Signal transduction systems of mycobacteria with special reference to *M. tuberculosis*

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Two-component systems and eukaryotic-like Ser/Thr protein kinases comprise major components of the signal transduction machinery of *Mycobacterium tuberculosis*. The elucidation of its genome sequence provided a tremendous fillip to their study. Multiple approaches embracing bioinformatics, genetics, biochemistry, cell and molecular biology and animal models are being used to characterize them and to decipher their role in the pathobiology of *M. tuberculosis*. Several response regulator proteins have been implicated in the survival, multiplication and persistence of *M. tuberculosis* in animals and cell models of virulence. Six additional mycobacterial genome sequencing projects are at advanced stages of completion. Bioinformatics tools have revealed that the two-component systems, eukaryotic-like Ser/Thr protein kinases and phosphatases are conserved to varying extents in different mycobacteria. This information will provide a jumpstart to their functional analysis. One of the best understood systems among the signal transduction proteins is the DevR–DevS two-component system. Studies from several laboratories have contributed to our present understanding of its role in the hypoxia response of pathogenic and non pathogenic mycobacteria. The current status of our knowledge of this and other signal transduction systems in mycobacteria is the subject of this review.

THE genus *Mycobacterium* encompasses 71 validly named species and an additional three subspecies that include the principal human pathogens, *M. tuberculosis* and *M. leprae*, and the cattle pathogen, *M. bovis*. In all, 32 species are known to be pathogenic to humans or animals. Mycobacteria other than *M. tuberculosis* and *M. leprae* are often referred to as 'atypical mycobacteria'. The most commonly encountered pathogens among the atypical mycobacteria are species of the *Mycobacterium avium* complex (MAC) that includes *M. avium* and *M. avium* subspecies *paratuberculosis*, common pathogens of birds and cattle respectively. *M. avium* has been implicated in pulmonary and nonpulmonary infections and MAC strains are the most frequent mycobacterial isolates from patients with AIDS. *M. marinum* is one of seven *Mycobacterium* species that causes fish mycobacteriosis. All these also cause disease in humans.

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The era of whole genome sequencing ushered in hopes of harnessing the genetic blueprint of microbial pathogens towards developing novel and knowledge-based strategies for disease detection, prevention and treatment. It was natural therefore that the genome sequences of two strains of *M. tuberculosis* and the related obligate intracellular pathogen, *M. leprae*, were determined in the midst of great enthusiasm[1–3] and six mycobacterial genome sequencing projects including those of cattle, bird and fish pathogens are currently underway.

Brief overview of host–*Mycobacterium* interaction

All pathogenic mycobacteria are intracellular pathogens and survive within host phagocytes. Pulmonary tuberculous infection commences when tubercle bacilli within inhaled droplet nuclei are ingested by alveolar macrophages in the lung. If the macrophage fails to destroy or inhibit the inhaled bacteria, the bacilli multiply (as in resting macrophages) until the macrophage bursts. Evidence is accumulating that pathogenic mycobacteria survive in specially remodelled vacuoles within their host macrophages while resisting fusion with lysosomes[4]. The bacteria-containing vacuoles fuse with early endosomes and bacteria-derived compounds including cell wall lipids and proteins traffic through the host cell and are released by exocytosis. The molecules released by intracellular mycobacteria constitute antigens recognized by the immune system. A majority of infected people mount an effective immune response that culminates in granuloma formation around the infective foci and cessation of disease progression. Granuloma formation is a well-conserved host response in the face of most mycobacterial infections and serves to wall off the infection by rapid, extensive and sustained recruitment and trafficking of T cells and monocytes into and through the site that contains activated lymphoid cells and myeloid cells in various stages of cytokine-induced differentiation. These range from monocytes through tissue macrophages and epithelioid cells to multinucleated giant cells. Fibroblasts isolate the site by encircling it. As a consequence of tissue necrosis associated with cell-mediated cytotoxicity and cytokine production, central cavitation and liquefaction occurs in tuberculous granulomas. Bacilli can be found extracellularly in the liquefied areas and intracellularly in

tissue macrophages and perhaps in multinucleated giant cells. In response to host defences, bacillary growth stops and is replaced by bacillary persistence within the granulomas[5]. A strong cellular immunity successfully contains the tubercle bacilli that remain in persistent state for an indefinite period of time. In fact, culturable bacilli have been isolated from closed tuberculous lesions of human patients of tuberculosis[6]. However in the absence of good cellular immunity, the pathogen is not contained within the granulomas leading to disseminating or miliary disease. Approximately 10% of latent infections reactivate in the event of lowered immunity as in HIV infection, malnutrition, immunosuppressive drug therapy, etc. resulting once again in active infectious disease[7,8]. In summary, the continuum from infection to disease comprises many stages that include adhesion, invasion, intracellular survival and replication through dissemination to other body sites to sequestration in a non-replicative persistent form and finally, reactivation.

TCS, STPKs and phosphatases in mycobacteria

What are the systems available to the pathogen to perceive and overcome or neutralize the onslaught of host defence mechanisms? Protein phosphorylation is the principal means by which environmental signals are converted to appropriate cellular responses in both prokaryotes and eukaryotes. In bacteria the two-component systems (TCS) consisting of histidine sensor kinase/response regulator partner proteins play a key role in this process with phosphorylation occurring on histidine/aspartic acid residues. In contrast, in eukaryotes, the Ser/Thr protein kinases (STPKs) and phosphoprotein phosphatases constitute the mainstay of signal transduction pathways. Previously STPKs and their associated phosphatases were thought to be unique to eukaryotes; however sequence analysis of bacterial genomes has led to their discovery in bacteria[9] including *M. tuberculosis*[1]. Mycobacteria are characterized by the presence of both classes of signal transduction systems, namely, TCS and eukaryotic-like STPK proteins. The importance of STPKs in the biology of *M. tuberculosis* was reviewed recently[10]. The present article examines our current understanding of mycobacterial signal transduction systems, TCS in particular, gleaned from genome sequence analysis and experimental approaches.

TCS control a wide variety of physiological processes in bacteria ranging from nutrient uptake to the expression of the virulence phenotype[11]. They are built up from modular units: input sensing domains, output effector domains, transmitter domains and receiver domains for promoting protein–protein communication. The sensing domains of histidine kinases, that are typically membrane-associated, sense environmental signals and through a phosphorylation cascade involving response regulators modulate gene expression that culminates in appropriate bacterial responses[12]. The sensors are tailored to recognize one (or many) of a gamut of environmental signals that for mycobacteria would predictably comprise of conditions prevailing in the host including changes in oxygen tension and temperature, the hostile environment of the macrophage, levels of cations such as magnesium, calcium and iron, nutrient limitation and, last but not the least, the host immune response.

The availability of partially completed mycobacterial genome sequences together with those of *M. tuberculosis* H37Rv and CDC1551 and *M. leprae* provided us with an unprecedented opportunity to assess the potential similarities and uniqueness of the signal transduction systems encoded in their genetic make up using BLAST alignment tools (www.ncbi.nlm.nih.gov). The results are presented in Tables 1 and 2. At first glance *M. tuberculosis* seems to possess a limited repertoire of TCS comprising of only eleven complete TCS and seven orphan response regulator and sensor kinase proteins. The surprising discovery of 14 genes in the genome, encoding eukaryotic-like STPKs and phosphatases suggested that the deficiency in TCS might perhaps be offset by the contribution of the former to cellular adaptation processes[1].

The genes encoding these protein families were almost identical among the three sequenced strains of *M. tuberculosis* (H37Rv, CDC1551 and 210) except for the apparent absence of STPK gene *pknA* in strain 210 and a merging of putative histidine kinases Rv0601c (156 amino acids) and Rv600c (207 amino acids) as a 377 amino acids-long protein with a distinct N-terminal sequence in CDC1551. These differences between *M. tuberculosis* strains are striking but their impact, if any, on the adaptation properties of the tubercle bacilli is yet to be assessed. Approximately a third of the TCS proteins of the CDC1551 and H37Rv strains also showed a disparity in their predicted lengths (+ 52 to – 7 amino acids) which could be attributed to differences in the assignment of ORFs and not in the nucleotide sequence.

The eleven TCS and seven orphan response regulator/sensor kinase proteins were completely conserved in *M. tuberculosis* and *M. bovis* genome sequences. However, *M. marinum*, *M. avium* and *M. avium* subsp. *paratuberculosis* contained 10 of 11, *M. smegmatis* 9 of 11 and *M. leprae* the least of all, namely, 4 of 11 TCS (Table 1). Only a limited number of TCS have been subjected to biochemical characterization. These are SenX3–RegX3 (ref. 13), TrcR–TrcS[14] and DevR–DevS[15] of *M. tuberculosis*. Four TCS, namely, SenX3–RegX3, PrrA–PrrB, MprA–MprB, MtrA–MtrB and two orphan proteins, Rv1626 and Rv3220c, were conserved in all mycobacterial species (highlighted in orange, Table 1). Five TCS, namely, PhoP-PhoR, KdpD–KdpE, TrcR–TrcS, DevR–DevS and TcrX–TcrY, NarL and three orphans, Rv0260c, Rv0818 and Rv3143, were conserved in all mycobacteria except *M. leprae* (highlighted in blue, Table 1). The presence of only a partial set of signal transduction systems in the leprosy bacillus is consistent

Table 1. Profile of two-component systems in mycobacteria

Rv Gene[a] (Name)	Organization[b]	*Mbov*[c]	*Mmar*	*Mlep*	*Mav*	*Mptb*	*Msm*	Involvement/phenotype of *Mtb* mutant	Environmental signal (gene response)	Target genes
0490/ 0491 (SenX3/ RegX3)	HR	+/+[d]	+/+	+/+	+/+	+/+	+/+	No phenotype in BALB/c mouse[32]	Constitutive expression[32]	Auto regulation[13]
0600c/ 0601c/ 0602c (U/ U/ TcrA)	RHH	+/+/+	–/–/–	–/–/–	–/–/±	–/–/±	–/–/–		Hypoxia (Rep)[35]	
0757/ 0758 (PhoP/ PhoR)	RH	+/+	+/+	P/P	+/+	+/+	+/+	Intracellular multiplication in MP and BALB/c mouse, attenuated in mouse[30]	Constitutive expression[29]	
0844c/ 0845 (NarL/ NarS)	Divergent	+/+	+/+	–/–	+/+	+/+	+/–	No phenotype in SCID mouse[31]		
0902c/ 0903c (PrrB/ PrrA)	RH	+/+	+/+	+/+	+/+	+/+	+/+	Early intracellular multiplication in MP, no phenotype in BALB/c mouse[32]	Entry into MP (Ind)[32]	
0981/ 0982 (MprA/ MprB)	RH	+/+	+/+	+/+	+/+	+/+	+/+	Tissue-specific persistence in BALB/c mouse[29]	Ind in *Mbov* but not in *Mtb* within MP[29]	
1027c/ 1028c (KdpE/ KdpD)	HR	+/+	+/+	–/–	+/+	+/+	+/+	Hypervirulent in SCID mouse[31]	Nutrient starvation (Ind)[44]	
1032c/ 1033c (TrcS/ TrcR)	RH	+/+	+/+	P/P	+/+	+/+	+/+	No phenotype in BALB/c mouse[32], hypervirulent in SCID mouse[31]		Auto regulation[46]
3132c/ 3133c (DevS/ DevR)[e]	RH	+/+	+/+	–/–	±/+	±/+	+/+	Hypervirulent in SCID mouse[31], attenuated in guinea pig[33], impaired survival under hypoxia *in vitro* in *Mbov*[36] and *Msm*[23]	Hypoxia (Ind) in *Mtb*[35], *Mbov*[36], *Msm*[22,23] *in vitro*; Ind in *Mtb* but not in *Mbov* within MP[29]	Hypoxia-responsive genes in *Mtb*[48]; Acr, Rv2623 (Usp), Rv2626c in *Mbov*[36]; Acr, Rv2026c (Usp), Rv3134c/Rv2028c (Usp), Rv2032 orthologues in *Msm*[23]
3245c/ 3246c (MtrB/ MtrA)	RH	+/+	+/+	+/+	+/+	+/+	+/+	MtrA essential[27]	Iron (Ind)[28]	
3764c/ 3765c (TcrY/ TcrX)	RH	+/+	+/+	P/P	+/+	+/+	+/+	Hypervirulent in SCID mouse[31]		
0260c (U)	OR	+	+	–	+	+	+			
0818 (U)	OR	+	+	P	+	+	+		Constitutive expression[29]	
1626 (U)	OR	+	+	+	+	+	+			
2027c (DevS homologue)	OH	+	±	–	–	–	–			
2884 (U)	OR	+	+	P	+	+	–			
3143 (U)	OR	+	+	–	+	+	+		Ind in *Mbov* but not in *Mtb* within MP[29]	
3220c (U)	OH	+	+	+	+	+	+	No phenotype in SCID mouse[31]		

a, Refers to annotation according to Cole *et al.*[1]; 'U', no known annotation; 'P', pseudogene of *M. tuberculosis* orthologue (*M. leprae* web page at www. sanger.ac.uk).
b, H and R refer to histidine kinase and response regulator, respectively, in the direction of transcription. O refers to orphan protein.
c, *Mbov*, *M. bovis*; *Mmar*, *M. marinum*; *Mlep*, *M. leprae*; *Mav*, *M. avium*; *Mptb*, *M. avium* subsp. *paratuberculosis*; *Msm*, *M. smegmatis*; *Mtb*, *M. tuberculosis*; MP, macrophages; Ind and Rep, induction and repression of gene expression, respectively.
d, A gene is reported as orthologue '+', if its translated protein sequence shares ≥ 50% identity over 90% of the length of the protein, otherwise '–'. '±' represents the cases where the identity is between 40–50% over 90% of the length of the protein.
e, DevR is designated as DosR in *M. bovis* BCG[36].
The genes highlighted in orange are conserved amongst all the mycobacteria, while those highlighted in blue are conserved in all except *M. leprae*.

with the occurrence of massive gene decay and pseudogene accumulation[3]. It is tantalizing to think that the restricted tissue specificity of *M. leprae* exemplified by its strong neural predilection could be attributed, among other factors, to its abridged repertoire of signal transduction systems resulting in a limited adaptive capability[16].

Orthologues of all eleven STPKs of *M. tuberculosis* were present in *M. bovis* and *M. marinum* except for PknJ, which was absent in *M. marinum* and all other species of mycobacteria. It is noteworthy that none of the STPK or TCS proteins map in the chromosomal regions of difference that are deleted from certain *M. bovis* strains including the BCG vaccine strains[17]. *M. smegmatis*, *M. avium* and *M. avium* subsp. *paratuberculosis* each contain a subset of STPKs (6 of 11, 8 of 11 and 8 of 11 respectively) while *M. leprae* possesses only 4 of 11 orthologues of the STPK family. Four STPKs, namely, PknB, PknA, PknG and PknL were conserved amongst all mycobacteria including *M. leprae* and the nonpathogenic species, *M. smegmatis* (highlighted in orange, Table 2). Virulent and attenuated strains of *M. bovis* contain a truncated orthologue of PknD that lacks the C-terminal domain and is predicted to contain kinase activity in its N-terminal cytoplasmic domain[18]. Functional polymorphisms of this nature may contribute towards phenotypic differences between *M. bovis* and other members of the *M. tuberculosis* complex and could also perhaps form the basis for a PCR–RFLP assay to distinguish between them. Five STPKs, namely, PknD, PknB, PknF, PknG and PknA, have been demonstrated to be functional kinases[10,19,20]. However the cellular pathways in which they are involved are not known except for PknA that is implicated in cell elongation/division[20]. Of the three phosphatases, Ppp (PtpB) was conserved among all mycobacteria while the others were present in all species except *M. leprae*. Two tyrosine phosphatases encoded by *ptpA* and *ptpB* genes of *M. tuberculosis* were catalytically active and were secreted proteins suggesting they may play a role in modulating host cell signal transduction pathways[21]. The presence or absence of orthologues to TCS, STPK and the phosphatase classes of proteins reported in this analysis of unfinished genomes should be interpreted with caution since confirmation awaits the verification and annotation of completed sequences.

All TCS shared the same genetic organization [in terms of the direction of transcription of histidine kinases (H) and response regulators (R)] in various mycobacterial species (Table 1). An exception was the *devR–devS* locus in *M. smegmatis* wherein the response regulator and histidine kinase genes were interrupted by a 1985 bp segment that contained two genes, *Rv3130c* and *Rv3129* (refs 22, 23).

M. tuberculosis histidine kinases and response regulators fall in two classes

Histidine kinases generally display rather low overall sequence homologies as their N-terminal sensor domains differ greatly, possibly a reflection of the wide variety of molecules/signals sensed by them. All histidine kinases contain a C-terminal kinase catalytic core that comprises

Table 2. Profile of Ser/Thr protein kinases and phosphatases in mycobacteria

Rv Gene[a] (Name)	*Mbov*[b]	*Mmar*	*Mlep*	*Mav*	*Mptb*	*Msm*	Environmental signal (gene response)
0014c (PknB)	+[c]	+	+	+	+	+	Hypoxia, nutrient starvation (Rep)[35,44]
0015c (PknA)	+	+	+	+	+	+	
0410c (PknG)	+	+	+	+	+	+	Hypoxia (Ind)[35]
0931c (PknD)	+[d]	+	P	+	+	–	Nutrient starvation (Rep)[44]
1266c (PknH)	+	+	–	+	+	–	
1743 (PknE)	+	+	P	–	–	–	
1746 (PknF)	+	+	P	±	±	+	
2088 (PknJ)	+	–	–	–	–	–	
2176 (PknL)	+	+	+	+	+	+	
2914c (PknI)	+	+	P	±	±	–	
3080c (PknK)	+	+	–	–	–	+	
0018c (Ppp; PtpB)	+	+	+[e]	+	+	+	Nutrient starvation (Rep)[44]
0153c (U)	+	+	–	+	+	+	
2234 (PtpA)	+	+	P	+	+	+	Hypoxia (Ind)[35]

a, Refers to annotation according to Cole *et al.*[1]; 'U', no known annotation; 'P', pseudogene of *M. tuberculosis* orthologue (*M. leprae* web page at www.sanger.ac.uk).
b, *Mbov*, *M. bovis*; *Mmar*, *M. marinum*; *Mlep*, *M. leprae*; *Mav*, *M. avium*; *Mptb*, *M. avium* subsp. *paratuberculosis*; *Msm*, *M. smegmatis*; Ind and Rep, induction and repression of gene expression, respectively.
c, A gene is reported as orthologue '+', if its translated protein sequence shares ≥ 50% identity over 90% of the length of the protein, otherwise '–'. '±' represents the cases where the identity is between 40–50% over 90% of the length of the protein.
d, Truncated orthologue.
e, *M. leprae* contained a *ptpB* gene orthologue that showed 76% identity with its counterpart in *M. tuberculosis*. This gene was not earlier annotated[3].
The genes highlighted in orange are conserved amongst all the mycobacteria, while those highlighted in blue are conserved in all except *M. leprae*.

a histidine-containing phosphotransferase subdomain and an ATP-binding subdomain. The catalytic core possesses several conserved amino acid motifs (H, N, D/G1 and G2) indicative of a common evolutionary origin[12]. The amino acid sequence of the region around the histidine residue that is phosphorylated was used to classify the sensor kinases of *M. tuberculosis* into Groups I and II (Figure 1). The involvement of the conserved histidine residue in the H box as the site of autophosphorylation has been experimentally confirmed in only a limited number of *M. tuberculosis* kinases that include SenX3 (ref. 13), DevS[15] and the DevS homologue Rv2027c (Saini *et al.*, manuscript submitted). All the histidine kinases are predicted to contain 1–5 transmembrane segments and have a membrane association (Figure 2). The intertransmembrane segments varied in length (from 3 to 380 residues) and were oriented on both sides of the cytoplasmic membrane. In order to predict the potential of the histidine kinases to sense signals, domain searches of their input domains were performed using the InterPro Scan domain search tool (http://us.expasy.org). Apart from two GAF domains each in DevS and Rv2027c, and a potassium channel sensor domain in KdpD, no other sensory motifs could be detected among the histidine kinases of *M. tuberculosis*. The mechanism of signal sensing by the histidine kinases is a challenging area of investigation that has lagged far behind the study of TCS. Advances in this field would certainly enhance understanding of mycobacterial acclimatization to a medley of intracellular environments prevailing in resting and activated macrophages, and granulomas of host tissues.

Group I (NarL)

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Rv0845 (NarS)             RIA--EAIHDGPLQDVLA
Rv2027c^(DevS homologue)  RIA--RDLHDHVIQRLFA
Rv3132c^(DevS)            RIA--RDLHDHVIQRLFA
Rv3220c (U)               RVSSIALVHDALSMSVDE
                          *::    :**     :
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Group II (OmpR)

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Rv0490^ (SenX3)           FVANVSHELKTPVG
Rv0601c (U)               FVADASHELRTPLA
RvO758 (PhoR)             FITDASHELRTPLT
Rv0902c (PrrB)            FAAVSSHELRTPLT
Rv0982 (MprB)             LVTDAGHELRTPLT
Rv1028c (KdpD)            LLSAVSHDLRTPLA
Rv1032c (TrcS)            FITDASHELRTPLA
Rv3245c (MtrB)            FTSDVSHELRTPLT
Rv3764c (TcrY)            FVADASHELRTPLA
                          : :  .*:*:**:
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Figure 1. Classification of *M. tuberculosis* kinases by the sequence around the histidine phosphorylation site (highlighted residues). Sensor kinases were assigned into groups I and II corresponding to group II (NarL) and IIIA (OmpR) respectively of *B. subtilis*[57] which were related to the homology of the output domain of their cognate response regulator proteins to those of *E. coli* proteins, NarL and OmpR respectively. *Rv0600c* of the atypical TCS *Rv0600c-Rv0601c-Rv0602c* could not be placed in either category. Alignment was performed using ClustalW at http://us.expasy.org; ^, experimentally verified phosphorylation site[13,15] (Saini *et al.*, manuscript submitted). U, no known annotation.

The response regulators were also classified by sequence similarities of their C-terminal output domains into two groups (Figure 3). Only two response regulators showed high homology with the C-terminal DNA-binding domain of NarL from *E. coli*. These response regulators were paired with a kinase classified as Group I (NarL) on the basis of homology around the phosphorylatable histidine residue. A similar analysis using OmpR C-terminal domain of *E. coli* identified 12 response regulators with high homology in those residues making up the hydrophobic core of the domain[24]. All the response regulators of the OmpR class were paired with a kinase of group II. None of the response regulators of a particular group were mispaired with a kinase of a different group. This classification might prove useful in determining the possibility of cross talk between members of the same group.

Composite kinases in which a phosphorylatable response regulator domain is contiguous with the kinase polypeptide (e.g. BvgS of *Bordetella pertussis* and LemA of *Pseudomonas* species) were absent in *M. tuberculosis*. However, two CheY-like proteins were encoded by *Rv1626* and *Rv3143* genes in *M. tuberculosis* and were well conserved in all mycobacteria except for *Rv3143* that was absent in *M. leprae*. The CheY protein in *E. coli* consists of only the phosphorylatable receiver domain without an output domain and interacts with the flagellar motor in chemotaxis[25] in contrast to most response regulators that are transcription factors dependent on phosphorylation for activity. These single-domain response regulators in mycobacteria may serve as phosphointermediates in phosphorelay as in the case of Spo0F of *B. subtilis*[26].

Role of TCS in mycobacterial virulence

Mycobacterial disease is characterized by the lack of involvement of classical virulence factors; rather a dynamic balance between host and pathogen defines the outcome of an infection. Therefore those mycobacterial genes that confer an advantage to the organism in this ongoing battle would qualify as virulence factors. Infection of macrophages constitutes an early stage in the host pathogen encounter. Obvious candidates among *M. tuberculosis* genes that can mastermind the intracellular survival and multiplication within macrophages as also the shutdown of mycobacteria during persistence are signal transduction systems, in particular TCS. Therefore *in vitro* infection models have been used extensively to delineate the role of TCS during the stage of pathogen–macrophage interaction. Animal models have also been used to study the effect of defined mutations in TCS on growth and virulence of the mycobacterial strains.

Several response regulator proteins have been implicated in the survival and multiplication of *M. tuberculosis* in

cell infection and animal models (see Table 1). The response regulator MtrA was found to be essential[27] and to be induced in iron sufficient conditions[28]. Inactivation of MprA response regulator affected growth of *M. tuberculosis* in BALB/c mice in a tissue-specific manner; mutant bacilli were unable to maintain persistent infection in lungs or establish infection in the spleens of infected mice indicating a role for MprA in persistence[29]. A *phoP* mutant strain of *M. tuberculosis* was also attenuated in virulence in a mouse model with impaired growth observed in the lungs, livers and spleens[30]. A recent study reported the effect of mutations in six TCS on their virulence in a SCID mouse model. Infection with two of the mutants (*narL* and *Rv3220c*) showed no phenotype. However, the remaining mutants (*devR*, *tcrXY*, *trcS* and *kdpDE*) were hypervirulent[31]. This contrasts with all previous studies with *M. tuberculosis*, in which gene mutation has resulted either in attenuation or no change in virulence. It has

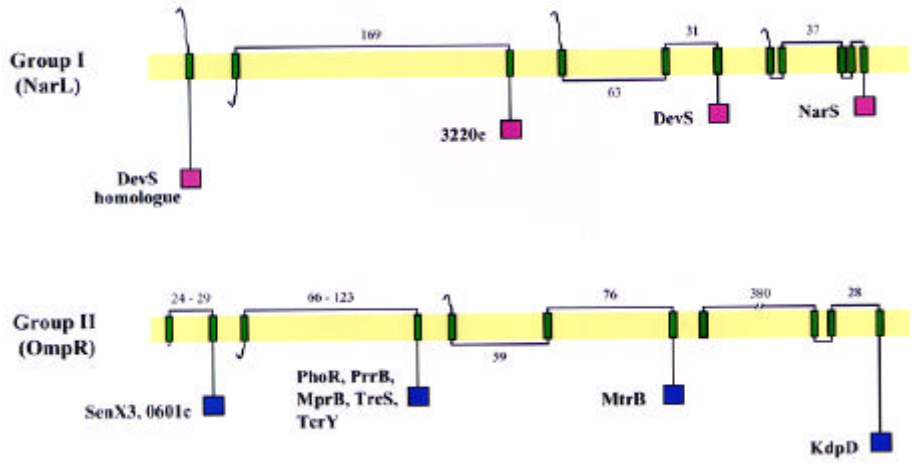

Figure 2. Schematic representation of the *M. tuberculosis* histidine kinases. Groups I and II were assigned on the basis of homology of the residues surrounding the histidine phosphorylation site. Related kinase domains are represented by boxes of the same colour (pink and blue). Green boxes are transmembrane segments predicted by TMpred (at http://us.expasy.org). Intertransmembrane segment lengths of > 20 amino acid residues are indicated.

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NarL (E. coli)  VNQLTPRERDILKLIAQGLPNKMIARRLDITESTVKVHVKHMLKKMKLKSRVEAAVWVHQERIF--

Rv0844c (NarL)  APVLSARERFVLNRIACGQSIPAIAAELYVAPSTVKTHVQRLYEKLGVSDRAAAVAEAMRQRLLD-
Rv3133c (DevR)  LSGLTDQERTLLGLLSEGLTNKQIADRMFLAEKTVKNYVSRLLAKLGMERRTQAAVFATELKRSRP
                   *: :** :*  :: * .   ** .: :: .*** :*.::  *: :. *. *.. . . :

OmpR  (E. coli)  EPMPLTSGEFAVLKALVSHPREPLSRDKLMNLARGREYSAMERSIDVQISRLRRMVEEDPAHPRYIQTVWGLG

Rv0260c (U)      SVKPLSPSGMAILRALVHRPGGVVSRGDLLRVLPGD--GSDTHAVDTAVLRLRTALG----DKNIVATVVKRG
Rv0491  (RegX3)  DTITLPLKEFDLLEYLMRNSGRVLTRGQLIDRVWGADYVGDTKTLDVHVKRLRSKIEADPANPVHLVTVRGLG
Rv0602c (U)      TEIRLSHKEFTLLEALIRRPGIVHTRAQLLERCWDAAYEARSNIVDVYIRYLRDKIDR-PFGVTSLETIRGAG
Rv0757  (PhoP)   QPVSLSPTEFTLLRYFVINAGTVLSKPKILDHVWRYDFGGDVNVVESYVSYLRRKIDT-GEKR-LLHTLRGVG
Rv0818  (U)      RPLDLTYKEFELLKYLAQHAGRVFTRAQLLHEVWGYDFFGGTRTVDVHVRRLRAKLG--PEHEALIGTVRNVG
Rv0903c (PrrA)   VDVDLTKREFDLLAVLAEHKTAVLSRAQLLELVWGYDFAADTNVVDVFIGYLRRKLEA-GGGPRLLHTVRGVG
Rv0981  (MprA)   RRISLTRTEFALLEMLIANPRRVLTRSRILEEVWGFDFPTSGNALEVYVGYLRRKTEA-DGEPRLIHTVRGVG
Rv1027c (KdpE)   AEVHLTPTEWGMLEMLARNRGKLVGRGELLKEVWGPAYATETHYLRVYLAQLRRKLEDDPSHPKHLLTESGMG
Rv1033c (TrcR)   TPISLSSTEFELLRFLMRNPRRALSRTEILDRVWNYDFAGRTSIVDLYISYLRKKIDS-DREP-MIHTVRGIG
Rv2884  (U)      RDLGLTLTEFKLMNFLVQHAGRAFTRTRLMREVWGYECHGRIRTVDVHVRRLRAKLG--AEHESMIDTVRGVG
Rv3246c (MtrA)   EQISLTPLEFDLLVALARKPRQVFTRDVLLEQVWGYRHPADTRLVNVHVQRLRAKVEKDPENPTVVLTVRGVG
Rv3765c (TcrX)   EPVSLTSTEFELLRFMMHNSKRVLSKAQILDRVWSYDFGGRSNIVELYISYLRKKIDN-GREP-MIHTLRGAG
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Figure 3. Relatedness of output domains of *M. tuberculosis* response regulators to the output domains of NarL and OmpR of *E. coli*. Amino acid sequences of the C-terminal domains of response regulators were compared to the sequence of the output domain of NarL and OmpR proteins of *E. coli*. The highlighted residues are crucial for correct folding in the domain of the NarL group and make up the hydrophobic core of the domain in the OmpR group[57]. Alignments were performed using ClustalW; U, no known annotation.

been reported earlier by another group that a *trcS* mutant was not altered in virulence[32] which is inconsistent with the findings of Parish *et al.*[31]. The discrepancy in findings was attributed to differences in the infection model and *M. tuberculosis* strains[31]. The hypervirulence of the *devR* mutant, reported in the same study[31] was inconsistent with our findings. We used a guinea pig model and found a *devR* disruption mutant to be significantly attenuated in virulence in terms of gross lesions and histology in livers and lungs and number of bacteria recovered from spleens[33]. The differences in the model used or in the nature of the mutant strains employed by the two laboratories may explain the contrasting results obtained. In summary, studies employing animal and cell models of virulence show that several TCS mutants show an altered phenotype (attenuated or hypervirulent) thereby underscoring the crucial role of TCS in determining the consequence of *M. tuberculosis* infection. The involvement of multiple TCS is perhaps a reflection of the diverse stimuli to which tubercle bacilli are exposed during macrophage entry, compartmentalization in pathogen-remodeled phagosomes and existence within granulomas.

Environmental signals and domains

Global analysis of gene expression in several bacteria including *M. tuberculosis* has revealed that large-scale changes occur upon *in vitro* exposure to environmental conditions that simulate the intracellular milieu[34]. The specific modulation of one or more signal transduction proteins is suggestive of its (their) role during such a challenge. The nature of environmental signals that modulate TCS and STPK activity of mycobacteria is compiled in Tables 1 and 2.

Intracellular macrophage environment

Following its ingestion by macrophages, *M. tuberculosis* inhibits the maturation of its phagosome, preventing progression to a bactericidal phagolysosome and allowing intracellular multiplication of bacteria. The macrophage-*M. tuberculosis in vitro* system has been exploited in two ways to assess the role of TCS in *M. tuberculosis*. First, expression analysis of response regulator genes within infected macrophages indicated specific intracellular patterns of expression that allowed their categorization into (i) those whose expression was not detected, (ii) those that were expressed constitutively, or (iii) those that were induced during growth in macrophages. Secondly, *M. tuberculosis* mutants in TCS were assessed for survival and multiplication within macrophages (Table 1). On this basis, PrrA–PrrB was implicated in the early intracellular multiplication of *M. tuberculosis*[32] while PhoP was involved in bacterial multiplication but not survival in resting macrophages[30]. The growth of an *mprA* mutant was significantly higher than that of parental H37Rv strain in resting but not in activated macrophages[29], while an early rise in the numbers of *devR* mutant bacteria within activated macrophages was reported by Parish *et al.*[31].

Hypoxia

Extensive redirection of gene expression was noted in *M. tuberculosis* cultures in response to a brief period of oxygen limitation; the expression of > 100 genes was altered under defined hypoxic conditions. Microarray analysis indicated that fifteen gene clusters containing four genes or more were upregulated under these conditions[35]. A moderate induction of *pknG* and *ptpA* and a dramatic induction of *devR–devS* were noted. DevR–DevS was part of a 12-gene cluster so regulated and was the only TCS induced by hypoxia suggesting a regulatory role for it in the hypoxia response. Induction of some genes was accompanied by repression, albeit not as dramatic, of several other *M. tuberculosis* genes. The repressed genes included STPK and TCS genes *pknB* and *Rv0601c–Rv0602c* and those that encoded proteins involved in DNA, protein, lipid and amino acid synthesis, production of polyketides and other complex molecules and aerobic metabolism. This study demonstrated that *M. tuberculosis* could rapidly redirect its gene expression profile in response to hypoxia and suggested that this ability could be crucial for its survival within granulomas. As mentioned earlier, pathogenic bacteria are believed to persist in inflammatory and necrotic lesions such as granulomas, in a stationary phase-like dormant state[6,8], as a consequence of bacterial adaptation to the conditions prevailing therein that perhaps include a decrease in oxygen tension and/nutrient supply. The DevR–DevS system is also upregulated in hypoxia in the non-pathogenic species *M. smegmatis*[22]. *devR* mutants of *M. bovis* BCG[36] and *M. smegmatis*[23] were severely compromised in viability under conditions of hypoxia underscoring the pivotal role of DevR in the adaptation of mycobacteria to hypoxia.

So how exactly is oxygen sensed by DevS is a mystery at present. Analysis of the sequence of DevS protein revealed many important aspects of its function and modularity. The N-terminal sensory domain is marked by the presence of two GAF domains that partially overlap with the putative transmembrane segments of the protein. This architecture provides membrane anchoring to the protein with the characteristic sensory domains being exposed to the milieu on either side. The C-terminal catalytic region is marked by the presence of conserved motifs important for stimulus-modulated autokinase activity. Proteins belonging to the GAF domain and the PAS domain families share a striking similarity in the structures of these domains and together constitute a huge number of proteins implicated in sensory and signalling pathways[37]. Intriguingly, two additional proteins of *M. tuberculosis*

encoded by *Rv2027c* and *Rv1354c*, also contained GAF domains. The former protein, which is homologous to DevS[38], also possesses two GAF domains placed similarly as in DevS, suggesting that perhaps both proteins may be involved in execution of similar outputs. The immediate question is whether the GAF domain(s) in DevS, and possibly Rv2027c and Rv1354c, sense oxygen and if so, how? GAF domains are reported to bind low molecular molecules such as cGMP and cAMP and modulate the activity of proteins that contain them[37,39]. While in mammals, cGMP has been well established as an intracellular mediator of a variety of cell functions[40], only a few studies have focused on the roles of cGMP in bacteria[41]. Of interest is the reported presence of cGMP, guanylate cyclase and cGMP phosphodiesterase in *M. smegmatis*[42]. An alternative possibility is that instead of oxygen DevS senses the redox state of the environment or metabolites produced as a consequence of oxygen deprivation as does the *E. coli* histidine kinase ArcB[43].

On the basis of sequence homology, we had earlier suggested that the orphan sensor, Rv2027c, may participate in an inter-connected regulatory circuit[38] and perform a 'back-up' function whereby it could functionally replace the cognate sensor kinase DevS in some scenario, including mutation-induced loss-of-function. That the orphan sensor Rv2027c may indeed play a 'substitute' role of transducing signal to DevR in the absence of DevS was suggested as a possible reason for the observed lack of phenotype in *devS* mutants of *M. tuberculosis* and *M. bovis* BCG[35,36]. Recent experiments have established Rv2027c to be a bonafide histidine kinase that engages in phosphotransfer with DevR. This suggests the possibility of a single response regulator DevR being modulated by two sensor kinases, DevS and Rv2027c, that could sense different environmental signals but generate a common adaptive response (Saini *et al*., manuscript communicated). Interestingly, BLAST analysis of various unfinished and finished mycobacterial genomes indicated that such a 'back-up' orthologue was absent in all mycobacteria except *M. tuberculosis*, *M. bovis* and *M. marinum* (Table 1).

Nutrient starvation

In a nutrient starvation model of persistence, a global analysis of gene expression revealed that among the signal transduction systems, only *kdpE* gene encoding KdpE response regulator was induced while *pknB*, *pknD* and *ppp* genes encoding PknB, PknD and PPP respectively were repressed[44]. The KdpD–KdpE system in *E. coli* controls expression of the potassium transport system[45] and although its function in *M. tuberculosis* has not been determined, it is likely to be involved in regulating expression of the *kdpF–kdpA–kdpB–kdpC* locus, encoding the putative K^+ transporter, from which it is divergently placed in the genome.

Targets of two-component systems

Response regulator proteins often regulate their own expression as well as that of their cotranscribed cognate sensor kinase genes. This facilitates a rapid response to an environmental signal. The two TCS of *M. tuberculosis* that have been biochemically characterized so far, namely, TcrR–TcrS and SenX3–RegX3, are both positively autoregulated by the respective response regulator via direct binding to DNA in the promoter region[13,46]. Genes that are modulated in a concordant manner with a TCS are candidate targets for control by that particular TCS. The identity of target genes regulated by TCS has been established for only one response regulator protein namely DosR (DevR). Several *M. tuberculosis* genes were induced under conditions of defined hypoxia including those coding for Acr (HspX), 6 Usp proteins (containing Universal stress protein domain) and a number of proteins of unknown function[35]. Acr is a member of the small heat shock protein family that has chaperone activity *in vitro* and is thought to confer protection to tubercle bacilli under adverse conditions including hypoxia[47]. In a mutant strain of *M. tuberculosis* where *dosR* (*devR*) gene was deleted, the hypoxic induction of the majority of the hypoxia-induced genes including that coding for Acr was abrogated, suggesting them to be under the control of *dosR*[48]. *acr* gene induction under hypoxic conditions was also noted in cultures of *M. smegmatis*[22] that was shown to be dependent on DevR[23]. *M. tuberculosis* cultured under anaerobic conditions showed wall thickening that was ascribed to Acr expression[49]. Although the presence of an *acr* homologue *M. smegmatis* was predicted[50], and its expression was induced in hypoxic cultures of *M. smegmatis* in a DevR-dependent manner[22,23], the phenomenon of wall thickening was not observed in anaerobically cultured *M. smegmatis*[49]. The reason for this difference in cell wall architecture of anaerobic cultures of *M. tuberculosis* and *M. smegmatis* is not known at present. An independent analysis of protein expression using two-dimensional gel technique revealed DevR-dependent hypoxic induction of several proteins in *M. bovis* BCG and *M. smegmatis*. The proteins included Acr, orthologues of Usp proteins and a putative nitroreductase (Table 1).

Signal transduction systems as novel drug targets

Although effective drugs for the treatment of tuberculosis are available, drug resistance has emerged as a major public health challenge. This is due in part to patient noncompliance resulting from the long duration of treatment. Directly observed therapy, short-course or DOTS, evolved through decades of research much of which was done in

India. Tuberculosis control programmes have adopted DOTS worldwide as it is the most effective mode of tuberculosis treatment. Despite the implementation of DOTS and the ensuing improvement in treatment success rates, the rise in prevalence of drug resistance with time is inevitable. No new class of anti-tubercular drugs has been introduced for nearly three decades. Conventional drugs target pathways required for bacterial growth and division such as cell-wall biosynthesis and DNA replication. Their poor activity against slow-growing or non-growing persistent bacteria is thought to be an important reason why currently used regimens take so long to eradicate infection[8] and is an impetus to the development of drugs to target this class of 'difficult-to-clear' bacteria. The development of an antitubercular drug active against persistent bacteria would have a profound impact on tuberculosis treatment programmes as such a drug would reduce the overall length of chemotherapeutic regimens, thereby reducing patient noncompliance and treatment failures. As mentioned earlier, *in vitro* models of persistence have provided exceedingly valuable insights into the adaptation response of tubercle bacilli to a dormancy-inducing stimulus. This body of knowledge has guided the use of strains mutated in specific genes to understand their function in TB pathogenesis. Several genes coding for proteins including enzymes and regulatory molecules including TCS response regulators have been shown to play key roles in persistence[29,47,51–54] and have unveiled molecules that can be targeted by new classes of drugs. TCS involved in bacterial survival and multiplication within the host (Table 1) can also be seen as a novel class of drug targets. Furthermore, since TCS are quite distinct from the regulatory systems of mammalian cells, drugs active against the former carry a minimum risk of toxic side effects. In principle, the signal transduction cascade can be intercepted at any one of four steps, namely, signal sensing by the sensor kinase, autophosphorylation of the sensor kinase, phosphotransfer to the response regulator and the binding of response regulator to the target genes.

The identification of the role of TCS and STPKs in mycobacterial virulence and persistence is a key goal for the future. As of now, the utilization of signal transduction systems for the development of new antitubercular drugs has been relatively unexplored. The elucidation of the structures of signal transduction proteins is being undertaken by several laboratories worldwide. The crystal structure of only one protein involved in signal transduction, namely, PknB Ser/Thr protein kinase of *M. tuberculosis* has been elucidated so far[55]. There exists only one report of the use of a conventional isoquinoline in inhibiting mycobacterial kinase activity and mycobacterial growth, suggesting that protein kinase inhibitors could be exploited in the development of antimycobacterial agents[56]. Structure determination and molecular modelling of the signal transduction proteins in conjunction with inhibitor designing and high throughput screening of libraries of synthetic compounds and natural products are rational approaches to the design of drugs targeting these novel classes of mycobacterial proteins.

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