Genes and regulatory networks involved in persistence of *Mycobacterium tuberculosis*

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The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is one of the most successful of human pathogens. It can evade the host immune response and establish a persistent infection or enter a dormant state within the host which can be reactivated if the host becomes immuno-compromised. Both of these features are major obstacles to tuberculosis eradication. Dormancy and reactivation of *M. tuberculosis* are tightly coordinated dynamic processes involving numerous genes and their products. Molecular mechanisms underlying *M. tuberculosis* persistence may provide an opportunity for the discovery of effective drug targets for tuberculosis control. Here, we review the genes required for *M. tuberculosis* persistence and propose a regulatory network for the action of these genes using text mining. This should provide fresh insights into the persistence mechanisms of *M. tuberculosis* and suggest candidates for new drug targets and immune intervention.

***Mycobacterium tuberculosis*, persistent infection, dormancy, network**

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The causative agent of tuberculosis, *Mycobacterium tuberculosis*, is one of the most successful of human pathogens, but much of its biology remains unclear. Archaeological findings show that *M. tuberculosis* is at least as old as human beings on earth. The application of a series of serendipitously discovered antibiotics from the 1940s onwards halted the rampant spread of *M. tuberculosis* and the incidence rate of TB declined. However, during the late 1980s, drug-resistant *M. tuberculosis* strains began to emerge and are now found worldwide. The World Health Organization has issued two major alerts regarding the seriousness of tuberculosis. Approximately eight million individuals develop active tuberculosis annually, and two million die from the disease. HIV co-infection, widespread application of immunosuppressive agents and an aging population often

exacerbate the difficulties of tuberculosis control. The vast majority of individuals infected with *M. tuberculosis* exhibit no overt symptoms of active disease. Most are latent carriers with a 2%–23% lifetime risk of developing reactivation tuberculosis [1]. Once the host immune function wanes, asymptomatic persistent infection may become active tuberculosis. However, little is known about the molecular mechanisms underlying *M. tuberculosis* persistence and reactivation. No clinical drugs specifically targeting persistent *M. tuberculosis* are available. A lack of well-recognized animal models or *in vitro* models to study the persistence of *M. tuberculosis* further complicates this problem. However, features of *M. tuberculosis* may be found via models simulating one or two aspects of *M. tuberculosis* persistence and reactivation. It is generally recognized that compounds capable of shortening the duration or accelerating activation of persistent *M. tuberculosis* may point to anti-tuberculosis

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therapies. In this study we propose a regulatory network of *M. tuberculosis* genes underlying persistence using text mining, which we believe may elucidate persistence mechanisms and novel drug targets, and facilitate discovery and identification of new immune intervention strategies for tuberculosis control.

1 *M. tuberculosis* genes implicated in persistent infection and their functions

Persistence of *M. tuberculosis* necessitates the coordinated expression of numerous virulence determinants, including those involved in intermediary and secondary metabolism, cell wall processes, stress responses, and signal transduction pathways. However, only a handful of these genes have been experimentally demonstrated and not all of them are present in all currently available models. This suggests that the models should also be further refined.

1.1 Metabolic pathway and persistence

Swift adaptation to environmental cues is a typical feature of the most successful *M. tuberculosis* strains. Many studies have shown that *M. tuberculosis* metabolic pathways are rapidly altered to acclimatize to the macrophage milieu. Pinpointing such alterations will be useful in understanding the organism's regulatory processes and leading to the discovery of novel drug targets. *M. tuberculosis* genes impli-

cated in persistence which have been reported in the literature are shown in Table 1.

1.1.1 *icl*, *gcvB* and glyoxylate shunt

Fatty acids are widely regarded as a major source of carbon and energy for persistent *M. tuberculosis* [2]. The anapleurotic pathway for the tri-carboxylic acid (TCA) cycle afforded by the glyoxylate shunt is vital for the survival of the bacteria [18]. The major source of carbon is furnished by β -oxidation of fatty acids when the generation of pyruvate from glycolysis is dramatically reduced. The *M. tuberculosis icl* gene, which encodes the isocitrate lyase, is required for persistence. Isocitrate lyase, the initial enzyme in the glyoxylate cycle, catalyzes the conversion of isocitrate to glyoxylate and succinate [1]. This enzyme plays a pivotal role in the regulation of isocitrate flux at the branch point between the glyoxylate shunt and the TCA cycle [19]. The *M. tuberculosis Δicl* mutant is markedly attenuated for survival in activated but not resting macrophages [2]. The disruption of *icl* attenuates persistence and virulence of *M. tuberculosis* in immune-competent mice without affecting bacterial growth during the acute stage of infection, and hence is also called a "persistence factor" [19]. However, this result has been challenged recently by studies that have found no strict correlation between the *icl* expression and whether the fatty acids are the sole carbon source (personal communication with Dr. Shi LanBo from New Jersey Medical School, USA). *gcvB* encodes a putative glycine dehydrogenase that catalyzes the reductive amination of

Table 1 Genes implicated in *Mycobacterium tuberculosis* persistence

Gene	Name	Function	Knockout or expression studies	References
Rv0467	<i>icl</i>	Isocitrate lyase	Reduced persistence in mice	[2]
Rv1832	<i>gcvB</i>	Glycine decarboxylase	Increased expression during hypoxia	[3]
Rv1161-4	<i>narGHJI</i>	Nitrate reductase	Reduced persistence in mice	[4]
Rv1736c	<i>narX</i>	Fused nitrate reductase	Increased expression during hypoxia and in human granulomas	[4]
Rv1737c	<i>narK2</i>	Nitrate/nitrite transport	Increased expression during hypoxia	[5]
Rv0470c	<i>pcaA</i>	Cyclopropane synthase	Reduced persistence in mice	[6]
Rv0126	<i>treS</i>	Trehalose synthase	Prolonged time-to-death in mice	[7]
Rv0981	<i>mprA</i>	Two-component regulator	Reduced persistence in mice	[8]
Rv3132c	<i>dosR</i>	Two-component regulator	Increased lethality in mice	[9]
Rv3764/5c	<i>trcY</i>	Two-component regulator	Increased lethality in mice	[9]
Rv1032c	<i>trcS</i>	Two-component regulator	Increased lethality in mice	[9]
Rv1027/8c	<i>kdpDE</i>	Two-component regulator	Increased lethality in mice	[9]
Rv3286c	<i>sigF</i>	RNA polymerase σ factor	Prolonged time-to-death in mice	[10]
Rv3223c	<i>sigH</i>	RNA polymerase σ factor	Reduced pathology, prolonged time-to-death in mice	[11]
Rv1221	<i>sigE</i>	RNA polymerase σ factor	Prolonged time-to-death in mice	[12]
Rv3416	<i>whiB3</i>	Transcriptional regulator	Reduced pathology, prolonged time-to-death in mice	[13]
Rv2583c	<i>relA</i>	(p)ppGpp synthase	Reduced persistence in mice	[14]
Rv2031c	<i>hspX</i>	α -crystallin	Increased expression during hypoxia and in mice	[15]
Rv0353	<i>hspR</i>	HspR repressor	Reduced persistence in mice	[16]
Rv0350	<i>hsp70</i>	Heat shock protein 70		[16]
Rv1908c	<i>katG</i>	Catalase-peroxidase	Reduced persistence in mice	[17]

glyoxylate, concomitantly oxidizing NADH to NAD. Its activity is significantly up-regulated upon the entry of *M. tuberculosis* into persistence *in vitro* [3].

1.1.2 *narGHJI*, *narK2*, *narX* and nitrate respiration

Many bacteria can use nitrate instead of oxygen as a terminal electron acceptor to maintain the proton motive gradient to sustain growth under anaerobic conditions. *M. tuberculosis* is the most active nitrate-reducer among all slow growing mycobacteria [20]. The activity of nitrate reductase encoded by *M. tuberculosis narGHJI* is low during aerobic growth, but sharply up-regulated by hypoxia [21]. This induction might serve a respiratory function for *M. tuberculosis* adaptation to non-replicating persistence [20]. *M. bovis* BCG lacking anaerobic nitrate reductase ($\Delta narG$) fails to persist in the lungs, livers, and kidneys of immune-competent BALB/c mice, but persistence and growth of BCG in the spleens of these mice appear largely unaffected, indicating that the role of the enzyme in pathogenesis is tissue-specific [4]. The marked activity increase is because of the induction of the nitrate transporter encoded by *narK2* instead of the up-regulation of the *narGHJI* [22]. Nitrite production increases markedly in *M. tuberculosis* cultures grown with nitrate during hypoxic adaptation. It may be secreted into the environment by NarK2, a nitrite-extrusion protein that has been shown to be up-regulated in response to hypoxia [5,23]. The *M. tuberculosis* gene *narX* encodes a “fused nitrate reductase”, a protein with homology to parts of the NarG, NarJ, and NarI proteins. Its real function is unknown. However, the nitrate reductase activity of the $\Delta narX$ mutant was comparable to that of the wild type [24].

1.2 Cell wall and persistence

The cell wall of *Mycobacterium* spp. contains unique glycolipids, mycolic acids and long-chain branched fatty acids that are covalently or non-covalently attached to the cell surface [1]. Cell wall components are *M. tuberculosis* important factors regulating persistence in *M. tuberculosis*. Cell wall determinants implicated in *M. tuberculosis* pathogenesis are well documented, and include phthiocerol dimycocerosate, phenolic glycolipid, oxygenated mycolate, mycolic acid, and mycolic acid cyclopropanation [25].

1.2.1 *pcaA*

pcaA encodes a cyclopropane synthase, an enzyme required for mycolic acid cyclopropane ring synthesis in the cell wall of both the tuberculosis vaccine, bacillus Calmette-Guérin (BCG) and *M. tuberculosis* [6]. Mycolic acids are long-chain branched fatty acids that are covalently attached to the cell wall or present in the form of a toxic glycolipid trehalose dimycolate. Cyclopropane rings are a modification of membrane lipids that are found in diverse bacterial species. The *pcaA* gene is required for long-term persistence and virulence of *M. tuberculosis* and the deletion of *pcaA* re-

duces the ability of *M. tuberculosis* to undergo long-term persistence in the host. The $\Delta pcaA$ mutant can be eliminated faster from mice although it does also grow more rapidly than wild-type *M. tuberculosis* during acute stages of infection [6]. Mice infected with the $\Delta pcaA$ mutant exhibit a significant increase in time-to-death compared to mice infected with wild-type *M. tuberculosis* [1]. The contribution of *pcaA* expression to *M. tuberculosis* persistence remains unclear. The inability to attach cyclopropane rings to cell wall mycolic acids may alter the host antigen presentation. The *pcaA* gene is required for the synthesis of the cord factor in the *M. tuberculosis* cell wall. Inhibition of cyclopropanation of cell wall mycolic acid can result in a change in lipid fluidity and cell wall permeability, and therefore a change in resistance to reactive oxygen intermediates (ROIs) and reactive nitrogen intermediates (RNIs) [23].

1.2.2 *treS*

Trehalose, encoded by *treS*, presents as a free disaccharide in the cytoplasm of mycobacteria and a component of cell-wall glycolipids implicated in tissue damage associated with mycobacterial infection [26]. It can participate in the biosynthesis of the cell wall of replicating *M. tuberculosis* and function as a stress protectant in non-replicating bacteria during exposure to a range of environmental stresses, including heat shock, dehydration, and hypoxia [7]. Trehalose 6,6'-dimycolate (TDM), also known as cord factor, occurs in the outer envelope of all mycobacteria and is the most abundant extractable toxic lipid on the surface of *M. tuberculosis*. TDM is capable of inducing granulomas that are similar to those in human tuberculosis, increasing chemokine and cytokine production and influencing the morphology of mycobacterial colonies [27–29]. On the surface of *M. tuberculosis*, TDM protects the bacteria from killing within macrophages by blockage of phagosome-lysosome fusion and decreasing the acidification of phagosomes [30].

1.3 Signal transduction and persistence

Signal transduction is indispensable for *M. tuberculosis* persistence within granulomas for handling various stresses including hypoxia, nutrient limitation, reactive oxygen and nitrogen intermediates, low pH, alveolar surfactants, and toxic proteins and fatty acids. *M. tuberculosis* two-component signaling systems and stress responsive sigma factors have been extensively studied.

1.3.1 Two-component systems

Two-component systems (TCS) usually consist of histidine kinase sensor proteins and cognate cytoplasmic response regulator proteins. Eleven complete and several orphaned two-component systems are present in the *M. tuberculosis* genome, many of which are implicated in persistent *M. tuberculosis* infection and are capable of regulating gene ex-

pression to adapt *M. tuberculosis* to environmental conditions that simulate the intracellular milieu.

(i) *mprA*

mprAB encodes a *M. tuberculosis* two-component system that is important for virulence during the persistent stage of infection [31]. *M. tuberculosis mprAB* is induced in response to nutrient starvation and low concentrations of sodium dodecyl sulfate (SDS). Inactivation of *mprA* enhances *M. tuberculosis* survival in resting macrophages but impairs *M. tuberculosis* persistence in lungs and spleens of mice [8]. The disruption of *mprA* is tissue-specific and affects the growth of *M. tuberculosis* during multiple stages of the infection process [8]. Compared to the parental strain, the *M. tuberculosis ΔmprAB* mutant is more resistant to SDS and grows better in human peripheral blood monocytes [32]. MprA binds to conserved motifs in the upstream of both *sigB* and *sigE* *in vitro* and directly regulates the *in vivo* expression of *M. tuberculosis sigB* and *sigE* [31]. *mprAB* is also regulated by sigma factors. For example, SDS exposure induces *sigE* expression, ensuing activation of 23 genes, including *sigB*, *mprA*, and *mprB* [32,33]. MprAB has either a directly positive or negative effect on the expression of *acr2*, which encodes a small molecule chaperone [34]. MprA auto-regulates its own expression and that of downstream gene *pepD*, which encodes trypsin-like serine protease [35]. During exponential growth and/or in the presence of SDS, the disruption of *mprA* also up-regulates multiple stress-associated genes of the DosR, SigD and IdeR regulons, including *rpf* (resuscitation-promoting factor), *narK2* (nitrate/nitrite transporter), *pkv4* (polyketide synthase), *mmpL10* (fatty acid transport), and iron responsive genes such as *hisE* (phosphoribosyl-ATP pyrophosphatase), *mbtB* and *mbtD* (mycobactin synthetase), *fdxA* (ferredoxin), and Rv2123 (PPE37) [32].

(ii) *dosR*

The Dos two-component system consists of two sensor histidine kinases, DosS and DosT, and the cognate response regulator DosR. DosS functions as a redox sensor and DosT functions as a hypoxia sensor [36]. Hypoxia, NO, CO, S-nitrosoglutathione (GSNO), ethanol and H₂O₂ can all induce the expression of *M. tuberculosis dosR* gene [37]. Parish *et al.* [9] found that the growth of the *ΔdosR* mutant was greater than that of the wild-type strain in IFN- γ -activated macrophages, and SCID mice infected intravenously with the *ΔdosR* mutant died more rapidly than mice infected with wild-type *M. tuberculosis*. This is the first report of an increase in mycobacterial virulence following targeted gene deletion. However, Malhotra *et al.* [38] found that a mutant expressing a truncated DosR protein showed reduced pathology and spleen bacillary burdens in subcutaneously infected guinea pigs. Recently, another study demonstrated that the disruption of *dosR* led to attenuation in resistant BALB/c and C57BL/6 mice and guinea pigs [39]. This is consistent

with previously reported findings of attenuation in guinea pigs, but is contrary to findings of hypervirulence in mice. Genes induced by DosR include *hspX* (14 kD heat shock protein), *fdxA* (ferredoxin), *pfkB* (phosphofructokinase), *narK2* (nitrate/nitrite transporter), *nrdZ* (ribonucleoside diphosphate reductase), *ctpF* (metal cation transporter P-type ATPase), *bfrB* (bacterioferritin), *narX* (fused nitrate reductase), and *otsB* (trehalose-6-phosphate phosphatase) [40].

(iii) *kdpDE*, *tcxY* and *trcS*

Kdp signal transduction, mediated primarily by K⁺ concentration, is a primary bacterial response to environmental osmotic stress. *E. coli* regulates its cytoplasmic K⁺ content through the *kdpFABC* and *kdpDE* operons to tackle the osmotic stress. The *kdpFABC* operon encodes the K⁺-transporting P-type ATPase [41]. The *kdp* genes encode for components of a mycobacterial signaling pathway by demonstrating the K⁺ dependence of *kdpFABC* expression in both *M. tuberculosis* and *Mycobacterium smegmatis* [42]. A yeast two-hybrid screen shows that the sensing module of *M. tuberculosis* KdpD interacts specifically with two membrane lipoproteins, LprJ and LprF [42]. The gene *tcxX* is markedly induced under iron-limitation and *tcxX* expression has been observed during the post-infection period in human macrophages infected by *M. tuberculosis* [43,44]. Consistent with the *dosR* gene, SCID mice infected intravenously with three of the *M. tuberculosis* mutants (deletions of *tcxXY*, *trcS*, and *kdpDE*) die more rapidly than mice infected with wild-type strains [9].

1.3.2 Sigma factors

The sigma factor reversibly associates with the prokaryotic core RNA polymerase, forming the RNA polymerase holoenzyme, and is responsible for promoter recognition. The *M. tuberculosis* genome harbors 13 different putative sigma factors. Several extracytoplasmic function sigma factors are induced upon the exposure of *M. tuberculosis* to various environmental stresses.

(i) *sigF*

The *M. tuberculosis sigF* gene is strongly induced upon exposure to nitrogen limitation, cold shock, nutrient starvation and entry into stationary phase [45,46]. The growth rate of the *M. tuberculosis ΔsigF* mutant is identical to that of the wild-type strain in culture and human macrophages during exponential phase, but the mutants grow to a higher density than the wild-type strain in stationary phase [10]. In BALB/c mice, the *ΔsigF* mutant and wild-type strains have nearly identical growth rates and similar degrees of granulomatous inflammation during the early stage after infection, but after eight weeks, the *ΔsigF* mutant persists in the lung and shows fewer lesions and less inflammation, while the wild type continues to proliferate slowly [47]. DNA microarray data shows that genes down-regulated significantly in the *M. tuberculosis ΔsigF* mutant strain during exponen-

tial phase are mostly involved in fatty acids and phospholipid metabolism, detoxification and protein folding, such as *acpP* (acyl carrier protein), *fabD* (malonyl CoA-acyl carrier protein), *pks16* (polyketide synthase pKS16), *efpA* (efflux protein), *groES* (10 kD chaperonin), *hsp* (Hsp20 homologue), *glnA1* (glutamine synthetase), *trxC* (thioredoxin), *kasA* (3-oxoacyl-(acyl carrier protein) synthase), *mdh* (malate dehydrogenase), *echA6* (enoyl-CoA hydratase/isomerase), Rv0297 (PE-PGRS5), and Rv0285 (PE5) [47]. Notably, the *acpC* gene, which encodes alkyl hydroperoxidase and is involved in isoniazid resistance, is strongly down-regulated by the loss of *sigF*. Genes down-regulated by the deletion of *sigF* during the stationary phase include those involved in energy metabolism, nucleotide synthesis, central intermediary metabolism and cell envelope synthesis, such as *esxQ* (esat-6-like protein *esxQ*), *trpG* (glutamine amidotransferase), *mmpL1* (transmembrane transport protein), *ndhA* (NADH dehydrogenase), *nrdZ* (ribonucleoside-diphosphate reductase), *bfrA* (bacterioferritin), *pknB* (serine/threonine protein kinase), *ufaA1* (cyclopropane-fatty-acyl-phospholipid synthase), *pimB* (mannose glycosyltransferase), *fadD30* (acyl-CoA synthetase), *lipW* (lipase), and *fgd2* (glucose-6-phosphate dehydrogenase) [47]. In addition, genes encoding several key transcriptional regulators such as TetR, GntR and MarR family regulators are also down-regulated. Moreover, the expression of *sigB* and *sigC* also appears to be SigF-dependent [47,48]. Overexpression of *sigF* results in significant up-regulation of genes encoding members of the MmpL family (*mmpL2*, *mmpL5* and *mmpL11*), lipoproteins (*lprA*, *lprE*, *lprK*) and other genes such as *pks14*, *murA*, *sigG*, *dnaA*, *embR*, *aceAb*, and *trxA*. The most highly up-regulated gene following *sigF* induction is *phoY1*, which encodes a transcriptional regulatory protein homologous to PhoU proteins involved in the regulation of phosphate uptake [49].

(ii) *sigH*

The expression of *M. tuberculosis sigH* is induced in response to heat shock and oxidative stress, and following uptake by macrophages [50]. The *M. tuberculosis ΔsigH* mutant is more sensitive than the wild-type strain to heat shock and to various oxidative stresses, but does not show decreased ability to grow inside macrophages [51]. In resistant C57BL/6 mice, the *ΔsigH* mutant achieves a high bacterial burden identical to those of wild-type strain in lung and spleen, but produces a blunted, delayed pulmonary inflammatory response, and recruits less CD4⁺ and CD8⁺ T cells to the lung [11]. In susceptible C3H mice, the *ΔsigH* mutant again shows diminished immunopathology in contrast to wild-type bacilli [11]. Microarray expression profiles of the wild-type *M. tuberculosis* and the *ΔsigH* mutant strain after exposure to the thiol-specific oxidizing agent diamide show that the deletion of *M. tuberculosis sigH* down-regulates *cysM* (cysteine synthase B), *cysA* (sulphate transport ATP-binding protein), *cysW* and *cysT* (sulphate

transport system permease protein), *papA4* (PKS-associated protein), *chA12* (enoyl-CoA hydratase/isomerase protein), *hsp* (heat-shock protein), *trxB2*, *trxB* (thioredoxin reductase), *trxC* (thioredoxin), *murl* (glutamate racemase), *moeZ* (molybdopterin biosynthesis protein), *rmlB* (dTDP-glucose 4,6-dehydratase), *rmlC* (dTDP-4-dehydrorhamnose 3,5-epimerase), *clpB* (endopeptidase ATP binding protein), *rpi* (dTDP-4-dehydrorhamnose 3,5-epimerase), Rv0355c (PPE8), and *sigB* and *sigE* [51]. The inducible expression of *sigB* is dependent on SigE and SigH [50]. The expression of the heat shock genes *dnaK* and *clpB* of the *ΔsigH* mutant is down-regulated under heat stress [50].

(iii) *sigE*

M. tuberculosis sigE shows increased expression following exposure to heat shock, SDS treatment, and oxidative stress, as well as after phagocytosis in macrophages [12]. The *M. tuberculosis ΔsigE* mutant is defective in its ability to grow inside macrophages but has identical bacterial burden to the wild-type strain in the lung of the C3H/HeJ mice [33]. Time-to-death analysis shows that 50% of C3H/HeJ mice and all the BALB/c-SCID mice infected with the *ΔsigE* mutant strains die more slowly than the wild-type *M. tuberculosis* [12]. DNA microarray data shows that 38 genes are down-regulated in *M. tuberculosis ΔsigE* mutant, among which five genes are involved in protein translation (*tuf*, *rpsJ*, *rplD*, *rplP* and *infC*), four in mycolic acid biosynthesis (*fbpC2* (mycolyl transferase 85C), *fbpB* (mycolyl transferase 85B), *fabD* (acyl-carrier-protein S-malonyltransferase) and *acpM* (acyl carrier protein)), three are transcriptional regulators (Rv0287, *sigB* and *cspA* (cold shock protein A)), three are involved in electron transport (*ctaE* (cytochrome C oxidase subunit III), *qcrA*, *qcrB* (ubiQ-cytB reductase component)), two in acyl-CoA biosynthesis (*aceE* (pyruvate dehydrogenase E1) and *fadD29* (acyl-CoA synthase)), and the last two are *glnA1* (glutamine synthase), *sodA* (superoxide dismutase) [33]. Only *aceA* (*icl*), Rv1130 and *gltA1* (citrate synthase 3) show a higher level of expression in the *ΔsigE* mutant than in the wild-type strain [33].

1.3.3 *whiB*

M. tuberculosis whiB3 encodes a homolog of the *Streptomyces coelicolor whiB* gene, whose function is required for sporulation [52]. *M. tuberculosis WhiB3* responds to signaling molecules NO and O₂ via its [4Fe-4S] cluster and is also essential for surviving the nutrient starvation [53]. As a physiological regulator of virulence lipid anabolism, WhiB3 differentially modulates the assimilation of propionate into complex virulence polyketides under defined oxidizing and reducing conditions [54]. WhiB3 interacts with the principal sigma factor RpoV to affect host survival but is dispensable for *in vivo* growth of *M. tuberculosis* [13]. In addition, WhiB3 binds to *pks2* and *pks3* promoter DNA [54]. *M. tuberculosis whiB3* is induced maximally during the early phase of mouse lung infection and *whiB3* is induced in

resting macrophages but repressed in IFN- γ -activated macrophages [55]. Mice infected with the *M. tuberculosis* $\Delta whiB3$ mutant have significantly longer mean survival time compared with mice challenged with wild-type strain. Remarkably, the bacterial organ burdens of both mutant and wild-type strains are identical during the acute and persistent phases of infection [13].

1.4 Other genes required for persistent *M. tuberculosis* infection

1.4.1 *relMtb*

The stringent response utilizes hyperphosphorylated guanine [(p)ppGpp] as a signaling molecule to control bacterial gene expression involved in long-term survival under starvation conditions [14]. In gram-negative bacteria, RelA and SpoT are responsible for the synthesis of (p)ppGpp. *M. tuberculosis* *relMtb* encodes a single RelA or SpoT homolog, designated Rel_{Mtb}, a dual-function enzyme, for both (p)ppGpp synthesis and hydrolysis [56]. The *M. tuberculosis* $\Delta relMtb$ mutant is unable to synthesize (p)ppGpp *in vitro* on starvation and severely defective in the maintenance of long-term viability. The mutant strain displays a significantly slower growth rate than the wild type in synthetic liquid media but shows an identical growth rate to that of the wild type within a human macrophage-like cell line [14]. The ability of the $\Delta relMtb$ mutant to sustain persistent infection in C57BL/6 mice infected via aerosol inoculation is severely impaired. Significant histopathologic differences are observed in lungs and spleens of mice infected with the $\Delta relMtb$ mutant compared with controls [57]. A DNA microarray was carried out to analyze differentially expressed genes between the *M. tuberculosis* $\Delta relMtb$ mutant and the wild-type strain. Genes displaying a higher level of expression in the wild type include *pbpA* and *pbpB* (penicillin-binding protein), *mceI*, *mce3B*, *mce4* (cell invasion factor), *narH* (nitrate reductase β chain), *narI* (nitrate reductase α chain), *murC* and *murG* (peptidoglycan synthesis protein), *fbpC* (mycolyl transferase 85C), *hspX* (14 kD heat shock protein), *lpqH* (19 kD lipoprotein antigen precursor), *pstS1* (periplasmic phosphate-binding lipoprotein), *esxA* (6 kD early secretory antigenic target ESAT-6), Rv3144c (PPE52), Rv1646 (PE17), *acpM*, *kasA*, *kasB*, *sigD*, *pks4*, and *rpf* [57]. Genes whose expression levels are higher in the $\Delta relMtb$ mutant strain include *groES* (10 kD chaperone), *groEL* (heat shock protein 65), *mmaA2* (methoxymycolic acid synthase 2), *fbpA*, *fbpB*, *fbpD* (mycolyl transferase), *lppO*, *lppS*, *lppU* (lipoprotein), *whiB3*, *eis*, *ompA*, *pks15* and genes encoding PE12, PE26, PE35, PPE27, PPE41, PE-PGRS42 [57].

1.4.2 *hspX*

The most dramatic transcriptional activation (~80-fold) by *M. tuberculosis* DosR during hypoxia is the induction of the *hspX* gene (*acr*), which encodes a 16-kD α -crystallin-like

heat shock protein. The HspX protein acts as a molecular chaperone, preventing thermally induced aggregation of other proteins, and may play a role in stabilizing cell structures during long-term survival of *M. tuberculosis* [58]. The *M. tuberculosis* $\Delta hspX$ mutant exhibits increased growth in both resting and activated macrophages *in vitro* and following infection of BALB/c mice *in vivo* [15]. The increased growth of the $\Delta hspX$ mutant in macrophages is equivalent to that of a $\Delta dosR$ mutant (as measured by the number of CFU (colony forming unit)), which suggests that the increased number of CFU of the $\Delta dosR$ mutant is largely due to loss of *hspX* expression [15]. Overexpression of *hspX* results in reduction in growth rates of both *M. tuberculosis* and *Mycobacterium smegmatis*, and constitutive expression of *hspX* leads to a decreased growth rate for *M. tuberculosis*, suggesting that the *hspX* gene is associated with slow growth [15,59].

1.4.3 *hsp70* and *hspR*

Exposure of *M. tuberculosis* to increased temperature results in elevated expression of heat-shock genes. The HspR repressor inhibits expression of only a small number of *M. tuberculosis* genes, including the *hsp70* operon and the gene encoding the ATPase ClpB. Although the *M. tuberculosis* $\Delta hspR$ mutant is fully virulent in the initial phase of infection, it is significantly impaired in its ability to persist during the persistent stage [16]. The deletion of *hspR* leads to constitutive overexpression of the Hsp70 proteins. The high level of the Hsp70 proteins within the cell might block some developmental program involved in mycobacterial adaptation and enhance the immune recognition of the $\Delta hspR$ mutant.

1.4.4 *katG*

Many isoniazid-resistant strains of *M. tuberculosis* have deletions or missense mutations in the *katG* gene, which encodes the catalase-peroxidase. Transformation of functional *M. tuberculosis* *katG* genes into the avirulent H37RvINH^R strain restores the ability of this recombinant to multiply and persist in spleens of mice and guinea pigs [17].

2 Construction of the gene regulatory network of persistent *M. tuberculosis* infection

Most events within cells cannot be accomplished with a single gene or its product. Gene interactions underpin complex gene expression regulatory networks. Functional genomics methodologies including DNA microarray and mass spectrometry provide unprecedented opportunity to address the intricacy of gene regulatory networks. Gene regulatory networks depict complicated gene interactions and consist of many nodes (genes) and links among nodes (positive regulation or negative regulation). A gene regulatory net-

work based on the genes and regulators previously reported to be implicated in *M. tuberculosis* persistence is shown in Figure 1.

This network indicates that genes encoding for sigma factors and two component systems play essential roles during the *M. tuberculosis* persistent infection stage. *M. tuberculosis* two component systems initially respond to various environmental stresses within the granuloma. Histidine kinase sensor proteins transmit stress signals to cytoplasmic response regulators to control the expression of sigma factors, and then mediate other genes to respond to environmental changes. The signal transduction pathway “*mprB/A-sigE-relA*” is a perfect example of this [60]. In principle, the signal transduction cascade can be intercepted at any one of these steps. Genes whose expressions are regulated include those encoding the MmpL protein family members, which are related to transport of lipids, such as *mmpL1*, *mmpL2*, *mmpL5*, *mmpL10*, *mmpL11*. MmpL-mediated lipid secretion both contributes to the intracellular survival of *M. tuberculosis* and also to the host-pathogen interaction that determines the ultimate outcome of infection [61]. Furthermore, there are many genes encoding PE/PPE/PE-PGRS protein family members and genes from this family account for nearly 5% of coding capacity of *M. tuberculosis*. This protein family is required for virulence and persistence of *M. tuberculosis* and several members of this family localize to the cell surface and influence interactions

between *M. tuberculosis* and the macrophages. PE-PGRS51 is a dominant antigen which is recognized by sera of latent carriers. In addition, this network also involves several salty ion transport genes, such as nitrate/nitrite transport gene *narK2*, sulfate transport genes *cysA*, *cysW* and *cysT*, and phosphate transport gene *pstS1*. Genes encoding for the Ag85 complex, which consists of three distinct trehalose dimycolyl transferases Ag85A, Ag85B and Ag85C, frequently appear in this network, including *fbpA*, *fbpB*, and *fbpC*. These antigens participate in mycolic acid biosynthesis and cell wall assembly. They also function as fibronectin-binding proteins (fbps) to attach to the macrophages. *fbp* genes are induced upon isoniazid treatment and suggestive of novel anti-tuberculosis drug targets [62].

Drug resistance of tuberculosis has recently emerged as a major public health challenge in China, and there has been an alarming increase in patients with multi-drug resistant tuberculosis (MDR-TB) and extensively drug resistant tuberculosis (XDR-TB). Resistance rates to isoniazid, rifampicin, and streptomycin are high. This network involves a large number of isoniazid resistance genes. The *katG* gene encodes catalase-peroxidase, an enzyme that converts isoniazid to an active form to inhibit the biosynthesis of the mycolic acid and cell wall. Mutations, deletions and insertions in the *katG* gene are responsible for isoniazid resistance. The *kasA* gene encodes 3-oxoacyl-(acyl carrier protein)

Figure 1 Gene regulatory network based on the genes and regulators reported to be implicated in *M. tuberculosis* persistence. The genes highlighted by red represent *M. tuberculosis* genes implicated in persistent infection which have been reported; the pink proteins represent genes which encode these proteins.

synthase, a key enzyme involved in the biosynthesis of mycolic acid, and the mutation in the *kasA* gene is related to isoniazid resistance. The *ahpC* gene encodes alkyl hydroperoxide reductase subunit C and the mutation of its promoter results in AhpC over-expression, thereby compensating the lack of catalase-peroxidase caused by *katG* mutation. The *ndh* gene encodes NADH dehydrogenase and mutations in the *ndh* gene lead to defective activity of NADH dehydrogenase, increased NADH/NAD⁺ ratio and inhibited peroxidation of isoniazid, suggesting that *ndh* is associated with isoniazid resistance.

There are interactions among sigma factors and certain sigma factors can regulate other sigma factors, achieving an internal regulation of sigma factors. From this network we know that *sigE*, *sigH*, *sigF* are able to positively regulate *sigB* and *sigB* may auto-amplify its expression under certain conditions, suggesting that the complex interplay of sigma factors converges at *sigB* [63]. The expression of other sigma factors is unchanged after *sigB* induction, suggesting that SigB functions as an end regulator in the sigma factor regulatory network [63]. The deletion of *sigB* in *M. tuberculosis* results in its higher sensitivity to SDS, heat shock, oxidative stress and hypoxic conditions and over-expression of *sigB* significantly upregulates *katG*, *kasA*, *whiB2*, and *ideR* [63,64]. Currently, the gene regulatory network of *M. tuberculosis* persistence is still far from complete. Also with such a complex organism, a small disturbance might well have a significant effect in this network and on the fate of the pathogen. Discovery of the pivotal nodes in this network will undoubtedly be important for the development of future intervention measures.

3 Vaccine design and drug targets screening

Long term persistence of *M. tuberculosis* is also a complex process requiring the coordinated expression of numerous *M. tuberculosis* virulence genes and adaptive immune evasion strategies. Understanding which genes and networks are implicated in *M. tuberculosis* persistence is instrumental in determining the underlying molecular mechanisms, and producing better drug targets for latent tuberculosis treatment.

3.1 Inspiration for vaccine design

The current tuberculosis vaccine BCG is largely ineffective in protecting adults against pulmonary tuberculosis. Most new generation tuberculosis vaccines are designed as prophylactic vaccines and are based on antigens that are expressed during the early phases of *M. tuberculosis* infection [65]. Persistent *M. tuberculosis* is unlikely to be targeted by such prophylactic vaccines, as they may not be effective in the already infected host during the late stage infection. One strategy is to develop therapeutic vaccines that can adequately activate

T-cell responses to late stage infection associated antigens. Such vaccines may target and help eliminate dormant or slowly replicating *M. tuberculosis*, thereby preventing later reactivation from latency [65]. Therapeutic vaccines alone or in combination with drugs may provide a more rapid and more effective strategy against persistent *M. tuberculosis*. Identifying genes required for persistent infection by *M. tuberculosis* and constructing gene regulatory networks should lead to the discovery of novel TB drug targets. The general nodes such as common genes and the hubs encoding two-component systems and sigma factors might be promising targets for vaccine design.

The heat shock protein HspX is required for persistence of *M. tuberculosis* within the macrophage. In the sera of tuberculosis patients, both T-cell and B-cell responses against HspX were found to be associated with persistent *M. tuberculosis* infection, indicating the importance of HspX as an antigenic target of immune responses during persistent infection. Subunit vaccines incorporating HspX and/or other latency antigens might be good vaccine candidates for latent tuberculosis [66]. Lowrie [67] demonstrated that post-therapy relapse of tuberculosis could be prevented by vaccinating the Cornell mice with a DNA vaccine encoding the mycobacterial Hsp65 heat shock protein, suggesting that the immune boost provided by vaccination promoted the complete eradication of *M. tuberculosis* from the tissues. Recombinant BCG expressing DosR can be aerobically cultured *in vitro* but fails to enter a non-replicating state, allowing evaluation of this strain as a latency-specific vaccine candidate [68]. In addition, the high survival rate in tissues with reduced lethality of the *M. tuberculosis* Δ *sigF* mutant suggests that this class of mutant may be valuable as live-attenuated vaccines for tuberculosis. The Δ *sigF* mutant strain appears to cause stronger immunity than BCG in the rabbit model, suggesting that alterations in the SigF regulon result in a superior set of immunogens with potentially more long-lived protection than those induced by BCG with potentially more long-lived protection [47].

3.2 Inspiration for drug targets discovery

Although some front-line antibiotics (isoniazid, ethambutol, pyrazinamide, and rifampicin) are still reasonably effective at killing *M. tuberculosis* which is actively replicating in culture, these antibiotics are often ineffective in eliminating *M. tuberculosis* during persistent stages of infection [1]. The dearth of specific drugs to target persistent *M. tuberculosis* is the major barrier to developing effective short-term treatment. Research into tuberculosis has placed an inappropriate emphasis on the need to discover genetic "essentiality" for any target, based on the misplaced assumption that genetic knockouts can be phenotypically copied by small-molecule inhibitors of metabolic pathways. We need to shift the emphasis from the discovery of essential genes to identifying the subtle holistic interactions between path-

ways and gene products that are required for survival in the dynamic host milieu. These interactions constitute a gene regulatory network; central nodes in this network are likely to be metabolic breakpoints that could be considered as drug targets. For example, DosR is a key transcriptional regulator for coordinating the response to host immune attack which eventually results in the entry into the non-replicating state. Therefore, it is widely recognized as a potential drug target against latent tuberculosis. However, by inhibiting *dosR*, hypervirulent strains of *M. tuberculosis* were produced. This precludes precise prediction of the effect of *dosR* inactivation on shortening the duration of tuberculosis treatment [69].

4 Concluding remarks

Significant advances have been made in identifying *M. tuberculosis* “persistence factors”, but it is not yet clear whether targeting these persistence factors with new drugs will fulfill the expectation of killing *M. tuberculosis* *in vivo*, nor is it clear whether persistence factors that are essential in the mouse model will likewise be important in human disease. A major challenge for future research will be the formulation of models of persistent infection relevant to human infection and replicating the behavior of *M. tuberculosis* in its natural niche [70]. Reporter enzyme fluorescence (REF) allows real-time imaging of pulmonary infections and rapid quantification of *M. tuberculosis* in living animals. This technology is based on near-infrared (NIR) fluorogenic substrates for β -lactamase, an enzyme expressed by *M. tuberculosis*, but not by their eukaryotic hosts [71]. Gene regulatory network study is an emerging area for which various models and computational tools are now available. Construction of gene regulatory networks will help us to understand the interactions among genes required for *M. tuberculosis* persistent infection, to study gene functions from a global perspective and to discover key therapeutic targets against tuberculosis. Much endeavor in this field should lead to better control of TB.

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