Adaptation to Environmental Stimuli within the Host: Two-Component Signal Transduction Systems of Mycobacterium tuberculosis

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INTRODUCTION

Adaptation to environmental stimuli in bacteria is mediated primarily through the expression of transcriptional regulators, including serine-threonine protein kinases (STPKs), extracytoplasmic function (ECF) sigma factors, and two-component signal transduction systems (TCSSs). Prototype TCSSs are comprised of a membrane-localized histidine sensor kinase (SK) and a cytoplasmically localized response regulator (RR). SKs and RRs are often identified based on their conserved domain architectures and their ability to mediate signal transduction events via phosphorylation. SKs are typically comprised of a sensor domain and one or more transmembrane domains connected via a flexible linker region to a cytoplasmic transmitter region containing a dimerization motif and a kinase domain. The kinase domain can be further divided into two subdomains, one containing the highly conserved site of histidine phosphorylation (H box) and the other containing 4 regions of highly conserved amino acids (N, D, F, and G boxes) that collectively form the ATP-binding pocket. RRs are also organized into discrete functional modules that include an N-terminal receiver domain containing a highly conserved aspartic acid that serves as the site of phosphorylation and a C-terminal DNA-binding domain.

Initiation of signal transduction events between SKs and RRs is mediated following the recognition of environmental stimuli by the SKs (Fig. 1A). Following signal recognition, SKs often dimerize and autophosphorylate in trans at a single conserved histidine residue present within each SK monomer. Transfer of this phosphate by the SK to the conserved aspartic acid residue in the receiver domain of the cognate RR results in conformation alterations that promote DNA binding and transcriptional regulation, RNA binding, protein-protein interactions, or other enzymatic activities (46, 65, 152–154, 171). In addition to serving as phosphoryl donors, certain SKs also function as RR phosphatases, an activity that helps regulate activation levels of the system. Recently, more complex versions of TCSSs with multiple histidine- or aspartate-containing phosphotransfer domains or phosphodonor intermediates (Fig. 1B) have been described (105).

While present in some plants, lower eukaryotes, and archaean, TCSSs are ubiquitous in bacteria (77). The number of systems present in a given organism generally correlates with its genome size and the complexity of the environment(s) in which the bacterium typically resides (46). TCSSs are usually genetically linked and transcriptionally coupled, though orphaned and independently transcribed systems also exist. Well-characterized TCSSs in bacteria include the Escherichia coli EnvZ-OmpR system, which regulates gene expression in response to osmotic stress (37), the E. coli and Bacillus subtilis CheA-CheY system involved in chemotaxis (81), and the Salmonella enterica PhoQ-PhoP system required for gene regula-
tion in response to magnesium concentrations (75). Beyond these well-characterized systems, TCSSs have also been shown to regulate many physiological processes, including sporulation, competence, antibiotic resistance, transition into stationary phase, virulence, and carbon, nitrogen, and phosphate utilization (82). The importance of TCSSs in bacterial survival and the absence of TCSSs in higher eukaryotes also make these systems attractive targets for therapeutic development against pathogenic organisms (7, 8). Consistent with this idea, mutant strains that are defective in specific TCSSs that lead to virulence attenuation are now being investigated as potential vaccine candidates (1, 102, 163).

TCSSs OF MYCOBACTERIUM TUBERCULOSIS

*Mycobacterium tuberculosis* is a facultative intracellular pathogen and is estimated to currently infect about one-third of the world’s population. It is the etiological agent of tuberculosis (TB) and is responsible for over 9 million new infections and 1.7 million deaths annually (177a). *M. tuberculosis* infects and survives within professional phagocytes, including macrophages, and is able to persist in the human host for decades within granulomatous lesions, where the organism is likely exposed to environmental stresses that include hypoxia, nutrient limitation, reactive oxygen and reactive nitrogen intermediates, pH alterations, and cell wall/membrane stress. To adapt to these and other stresses, *M. tuberculosis* encodes approximately 190 regulatory proteins, including 11 genetically linked TCSSs, five orphaned RRs, and two orphaned SKs (30, 161). The number of intact TCSSs in *M. tuberculosis* is lower than that typically found in other bacteria of similar genome size, possibly reflecting the evolution of this bacterium as a strict human pathogen and its adaptation to a predominantly intracellular lifestyle. Comparative genomic analyses of the 11 genetically linked TCSSs in *M. tuberculosis* indicate that homologs of these genes exist in other representative *Mycobacterium* species, including *Mycobacterium bovis, Mycobacterium avium, Mycobacterium leprae*, and *Mycobacterium smegmatis*, which is often used as a fast-growing surrogate for *M. tuberculosis* (Table 1) (30, 183). All of the paired TCSSs found in *M. tuberculosis* are conserved in their genetic arrangement and location within the closely related *Mycobacterium bovis* BCG vaccine strain. In contrast, only four TCSSs are present and predicted to be functional in *M. leprae*. Here, we discuss our current understanding of the 11 genetically linked TCSSs and the orphaned RRs and SKs of *M. tuberculosis* H37Rv, including the environmental signal(s) to which these systems are responsive, the regulons they control, and the importance of individual TCSSs in the physiology and/or virulence of *M. tuberculosis*.

*senX3 (Rv0490)-regX3 (Rv0491)*

*senX3-regX3* was the first TCSS to be described in *M. tuberculosis* (178). It is comprised of SK SenX3 and RR RegX3. Homologs of *senX3* and *regX3* are present in the genomes of various mycobacterial species, including *M. leprae* (Table 1) (183), suggesting that this TCSS is evolutionarily conserved and may regulate fundamental physiological processes. *senX3* and *regX3* are transcriptionally coupled in *M. tuberculosis*, with *senX3* encoding the first gene product in the operon (155). The intergenic region of these genes contains repetitive DNA se-
TABLE 1. Conservation of M. tuberculosis H37Rv TCSSs in other Mycobacterium species

<table>
<thead>
<tr>
<th>TCSS</th>
<th>Mab</th>
<th>Mav</th>
<th>Mb</th>
<th>BCG</th>
<th>Ml</th>
<th>Mmar</th>
<th>Mpar</th>
<th>Msm</th>
<th>Mtb</th>
<th>MRa</th>
<th>Mul</th>
</tr>
</thead>
<tbody>
<tr>
<td>regX3-senX3</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Rv0609c-Rv0601c-tcrA</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>phoP-phoR</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>narL-Rv0845</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>prrA-prrB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>mmpA-mmpB</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>kdpD-kdpE</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>trcR-trcS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>dosR-dosS</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>tcrX-tcrY</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>pdaR-pdaS</td>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

a Mab, Mycobacterium abscessus ATCC 19977; Mav, Mycobacterium avium 104; Mb, Mycobacterium bovis AF212297; BCG, Mycobacterium bovis BCG Pasteur 11732P; Ml, Mycobacterium leprae strain M; Mpar, Mycobacterium paratuberculosis K-10; Msm, Mycobacterium smegmatis; Mtb, Mycobacterium tuberculosis CDC1551; MRa, Mycobacterium ulcerans Ag99. +, genes encoding the sensor kinase and response regulator are present; -, genes encoding both the sensor kinase and response regulator are absent; *, genes encoding the two sensor kinases have been fused and the gene encoding this fused sensor kinase is genetically linked to the response regulator; †, one of the two sensor kinase genes is absent and the gene encoding the other kinase is genetically fused to the gene encoding the response regulator.

b pdtA7 and pdtD are not genetically linked but are present in all genomes listed.

TABLE 2. Sensor kinases in M. tuberculosis H37Rv

<table>
<thead>
<tr>
<th>SK</th>
<th>Gene</th>
<th>Size of SK (aa)</th>
<th>Predicted extracytoplasmic domain(s) (length of aa sequence)</th>
<th>Site of phosphorylation</th>
<th>Metal ion cofactor</th>
<th>Signal</th>
</tr>
</thead>
<tbody>
<tr>
<td>SenX3</td>
<td>Rv0490</td>
<td>118</td>
<td>22–28, 288–410</td>
<td>His-167</td>
<td>Mg2+</td>
<td>Pi (Msm); ND (Mtb)</td>
</tr>
<tr>
<td>HK1</td>
<td>Rv0600c</td>
<td>168</td>
<td>None</td>
<td>None</td>
<td>Mg2+</td>
<td>ND</td>
</tr>
<tr>
<td>HK2</td>
<td>Rv0601c</td>
<td>157</td>
<td>1–44</td>
<td>His-131</td>
<td>Mn2+</td>
<td>ND</td>
</tr>
<tr>
<td>PhoR</td>
<td>Rv0758</td>
<td>485</td>
<td>38–157</td>
<td>His-259</td>
<td>Mn2+</td>
<td>ND</td>
</tr>
<tr>
<td>Rv0845</td>
<td>Rv0845</td>
<td>426</td>
<td>63–66, 128–130, 175–183</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>PrrB</td>
<td>Rv0902c</td>
<td>446</td>
<td>43–149</td>
<td>ND</td>
<td>Mg2+ or Mn2+</td>
<td>ND</td>
</tr>
<tr>
<td>MprB</td>
<td>Rv0982</td>
<td>504</td>
<td>64–33, 48–161</td>
<td>His-249</td>
<td>Mg2+ or Mn2+</td>
<td>ND</td>
</tr>
<tr>
<td>KdpD</td>
<td>Rv1028c</td>
<td>860</td>
<td>419–429, 453–475</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>TccB</td>
<td>Rv1032c</td>
<td>509</td>
<td>48–187</td>
<td>His-280</td>
<td>Ca2+ or Mn2+</td>
<td>Low O2, NO, CO, ascorbate</td>
</tr>
<tr>
<td>DosS</td>
<td>Rv3132c</td>
<td>578</td>
<td>None</td>
<td>His-395</td>
<td>Mg2+</td>
<td>ND</td>
</tr>
<tr>
<td>DosT</td>
<td>Rv3202c</td>
<td>573</td>
<td>None</td>
<td>His-392</td>
<td>Mg2+, Mn2+, or Ca2+</td>
<td>ND</td>
</tr>
<tr>
<td>MtrB</td>
<td>Rv3245c</td>
<td>567</td>
<td>63–119</td>
<td>His-305</td>
<td>Mg2+</td>
<td>ND</td>
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<tr>
<td>TcrY</td>
<td>Rv3764c</td>
<td>475</td>
<td>1–152</td>
<td>His-256</td>
<td>Mg2+ or Ca2+</td>
<td>ND</td>
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<tr>
<td>PdtaS</td>
<td>Rv3220c</td>
<td>501</td>
<td>None</td>
<td>ND</td>
<td>Mg2+</td>
<td>ND</td>
</tr>
</tbody>
</table>

a aa, amino acids; ND, not defined.

b The size(s) of the predicted extracytoplasmic domain(s) of each sensor kinase was determined using transmembrane prediction servers, including TMHMM2.0 (83) and TMpred (67).

c The site was predicted based on crystal structure, sequence alignment, or molecular modeling with other sensor kinases.

d Magnesium (Mg), manganese (Mn), and calcium (Ca) divalent cations able to mediate autophosphorylation of the sensor kinase in vitro.

e Environmental stimulus shown to activate the TCSS. Information in parentheses indicates that the signal activates only that species. Msm, Mycobacterium smegmatis; Mtb, Mycobacterium tuberculosis; NO, nitric oxide; CO, carbon monoxide.
is direct and is initiated through recognition of a loosely conserved, inverted-repeat element present in the promoter regions of these genes (Table 3) (49). Structural analysis of RegX3 dimers supports the ability of this protein to recognize an inverted-repeat element when the protein is in an active state (80). In the presence of elevated Pi levels, SenX3 acts as a RegX3 phosphatase, preventing the accumulation of phosphorylated RegX3 and subsequent activation of downstream gene targets (49). Interestingly, RegX3 and downstream targets can be activated in the absence of SenX3, suggesting that other mechanisms for RegX3 phosphorylation may exist (49).

In M. tuberculosis, RegX3 both positively and negatively regulates a large and functionally diverse regulon comprising of ~100 genes (115). Several of these genes encode hypothetical proteins, while others are involved in important physiological activities, including energy metabolism, cell envelope maintenance, and regulatory functions (115). RegX3 also positively regulates HK2, a novel histidine phosphotransfer (Hpt) monodomain (141). Functional interaction between HK2 and Hpt proteins as a cognate TCSS (143). HK2 does not bind ATP, but it is phosphorylated at His-131 in the presence of HK1 and ATP (Table 2) (143). Neither HK1 nor HK2 undergoes autophosphorylation (143). Finally, HK2 does not bind ATP, but it is phosphorylated at His-131 in the presence of HK1 and ATP (Table 2) (143). Neither HK1 nor HK2 undergoes autophosphorylation (143). Functional interaction between these proteins is also observed in vivo, as determined by yeast two-hybrid assays (142). It has been proposed that HK2 exists as a dimer and that each monomer of HK2 is anchored in the membrane by a single transmembrane domain. Following recognition of an as-yet-unknown environmental signal, HK1 binds ATP with high affinity and mediates ATP hydrolysis (143).

Both in vitro and in vivo studies indicate that senX3-regX3 is required for aspects of M. tuberculosis virulence (Table 4) (115, 123, 124). An H37Rv Δ(senX3-regX3) mutant is attenuated for growth in the human THP-1 macrophage-like cell line and in gamma interferon (IFN-γ)-activated, murine bone marrow-derived macrophages (115). Significant attenuation of this mutant strain is also observed in the lungs, spleens, and livers of DBA/2 mice following intravenous infection with a high-dose inoculum (115) and in time-to-death assays following infection of SCID mice (115). A similarly attenuated phenotype is observed in the lungs and spleens of BALB/c mice following intravenous infection with individual ΔsenX3 or ΔregX3 mutant strains of H37Rv (123). Finally, an M. tuberculosis CDC1551 regX3-transposon (Tn) insertion mutant is attenuated for growth in the lungs of BALB/c mice and Hartley guinea pigs following a low-dose aerosol infection (124). Defining the specific contribution of the SenX3-RegX3 system to the M. tuberculosis life cycle remains a priority and is expected to provide novel insights into determinants that contribute to the pathogenesis of this bacterium.

### TABLE 3. Response regulators in M. tuberculosis H37Rv

<table>
<thead>
<tr>
<th>RR</th>
<th>Gene</th>
<th>Size of RR (aa)</th>
<th>Recognition sequence</th>
<th>Site of phosphorylation</th>
<th>No. of genes regulated</th>
<th>Autoregulated</th>
</tr>
</thead>
<tbody>
<tr>
<td>RegX3</td>
<td>Rv0491</td>
<td>227</td>
<td>23-bp inverted repeat</td>
<td>Asp-52</td>
<td>~100</td>
<td>Yes</td>
</tr>
<tr>
<td>TerA</td>
<td>Rv0602c</td>
<td>253</td>
<td>ND</td>
<td>Asp-71</td>
<td>~150</td>
<td>Yes</td>
</tr>
<tr>
<td>PhoP</td>
<td>Rv0757</td>
<td>247</td>
<td>22-bp direct repeat</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>NarL</td>
<td>Rv0844</td>
<td>216</td>
<td>ND</td>
<td>Asp-71</td>
<td>~150</td>
<td>Yes</td>
</tr>
<tr>
<td>PrnA</td>
<td>Rv0903c</td>
<td>236</td>
<td>ND</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>MprA</td>
<td>Rv0981</td>
<td>230</td>
<td>17-bp direct repeat</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
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<tr>
<td>KdpE</td>
<td>Rv1027c</td>
<td>226</td>
<td>ND</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>TscR</td>
<td>Rv1033c</td>
<td>257</td>
<td>28-bp AT-rich region</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
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<tr>
<td>DosR</td>
<td>Rv3133c</td>
<td>217</td>
<td>18/20-bp palindromic sequence</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>MtrA</td>
<td>Rv3246c</td>
<td>228</td>
<td>20-bp direct repeat</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>TcrX</td>
<td>Rv3765c</td>
<td>234</td>
<td>ND</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
<tr>
<td>PdtaR</td>
<td>Rv1626</td>
<td>205</td>
<td>ND</td>
<td>Asp-54</td>
<td>~200</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* aa, amino acids; ND, not defined.

* The site was predicted based on crystal structure, sequence alignment, or molecular modeling with other response regulators.

* Number of genes in the regulon in M. tuberculosis, as determined by DNA microarrays.

* The number of genes in the regulon was determined via quantitative reverse transcription-PCR (qRT-PCR).
<table>
<thead>
<tr>
<th>TCSS Mutations(s)</th>
<th>Strain(s)</th>
<th>In vitro model(s)</th>
<th>In vitro phenotype(s)</th>
<th>In vivo model(s)</th>
<th>In vivo phenotype(s)</th>
<th>Route(s)</th>
<th>In vivo phenotype(s)</th>
<th>Reference</th>
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<tbody>
<tr>
<td><strong>senX3-regX3</strong></td>
<td>H37Rv</td>
<td>mBMDM and hTHP1</td>
<td>Attenuated</td>
<td>BALB/c mice</td>
<td>ND</td>
<td>i.v.</td>
<td>Attenuated in lung, spleen, and liver</td>
<td>115</td>
</tr>
<tr>
<td><strong>ΔsenX3 or ΔregX3</strong></td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>SCID mice</td>
<td>i.v.</td>
<td>No death</td>
<td>Yes</td>
<td>123</td>
</tr>
<tr>
<td><strong>regX3-Tn</strong></td>
<td>CDC1551</td>
<td>ND</td>
<td>BALB/c mice and guinea pigs</td>
<td>Aerosol for both models</td>
<td>ND</td>
<td>124</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>scsK-Tn</strong></td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>BALB/c mice</td>
<td>ND</td>
<td>117</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>phoP-phoR</strong></td>
<td>MT03</td>
<td>mBMDM</td>
<td>Attenuated early</td>
<td>SCID mice</td>
<td>i.v.</td>
<td>No death</td>
<td>Yes</td>
<td>102</td>
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<tr>
<td><strong>phoP-Km&lt;sup&gt;+&lt;/sup&gt;</strong></td>
<td>MT03</td>
<td>ND</td>
<td>ND</td>
<td>BALB/c mice</td>
<td>i.v.</td>
<td>No phenotype for either route</td>
<td>Yes for i.v., ND for aerosol</td>
<td>38</td>
</tr>
<tr>
<td><strong>nrl-L-Rv0845</strong></td>
<td>H37Rv</td>
<td>mBMDM</td>
<td>No phenotype</td>
<td>SCID mice</td>
<td>i.v.</td>
<td>No phenotype</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td><strong>prrA-prrB</strong></td>
<td>MT03</td>
<td>mBMDM</td>
<td>Attenuated early</td>
<td>SCID mice</td>
<td>i.v.</td>
<td>No phenotype</td>
<td>ND</td>
<td>183</td>
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<tr>
<td><strong>mprA-mprB</strong></td>
<td>H37Rv</td>
<td>J774A.1 and mBMDM</td>
<td>Hypervirulent for both models</td>
<td>BALB/c mice</td>
<td>i.v.</td>
<td>Attenuated in lung and spleen, no phenotype in liver</td>
<td>ND</td>
<td>113</td>
</tr>
<tr>
<td><strong>kdpD-kdpE</strong></td>
<td>H37Rv</td>
<td>mBMDM</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td><strong>trcR-trcS</strong></td>
<td>MT03</td>
<td>mBMDM</td>
<td>ND</td>
<td>ND</td>
<td>SCID</td>
<td>i.v.</td>
<td>Enhanced time to death</td>
<td>ND</td>
</tr>
<tr>
<td><strong>dosR</strong> &lt;sup&gt;[devR]&lt;/sup&gt; &lt;sup&gt;[dosS]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>Human monocytes</td>
<td>Hypervirulent</td>
<td>SCID</td>
<td>i.v.</td>
<td>Enhanced time to death</td>
<td>ND</td>
<td>114</td>
</tr>
<tr>
<td><strong>devR</strong> &lt;sup&gt;[devR]&lt;/sup&gt; &lt;sup&gt;[dosS]&lt;/sup&gt;</td>
<td>H37Rv</td>
<td>mBMDM</td>
<td>Hypervirulent</td>
<td>SCID mice and DBA/2 mice</td>
<td>i.v.</td>
<td>Enhanced time to death for SCID mice, hypervirulent phenotype in lung, spleen, and liver</td>
<td>Yes for SCID mice, ND for DBA/2 mice</td>
<td>114</td>
</tr>
<tr>
<td><strong>evs313s-dosS</strong></td>
<td>H37Rv</td>
<td>ND</td>
<td>ND</td>
<td>C57BL/6 mice</td>
<td>Aerosol</td>
<td>No phenotype in lung or spleen for any model</td>
<td>ND</td>
<td>127</td>
</tr>
</tbody>
</table>
### Table 1. Phenotype of Murine Bone Marrow-Derived Macrophages infected with Defined Mutants of Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>mtrA-mtrB</td>
<td>Enhanced time to death</td>
<td>H37Rv</td>
<td>(58)</td>
</tr>
<tr>
<td>mtrA overexpression</td>
<td>No phenotype</td>
<td>hMDM</td>
<td>(58)</td>
</tr>
<tr>
<td>mtrB overexpression</td>
<td>No phenotype</td>
<td>mJ774A.1</td>
<td>(58)</td>
</tr>
<tr>
<td>tcrX-tcrY</td>
<td>Enhanced time to death</td>
<td>H37Rv</td>
<td>(58)</td>
</tr>
<tr>
<td>Rv3220c</td>
<td>No phenotype</td>
<td>SCID mice</td>
<td>(58)</td>
</tr>
</tbody>
</table>

### Table 2. Phenotype of Human THP-1 Macrophage-like Cell Line infected with Defined Mutants of Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>pdtaR-pdtaS</td>
<td>No phenotype</td>
<td>H37Rv</td>
<td>(58)</td>
</tr>
<tr>
<td>Rv0600c</td>
<td>No phenotype</td>
<td>SCID mice</td>
<td>(58)</td>
</tr>
</tbody>
</table>

### Table 3. Phenotype of Rat Normal Kidney Fibroblast-like Cell Line infected with Defined Mutants of Mycobacterium tuberculosis

<table>
<thead>
<tr>
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<th>Phenotype</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoP-phoR</td>
<td>No phenotype</td>
<td>NRK-49F</td>
<td>(58)</td>
</tr>
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</table>

### Table 4. Phenotype of Human Lung Fibroblasts infected with Defined Mutants of Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoP-phoR</td>
<td>No phenotype</td>
<td>MRC-5</td>
<td>(58)</td>
</tr>
</tbody>
</table>

### Table 5. Phenotype of Human Fetal Lung Fibroblast-like Cell Line infected with Defined Mutants of Mycobacterium tuberculosis

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Phenotype</th>
<th>Strain</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>phoP-phoR</td>
<td>No phenotype</td>
<td>MRC-5</td>
<td>(58)</td>
</tr>
</tbody>
</table>

### Discussion

The phoP-phoR system was initially named for its sequence similarity to PhoP-PhoR from *B. subtilis* and PhoP-PhoQ from *Salmonella enterica* serovar Typhimurium (30). This system is comprised of RR PhoP and SK PhoR. *phoP* and *phoR* are genetically linked and appear to be present in all *Mycobacterium* species examined to date except *M. leprae* (Table 1). In *M. tuberculosis*, PhoR is phosphorylated at residue His-259 following its activation (Table 2) (58). Once phosphorylated, PhoR transfers this phosphate to Asp-71 of PhoP (Table 3) (58).

Biomedical and genetic studies in *M. tuberculosis* indicate that *phoP-phoR* is autoregulated and that expression of these genes is transcriptionally coupled (Table 3) (52). However, *phoR* may also be expressed from its own promoter element (52). PhoP positively regulates the expression of itself and phoR through recognition of a 22-bp sequence that contains two 9-bp repeat elements present in the *phoP* upstream region (Table 2) (52, 57). PhoP likely binds to its consensus sequence in a cooperative manner, binding one direct repeat and then the other in a head-to-head fashion (57). Phosphorylation of PhoP results in a conformational change that is thought to enhance protein dimerization and subsequent DNA-binding stability (147). The structure of the PhoP DNA-binding domain has been solved. It contains a winged helix-turn-helix motif that includes three α-helices flanked by two β-sheets (169). Several amino acids within the α-helices appear critical for DNA binding and sequence recognition, including Asn-212, Val-213, Glu-215, Ser-219, Tyr-220, Tyr-222, and Lys-224 (33). Interestingly, PhoP from the attenuated *M. tuberculosis* strain H37Ra (PhoP<sub>H37Ra</sub>) contains a single nucleotide polymorphism that results in an amino acid substitution at position 219 from serine to leucine (27). Relative to PhoP<sub>H37Ra</sub>, PhoP<sub>H37Ra</sub> exhibits a significantly diminished capacity to bind its recognition sequence from the *phoP* upstream region (27, 87). Consistent with this observation, a large number of genes that are downregulated in a Δ*phoP* mutant of *M. tuberculosis* H37Rv are also expressed at lower levels in wild-type H37Ra (87). Additionally, of the 50 genes that are differentially expressed between wild-type *M. tuberculosis* H37Rv and wild-type *M. tuberculosis* H37Ra during intracellular growth in murine bone marrow-derived macrophages, 12 of them are regulated by PhoP-PhoR (91). The effect of this mutation in *M. tuberculosis* H37Ra on transcriptional regulation at the *phoP-phoR* promoter region remains unclear, as there are conflicting reports regarding whether PhoP<sub>H37Ra</sub> positively or negatively regulates its own expression (51, 52, 58, 168).

The generation of defined mutations in *phoP* and/or *phoR* has provided important insights into the genes regulated by this TCSS and the role that this system plays in the physiology and pathogenesis of *M. tuberculosis*. PhoP-PhoR regulates a...
diverse regulon of >150 genes in *M. tuberculosis* (Table 3) (51, 168). Functional gene classes regulated by PhoP include those involved in lipid metabolism, general metabolism, and respiration (51, 168). A number of genes encoding membrane/secreted proteins and those comprising the initial hypoxic response (dosRS and constituents of its regulon) and enduring hypoxic response are also differentially regulated, as are genes from the PE/PPE/PE-PGRS protein families (51, 168). Interestingly, a number of genes from the virulence-associated region of difference 1 (RD1), including *espB* and *espR*, are also regulated by PhoP (51, 91). Recent studies have indicated that both EspB and EspR are necessary for the secretion of proteins (notably, the major antigens ESAT-6 and CFP-10) via the ESX-1 secretion pathway (104, 120). Consistent with this observation, *M. tuberculosis* phoP::Km′ mutants and wild-type *M. tuberculosis* H37Rv synthesize ESAT-6 but only marginally secrete this protein. These alterations in ESAT-6 secretion also result in low T-cell responses to this protein following infection by these strains (45).

Given the diverse regulon controlled by PhoP-PhoR, it is not surprising that *M. tuberculosis* phoP and/or phoR mutants exhibit pleiotropic phenotypes, compared to their wild-type counterparts (see reference 129 for a review). Morphologically, these mutants are smaller than the wild type and exhibit altered colony characteristics, including inabilities to form serpentine cords, fix neutral red, and stain acid-fast (53, 117, 168). Some of these differences are due to the lack of specific lipids within the cell membrane/cell wall of the phoP mutant, including 2,3-di-O-acyl-trehaloses (DATs), polyacyltrehaloses (PATs), and sultolipids (SLs) (53, 168). Wild-type *M. tuberculosis* H37Rv exhibits similar colony characteristics and also lacks DATs, PATs, and SLs in its cell membrane/cell wall (27). Interestingly, these phenotypes are reversed by expression of *M. tuberculosis* phoP in H37Rv (27). *M. tuberculosis* phoP mutants are also more sensitive than the wild type to cumene hydroperoxide (an organic peroxide), CdCl2 (a superoxide generator and toxic heavy metal), and several cell wall-perturbing antibiotics, including vancomycin and claxocillin (168).

Several lines of evidence also support a role for PhoP-PhoR in the virulence of *M. tuberculosis* (Table 4). phoP expression is upregulated following exposure of *M. tuberculosis* to iron (100), a metal that is essential for the *in vivo* growth of numerous bacterial pathogens, including *M. tuberculosis*. In addition, several genomic alterations in the phoP-phoR locus have occurred in various *M. bovis* BCG derivatives, perhaps explaining, in part, the virulence variability observed in these vaccine strains (17, 89). Similarly, a multidrug-resistant strain of *M. bovis* BCG that carries an IS6110 insertion sequence in the phoP promoter region that enhances its transcription was responsible for a large outbreak of tuberculosis in Spain (148). Importantly, phoP and phoP-phoR mutants of *M. tuberculosis* are attenuated for growth in various cultured or primary cell types, including murine bone marrow-derived macrophages (117), murine alveolar macrophages (168), murine J774A.1 macrophage-like cells (168), and human THP-1 macrophage-like cells (53, 168) (Table 4). Interestingly, this attenuation phenotype may be cell-type specific, as a phoP mutant of *M. tuberculosis* is not altered for growth in fibroblasts (41). Compared with wild-type *M. tuberculosis, phoP* mutants also exhibit lower bacterial burdens in target organs of infection when assayed in several animal model systems of infection, including mice and guinea pigs (Table 4) (1, 102, 168). Finally, mice infected with phoP or phoP-phoR mutants also exhibit delayed times to death compared to those of mice infected with the wild-type parent (1, 102). Thus, a loss of PhoP-PhoR is detrimental to the growth/survival of *M. tuberculosis in vivo*.

The observed attenuation of phoP and/or phoR mutant strains has led to investigations of the potential utility of an *M. tuberculosis* phoP (SO2 strain, a derivative of clinical isolate MT103) as a possible live-vaccine candidate. Subcutaneous vaccination of BALB/c mice with SO2 provides the same level of protection as vaccination with *M. bovis* BCG following intravenous or intratracheal challenge with *M. tuberculosis* H37Rv (1, 102). Similarly, subcutaneous vaccination with SO2 affords guinea pigs an increased level of protection against a high-dose aerosol challenge with *M. tuberculosis* H37Rv compared to that of animals vaccinated with BCG (102). Vaccination with SO2 also induces less lung pathology than vaccination with BCG (102) following challenge with H37Rv and reduces the production of several cytokines, including interleukin 4 (IL-4), tumor necrosis factor alpha (TNF-α), and IFN-γ (1). Furthermore, vaccination results in high levels of inducible nitric oxide synthase (iNOS) (1), increases numbers of CD4+ and CD8+ T cells in the spleen, and increases the proportion of CD4+/CD8- cells that express IFN-γ (1, 102). Importantly, the SO2 strain is fully susceptible to four frontline antimycobacterial drugs (ethambutol, isoniazid, rifampin, and streptomycin) *in vivo* and does not induce adverse effects following vaccination in guinea pigs previously exposed to *M. tuberculosis* (20). The SO2 strain is also protective in rhesus monkeys, suggesting real promise that a phoP mutant could be further evaluated for use as an effective live vaccine in humans (163).

Despite extensive characterization of this TCSS, the environmental signal(s) to which PhoP-PhoR is responsive remains unknown. phoP mutants of *M. tuberculosis* are unable to grow in the absence of the divalent cation magnesium (168). Interestingly, supplementing tissue culture medium with Mg2+ partially restores intracellular growth characteristics to phoP mutants of *M. tuberculosis* within THP-1 macrophage-like cells (168). Whether magnesium is the signal to which PhoP-PhoR responds has yet to be delineated. Clearly, further work to define the molecular mechanism(s) underlying the attenuation of these strains *in vitro* and *in vivo* is required before phoP mutants can be fully assessed for their potential as vaccine candidates.

**narL** (*Rv0844c*)-*Rv0845**

While genes encoding homologs of NarL-Rv0845 exist in most *Mycobacterium* species sequenced to date, except *M. leprae* (Table 1), very little is known about the biochemical activity or functional significance of the *narL-Rv0845* TCSS. In *M. tuberculosis, narL* is divergently transcribed from *Rv0845*, and its gene product exhibits sequences and structures in the receiver domain that are homologous to those of NarL from *E. coli* (136). In *E. coli*, *narL* encodes the RR component of the NarQ-NarL TCSS. This system regulates genes in response to nitrite concentrations, including determinants involved in nitrate metabolism during anaerobic respiration (150). Whether Rv0845 senses a similar signal remains to be demonstrated,
and characterization of NarL-Rv0845 as a bona fide TCSS in mycobacteria awaits.

Several studies suggest that this system may not contribute to the virulence of *M. tuberculosis*. Expression of narL is below detection limits following infection of *M. tuberculosis* H37Rv in human macrophages (60), and exposure of peripheral blood mononuclear cells isolated from tuberculosis (TB) patients to recombinant NarL does not stimulate significant levels of IFN-γ production (160). Importantly, a ΔnarL mutant of *M. tuberculosis* H37Rv exhibits no apparent growth or survival defect following infection of activated murine bone marrow-derived macrophages (Table 4) (114), and SCID mice infected with this strain display time-to-death kinetics similar to those of the wild-type H37Rv parent (Table 4) (114). Thus, further studies are needed to better define the NarL-Rv0845 TCSS and to delineate the role of this TCSS in *M. tuberculosis* physiology and pathogenesis.

**pprA (Rv0903c)-pprB (Rv0902c)**

*pprA*-pprB is one of four TCSSs that is present and genetically linked in all *Mycobacterium* species examined to date (Table 1). Bioinformatic and biophysical studies indicate that PrrA and PrrB possess amino acid sequences and structural motifs highly characteristic of RRs and SKs, respectively (110, 111). In vitro, PrrA-PrrB has been biochemically characterized and shown to function as an intact TCSS. The cytoplasmic domain of SK PrrB autophosphorylates in the presence of Mn²⁺ or Mg²⁺, and this domain participates in phosphotransfer reactions with RR PrrA (39, 111). However, sites of phosphorylation for both PrrB and PrrA have yet to be defined. Electromobility shift assays (EMSAs) and reporter fusions have demonstrated that PrrA binds to its own promoter region and positively autoregulates its own expression (Table 3) (39). The binding of DNA by PrrA is phosphorylation independent, but it is enhanced by phosphorylation (39). It has been suggested that PrrA initially assumes an inhibitory, closed conformation that is poorly phosphorylatable yet shifts to a more open, DNA-binding-favored conformation upon phosphorylation (110) (6). The stimuli recognized by PrrB and the genes regulated by PrrA have yet to be defined.

Several studies indicate that *pprA*-pprB may play a role in *M. tuberculosis* virulence. *pprA*-pprB is expressed by *M. tuberculosis* H37Rv during growth in human peripheral blood monocyte-derived macrophages but not during growth in standard laboratory medium, indicating that these genes may be specifically upregulated following infection (54). In agreement with these observations, infection of murine bone marrow-derived macrophages with an *M. bovis* BCG derivative containing an *M. tuberculosis* *pprA (pprAΔcat,Δgfp)* promoter fusion plasmid is transiently upregulated at 4 h postinfection (38). Consistent with these observations, while strain MT103 with a Tn9563 transposon insertion mutation of *pprA* exhibits no phenotype during the growth of *M. tuberculosis* in 7H9 or Sauton’s medium, this mutant strain is attenuated during initial time points following infection of murine bone marrow-derived macrophages in *vitro* (Table 4) (38). Interestingly, growth of the mutant recovers to wild-type levels at later time points, suggesting that signaling through PrrA-PrrB may be important only for early stages of infection in this cell type. However, when assayed in *vivo*, the *pprA::Tn* mutant exhibited no differences in virulence levels in the lungs, spleens, or livers of BALB/c mice following either intravenous or aerosol infection from those of the wild-type parent (Table 4) (38). Thus, the role of *pprA*-pprB in *M. tuberculosis* pathogenesis remains unclear.

**mprA (Rv0981)-mprB (Rv0982)**

The Rv0981-Rv0982 TCSS system was originally described as being necessary for the establishment and maintenance of persistent infection by *M. tuberculosis* in mice and was therefore named *mpr* for *mycobacterium persistence regulator* (183). This system consists of RR MprA and SK MprB. Genes encoding these determinants are highly conserved in all *Mycobacterium* species, including *M. leprae* (Table 1) (183). In addition to *mprA* and *mprB* being genetically coupled, their chromosomal positioning adjacent to *pepD* (encoding an HtrA-like serine protease) and *moaB2* (encoding a predicted molybdenum cofactor synthase) are also conserved among these species (183). Biochemical studies have established MprA and MprB as a functional TCSS in *M. tuberculosis* H37Rv both in *vitro* and in *vivo*. MprB autophosphorylates at residue His-249 and transfers this phosphate to MprA at residue Asp-48 (Tables 2 and 3) (184). In addition to having kinase activity, MprB acts as an MprA phosphatase to regulate levels of phospho-MprA within the cell (184). In *vivo*, activation of downstream signaling pathways by MprA is dependent on the extracytoplasmic domain of MprB, as strains of *M. tuberculosis* expressing mutant *mprB* alleles carrying deletions in this domain are unable to initiate the signaling cascade (63).

The generation of *mprA::Km* and Δ*mprAB* mutant strains of *M. tuberculosis* H37Rv has led to the identification of determinants regulated by the *mprAB* TCSS and the environmental signals to which this system is responsive (63, 113). Comparative microarray analyses between wild-type and mutant strains indicate that MprA both positively and negatively regulates a diverse regulon of >200 genes (63, 113). MprA positively regulates its own expression and that of downstream genes *pepD* and *moaB2* by directly binding a 17-bp sequence containing two 6-bp direct-repeat motifs separated by 5 nucleotides (Table 3) (64, 112, 173). MprA also directly regulates downstream genes *pepD* and *moaB2* by binding three tandemly positioned MprA recognition sequences located in the end of *mprB* and extending into the *mprB-pepD* intergenic region (64, 173), and MprA directly regulates, apart from its own locus, the expression of other determinants, including those encoding the Acr2 alpha-crystallin-like protein (112), a predicted 18-kDa chaperone.

Defining the core regulon directly controlled by MprA in *M. tuberculosis* is complicated by the fact that MprA directly regulates the expression of two ECF sigma factors, *sigE* and *sigB*, which have extensive regulons of their own (36, 63, 113). Regulation of *sigE* by MprA seems especially critical, since MprA and SigE participate in a positive-feedback loop in response to cell wall/membrane-perturbing agents, such as SDS (63, 101, 113, 173, 174). Consistent with this observation, both *mprA* and *sigE* mutants exhibit increased sensitivity to SDS (101, 173). SigE also upregulates other determinants that may influence MprAB activation indirectly. *ppk1* is regulated by SigE and generates polyphosphate molecules that are phosphoryl donors for MprB activation under conditions of low ATP concentrations (156). Additionally, the MprA-regulated determinant PepD associates with and proteolytically cleaves the
SigE-regulated determinant Rv2744c (the 35-kDa antigen) (174), an interaction that influences susceptibility to cell wall-targeting antibiotics (174). MprA is also responsive to stimuli other than SDS which are likely to affect cell wall/cell membrane homeostasis, including Triton X-100, alkaline pH, and nutrient limitation (12, 63, 113).

While several in vitro conditions activate MprA-MprB signaling, mprA-mprB expression is also upregulated in M. tuberculosis in vivo. mprA-mprB is part of an in vivo-expressed genomic island (iVEGI) of 49 genes that is highly upregulated in M. tuberculosis within the lungs of BALB/c mice during acute and chronic stages of infection (157, 158). mprA is also upregulated in M. tuberculosis within an artificial hollow-fiber granuloma model system (74). Finally, mprA expression is upregulated following infection by M. tuberculosis in human monocyte-derived macrophages (60). Consistent with activation of MprA-MprB by in vivo signals, mprA and/or mprA-mprB mutants of M. tuberculosis also exhibit altered virulence characteristics in model systems of infection (Table 4). mprA:Km’ and ΔmprAB mutants of M. tuberculosis H37Rv are hypervirulent in macrophages (113, 183) and are attenuated for virulence within the lungs and spleens of BALB/c mice following intravenous infection (183). Thus, MprA-MprB transcriptionally regulates adaptation programs in response to several environmental stimuli and is required by M. tuberculosis for aspects of virulence in vivo.

\[ kdpD \text{ (Rv1028c)-kdpE (Rv1027c)} \]

SK KdpD and RR KdpE have been named based on the similarity of their amino acids to those of the KdpD-KdpE TCSS in E. coli and on their conserved genetic location adjacent to determinants (kdpFABC) encoding a predicted potassium uptake system. kdpD-kdpE is present in many species of Mycobacterium but is absent from M. leprae and M. ulcerans (Table 1). In M. tuberculosis and M. bovis, kdpD-kdpE is divergently transcribed from kdpFABC, while these genes are transcriptionally coupled in other species, including M. avium, Mycobacterium marinum, and M. smegmatis.

Only a few studies have examined the biochemical activity of KdpD-KdpE in M. tuberculosis or the importance of this system in the physiology and/or pathogenesis of the bacterium. Expression of an M. tuberculosis kdpF promoter-lacZ fusion is reduced in M. smegmatis and M. tuberculosis when grown under conditions of increasing K⁺ concentrations (151). However, it is unclear whether K⁺ is the stimulus to which kdpD-kdpE is responsive. Similarly, while yeast two-hybrid assays indicate that KdpD and KdpE physically interact with each other (145, 151), it is not yet established whether these proteins function as a cognate SK/RR pair, mediate phosphate transfer reactions between each other, or autogenously regulate their own expression. KdpD has been shown to associate with two membrane lipoproteins, LprF (Rv1368) and LprJ (Rv1690) (151). While the significance of these interactions awaits further delineation, overexpression of lprF or lprJ enhances expression of the kdpF promoter-lacZ fusion, suggesting that these proteins may modulate the activity of KdpD-KdpE signaling (151). KdpD has an unusual structure for an SK, with large N- and C-terminal cytoplasmic domains separated by four small, transmembrane-spanning regions (151). This structure is consistent with a model that predicts that KdpD likely senses osmotic changes in the cytoplasm. Physiological stimuli other than K⁺ may also regulate activation of KdpD-KdpE. kdpD is induced in M. tuberculosis H37Rv under conditions of starvation (12), and kdpE is required for the optimal growth of M. tuberculosis in vitro (134). Importantly, kdpD-kdpE may also contribute to M. tuberculosis virulence, as these genes are differentially expressed in human macrophages (60), and SCID mice intravenously infected with an M. tuberculosis ΔkdpDE mutant exhibit enhanced times to death (Table 4) (114). Clearly, additional work is needed to further characterize these genes and delineate mechanisms underlying the hypervirulent phenotype seen in the mouse model of infection.

\[ \text{trc}R \text{ (Rv1033c)-trc}S \text{ (Rv1032c)} \]

The trcR-trcS system is comprised of SK TrcS and RR TrcR and was one of the first M. tuberculosis systems shown to function as a cognate TCSS in M. tuberculosis (62). In vitro, TrcS autophosphorylates in the presence of Ca²⁺ or Mn²⁺ and acts as a TrcR kinase (62). Amino acid residues that are phosphorylated in both TrcS and TrcR have yet to be defined. trcR and trcS are genetically linked, transcriptionally coupled, and autogenously regulated (Table 3) (59). TrcR binds directly to its own upstream region at an AT-rich stretch of nucleotides (Table 4) (59). A bioinformatic search of the M. tuberculosis genome identified a similar 28-bp, AT-rich sequence upstream of Rv1057, encoding a seven-bladed β-propeller protein (61). While TrcR negatively regulates expression of Rv1057 by directly binding to the promoter region (61), the function of this gene product, as well as the significance of this regulation, awaits further investigation. Microarray analyses comparing M. tuberculosis H37Rv and a ΔtrcS mutant have identified approximately 50 genes that are differentially regulated by this TCSS (172). Whether these genes are directly regulated by TrcR via binding to a similar AT-rich sequence has yet to be determined. trcR-trcS is expressed during the growth of M. tuberculosis in broth medium and shortly after infection in primary human peripheral blood-derived macrophages (59). These genes are also upregulated during the growth of M. tuberculosis in an artificial hollow-fiber granuloma model system (74).

While trcR-trcS is expressed by M. tuberculosis in physiologically relevant environments, there are conflicting reports as to the importance of this system in the virulence of the tubercle bacillus (Table 4). While a trcS transposon insertion mutant of M. tuberculosis MT103 is unaltered for growth/survival in murine bone marrow-derived macrophages and in an aerosol infection model of C57BL/6 mice (38), a ΔtrcS mutant of M. tuberculosis H37Rv is slightly hypervirulent in SCID mice (114). Therefore, further work on this system is still needed to identify the stimulus processed by TrcS, to characterize the regulon controlled by TrcR, and to delineate the contribution of these proteins to M. tuberculosis-host-pathogen interactions.

\[ \text{devR-devS-Rv2027c or dosR-dosS-dosT} \]

The RR comprising the devR-devS-Rv2027c TCSS was initially identified in a screen for determinants in the virulent M. tuberculosis strain H37Rv that were differentially expressed in...
its avirulent counterpart H37Ra, prompting its initial designation as devR (differentially expressed in the virulent strain) (34, 79). Subsequent studies have demonstrated a role for devR as a regulator of dormancy survival (i.e., dos) in M. bovis BCG when grown in the Wayne culture model for nonreplicating persistence (15, 16), leading to the alternate designation of this determinant as dosR. Both gene designations remain in use today. Here, the dos designation will be utilized, given the demonstrated functional role for these genes in mediating the transition to nonreplicating persistence in vitro.

DosR-DosS-DosT represents an unusual TCSS in that it consists of two soluble, full-length SKs (DosS and DosT) and a single RR (DosR). In M. tuberculosis, dosR and dosS are genetically linked and transcriptionally coupled with each other, as well as with upstream gene Rv3134c (34). In contrast, dosT is encoded elsewhere in the genome. While dosR and dosS are conserved and tandemly arranged in many mycobacterial species (except M. leprae and M. ulcersans) (Table 1), dosT appears to be less well conserved. Interestingly, M. smegmatis contains homologs of DosR and DosT but lacks a DosS homolog. Biochemical and genetic studies indicate that DosR-DosS-DosT constitutes a functional TCSS. DosS and DosT autophosphorylate at conserved histidine residues (DosS at His-395 and DosT at His-392), and both proteins phosphorelay to Asp-54 of DosR (Tables 2 and 3) (125, 130–132).

The DosR-DosS-DosT system has been extensively studied over the years due, in part, to its association with bacterial dormancy and the demonstration that environmental signals activating this system in vitro are likely to be encountered by M. tuberculosis in vivo. dosRS is upregulated in M. tuberculosis and M. bovis BCG when bacteria are cultured under conditions of low-oxygen tension (Table 2), an observation that was initially reported in 2001 (16, 138). A similar phenotype is also observed when these M. tuberculosis genes and their upstream promoter region are introduced into surrogate host M. smegmatis cells that are cultured under microaerophilic or anaerobic conditions (5, 103). Exposure of M. tuberculosis H37Rv to hypoxia alters the expression of more than 100 genes (138), including a subset of 48 genes whose upregulation is dependent on DosR (Table 3) (116). The same 48-gene regulon is also upregulated following exposure of M. tuberculosis to nitric oxide (NO) (165) or during the transition of M. tuberculosis from aerobic respiration to nonreplicating persistence induced via gradual oxygen depletion (165, 166, 170). The 48 genes upregulated by DosR have been collectively termed the “dormancy regulon.” They are generally clustered into a small number of discrete transcriptional units that are scattered around the chromosome (116, 165). This type of arrangement may facilitate a more rapid, pronounced, and coordinated response to environmental stimuli. It is predicted that DosR directly regulates the vast majority of genes comprising this regulon. EMSAs, DNase I footprinting, and gene reporter expression studies carried out thus far generally support this contention (4, 24–26, 42, 55, 116, 125, 162). In particular, DosR recognizes a fairly conserved 18/20-bp palindromic sequence that is present with some variation upstream of the transcriptional units for these genes (Table 3) (4, 25, 26, 42, 55, 116, 125, 162). Similar sequences are also found upstream of other genes outside this regulon, although their regulation by DosR remains unclear (116, 185). Promoter regions from DosR-regulated genes often contain two or more sets of recognition sequences, and cooperative binding by DosR to these sites is necessary for their full induction (24–26). Binding by DosR to its recognition sequences is mediated primarily through the C-terminal domain (176, 177), and amino acid residues within this region that are important for contacting DNA have been defined and characterized (55, 176). However, the N-terminal domain also regulates aspects of DosR binding and subsequent gene regulation. This domain influences the ability of DosR to act cooperatively (47), particularly at sites in which the recognition motif varies from the optimal consensus (25, 26). However, the N-terminal domain of DosR also regulates DNA binding by “locking” the C-terminal domain in a conformation that limits its ability to bind DNA in the absence of phosphorylation (47, 177). Interestingly, a phenylcoumarin-based compound that inhibits DosR binding to its recognition sequence was recently identified (56). Exposure of M. tuberculosis to this compound prevents the upregulation of genes from the DosR regulon, even under inducing conditions (56). Furthermore, exposure to this compound reduces the ability of M. tuberculosis to establish nonreplicating dormancy in vitro following gradual oxygen depletion (56). Thus, the development of small molecules inhibiting DosR-mediated binding or regulation may represent novel therapeutic strategies for combating infection by M. tuberculosis.

Exposure of M. tuberculosis to carbon monoxide (CO), high NO levels, and possibly other stimuli also induce expression of dosR-dosS and genes from this regulon (Table 2) (76, 84, 85, 140). While the differential regulation of dosR-dosS and genes from the DosR regulon is dependent on dosR (15, 138), dosS is dispensable for this regulation (138). This observation led to the discovery of the second DosR kinase, DosT, which is encoded outside the DosR regulon (125, 131), dosT appears to be constitutively expressed (60, 125, 131), and mutational analysis has suggested that this SK may mediate initial adaptation events to hypoxia (69). Consistent with a role for DosT as a second DosR kinase, M. tuberculosis ∆dosS ∆dosT double mutants fail to increase the expression of DosR-regulated determinants during hypoxia (125). More recently, dosR-dosS and members of its regulon were shown to be upregulated in M. tuberculosis in response to ascorbic acid (vitamin C) in the presence of oxygen (68, 159). However, this effect is likely due to the ability of this powerful cytochrome c reductant to inhibit respiration, scavenge oxygen, and induce hypoxia (159). Finally, it has recently been reported that M. tuberculosis H37Rv displays constitutive DosR-mediated regulation when grown aerobically in medium containing asparagine (99) and that DosR-DosS-DosT signaling is regulated by the serine/threonine protein kinase PknH, which phosphorylates DosR on Thr-198 and Thr-205 and enhances DosR-DNA interactions (22). While these observations await further characterization, they solidify the contention that DosR-DosS-DosT receives and processes regulatory inputs from multiple sources.

Structural and biochemical analyses have provided important insights into the nature of gas ligand binding by SKs DosS and DosT (28, 29, 71, 72, 78, 85, 86, 118, 133, 149, 165, 179–181). Based on this extensive structural information, it has been proposed that DosT is inactive when bound to oxygen under normoxic conditions. Oxygen binding occurs via a heme molecule contained within the first of two cyclic GMP (cGMP)
phosphodiesterases, adenylate cyclases, and EthA (GAF) domains found near the N terminus of the protein. No ligand to date has been identified for the second GAF domain, although the crystal structure suggests that it does not bind cyclic nucleotides, which are prototypical ligands of GAF domains (86).

During hypoxia or, alternatively, upon displacement of oxygen by NO or CO, DosT is activated and initiates signaling through DosR. Similar gas binding can also be attributed to DosS. However, DosS may respond to the redox state of the cell, as DosS is oxidized more rapidly than DosT following oxygen binding (85, 149). Biochemical analysis supports this conclusion, as Fe^{2+}-DosS does not autophosphorylate, while Fe^{2+} DosS does (85). Further evidence supporting a role for DosS as a redox sensor comes from studies demonstrating that flavin mononucleotides (FMNs) and flavin adenine dinucleotides (FADs) reduce the first GAF domain of DosS (29). In addition, DosS-mediated activation of DosR is observed following the disruption of the electron transport chain, resulting in increased concentrations of reduced components of this pathway (including FMNs, FADs, and quinones) (68).

Numerous studies have implicated DosR-DosS-DosT in aspects of M. tuberculosis pathogenesis (for further discussion on the DosS-dependent response, see reference 128). DosR and/or members of the DosR regulon are upregulated in M. tuberculosis following infection of human monocytes (34), human monocyte-derived macrophages (60), and murine bone marrow-derived macrophages (135). Genes or proteins from this regulon are also expressed or produced in M. tuberculosis within an artificial hollow-fiber granuloma model (74). Finally, dosS-dosT members of the DosR regulon are expressed in M. tuberculosis during late stages of infection in mice (139) and in the lungs of guinea pigs following infection with the tubercle bacillus (137). Consistent with the in vivo expression of these determinants, a subset of DosR regulon determinants are immunodominant antigens recognized during infection. Antibody production and Th1 T-cell responses are observed in mice vaccinated with plasmid DNA encoding select dormancy genes (1, 11, 126). Antibodies to the M. tuberculosis 14-kDa alpha-crystallin-like antigen HspX (Acr; Rv2031c) are also observed in the cerebrospinal fluid of humans with TB meningitis (21) and in the sera of BCG-vaccinated individuals (73). Furthermore, purified proteins from several DosR regulon members induce IFN-γ production in T-cell lines (90) and in peripheral blood monocytes from M. tuberculosis-infected individuals, with a greater response observed in latently infected individuals (90). Immunological responses to DosR regulon antigens are also observed in TB-positive individuals from geographically diverse regions of Africa (14), in individuals with prior exposure to M. tuberculosis or nontuberculosis mycobacteria (94, 95), and in individuals with remote latent tuberculosis infection or who have been cured of the disease (50). In contrast, peripheral blood mononuclear cells from adolescents vaccinated with M. bovis BCG exhibit low responsiveness when stimulated with proteins from the dormancy regulon (94). This may be due to observed differences in dormancy regulon expression between the M. tuberculosis and M. bovis strains (70). Recent evidence also indicates that strains of the highly virulent M. tuberculosis W-Beijing lineage express dosR at elevated levels (3, 40, 122). Additionally, a large number of isolates sequenced from this lineage contain a frameshift mutation in dosT that results in the introduction of a premature stop codon (40). Some of these strains also carry a large gene duplication in the region of DNA that includes dosR-dosS (35). The impact of these genomic events on the virulence of these strains is not yet understood.

Despite evidence demonstrating that dosR-dosS-dosT and proteins from this regulon are produced during infection and are immunogenic, the role for this TCSS in the virulence of M. tuberculosis remains unclear. M. tuberculosis derivatives containing mutations in dosR, dosS, and/or dosT either are attenuated (31, 98), are hypervirulent (9, 114), or exhibit no phenotype (31, 127) when examined in vitro and/or in vivo model systems of infection (Table 4). These observed virulence differences may reflect the use of different M. tuberculosis strain backgrounds, mutation types, and/or model systems of infection. There is also debate regarding the importance of the DosR regulon to bacterial dormancy (23). While the expression of genes comprising this regulon is rapid following activation of DosR-DosS-DosT by hypoxia (127, 138), the expression of these genes wanes over time (127). Furthermore, growth attenuation of M. tuberculosis ΔdosR under hypoxic conditions occurs only after DosR-regulated gene expression has waned (15, 88, 127). An enduring hypoxic response in which numerous DosR-independent genes are upregulated following prolonged exposure to hypoxia has been observed (127). In this response, expression of DosR and the dormancy regulon is predicted to “prime” the M. tuberculosis response to hypoxia and allow M. tuberculosis to consume oxygen at an appropriate rate, allow maintenance of appropriate NAD/NADH ratios (and thus the redox environment), and allow for improved recovery following the reactivation of M. tuberculosis from anaerobic to aerobic growth (88). Furthermore, NO induction of the dormancy regulon does not sensitize M. tuberculosis to a second NO response (165), suggesting that DosR-DosS-DosT may be important within the dynamic environment of the granuloma.

Since its first description, the DosR-DosS-DosT system has generated a lot of interest. Despite this, further work is still needed to better elucidate the contribution of this TCSS to M. tuberculosis physiology and pathogenesis and to define the biological contribution of dosR regulon members in the various phenotypes attributed to this system.

mtrA (Rv3246c)-mtrB (Rv3245c)

Genes comprising the mtrA-mtrB TCSS are present in all Mycobacterium species characterized thus far (Table 1) (183) and regulate functions essential for the growth of M. tuberculosis on laboratory medium (164, 182). This system was first identified in 1994 (32). At that time, RR Rv3246c and SK Rv3245c were designated MtrA and MtrB (Mycobacterium tuberculosis response regulator), respectively, based on the observed amino acid similarity of this system to other TCSSs (164). Biochemical studies have since confirmed that MtrB autophosphorylates and participates in phosphotransfer reactions with MtrA (92). In particular, MtrA is phosphorylated at Asp-56 (Table 3) (44). The site of MtrB autophosphorylation remains undescribed but is predicted to occur at His-305 based on sequence alignment with other SKs (Table 2) (164). Like
PrrA, MtrA is classified as a poorly phosphorylable RR based on protein structure (6, 44).

Gene expression studies indicate that mtrA-mtrB is expressed in vivo and under physiologically relevant conditions in vitro. An M. tuberculosis mtrA promoter fusion is expressed at low levels in M. bovis BCG during growth on laboratory medium (32, 164) and is induced in this strain following infection of murine 774A.1 macrophase-like cells (164, 182). Interestingly, mtrA-mtrB is constitutively expressed in M. tuberculosis in vitro, within 774A.1 macrophase-like cells, and in human peripheral blood monocyte-derived macrophages (60, 182). Thus, expression of this locus may be differentially regulated between M. tuberculosis and BCG. Consistent with MtrA’s intracellular expression, sera obtained from preclinical tuberculosis patients, but not purified protein derivative-positive (PPD+) or healthy controls, contain antibodies to MtrA (146). This suggests that mtrA is expressed and recognized by the immune system during acute infection when the bacteria are growing but not in PPD+ patients in whom the infection is latent and there is little to no growth of the tubercle bacillus (146).

Due to the inability to generate mtrA and/or mtrB mutants of M. tuberculosis, investigations into the function of the mtrA-mtrB TCSS have relied on the use of alternative model organisms containing homologs of MtrA-MtrB that are dispensable for growth. Additionally, MtrA-MtrB function has been assessed directly in M. tuberculosis using genetic strategies that result in overexpression or repression of mtrA-mtrB. mtrA-mtrB mutants of Corynebacterium glutamicum, a related actinomycete, exhibit a number of pleiotropic phenotypes, including an altered transition into stationary phase, abnormal cellular division, and increased sensitivity to certain cell wall-targeting antibiotics or enzymes, including penicillin, vancomycin, and lysozyme (106). Similar phenotypes are also observed with an mtrB::Tn mutant of M. avium (19). This mutant is also avirulent in human THP-1 macrophase-like cells (19). In contrast, while overexpression of mtrc in wild-type M. tuberculosis does not result in observable growth phenotypes in vitro, this derivative fails to prevent phagolysosomal fusion in THP-1 cells. This strain also exhibits intracellular attenuation in this cell type as well as other cell types, including murine 774A.1 macrophages, human peripheral blood monocytes, and human peripheral blood monocyte-derived macrophages (Table 4) (43, 121). These observed phenotypes are likely the consequences of elevated levels of phosphorylated MtrA, as M. tuberculosis strains overexpressing an allele of mtrA that cannot be phosphorylated (MtrAD53N), or expressing mtrA and mtrB together, exhibit normal intracellular trafficking and growth characteristics (43, 121). Regardless, M. tuberculosis strains overproducing either wild-type MtrA or MtrAD53N are attenuated in the lungs and spleens of C57BL/6 mice following infection even though these mutants maintain the ability to persist, albeit at lower levels (Table 4) (43). Thus, MtrA-MtrB also participates in aspects of M. tuberculosis virulence in vivo.

MtrA recognizes a 20-bp sequence that contains two 9-bp direct-repeat elements that are upstream of as many as 150 genes in M. tuberculosis, including dnaA, an essential replication factor, and fbpABC, encoding components of the antigen 85B, which is critical for cord formation and cell wall biogenesis (Table 3) (93, 121). MtrA is also autoregulatory, binding to its own promoter region (Table 3) (121). Additionally, MtrA binds to the promoter region of iniB, an isoniazid-inducible gene that is a predicted transporter and may be involved in efflux (93). Perhaps most intriguingly, MtrA binds to the Mycobacterium origin of replication, oriC, suggesting that this TCSS may directly influence replication.

While genes regulated by MtrA-MtrB continue to be elucidated, the signal detected and processed by this TCSS remains unknown (Table 2). It appears that interaction between MtrB and the membrane protein LpqB, a determinant encoded immediately downstream of mtrA-mtrB, may be important (109). Consistent with this observation, an M. smegmatis ipqB transposon mutant exhibits phenotypes similar to those seen with mtrA-mtrB mutants of other organisms, including an altered colony morphology, increased cell wall permeability, defective septation, and increased SDS sensitivity (109). Specifically, interaction between MtrB and LpqB may be necessary to activate signaling through the MtrA-MtrB system, as decreased levels of phosphorylated MtrA are observed in the ipqB transposon mutant even though total protein levels are similar (109). Additionally, ipqB, like mtrA, is thought to be essential in M. tuberculosis (134).

MtrA-MtrB signaling may represent a novel therapeutic target, given that this system regulates essential processes, including aspects of DNA replication and cell wall integrity in the bacterium. However, there is still much to be delineated regarding this TCSS, including the environmental signals that MtrA-MtrB senses, the manner in which genes are regulated by MtrA, and the genes that comprise the MtrA-MtrB regulon. Importantly, additional studies may also help clarify the role that this TCSS plays in M. tuberculosis physiology and pathogenesis.

tcrX (Rv3765c)-tcrY (Rv3764c)

Bioinformatic and biochemical studies have recently demonstrated that the tcrX and tcrY determinants encode a functional TCSS in M. tuberculosis (13). tcrY encodes an SK that contains an N-terminal sensing domain, a single transmembrane domain, and a cytoplasmic C-terminal effector domain. TcrY dimerizes, autophosphorylates in the presence of Mg2+ or Ca2+, and transfers phosphate to the corresponding RR TcrX (13). While the sites of phosphorylation in TcrY and TcrX have not yet been determined biochemically, sequence alignments and molecular modeling predictions point to residues His-256 in TcrY and Asp-59 in TcrX as likely sites of modification (Tables 2 and 3) (13). Little is known about the conditions or environments which influence the expression and/or activation of tcrX-tcrY. tcrX is transiently expressed in human macrophages (60), and an M. tuberculosis tcrX deletion mutant exhibits increased virulence in SCID mice (Table 4) (114). Clearly, more work is necessary to define the mechanism underlying these phenotypes, the regulon controlled by TcrX, and the environmental signals processed by TcrY.

pdtA (Rv1626)-pdtS (Rv3220c) and Other Orphaned RRs and SKs

The original sequencing of M. tuberculosis H37Rv revealed that, in addition to the 11 genetically linked TCSSs, there are at least five orphaned RRs (Rv0260c, Rv0818, Rv1626, Rv2884, and Rv3143) and two orphaned SKs (Rv2027c and Rv3220c).
Subsequent sequence analysis has also revealed the presence of an open reading frame (Rv2998A) encoding a putative sensor portion of an SK (18). Compared to what is known about the genetically linked TCSSs, very little is known about these orphaned RRs and SKs. However, it is likely that these TCSS components influence the physiology and virulence of \textit{M. tuberculosis}.

Rv2027c was originally described as an orphaned SK; however, it is now appreciated that Rv2027c, i.e., DosT, is a second SK of the DosR-DosS TCSS. More recently, a novel TCSS comprised of the orphaned RR Rv1626 and the orphaned SK Rv3220c has been elucidated (107, 108). Rv3220c is a soluble SK that autophosphorylates in the presence of Mg$_2^+$ and can transfer phosphate to Rv1626 but not to other \textit{M. tuberculosis} RRs, including PrrA, NarL, and RegX3 (108). While Rv3220c contains a single GAF domain and a single period clock protein, aryl hydrocarbon receptor, and single-minded protein (PAS) domain, the environmental signals activating this SK have yet to be defined. Similarly, Rv1626 has an unusual domain structure. While the N-terminal receiver domain of this protein shares high homology with prototypical RRs, including the putative phosphorylation site (107), the C-terminal domain is unlike any other RR of \textit{M. tuberculosis}. It contains an AmiR and$\alpha$NasR transcription antitermination regulator (ANTAR) domain, which is found only in prokaryotic proteins (144). This domain has been shown to be important for RNA binding (175). Given this domain architecture, it has been speculated that Rv1626 may bind to RNA and regulate gene expression by acting as an antiterminator to prevent stem-loop structure formation in the leader region of newly transcribed RNA (107). Consequently, Rv1626 was renamed the phosphorylation-dependent transcriptional antitermination regulator (PtdaR), and Rv3220c was renamed PdtAS (108). Whether Rv1626 indeed acts as an antiterminator remains to be defined. Interestingly, ptdaR is an essential gene in \textit{M. tuberculosis} (134), but deletion of ptdaS can be tolerated (114). Additionally, ptdaR and ptdaS appear to be conserved in all \textit{Mycobacterium} species (Table 1) (108). While nothing is currently known about the genes/operons that are controlled by PtdaR-PtdaS, a ptdaS mutant of \textit{M. tuberculosis} exhibits no phenotype following infection of SCID mice (114), indicating that this system may not participate in aspects of virulence. Regardless, further investigations into this novel TCSS and the remaining orphaned RRs are needed.

**CONCLUDING REMARKS**

The circulation of multidrug-resistant and extensively drug-resistant strains of \textit{M. tuberculosis} that are recalcitrant to current antibiotics and the increase in comorbidity of patients infected with \textit{M. tuberculosis} and HIV continue to threaten our ability to control tuberculosis. The development of new antimycobacterial agents and vaccines with improved efficacy against \textit{M. tuberculosis} is an important goal for the effective control of this disease. The TCSSs of \textit{M. tuberculosis} have been proposed as targets for antimycobacterial drug design, and attenuated mutants have been suggested as possible vaccine candidates. The recent expansion of \textit{M. tuberculosis} TCSS research and the utilization of powerful techniques, such as global transcriptional profiling using DNA microarrays, have generally validated beliefs that the expression of TCSSs plays an important role in facilitating the successful adaptation of \textit{M. tuberculosis} to diverse environmental conditions encountered within the host. Such stress signals include nutrient starvation, hypoxia, fluctuating P$_i$ and K$^+$ concentrations, altered pH, NO and CO exposure, and cell wall/membrane stress. Undoubtedly, more signals exist and await identification. Studies of \textit{M. tuberculosis} SK and RR mutants in tissue culture and animal model systems of infection indicate that several TCSSs play substantive roles in virulence. Genetic and biochemical characterizations of these genes and the downstream determinants comprising their regulons will continue to aid in our understanding of the roles that individual TCSSs play in \textit{M. tuberculosis} physiology and virulence. Such studies are also expected to provide new insights into the environmental signals recognized by these systems and the molecular mechanisms maintaining them in an inactive state in the absence of stimulation. Importantly, continued investigation of the TCSSs of \textit{M. tuberculosis} is likely to lead to novel therapeutics or vaccine candidates which may aid in the successful control of this significant global pathogen.

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