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MICRO REVIEW

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# Two-component sensor histidine kinases of *Mycobacterium tuberculosis*: Beacons for niche navigation

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## Abstract

Intracellular bacterial pathogens such as *Mycobacterium tuberculosis* are remarkably adept at surviving within a host, employing a variety of mechanisms to counteract host defenses and establish a protected niche. Constant surveying of the environment is key for pathogenic mycobacteria to discern their immediate location and coordinate the expression of genes necessary for adaptation. Two-component systems efficiently perform this role, typically comprised of a transmembrane sensor kinase and a cytoplasmic response regulator. In this review, we describe the role of two-component systems in bacterial pathogenesis, focusing predominantly on the role of sensor kinases of *M. tuberculosis*. We highlight important features of sensor kinases in mycobacterial infection, discuss ways in which these signaling proteins sense and respond to environments, and how this is attuned to their intracellular lifestyle. Finally, we discuss recent studies which have identified and characterized inhibitors of two-component sensor kinases toward establishing a new strategy in anti-mycobacterial therapy.

**KEYWORDS**
antibiotic targets, histidine sensor kinase, *Mycobacterium tuberculosis*, TB, two-component transcriptional regulation

## 1 | INTRODUCTION

The ability of pathogenic bacteria to colonize and survive within a host is dependent on a highly complex host-pathogen interplay. The bacterial mechanisms that contribute to this interplay range from structural and enzymatic proteins to protein secretion systems, and are necessary to resist host-defense mechanisms, permit access to new tissue sites, and facilitate transmission to a naïve host. The timely expression of these genes within the correct environment is critical for successful infection; therefore, pathogenic bacteria require sensitive response systems of gene regulation to efficiently perform this function.

A two-component system (TCS) is a signal transduction pathway comprised of two proteins that translate environmental information into a physiological response (Capra & Laub, 2012). The prototypical TCS is comprised of a transmembrane sensor kinase (SK) and a cytoplasmic response regulator (RR) (Figure 1). The SK is responsible for detecting environmental changes and relaying this information to its cognate RR partner through a phosphotransfer event. Most RRs, particularly those found in human pathogens, are DNA-binding proteins whose phospho-activation is translated into an altered transcriptional state for the bacterial cell and subsequent phenotypic responses.

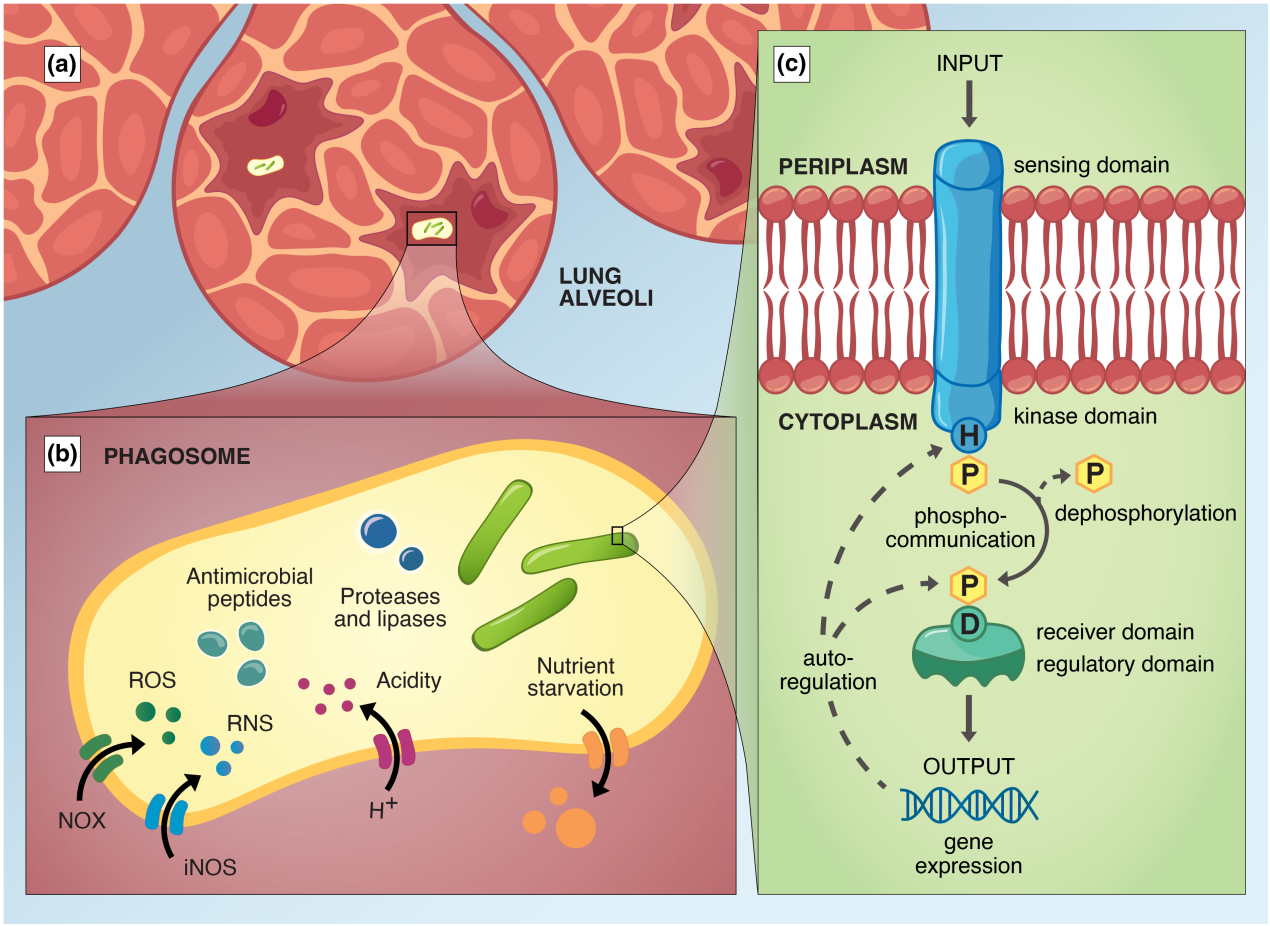


FIGURE 1 The niche of *Mycobacterium tuberculosis* and the mechanism of two-component signal transduction for intracellular adaptation. Inhalation will deposit *M. tuberculosis* in the alveoli of the lungs, where it is engulfed into a phagosome of an alveolar macrophage (a). Phagocytosed *M. tuberculosis* is exposed to inhospitable environmental factors, such as acid stress and iron starvation, proteases, lipases, and antimicrobial peptides (b). Moreover, host proteins NADPH oxidase (NOX) and inducible nitric oxide synthase (iNOS) produce reactive oxygen or nitrogen species (ROS or NOS), respectively. These intracellular conditions represent important stimuli *M. tuberculosis* must recognize to co-ordinate a protective response. Two-component systems efficiently perform this role (c), typically comprised of a transmembrane sensor kinase and a cytoplasmic response regulator. The sensor kinase autophosphorylates upon stimulus detection on a histidine (H) residue and relays this phosphoryl group (P) to an aspartate residue (D) of the receiver domain located on its cognate response regulator, subsequently acting as a transcriptional regulator to modulate the expression of genes required for successful adaptation. Two-component systems generally self-regulate in a positive manner, whereby the phosphorylated RR promotes the transcription of genes encoding the two-component system

Two-component systems have been strongly implicated in the pathogenesis of *Mycobacterium tuberculosis*, the causative agent of tuberculosis (TB). The genome of *M. tuberculosis* contains 12 paired TCSs, 5 orphaned RRs, and 2 orphaned SKs (Table 1) (Cole et al., 1998). Several TCSs, namely DosRST, PhoPR, MprAB, and SenX3-RegX3, have well-defined contributions to in vivo virulence, as defined by infection models utilizing immune-competent and -compromised mice as well as guinea pigs, rabbits, and non-human primates (Converse et al., 2009; Mehra et al., 2015; Parish, Smith, Kendall, et al., 2003; Parish, Smith, Roberts, et al., 2003; Pérez et al., 2001; Rifat et al., 2014; Tischler et al., 2013; Walters et al., 2006; Zahrt & Deretic, 2001). Recently, studies have illuminated the importance of two additional TCSs, i.e., PdtaRS and MtrAB, during infection of mice (Banerjee et al., 2019; Buglino et al., 2021). Other TCSs of *M. tuberculosis*, namely TcrXY, HK1-HK2-TcrA, KdpED, NarLS, PrrAB, and TrcRS, and the orphaned SKs and RRs, remain poorly characterized, however, some evidence exists for their in vivo virulence potential, albeit in immune-compromised mice and in vitro infection models of macrophages (Ewann et al., 2002; Parish, Smith, Kendall, et al., 2003). Nonetheless, it is clear that TCSs have important roles in the pathogenesis of *M. tuberculosis*, and thus their characterization will be essential for the development of new therapies to treat TB.

## 2 | SIGNAL PERCEPTION BY SENSOR KINASES

SKs are typically homo-dimeric transmembrane proteins whose activities are modulated by input signals to the sensing domain (Figure 1). The sensing domains of SKs are most commonly located

**TABLE 1** *Mycobacterium tuberculosis* TCS sensor kinase knowledge base; regulation, interaction, and inhibition

| Sensor kinase | Regulatory stimuli | Available structures | Sensor kinase signaling partner | | | Sensor kinase inhibitors |
|---|---|---|---|---|---|---|
| | | | Cognate RR | Other RRs | Non-RRs | |
| SenX3 | CO, NO, $O_2$ (Singh & Kumar, 2015), phosphate starvation (Glover et al., 2007; Rifat et al., 2014; Tischler et al., 2013), pH (Mahatha et al., 2020) | NCA | RegX3 (Himpens et al., 2000) | No (Agrawal et al., 2015) | NCA | NCA |
| HK1/HK2 | NCA | NCA | TcrA (Shrivastava et al., 2007) | No (Agrawal et al., 2015) | NCA | NCA |
| PhoR | pH (Abramovitch et al., 2011; Baker et al., 2014; Feng et al., 2018), chloride (Tan et al., 2013), magnesium[a] (Walters et al., 2006) | Yes (Xing et al., 2017) | PhoP (Gupta et al., 2006) | DosR, TcrX, TcrA (Agrawal et al., 2015) | NCA | Ethoxzolamide[b] (Johnson et al., 2015) |
| NarS | Nitrate/Nitrite (Malhotra et al., 2015) | NCA | NarL (Malhotra et al., 2015) | No (Agrawal et al., 2015) | NCA | NCA |
| PrrB | Nitrogen starvation (Haydel et al., 2012) | Yes (Nowak et al., 2006) | PrrA (Ewann et al., 2004) | MprA (Agrawal et al., 2015) | NCA | Diarylthiazole[c] (Bellale et al., 2014) |
| MprB | Membrane stress (Bretl et al., 2014; He et al., 2006; Pang et al., 2007) | NCA | MprA (Zahrt et al., 2003) | No (Agrawal et al., 2015) | NCA | BTP15 (Rybniker et al., 2014) |
| KdpD | Osmotic stress (Steyn et al., 2003) | NCA | KdpE (Agrawal & Saini, 2014) | NarL (Agrawal et al., 2015) | NCA | NCA |
| TrcS | NCA | NCA | TrcR (Haydel et al., 1999) | No (Agrawal et al., 2015) | NCA | NCA |
| DosT | CO, NO, $O_2$ (Kumar et al., 2007, 2008; Roberts et al., 2004; Shiloh et al., 2008; Sousa et al., 2007; Vos et al., 2012) | Yes (Podust et al., 2008) | DosR (Roberts et al., 2004) | NarL[d] (Lee et al., 2012) | NCA | Artemisinin, HC106A, HC102A, HC103A (Zheng et al., 2017, 2020) |
| Rv2998A | NCA | NCA | NCA | NCA | NCA | NCA |
| DosS | CO, NO, $O_2$ (Barreto et al., 2019; Basudhar et al., 2016; Ioanoviciu et al., 2007; Kumar et al., 2007, 2008; Lobão et al., 2019; Sardiwal et al., 2005; Shiloh et al., 2008; Sousa et al., 2007; Vos et al., 2012; Yukl et al., 2011) | Yes (Cho et al., 2009; Madrona et al., 2016) | DosR (Saini et al., 2004) | NarL (Agrawal et al., 2015; Cho & Kang, 2014) | GroEL2, Rv2859c, MoeA1, Rv0260c (Gautam et al., 2019) | Artemisinin, HC106A, HC102A, HC103A (Zheng et al., 2017, 2020), DevRS A-ext, DevRS D (Kaur et al., 2014) |
| PdtaS | Copper, Zinc, NO (Buglino et al., 2021), Cyclic di-GMP (Hariharan et al., 2021) | Yes (Preu et al., 2012) | PdtaR (Morth et al., 2005) | No (Agrawal et al., 2015) | NCA | NCA |
| MtrB | NCA | NCA | MtrA (Friedland et al., 2007) | PhoP, NarL, KdpE, TcrX, TcrA (Agrawal et al., 2015; Singh et al., 2019) | NCA | NCA |
| Rv3365c | NCA | NCA | NCA | NCA | NCA | NCA |
| TcrY | NCA | NCA | TcrX (Bhattacharya et al., 2010) | No (Agrawal et al., 2015) | NCA | NCA |

*Note*: NCA—Information not currently available.

[a]Unclear based on transcriptomic analysis and homology modeling.

[b]Mechanism of action is unclear, proposed to indirectly inhibit PhoR sensing.

[c]Hypothesised target based on mutational analysis.

[d]Phosphotransfer not observed by Agrawal et al. (2015).

on the N-terminus of the protein and are exposed to the extracytoplasmic or periplasmic space, though can also exist within the membrane or cytoplasm (Cheung & Hendrickson, 2010; Krell et al., 2010; Preu et al., 2012). In the presence of an inducing stimulus, either a physical signal (e.g., light, temperature) or a chemical ligand (e.g., small molecule), the SK adopts an on-state conformation that promotes its autokinase activity. SK autophosphorylation is mediated via the catalytic and ATP-binding (CA) domain, which binds ATP and phosphorylates a highly conserved histidine residue located within the dimerization and histidine phosphotransfer (DHp) domain (Jacob-Dubuisson et al., 2018). Phosphorylated SKs then act as substrates for the phosphorylation of their cognate RRs to activate their output response. Under conditions whereby the inducing stimulus is absent or the organism transitions from a SK inducing to a non-inducing environment, dephosphorylation of the RR is critical to ensure the TCS pathway is reset. For the majority of TCSs, this occurs through the phosphatase activity of the off-state SK (Jacob-Dubuisson et al., 2018).

For the majority of encoded SKs, the environmental signals that directly regulate their activity are unknown. In the context of mycobacterial pathogenesis, this knowledge gap hinders our understanding of the contribution of these systems to different host niches. The best characterized mycobacterial SKs are DosS and DosT, which are part of the DosRST TCS (Table 1). In this system, two SKs phosphorylate the RR DosR, a key mediator of the initial hypoxic response of *M. tuberculosis* (Boon & Dick, 2012; Roberts et al., 2004; Saini et al., 2004). DosS is a redox sensor that contains a heme-based sensory domain that exists in the ferric ($Fe^{3+}$) form under aerobic conditions (off-state) and is reduced to a ferrous ($Fe^{2+}$) form under anaerobic conditions to shift to its on-state, enhancing its autokinase activity (Ioanoviciu et al., 2007; Kumar et al., 2007). DosT is predominantly a hypoxia sensor that also contains a heme-based sensory domain, but which exists in the oxy ($Fe^{2+}$-$O_2$) form during aerobic conditions that shift into the on-state deoxy ($Fe^{2+}$) form during hypoxia to enhance its autokinase activity (Kumar et al., 2007). The crystal structures of the N-terminal sensory domains of DosS and DosT have been solved, revealing the direct mechanism of oxygen sensing via the bound heme group (Cho et al., 2009; Podust et al., 2008). Moreover, the sensors DosS and DosT are capable of directly sensing nitric oxide and carbon monoxide, in addition to diatomic oxygen, to modulate DosR activation (Honaker et al., 2009; Kumar et al., 2008; Voskuil et al., 2003). The gases NO and CO directly bind to the heme ferrous form of DosS and the deoxy form of DosT, enhancing their autokinase activity (Kumar et al., 2007; Sousa et al., 2007; Vos et al., 2012). Notably, the crystal structure of the NO- and CO-heme complexes of the DosS N-terminal sensory domain have been recently solved (Madrona et al., 2016). For several other SKs of mycobacteria, strong biochemical evidence for their regulatory stimuli also exists (Table 1). Diatomic gases $O_2$, NO, and CO are also able to bind to the heme-based sensory domain of the SenX3 SK to modulate its activity (Singh & Kumar, 2015). The basal autokinase activity of SenX3 in its deoxy form is enhanced in the presence of $O_2$, whereas NO and CO inhibit the activity of SenX3 (Singh & Kumar, 2015). The sensory domain of the MprB SK interacts with the surface-associated chaperone DnaK involved in resolving misfolded proteins and protein aggregates (Bretl et al., 2014). MprB activation is inhibited when bound to DnaK, however, it is proposed that under conditions of cell envelope stress, dissociation of DnaK from MprB promotes SK activity (Bretl et al., 2014; Rao et al., 2021). The structure of the cytoplasmically-localized SK PdtaS has been solved (Preu et al., 2012), and two recent reports suggest cyclic diguanosine monophosphate (di-GMP), copper, zinc, and NO may be regulatory ligands of PdtaS activity (Buglino et al., 2021; Hariharan et al., 2021). Cyclic di-GMP binds to purified PdtaS with high affinity and enhances its autokinase activity in a dose-dependent manner. In contrast, metal ions copper and zinc, and the diatomic gas NO, potently inhibit PdtaS autokinase activity in a dose-dependent manner, and this inhibition is dependent on the N-terminal sensory domain of the SK. This suggests that multiple regulatory ligands likely act to modulate PdtaS activity, as is potentially the case for other mycobacterial SKs discussed below. However, for the remaining SKs of *M. tuberculosis*, little biochemical and structural evidence exists for the signals that regulate their activity, and evidence for their regulatory stimuli is derived primarily from transcriptomic analyses (see Section 3).

The complexity of SK sensing is highlighted by the observation that individual SKs can sense multiple signals, acting synergistically or antagonistically to fine-tune TCS output. For example, evidence that DosS and DosT may respond synergistically to diatomic gases is based on transcriptomic data which demonstrated that *M. tuberculosis* cultured under anaerobic conditions, supplemented with diethylenetriamine/nitric oxide adduct (DETA-NO) as a source for free NO radicals, potently enhanced DosR regulon induction compared to *M. tuberculosis* cultured under aerobic conditions exposed to DETA-NO (Voskuil et al., 2003). That is, anaerobic conditions appear to prime DosS and DosT to directly respond to NO. Indeed, the autokinase activity of purified DosS in the presence of NO is enhanced when the SK is in its ferrous form compared to its ferric state (Kumar et al., 2007). The autokinase activity of purified DosT in the presence of NO is slightly enhanced in its oxy form; however, this activity is far greater when DosT is in its de-oxy state (Kumar et al., 2007). Such synergistic responses have been seen in other SKs of intracellular pathogens such as *Salmonella typhimurium* where the SK PhoQ of the PhoPQ TCS can potentially sense low pH conditions in addition to the divalent cations magnesium and calcium (Martin-Orozco et al., 2006). We propose that this multi-stimulus SK regulation likely reflects the pathogens' need to fine-tune its physiology in light of prevailing stimuli within the colonized niche; for example, the granuloma where *M. tuberculosis* likely responds to hypoxia and oxidative/nitrosative stress. As discussed below, it appears that several mycobacterial SKs are able to sense multiple signals, suggesting that multi-stimulus regulation of SK activity is a commonality among these signaling proteins rather than an exception. This has significant implications for *M. tuberculosis* infection biology, shedding light on a previously underappreciated feature of SKs that we believe is critical to *M. tuberculosis* survival within its host niche.

## 3 | LINKING TWO-COMPONENT SYSTEMS TO HOST ENVIRONMENTS

### 3.1 | Environments encountered by *M. tuberculosis* during its infectious lifecycle

Our ability to identify the sensory ligands of mycobacterial SKs is limited by our understanding of the environments pathogenic mycobacteria encounter during their infectious lifecycle. Upon inhalation, *M. tuberculosis* is internalized by alveolar macrophages resident in the lungs of an individual (Cohen et al., 2018). Conventionally, phagocytosed particles are delivered to the lysosome of these phagocytes, which serves as a hydrolytic and acidic environment for the destruction of invading bacteria. However, *M. tuberculosis* employs antagonistic strategies to circumvent this cellular defense (see Carranza and Chavez-Galan (2019) for a recent review). As a result, the compartment in which *M. tuberculosis* resides is only mildly acidic (~pH 6.4), remains accessible to essential nutrients, and is devoid of antimicrobial enzymes; ultimately, an environment that is permissive for bacterial growth (Figure 1) (Podinovskaia et al., 2013; Rohde et al., 2012). Eventually, interferon-gamma (IFN-$\gamma$)-induced activation of the infected macrophage overcomes the mycobacteria-induced phagosome maturation block, delivering the bacillus to a highly acidic phagolysosomal compartment that is accessible to antimicrobial attack from reactive oxygen/nitrogen species and starved of essential nutrients such as iron (Baker et al., 2019; Chao et al., 2019; Shastri et al., 2018). Although much work has been dedicated to understanding *M. tuberculosis* responses to macrophage infection, the pathogen is known to reside intracellularly in a variety of immune and endothelial cell types during infection, e.g., granulocytes and lymphatic endothelial cells (Lerner et al., 2016; Lovewell et al., 2021). It is important to note that each of these cell types serves as a different niche to that described for the macrophage and thus likely induces different bacterial responses (Srivastava et al., 2014).

The hallmark of TB is the granuloma, a complex immune structure that serves as the host's attempt to control mycobacterial replication (Pagán & Ramakrishnan, 2018; Ramakrishnan, 2012). The granuloma, despite being a niche of significant importance to *M. tuberculosis* infection, is a hostile environment comprised of a variety of immune stressors including hypoxia, oxidative and nitrosative stress, and broad nutrient starvation (Berney & Berney-Meyer, 2017; Jamaati et al., 2017; Rustad et al., 2009; Shastri et al., 2018). The granuloma is considered the primary niche of *M. tuberculosis* within an immunocompetent host, as the organism predominantly resides within this environment for the majority of its infectious lifecycle. Granuloma presentation in TB patients is highly heterogeneous, encompassing a range of pathological subtypes with differing immunological behavior and bacterial sterilization efficacy, even within the same host (Lin et al., 2014). The classical *M. tuberculosis*-containing granuloma is comprised of foci of epithelioid macrophages organized around an acellular necrotic core, known as the caseum. Neutrophils are regularly observed within this structure, which is enclosed by a fibrous cuff and peripheral B and T lymphocytes (Russell et al., 2009). Numerous other granuloma types have been identified, including non-necrotic, necrotic neutrophilic, fibrotic, and calcified granulomas, all of which determine the replicative ability, metabolism, and drug susceptibility of the contained mycobacteria, as well as alter host clinical outcomes (Lenaerts et al., 2015). In light of the numerous possible niches that *M. tuberculosis* can occupy, it is critical for the bacterium to have the ability to rapidly sense and respond to a large variety of immune microenvironments for successful adaptation and persistence within the host.

### 3.2 | TCS activity: What has been learned from in vitro models of infection

Much of our understanding of the role of TCSs in different host environments comes from studying *M. tuberculosis* physiological responses under defined in vitro culture conditions thought to mimic the conditions encountered by the organism during infection. Early transcriptomic studies that assess the response of *M. tuberculosis* to a variety of stress conditions have been highly valuable in dissecting genetic pathways important for mycobacterial adaptation (Table 1). Several studies of the initial *M. tuberculosis* response to hypoxia revealed the importance of the *dosRS* operon (Park et al., 2003; Saini et al., 2004; Sherman et al., 2001), and subsequently in the response to NO and CO (Kendall et al., 2004; Kumar et al., 2008; Ohno et al., 2003; Shiloh et al., 2008; Voskuil et al., 2003), supporting the available biochemical and structural data of the DosS and DosT kinases in their role in sensing and responding to these gases within the host. *M. tuberculosis* likely encounters phosphate starvation during its infectious lifecycle (Tischler et al., 2013), and *M. tuberculosis* cultured in a phosphate-depleted medium induces transcription of an operon encoding the SenX3-RegX3 TCS (Glover et al., 2007; Rifat et al., 2009; Tischler et al., 2013). Several genes involved in *M. tuberculosis* virulence are induced in a phosphate-depleted medium in a SenX3-RegX3 dependent manner, notably members of the ESX-5 secretion system (Elliott & Tischler, 2016; Elliott et al., 2019; Ramakrishnan et al., 2015; Sanyal et al., 2013), highlighting the importance of this TCS in regulating *M. tuberculosis* physiology in such an environment. Interestingly, $O_2$, NO, and CO have been shown to be regulatory ligands of purified SenX3 autokinase activity (Singh & Kumar, 2015), suggesting this TCS may have broader roles in *M. tuberculosis* physiology than simply adaptation to phosphate starvation. *M. tuberculosis* is thought to encounter varying gradients of charged ions during its residence within the macrophage phagosome (Tan, 2021), and the PhoPR TCS has been implicated in responding to several different physiologically relevant ions including protons (Abramovitch et al., 2011; Baker et al., 2014; Bansal et al., 2017; Feng et al., 2018), chloride (Tan et al., 2013) and magnesium (Walters et al., 2006). *M. tuberculosis* encounters cell wall stress during infection (Schnappinger et al., 2003), and the transcription of an operon encoding the MprAB TCS is upregulated in *M. tuberculosis* when cultured in the presence of surfactants such as sodium dodecyl

sulfate (SDS) and Triton X-100 (Bretl et al., 2014; He et al., 2006; Pang et al., 2007). The MprB SK appears to sense cell wall stress indirectly through interactions with DnaK, implicating the MprAB as a general stress response TCS that can respond to a variety of cell wall damaging agents. The importance of the MprAB TCS is exemplified by the observation that it is required for the regulation of genes involved in several *M. tuberculosis* stress response pathways when cultured in the presence of SDS, including the stress response sigma factor E pathway (He et al., 2006; Pang et al., 2007). In contrast, PdtaS is a completely cytoplasmic SK and thus responds to intracellular signals to regulate the activity of the PdtaRS pathway. PdtaR has been shown to be required for the regulation of genes involved in NO resistance when *M. tuberculosis* is cultured in the presence of DETA-NO (Buglino et al., 2021), suggesting this TCS is also important in the adaptation of *M. tuberculosis* to this environment when encountered within the host. The kinase activity of purified PdtaS was also shown by Buglino et al. (2021) to be regulated by zinc and copper, indicating that the PdtaRS pathway may have additional functions in regulating *M. tuberculosis* responses to these metals. Recently, the activity of the PdtaRS pathway has been shown to be regulated by intracellular levels of cyclic di-GMP, which increases when *Mycobacterium smegmatis* is cultured in a nutrient-poor medium to activate the PdtaRS TCS (Hariharan et al., 2021), implicating this system in the adaptation of *M. tuberculosis* to nutrient starvation during infection in addition to the aforementioned environments. Taken together, these in vitro models have begun to shed light on the role of TCSs in host adaptation during *M. tuberculosis* infection. In conjunction with biochemical and structural data available for the purified SKs of *M. tuberculosis*, whole-cell in vitro studies of TCS activity have been useful in delineating the environments where these TCSs are likely important. However, translating these in vitro studies of TCS activity to in vivo models of infection is a challenging, albeit essential, task.

### 3.3 | TCS activity: Translating in vitro data to in vivo models of TB

Several studies have employed global approaches to directly studying the expression of TCSs within macrophage infection models of TB. An early investigation by Zahrt and Deretic (2001) into the expression of *M. tuberculosis* TCSs within the phagosome of human macrophages was performed by using fluorescent promoter-reporter strains of TCS genes, whereby TCS promoter activities were transcriptionally linked to GFP expression. The regulatory genes were assigned to three categories based on their temporal expression profiles during macrophage infection: (i) genes constitutively expressed (*phoP*, *Rv0818*, *mtrA*), (ii) genes differentially expressed (*dosR*), and (iii) genes that were not expressed during infection (*narL*, *prrA*, *tcrR*, *tcrX*, *pdtaR*, *Rv2884*). A similar investigation was subsequently performed by Haydel and Clark-Curtiss (2004) using an approach known as selective capture of transcribed sequences (SCOTS). The regulatory genes were similarly assigned to three categories: (i) genes constitutively expressed (*pdtaR*, *dosT*, *mtrA*), (ii) genes differentially expressed (*pdtaS*, *regX3*, *phoP*, *prrA*, *mprA*, *kdpE*, *trcR*, *dosR*, *tcrX*), and (iii) genes that were not expressed during infection (*Rv1095*, *Rv0260c*, *Rv0818*, *Rv2884*, *Rv3143c*), which predominantly represented orphaned RRs and SKs. Overall, very little overlap in TCS expression profiles was observed between these studies, likely reflecting differences in the infection model used and limitations in the techniques themselves. Nonetheless, these studies are largely concordant with the aforementioned in vitro observations of bacterial responses to environments *M. tuberculosis* is thought to encounter during macrophage infection (i.e., PhoPR, MprAB, DosRST, PdtaRS, SenX3-RegX3), highlighting the active role these TCSs likely play for *M. tuberculosis* adaptation to the intraphagosomal environment, but also the need for further work to clearly delineate the importance and activity of each regulator during macrophage infection.

The study of TCS activity within animal models of TB is highly complex, given the inherent heterogeneity of pathological sites (and thus bacterial responses) associated with disease, the lack of known environments and stressors *M. tuberculosis* encounters within the host, and the absence of appropriate tools/techniques to effectively monitor TCS activity. Indeed, there are very few studies of *M. tuberculosis* TCS activity within in vivo models of infection, reflecting the difficulty of this endeavor. Expression of *dosR* and several genes potently induced transcriptionally during in vitro hypoxia in a DosR-dependent manner have been shown to also be highly expressed in infected mouse lungs by qRT-PCR (Gautam et al., 2015; Voskuil et al., 2003). However, techniques such as qRT-PCR and other gene expression analyses have had limited use for TCS studies, as these techniques are reliant on highly expressed genes for adequate analysis, and thus not suitable for genes that are expressed at low levels or genes which are only expressed in certain pathological sites. Promoter-reporter systems, on the contrary, have the potential to overcome many challenges associated with bacterial RNA isolation and pathological heterogeneity, providing single-cell resolution of TCS activity. When coupled with luminescence or fluorescence technology, *M. tuberculosis* reporter strains can be very useful to discern TCS activation in different pathological sites. Notably, a seminal study by Tan et al. (2013) generated PhoPR reporter (*Rv2390c* promoter-GFP fusion) and DosRST reporter (*hspX* promoter-GFP fusion) strains to probe the activity of these TCSs in mouse models of TB. The activity of the *Rv2390c* promoter, as an indicator of PhoPR activity, was enhanced in *M. tuberculosis* cultured under levels of low $H^+$ and high $Cl^-$, and this response was abolished in a *phoPR* knockout background. In IFN-$\gamma^{-/-}$ mice infected with the PhoPR reporter strain, *Rv2390c* promoter activity was strongly reduced when compared to levels in wild-type mice, indicating that *M. tuberculosis* likely encounters an IFN-$\gamma$-dependent low $H^+$/high $Cl^-$ environment during infection that may require the PhoPR TCS for successful adaptation. Additionally, GFP expression in the DosRST reporter strain was strongly induced when *M. tuberculosis* was cultured in hypoxia or the presence of DETA-NO, indicating high levels of *hspX* promoter activity under these conditions. Similarly, in IFN-$\gamma^{-/-}$ mice infected with the DosRST reporter strain, *hspX* promoter activity was significantly

reduced when compared to levels in wild-type mice. Promoter activity was also shown to be significantly higher in regions positive for the intracellular nitric oxide synthase (iNOS) (i.e., co-localized) compared to iNOS-negative regions. This indicates that *M. tuberculosis* encounters hypoxic and/or nitrosative environments in the lungs of infected mice in an IFN-$\gamma$-dependent manner, and adaptation to these environments likely requires the activity of the DosRST TCS. Notably, the authors observed large variability in GFP expression levels from infected tissue, highlighting the heterogeneity in bacterial responses during infection, likely reflective of the diverse host niches in which they reside in.

Given the broad applicability of reporter strains to decipher TCS activity in complex models of infection, we expect this technology will be useful in the study of other TCSs of *M. tuberculosis* to translate observations from in vitro responses and macrophage models of infection to the environments that *M. tuberculosis* encounters in animal models of TB. A major benefit to this approach will be the ability to study the impact of the host environment on the TCS pathway of interest through genetic interference or chemotherapeutic inhibitors of macrophage processes, thus providing a means to better understand the role of TCSs in the host-pathogen interaction. We see this as an essential "next step" in TCS biology to illuminate their necessity in the pathogenesis of TB.

## 4 | SENSOR KINASES: PROMISCUOUS SIGNALING PROTEINS IN MYCOBACTERIA?

The high degree of sequence and structural similarity in members of the SK and RR gene families raises the possibility of cross-communication between TCS signaling pathways. Cross-talk between TCS proteins (Figure 2) has been recognized in the literature for some time and considered a potential mechanism for complex stimulus integration and signal transduction in bacteria (Hellingwerf et al., 1995; Wanner, 1992). The majority of studied TCSs appear to behave as insulated two-protein signal transduction pathways (Laub & Goulian, 2007). However, there are many examples in microbiology where this two-protein paradigm is subverted (Drepper et al., 2006; Howell et al., 2006; Matsubara et al., 2000; Rabin & Stewart, 1993), and in some instances, these non-canonical signaling pathways are critical for successful environmental adaptation. A recent example of beneficial cross-talk is between the PmrAB and QseBC TCSs in uropathogenic *Escherichia coli*, contributing to the organism's response to ferric iron (Guckes et al., 2017).

Several groups have investigated the potential for cross-communication between mycobacterial TCS proteins. Lee et al. (2012) employed a yeast two-hybrid assay to investigate the interaction between all SK/RR pairs in *M. tuberculosis*. The study identified NarL, the RR of the NarLS TCS, as a phospho-acceptor of DosT, linking the activities of the DosRST and NarLS TCS pathways. A subsequent comprehensive study by Agrawal et al. (2015) investigated in vitro interactions between all purified SK/RR pairs by phosphotransfer profiling. Interestingly, 40% of tested SKs were

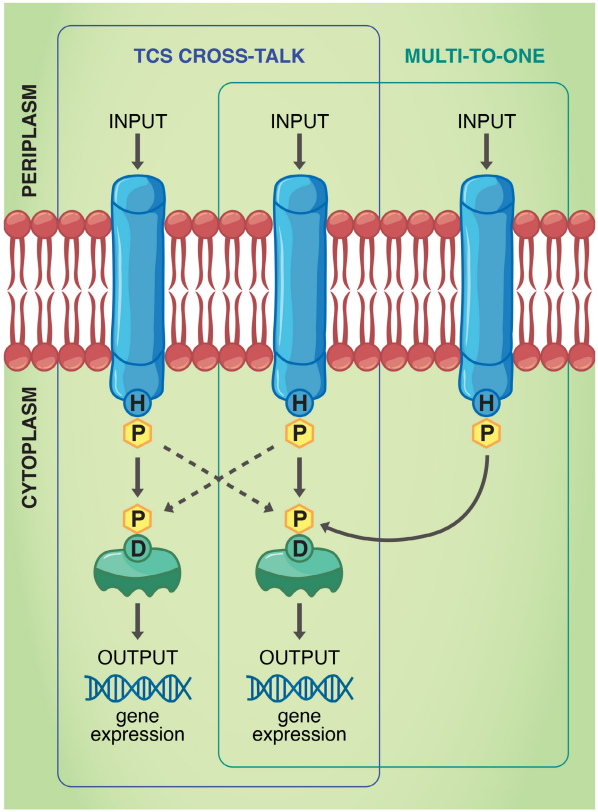


FIGURE 2 Signal transduction pathways afforded by sensor histidine kinases of *Mycobacterium tuberculosis*. Cross-talk between two-component system (TCS) proteins represents a mechanism to expand the signaling repertoire of mycobacteria, whereby a sensor kinase (SK) from one pathway communicates with a non-cognate response regulator (RR) from another pathway by phosphorylation. Promiscuous SKs that are capable of phosphorylating more than one substrate represent multi-to-one signaling pathways, further increasing the input recognition capabilities of RRs, thereby expanding and/or tuning the subsequent responses via the phosphorylated RR

able to serve as phospho-donors to more than one RR (KdpD, PrrB, PhoR, MtrB, DosS) and 60% of tested RRs could serve as phospho-acceptors to more than one SK (TcrX, MprA, NarL, TcrA, DosR, PhoP, KdpE) (Table 1). The physiological consequences of these SK-RR interactions remain unclear; nonetheless, as more studies aim to investigate the potential of these TCS proteins to modulate mycobacterial physiology, it appears likely "multi-to-one" signaling relationships (Figure 2) may facilitate important cellular functions beyond the classically studied SK-RR insulated signaling pathway.

A recent study by Gautam et al. (2019) has expanded the notion of cross-communication to include non-TCS proteins, whereby a SK phosphorylates other non-RR proteins. Using a number of complementary approaches to identify SK-interacting proteins, DosS was shown to catalyze the phosphotransfer to GroEL2 (Rv0440), Rv2859c, MoeA1 (Rv0994), and Rv0260c (Table 1). These proteins in themselves have been reported to have important cellular functions that likely contribute to the *M. tuberculosis* hypoxic response but are not directly transcriptionally controlled by DosR. Therefore,

this finding expands the repertoire of physiological adaptations available to *M. tuberculosis* when encountering a hypoxic environment, i.e., responses in a DosR-independent manner. This study provides evidence for the broader roles that SKs may have in regulating mycobacterial physiology and highlights the importance of studying other SKs which may have similar promiscuous phospho-donating properties to facilitate successful environmental adaptation.

It is evident that our understanding of the contribution of TCS proteins to mycobacterial physiology is only just beginning, as these systems potentially participate in extensive signaling pathways that may even constitute non-TCS proteins. Such signaling networks may have important consequences for mycobacterial pathogenesis, particularly considering that many of the aforementioned environments encountered by *M. tuberculosis* are colocalized within the same niche, suggesting that many TCSs may be active simultaneously, and cross-communication/cross-regulation may be a mechanism pathogenic mycobacteria rely on to permit successful environmental adaptation. Indeed, the reduced number of TCSs in *M. tuberculosis* as compared to other notable intracellular human pathogens (e.g., *S. typhi*) suggests a highly refined efficiency within the regulatory system of *M. tuberculosis*. In addition, there is growing evidence that the few TCSs encoded within the *M. tuberculosis* genome may employ cross-communication between TCSs and other non-TCS proteins as a strategy to enhance capacity. Knowledge gain in this area is currently expanding, with insights leading to an appreciation of host adaptation but also potential vulnerabilities in bacterial protein communication that can be exploited. This is an area of growing interest and in the author's opinion, one which holds significant potential for adjunct therapy development.

## 5 | DISCOVERY OF INHIBITORS OF TWO-COMPONENT SYSTEMS

Given the importance of TCSs for TB pathogenesis, TCS proteins represent an attractive target for the development of new antibiotics or adjunct therapies as discussed above. Inhibition of TCS proteins may provide a blockade to the organism's ability to sense and respond appropriately to environmental stimuli, leaving the bacteria vulnerable to innate and cellular immunity or increase their susceptibility to antibiotics. Although RRs represent valid targets for chemical inhibition, here we will focus on recent advances in the development of SK inhibitors and provide insights into their suitability for anti-TB therapy.

### 5.1 | Advantages of chemotherapeutic targeting of sensor kinases

SK activities are attractive inhibitory targets for the dysregulation of TCS function. Several features of mycobacterial SKs make these proteins appealing for chemical inhibition: (i) sensory domains are typically localized extracytoplasmically, thus circumventing issues surrounding drug efflux, (ii) conservation of the CA domain (necessary for catalytic activity) and the DHp domain (which contains the conserved His residue for autophosphorylation) may enable targeting of multiple SKs simultaneously, and (iii) structures of the sensory and catalytic domains of several mycobacterial SKs have been solved, thus paving the way for targeted rational design.

An advantage to targeting SKs is their multi-enzymatic functionalities. SK autophosphorylase, phosphotransferase, and dephosphorylase activities all represent viable targets amenable to inhibition, blocking the capacity of the SK to sense environmental stimuli and regulate the activity of its cognate RR (or other phosphotargets of the SK). Furthermore, we foresee that agonists that enhance SK activity will similarly be an effective mechanism to dysregulate TCS pathways, locking SKs in an "on-state" conformation. The activation of the SK's cognate RR in the absence of the appropriate stimulus may have a significant impact on bacterial fitness by promoting the expression of genes that are not suitable for the organism's niche, therefore, blocking its ability to successfully adapt.

There are notable challenges, however, to targeting mycobacterial SKs as modes for anti-TB therapy. One potential pitfall is that eukaryotic kinases may have biochemical or structural features that are similar to mycobacterial SKs. For instance, the ATP-binding Bergerat fold present in SK CA domains is also found in several mammalian proteins, namely those of the GHKL (gyrase, Hsp90, histidine kinase, MutL) family (Bem et al., 2015; Fihn & Carlson, 2021). In this case, targeting the CA domain could be accompanied by the off-target risk of host cytotoxicity. Despite this, we presume this will not be a major barrier to SK inhibitor design and development, given several research groups have successfully identified potent mycobacterial SK inhibitors thus far with no apparent off-target effects (Table 1).

### 5.2 | Inhibitors of mycobacterial SKs

Ethoxzolamide, an FDA-approved drug currently used for the treatment of glaucoma and duodenal ulcers, has been shown to inhibit the PhoPR virulence-associated regulon in *M. tuberculosis* (Johnson et al., 2015). Ethoxzolamide was able to significantly reduce *M. tuberculosis* burden in both infected macrophages and mice. Although the mechanism of action is unclear, the authors propose a model whereby ethoxzolamide indirectly inhibits PhoR sensing by targeting cell surface carbonic anhydrases that may modulate the local extracellular environment.

The DosRST TCS has also attracted attention for chemical inhibition. A recent study that screened ~540,000 compounds reported that one of the most effective antimalarial drugs, artemisinin, and several other small molecules (HC102A-HC106A) are able to inhibit DosRST activity (Zheng et al., 2017). Transcriptomic analyses showed that the molecules potently inhibit the DosRST regulon while having no influence on bacterial viability in vitro. Artemisinin, HC102A, and HC103A all significantly reduced the survival of *M. tuberculosis* during hypoxia. Investigation of the mode of action revealed that artemisinin degrades ferrous heme and generates heme-artemisinin

adducts, thereby inactivating the heme-based SKs DosS and DosT. HC106A is also thought to interact with SK heme, however, in a mechanism distinct from artemisinin (Zheng et al., 2020). In addition, HC102A and HC103A were also shown to inhibit the autokinase activity of DosS and DosT, with little to no cytotoxicity reported against several mammalian cell lines (Zheng et al., 2017).

In a study aimed at finding small molecule inhibitors of *M. tuberculosis* protein secretion systems that facilitate bacteria-induced cytolysis of cultured lung fibroblasts, the authors identified BTP15, a benzothiophene inhibitor of the SK MprB (Rybniker et al., 2014). The MprAB TCS positively regulates substrates of the major *M. tuberculosis* virulence protein secretory system ESX-1 (Cao et al., 2015; Pang et al., 2013). BTP15 was found to potently inhibit MprB autokinase activity in vitro in a dose-dependent manner, and the treatment of cultured macrophages infected with *M. tuberculosis* with BTP15 significantly reduced the intracellular bacterial load.

Taken together, these studies demonstrate the viability of targeting mycobacterial SKs for anti-TB therapy. In the author's opinion, SK inhibition represents a new paradigm in antimycobacterial therapy, and we foresee the development of "anti-sensory" agents as valuable tools in the growing arsenal of TB chemotherapies. A notable commonality between the aforementioned studies is the repurposing of compounds that may have value as anti-mycobacterial agents, thus significantly reducing the time between hit identification and translation into the clinic. This is an emerging and exciting area of study, and given the importance of SKs in mycobacterial pathogenesis, one which will have significant implications in the global control of TB.

## 6 | FINAL REMARKS

The vital contribution of TCSs to successful infection and transmission of *M. tuberculosis* is without question. This is an exciting area of an ongoing investigation which is revealing an unexpected level of complexity within and between TCSs. For the intracellular pathogen, this complexity is apparent in the breadth of sensing mechanisms, growing evidence of cross-interaction, the predominance of systems, and also complex genetic regulation within. An increased understanding of the signaling interplay of, and the pathogens' reliance on TCSs will continue to attract efforts toward targeted therapeutic interventions in a space urgently in need of new treatment paradigms.

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### CONFLICT OF INTEREST

The authors declare no conflict of interest.

### AUTHOR CONTRIBUTIONS

Miljan Stupar, Juanelle Furness, Christopher J. De Voss, Lendl Tan, and Nicholas P. West wrote the manuscript. Juanelle Furness created the figures with input from all authors. All authors read and approved the final manuscript prior to publication.

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