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The Mycobacterium DosR Regulon Structure and Diversity Revealed by Comparative Genomic Analysis

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ABSTRACT

Tuberculosis (TB), caused by *Mycobacterium tuberculosis* (*Mtb*), which claims approximately two million people annually, remains a global health concern. The non-replicating or dormancy like state of this pathogen which is impervious to anti-tuberculosis drugs is widely recognized as the culprit for this scenario. The dormancy survival regulator (DosR) regulon, composed of 48 co-regulated genes, is held as essential for *Mtb* persistence. The DosR regulon is regulated by a two-component regulatory system consisting of two sensor kinases—DosS (Rv3132c) and DosT (Rv2027c), and a response regulator DosR (Rv3133c). The underlying regulatory mechanism of DosR regulon expression is very complex. Many factors are involved, particularly the oxygen tension. The DosR regulon enables the pathogen to persist during lengthy hypoxia. Comparative genomic analysis demonstrated that the DosR regulon is widely distributed among the mycobacterial genomes, ranging from the pathogenic strains to the environmental strains. In-depth studies on the DosR response should provide insights into its role in TB latency in vivo and shape new measures to combat this exceeding recalcitrant pathogen. J. Cell. Biochem. 114: 1–6, 2013.

KEY WORDS: *Mycobacterium tuberculosis*; DosR REGULON; DORMANCY; COMPARATIVE GENOMIC ANALYSIS

Tuberculosis (TB) remains one of the deadliest infectious diseases with global significance. Almost one-third of the world's population is latently infected with *Mycobacterium tuberculosis* (*Mtb*), and about two million deaths occur due to active TB annually. The unique switch capacity of *Mtb* between active and non-replicating status was widely recognized as the crux for its infamous drug resistance [Boon and Dick, 2002]. Intensive studies are being undertaken to discover the mechanisms underlying the shift of *Mtb* between dormancy and active metabolism. The dormancy survival regulator (DosR) regulon is unique for its role in the above-mentioned metabolic state transition. The constituents, expression, and underlying regulation have been summarized following Mycobacterium comparative genomic analysis to provide clues for better characterization of this important regulon.

THE COMPONENTS AND DISTRIBUTION OF THE DosR REGULON

The survival and persistence of *Mtb* in extreme environment necessitates the swift external signals sense and subsequent cognate responses [Chao et al., 2010]. Low oxygen tension (hypoxia) is one of the leading factors frequently associated with the establishment and maintenance of latent TB [Wayne and Sohaskey, 2001], which presumably forces the pathogen to adopt a non-replicating but viable state in an anaerobic environment. The initial response to hypoxia of MTB includes the altered expression of about 100 genes, including the DosR regulon. The DosR regulon composes of 48 co-regulated genes, putatively essential for *Mtb* survival during latency [Fallow et al., 2010; Rustad et al., 2009], such as the genes involved

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in alternative electron transport pathways (*fdxA*), nitrate metabolism (*narK2* and *narX*), triglyceride synthetase (*tgs1*), and deoxynucleoside triphosphate synthesis under microaerophilic conditions (*nrdZ*) [Fallow et al., 2010; Rustad et al., 2009]. The DosR regulon is regulated by a two-component regulatory system consisting of two sensor kinases—DosS (Rv3132c) and DosT (Rv2027c), and a response regulator DosR (Rv3133c). DosR is homologous to NarL/UhpA/FixJ subfamily responsive regulator, and DosS is a functional kinase. Both are conserved in *Mtb* and *M. bovis* BCG but not in other mycobacteria [Dasgupta et al., 2000]. DosS has one histidine kinase of the two-component transcriptional regulatory system which can transfer phosphate to DosR in vitro. DosT, a second putative kinase, also can phosphorylate DosR [Roberts et al., 2004].

The DosR regulon is widely distributed in the mycobacterial genomes (Fig. 1). It is tempting to speculate that the genes of DosR regulon are very conserved, which is essential to the mycobacteria survival during anaerobic dormancy and also required for metabolic processes that occur upon entry into and throughout the dormant state [Park et al., 2003]. It is worth noting that some DosR regulon genes are also conserved in the environmental mycobacteria, suggestive of a more broad role of these regulon genes for response to environmental cues such as hypoxia, rather than drug resistance or virulence. The DosR regulon is highly conserved among all sequenced isolates of *Mtb*, an indication of crucial role in latency [Bartek et al., 2009]. Our result is consistent with a recent publication [Gerasimova et al., 2011] that *Mycobacterium leprae* is the only mycobacterium which lacks DosR two-component system, together with universal stress proteins and diacylglycerol acyltransferase. Some unique hypoxia adaptation stratagem might exist within *M. leprae* genome. One surprising finding is that the nucleotide sequence similarity of DosR (Rv3133c) between *Mtb* F11 strain and H37Rv strain is astonishingly low—only 43%. *Mtb* F11 strain is a clinical virulent isolate from South Africa. The significance of this unexpected finding remains to be determined.

DosS and DosT share 62.5% amino acid sequence similarity. These two kinases contain several similar domains: histidine kinase domain and an ATP-binding domain in the C-terminal, and two tandem GAF (cGMP, adenylyl cyclase, FhlA) domains (GAF A and GAF B) in the N-terminal. GAF domains are regulatory domains which can bind small molecules and widely distributed among prokaryotes and eukaryotes [Honaker et al., 2010]. The three-dimensional structures of GAF-A and GAF-B have been determined: a *b*-type heme is embedded into the GAF-A domain, which is composed of five strand anti-parallel β-sheet and four α-helices. The heme is positioned nearly perpendicular to the β-sheet [Ioanoviciu et al., 2007; Podust et al., 2008; Sardiwal et al., 2005]. H149 and H147 function as the proximal axial ligands for DosS and DosT, respectively [Sardiwal et al., 2005]. The binding state of distal ligand and the redox state of heme iron are responsible for regulating the kinase activity of DosS and DosT: the heme iron in ferric form would make the kinase inactive; once the iron is de-oxidization or bound with NO/CO, the kinase activity restores. Further, it is reported that flavin nucleotides could help to reduce the heme iron of DosS. The heme iron of DosT can withhold the conversion of the Fe^{2+} to Fe^{3+} in the presence of oxygen [Honaker et al., 2009], suggesting that DosT could play a more important role than DosS in the early phase of hypoxic conditions especially when transferred from aerobic to hypoxic conditions.

The C terminus of DosR can bind DNA, and the 54th conserved aspartate is essential for the binding. There is a 20-mer degenerate palindromic motif associating with DosR regulon [Park et al., 2003; Rustad et al., 2009; Vasudeva-Rao and McDonough, 2008]. One variant of this consensus sequence locates upstream of nearly all

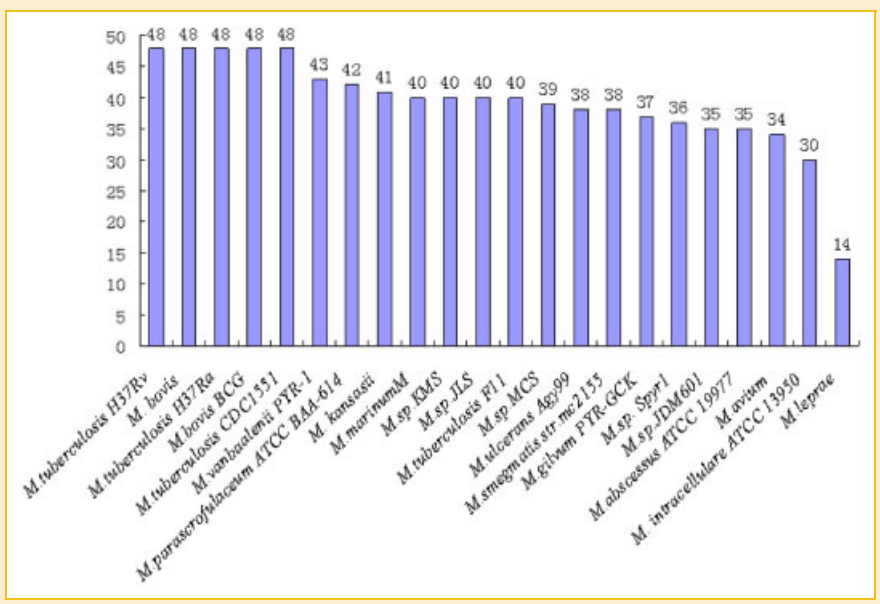

Fig. 1. Distribution of DosR regulon among *Mycobacterium*. Each column indicates the number of DosR regulon members in particular *Mycobacterium*. The data were obtained through Blastn against *M. tuberculosis* H37Rv homolog.

MTB operons. Mutations within this site would abrogate the abilities of DosR to bind and induce downstream reporter gene under hypoxia.

THE EXPRESSION AND REGULATION OF DosR REGULON

DosR regulon are required for *Mtb* survival during persistent infection, whose expression is modulated by the following factors (Fig. 2): (1) divalent gas: when stimulated by hypoxia [Park et al., 2003; Sherman et al., 2001], nitric oxide [Sherman et al., 2001], and carbon monoxide [Kumar et al., 2008; Shiloh et al., 2008], the expression level of DosR regulon is upregulated, followed by the oxygen consumption shifting, ATP levels maintenance and redox state (NAD/NADH ratio) homeostasis. Its expression level is also vital to the optimal transition of *Mtb* from the anaerobic non-replicating state back to aerobic growth state [Leistikow et al., 2009]. (2) menaquinone [Honaker et al., 2010]: the expression levels of DosR regulon are proportional to the amount of menaquinone. Menaquinone is central to the electron transport system (ETS) and provides a mechanistic link between the respiratory state of the bacilli and DosS signaling. During anaerobic dormancy, the amount decreases in menaquinone levels likely causes the gradual decrease in DosR regulon expression. During hypoxia, compounds that preclude electron flow into the menaquinone pool or decrease the size of the menaquinone pool can attenuate the induction amplitude of the regulon, however, exogenous menaquinone can increase the regulon expression during hypoxia. (3) Other conditions such as GSNO (NO donor), ethanol, and H_2O_2: Microarray data show that the expression levels of dosR regulon are different when exposure to GSNO, ethanol, or H_2O_2 after 30 min. The treatment with H_2O_2 results in relatively lower expression than with the other agents, suggesting H_2O_2 is not the optimal induce condition [Kendalla et al., 2003]. The DosR regulon is regulated by the response regulator DosR. PhoP(Rv0757) can restore the DosR regulon to basal level during aerobic growth too [Gonzalo-Asensio et al., 2008; Honaker et al., 2009].

The insights of activation mechanism which DosR adopts would facilitate the development of more potential inhibitors against this target, which should be a very intriguing field. The activation of DosR transcriptional factor is modulated by DosS and DosT. These two histidine kinases with different functions are susceptible to the redox state and the oxygen levels, respectively. DosT responds to hypoxia first, which can autophosphorylate at the 54th aspartate and then transfer phosphate to the aspartate residue of DosR in order to activate it [Roberts et al., 2004], followed by the activation of DosS, a member of the DosR regulon that can further amplify the regulon. As oxygen becomes limited, DosT loses its function, DosS maintains the induction state of DosR regulon alone [Honaker et al., 2009; Kim et al., 2010]. Upon exposure to 0.2% oxygen 2 h, the expression level of DosR regulon in △dosS and △dosT single mutants reduced to 40–45% of the normal level. The regulon induction is abolished in bacteria lack both sensors. These results demonstrate that both DosS and DosT are required for full activation of DosR under hypoxia. In brief, DosS and DosT of *Mtb* play different role in hypoxia: DosT is a hypoxia sensor which can respond to the decrease of oxygen tension more sensitively and strongly than DosS, whose function is a redox sensor. Under aerobic conditions, DosR regulon could be induced by DosS not DosT after the addition of ascorbate, a powerful cytochrome *c* reductant, indicating that DosS can respond to redox signal even in high oxygen tension [Honaker et al., 2010]. This might results from their different autooxidation rates due to the different hemes containing GAF-A. This sentence should be replaced with In addition, DosR is a substrate of Ser/Thr kinase PknH, which can phosphorylate DosR on the Thr^{198} and Thr^{205} sites[Chao et al., 2010]. It is speculated that phosphorylation of DosR Thr and Asp by PknH and DosS, respectively, can enhance the binding of DosR to cognate DNA sequences.

DosR itself contains all the conserved residues, including Asp8 (D8), Asp9 (D9), Asp54 (D54), Thr82 (T82), Tyr101 (Y101), and Lys104 (K104). D8 and D9 residues, together with D54 and

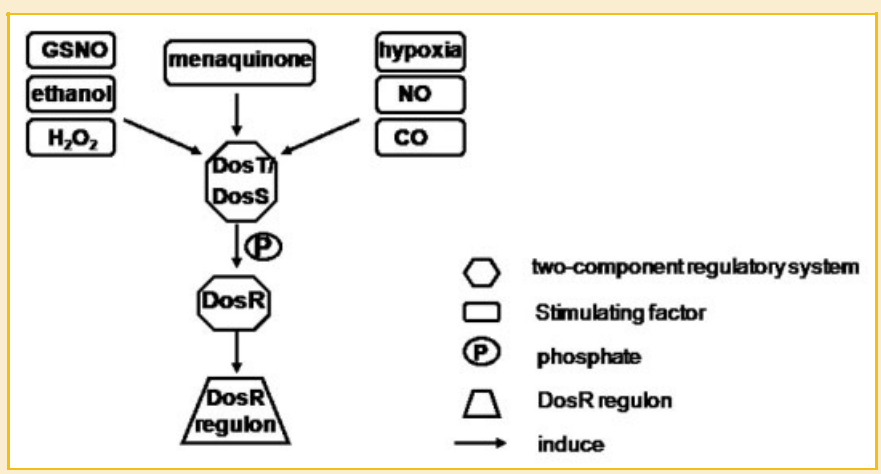

Fig. 2. The inducing factor of DosR regulon in vitro and its activation process.

coordinate Mg^{2+}, are functionally important for DosR phosphorylation. It has been established the essential function of Thr82 residue in the activation of DosR by in vivo and in vitro approaches, including the phosphorylation of DosR, cooperative binding with the DNA promotors and subsequent autoregulation and the activation of DosR regulon. The ability of autoregulation and the activation of DosR regulon in *Mtb* expressing a DosR T82A mutant protein is very defective, which results from the slow and partial phosphorylation and the disability of T82A mutant protein binding with DNA [Gautam et al., 2011].

The regulation of DosR regulon expression is very complex. There are multiple regulatory sequences upstream of Rv3134, immediately upstream of dosR. If such a regulatory sequence is lost in a complemented strain, the dysregulation of dosR and poor complementation would occur [Leistikow et al., 2010]. The DosR expression level is also related to bacteria virulence [Badillo-Lopez et al., 2010]. The expression of DosR in the exponential growth phase M.W/Beijing increases 17 times and 195 times, respectively, compared with H37Rv and *Mycobacterium canettii*. *Mtb* H37Rv and *M. canettii* dosR expression were upregulated under hypoxia, while DosR expression in *Mtb* Beijing is lowered, about 53- and 25-fold of the above two strains [Badillo-Lopez et al., 2010]. Interestingly, DosR regulon expression in W/Beijing strain is constitutive under normal conditions, somewhat relevant to a natural frameshift mutation in the DosT gene [Fallow et al., 2010]. However, role of dosT mutation in the constitutive DosR regulon phenotype is controversial [Honaker et al., 2010]. DosT mutation can attenuate DosR regulon expression under certain conditions. Abnormal metabolism might constitutively reduced ETS, accordingly the overexpression of the regulon through DosS. Duplications of some other gene within this strains, specifically the *nuo* gene cluster encoding the type I oxidoreductase, and the succinate dehydrogenase gene, lend additional creditability to this hypothesis [Honaker et al., 2010]. Both enzyme clusters are involved in the ETS. The capability of W/Beijing strains to constitutively overexpress dosR regulon prior to stimuli such as NO might be the secret for mycobacterium's super surviving ability [Fallow et al., 2010]. The poor protection offered by BCG vaccination against W/Beijing strain might be stemmed from this unique feature.

THE STRUCTURE OF DosR REGULON

DosS and DosT each contains two tandem GAF domains that are responsible for detecting oxygen tension at the N-terminal sensory domain and a histidine kinase-like ATPase (HATPase) domain at the C terminus [Cho et al., 2009]. The overall structure of GAF consists of 5-stranded anti-parallel β-sheets and four α-helices. The α-helices are named as α1 to α4 from N terminus to C terminus. The long anti-parallel helices α1 and α4 locate on the same side of the sheet, while the short one α3 on the opposite side. A pocket formed by the helix α3 and the β-sheet could accommodate the heme which directly binds O_2, NO, and CO. Virtually, the heme locates in a orthogonal orientation with regard to the twisted surface of the β-sheet, and it is shielded from the solvent by short helix α2 and α2–α3 connecting loop [Podust et al., 2008].

The full length DosR structure is composed of N-terminal domain (residues 1–97), C-terminal domain (residues 150–210) and the linker region (residues 98–149), the three domains are all topologically different from other NarL subfamily members. The N-terminal domain of DosR contains an α/β fold with a $(\beta\alpha)_4$ arrangement unlike the canonical $(\beta\alpha)_5$ arrangement. The C-terminal domain contains four α-helices named α7, α8, α9, and α10. This domain has an unusual conformation where the first α-helix in the N-terminal domain packs against helix α10 which moves away from the core constructed by helices α7 to α9. The linker region is consisting of helices α5 (residues 101–121) and α6 (residues 127–143), helix α6 has high flexibility. In dimer, two helices of the linker region form a four-helix bundle with two helices from another subunit, and α10 and α7–α8 loop of C-terminal domain in two subunits have a dimerization. A sulfate molecule is found buried in the dimer interface. DosR can forms a tetramer by the dimerization of C terminus. In the DosR-DNA complex, DosR interacts with DNA at Lys179, Lys182, and Asn183 [Wisedchaisri et al., 2005, 2008].

DosR molecule conformation changes upon the transfer of a phosphoryl group from histidine kinases DosS or DosT to Asp54 residue in the N-terminal domain. Rearrangement activation might underlie helices α4, α5, and α10 [Wisedchaisri et al., 2008]. Phosphorylation of the catalytic Asp54 presumably leads to a series of conformational change. First, helix α10 is precluded from binding to the N-terminal domain due to the steric hindrance between the phosphorylated Asp54 and Gln199 side chains. Second, the N-terminal domain conformation changes after phosphorylation. The change is that the $(\beta\alpha)_4$ domain and α5–α6 linker will rearrange and become the canonical $(\beta\alpha)_5$ fold. Third, the conserved Thr82 side chain may not interact with the C-terminal domain and form a hydrogen bond with phosphorylated Asp54 [Wisedchaisri et al., 2008].

IMMUNOLOGICAL FEATURE OF DosR REGULON

DosR regulon exert an important role in the persistence and intracellular survival of *Mtb* and are targeted by the immune system during latent infection in humans. The upregulation of DosR might be involved in the virulence of the strains. The mutants were attenuated in mice, guinea pig, and rabbit model. No discernable growth or survival effect can be found for DosR regulon gene *Rv1996*, *Rv2005c*, and *Rv2028c* mutants under stress conditions. However, *Mtb* mutant within the gene encoding the response regulator DosR *Rv3133c* is defective for survival under hypoxia, a dramatic reduction about 10,000-fold [Boon and Dick, 2002]. This phenotype holds true for *M. bovis* BCG and *M. smegmatis* [Hingley-Wilson et al., 2010]. However, the experimental data concerning the virulence of the mutants are conflicting. No distinguishable colony forming units (cfu) and histopathology difference can be spotted between dosR mutant and the parent strain H37Rv when C57BL/6, DBA2, and C3He/FEJ mice were challenged, respectively [Rustad et al., 2008]. The underlying cause for this discrepancy needs to be determined yet.

The immunogenicity of eight DosR regulon-encoded antigens, namely *Rv1733c*, *Rv1738*, *Rv2029c (pfkB)*, *Rv2031c/hspX (acr)*, *Rv2032 (acg)*, *Rv2626c*, *Rv2627c*, and *Rv2628*, has been explored in BALB/c and C57BL/6 mice [Roupie et al., 2006]. The DNA vaccination of Rv2031c and Rv2626c can elicit the strongest Th1-type responses. The immune responses against Rv1733c, Rv2031c, and Rv2626c are also induced in BALB/c and (B6D2)F1 mice after *Mtb* challenge. DosR regulon antigens were found to be preferentially recognized by T-cell in latent TB patients instead of active TB cases. Immune responses against DosR regulon also exist in latent TB patients, suggestive of partial protection for host to repress the *Mtb* [Demissie et al., 2006; Leyten et al., 2006]. In contrast, T-cell responses against DosR regulon-encoded antigens are very low in BCG-vaccinated mice and humans, possibly emphasizing the importance of unrelenting low dose stimulation from the dormancy pathogen released antigens. The immune responses to these latent antigens might be harnessed to promote the natural protection against TB, and help to control latent *Mtb* infection.

T-cell can response to three DosR antigens, namely Rv1733c, Rv2029c, Rv2031c, the top 10 most frequently recognized *Mtb* DosR proteins in *Mtb*-exposed individuals [Black et al., 2009; Commandeur et al., 2011; Leyten et al., 2006; Schuck et al., 2009]. Strong DosR antigen-specific single and double functional $CD4^+$ and $CD8^+$ T-cell responses are detected in LTBI. The highest responses are occurring among the single cytokine producing $CD4^+$ and $CD8^+$ T-cell subsets, and then is the double producing $CD4^+$ and particularly $CD8^+$ T-cells. The majority of multiple cytokine producing T-cells are IFN-γ^+TNF-$\alpha^+$$CD8^+$ T-cells, which belong to effector memory ($CCR7^-$ and $CD45RA^-$) or effector ($CCR7^-$ and $CD45RA^+$) T-cells [Bruns et al., 2009]. These findings can facilitate screen for better vaccine components. However, above studies did not consider the significant homology of these antigens between pathogenic and environmental Mycobacterium. For vaccine components inclusion, this consideration is indispensable.

THE RESPONSE OF BACTERIA TO ENDURING HYPOXIA STRESS

Transcriptional profiling [Rustad et al., 2008] reveals that the induction of the DosR regulon in hypoxia in vitro is transient, as the expression of nearly half of the regulon genes would return to baseline within 24 h, followed by a significant additional transcriptional response consisting of almost 230 genes strongly induced at 4 and 7 days. This long-lasting response is named as enduring hypoxic response (EHR), which is both more extensive and more stable than the DosR response, and is largely independent of the DosR regulon. Further study in more depth of both the EHR and the initial hypoxic response controlled by DosR are needed to elucidate the mechanisms of MTB to enter, persist in and exit from the latency.

The past several years have witnessed the intensive exploration in the patterns of expression and the regulatory of the DosR system. However, the detailed mechanisms, such as the link between DosR regulon expression and virulence or persistence, remained to be determined.

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