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***Mycobacterium tuberculosis mtrA* merodiploid strains with point mutations in the signal-receiving domain of MtrA exhibit growth defects in nutrient broth**

Maha Al Zayer[¶], Dorota Stankowska[¶], Renata Dziedzic, Krishna Sarva, Murty V. Madiraju, and Malini Rajagopalan^{*}

Biomedical Research, The University of Texas Health Science Center at Tyler, Tyler, TX- 75708

Abstract

The genetic and biochemical aspects of the essential *Mycobacterium tuberculosis* MtrAB two-component regulatory signal transduction (2CRS) system have not been extensively investigated. We show by bacterial two-hybrid assay that the response regulator (RR) MtrA and the sensor kinase MtrB interact. We further demonstrate that divalent metal ions [Mg(2+), Ca(2+) or both] promote MtrB kinase autophosphorylation activity, but only Mg(2+) promotes phosphotransfer to MtrA. Replacement of the conserved aspartic acid residues at positions 13 and 56 with alanine (D13A), glutamine (D56E) or asparagine (D56N) prevented MtrA phosphorylation, indicating that these residues are important for phosphorylation. The MtrA_{D56E} and MtrA_{D13A} proteins bound to the promoter of *fbpB*, the gene encoding antigen 85B protein, efficiently in the absence of phosphorylation, whereas MtrA_{D56N} did not. We also show that *M. tuberculosis mtrA* merodiploids overproducing MtrA_{D13A}, unlike cells overproducing wild-type MtrA, grow poorly in nutrient broth and show reduced expression of *fbpB*. These latter findings are reminiscent of a phenotype associated with MtrA overproduction during intramacrophage growth. Our results suggest that MtrA_{D13A} behaves like a constitutively active response regulator and that further characterization of *mtrA* merodiploid strains will provide valuable clues to the MtrAB system.

Keywords

Mycobacterium tuberculosis; response regulator; signal transduction; mutants; merodiploids; real-time PCR; EMSA

1. Introduction

The survival of *Mycobacterium tuberculosis*, the causative agent of tuberculosis, upon infection is believed to depend, in part, on the activity of several regulatory networks, one of which is a paired histidine-aspartate two-component regulatory system (2CRS). The 2CRS includes a sensor histidine kinase (which is a membrane-bound protein) and a response regulator protein (which is often a cytosolic protein) [reviewed (Hoch, 2000; Stock et al.,

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^{*}Address for Correspondence: murty.madiraju@uthct.edu.

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2000)]. Upon detecting an environmental signal, sensor kinases undergo autophosphorylation by transferring the γ -phosphoryl group from ATP to a highly conserved histidine residue located in its transmitter domain. The phosphoryl group is then transferred to the regulatory domain of the cognate RR by a phospho-relay process. This, in turn, results in the activation of the output domain of the RR, which usually functions as a transcriptional regulator [reviewed in (Hoch, 2000; Stock et al., 2000)]. Phosphorylation of an RR often leads to its dimerization, although some RRs such as CheA remain monomeric.

The *M. tuberculosis* genome contains 11 paired 2CRSs; however, MtrAB is the only essential 2CRS (Cole et al., 1998; Via et al., 1996; Zahrt and Deretic, 2000). The *mtrB* gene of *M. avium* is implicated in the regulation of cell wall permeability and the colony-switch phenotype (Cangelosi et al., 2006). The MtrAB system of *Corynebacterium glutamicum* is implicated in the osmo-stress response (Moker et al., 2004; Moker et al., 2007). Recent studies indicate that elevated intracellular levels of phosphorylation-competent MtrA are detrimental for *M. tuberculosis* growth in macrophages (M μ) (Fol et al., 2006), whereas elevated levels of *mtrB* along with *mtrA* reverse the growth defect, indicating that one role of MtrB kinase is to help regulate the phosphorylation status of MtrA. Presumably, it is the ratio of MtrA to MtrA~P, rather than their absolute levels, that is critical for optimal proliferation. These studies also revealed that the *dnaA* promoter is MtrA target (Fol et al., 2006). The above studies assumed that MtrB kinase phosphorylates MtrA and that signals for MtrA phosphorylation are enriched in the intracellular growth environment. Other recent studies identified MtrA motifs in the replication origin (or *oriC*) and the promoter for Ag85B (Rajagopalan et al., 2010), confirmed *dnaA* promoter is MtrA target (Li et al., 2010), and that the gene product encoded by *lpq*, which is located immediately downstream of *mtrB*, interacts with MtrB and possibly regulates its phosphorylation status (Nguyen et al., 2010).

Although the above studies suggest that the MtrAB system plays an important role in *M. tuberculosis* survival, several key questions remain with respect to the biochemical properties of the MtrAB system. For example, do MtrA and MtrB proteins interact and act as an intact signal transduction pair? Does MtrB exhibit autophosphorylation and phosphotransfer activities? What are the residues in MtrA that are critical for phosphorylation? Does a MtrA protein defective for phosphorylation interact with its targets proficiently? Finally, do MtrA merodiploid strains producing altered MtrA phosphorylation activity exhibit growth defects in the absence of phosphorylation activation signals? It is hoped that the answers to these questions as well as the identification of the conditions that cause MtrA to be constitutively active for phosphorylation could provide valuable insights into MtrA function.

2. Materials and methods

2.1 Strains and bacterial growth conditions

Escherichia coli strains were grown in Luria-Bertani (LB) broth or LB agar supplemented with ampicillin (Amp, 50 $\mu\text{g ml}^{-1}$), hygromycin (Hyg, 200 $\mu\text{g ml}^{-1}$) or ampicillin and 0.2% glucose. *M. tuberculosis* strains were propagated in Middlebrook 7H9 broth supplemented with OADC (oleic acid, albumin, dextrose, catalase and sodium chloride), 0.05% Tween 80 and hygromycin (Hyg at 50 $\mu\text{g ml}^{-1}$). Growth was monitored by measuring absorbance at 600 nm.

2.2 Mutagenesis and construction of *mtrA* merodiploid strains

For overproduction of MtrA in *M. tuberculosis*, we used the integration-proficient plasmid pJfr19 and placed the *mtrA* coding region downstream of the constitutively active

acetamidase promoter as an *NdeI-XbaI* fragment, as previously described (Fol et al., 2006). Unless otherwise noted, PCR amplifications were carried with high fidelity deep vent DNA polymerase and genomic DNA as a template to replace the aspartic acid residues at the 13th and 56th positions. The primer combination MtrAD10A-F and MVM410-R was used to replace the aspartic acid at the 13th codon with alanine to create plasmid pMZ3, whereas the primer combinations MVM409-F-MVM410-R and D53E(F)-D53(R) were used to replace the aspartic acid at the 56th codon with glutamic acid, using an overlay PCR technique to create plasmid pDS3. Primers MtrAD10A-F and MVM410-R and template pMG129 (Fol et al., 2006) were used to replace the aspartic acids at codons 13 and 56 with alanine and asparagine, respectively, to create pMZ5, whereas the primer combination MVM-409-F-MVM-410R and the template pDS3 were used to replace the aspartic acids at codons 13th and 56th with alanine and glutamic acid, respectively, to create pDS6. All PCR products were sequenced to confirm sequence identity. The recombinant plasmids pMZ3, pMZ6, pMZ5, pDS3 and pDS6 were stably integrated at the *attB* locus of *M. tuberculosis* by electroporation to create the merodiploid strains Rv-MZ3, Rv-MZ5, Rv-DS3 and Rv-DS6, respectively. The sources of the *M. tuberculosis* *mtrA* merodiploid strain overproducing wild-type MtrA (Rv-78) and the phosphorylation-defective MtrA (Rv-129) were previously reported (Fol et al., 2006).

2.3 Purification of recombinant wild-type and mutant MtrA, MtrB and EnvZ proteins

For the overproduction and purification of recombinant wild-type and mutant MtrA proteins, respective *mtrA* coding regions were cloned into the pET-19b vector (Novagen) and transformed into the *E. coli* expression strain Arctic express (DE3) RIL (Stratagene). For protein overproduction, cultures were grown to an OD₆₀₀ of 0.6 and protein production was induced with 1 mM isopropyl-β-d-thiogalactopyranoside for 20 h at 10°C. MtrA proteins were purified under soluble conditions on Ni-NTA affinity columns (Qiagen) essentially following the manufacturer's recommendations. A truncated *mtrB* gene lacking the first 233 codons from the 5' end was amplified using the primers MVM877-F and MVM878-R and then cloned as a fusion to gene for maltose binding protein. A recombinant plasmid expressing the MalE-EnvZ construct was a generous gift from Dr. M. Igo, University of California at Davis, CA. Soluble MtrB and EnvZ were purified as MalE-MtrB and MalE-EnvZ fusion proteins, respectively, from *E. coli* strain (DE3) RIL on amylose affinity columns following the manufacturer's instructions (NEB). MtrA, MtrB and EnvZ proteins were dialyzed for 4 h in storage buffer (20 mM sodium phosphate, 150 mM NaCl, 20% glycerol, 0.1 mM EDTA, and 1 mM DTT) and stored at -80°C.

2.4 Bacterial two-hybrid (BACTH) assay

Bacterial Adenylate Cyclase-based Two-Hybrid (BACTH) constructs were produced using the primers described in Table 1. The BACTH assay was carried out essentially as described by Karimova et al. (Karimova et al., 2005). *E. coli* BTH101 recombinants with *mtrA* or *mtrB* plasmids or with control plasmids were selected on MacConkey agar supplemented with 100 mg/ml Amp and 50 µg/ml Km at 30°C for 24 to 36 h. For β-galactosidase activity measurements, cells grown in LB broth were permeabilized with 0.1% toluene and 0.01% sodium dodecyl sulfate, mixed with an equal volume of PM2 buffer (70 mM Na₂HPO₄, 30 mM NaH₂PO₄, 1 mM MgSO₄ and 0.2 mM MnSO₄, pH 7.0) and 100 mM β-mercaptoethanol. The reaction was started by the addition of 0.25 ml of 0.4% O-nitrophenol-β-galactoside (ONPG) in PM2 buffer, and the tube was incubated at 28°C for 5 min or until a visible yellow color developed. The reaction was stopped by the addition of 0.5 ml of 1 M Na₂CO₃, and the OD₄₂₀ was recorded. The enzymatic activity was defined as units per milliliter: $200 \times [(OD_{420} \text{ of the culture} - OD_{420} \text{ in the control tube}) / \text{minutes of incubation}] \times \text{dilution factor}$. The specific activity of β-galactosidase is defined as units/mg dry weight bacteria, and 1 unit = 1 nmol of ONPG hydrolyzed per min at 28°C. At least 5-

fold higher b-galactosidase activity than that measured for BTH101 carrying a single gene and an empty vector was considered indicative of an interaction.

2.5 Autophosphorylation of MtrB and phosphotransfer to MtrA

MtrB autophosphorylation reactions (2 μ M) were carried out in phosphorylation buffer (PB) containing 50 mM Tris-HCl, pH 7.5, 50 mM KCl and 1 mM DTT supplemented with 20 mM MgCl_2 (Mg^{2+}), 10 mM CaCl_2 (Ca^{2+}) or both (Mg^{2+} and Ca^{2+}). The reactions were initiated by adding ^{32}P -ATP and incubated for 2, 5, 10, 20 and 30 min at 37°C. The samples were removed, diluted with SDS-PAGE sample buffer and resolved in a 12% polyacrylamide gel under denaturing conditions, and protein bands were visualized using a BioRad Molecular Imager.

Phosphotransfer reactions using 2 μ M MtrB were carried out in PB buffer supplemented with 20 mM MgCl_2 or 10 mM CaCl_2 plus 20 mM MgCl_2 . MtrB autophosphorylation was initiated by adding ^{32}P -g-ATP; this was incubated at 37°C for 30 min and subsequently used in phosphotransfer reactions with 3 μ M MtrA for 2 and 10 min. The samples were removed, diluted with SDS-PAGE sample buffer and resolved in a 12% polyacrylamide gel under denaturing conditions, and protein bands were visualized using a BioRad Molecular Imager.

2.6 Phosphorylation of MtrA by EnvZ Kinase

EnvZ kinase was phosphorylated for 5 min in the phosphorylation buffer described above. An aliquot of 2.5 μ M phosphorylated EnvZ was mixed with 4 μ M of either MtrA, MtrA_{D13A}, MtrA_{D56E}, MtrA_{D10AD56E}, MtrA_{D13AD56N} or MtrA_{D56N}, incubated for 15 min, resolved by SDS-PAGE, dried, exposed to a phosphoimager screen and then scanned in a Bio-Rad Molecular Imager.

2.7 Protein - DNA binding assay

EMSA experiments to detect MtrA binding to *PfhpB* were carried out using FITC-labeled promoter in buffer containing 50 mM Tris-HCl pH 7.0, 50 mM sodium chloride, 10 mM magnesium chloride, 10 mM calcium chloride, 1 mM DTT, 0.1 mM EDTA, 5% glycerol, 0.01% NP-40, 0.05 ng/ μ l sheared salmon sperm DNA, 250 nM poly dI/poly dC, 100 nM FITC-labeled *PfhpB* and 0.5 to 5 μ M phosphorylated or non-phosphorylated MtrA as described (Rajagopalan et al., 2010). The samples were incubated for 15 min at 37°C and resolved by 5% TAE native gel electrophoresis (120 volts, 4C, 40 min). MtrA protein was phosphorylated by EnvZ in the presence of 20 mM cold ATP in PB for 15 min at 37°C. FITC-labeled *PfhpB* was prepared by amplification using the cloned *PfhpB* promoter and FAM-labeled T7 universal and Sp6 primers.

2.8 High performance gel filtration chromatography

MtrA proteins were separated on Superdex 200 10/300 GL (GE) with optimal resolution in the molecular weight range from 1.3 – 669 kDa. The column was equilibrated with buffer containing 50 mM Tris-HCl, 50 mM KCl, 20 mM MgCl_2 and 1 mM DTT. Approximately 70 μ g of MtrA protein was injected and resolved. BioRad gel filtration standards (Cat #151–1901) containing thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.3 kDa) were resolved, and their peak elution volumes plotted in a standard curve of V_e/V_o (elution volume/void volume) versus the log of molecular masses of proteins from gel filtration standards. Using this curve, the molecular size of MtrA protomer species was calculated.

2.9 RNA extraction and quantitative real-time PCR

Extraction of total RNA from broth-grown cultures of *M. tuberculosis* incubated for different periods of time was performed as described previously (Fol et al., 2006; Nair et al., 2009). DNA contamination was removed by treatment with DNase I (Ambion). cDNA was synthesized using 100 nM RT16S3 with RTAg85A2 primers and Superscript II reverse transcriptase (Invitrogen). Real-time PCR (Taqman chemistry) was performed in a Bio-Rad IQ5 Cyclor using Taq DNA polymerase (NEB), the Taqman probes 16S-TP and 85B-TP (Biosearch Technologies), and reverse and forward primers (see Table 1). The calculated threshold cycle (Ct) value for each gene of interest was normalized to the Ct value for the 16S, and the fold expression was calculated using the formula: fold change = $2^{\Delta(\Delta C_t)}$ (Fol et al., 2006; Nair et al., 2009). No reverse transcriptase (RT) reactions were included as negative controls. Expression data are the average from at least three independent RNA preparations, each reverse-transcribed and quantified by real-time PCR in triplicate. The real-time PCR conditions include initial denaturation at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing and extension at 60°C for 1 min.

3. Results and discussion

3.1 MtrB interactions with MtrA

A BACTH assay was used to evaluate interactions between the *mtrA* and *mtrB* gene products (Karimova et al., 2005). In these assays, the *mtrA* and *mtrB* genes were cloned as fusions to the T25 and T18 fragments of adenylate cyclase in two separate vectors and transformed into the reporter *E. coli* strain BTH101 (Karimova et al., 2005). Functional complementation of adenylate cyclase activity due to interactions between the partners leads to cAMP production and subsequent transcription of the *lac* reporter gene, which gives a distinct color to colonies growing on indicator agar plates. The strength of the interaction was also measured by assaying for b-galactosidase activity. As can be seen, transformants expressing *mtrA* from both vectors were blue, indicating a self-interaction similar to that observed when *gcn4* is expressed from both vectors (Fig. 1A). Similarly, transformants expressing *mtrA* and *mtrB* from two different vectors were also blue, whereas those expressing *mtrA* plus a control vector or carrying two control vectors, pUT18C and pKNT25, were not (see Fig. 1). We also found that transformants expressing *mtrB* from both pUT18C and pKNT25 were not blue. Presumably, the self-interaction between MtrB protomers could be facilitated by their phosphorylation status, and signals that promote MtrB phosphorylation could be missing in the assay system used here. The extent of interaction between these proteins was evaluated by measuring the amount of b-galactosidase activity produced (Fig. 1B). Consistent with the observed interactions, constructs expressing *mtrB-mtrA* or *mtrA-mtrA* yielded b-galactosidase activity comparable to that of the positive control *gcn4-gcn4*. As expected, white transformant colonies did not show b-galactosidase activity (Fig. 1).

3.2 MtrB autophosphorylation and transphosphorylation activities

Histidine-aspartate sensor kinases exhibit autophosphorylation and transphosphorylation activities (Hoch, 2000). Our earlier studies showing that overexpression of *mtrB* along with *mtrA* reverses growth defects associated with *mtrA* overexpression in macrophages support the notion that MtrB kinase regulates the phosphorylation potential of MtrA RR (Fol et al., 2006). These studies assumed but have not proven that MtrA and MtrB work as an intact signal-transducing pair. To address this issue, we evaluated the autophosphorylation and transphosphorylation activities of MtrB kinase. MtrB is a 567-residue membrane-binding protein with two potential transmembrane domains (residues spanning 42–62 and 213–233), a HAMP linker (residues 215–284) and a His Kinase A phosphoacceptor (residues 302–519) domain (Aravind and Ponting, 1999; Bilwes et al., 1999). Hence, to evaluate

phosphorylation activity, we amplified a fragment encoding a soluble derivative of the protein spanning residues 234 to the end, cloned it as a fusion to Maltose Binding Protein, purified the recombinant protein on amylose resin and characterized it. Initial phosphorylation experiments were performed in buffers containing the divalent cations Mg^{2+} , Ca^{2+} or Mg^{2+} plus Ca^{2+} (Fig. 2A). A time-dependent increase in MtrB phosphorylation was noted with all metal ions (Fig. 2, panels i and ii). Densitometric analysis revealed that buffers containing Mg^{2+} plus Ca^{2+} showed higher levels of autophosphorylation activity compared to buffer containing Mg^{2+} alone (Fig. 2A-ii). We noted that at longer incubation times, i.e., 10 min and beyond, MtrB protein aggregated in buffers containing Ca^{2+} (data not shown).

Next, we examined the phosphotransfer activity of MtrB. In these experiments, MtrB was first phosphorylated by incubating in the presence of ^{32}P -ATP for 30 min followed by the addition of MtrA. Controls received buffer in place of MtrA. Samples were removed after 2 and 10 min of contact with MtrB. As a positive control, EnvZ protein was phosphorylated for 5 min and then incubated with MtrA. Consistent with the earlier published data (Fol et al., 2006), incubation of radio-labeled ATP with EnvZ led to MtrA phosphorylation (Fig. 2B, lane marked MtrA+EnvZ). Similar to EnvZ, MtrB kinase also promoted phosphorylation of MtrA in buffer containing Mg^{2+} , but not in buffer containing both Mg^{2+} and Ca^{2+} . These results indicate that although the divalent metal ion Ca^{2+} promotes MtrB phosphorylation, it interferes with MtrB transphosphorylation activity. This intriguing result signals a possibility that the phosphorylation potential of MtrB kinase could be regulated by MtrB interactions with divalent Ca^{2+} ions. This could in turn dictate the level and extent of MtrA phosphorylation and possibly the signal output. This finding may have important implications for understanding *Mtb* pathogen survival in vivo. For example, it is believed that *Mtb* survival upon infection is critically dependent on the metal ion composition of the intraphagosomal environment (Wagner et al., 2005a; Wagner et al., 2005b). Reports indicate that the metal ion composition of vacuoles containing pathogenic and nonpathogenic mycobacterial species differs (Wagner et al., 2005a). It is unknown whether divalent ions other than Ca^{2+} interfere with MtrB transphosphorylation activity. Presumably, the ratio of Ca^{2+} to other metal ions influences the signal output of the MtrAB system during intracellular growth. Alternatively, in the presence of excess Ca^{2+} , MtrB~P could phosphorylate other RR proteins and/or perform functions independently of MtrA. These possibilities are not mutually exclusive. Further studies are required to address these issues.

3.3 Amino acid residues important for MtrA phosphorylation

Mutagenesis studies with several bacterial RRs have shown that replacement of the conserved D residues with select amino acids modulates RR phosphorylation potential and associated phenotypes (reviewed in (Hoch, 2000; Stock et al., 2000)). Such replacements in some cases could lead to constitutive activation of the RR. Constructing and characterizing MtrA proteins with altered phosphorylation properties that potentially influence MtrA activity, and hence *M. tuberculosis* survival, could provide valuable insights into the MtrAB signal transduction system. Recent MtrA crystal data indicate that D13 and D56 are conserved residues in the receiver domain (Friedland et al., 2007). To begin addressing this issue, we initially focused on the D residues at the 13th and 56th positions, overproduced mutant proteins, purified them as His-fusion proteins and determined their phosphorylation activity. Unlike wild-type MtrA, all the mutant proteins failed to be phosphorylated by the EnvZ kinase (Fig. 3). These results confirm that the D residues at positions 13 and 56 are important for MtrA phosphorylation (Fig. 3). Presumably, replacement of these aspartic acid residues with alanine, glutamine or asparagine inactivates MtrA protein for phosphorylation or keeps it in a state that no longer accepts the phosphate group. The latter possibility

implicates that the mutant proteins behave differently compared to the wild-type protein with respect to their ability to bind targets and possibly modulate target gene transcription.

3.4 Interaction of MtrA proteins with the *fbpB* promoter

One consequence of RR phosphorylation is activation of its output domain that promotes RR binding to DNA and modulates target gene expression (Hoch, 2000). We recently demonstrated that promoters for the secreted antigen 85B (*PfbpB*) and *dnaA* (*PdnaA*) and the origin of replication (*oriC*) are bona fide MtrA targets and that phosphorylated MtrA binds preferentially to *PfbpB* and *oriC* (Rajagopalan et al., 2010). Accordingly, we evaluated MtrA binding to *PfbpB* by EMSA. Preliminary experiments revealed that MtrA_{D13AD56N} and MtrA_{D13AD56E} proteins aggregated during incubation with DNA, and thus, the DNA-protein complexes remained in the wells (not shown). Consequently, EMSA experiments were carried out with MtrA_{D56E}, MtrA_{D13A} and MtrA_{D56N} (Fig. 4). As expected, MtrA and its phosphorylated derivative MtrA~P (produced by incubation with EnvZ and ATP) bound to *PfbpB* and retarded its mobility, although MtrA~P resulted in greater retardation at all concentrations tested (Fig. 4). MtrA_{D13A} bound *PfbpB* both in the presence and absence of EnvZ and ATP; however, binding appeared to be favored in the absence of ATP and EnvZ. Binding under these conditions was as proficient as that with wild-type MtrA in the presence of ATP and EnvZ (Fig. 4).

MtrA_{D56E} showed significant binding, but only in the absence of ATP and EnvZ (Fig. 4). Also, two shifted bands were detected at some protein concentrations (see Fig. 4, lanes marked 2 and 3 corresponding to no ATP and EnvZ). On the other hand, phosphorylation-defective MtrA_{D56N} did not bind *PfbpB* (Fig. 4). Presumably, the ability of MtrA to undergo phosphorylation could be critical for its interaction with the target and that replacement of the conserved aspartic acid residue with asparagine made the protein inert for interaction with its target. On the other hand the analogous D56E mutation at the phosphorylation site in other RR that have been investigated, i.e. NtrC, OmpR and CtrA, mimics aspartate-phosphoryl state, activates RR and bypasses the requirements for the cognate sensor kinase (Domian et al., 1997; Klose et al., 1993; Lan and Igo, 1998). Phosphorylation of RR often enhances its DNA binding affinities and promotes cooperative binding at two adjacent recognition sequences. The *fbpB* promoter region has been predicted to have four direct MtrA repeat binding motifs (Rajagopalan et al., 2010). Thus, the appearance of two distinct shifted bands in reactions incubated in the absence of EnvZ supports the notion that D53E mutation affected protein conformation in the signal receiving domain of RR, possibly mimics phosphorylation state, hence promotes cooperative DNA-binding. The observed differences in the *PfbpB* DNA-binding between MtrA_{D13A} and MtrA_{D56E} could reflect differences in the activities of the protein preparations.

The observed differences in the binding of MtrA proteins to *PfbpB* (notably in the absence of ATP and EnvZ) could reflect changes in protein conformation. Such changes could involve protein dimerization and higher order oligomerization upon phosphorylation. This is not entirely unexpected because RRs, specifically OmpR/PhoP family proteins, often exhibit a propensity to dimerize upon phosphorylation (Bilwes et al., 1999; Hoch, 2000), and MtrA belongs to the OmpR/PhoP family of RRs. Hence, to address the consequences of mutations on protein conformation, we compared the elution profiles of the different MtrA proteins by size exclusion chromatography on a Superdex S200 column in buffers lacking ATP and EnvZ kinase. A protein standard mix containing tyroglobulin (670 kDa), g-globulin (158 kDa), ovalbumin (44 kDa) and myoglobulin (17 kDa) resolved into distinct peaks (Fig. 5, panel A). Wild-type and mutant MtrA proteins resolved into single peaks, and the positions of the peaks corresponded to monomers (Fig. 5, panels B–E). These results indicate that replacement of the D residues at 13 and 56 positions with select amino acids does not significantly affect protein conformation. MtrA incubated with ATP and EnvZ also failed to

show a significant change in peak position, indicating the absence of visible dimers and oligomers, although MtrA~P produced under these conditions showed enhanced binding to its target (Fig. 4). Presumably, the extent of MtrA phosphorylation provided by EnvZ is not sufficient to promote either dimerization or higher order oligomerization. Notably, the recent MtrA crystal studies suggest that the orientation of the regulatory and DNA-binding domains is such that it stabilizes the inactive conformation of MtrA and reduces the rate of phosphorylation and thus regulates the MtrA activity (Friedland et al., 2007).

3.5 *M. tuberculosis* mtrA merodiploid strains show growth defects in nutrient broth

Above, we showed that although MtrA_{D13A}, MtrA_{D56E} and MtrA_{D56N} proteins are each defective for phosphorylation, their interactions with their PAg85B target differ. MtrA RRs can have multiple targets, and it is likely that the mutant MtrA proteins both interact with and modulate the expression of these targets differently, which could in turn affect the growth and proliferation of the respective strains. Because *mtrA* is an essential gene, to address this possibility, we created merodiploid strains and evaluated their growth patterns in nutrient broth (Fig. 6). Consistent with our previously published data, wild-type *M. tuberculosis* (Rv-19) and *M. tuberculosis* overproducing phosphorylation-proficient (Rv-78) and phosphorylation-defective (Rv-129) MtrA merodiploids grew similarly in nutrient broth (Fig. 6A) (Fol et al., 2006). In contrast, Rv-MZ3 and Rv-DS3 merodiploids producing MtrA_{D13A} and MtrA_{D56E} grew normally during early periods but showed a growth defect at later periods and reached stationary growth early.

3.6 The expression of *fbpB* is downregulated in Rv-DS3

Earlier studies with Rv-78 merodiploids indicated that signals promoting MtrA phosphorylation are enriched during intramacrophage growth (Fol et al., 2006) and that *fbpB* expression is sharply downregulated in Rv-78 upon infection (Rajagopalan et al., 2010). It is likely that the mutant MtrA proteins defective for phosphorylation modulate the expression of target genes independent of phosphorylation signals. Accordingly, we hypothesized that the expression of MtrA target genes (including *fbpB*) could be affected in Rv-MZ3 and Rv-DS3 merodiploids relative to wild-type and Rv-78. To gain insight into this issue, we characterized one of the merodiploids (Rv-MZ3) further as a proof of principle, and we evaluated *fbpB* expression relative to 16S rRNA at 3 and 8 days of growth in both Rv-19 and Rv-78 (Fig. 6B). As shown in Fig. 6B, *fbpB* expression was downregulated 4.5- and 16.8 -fold relative to wild type at days 3 and 8, respectively, whereas it was modestly lower in Rv-78 by 1.8- and 2.5-fold at days 3 and 8, respectively, relative to wild-type.

We conclude from the above results that certain mutations in the signal-receiving domain of MtrA that impair its phosphorylation potential interfere with growth and possibly exhibit a dominant-negative phenotype. Although more detailed studies with other mutations are required, our results showing growth retardation and downregulation of *fbpB* expression in broth – grown Rv-MZ3 tend to indicate that the observed phenotypes are reminiscent of Rv-78 in macrophages. Because MtrA_{D13A} is defective for phosphorylation and binds to *PfbpB* similarly with or without EnvZ and ATP (Fig. 4), we propose that MtrA_{D13A} acts as a constitutively active protein and exerts its effects in the absence of signals that activate the MtrAB system. MtrA could have many targets, and in the current study, only its interaction with the *PfbpB* target was evaluated. It is likely that the interactions of MtrA_{D13A} with other target promoters are also modulated in vivo, thereby influencing growth. Further studies are required to evaluate this possibility. Nonetheless, our studies identified a collection of useful mutants, and their detailed characterization in vitro and in vivo could improve our understanding of the MtrAB signal transduction process.

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Fig. 1.

BACTH assays showing MtrB and MtrA interactions. *E. coli* cells producing MtrA and MtrB proteins fused to the T18 and T25 subunits of adenylate cyclase were assessed for interaction strength by quantitating beta-galactosidase activity. The graph presents the average from three independent experiments $\pm$ standard deviation. The respective *E. coli* BTH101 recombinants bearing the indicated combinations of plasmids were also spotted on LB agar containing X-gal and IPTG. Blue colonies indicate strong positive interactions.

Fig. 2.

Autophosphorylation and phosphotransfer properties of MtrB in the presence of magnesium and calcium. **A.** Enhanced autophosphorylation of MtrB in the presence calcium and magnesium. **i)** Phosphorylation reactions were carried for 2, 5, 10, 20 or 30 minutes in buffer containing 20 mM MgCl₂ (Mg²⁺), 10 mM CaCl₂ (Ca²⁺) or both (Mg²⁺ and Ca²⁺). Reactions were run on SDS-PAGE, dried, exposed to a phosphorimager screen and then scanned in a Bio-Rad Molecular Imager **ii)** Time course of phosphotransfer. Phosphorylated MtrB in panel 'i' was quantitated using QuantityOne software and data plotted. Time-dependent changes in the level of phosphorylation of MtrB in the presence of calcium and magnesium are shown. Representative data from one of three experiments are shown. **B.** Calcium ions inhibit the phosphotransfer from MtrB to MtrA. MtrB autophosphorylation was carried for 30 min in buffer containing 20 mM MgCl₂ (Mg²⁺) in presence or absence of 10 mM Ca²⁺ and transfer to MtrA examined for indicated time intervals (see Materials and Methods for details). Reactions were resolved by SDS-PAGE and gels processed as in panel A-'i'. Phosphorylation of the EnvZ control was done for 5 min.

Fig. 3.

In vitro phosphorylation activity of various MtrA proteins. Recombinant mutant MtrA proteins were purified, and approximately 3 μ M of each protein was evaluated for its ability to exhibit phosphorylation in the presence of 2 μ M EnvZ and 32 P-g-ATP. The reactions were incubated for 15 min at 37°C, the samples were resolved by SDS-PAGE, and the radioactive bands were visualized using a BioRad Molecular Imager.

Fig. 4.

EMSA showing interactions between MtrA and *PfbpB*. Approximately 100 femtomoles of FITC-labeled *PfbpB* DNA was incubated with increasing amounts of MtrA protein mixed with or without EnvZ and ATP was incubated for 15 min in the presence of buffer containing 10 mM $MgCl_2$ and 10 mM $CaCl_2$. The protein-DNA samples were resolved in 5% polyacrylamide gels and imaged on a BioRad molecular imager.

Fig. 5.

Size exclusion chromatography of recombinant MtrA proteins on Superdex 200 10/300 GL. **A.** Top graph: Elution profiles of the molecular mass standards. Bottom graph: Peak elution volumes of protein standards [thyroglobulin (670 kDa), γ -globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B₁₂ (1.3 kDa)] were plotted against the $\log kDa$. This calibration curve was used for the determination of molecular weight of MtrA proteins following gel filtration chromatography. **B–E.** Elution profiles of the MtrA proteins, wild type, D56N, D13A and D56E, respectively. Peak fractions were resolved by SDS-PAGE and coomassie stained gel is shown as inset in each panel. Arrow indicates the respective MtrA protein band. The experiment was repeated twice, and representative data are shown.

Fig. 6.

Phenotypes of *mtrA* merodiploid strains. (A). The growth of *M. tuberculosis* merodiploid strains was monitored by measuring absorbance at 600 nm. Representative data from three experiments are shown. (B) Quantitative real-time PCR analysis of *Ag85B* gene expression. cDNAs specific to the *fbpB* and 16S rRNA genes were synthesized from RNA samples from the strains Rv-19, Rv-MZ3, and Rv-78 grown in 7H9 broth for 3 or 8 days and was used to evaluate *ag85B* expression relative to 16S rRNA. The data presented are fold-change in the *ag85B* expression in Rv-MZ3 and Rv-78 relative to wild-type Rv-19 on day 3 and day 8th (see details in materials and methods). Averaged data from three independent experiments are shown. The bars indicate the standard deviation.

Table 1**PCR Primers**

Oligo name	Oligo sequence (5'→3')	Description
D10A-F	CCG GAA TTC CATATGAGGCAAAGGATTTGGTCGTT	Mutagenic forward primer for <i>mtrA</i> D13A
D53E-F	GATCTGGTGTATTGGAGTTGATGCTGCCCGGT	Mutagenic forward primer for <i>mtrA</i> D56E
MVM409F	GATTCCATATGAGGCAAAGGATTTGGTC	Forward primer for <i>mtrA</i>
MVM410R	CTCGAGTCTAGATCACGGAGGTCCGGCCTTGT	Reverse primer for <i>mtrA</i>
D53E R	ACCGGGCAGCATCAACTCCAATAACACACCAGATC	Mutagenic reverse primer for <i>mtrA</i> D56E
MVM877-F	CGGGGGTACCGATGTCGCGTCAGGTGGTGGTGCC	Forward primer for MalE–MtrB
MVM878-R	CCCAAGCTTTCAACCGCTCCACTCCGCGTG	Reverse primer for MalE–MtrB
MVM 846	TTGCTCTAGAGATGATCTTCGGCTCGCGCCG	MtrBXbaI – B2H assay
MVM 847	TTACTTAGGTACCCGACCGCTCCACTCCGCGTGCT	MtrBKpnI – B2H assay
MVM 848	TTACTTAGGTACCCGCGGAGGTCCGGCCTTGTACC	MtrAKpnI – B2H assay
MVM 849	TTGCTCTAGAGAGATGGACACCATGAGGCAAAG	MtrXbaI – B2H assay
MVM54685RTY1	TCAGGGGATGGGCCTAGCC	qRT-PCR forward primer for Ag85B
RTAg85A2	GCTTGGGGATCTGCTGCGTA	qRT-PCR reversed primer for Ag85B
MVM545A85BRTY3	GCCGGCGCCTAACGAACCTCTGC	RT primer for Ag85B
85B-TP	FAM-TCGAGTGACCCGGCATGGGAGCGT-BHQ-1	Taqman probe for Ag85B
RT16S1	GAGTGGCGAACGGGTGAGTAACA	qRT-PCR forward primer for 16S rRNA
RT16S2	CACCCACCAACAAGCTGATAGG	qRT-PCR reversed primer for 16S rRNA
RT16S3	CCGCACGCTCACAG	RT primer for 16S rRNA
16S-TP	FAM-TCCACCACAAGACATGCATCCCGTG-BHQ-1	Taqman probe for 16S rRNA