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The Interaction Topology of *Mycobacterium tuberculosis* Genes Response to Capreomycin and Novel Clues for More Drug Targets

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ABSTRACT

The resurgence of tuberculosis (TB) and emergence of multidrug-resistant TB (MDR-TB) are significant obstacles to stop TB treatment. Capreomycin (CPM) is regarded as an ideal second-line treatment for TB as well as for MDR-TB. However, the inexorable emergence of capreomycin resistant TB cases accentuates the urgent need for more detailed characterization of CPM targets. Most of these are single gene mutation, such as those involved in the complex formation of ribosomal 30S initiation, inhibit protein synthesis, affect 50S ribosomal protein L10, control transcription and translation of operon rplJL-rpoBC. A new paradigm integrating gene, small metabolites, protein and underlying signaling pathway to shed light on the physiology, pathogenesis, and network of pathogen response is emerging. This model holds great promise to unravel the intricacy of drug action. However, to our knowledge, no such work regarding *Mycobacterium tuberculosis* response to capreomycin exposure was ever reported. We employed the data mining to construct an interaction topology of *M. tuberculosis* genes response to capreomycin. Most valuable genes were summarized for further experimental validation based on this topology. Dampening the virulence factors and respiratory of *M. tuberculosis* might be the new targets of CPM beyond *Rv1364c*, *pe_pgrs38*, *pe_pgrs51* which are the salient nodes of the network and represent most promising new capreomycin targets meriting further exploration. This work will facilitate further investigation of capreomycin targets against *M. tuberculosis* and be conducive to novel TB drug discovery. J. Cell. Biochem. 112: 2716–2720, 2011.

KEY WORDS: CAPREOMYCIN; TOPOLOGY; DRUG TARGETS; *MYCOBACTERIUM TUBERCULOSIS*

Resurgence of tuberculosis (TB), largely resulting from *Mycobacterium tuberculosis* (MTB) persistence, multidrug-resistant MTB, extensive drug resistant MTB and HIV co-infection, are new obstacles to stop TB treatment. The traditional first-line anti-TB drugs such as rifampicin, streptomycin, cannot meet the clinical requirements. The relative contribution of capreomycin to the treatment of active TB is rather insignificant; its role in combating persistent TB is gaining increasing concern [McClatchy et al., 1977]. Besides, as a more popular second-line drug, CPM is effective against drug-resistant TB. The understanding of the mechanisms of action of CPM are continually deepened. In the 1970s, the mechanisms of action of CPM largely attributed to interference with the synthesis of ribosomal 30S initiation complex [Garcia-Contreras et al., 2007]. In 1977, it was found that CPM blocks the tRNA transferring from the A to the P-site, which blocks the elongation of peptide chains, thereby inhibiting the protein synthesis [Modolell and Vazquez, 1977]. Genome-wide exploration of drug action of CPM against *M. tuberculosis* using Oligonucleotide GeneChips revealed a variety of possible novel CPM targets beyond 16S rRNA [Li and Fu, 2007].

During the past few decades, individual gene has been the focus such as identify specific gene defects, together with single nucleotide polymorphism, and copy number variations. This pattern is very successful, but far from comprehensive, especially when systems biology increasingly unveils that most cellular components exert their functions by fine-tuning underlying network [Lee et al., 2008]. With oceans of "-omics" data available, the more holistic network analysis is attracting more and more attention to elucidate

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the intricacy of biological systems. This methodology is especially helpful to unveil the mechanisms of action of drugs. In light of the systems biology philosophy, an interaction topology of CPM action based on microarray data was constructed; genes involved in virulence of MTB, antigen, cell well permeable barrier, efflux of drug are highlighted as tempting additional CPM targets.

FUNCTION ANALYSIS OF *M. tuberculosis* GENES RESPONSE TO CPM EXPOSURE

SAM (Significance Analysis of Microarrays) program was employed by Fu et al. to extract the response of specific genes to CPM via comparison of the original data of *M. tuberculosis* genes response to CPM exposure, which was conducted by Shinnick et al., with those of *M. tuberculosis* H37Rv exposed to diverse antibiotics such as rifampicin, isoniazid, streptomycin, ethionamide, and PA-824 [Fu and Tai, 2009]. Forty-two upregulated and 196 downregulated genes after CPM exposure were manually curated among 42 upregulated genes. Six genes belong to *pe_pgrs* multiple-gene subfamily member: *Rv2634c*, *Rv1087*, *Rv0834c*, *Rv2162c*, *Rv3367*. Twenty-six genes encode conserved hypothetical proteins. The genes described in Table I are, what we think, the significant ones. Table I shows that genes are upregulated and downregulated after CPM exposure.

Based on Table I, we proposed that *M. tuberculosis* might avert the attack of CPM largely through the synthesis of more virulence factor, mainly phthiocerol dimycocerosate (PDIM), and decreasing respiration rate. However, this is a tempting hypothesis awaiting further experimental validation.

INCREASING THE SYNTHESIS OF A PUTATIVE VIRULENCE FACTOR PDIM

PDIM is one of the main constituents of *M. tuberculosis* cell well permeable barrier. Though controversial and the mechanisms underlying PDIM virulence remain unknown [Goren et al., 1974], PDIM was regarded as a virulence factor of *M. tuberculosis* [Parida et al., 2005]. PDIM-less mutant of *M. tuberculosis* H37Rv was attenuated during the infection of the guinea pig. The biosynthesis and the precise location of PDIM involve multiple molecules and their interaction. FadD26 is a fatty-acid-CoA ligase involved in the synthesis of PDIM [Scandurra et al., 2007]. Both MmpL7 and DrrC (probable daunorubicin-dim-transport integral membrane protein ABC transporter) are transporters for PDIM. Insertional mutation of *drrC* gene resulted in the accumulation of PDIM in the bacterial cytosol or plasma membrane, instead of their original cell wall destination [Camacho et al., 2001]. Upon CPM exposure, the expression of Rv1364c was upregulated, and the expression of mmpL7 was downregulated. Rv1364c encodes a heat stress-related protein and annotated as a potential anti-sigma factor antagonist. It consists of a sensory domain (PAS, a protein domain contained many signaling proteins and was named after three proteins: P—period circadian, A—aryl hydrocarbon receptor nuclear translocator protein, and S—single-minded protein) at the N-terminal end, a PP2C phosphatase (RsbU Regulator of Sigma B U) domain, and an RsbW (Regulator of Sigma B W) kinase domain in the middle region and an anti-sigma factor (RsbV Regulator of Sigma B V) domain at the C-terminal region. The yeast two-hybrid experiment demonstrated that it can interact with both RsbW and SigF directly. SigF is one of the 12 alternative sigma factors. *M. tuberculosis* sigF deletion mutant was attenuated in the mouse infection model [Geiman et al., 2004]. *M. tuberculosis* SigF is nonessential for survival in macrophages and is not involved in response to the heat shock, cold shock, low oxygen pressure [Riccardo Manganelli, 2004]. Anti-σ factor releases sigma factor. The released sigma factor binds to core RNA polymerase instead of anti-σ factor to regulate transcription. The upregulation of sigF can increase the expression of FadD26 [Williams et al., 2007]. We hypothesized that upon exposure to CPM, *M. tuberculosis* might produce more PDIM to neutralize the detrimental effect of CPM. However, CPM simultaneously downregulated the expression of mmpL7, which might intercept the timely transfer of PDIM to their destination. The possible remedy that *M. tuberculosis* might count on is the seemingly unaltered expression of DrrC. This speculation is

TABLE I. Genes That Upregulated and Downregulated After CPM Exposure

ORF	Gene	Protein	Fold
Rv1258	*cysD*	Probable sulfate adenylyl-transferase subunit 2	2.82
Rv2878c	*mpt53*	Soluble secreted antigen mpt53 precursor	2.74
Rv1687c	–	Probable conserved ATP-binding protein ABC transporter	2.41
Rv3260c	*whiB2*	Probable transcriptional regulatory protein	2.1
Rv1364c	–	Conserved hypothetical protein	3.54
Rv2634c	*Pe_pgrs46*	PE_PGRS family protein	2.54
Rv3367	*Pe_pgrs51*	PE_PGRS family protein	2.26
Rv2126c	*Pe_pgrs38*	PE_PGRS family protein	2.30
Rv2942	MmpL7	Conserved transmembrane transport protein	−2.9
Rv2195	qcrA	Probable Rieske iron–sulfur protein	−2.1
Rv2196	qcrB	Probable ubiquinol-cytochrome C reductase, cytochrome B subunit	−3.2
Rv3150	*nuoF*	Probable NADH dehydrogenase i chain f	−2.9
Rv3151	*nuoG*	Probable NADH dehydrogenase i chain g	−3.9
Rv3152	*nuoH*	Probable NADH dehydrogenase i chain h	−3.9
Rv3153	*nuoI*	Probable NADH dehydrogenase i chain i	−4.1
Rv3154	*nuoJ*	Probable NADH dehydrogenase i chain j	−4.4
Rv3155	*nuoK*	Probable NADH dehydrogenase i chain k	−4.6
Rv3156	*nuoL*	Probable NADH dehydrogenase i chain l	−4.4
Rv3157	*nuoM*	Probable NADH dehydrogenase i chain m	−5.5

tempting, but more evidences are needed, such as the dynamic of PDIM level upon CPM exposure, and the transcriptional and translational levels of drrC.

UPREGULATING OF EXPRESSION OF MOLECULES RELATED TO *M. TUBERCULOSIS* VIRULENCE

Mycothiol (MSH) is the major low-molecular-mass thiol in *mycobacteria*. MSH is essential for *M. tuberculosis* anti-oxidant defense and degradation of toxic substances due to the absence of gluathione, the major low-molecular-mass thiol in many other bacteria [Newton et al., 2000]. Experiments with *M. tuberculosis* mutant defective in MSH biosynthesis demonstrated that MSH is vital for maximum growth of *M. tuberculosis* in oxygen-rich environments, sensitivity to first-line drugs isoniazid and rifampin [Buchmeier et al., 2003]. The ultimate formation of MSH associates with sulfur metabolism. Upon CPM exposure, the expression of CysD was upregulated. *CysD* and *cysNC* orthologs constitute an operon that encodes a multifunctional enzyme which has ATP sulfurylase, GTPase, and adenosine 6'-phosphosulfat (APS) kinase activities. *CysD* gene encodes the adenylyl-transferase subunit of ATP sulfurylase which can catalyze phosphate and GDP to form APS and pyrophosphate. This enzyme is one of the several sulfur metabolism enzymes implicated in the virulence, antibiotic resistance, and anti-oxidant defense of *M. tuberculosis* [Pinto et al., 2004]. Enzymes related to sulfur metabolism, such as *cysD*, might be mobilized by *M. tuberculosis* upon CPM exposure to countermeasure the antibiotic's detrimental effect. MSH temporarily increase might also be involved in CPM degradation. To our knowledge, no previous studies of CPM mechanisms have hinted at the role of sulfur metabolism. However, this bold guess needs more empirical evidence, so as to test the CPM efficacy against MSH mutant of *M. tuberculosis* and the mutants' *cysD* expression upon CPM treatment.

Mpt53, PE_PGRS51, and PE_PGRS38 induced by CPM are associated with the virulence of *M. tuberculosis*. Mpt53, is known as a 15 kDa secreted protein and is also annotated as DsbE. It contains a thioredoxin active site and is a strong oxidant. It may be required for proper folding of reduced unfolded secreted proteins [Goulding et al., 2004]. So, it might be associated with the virulence of *M. tuberculosis*. SigL is one of the 12 alternative sigma factors regulating virulence. Four operons, *sigL* (*Rv0735*)-*Rv0736*, *mpt53* (*Rv2878c*)-*Rv2877c*, *pks10* (*Rv1660*)-*pks7* (*Rv1661*), and Rv1139c-Rv1138c, are significantly upregulated in the sigL-overexpressing strain [Hahn et al., 2005]. SigL-overexpression also indirectly induces four pe_pgrs family members: *pe_pgrs27*, *pe_pgrs46*, *pe_pgrs48*, *pe_pgrs51* [Hahn et al., 2005]. Pe_pgrs is a multiple-gene family. PE_PGRS proteins contain two domains: PE domain and PGRS domain. PGRS domain has large Gly-Ala-X repeat. PE_PGRS proteins are hitherto found only in *Mycobacterium*, most PE-PGRS proteins limited to pathogenic *Mycobacterium*. CPM induced Pe_pgrs 51 is one of the four important *M. tuberculosis* antigens that are expressed in vivo in aerosol infection rabbits [Singh et al., 2001]. This protein was also recognized as a potential diagnostic marker for preclinical TB due to its response to preclinical TB sera obtained, prior to the clinical manifestation of TB. Rv0834c (PE_PGRS14), another PE_PGRS protein induced by CPM is on the list of the top 100 persistence targets selected by the TB Structural Genomics Consortium. Rv0834c participates in the acid stress response. Its expression was detectable in lower pH condition. The experiment employs RT-PCR-based *acr* (16 kDa α crystalline protein) transcription as an indicator for latent *M. tuberculosis* in "in vitro" and "in vivo" experimental models. The exponential phase (EP) and stationary phase (SP) *M. tuberculosis* were exposed to different pH in an unstirred culture, the expression of genes was monitored [Gordillo et al., 2006]. Pe_pgrs38, a *M. tuberculosis* virulence factor *pe_pgrs33* (identity 54.5%) homolog located at cell surface associated with cell death, can increase IL-10 level and release of lactate dehydrogenase in macrophage cultures [Singh et al., 2008]. In brief, multiple established *M. tuberculosis* virulence factors involved in CPM exposure. However, the master regulator of this complex virulence factor response remains unknown.

DECREASE OF AEROBIC RESPIRATION RATE

Microbes usually survive hardships such as starvation, antibiotics exposure, hypoxia, and other stresses via lowered metabolic activity [Smeulders et al., 1999]. This is particularly the case for *M. tuberculosis*, the hitherto most successful pathogen of mankind. CPM exposure downregulated a plethora of *M. tuberculosis* genes associated with respiration, such as *nuoF/M* encoding NADH dehydrogenase subunit and *qcrA*, *qcrB* encoding cytochrome reductase subunit. The gross effect of these downregulation might lead to weakened transmission of electronic and hydrion and much lower aerobic respiration rate of *M. tuberculosis* [Sone et al., 2003]. Decrease of the oxidative phosphorylation rate due to reduced ATP synthase level also contributes to this. Respiratory chain, or the nitrite reduction respiratory relevant genes, might be the targets of CPM too [Honer zu Bentrup and Russell, 2001].

THE INTERACTION TOPOLOGY OF *Mycobacterium tuberculosis* GENES RESPONSE TO CAPREOMYCIN

Based on Figure 1, upon exposure to CPM, the expression of many genes in *M. tuberculosis* is regulated by a variety of cellular transcription factors, which play a major role in SigL, SigF, and two-component regulatory system MprAB. The genes pks7, pks11, pks8, pks10, ppsA induced by SigL, and FadD26, FadD21, *etc* induced by SigF are the genes associated with the synthesis of cell wall components PDIM.

In addition to pe_pgrs51, pe_pgrs46, pe_pgrs38 induced by CPM in particular, various pe_pgrs family proteins are also present in the control network. The protein family is a rather special family because of escaping the immune response. Meanwhile, in the regulatory network of MprAB-SigE-SigB-WhiB2, we are interested in WhiB2. The gene belongs to WhiB family, whose function is involvement in cell differentiation, nutritional deficiencies, antibiotic resistance, treatment mechanisms, and pressure sensor [Alam et al., 2009]. ΔwhmD mutant of *Mycobacterium smegmatis* exhibited irreversible filamentous branched growth with diminished septum formation and aberrant septal placement [Gomez and Bishai, 2000]. WhiB2 is a hypothetical transcription factor, but there is no report about which genes are regulated by genes. Research on the

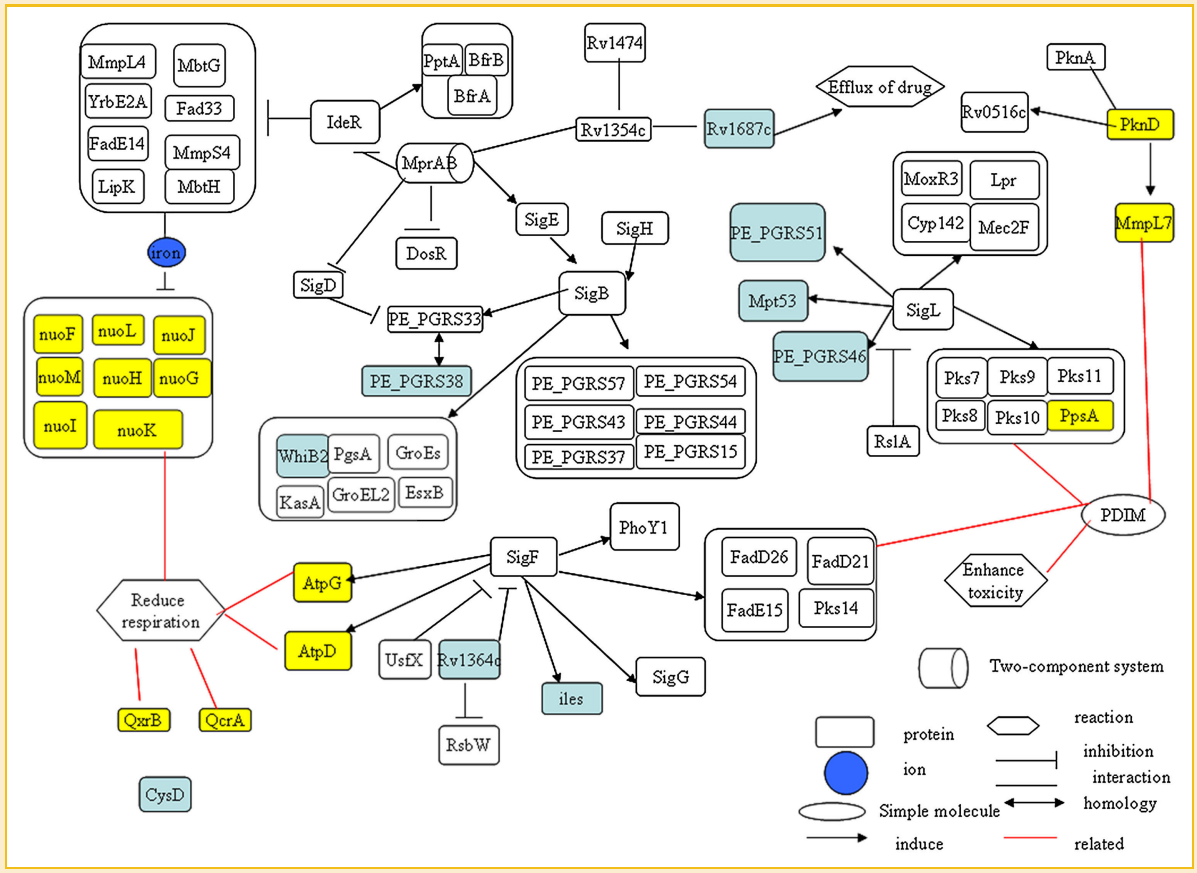

Fig. 1. The interaction topology of *Mycobacterium tuberculosis* genes response to capreomycin. Proteins highlighted in light green denote those induced by capreomycin, whereas proteins highlighted in yellow denote those repressed by capreomycin. [Color figure can be seen in the online version of this article, available at http://wileyonlinelibrary.com/journal/jcb]

regulatory network of WhiB2 will play an important role in finding a new target of CPM.

THE PROSPECTS OF THE CAPREOMYCIN TREATMENT OF TUBERCULOSIS

THE SIDE EFFECTS OF CAPREOMYCIN

Use of aminoglycosides and capreomycin causes renal wastage of electrolytes, consisting of potassium, magnesium, and calcium. The secondary hyperaldosteronism would be induced by aminoglycosides and capreomycin, which would lead to urinary loss of potassium and magnesium. In the adenosine triphosphatase-dependent mechanism for active transport of sodium and potassium across the cell membrane, magnesium serves as a co-factor, so loss of magnesium can lead to further potassium wastage which occurs as a consequence of resultant intracellular magnesium deficiency. Lastly, to a certain extent through the suppressive effect of low magnesium levels on the parathyroid hormone, hypomagnesemia can induce hypocalcemia [Holmes et al., 1970; Shin et al., 2004]. Though capreomycin has side effect, it has an important role in the treatment of MDR-TB. Moreover, apply inhaled large porous particles of CPM to human with MTB, might simplify drug delivery by eliminating injections and might reduce adverse effects through lowering the dose [Garcia-Contreras et al., 2007].

THE ROLE OF TOPOLOGY IN SEARCHING FOR NEW DRUG TARGET OF CPM

With the use of CPM, the TB resistant to CPM has emerged. The major molecular mechanism of *M. tuberculosis* drug resistance to CPM is as follows. Mutation of the gene that encodes rRNA modification enzyme—2′-*O*-methyltransferase *tlyA* is the important reason [Maus et al., 2005]. A mutation in the decoding center of thermus thermophilus 16S rRNA, A1408G, is another reason of CPM resistance [Gregory et al., 2005]. Also, it is the mechanism of streptomycin resistance. The emergence of multidrug-resistant *M. tuberculosis* is a big obstacle to TB treatment. It is imminent to search for new drug targets and develop new anti-TB drugs.

The viewpoint of systems biology is mainly a great leapfrog to tackle the complex systematic response such as to the drug exposure. This can avoid the setbacks of pure reductionism and bring about more integrative picture of life and disease [Chautard et al., 2009]. Our initial attempt began to unveil some intriguing features of *M. tuberculosis* response to CPM exposure, such as the altering of aerobic respiration rate and increased virulence factors level. Of note, Rv1364c, Rv1687c, and a handful of PE-PGRS family member are justified for further investigation.

The interaction topology of *M. tuberculosis* genes response to CPM will help us better understand the response of *M. tuberculosis* exposure to CPM, and reveal the mechanism of its tolerance of CPM

further. It is an inescapable step to identify molecular interactions within cells to appreciate the delicacy of life. This is the case for a thorough understanding of the mechanisms of action of CPM. When we search for new drug targets, we can consider not only a single gene, but also a series of regulatory and physiological biochemical process in *M. tuberculosis* so as to develop a new road for the TB treatment.

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