA binding domain is known, comprising the winged helix-turn-helix of the OmpR family (12). PhoR is the cognate sensor and phosphorylates PhoP (13). PhoP and PhoR are co-transcribed; the operon is auto-regulated, with PhoP binding to direct repeats in its own promoter (13) and positively autoregulating its expression (14).

As with other regulators, PhoP can bind DNA in a nonphosphorylated state, although phosphorylation induces conformational changes and alters the protein-protein interface (15). PhoP recognizes a 23-bp sequence in its own promoter, composed of three direct

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**TABLE 1** The paired two-component regulatory systems of *M. tuberculosis*

<table>
<thead>
<tr>
<th>Gene</th>
<th>Rv #</th>
<th>Identity</th>
<th>Role</th>
<th>Stimulus</th>
</tr>
</thead>
<tbody>
<tr>
<td>SenX3</td>
<td>Rv0490</td>
<td>Sensor</td>
<td>Virulence, phosphate uptake, aerobic respiration</td>
<td>Phosphate</td>
</tr>
<tr>
<td>RegX3</td>
<td>Rv0491</td>
<td>Regulator</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>tcrA</td>
<td>Rv0602c</td>
<td>Regulator</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>HK1</td>
<td>Rv0600c</td>
<td>Sensor</td>
<td>Virulence, cell wall components, secretion of</td>
<td></td>
</tr>
<tr>
<td>HK2</td>
<td>Rv0601c</td>
<td>Sensor</td>
<td>Esx-1 components</td>
<td>pH</td>
</tr>
<tr>
<td>PhoP</td>
<td>Rv0757</td>
<td>Regulator</td>
<td>Virulence, cell wall components, secretion of</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Esx-1 components</td>
<td></td>
</tr>
<tr>
<td>PhoR</td>
<td>Rv0758</td>
<td>Sensor</td>
<td>Unknown</td>
<td></td>
</tr>
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<td>NarS</td>
<td>Rv0845c</td>
<td>Sensor</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>NarL</td>
<td>Rv0844c</td>
<td>Sensor</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>PrrA</td>
<td>Rv0903c</td>
<td>Sensor</td>
<td>Adaptation to intracellular infection</td>
<td>Macrophage infection</td>
</tr>
<tr>
<td>PrrB</td>
<td>Rv0902c</td>
<td>Sensor</td>
<td>Unknown</td>
<td></td>
</tr>
<tr>
<td>MprA</td>
<td>Rv0981</td>
<td>Sensor</td>
<td>Sigma factors, persistence in vivo, stress response, cell envelope</td>
<td>Detergents</td>
</tr>
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<td>MprB</td>
<td>Rv0982</td>
<td>Sensor</td>
<td>Potassium uptake</td>
<td></td>
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<td>KdpD</td>
<td>Rv1028c</td>
<td>Sensor</td>
<td>Controls expression of Rv1057</td>
<td>Unknown</td>
</tr>
<tr>
<td>KdpE</td>
<td>Rv1027c</td>
<td>Sensor</td>
<td>Controls expression of Rv1057</td>
<td>Unknown</td>
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<tr>
<td>TrcR</td>
<td>Rv1033c</td>
<td>Regulator</td>
<td>DNA replication, cell division</td>
<td>Unknown</td>
</tr>
<tr>
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<td>Sensor</td>
<td>DNA replication, cell division</td>
<td>Unknown</td>
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<tr>
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<td>Rv3246c</td>
<td>Regulator</td>
<td>Unknown</td>
<td>Low iron, starvation</td>
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<td></td>
</tr>
<tr>
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<td>Rv3764c</td>
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<td></td>
</tr>
<tr>
<td>PdtaS</td>
<td>Rv3220c</td>
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<tr>
<td>PdtaR</td>
<td>Rv1626</td>
<td>Regulator</td>
<td>Unknown</td>
<td></td>
</tr>
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</table>

The gene annotations for the laboratory reference strains H37Rv (Rv#) and common names are given. The role describes the pathways or genes controlled by each system. The stimulus is the environmental signal that causes either upregulation or activation of the regulator.
repeats. PhoP dimerization can be promoted by DNA binding (as well as phosphorylation), and modeling suggests that the two molecules bind in a head-to-head orientation (15, 16). Dimerization occurs between the receiver domains, which are linked to the effector domain via a short flexible region (17). Phosphorylation may stabilize the dimerization process and increase DNA binding affinity by bringing the two DNA binding domains closer together (17).

The PhoP regulon has been characterized in some detail; the members include genes involved in the synthesis of diacyltrehalose, polyacyltrehalose, and sulfolipids in the pkg2-mmpL8 and msl3 gene cluster (18). Over 40 genes are upregulated, and 70 are downregulated in the absence of PhoP (18). PhoP controls the hypoxic response through cross-talk with the DosR system (19). PhoP expression is controlled by the ArsF family transcriptional factor in M. smegmatis (19), suggesting that it forms part of a regulatory cascade. Rv2034 also regulates DosR expression, suggesting another mechanism for cross-talk between different regulons (20).

The control of transcription by PhoP is complex, since its phosphorylation state determines the specificity and strength of DNA binding; for example, nonphosphorylated PhoP can bind to its own promoter, but only phosphorylated PhoP can bind to the msl3 promoter (21, 22) or to the pks promoter (22). In contrast, PhoP binding to the mce1 promoter is reduced by phosphorylation (23). A consensus DNA binding motif for PhoP has been constructed that consists of two direct repeats, sometimes associated with a third direct repeat (24); the motif is found upstream of 87 genes (24).

The PhoPR system controls cell wall composition, and deletion of PhoP has pleiotropic effects. Changes in the relative amounts of mannosylated lipoarabinomannan (manLAM) are seen in the PhoP mutant strain, with monoacetylated forms predominating over the triacetylated forms (25). LAM is a major, essential component of the M. tuberculosis cell wall (26, 27) which plays a key role in immunomodulation and virulence by preventing phagolysosome fusion (28). PhoP mutants also have altered colony morphology, smaller cell size, and reduced cording properties resulting from a lack of synthesis of diacyltrehalose, polyacyltrehalose, and sulfolipids, all implicated in virulence (18, 29).

PhoP may also play a role in sensing and responding to pH; at least one pH-responsive gene cluster, aprABC, is controlled by PhoP (30). AprABC expression is induced by low pH in macrophages and in vitro and is required for survival in macrophages; disruption of AprABC leads to changes in cell wall and storage lipids (30). PhoP also regulates lipF, another pH-responsive gene (24).

A single point mutation in PhoP is sufficient to attenuate the virulence of M. tuberculosis. The avirulent strain H37Ra has the mutation S219L in the DNA binding domain of PhoP, and this single nucleotide polymorphism (SNP) is partly responsible for loss of virulence (31–33). Mutant PhoP is no longer able to bind to its own promoter (33). Among other changes, the mutation leads to a loss of secretion of the major T cell antigen, ESAT-6. Complementation with a PhoP wild-type allele restores ESAT-6 secretion and induces T cell responses during infection, as well as increasing virulence; in addition, changes in colony morphology are seen (31). Transcriptome profiles show that many members of the PhoP regulon are underexpressed during intracellular growth in H37Ra (as compared to H37Rv); these include genes involved in complex lipid biosynthesis, such as papA3 and fadD21 (34).

Given PhoP’s role in regulating key virulence pathways, many studies have characterized PhoP mutant strains in infection models. In particular, much interest has been paid to the potential of PhoP deletion strains for use as live vaccines with superior efficacy compared to the current vaccine, M. bovis BCG. PhoP mutants show increased adhesion to macrophages, suggesting increased uptake, as well as reduced intracellular replication that is likely due to the loss of the ability to prevent phagolysosome fusion (33). The latter ability may be affected by changes in the composition of LAM, since it is known to play a major role in phagosome maturation arrest (25). In addition, PhoP mutants fail to induce apoptosis in macrophages in vitro or during infection in the lung (36). PhoP mutants are unable to grow in low Mg2+ medium, and at least part of the attenuation seen in macrophages is due to this requirement, since growth can be partially restored by adding excess Mg2+ (18).

The SO2 strain (PhoP disruption) (37) has been widely tested and is attenuated in murine macrophages and in both immunocompetent and immunocompromised mice (18, 37–39). The strain shows greater attenuation in SCID mice than M. bovis BCG (39). Protection from challenge infection in the mouse model is comparable to BCG vaccination, whereas protection in the susceptible guinea pig is greater than that with BCG (39). SO2 showed protective efficacy against M. tuberculosis in the rhesus macaque model of infection and is well tolerated, supporting its further development as a live vaccine (40). The SO2 strain is stable in vitro and in vivo, with no reversion to wild type, even after 6 months, and was demonstrably safe in guinea pigs, with
no lesions or histology after 6 months of dosing with 50 times a normal vaccine dose (41). These studies underscore the essential role of the PhoPR 2CRS in virulence.

There is little information about PhoP variation in clinical isolates, although upregulation of PhoP after IS6110 insertion into the promoter has been seen in a particularly virulent clinical isolate (42). However, numerous mutations have been noted in strains of BCG; several mutations would lead to frameshifts in PhoR, with one frameshift in PhoP, and one IS6110 insertion upstream (and divergently transcribed) (43). It is presumed that all mutations lead to inactivation or disruption of the PhoPR system and contribute to the lack of virulence in the vaccine strains (43).

MprAB

The MprAB system appears to be involved in the response to stress conditions, in particular those that affect the cell envelope. MprA is a member of the OmpR family of RR, containing a winged helix-turn-helix DNA binding domain and the conserved aspartate site of phosphorylation (44). MprB is the cognate HK sensor, which autophosphorylates and transduces the signal to MprB (45). MprB activity is dependent on Mg$^{2+}$, although this HK is one of a small group that can also utilize Mn$^{2+}$ (45).

The mprAB operon is autoregulated, and MprA binds to its own promoter; a binding motif (the “Mpr box”) consisting of two short direct repeats is located upstream of mprA (46). MprA binds to the motif as two monomers and presumably dimerizes (46). Although nonphosphorylated RR can bind to this motif, the affinity is enhanced by phosphorylation, and activation is required for regulation (46).

MprAB is part of a complex regulatory cascade involving several sigma factors that respond to environmental stresses, in particular to envelope stress. MprA controls the expression of the sigma factors sigE and sigB (47, 48), as well as the serine protease PepD (46), in response to sodium dodecyl sulfate exposure. In view of this, it is surprising that MprAB deletion strains are more resistant to sodium dodecyl sulfate (48), although this may result from upregulation of genes via a compensatory mechanism within the cascade. The interaction between MprAB and SigE/B/H is complex, with many genes in the regulatory cascade being controlled by more than one of the regulators. For example, PepD is controlled by both MprA and SigE (49–51), whereas SigE is controlled by MprA and SigH (47, 48). In M. smegmatis, the MprAB-SigE cascade is controlled by the availability of polyphosphate, suggesting this may be the source of the phosphoryl groups utilized by the sensor MprB under stress conditions, rather than ATP (49).

Members of the MprA regulon play key roles in the cell envelope. PepD is a member of the HtrA family and has both chaperone and protease activity, so it can participate in protein refolding and degradation under stress conditions (51, 52). A major substrate of PepD is a homolog of the phage shock protein, PspA (52). Deletion of PepD results in increased sensitivity to cell wall antibiotics and induction of the SigE stress response pathway (51, 52).

Other members of the MprA regulon have been characterized. In particular, two operons originally identified as being in the “Dos” regulon—the formate hydrogenlyase complex (Rv0081 to Rv0088) (53) and the Rv1812c-Rv1813c operon (54)—can be regulated by both MprA and DosR. In both cases there are two motifs upstream, a Dos box, and an Mpr box allowing for binding of either RR independently. Rv0081 negatively autoregulates by binding to its own operon promoter; DosR and MprA have independent binding sites that overlap the Rv0081 binding site and can therefore prevent Rv0081 binding and positively regulate expression (53). Thus, the operon can be switched on by either one of the two systems, thereby responding to alternative environmental signals. The Rv1812c operon is also positively regulated by either DosR or MprA binding (54); although the role of these two proteins is unknown, they are required for virulence in the mouse model of infection.

MprA partly controls the expression of Acr2, a member of the alpha-crystallin family of chaperones (55) involved in stress responses. Rv1057 is controlled by MprA and TrcR, with MprA being responsible for upregulation of Rv1057 under detergent stress and in macrophages (56). MprA may also control the secretion of components of the Esx-1 system, most notably the major T cell antigens ESAT-6 and CFP10. MprA negatively regulates the expression of EspA, binding to several Mpr boxes in the promoter region (57). Deletion of MprAB leads to reduced secretion of a number of proteins, including EspA, ESAT-6, and EspB (57); these changes lead to altered immune responses in macrophages (57).

MprAB has a role during infection with M. tuberculosis. Early studies demonstrated that MprA is required to establish a persistent infection in mice, at least in competition experiments with the wild-type strain (44). MprAB expression differs between M. tuberculosis and M. bovis BCG, and this leads to different phenotypes for deletion strains; for example, the M. bovis BCG deletion
strain is attenuated in macrophages (45), whereas the *M. tuberculosis* strain is hypervirulent (44).

SenX3-RegX3

The SenX3-RegX3 system was one of the first mycobacterial 2CRSs identified (58). The operon is slightly unusual in that the sensor gene is upstream of the regulator gene, and the two are separated by several copies of a repeat unit (mycobacterial interspersed repeat unit, or MIRU) (59). The number of repeats in MIRUs has been used as a genetic typing method for many studies (60). The biological consequences of the repeat units are unknown, but the number of repeats varies in clinical isolates, and there are no repeats in the *M. smegmatis* operon.

SenX autophosphorylates at His167 in the cytoplasmic domain (61), followed by phosphotransfer to Asp52 in the receiver domain of RegX3 (61). SenX3 is a member of the PhoR/EnZ subfamily of sensors; the protein has at least one transmembrane domain located N-terminal to the sensor domain (62). The stimulus for SenX3 is unknown, although the system responds to low phosphate mediated by a phosphate transporter (63, 64). The sensor has homology with ArcB, the global regulator of aerobic metabolism in *Escherichia coli* (62), and contains an atypical sensor PAS domain, which often acts as sensors of oxygen or redox states.

RegX3 is a member of the OmpR subfamily of regulators with an N-terminal receiver domain and a winged helix-turn-helix DNA binding motif in the C-terminal effector domain (65–67). For most regulators, dimers are the active DNA binding form (68, 69). The structure of full-length RegX3 reveals that the protein dimerizes, as expected for RRs, but that the active form appears to be an unusual dimer in which three-dimensional domain swapping has occurred (70). In this form a domain from each of the two monomers is swapped, a more complex interaction than simple dimerization by interaction at an interface. Achieving domain swapping is likely to be a highly energetic process, since it requires disruption of the interactions within a monomer, as well as partial protein unfolding (in the receiver domain). This leads to the possibility that there may be alternative dimers and these may have different DNA binding motifs and thus regulate alternative regions of the regulon members (70). The stimuli that could promote domain swapping are unknown.

The regulon controlled by RegX3 is not fully characterized and differs between the pathogenic and nonpathogenic strains *M. tuberculosis* and *M. smegmatis*. Data using deletion strains and gene expression analysis suggest that RegX3 may have a dual role in controlling respiration and phosphate metabolism (63, 64, 71–73). Interestingly, the system is essential in *M. smegmatis*, but not in *M. tuberculosis* (74), and it is one of the five 2CRSs retained in *M. leprae*, suggesting its importance for bacterial survival.

Autoregulation has been demonstrated in *M. smegmatis* (61) with RegX3 binding upstream of the operon promoter, although the fold change is small in comparison to other systems—only about 2-fold upregulation. Autoregulation was not seen in *M. tuberculosis* (71), where deletion of RegX3 resulted in increased promoter activity. The expression of the operon is controlled by growth, with transient upregulation during log phase aerobic growth, but the level of expression is generally low (as compared to sigA), and changes in expression levels are small (less than 2-fold) (72).

The regulon controlled by RegX3 is not fully defined; early microarray analysis suggested a group of 50 genes controlled by the regulator under aerobic conditions in *M. tuberculosis* (71). The majority of changes in a SenX3-RegX3 deletion strain were associated with general growth, for example, downregulation of ribosomal proteins (71). Characterization of global gene expression under nonreplicating conditions and complete nutrient starvation demonstrated that genes associated with low-oxygen environments are differentially expressed (71, 72). In contrast to RegX3 binding to its own promoter, the regulator binds to the promoter regions of the regulon members *ald, cydB*, and *gltA1* in a phosphorylation-dependent manner (72). Under static conditions, RegX3 acts as a positive regulator of the *cydB* and *gltA1* operons (72).

In *M. smegmatis*, RegX3 controls the expression of genes involved in phosphate uptake. SenX3 acts as a phosphodonor or as a phosphatase of RegX3, depending on phosphate availability; phosphorylated regX3 binds to an inverted repeat upstream of *pboA* and *pstSCAB* (and its own promoter) genes to activate transcription (63). PhoA encodes an exported alkaline phosphatase, which can release inorganic phosphate from a range of macromolecules (75), whereas the Pst operon encodes a high-affinity phosphate transport system. In addition, RegX3 is implicated in the regulation of an alternative phosphate uptake system, PnhDCE (76).

In *M. tuberculosis* the operon is upregulated by phosphate starvation (2- to 4-fold). In contrast to *M. smegmatis*, there are three Pst high-affinity phosphate uptake systems (PstS1, PstS2, and PstS3) but no Pnh system. RegX3 controls the induction of the *pstS3* operon (*pstS3-pstC2-pstA1*) but not *pstS1* or *pstS2* (64). PstA controls expression of the SenX3 operon, exerting a negative effect
when phosphate is abundant and preventing the expression of genes required for phosphate acquisition (73). *M. tuberculosis* PstA1 deletion strains are attenuated in vivo in an interferon-γ-dependent fashion, but full virulence can be restored by deleting regX3, suggesting that dysregulation of phosphate-responsive genes is the cause (73).

The changes induced by phosphate limitation dependent on RegX3 have also been determined, but there is little overlap with those identified in aerobic culture (64, 71–73); the two studies conducted with *M. tuberculosis* in phosphate limitation showed little agreement in differentially expressed genes, except for the Pst transporters (64, 73). These studies demonstrate the difficulty of finding appropriate conditions for expression analyses and the identification of the true regulon. Differences may be explained by the possibility that the alternative dimer forms seen in structural studies control different regulons (70).

The role of the RegX3 system must differ between the nonpathogenic and the pathogenic species, since RegX3 is essential in *M. smegmatis* (63, 74) but not in *M. tuberculosis* (62, 71, 77). One major difference is that *M. tuberculosis* lacks a PhoA homolog, whose expression is RegX3-dependent in *M. smegmatis* (63).

The model for regulation of phosphate-dependent genes is not complete. The sensor of phosphate is the Pst transporter, but it is not clear how the signal is transduced to SenX3. In addition, mutants with deletion of SenX3 or with transposon disruptions displayed different PhoA expression profiles; deletion led to a complete lack of PhoA expression, whereas transposon insertion lead to upregulation of PhoA under phosphate-replete conditions. Since SenX3 may act as both a kinase and a phosphatase for RegX3, it is possible that the system is very sensitive to the amount of RegX3 that is changed in the transposon mutant (63).

Deletion of the complete SenX3-RegX3 operon was achieved in *M. smegmatis* at a low frequency, suggesting that suppressor mutations can compensate for loss of the complete operon (74). Mutations in NhaA, among others, are proposed to relieve the essentiality. In this context it is interesting to note that *M. tuberculosis* does not have a homolog of NhaA and that regulation of intrabacterial pH differs between the species, with *M. tuberculosis* being much more susceptible to weak acids than *M. smegmatis* (78). The system is required for normal growth in low phosphate, with SenX3 or operon deletions showing major defects in growth when phosphate is limiting.

The RegX3 system undoubtedly plays a role during infection; several studies have demonstrated that deletion strains have defects in macrophages or in mice (62, 64, 71, 73, 79). Phosphate limitation occurs in macrophages infected with *M. tuberculosis*, suggesting that these phenotypes relate to the inability to acquire sufficient exogenous inorganic phosphate (64). A partial deletion of SenX3 and RegX3 resulted in attenuation in mouse bone marrow–derived macrophages and human macrophages (THP-1 cell line) (71); attenuation during infection of immune-compromised, SCID mice and immune-competent, DBA mice was seen. In addition, deletion of either RegX3 or SenX3 resulted in reduced replication in BALB/c or nude mice (62). Transposon disruption of RegX3 in the CDC1551 strain also resulted in a reduction in the persistence of the strain in guinea pig and mouse infections (64). In contrast, in the only study to show no attenuation, disruption of RegX3 in *M. tuberculosis* MT103 by transposon insertion had no detectable effect on intracellular replication in mouse bone marrow–derived macrophages or in the replication of the organism after aerosol infection of BALB/c mice (77).

**PrrAB**

The PrrAB system is composed of the regulator PrrA and the sensor PrrB; the structures of both the sensor (80) and the regulator have been determined (81). PrrA is a member of the OmpR family and contains the characteristic winged helix-turn-helix DNA binding domain.

Structural studies reveal that the regulator can exist in either a “closed” or “open” form in which the accessibility of the DNA binding recognition helix changes (81). Activation by phosphorylation results in increased formation of the open form (in which the helix is accessible), presumably resulting in increased DNA binding. As with other members of the OmpR family, PrrA is able to bind to DNA in a nonphosphorylated state, although phosphorylation increases its affinity for DNA, and autoregulation occurs (82), with the regulator being required for transient intracellular induction in macrophages (77, 82).

The PrrAB 2CRS plays a role in early adaptation to intracellular infection (77, 82) and is essential for in vitro survival (83). Disruption of the operon by transposon mutagenesis leads to reduced expression of PrrA and PrrB (83); in this strain, the replication rates in murine bone marrow–derived macrophages are reduced (77). This attenuation is only seen at early time points post-infection (up to 6 days), and strains eventually reach the same plateau numbers as wild type (after 9 days) (77). PrrAB is expressed as a bicistronic operon at low levels during in vitro growth (83, 84). Expression of the
prrAB operon is transiently induced after macrophage infection, with a 2-fold increase in expression seen over the first 3 days of infection, followed by a gradual reduction to baseline (77). In vitro, expression is induced by nitrogen limitation but not by carbon starvation, hypoxia, or acidic pH, suggesting that this may be the stimulus seen in macrophages (83).

MtrAB

MtrA was originally identified as a homolog of Pseudomonas aeruginosa PhoB by DNA hybridization (85) and forms a 2CRS with the sensor MtrB. The genes are located in an apparent operon, with the RR upstream of the HK (86). Phosphorylation of MtrA by a noncognate HK (CheA) was demonstrated, confirming its role as an RR (83), and the RR directly interacts with MtrB and DNA (87). MtrB autophosphorylation and phosphorylation transfer to MtrA has been demonstrated and requires divalent metal ions, with Mg2+ essential for the latter activity (88).

MtrA is a member of the OmpR family and has the characteristic winged helix-turn-helix DNA binding motif. The structure of inactive MtrA has been determined; the two domains of the protein interact extensively (89). Activation of MtrA via phosphorylation and dimerization would require a significant disruption of this interaction, suggesting that activation may be more difficult than for other members of the family and that phosphorylation may be relatively slow (89).

The MtrAB system is essential in M. tuberculosis as demonstrated by several studies in which deletion mutants were not viable (86, 90, 91). The MtrAB system is not essential in all mycobacteria. In Mycobacterium avium, disruption of MtrB leads to changes in cell morphology, with cells losing the ability to switch from the red to the white morphotype (92). The red morphotype (defined as staining with Congo red) was exhibited by the MtrB mutant strain, which was also more permeable and more sensitive to antibiotics, presumably due to changes in the cell wall composition controlled by MtrB (92). In addition, the mutant strains were unable to survive in macrophages, showing loss of virulence (92). In M. smegmatis MtrB mutants are filamentous, with increased cell clumping and defective septum formation and cell division; in addition, cell wall defects were noted, which gave rise to increased susceptibility to lysozyme (93).

MtrA expression is controlled by sigma factor C (94), and there is at least one accessory protein, the lipoprotein LpqB, which interacts with the MtrB extra-cellular domain. This interaction affects phosphotransfer to MtrA and subsequent downstream effects on the regulon, including changes in dnaA expression (95). Disruption of LpqB in M. smegmatis leads to pleiotropic effects arising from cell wall changes, including increased cellular aggregation and loss of biofilm formation, as well as changes in motility and cell morphology during growth (95). These changes are dependent on the interaction with MtrB, since they can be reversed by expressing constitutively phosphorylated MtrA (95).

MtrB is localized to the septa and poles in an FtsX-dependent manner (93) consistent with its proposed role in controlling cell division. Unusually, both phosphorylated and nonphosphorylated forms show the same location, although it is proposed that MtrB must locate to the septum for activation and autophosphorylation (93).

Expression of the system differs between the virulent and avirulent species. For example, expression of the operon is induced after infection of macrophages in M. bovis BCG (85, 86), but it is constitutively expressed in M. tuberculosis (86), consistent with it being essential in vitro in the latter. Overexpression of MtrA does not affect growth in vitro but has profound effects on virulence, leading to reduced growth in macrophages and reduced virulence in the mouse model of infection (96). Attenuation is associated with loss of the ability to prevent phagolysosome fusion, a hallmark of infection by M. tuberculosis (96). MtrA positively regulates the expression of DnaA by binding to the promoter region in a phosphorylation-dependent manner (96). DnaA is required for DNA replication, and overexpression leads to a decreased growth rate (96). Coexpression of MtrB is sufficient to relieve the attenuation in macrophages, suggesting that the ratio of phosphorylated to non-phosphorylated RR is key (96).

The complete regulon of MtrA is not defined, but aside from DnaA, the RR also binds to the promoter of the mycolyl transferase fbpB and regulates its expression (97). The MtrA DNA binding motif is comprised of two direct repeats of GTCACAgcg, and these are also found in the origin of replication (oriC). MtrA binds to the motifs in the oriC region that are required for replication of autonomous plasmids, suggesting a key role for MtrA in chromosomal replication (97). Studies in M. smegmatis using unphosphorylated MtrA suggest that the RR may bind to an alternative motif CACGCCG (98); this motif is found upstream of over 400 mycobacterial genes, some of which may represent the true regulon members (98). In M. smegmatis the cell wall hydrolase ripA is controlled by MtrA (93).
TrcRS

The TrcRS system is another 2CRS that was identified by degenerate PCR before the availability of the complete genome sequence (99). TrcR and TrcS form a classical two-component system; the genes are arranged in a bicistronic operon, with the regulator gene (trcR) located upstream of the sensor kinase gene (trcS) (100). The sensor kinase, TrcS, has two predicted transmembrane regions that flank the proposed sensor domain in the N-terminal region; the C-terminus carries the kinase transmitter domain with the expected conserved regions and His-287, the site of autophosphorylation (99). The regulator, TrcR, is a member of the OmpR family of regulators. The N-terminal receiver domain contains several conserved residues required for function in this family and the Asp-82 residue, which is the site of phosphotransfer from TrcS (99). The C-terminal effector domain contains a helix-turn-helix DNA binding motif characteristic of the OmpR family (99).

Autophosphorylation and phosphotransfer have been shown using recombinant proteins in vitro with a truncated version of TrcS carrying the cytoplasmic effector domain alone (99). TrcS kinase activity is dependent on the divalent cations Mn$^{2+}$ or Ca$^{2+}$, but not Mg$^{2+}$ (99). Interestingly, the transmembrane domain of TrcS is toxic for E. coli and induces cellular lysis whether expressed as a full-length protein or alone. Thus, the stimulus for TrcS remains unknown, although the system is expressed during aerobic growth in culture and at low levels early after infection of human macrophages (but not at later stages of infection) (100). Transposon mutants with disruption of trcS were fully virulent in both murine macrophages and in the acute mouse model of infection, suggesting that this regulon is not involved in response to the intracellular environment, or for expression of pathogenic traits (77), consistent with its lack of expression under those conditions.

The trcRS operon is autoregulated, with TrcR binding to an AT-rich region upstream of the promoter leading to >500-fold induction of the operon (100). The M. tuberculosis genome is GC-rich (65% GC) (101), such that the AT-rich region of 78% is unusual. Regions of high AT are often associated with promoters or regulatory regions, since the energy required for unwinding and generating the open complex are lower. The minimal binding region for TrcR is a 28-bp region, although footprinting assays reveal that the regulator protects a larger fragment of 58 to 86 bp. Unphosphorylated TrcR is able to bind to the operon promoter, but the phosphorylated regulator binds with a 10-fold higher affinity and protects a larger region of DNA (100). This suggests that, as with other regulators, TrcR dimerizes upon activation.

A single member of the regulon is known; Rv1057 encodes a protein of unknown function, but with an unusual structure of a seven-bladed β-propeller (56, 102). TrcR represses expression of Rv1057, and TrcRS deletion mutants show increased expression (∼30-fold) of Rv1057 in culture. Rv1057 is induced during infection of macrophages when expression of TrcRS is expected to be decreasing. As expected, TrcR binds to the AT-rich region upstream of the transcriptional start site (102), although a binding motif is not clearly defined. Regulation of Rv1057 is complex, since it is also dependent on SigE (103) and MprAB (56), both of which are involved in the response to cell envelope stress.

KdpDE

The KdpDE 2CRS is one of the few systems identified with a clear homolog in other bacteria; the sensor KdpD interacts with the regulator KdpE (104, 105). KdpD is unusual, since it has a large cytoplasmic sensor domain. The N-terminal cytoplasmic domain of the sensor KdpD interacts with the lipoproteins LprF and LprJ (105). The role of these accessory proteins may be during signal relay or signal recognition, although this is not yet established (105).

KdpDE controls the expression of the adjacent and divergently transcribed kdpFABC operon; expression is controlled in response to K+ concentration as in other bacterial species (105). The role of KdpDE in other bacteria is to control K+ uptake by the KdpFABC transport system, and it is assumed that the mycobacterial system performs the same function. The four proteins make up a P-type ATPase K+ transporter whose role is to control the intracellular concentration of potassium ions to maintain turgor pressure (106).

LprF, LprJ, and KdpE are upregulated in response to starvation, suggesting that they may play a role in persistence (107). Potassium ions may play a role during infection, since they are likely to be at limiting concentrations, although no defect in growth under low K+ was evident in a deletion strain (90). Deletion of KdpDE resulted in increased virulence in immunocompromised mice (90).

NarL(Rv0844)/Rv0845 2CRS

NarL (Rv0844) is a member of the NarL subfamily of RR; the structure of the receiver domain has been determined (108). The protein has the typical ($β_6$)5 fold of signal receiver domains, with the phosphorylation site...
located in the loop after the third β strand. As with other members of the family, the active site is structurally similar to haloacid dehalogenases, but with a key substitution of aspartate by arginine.

The stimulus and regulon for NarL remain uncharacterized. The cognate sensor Rv0845 lacks homology with nitrate or nitrite sensors in other bacteria, although it is upregulated in the stationary phase (109). Protein interaction with the HK Rv0845 has been shown, but there also appears to be cross-talk with the DosRST system. NarL can interact with DosT, but not DosS, and in vitro phosphotransfer from DosT to NarL occurs, although the rate is relatively slow (10); the specificity of this interaction is controlled by the DHp domain (10). This raises the possibility that the NarL regulon could be responsive to hypoxia as well as to other environmental stimuli sensed by Rv0845.

### Other Systems

Several 2CRSs remain poorly characterized in mycobacteria, with little known of the stimulus or regulon. These include TcrXY, the split TcrA system, PdtaRS, and the orphan components.

The HK TcrY and the RR TcrX form a bona fide system, with phosphotransfer from the sensor to the kinase demonstrated in vitro (110). TcrX binds to two inverted repeats in its own promoter and may auto-regulate, although, unusually, binding affinity appears to be the same regardless of phosphorylation state (111). Little is known of the function of the system, although its deletion results in hyper-virulence in immune-compromised mice (90), and the components are expressed in vivo (112) and may be upregulated under low iron (113).

Rv0600c (HK1), Rv0601c (HK2), and Rv0602c (TcrA) form an unusual system (114–117); in some strains of *M. tuberculosis*, Rv0600c and Rv0601c are expressed as a single, functional HK, whereas in others two proteins are expressed that can functionally complement each other to generate functional HK activity (118). Rv0601c encodes the membrane sensor domain that is proposed to dimerize and interact with monomers of Rv0600c (114–117). In either case, phosphotransfer to the RR is seen (117).

PdtaR/S form an unusual system in that the RR and HK are not coexpressed. PdtaS (Rv3220) autophosphorylates and then mediates phosphotransfer to PdtaR (Rv1626) (119). There are four orphan RRs found in the genome of *M. tuberculosis*. Rv0195 is a member of the LuxR family; it appears to be induced by sodium azide treatment (120) or nutrient starvation (107). Rv0260c has a transcriptional regulatory motif characteristic of RRs but also has homology with HemD, a uroporphyrinogen-II synthase, and is located upstream of CbiX, which is involved in cobalamin biosynthesis, suggesting it may have a dual function. Rv2884 is a possible homolog of GlnR involved in control of nitrogen metabolism. Rv0818 is an RR of unknown function. In addition, Rv3143 has a receiver domain, but no effector DNA binding domain, suggesting it may play a role as an ancillary protein or in a phosphorelay system.

### CONCLUSION

Two-component systems undoubtedly play major roles in the ability of mycobacteria to adapt to external conditions. In particular, several systems are required for pathogenicity and thus pose interesting targets for further study. Although much work has focused on identifying regulon members and understanding the structure and function of the RRs, much less insight has been gained on the role of the sensory proteins and the stimuli to which they respond. Future work to understand the sensor domains and the identifying additional accessory proteins is likely to answer several questions about the role of these important global regulatory systems.

### REFERENCES

Two-Component Regulatory Systems of Mycobacteria


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