




Identification of the Thioredoxin Partner of Vitamin K Epoxide Reductase in Mycobacterial Disulfide Bond Formation

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ABSTRACT Disulfide bonds influence the stability and activity of many proteins. In *Escherichia coli*, the DsbA and DsbB enzymes promote disulfide bond formation. Other bacteria, including the *Actinobacteria*, use instead of DsbB the enzyme vitamin K epoxide reductase (VKOR), whose gene is found either fused to or in the same operon as a *dsbA*-like gene. *Mycobacterium tuberculosis* and other Gram-positive actinobacteria secrete many proteins with even numbers of cysteines to the cell envelope. These organisms have predicted oxidoreductases and VKOR orthologs. These findings indicate that such bacteria likely form disulfide bonds in the cell envelope. The *M. tuberculosis* *vkor* gene complements an *E. coli* *dsbB* deletion strain, restoring the oxidation of *E. coli* DsbA. While we have suggested that the *dsbA* gene linked to the *vkor* gene may express VKOR's partner in mycobacteria, others have suggested that two other extracytoplasmic oxidoreductases (DsbE or DsbF) may be catalysts of protein disulfide bond formation. However, there is no direct evidence for interactions of VKOR with either DsbA, DsbE, or DsbF. To identify the actual substrate of VKOR, we identified two additional predicted extracytoplasmic DsbA-like proteins using bioinformatics analysis of the *M. tuberculosis* genome. Using the five potential DsbAs, we attempted to reconstitute disulfide bond pathways in *E. coli* and in *Mycobacterium smegmatis*, a close relative of *M. tuberculosis*. Our results show that only *M. tuberculosis* DsbA is oxidized by VKOR. Comparison of the properties of *dsbA*- and *vkor*-null mutants in *M. smegmatis* shows parallels to the properties of *dsb* mutations in *E. coli*.

IMPORTANCE Disulfide bond formation has a great impact on bacterial pathogenicity. Thus, disulfide-bond-forming proteins represent new targets for the development of antibacterials, since the inhibition of disulfide bond formation would result in the simultaneous loss of the activity of several classes of virulence factors. Here, we identified five candidate proteins encoded by the *M. tuberculosis* genome as possible substrates of the *M. tuberculosis* VKOR protein involved in disulfide bond formation. We then reconstituted the mycobacterial disulfide bond formation pathway in *E. coli* and showed that of the five candidates, only *M. tuberculosis* DsbA is efficiently oxidized by VKOR in *E. coli*. We also present evidence for the involvement of VKOR in DsbA oxidation in *M. smegmatis*.

KEYWORDS Dsb proteins, VKOR, disulfide bond, mycobacteria, thioredoxin-like protein

Disulfide bond formation plays an important role in protein folding and function. In Gram-negative bacteria, the formation of disulfide bonds in proteins is directly catalyzed by enzymes that are localized to the periplasmic space. In Gram-positive bacteria, such enzymes are anchored to the cytoplasmic membrane, with their active domains located in the extracytoplasmic space. So far, all of the many bacterial enzymes identified that promote disulfide bond formation are members of the thioredoxin superfamily, containing the Cys-X-X-Cys active-site motif characteristic of this

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family. The *Escherichia coli* pathway consists of the periplasmic enzyme DsbA, which catalyzes the formation of disulfide bonds when its two active-site cysteines are joined in a disulfide bond. DsbA donates its disulfide bond to substrate proteins and in the process becomes reduced. Therefore, a cytoplasmic transmembrane protein, DsbB, oxidizes this reduced DsbA, regenerating DsbA's disulfide bond to start a new round of substrate oxidation. In this oxidative step, electrons are transferred from DsbA to DsbB's cysteines and thence to quinones and, ultimately, to oxygen under aerobic growth conditions (1).

In many bacteria, the enzyme that oxidizes DsbA has been identified as DsbB. However, a bioinformatic analysis based on the genome sequences of nearly 400 bacteria revealed a somewhat different pathway for disulfide bond formation. Bacteria such as *Actinobacteria*, *Cyanobacteria*, and some deltaproteobacteria use vitamin K epoxide reductase (VKOR) instead of DsbB, whose gene is found either fused to or in the same operon as a *dsbA* homolog. Several lines of evidence are consistent with the proposal that VKOR performs the same function as DsbB, even though the two proteins are not homologs. First, the existence of *vkor* in an operon with or fused to a *dsbA* homolog in many bacteria is suggestive. Second, we showed previously that when the *vkor* gene is cloned from *Mycobacterium tuberculosis*, it complements an *E. coli dsbB* deletion strain, restoring the oxidation of *E. coli* DsbA and disulfide bond formation (2). Third, the crystal structure of fused VKOR-DsbA from *Synechococcus* sp. shows interactions and a disulfide bond joining the two parts of the protein, consistent with a mechanism for the oxidation of DsbA similar to that for DsbB (3). Finally, a detailed comparison of the mechanism of electron transfer between *M. tuberculosis* VKOR and *E. coli* DsbA reveals strong parallels (4).

M. tuberculosis causes tuberculosis, which results in 1.5 million deaths every year, and recently, the emergence of strains resistant to multiple antibiotics has posed a great threat to the treatment of the disease. Proteins, including virulence factors as well as other exported proteins, from the *M. tuberculosis* predicted proteome contain even numbers of cysteines, which suggests that the formation of disulfide bonds might be important for the folding and functioning of these proteins (5, 6). The *vkor* gene is essential for the growth of *M. tuberculosis* (7), and the deletion of a *vkor* homolog in a close relative and oft-used, faster-growing surrogate for *M. tuberculosis*, *Mycobacterium smegmatis*, causes a severe growth defect (5). In *M. tuberculosis*, there are other potential extracytoplasmic thioredoxin homologs that might be the direct catalysts of protein disulfide bond formation and possible substrates of VKOR. These potential candidates include DsbE, DsbF, and the product of Rv2969c (which is located upstream of the *vkor* gene, Rv2968c [for the reasons mentioned above, the protein product of this gene has been named DsbA]). Both DsbE and DsbF display oxidase activity *in vitro* (8, 9). DsbA is a membrane-anchored protein and itself contains a structural disulfide bond (10–12). DsbA also shows oxidase activity *in vitro* and binds in a cysteine-dependent manner to a hexapeptide derived from the periplasmic loop of VKOR (10). Another report shows that the DsbA protein exhibits only isomerase activity *in vitro* (12). However, none of these proteins have yet to be shown to interact with *M. tuberculosis* VKOR (*MtVKOR*).

Thus, here, in addition to the three above-mentioned thioredoxin candidates, we have identified two other likely exported thioredoxin-like proteins in the *M. tuberculosis* genome by a bioinformatics approach, and we seek to identify the substrate of VKOR by reconstituting a disulfide bond pathway in an *E. coli* strain missing both its DsbB and DsbA proteins by expressing *M. tuberculosis* VKOR and each of the *M. tuberculosis* extracytoplasmic thioredoxin-like candidates. Our results show that only *MtDsbA* is efficiently oxidized by *MtVKOR*. Furthermore, the expression of *MtDsbA* and *MtVKOR* in *M. smegmatis* presents additional direct evidence for the involvement of VKOR in DsbA oxidation. A comparison of the properties of *dsbA*- and *vkor*-null mutants in *M. smegmatis* shows parallels to the properties of *dsbA* and *dsbB* mutations in *E. coli*.

RESULTS

Identification of five thioredoxin-like exported proteins of *M. tuberculosis* as potential substrates of MtVKOR. We used a bioinformatics approach to predict the DsbA-like candidates in *M. tuberculosis* (see Materials and Methods). First, we assumed that since *E. coli* DsbA (*EcDsbA*) is efficiently oxidized by MtVKOR in *E. coli*, VKOR's substrate would be a member of the thioredoxin family, as is DsbA (2). Second, we assumed that a candidate DsbA would be a protein exported to the cell envelope since this is the location of other DsbAs and of VKOR's substrate, *EcDsbA*, when it is functional in *E. coli*. Therefore, using a hidden Markov model (13), we sought candidate proteins in *M. tuberculosis* that were members of the thioredoxin family and that exhibited signal sequences or transmembrane components when assessed by the Web-based prediction program Phobius (14), which predicted such proteins to have the active enzymatic portion of the protein localized to the extracytoplasmic space. We identified five proteins in the *M. tuberculosis* genome that fulfilled these criteria (see Table S1 in the supplemental material): (i) Rv2969c (*dsbA* product), which has a transmembrane sequence and was shown to be located in the membrane fraction (11), and (ii) Rv0526, Rv0816c, Rv1677, and Rv2878c, which have a signal peptide or transmembrane segment, indicating the export of their thioredoxin domains to the cell envelope. Rv0526 has been annotated as a possible thioredoxin and was found in a proteomics study to be secreted by cultured *M. tuberculosis* cells (15). Rv0816c (*thiX* product) has also been annotated as a thioredoxin family member, and it is conserved in mycobacteria. Rv1677 (*dsbF* product) is a conserved lipoprotein possibly involved in thiol-disulfide interchange, and it shares high similarity with the C terminus of Rv2878c (*dsbE* product). The crystal structures of Rv1677 and Rv2878c indicate that both proteins exhibit the typical thioredoxin fold (8, 9).

Reconstitution of the *M. tuberculosis* disulfide bond formation pathway in *E. coli*. Initially, we attempted to reconstitute the disulfide bond formation pathway of *M. tuberculosis* in *E. coli* by cloning into a pBAD33 plasmid the predicted gene of each thioredoxin candidate with a myc tag at the carboxy termini. However, with the full arabinose induction of this promoter, neither tagged *dsbF* nor tagged *dsbE* was expressed in *E. coli* at detectable levels by Western blotting using anti-myc antibody. These low expression levels may be due to poor translation or to problems in recognizing possible N-terminal mycobacterial signal sequences properly. Thus, we constructed versions of these genes in which the signal sequences or transmembrane sequences were truncated from the N termini of the proteins and replaced with the *E. coli* TorT cotranslational signal sequence (16). With these constructs, each thioredoxin homolog should be translocated into the *E. coli* periplasm and expressed as a soluble periplasmic protein with its signal sequence removed. In addition, the truncated proteins are all fused to a FLAG tag at their C termini. These new constructs were introduced into the pBAD33 plasmid, where the proteins were all expressed by the addition of arabinose to the growth medium.

We first tested the ability of each His-MtVKOR/*M. tuberculosis* thioredoxin-FLAG pair to restore disulfide bond formation to an *E. coli* strain deleted for its *dsbA* and *dsbB* genes by assessing the motility of such strains. Motility promoted by the flagella of the bacteria is dependent on the protein FlgI, a component of *E. coli* flagella, which requires disulfide bonds for its stability and activity. Thus, the $\Delta dsbA \Delta dsbB$ strain is nonmotile. Previously, we have shown that MtVKOR can restore the motility of *E. coli* $\Delta dsbB$ due to its ability to oxidize *EcDsbA* (2). We proceeded to express both MtVKOR (from a pTrc plasmid, induced with isopropyl- β -D-thiogalactopyranoside [IPTG]) and, independently, each of the five predicted exported thioredoxins in the *E. coli* $\Delta dsbA \Delta dsbB$ strain induced by the addition of arabinose (Fig. 1). We found that only MtDsbA, when coexpressed with MtVKOR in a $\Delta dsbA \Delta dsbB$ mutant, can confer motility similar to that of the control strain coexpressing *EcDsbA* with MtVKOR (Fig. 1). Motility was dependent on the presence of both mycobacterial proteins. This result is consistent with the suggestion that the MtDsbA protein is the only one of the thioredoxin-like proteins that is an effective substrate of MtVKOR and that can promote disulfide bond formation.

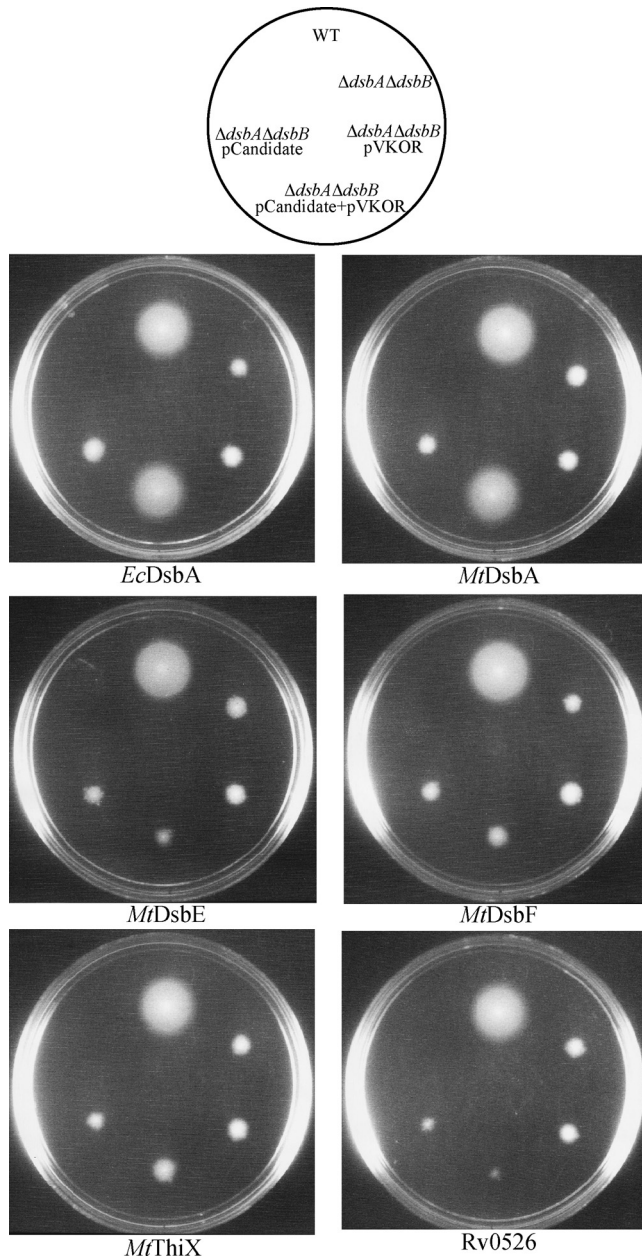


FIG 1 *MtDsbA* restores motility of the *E. coli* $\Delta dsbAB$ strain when coexpressed with *MtVKOR*. Shown are data from motility assays to test the five thioredoxin candidates as partners of *MtVKOR* in their ability to restore disulfide bond formation in *E. coli*. Strains used were as follows: WT (wild type), NK225 (HK295 with pTrc99a and pMER200); $\Delta dsbA \Delta dsbB$ strain, NK211 (HK329 with pTrc99a and pMER200); $\Delta dsbA \Delta dsbB$ pVKOR strain, NK212 (HK329 with pTrc99a-*MtVKOR* and pMER200); $\Delta dsbA \Delta dsbB$ pVKOR + pCandidate strains, NK220, NK218, NK214, NK216, NK222, and NK224 (HK329 with pTrc99a-*MtVKOR* and pMER200 carrying each thioredoxin gene); and $\Delta dsbA \Delta dsbB$ pCandidate strains, NK219, NK217, NK213, NK215, NK221, and NK223 (HK329 with pTrc99a and pMER200 carrying each thioredoxin). Cells were grown on soft-agar M63 plates (0.3% agar) with 0.2% maltose as the carbon source, 100 $\mu\text{g}/\text{ml}$ ampicillin, 10 $\mu\text{g}/\text{ml}$ chloramphenicol, 1 mM IPTG to induce the expression of *MtVKOR*, and 0.2% arabinose to induce the expression of thioredoxins. The plates were incubated at 30°C for 2 days. Images show representative results from at least three independent experiments.

Oxidation of *MtDsbA* depends on *MtVKOR* in *E. coli*. There are alternative explanations for the negative results observed with four of the five thioredoxin-like proteins in the motility assay. Two of these explanations are (i) that the four thioredoxins are expressed at a much lower level than *MtDsbA* and (ii) that the four thioredoxins can be oxidized by *MtVKOR*, but they are unable to oxidize FlgI (see Discussion for an additional alternative

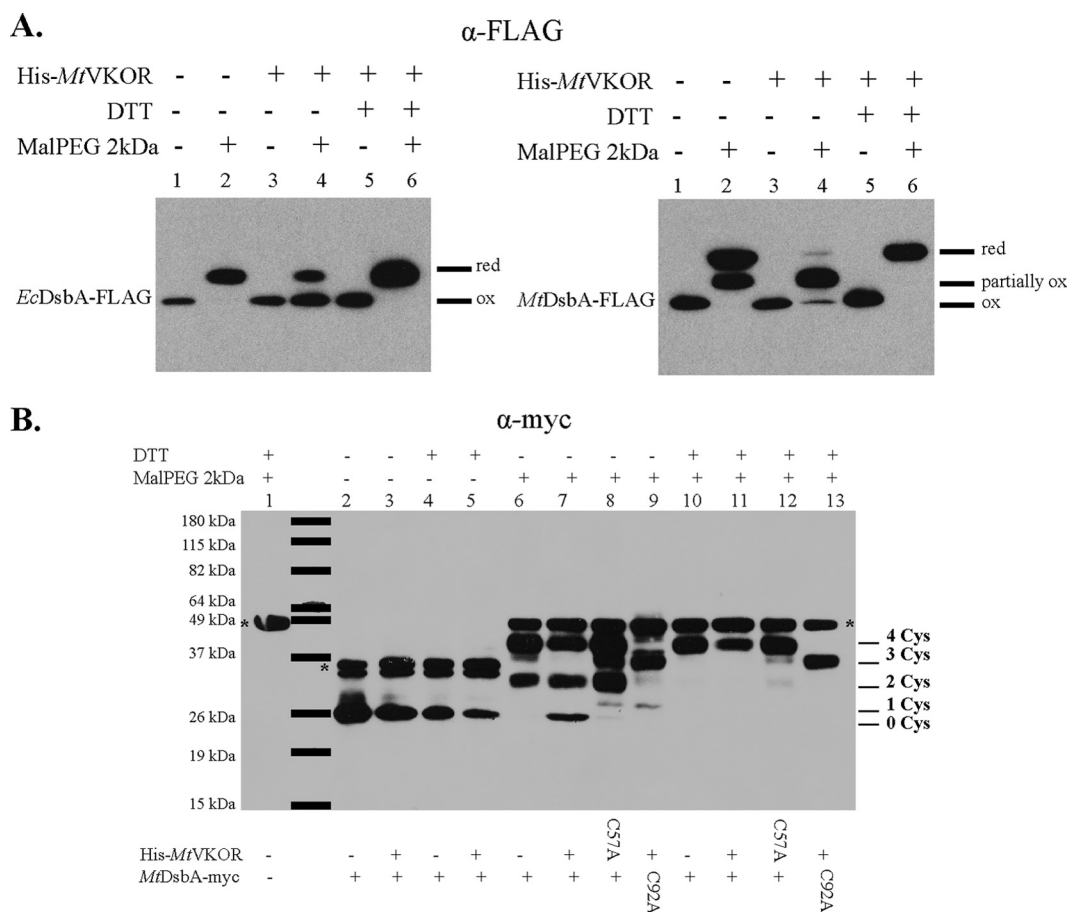


FIG 2 Oxidation of *MtDsbA* is dependent on *MtVKOR* in *E. coli*. (A) *In vivo* oxidation states of *MtDsbA* and *EcDsbA* with or without *MtVKOR* in *E. coli*. *MtDsbA* and *EcDsbA* were cloned into the pBAD33 vector and expressed in a truncated form (deletion of its original signal peptide or transmembrane sequence) using an *E. coli* TorT signal sequence in the $\Delta dsbA \Delta dsbB$ strain. His-*MtVKOR* was expressed from the pTrc99a plasmid. Cells were grown in M63 medium with antibiotics and 0.2% arabinose to induce the expression of the FLAG-tagged proteins. A total of 1 mM IPTG was used to induce the expression of His-*MtVKOR*. Proteins were TCA precipitated and then alkylated with Mal-PEG2k. DsbA proteins were detected with anti-FLAG antibody. The sample without Mal-PEG2k treatment shows the position of the oxidized protein, which is the same as that of the reduced protein with no alkylating agent present. The sample with Mal-PEG2k refers to the position of the protein with reduced cysteines that are alkylated with Mal-PEG2k, which adds to the molecular weight of the protein. red, reduced; ox, oxidized. Blots show representative results from at least two independent experiments. (B) Oxidation states of full-length *MtDsbA* in the *E. coli* $\Delta dsbA \Delta dsbB$ strain. *MtDsbA*-myc or *MtDsbA*_{C92A}-myc was cloned with its native transmembrane sequence into pBAD33. His-*MtVKOR* or His-*MtVKOR*_{C57A} was cloned into pTrc99a. Cell growth and sample preparation were done as described in Materials and Methods. *MtDsbA* was detected with anti-myc antibody. *, nonspecific band (lane 1) when alkylated at 49 kDa and when not alkylated at 37 kDa. 0, 1, 2, 3, and 4 indicate the numbers of cysteines (Cys) labeled with Mal-PEG2k. The blot shows a representative result from two independent experiments.

explanation). In order to test these explanations, we determined the expression level and the oxidation state of each candidate protein in the presence of the cysteine-alkylating agent α -[3-(3-maleimido-1-oxopropyl)amino]propyl- ω -methoxy polyoxyethylene (Mal-PEG2k) in these strains when coexpressed with *MtVKOR*. With this agent, one can distinguish between proteins with free cysteines and those with cysteines joined in disulfide bonds via the substantial molecular weight differences that the alkylating agent (Mal-PEG2k) would add to the molecular weight of proteins with free cysteines. These differences are readily observable by immunoblotting.

Our results show that there are no dramatic differences in expression levels of the five *M. tuberculosis* thioredoxins when fully induced by arabinose (Fig. 2A; see also Fig. S1A in the supplemental material), thus confirming that the lack of motility of the $\Delta dsbA \Delta dsbB$ strain was not due to a lack of expression of the candidates. In addition, we observed that without the presence of *MtVKOR*, all six thioredoxins (including *EcDsbA*) are mostly in the reduced form (Fig. 2A and Fig. S1, lane 2), as expected, since

there is no reoxidizing partner in the strain. However, when *MtVKOR* is coexpressed, *MtDsbA* and *EcDsbA* are present mostly in the oxidized form, while the other thioredoxins remain in the reduced state (Fig. 2A and Fig. S1, lane 4). We note that in addition to the two cysteines in the active-site $C_{89}XXC_{92}$ motif, *MtDsbA* has two additional cysteines that form a structural disulfide bond (10, 12). Thus, three bands of *MtDsbA* appear in the Western blot; the middle band is the form of the protein that carries one disulfide bond and two free cysteines (partially oxidized). For this middle band, we cannot determine whether the disulfide bond is formed between the two cysteines in the $C_{89}XXC_{92}$ motif or is a structural disulfide bond between the non-active-site cysteines ($C_{140}-C_{192}$). Even in the presence of *MtVKOR*, not all *MtDsbA* is fully oxidized, but the appearance of both the fully oxidized form and a form with only one disulfide bond indicates that this VKOR/thioredoxin pair is restoring disulfide bond formation. The degree to which *EcDsbA* and *MtDsbA* are oxidized in these experiments may be due to the levels of expression of *MtVKOR* or due to a decreased efficiency of *MtVKOR* (2). Three of the other thioredoxin-like proteins exhibit either none or an extremely small amount of the oxidized form in the presence of *MtVKOR* (Fig. S1, lane 4).

Because *MtDsbA* is the only one of the *M. tuberculosis* proteins that was oxidized in the presence of *MtVKOR*, we further investigated the steps in its oxidation. For this, we expressed *MtDsbA* as a full-length protein with its transmembrane sequence and a C-terminal myc tag in *E. coli* from a pBAD plasmid. The oxidation of *MtDsbA* depends on *MtVKOR*, as shown in Fig. 2B (lanes 6 and 7). Without the expression of *MtVKOR*, there is reduced (form 4) (Fig. 2B, lane 6) and partially oxidized (form 2) (Fig. 2B, lane 6) *MtDsbA*, and there is no fully oxidized *MtDsbA* (form 0) (Fig. 2B, lane 6). With the expression of *MtVKOR*, the oxidized band of *MtDsbA* appears (form 0) (Fig. 2B, lane 7), and there is still some amount of partially oxidized (form 2) (Fig. 2B, lane 7) and fully reduced (form 4) (Fig. 2B, lane 7) *MtDsbA*.

When the active cysteine C57 in *MtVKOR*, involved in binding to reduced DsbA (4), was mutated to alanine, the amount of fully oxidized *MtDsbA* decreased (forms 0 and 1) (Fig. 2B, lane 8). Most *MtDsbA* is found in the fully reduced state (forms 3 and 4) (Fig. 2B, lane 8) or a partially oxidized form (form 2) (Fig. 2B, lane 8). When the second cysteine in the $C_{89}XXC_{92}$ motif of *MtDsbA* was mutated to alanine, *MtDsbA* cysteines were mostly reduced (form 3 compared to the faint form 1) (Fig. 2B, lane 9), suggesting that the structural disulfide bond between C140 and C192 of *MtDsbA* relies on the catalytic activity of the $C_{89}XXC_{92}$ disulfide bond of *MtDsbA*, which is in turn reoxidized by *MtVKOR*.

Oxidation of *MtDsbA* depends on *MtVKOR* in *M. smegmatis*. *M. smegmatis* is a fast-growing, nonpathogenic mycobacterium species widely used as a host to study genes and proteins from *M. tuberculosis*. Deletion of *vkor* (MSMEG_2411) in *M. smegmatis* causes a growth defect that can be complemented by the expression of *MtVKOR* from a plasmid (5). In order to determine whether *MtVKOR* affects the oxidation state of *MtDsbA*, we integrated myc-tagged *MtDsbA* into a phage L5 attachment site on the chromosome (using the pJEB402 plasmid) (17) of the *M. smegmatis* $\Delta vkor$ strain, which expresses *MtVKOR* from the pTetG plasmid. The expression of *MtVKOR* was regulated by the Tet promoter and induced with anhydrotetracycline (ATc), while myc-tagged *MtDsbA* was under the control of a constitutive promoter (17). The *M. smegmatis* strain with a deletion of *vkor* grows slowly in 7H9 medium with 1.6 mM cysteine (or oxidized cysteine) (5). When this strain is complemented instead with the pTetG plasmid expressing *MtVKOR*, the strain grows similarly to the wild type, even without the addition of ATc. Thus, the basal *MtVKOR* expression level from the pTetG plasmid is sufficient to support the growth of the $\Delta vkor$ strain.

We determined the redox state of *MtDsbA* *in vivo* in *M. smegmatis* in the presence or absence of *MtVKOR* using the same cysteine-alkylating agent, Mal-PEG2k. We observed that when *MtVKOR* expression was induced with ATc in the $\Delta vkor$ strain (Fig. 3B, lanes 3 and 4), *MtDsbA* was found fully oxidized, with some amount of reduced *MtDsbA* (Fig. 3A, lane 4). However, when there is no VKOR in the cell (Fig. 3B, lanes 1

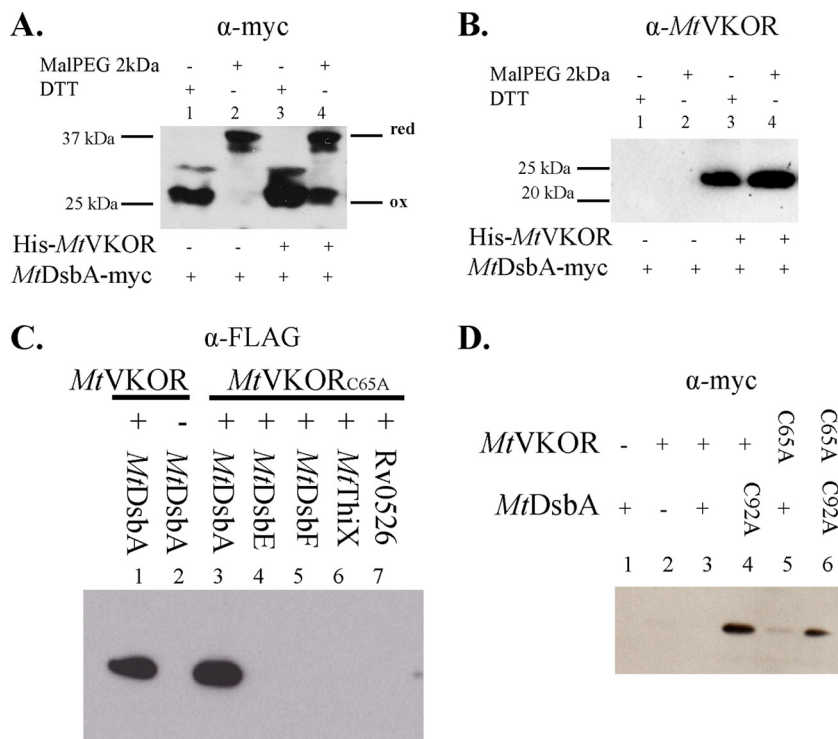


FIG 3 Oxidation of *MtDsbA* is dependent on *MtVKOR* in *M. smegmatis*. (A and B) Oxidation states of *MtDsbA* in the *M. smegmatis* Δ *vkor* strain with pJEB402-*MtDsbA*-myc inserted into the chromosome and complemented with either the pTetG (lanes 1 and 2) or pTetG-His-*MtVKOR* (lanes 3 and 4) plasmid. Strains were grown in 7H9 medium (plus 1.6 mM cystine for pTetG or 50 ng/ml of ATc for pTetG-His-*MtVKOR*), and proteins were TCA precipitated, followed by alkylation with Mal-PEG2k. *MtDsbA* (A) was detected with anti-myc antibody, and *MtVKOR* (B) was detected with anti-VKOR antibody. (C) *MtDsbA* forms a complex with *MtVKOR* in *E. coli*. Wild-type *MtVKOR* (pTrc99a-His-*MtVKOR*) or the C65A mutant (pTrc99a-His-*MtVKOR*_{C65A}) was coexpressed with *MtDsbA* or other mycobacterial thioredoxins (pMER200-*MtThioredoxin*-FLAG) in the *E. coli* Δ *dsbA* Δ *dsbB* strain. Cell lysates were loaded onto a nickel affinity spinning column. The bound proteins were eluted with 0.5 M imidazole, and eluted samples were analyzed by Western blotting with anti-FLAG antibody. Lane 1, coexpression of *MtVKOR* and *MtDsbA*; lane 2, expression of *MtVKOR* only; lanes 3 to 7, coexpression of the *MtVKOR*_{C65A} mutant with the five thioredoxin candidates. (D) *MtDsbA* forms a complex with *MtVKOR* in *M. smegmatis*. Wild-type *MtVKOR* (pTetG-His-*MtVKOR*) or the C65A mutant (pTetG-His-*MtVKOR*_{C65A}) was coexpressed with wild-type *MtDsbA* (pJEB402-*MtDsbA*-myc) or the *MtDsbA*_{C92A} mutant (pJEB402-*MtDsbA*_{C92A}-myc) in *M. smegmatis*. Samples were treated with anti-myc antibody as described above for panel B. Blots show representative results from two independent experiments.

and 2), all the *MtDsbA* protein was found in the fully reduced state (Fig. 3A, lane 2). Therefore, in the absence of *MtVKOR*, *MtDsbA* is not reoxidized in *M. smegmatis*, which is consistent with our results that indicate that *MtDsbA* requires *MtVKOR* to start a new catalytic cycle.

Only *MtDsbA* forms a complex with *MtVKOR* among the five thioredoxin candidates. Based on previous studies on the structure of cyanobacterial VKOR and the topology of *MtVKOR*, a model of the oxidation of the disulfide bond formation protein by *MtVKOR* was established in *E. coli* (3, 4). During the oxidation of *MtDsbA*, the first cysteine (C57) of *MtVKOR* forms an intermolecular disulfide bond with the first cysteine of the C₈₉XXC₉₂ motif of *MtDsbA* (4). The second cysteine (C65) of *MtVKOR* then attacks this disulfide bond and resolves the intermediate. Therefore, a C65 mutant of *MtVKOR* would help to trap the intermediate with *MtDsbA* by slowing the resolution of the intermediate complex.

To test whether *MtDsbA* and *MtVKOR* can interact specifically, we constructed *MtVKOR* with a C-to-A mutation at residue 65 (*MtVKOR*_{C65A}) and coexpressed it with each of the five thioredoxin candidates in an *E. coli* Δ *dsbA* Δ *dsbB* strain. We then used nickel affinity resin to pull down His-tagged *MtVKOR* and determine whether the

proteins are pulled down together with *MtVKOR*_{C65A} by immunoblotting with anti-myc antibody. As shown in Fig. 3C (lanes 1 and 3), both *MtVKOR* and *MtVKOR*_{C65A} can form a complex with *MtDsbA* but not with the other thioredoxin proteins expressed in *E. coli* (lanes 4 to 7). The size of the DsbA-VKOR complex is around 50 kDa, similar to the predicted size of the two proteins. When the sample is treated with dithiothreitol (DTT), the disulfide bond that links the two proteins is reduced, and the complex is dissociated into two smaller bands (data not shown). Even when the expression levels of the other four thioredoxin candidates were similar to that of *MtDsbA* (Fig. 2A), no complexes were detected with the *MtVKOR*_{C65A} mutant (Fig. 3C). Hence, *MtDsbA* is the only protein among the five thioredoxins that can directly interact with *MtVKOR* in *E. coli*. Additionally, we looked at the interaction between *MtDsbA* and *MtVKOR* in *M. smegmatis* by coexpressing *MtDsbA* with the *MtVKOR*_{C65A} mutant. We also used a mutant of the second cysteine in the DsbA active-site motif (*MtDsbA*_{C92A}), since this cysteine is also important to resolve the intermediate formed between C89 of *MtDsbA* and C57 of *MtVKOR*. Either *MtDsbA*_{C92A} or *MtVKOR*_{C65A} pulled down DsbA with His-tagged VKOR (Fig. 3D, lanes 4 to 6). Thus, our results confirm the specific interaction between mycobacterial VKOR and DsbA in *M. smegmatis*.

Deletion of the *MtDsbA* homolog in *M. smegmatis* causes a growth defect. In *M. tuberculosis*, both *MtDsbA* and *MtVKOR* are essential for growth (7). A strain of *M. smegmatis* deleted for *vkor* (MSMEG_2411), showing a growth defect, is unable to grow on 7H10 medium and forms tiny colonies on rich medium (5). We hypothesize that VKOR is essential for proper growth because the cell cannot form disulfide bonds efficiently in some essential protein(s) required for cell survival. The *MtDsbA* homolog in *M. smegmatis*, annotated as MSMEG_2410, is 80% identical to that of *M. tuberculosis* (see Fig. S2 in the supplemental material) and is located upstream of the *vkor* gene, forming an operon similar to that of the *M. tuberculosis dsbA-vkor* genes.

In order to determine whether *M. smegmatis* DsbA (*MsDsbA*) is also essential, we made a clear gene deletion in *M. smegmatis* by using a *sacRB* method described previously (18). The deletion was made by using a pTetG plasmid that expresses the *MsDsbA* gene from a Tet promoter. All our attempts to make a deletion without the complementing plasmid failed, which suggests that *MsDsbA* is also essential for the growth of *M. smegmatis* on 7H10 medium. The deletion of *MsDsbA* causes a more severe growth defect than does the deletion of *MsVKOR* (5), since this strain cannot grow on either 7H10 medium (Fig. 4A, top) or N-Z-Amine (NZ) medium (Fig. 4A, bottom) without inducing the expression of *MsDsbA* from the plasmid (i.e., it requires the addition of ATc). Unlike the $\Delta vkor$ strain, the addition of 1.6 mM cysteine did not restore the growth of the $\Delta dsbA$ strain. This may be due to the fact that small-molecule oxidants cannot substitute for DsbA, while they can reoxidize DsbA when VKOR is not present.

We then determined the essentiality of the four cysteines present in *MsDsbA* by mutating each residue to alanine. We cloned *MsDsbA* and its cysteine variants into the pJEB402 plasmid and then integrated the plasmid into the *M. smegmatis* $\Delta dsbA$ strain complemented with pTetG*MsdsbA*. We tested for growth in the presence or absence of ATc to determine whether the cysteine mutants are able to complement and restore growth. Similarly to *MtDsbA*, *MsDsbA* harbors four cysteines (two disulfide bonds), one in the catalytic C₈₆XXC₈₉ motif and one predicted structural disulfide bond between C136 and C188. Disrupting the cysteines of the structural disulfide bond permitted growth of the *M. smegmatis* conditionally lethal strain, as strains carrying C136 and C188 mutants grew when no ATc was added to the medium (Fig. 4B). However, the C89A mutant did not grow in the absence of ATc (Fig. 4B), suggesting that C86 and C89 are the essential catalytic cysteines of *MsDsbA* and that the disulfide bond in the active-site motif is essential for the growth of *M. smegmatis*.

DISCUSSION

M. tuberculosis as well as other Gram-positive actinobacteria, including *Corynebacterium* and *Streptomyces*, secrete many proteins with an even number of cysteines to the cell envelope. These organisms have predicted oxidoreductases and VKOR or-

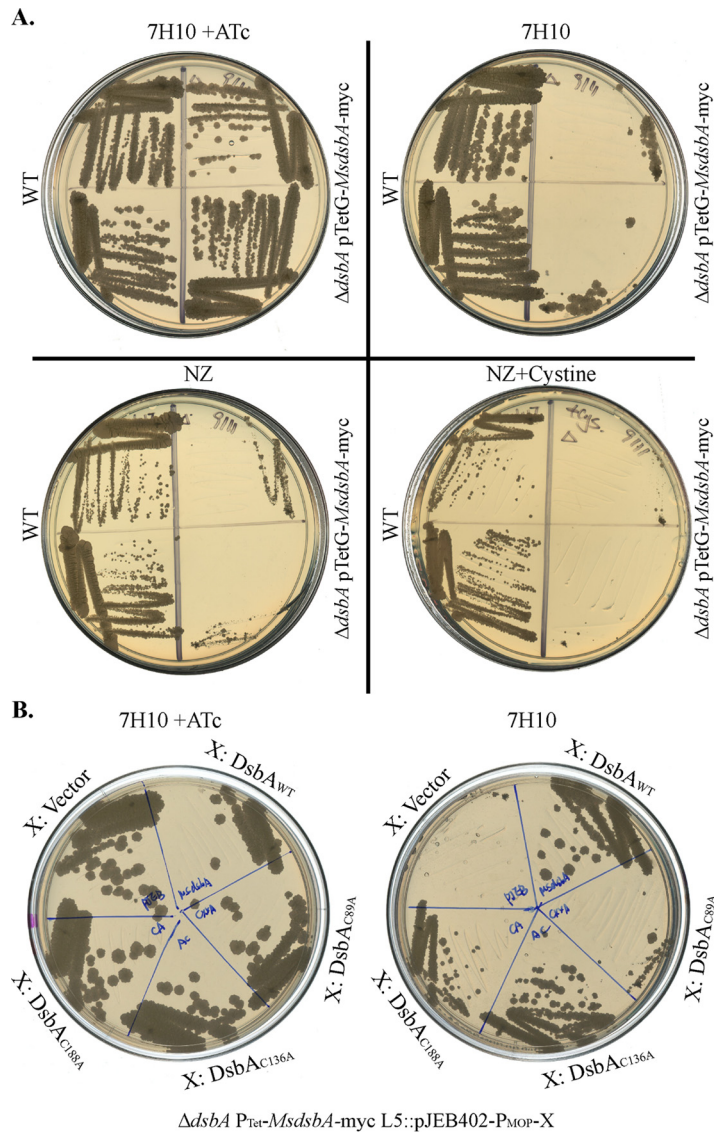


FIG 4 Deletion of the DsbA homolog causes a growth defect in *M. smegmatis*. (A) Deletion of *MsdsbA* in *M. smegmatis* expressing *MsDsbA* from a plasmid (pTetG-*MsDsbA*). *M. smegmatis* wild-type and conditionally lethal *ΔdsbA* strains were streaked on 7H10 plates with (top left) or without (top right) 100 ng/ml ATc to induce the expression of *MsDsbA* and with NZ medium (bottom left) or NZ medium supplemented with 1.6 mM cystine (bottom right). Strains were streaked in duplicate onto each plate. (B) Wild-type *MsDsbA*, *MsDsbA*_{C89A} (C₈₆XXA mutant), *MsDsbA*_{C136A} or *MsDsbA*_{C188A} (mutants in the structural disulfide bond) was cloned under the control of a constitutive mycobacterial optimized promoter (MOP) present in the pJEB402 plasmid and expressed in the conditionally lethal *M. smegmatis* *ΔdsbA* strain with plasmid pTetG-*MsDsbA*, which requires ATc to induce the expression of *MsDsbA* in order to grow. Strains were streaked on 7H10 medium plates with 100 ng/ml ATc (left) or without ATc (right). Images show representative results from two independent experiments.

thologs in their genomes. These properties together indicate that these bacteria likely form disulfide bonds in the cell envelope (2, 6). We have previously shown that the *vkor* gene from *M. tuberculosis* restores the oxidation of *E. coli* DsbA and, thus, disulfide bond formation in an *E. coli dsbB* deletion strain (2). A recent study has shown that *M. tuberculosis* VKOR can restore disulfide bond formation in the Gram-positive bacterium *Actinomyces oris* by interacting with the *A. oris* DsbA-like protein, MdbA (19). While it has been suggested that DsbA may be the VKOR partner in mycobacteria, no evidence for an interaction between the two proteins was shown (10, 19). In fact, two other oxidoreductases (*M. tuberculosis* DsbE and DsbF) that exhibit oxidase activity *in vitro* have been suggested to be the catalysts of protein disulfide bond formation (8, 9).

Therefore, in the present study, we investigated the functionality of five candidate *M. tuberculosis* proteins that belong to the thioredoxin superfamily (DsbA, DsbE, DsbF, ThiX, and Rv0526) and their interactions with *M. tuberculosis* VKOR in *E. coli* and in a close relative of *M. tuberculosis*, *M. smegmatis*.

When mycobacterial DsbA and VKOR, which are genetically linked in *M. tuberculosis*, are coexpressed in an *E. coli* *dsbAB* double-deletion mutant, they confer motility, a phenotype dependent on a functional disulfide bond pathway (Fig. 1). Motility is not restored when *MtVKOR* is coexpressed with mycobacterial DsbE, DsbF, ThiX, or Rv0526, consistent with the proposal that mycobacterial DsbA and VKOR are the proteins that act as partners in introducing disulfide bonds into substrate proteins. The failure to confer motility with each of the mycobacterial proteins DsbE, DsbF, ThiX, and Rv0526 when combined with mycobacterial VKOR was not due to poor protein expression. In contrast, the expression of *MtDsbA* and *MtVKOR* provided the only combination that resulted in oxidation by *MtVKOR* in *E. coli* (Fig. 2A). Consistently, when His-tagged *MtVKOR* or *MtVKOR*_{C65A} was used to pull down the complex with the thioredoxin candidates in *E. coli*, only *MtDsbA* was detected, thus indicating a direct interaction with *MtVKOR* (Fig. 3C). In addition, the oxidation of *MtDsbA* was dependent on C57 of *MtVKOR*, which is involved in binding to reduced DsbA (Fig. 2B). Similarly, the second cysteine in the catalytic C₈₉XXC₉₂ motif of *MtDsbA* was necessary for the oxidation of *MtDsbA* (Fig. 2B). While our results show that *MtDsbA* interacts with *MtVKOR*, we cannot rule out the possibility that one or more of these thioredoxin candidates are functional, but their functionality in disulfide bond formation does not show up under the several conditions that we have examined. Also, we cannot discard the possibility that these candidates are able to oxidize substrates in *M. tuberculosis* by a different mechanism and not via *MtVKOR*, e.g., by small-molecule oxidants.

We have used *M. smegmatis* as a model for studying the proteins involved in disulfide bond formation of *M. tuberculosis*. The deletion of *vkor* (MSMEG_2411) in *M. smegmatis* complemented with tagged *MtDsbA* displays only reduced *MtDsbA*, while the $\Delta vkor$ strain complemented with *MtVKOR* shows some *MtDsbA* in the oxidized state (Fig. 3A), indicating that oxidation of *MtDsbA* by *MtVKOR* takes place in *M. smegmatis*. The reason for the incomplete *MtDsbA* oxidation may be due to the levels of *MtVKOR* expression. The interaction between *MtDsbA* and *MtVKOR* can also be trapped in *M. smegmatis* by expressing the *MtDsbA*_{C92A} mutant or, to a lesser extent, the *MtVKOR*_{C65A} mutant (Fig. 3C). However, wild-type *MtVKOR* did not pull down *MtDsbA* in *M. smegmatis* as it did in *E. coli*. Perhaps *MtVKOR* and *MtDsbA* interact more efficiently with each other in *M. smegmatis* than in *E. coli* because the transmembrane segments are still intact when expressed in *M. smegmatis*. This less efficient interaction between *MtDsbA* and *MtVKOR* in *E. coli* may help to trap *MtDsbA* in *E. coli* pulldown assays. Additionally, the *vkor* deletion in *M. smegmatis* causes a severe growth defect in rich medium, and this strain does not grow in 7H10 medium unless it is supplemented with cysteine (5). On the other hand, the DsbA homolog of *M. smegmatis* cannot be deleted unless a second copy of the gene is expressed in the strain (by the addition of ATc); if induction is suspended, the strain is unable to grow, indicating *MsDsbA* essentiality (Fig. 4A). Expressing the *MsDsbA*_{C89A} mutant in the conditionally lethal *dsbA* strain cannot restore growth, while the *MsDsbA*_{C136A} and *MsDsbA*_{C188A} mutants complement growth (Fig. 4B). This indicates that *MsDsbA* catalyzes disulfide bond formation via its C₈₆XXC₈₉ motif. Consistent with *M. smegmatis* phenotypes, *MtVKOR* and *MtDsbA*, but not *MtDsbE* or *MtDsbF*, are essential for growth in *M. tuberculosis* (7). This is in contrast to the *E. coli* DsbA and DsbB proteins, which are not essential under aerobic growth conditions, although the mutants do not grow anaerobically (20). The reason why these mutants grow aerobically is that the background oxidation of proteins, such as the cell division protein FtsN and the lipopolysaccharide assembly protein LptD, is sufficient to maintain a significant pool of active essential proteins to permit *E. coli* growth (20, 21). On the other hand, anaerobic growth has no such background oxidation, and *E. coli* eventually stops growing (20). Thus, altogether, our results demonstrate that *MtDsbA* is the redox partner of *MtVKOR* participating in the formation of disulfide bonds in mycobacteria.

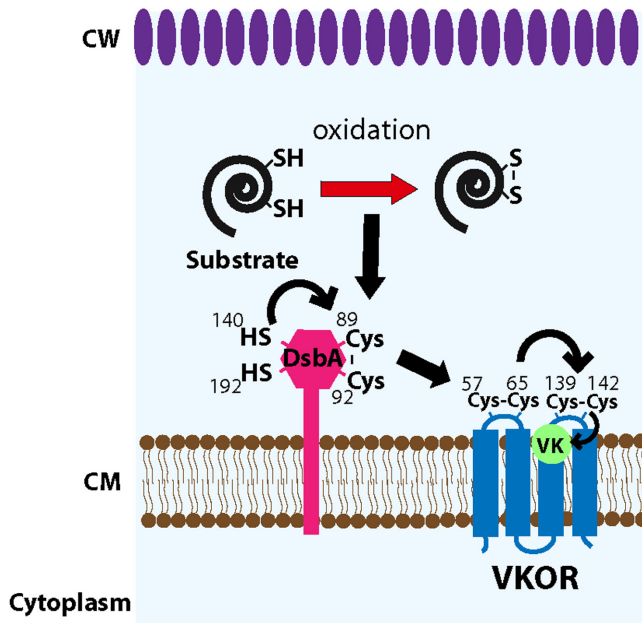


FIG 5 Proposed model of disulfide bond formation in *M. tuberculosis*. From its C₈₉XXC₉₂ motif, DsbA oxidizes a substrate that requires disulfide bonds to be properly folded and presumably essential for the growth of *M. tuberculosis*. A substrate of the pathway identified in our studies is the structural disulfide bond (C₁₄₀-C₁₉₂) of DsbA itself, which is formed by the catalytic activity of the C₈₉XXC₉₂ motif. Once DsbA transfers its disulfide to substrate proteins, DsbA becomes reduced, and VKOR is required to reoxidize DsbA's cysteines by transferring electrons to quinone. Therefore, the transfer of electrons goes from the substrate to DsbA to VKOR and thence to quinones. Black arrows indicate electron transfer. VK, vitamin K or menaquinone; Cys, cysteine; SH, reduced cysteine; S-S, oxidized cysteines; CW, cell wall; CM, cytoplasmic membrane.

Our results also indicate that the structural disulfide bond between C140 and C192 of MtDsbA relies on the reoxidation activity of MtDsbA by MtVKOR, as observed by the fully reduced state of cysteines in the MtDsbA_{C92A} mutant (Fig. 2B, lane 9), suggesting that the C140-C192 disulfide bond of MtDsbA is a substrate of the pathway and that it is formed by the catalytic cysteines C89 and C92, which are then reoxidized by MtVKOR cysteines. Our studies together with others allow us to propose a model of the interaction between MtDsbA and MtVKOR (depicted in Fig. 5); in this model, DsbA donates its Cys89-Cys92 disulfide bond to the substrate (e.g., C140-C192 of MtDsbA) and becomes reduced. Reduced Cys89 of DsbA then interacts with Cys57-Cys65 of VKOR, and by thiol-disulfide exchange reactions with Cys139-Cys142 of VKOR, the electrons are shuffled to quinones, releasing oxidized DsbA and VKOR.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Strains and plasmids used in this study are listed in Table 1. *E. coli* was grown in NZ medium supplied with suitable antibiotics at 37°C. *M. smegmatis* mc²155 was grown in either NZ medium supplied with 0.5% Tween 80, 7H9 medium, or 7H10 medium plus oleic acid-albumin-dextrose-catalase (OADC) at 37°C. The antibiotics used were ampicillin at 100 µg/ml, chloramphenicol at 10 µg/ml, kanamycin at 40 µg/ml for *E. coli* and 20 µg/ml for *M. smegmatis*, and hygromycin at 100 µg/ml. Mycobacterial genes were cloned by standard molecular biology techniques. The sequences of the identified mycobacterial thioredoxins cloned into pMER200 are given in the supplemental material.

Identifying candidate disulfide bond formation enzymes in *M. tuberculosis*. A hidden Markov model of *dsbA* was constructed based on a few known DsbA protein sequences (13). The HMMsearch program in the HMMER suite took the model and searched for homologs of the model in the *M. tuberculosis* genome (14). The hits with E values of <10⁻⁵ were used to predict their cellular location by Phobius (<http://phobius.sbc.su.se/>) (14). The ones that were predicted to be a secreted protein or an exported membrane protein were taken as the candidates for disulfide-bond-forming enzymes.

Motility assay. A motility assay was done on M63 medium soft-agar plates (0.3% agar). The colonies used for the motility assay were picked from a single colony by a pointed toothpick and pricked into a soft-agar plate containing proper antibiotics, 0.5 to 1 mM IPTG (to induce VKOR

TABLE 1 Strains and plasmids

Strain or plasmid	Genotype or description	Reference or source
Strains		
<i>E. coli</i>		
HK295	MC1000 $\Delta ara714 leu^+$	23
HK329	HK295 $\Delta dsbA \Delta dsbB$	H. Kadokura
NK225	HK295 with pMER200 (Cm ^r) and pTrc99a (Amp ^r)	This study
NK211	HK329 with pMER200 (Cm ^r) and pTrc99a (Amp ^r)	This study
NK212	HK329 with pMER200 (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK213	HK329 with pMER201 (pMER200-MtDsbE-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK214	HK329 with pMER201 (pMER200-MtDsbE-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK215	HK329 with pMER202 (pMER200-MtDsbF-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK216	HK329 with pMER202 (pMER200-MtDsbF-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK217	HK329 with pMER203 (pMER200-MtDsbA-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK218	HK329 with pMER203 (pMER200-MtDsbA-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK219	HK329 with pMER204 (pMER200-EcDsbA-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK220	HK329 with pMER204 (pMER200-EcDsbA-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK221	HK329 with pNK188 (pMER200-MtThiX-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK222	HK329 with pNK188 (pMER200-MtThiX-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
NK223	HK329 with pNK190 (pMER200-Rv0526-FLAG) (Cm ^r) and pTrc99a (Amp ^r)	This study
NK224	HK329 with pNK190 (pMER200-Rv0526-FLAG) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-1	HK329 with pXW251 (pBAD33-MtDsbA-myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-13	HK329 with pXW264 (pBAD33-MtDsbA _{C92A} -myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-11	HK329 with pXW260 (pBAD33-MtDsbA _{C89A} -myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-15	HK329 with pXW261 (pBAD33-MtDsbA _{C140A} -myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-16	HK329 with pXW262 (pBAD33-MtDsbA _{C192A} -myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW173-14	HK329 with pXW263 (pBAD33-MtDsbA _{C140A-C192A} -myc) (Cm ^r) and pRD33 (pTrc99a-6XHis-MtVKOR) (Amp ^r)	This study
XW169-5	HK329 with pMER201 (pMER200-MtDsbE-FLAG) (Cm ^r) and pXW169 (pTrc99a-6XHis-MtVKOR _{C65A}) (Amp ^r)	This study
XW169-6	HK329 with pMER202 (pMER200-MtDsbF-FLAG) (Cm ^r) and pXW169 (pTrc99a-6XHis-MtVKOR _{C65A}) (Amp ^r)	This study
XW169-1	HK329 with pMER203 (pMER200-MtDsbA-FLAG) (Cm ^r) and pXW169 (pTrc99a-6XHis-MtVKOR _{C65A}) (Amp ^r)	This study
XW169-9	HK329 with pNK188 (pMER200-MtThiX-FLAG) (Cm ^r) and pXW169 (pTrc99a-6XHis-MtVKOR _{C65A}) (Amp ^r)	This study
NK392	HK329 with pNK190 (pMER200-Rv0526-FLAG) (Cm ^r) and pXW169 (pTrc99a-6XHis-MtVKOR _{C65A}) (Amp ^r)	This study
<i>M. smegmatis</i>		
NK168	<i>Mycobacterium smegmatis</i> mc ² 155	E. J. Rubin's laboratory collection
RD149	<i>M. smegmatis</i> Δvkr	5
XW311	<i>M. smegmatis</i> Δvkr with pTetG (empty) (Hyg ^r) L5::pNK341 (pJEB402-MtDsbA-myc) (Km ^r)	This study
XW312	<i>M. smegmatis</i> Δvkr with pNK307 (pTetG-6XHis-MtVKOR) (Hyg ^r) L5::pNK341 (pJEB402-MtDsbA-myc) (Km ^r)	This study
NK351	<i>M. smegmatis</i> with pNK307 (pTetG-6XHis-MtVKOR) (Hyg ^r) L5::pNK341 (pJEB402-MtDsbA-myc) (Km ^r)	This study
NK352	<i>M. smegmatis</i> with pNK307 (pTetG-6XHis-MtVKOR) (Hyg ^r) L5::pXW307 (pJEB402-MtDsbA _{C92A} -myc) (Km ^r)	This study
NK354	<i>M. smegmatis</i> with pNK308 (pTetG-6XHis-MtVKOR _{C65A}) (Hyg ^r) L5::pNK341 (pJEB402-MtDsbA-myc) (Km ^r)	This study
NK355	<i>M. smegmatis</i> with pNK308 (pTetG-6XHis-MtVKOR _{C65A}) (Hyg ^r) L5::pXW307 (pJEB402-MtDsbA _{C92A} -myc) (Km ^r)	This study
NK317	<i>M. smegmatis</i> $\Delta dsbA$ with pNK252 (pTetG-MsDsbA-myc) (Hyg ^r)	This study
NK328	<i>M. smegmatis</i> $\Delta dsbA$ with pNK252 (pTetG-MsDsbA-myc) (Hyg ^r) L5::pJEB402 (Km ^r)	This study
NK394	<i>M. smegmatis</i> $\Delta dsbA$ with pNK252 (pTetG-MsDsbA-myc) (Hyg ^r) L5::pNK338 (pJEB402-MsDsbA-myc) (Km ^r)	This study
NK393	<i>M. smegmatis</i> $\Delta dsbA$ with pNK252 (pTetG-MsDsbA-myc) (Hyg ^r) L5::pNK395 (pJEB402-MsDsbA _{C89A} -myc) (Km ^r)	This study
NK336	<i>M. smegmatis</i> $\Delta dsbA$ with pNK252 (pTetG-MsDsbA-myc) (Hyg ^r) L5::pNK339 (pJEB402-MsDsbA _{C136A} -myc) (Km ^r)	This study
NK335	<i>M. smegmatis</i> $\Delta dsbA$ with pTetG-MsDsbA-myc (Hyg ^r) L5::pNK340 (pJEB402-MsDsbA _{C188A} -myc) (Km ^r)	This study
Plasmids		
pTrc99a	Expression vector; Amp ^r	24
pRD33	pTrc99a-6XHis-MtVKOR (Amp ^r)	2
pJEB402	Chromosomal integration (L5 attachment site) vector, mycobacterial constitutive promoter MOP (Km ^r)	17
pTetG	Mycobacterial expression vector (Hyg ^r)	25
pMER200	pBAD33-TorTss-FLAG (Cm ^r)	This study
pMER201	pMER200-TorTss-MtDsbE-FLAG (Cm ^r)	This study
pMER202	pMER200-TorTss-MtDsbF-FLAG (Cm ^r)	This study
pMER203	pMER200-TorTss-MtDsbA-FLAG (Cm ^r)	This study
pMER204	pMER200-TorTss-EcDsbA-FLAG (Cm ^r)	This study
pNK188	pMER200-TorTss-MtThiX-FLAG (Cm ^r)	This study
pNK190	pMER200-TorTss-Rv0526-FLAG (Cm ^r)	This study
pXW251	pBAD33-MtDsbA-myc (Cm ^r)	This study
pXW264	pBAD33-MtDsbA _{C92A} -myc (Cm ^r)	This study
pXW260	pBAD33-MtDsbA _{C89A} -myc (Cm ^r)	This study
pXW261	pBAD33-MtDsbA _{C140A} -myc (Cm ^r)	This study
pXW262	pBAD33-MtDsbA _{C192A} -myc (Cm ^r)	This study
pXW263	pBAD33-MtDsbA _{C140A-C192A} -myc (Cm ^r)	This study
pXW168	pTrc99a-6XHis-MtVKOR _{C57A} (Amp ^r)	This study
pXW169	pTrc99a-6XHis-MtVKOR _{C65A} (Amp ^r)	This study
pNK307	pTetG-6XHis-MtVKOR (Hyg ^r)	This study
pNK308	pTetG-6XHis-MtVKOR _{C65A} (Hyg ^r)	This study
pNK341	pJEB402-MtDsbA-myc (Km ^r)	This study
pXW307	pJEB402-MtDsbA _{C92A} -myc (Km ^r)	This study
pNK252	pTetG-MsDsbA-myc (Hyg ^r)	This study
pNK338	pJEB402-MsDsbA-myc (Km ^r)	This study

(Continued on next page)

TABLE 1 (Continued)

Strain or plasmid	Genotype or description	Reference or source
pNK395	pJEB402- <i>MsdSbA</i> _{C89A} -myc (Km ^r)	This study
pNK339	pJEB402- <i>MsdSbA</i> _{C136A} -myc (Km ^r)	This study
pNK340	pJEB402- <i>MsdSbA</i> _{C188A} -myc (Km ^r)	This study

expression), and 0.2% arabinose (to induce the expression of thioredoxin proteins). The plates were incubated at 30°C for 48 h.

Determining *in vivo* redox states of the mycobacterial candidates in *E. coli*. *E. coli* cultures were trichloroacetic acid (TCA) precipitated and washed with acetone as described previously (22). Mal-PEG2k { α -[3-(3-maleimido-1-oxopropyl)amino]propyl- ω -methoxy polyoxyethylene; NOF America Corporation} was used as an alkylating reagent to block reduced thiol, adding 2 kDa to the molecular weight of each cysteine. Controls were done by adding DTT (dithiothreitol) and then Mal-PEG2k in order to show the oxidized/nonalkylated (DTT only) or alkylated (DTT and Mal-PEG) protein.

Determining *in vivo* redox states of *MtDsbA* in *M. smegmatis*. Cultures were started in NZ medium supplemented with 0.5% Tween 80 and either 1.6 mM cysteine for the $\Delta vkor$ strain or 50 ng/ml of ATc to induce the expression of the $\Delta vkor$ strain complemented with a plasmid expressing *Mtvkor*. Cells were grown at 37°C to an optical density at 600 nm (OD₆₀₀) of 1. Cells were harvested, washed with 7H9 medium plus OADC three times, and resuspended in medium with either 1.6 mM cysteine for the $\Delta vkor$ strain or 50 ng/ml of ATc to induce the expression of *MtVKOR*. Cells were continually grown for 4 to 8 h at 37°C. The cells were then pelleted and resuspended in 50 mM Tris–5 mM EDTA (pH 8) with a protease inhibitor. Cell lysis was done by bead beating cells for five times during 30 s with 1-min ice incubation intervals. Lysates were centrifuged for 10 min at 13,000 rpm, and the supernatants were subjected to TCA precipitation and alkylation as described above. *MtVKOR* antibody was raised in rabbit by using the peptide PMRENRGSQERVGAR (GenScript, USA).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <https://doi.org/10.1128/JB.00137-18>.

SUPPLEMENTAL FILE 1, PDF file, 0.9 MB.

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