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MnoSR Is a Bona Fide Two-Component System Involved in Methylotrophic Metabolism in *Mycobacterium smegmatis*

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ABSTRACT *Mycobacterium smegmatis* and several other mycobacteria are able to utilize methanol as the sole source of carbon and energy. We recently showed that *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA)-dependent methanol dehydrogenase (Mno) is essential for the growth of *M. smegmatis* on methanol. Although Mno from this bacterium shares high homology with other known methanol dehydrogenases, methanol metabolism in *M. smegmatis* differs significantly from that of other described methylotrophs. In this study, we dissect the regulatory mechanism involved in the methylotrophic metabolism in *M. smegmatis*. We identify a two-component system (TCS), *mnoSR*, that is involved in the regulation of *mno* expression. We show that the MnoSR TCS is comprised of a sensor kinase (MnoS) and a response regulator (MnoR). Our results demonstrate that MnoS undergoes autophosphorylation and is able to transfer its phosphate to MnoR by means of phosphotransferase activity. Furthermore, MnoR shows specific binding to the putative *mno* promoter region *in vitro*, thus suggesting its role in the regulation of *mno* expression. Additionally, we find that the MnoSR system is involved in the regulation of *MSMEG_6239*, which codes for a putative 1,3-propanediol dehydrogenase. We further show that *M. smegmatis* lacking *mnoSR* is unable to utilize methanol and 1,3-propanediol as the sole carbon source, which confirms the role of MnoSR in the regulation of alcohol metabolism. Our data, thus, suggest that the regulation of *mno* expression in *M. smegmatis* provides new insight into the regulation of methanol metabolism, which furthers our understanding of methylotrophy in mycobacteria.

IMPORTANCE Methylotrophic metabolism has gained huge attention considering its broad application in ecology, agriculture, industries, and human health. The genus *Mycobacterium* comprises both pathogenic and nonpathogenic species. Several members of this genus are known to utilize methanol as the sole carbon source for growth. Although various pathways underlying methanol utilization have been established, the regulation of methylotrophic metabolism is not well studied. In the present work, we explore the regulation of methanol metabolism in *M. smegmatis* and discover a dedicated two-component system (TCS), MnoSR, that is involved in its regulation. We show that the loss of MnoSR renders the bacterium incapable of utilizing methanol and 1,3-propanediol as the sole carbon sources. Additionally, we establish that MnoS acts as the common sensor for the alcohols in *M. smegmatis*.

KEYWORDS methylotrophic metabolism, *Mycobacterium*, alcohol metabolism, histidine kinase, methanol oxidation, two-component system

Methanol is one of the major C_1 compounds found in nature and is a crucial carbon source for methylotrophic bacteria (1–3). Methanol metabolism has remained a topic of interest due to its wide range of industrial and agricultural applications (4). Methylotrophs also play an important role in interaction with plants to execute promising ecological applications (5). Methanol dehydrogenase is required for the conversion of methanol into formaldehyde, which is the primary and critical step in

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methanol utilization as the sole carbon source (2, 3). Notwithstanding the differences in the biochemical and structural properties of methanol dehydrogenases from different bacteria, several studies on the regulation of methylotrophic metabolism have shown that methanol dehydrogenases are overproduced by bacteria during growth on methanol (6–13). This suggests that the production of methanol dehydrogenase is the underlying cellular response to the presence of methanol in the extracellular environment and is conserved among the majority of methylotrophs. Thus, it is both important and interesting to explore the regulatory mechanism(s) involved in C_1 metabolism.

The mode of upregulation of methanol dehydrogenase expression in the presence of methanol differs among the known methylotrophs and involves different mechanisms. Earlier studies have suggested the involvement of a two-component system (TCS) in the regulation of methanol dehydrogenase expression in Gram-negative methanol-utilizing bacteria, such as *Methylobacterium extorquens* and *Paracoccus denitrificans* (14–16). In addition to the C_1 compounds, the expression of methanol dehydrogenase has also been shown to be regulated by the lanthanides (17, 18). In *Mycobacterium* sp. strain JC1, methanol dehydrogenase expression has been shown to be positively regulated by the TetR family of transcriptional regulator MdoR; additionally, MdoR has also been reported to be essential for the growth of bacteria on methanol (19). In methylotrophs, methanol dehydrogenase is not the only protein overproduced during growth on methanol. Several reports suggest that in methylotrophic metabolism, genes involved in the serine cycle and RuMP pathway are overexpressed in *M. extorquens* and *Bacillus methanolicus*, respectively (13, 20, 21). Whereas QscR, a LuxR family of transcriptional regulators, has been shown to regulate serine cycle genes in *M. extorquens*, little is known about the regulation of RuMP cycle genes in *B. methanolicus* (20). Thus, there are multiple means by which bacteria regulate methanol metabolism genes in the presence of methanol.

Growth of *Mycobacterium smegmatis* and other mycobacteria is supported by a range of carbon sources, and the metabolic pathways for their utilization in the majority of cases have been elucidated (22). We have previously shown that *M. smegmatis* harbors an *N*,*N*-dimethyl-*p*-nitrosoaniline (NDMA)-dependent methanol dehydrogenase (Mno), which is required for methanol utilization by the bacterium (23). However, the factors governing *mno* expression in the presence of methanol are not known. In the present study, we identify and characterize the factors that regulate methanol metabolism in *M. smegmatis*. Among the various regulators found in *M. smegmatis* that control gene expression, the TetR family of transcription regulators (TFTRs) is the most studied (24), and several of the characterized TFTRs present in *M. smegmatis* are required for the regulation of oxidoreductases (24). Additionally, TCSs in *M. smegmatis* are also known to regulate genes involved in essential cellular processes, such as nutrient acquisition, physiological response to hypoxia, and virulence in certain cases (25–27). Here, we identify and characterize a two-component system, MnoSR, which is involved in methylotrophic metabolism regulation in *M. smegmatis*. We report that the MnoSR TCS is composed of a sensor kinase, MnoS, which phosphorylates its cognate response regulator, MnoR. Together, these two proteins in the presence of methanol in the culture medium regulate *mno* expression. Our data suggest that the *mnoSR* two-component system is dedicated for the metabolism of alcohols in *M. smegmatis*. Our study forms the first report on the identification of a TCS involved in the regulation of an alcohol dehydrogenase in *M. smegmatis*, which will further enhance our understanding of the regulation of methylotrophic metabolism in bacteria.

RESULTS

Identification of a two-component system involved in the regulation of methanol metabolism in *Mycobacterium smegmatis*. We previously showed that *M. smegmatis* produces an NDMA-dependent methanol dehydrogenase (Mno), encoded by *MSMEG_6242* or *mno*, that is essential for bacterial growth on methanol as the sole carbon source (23). We further showed that *mno* is induced in the presence of methanol in the culture medium, irrespective of the presence of glucose (as an

FIG 1 Arrangement of *mnoSR* two-component system and the neighboring genes on the *M. smegmatis* mc²155 genome. The distribution of various genes on the *M. smegmatis* genome is shown. The direction of gene expression is marked with arrows. Genes considered for the present study are shown in different colors and are labeled; their protein products are also mentioned as sensor kinase (*MSMEG_6238*), response regulator (*MSMEG_6236*), Mno (*MSMEG_6242*), putative 1,3-propanediol dehydrogenase (*MSMEG_6239*), and a TetR family of transcriptional regulator (*MSMEG_6244*) divergent to *mno*.

additional carbon source) (23). Here, we asked what regulates *mno* expression and carried out a detailed *in silico* analysis of the *M. smegmatis* genome available at Mycobrowser (https://mycobrowser.epfl.ch) (28). We identified a gene, *MSMEG_6244*, that codes for a putative TetR family of transcriptional regulators (TFTRs) and is divergent to *mno* (Fig. 1). This is similar to a previous report that suggested the involvement of MdoR, a TFTR, in the regulation of methanol dehydrogenase of *Mycobacterium* strain JC1 (19). However, we could not find any significant sequence similarity between MSMEG_6244 and MdoR from *Mycobacterium* JC1 (data not shown). Nevertheless, to verify the role of *MSMEG_6244* in *mno* expression regulation, we generated an *MSMEG_6244* knockout in *M. smegmatis* by following the method as described previously (23) and confirmed the same by PCR (see Fig. S1 in the supplemental material) and DNA sequencing. We found that a deletion of *MSMEG_6244* hampered neither the growth of *M. smegmatis* on methanol nor the production of Mno (Fig. 2A and B), suggesting that *MSMEG_6244* has no role in methanol metabolism in *M. smegmatis*.

Our *in silico* analysis of the *M. smegmatis* genome also revealed the presence of a putative two-component system (TCS) formed by the products of two genes coding for a sensor kinase (*MSMEG_6238*; hereafter referred to as *mnoS*) and a response regulator (*MSMEG_6236*; hereafter referred to as *mnoR*) in the vicinity of *mno* (Fig. 1). A DOOR database (29) analysis confirmed that both of the genes form an operon (*mnoSR*). Furthermore, the stop codon of *mnoS* overlaps with the start codon of *mnoR*. TCSs are generally known to be present as operons in mycobacteria and other bacteria (30, 31). Methanol oxidation in several methylotrophs has been shown to be regulated by TCSs (14, 15). Thus, in order to validate whether *mno* in *M. smegmatis* is regulated by this TCS,

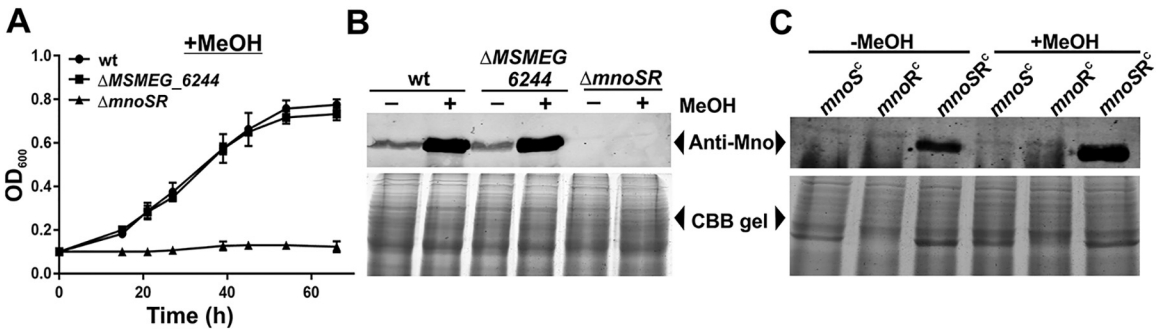

FIG 2 *mnoSR* regulates *mno* expression and is essential for methanol-dependent growth of *M. smegmatis*. (A) Growth of wild-type (wt), Δ*MSMEG_6244*, and Δ*mnoSR* strains of *M. smegmatis* in the presence of methanol (+MeOH) as the sole carbon source. OD_{600} of the culture medium was recorded at the given time points and plotted. Time 0 represents the addition of methanol at an OD_{600} of ~0.1. The error bars denote the standard deviation in the readings among the three sets of independent experiments. An increase in OD_{600} is observed only in the case of the wt and Δ*MSMEG_6244* strains. (B) Western blotting data to examine Mno production in the presence (+) and absence (–) of methanol (MeOH). Two percent glucose is present in all of the cases. Mno production is observed in the presence of methanol in the wild-type (wt) and Δ*MSMEG_6244* strains but not in the *mnoSR* knockout (Δ*mnoSR*) strain. (C) Western blot of Mno production in the absence (–MeOH) and presence (+MeOH) of methanol after complementing the *mnoSR* knockout with *mnoS* (*mnoS*^{C}), *mnoR* (*mnoR*^{C}), or both *mnoS* and *mnoR* (*mnoSR*^{C}). Methanol-dependent induction of Mno is observed only when complementation is carried out with both *mnoS* and *mnoR*. In both panels B and C, the Coomassie blue (CBB)-stained gel is shown to confirm protein loading.

we proceeded with constructing an *mnoSR* knockout strain of *M. smegmatis* (Δ*mnoSR*), having the deletion of both *MSMEG_6236* and *MSMEG_6238*. The knockout was prepared by following the method as described previously (23, 32) and confirmed by PCR (see Fig. S1) and DNA sequencing. Interestingly, our *M. smegmatis* Δ*mnoSR* strain is unable to grow when methanol is present as the sole carbon source (Fig. 2A). Additionally, in the Δ*mnoSR* strain, methanol-dependent Mno production is completely lost as judged by Western blotting (Fig. 2B). These results suggest that Mno production in *M. smegmatis* is regulated by the MnoSR TCS. To further confirm that both MnoS and MnoR are together required for Mno production and that there is no other cross-reacting TCS, we performed the complementation of *M. smegmatis* Δ*mnoSR* by expressing either *mnoS* (pADatMnoS) or *mnoR* (pADatMnoR) or both *mnoS* and *mnoR* (pADatMnoSR) from an acetamide-inducible promoter (33, 34). Our data show that Mno production could be restored only when the cells were transformed with pADatMnoSR, which coexpressed *mnoS* and *mnoR* (Fig. 2C). This, thus, confirms that MnoS and MnoR together form a cognate TCS, which is required for Mno production. The complementation data further rule out the possibility of any cross-reacting TCS component. In these experiments, the expression of MnoS, MnoR, and MnoSR from the pADatMnoS, pADatMnoR, and pADatMnoSR vectors, respectively, in Δ*mnoSR* cells was confirmed by Western blotting using an anti-His antibody (see Fig. S2 in the supplemental material). Taken together, our data strongly indicate that the MnoSR TCS carries out the positive regulation of Mno and is essential for the growth of *M. smegmatis* on methanol.

Since the *mnoSR* knockout and complementation data correctly identified them as the TCS involved in *mno* regulation, we did not attempt to decipher the role of other genes, such as *MSMEG_6240*, *MSMEG_6241*, and *MSMEG_6243*, present in the vicinity of *mno* (Fig. 1). While *MSMEG_6240* and *MSMEG_6241* code for conserved hypothetical protein and putative ATPase, respectively, *MSMEG_6243* (located upstream of *mno*) encodes a hypothetical protein containing the DUF1348 domain. It is likely that the products of these genes are involved in other aspects of mycobacterial physiology, and an examination of them is beyond of the scope of this study.

MnoS and MnoR form the cognate proteins of the two-component system. TCSs are involved in the signal transduction process in bacteria. The signaling between the factors of the TCS generally occurs by the autophosphorylation of sensor kinase, which then transfers the phosphate group to its response regulator protein. In most cases, the regulatory protein then binds to DNA and modulates the target gene expression (35). Our *in silico* analysis, carried out by the Conserved Domain Database (CDD) and Simple Modular Architecture Research Tool (SMART) (36, 37), shows that MnoS contains a GAF domain (named after the cyclic GMP [cGMP]-specific phosphodiesterase, adenylyl cyclase, and FhlA proteins) at its N terminus. This is followed by a histidine kinase domain (HisKA_3) comprising the kinase core and a histidine kinase-like ATPase (HATPase) domain responsible for the ATPase activity of the protein (Fig. 3A). Furthermore, the domain architecture of MnoS was found to be similar to that of other known sensor kinases, *viz*., Rv2027c and DevS of *Mycobacterium tuberculosis* (38). In order to confirm whether MnoS is indeed a sensor kinase, we examined the autophosphorylation activity of MnoS in the presence of [γ-^{32}P]ATP. We first cloned, expressed, and purified 6×histidine-tagged MnoS, and purity was checked by SDS-denaturing PAGE (see Fig. S3 in the supplemental material). We next incubated the purified MnoS with [γ-^{32}P]ATP and performed phosphorimaging of the proteins separated by SDS-PAGE. Our data show that MnoS indeed undergoes phosphorylation, thus confirming that MnoS is a sensor kinase that can undergo autophosphorylation (Fig. 3B).

The phosphotransferase reaction between the sensor kinase and cognate response regulator is the basis of the two-component-based signaling process (39). To confirm whether the MnoR is the response regulator protein that can accept phosphate from the sensor kinase MnoS, we cloned, expressed, and purified MnoR and performed the autophosphorylation and the phosphotransferase reactions in the absence and presence of MnoS. Our data show that MnoR, unlike MnoS, is incapable of demonstrating

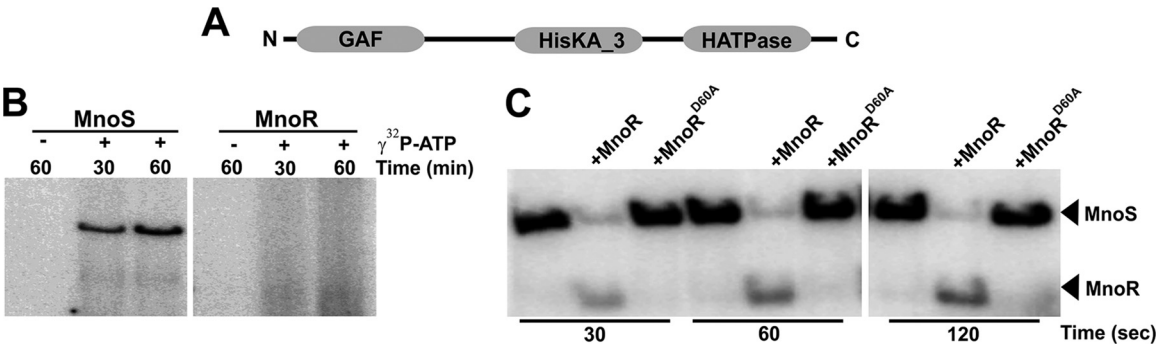

FIG 3 MnoS shows autophosphorylation and phosphotransferase activity *in vitro*. (A) Conserved domains present in the MnoS protein from the N to C terminus. GAF domain corresponds to a domain present in cGMP-specific phosphodiesterase, adenylyl cyclase, and FhlA proteins. The histidine kinase domain is represented as HisKA_3, whereas the HATPase domain is responsible for ATPase activity of the protein. (B) Autoradiogram for the autophosphorylation activities of MnoS and MnoR. The phosphorylation activity was carried out for the specified time (in minutes) in the presence or absence of [γ-^{32}P]ATP. The data show that MnoS undergoes autophosphorylation upon incubation with [γ-^{32}P]ATP, whereas MnoR is unable to perform autophosphorylation reaction under similar conditions. (C) Autoradiogram for the phosphotransferase activity of MnoS (presented in all of the lanes) toward either MnoR or MnoR^{D60A} proteins. The position of both MnoS and MnoR proteins in the autoradiogram is marked. The reaction was carried out for the specified time (in seconds). Only the wild-type MnoR undergoes phosphorylation upon incubation with phosphorylated MnoS, which is accompanied by the loss of signal from MnoS.

autophosphorylation activity (Fig. 3B). However, when incubated with the phosphorylated MnoS, MnoR is able to accept the phosphate group from MnoS, resulting in the dephosphorylation of MnoS and consequent phosphorylation of MnoR (Fig. 3C). This experiment confirms the phosphotransferase activity between the two proteins.

Sensor kinase in the majority of cases phosphorylates the conserved aspartate residue on the response regulator protein that eventually undergoes a conformational change and performs required function (35). A previous report suggests that Asp54 in DevR is the key residue that undergoes phosphorylation upon incubation with the cognate sensor kinase DevS, and mutation of Asp54 to valine renders DevR incapable of undergoing phosphorylation (40). A sequence alignment between DevR and MnoR using the EMBOSS Needle pairwise alignment tool (41) revealed the presence of conserved Asp60 in the latter (see Fig. S4 in the supplemental material), which also corresponded to the Asp54 of DevR that has been shown to undergo phosphorylation (40). The phosphorylation site of MnoR was further confirmed to be Asp60 as indicated by the prediction carried out at UniProt (accession number A0R5L8). Thus, in order to examine the specific phosphate acceptor site on the MnoR protein, we generated an MnoR D60A mutant (MnoR^{D60A}) and carried out the phosphotransferase activity. We observed that the MnoR^{D60A} mutant was not phosphorylated by MnoS (Fig. 3C), indicating the significance of the D60 residue in MnoR. Our data, thus, suggest that MnoR is the cognate response regulator of MnoS and that Asp60 is the site for the phosphorylation on MnoR. Thus, together, these proteins form the cognate proteins of the two-component system.

MnoR specifically binds to the *mno* promoter region. In order to successfully induce *mno* expression, MnoR must bind to the *mno* promoter region. We carried out a detailed analysis of 200 bp upstream of the *mno* translation start site and identified one inverted repeat sequence (Fig. 4A), which is likely the MnoR binding site. We next cloned this DNA segment upstream of a *lacZ* reporter gene in the promoterless *Escherichia coli-Mycobacterium* shuttle vector pSD5b (42), thus generating pSDmno, which was used for the transformation of *M. smegmatis*. Our experiments show the methanol-dependent *lacZ* expression (measured as β-galactosidase activity) from the 200-bp region, indicating the presence of an inducible promoter upstream of *mno* (Fig. 4B). We next cloned a 150-bp DNA segment upstream of the *mno* translation start site in pSD5b in order to truncate the inverted repeat sequence identified above and generated pSD150mno. Interestingly, β-galactosidase activity data suggest that pSD150mno is no longer methanol inducible (Fig. 4B). We conclude that the loss of

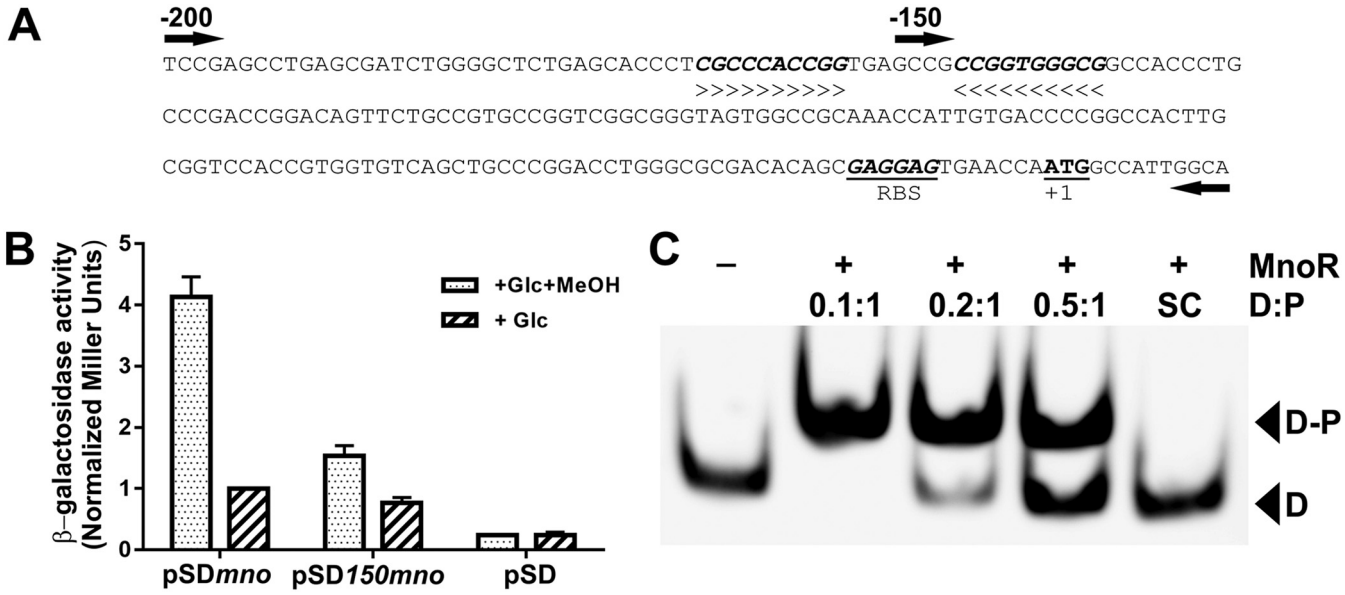

FIG 4 MnoR binds specifically to the *mno* promoter region. (A) DNA sequence upstream of *mno* start codon ATG (marked as +1). The ~200-bp region used for the promoter assays and the EMSA is marked as -200, whereas a smaller truncated promoter region of ~150 bp is marked as -150. The identified inverted repeats are bold and italicized. The putative ribosome binding site for the translation process is marked as RBS. (B) Promoter activity of 200-bp and 150-bp DNA segments upstream of *mno* translation start site in the presence and absence of methanol (MeOH); 2% glucose (Glc) is present in all of the conditions. Empty vector pSD was used as negative control. β-Galactosidase activity in the form of normalized Miller units is plotted. Higher promoter activity is observed in the case of the 200-bp *mno* promoter region in the presence of methanol, whereas the 150-bp promoter region shows poor methanol-dependent induction. (C) Electrophoretic mobility shift assay (EMSA) carried out in the absence (–) or presence (+) of MnoR and the ~200-bp biotinylated *mno* promoter region. Various mole ratios of DNA/protein (D:P) were used in the experiment. MnoR-bound promoter region complex (D-P) moves more slowly on the nondenaturing acrylamide gel than the free DNA (D). Nonbiotinylated DNA from the same region was used as specific competitor (SC), which confirms that the binding of MnoR to the probe is specific.

methanol-dependent induction from pSD150mno is due to the disruption of the inverted repeat sequence.

We next examined the binding of MnoR to the *mno* promoter region by carrying out an electrophoretic mobility shift assay (EMSA) with purified MnoR and the biotin-labeled 200-bp DNA segment used in promoter assays. Our data show the successful binding of MnoR to the 200-bp *mno* promoter region in a concentration-dependent manner (Fig. 4C). Furthermore, this binding is found to be specific since the interaction between DNA and protein could be masked by the use of nonbiotinylated 200 bp *mno* DNA as a specific competitor (Fig. 4C). Our observations allow us to infer that MnoR binds to the *mno* promoter region.

MnoSR TCS also regulates *MSMEG_6239* expression but is not required for formaldehyde detoxification. The bioinformatics analysis of the *M. smegmatis* genome carried out here also revealed *MSMEG_6239*, which codes for a putative 1,3-propanediol dehydrogenase, in the vicinity of *mnoSR*. MSMEG_6239 shares 77% sequence similarity with 1,3-propanediol dehydrogenase from *Saccharopolyspora erythraea* (UniProt accession number A4FCA4). This observation tempted us to monitor the effect of the *mnoSR* deletion on the expression of *MSMEG_6239*. We, thus, measured the relative expression of *MSMEG_6239* in both wild-type and Δ*mnoSR M. smegmatis* strains using reverse transcriptase quantitative PCR (RT-qPCR). We observed that the expression of *MSMEG_6239* was drastically reduced in the Δ*mnoSR* strain compared to the level in the wild type (Fig. 5A). Additionally, the growth of the Δ*mnoSR* strain on 1,3-propanediol as the sole carbon source was found to be hampered compared to that of the wild type, suggesting that the Δ*mnoSR* strain is unable to utilize 1,3-propanediol (Fig. 5B). Taken together, our data suggest that the MnoSR TCS regulates the expression of both *mno* and *MSMEG_6239*, and thus, it is essential for *M. smegmatis* growth on methanol and 1,3-propanediol.

It is worth mentioning here that the TCS-mediated regulatory mechanism is not just confined to the regulation of methanol oxidation in methylotrophs. A previous report

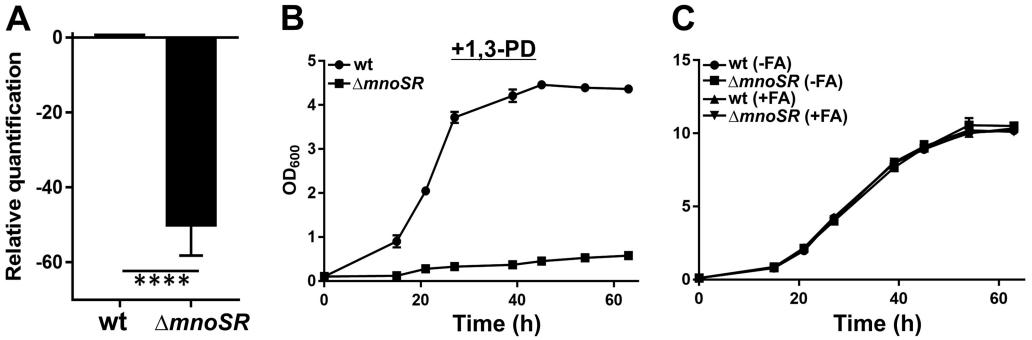

FIG 5 *mnoSR* TCS is essential for *MSMEG_6239* expression and 1,3-propanediol utilization but is not required for formaldehyde metabolism. (A) RT-qPCR-based expression data of *MSMEG_6239* (which codes for putative 1,3-propanediol dehydrogenase) in both the wild-type (wt) and the Δ*mnoSR* cells. Drastic downregulation of *MSMEG_6239* in the Δ*mnoSR* strain is observed compared to the wild type. RT-qPCR was performed thrice as independent sets of experiments. The error bars denote the standard deviation among the three experiments. ****, *P* value = highly significant. (B) Growth of *M. smegmatis* wild-type (wt) and Δ*mnoSR* cells in the presence of 1,3-propanediol (+1,3-PD) as the sole carbon source in the culture medium. The growth was monitored by measuring OD_{600} with time. Plot shows that Δ*mnoSR* cells are unable to utilize 1,3-propanediol. (C) Growth of wild-type (wt) and Δ*mnoSR* cells in absence (-FA) and presence (+FA) of 1 mM formaldehyde measured as OD_{600} with time. Two percent glucose is present in all of the conditions. Growth rates of both the wild-type and the Δ*mnoSR* cells remain similar at the formaldehyde concentration used in this experiment. In both panels B and C, data presented are an average of three independent experiments with error bars denoting standard deviation.

on *P. denitrificans* suggests that the TCS is known to regulate both methanol and formaldehyde oxidation (14). We, therefore, examined the role of the MnoSR TCS in formaldehyde oxidation by challenging Δ*mnoSR* and wild-type *M. smegmatis* strains to sublethal concentrations of formaldehyde (23). Our results show that the loss of *mnoSR* does not affect the growth of *M. smegmatis* in the presence of formaldehyde (Fig. 5C). This allows us to conclude that the *mnoSR* TCS is confined to the regulation of only alcohol metabolism.

MnoSR functions as a bona fide alcohol-sensing two-component system that allows cross-induction of gene expression. Loss of MnoSR leads to the downregulation of both Mno and putative 1,3-propanediol dehydrogenase production, which strongly indicates that expression of both of the genes is regulated by similar mechanisms. Since, here, one TCS is regulating different alcohol dehydrogenases, conceivably, these alcohol dehydrogenases should express even in the presence of their noncognate alcohols in the culture medium. Therefore, we first assessed the production of Mno in the presence of various alcohols such as methanol, ethanol, and 1,3-propanediol. Interestingly, we observed production of Mno in all of the alcohols (Fig. 6A); moreover, this expression required the presence of MnoSR since in its absence, the alcohol-dependent Mno production was lost in the Δ*mnoSR* strain (Fig. 6A). These data suggest that MnoSR functions as a dedicated TCS for various alcohols in *M. smegmatis*. We wish to add here that Mno is able to act upon various different alcohols in the oxidation reaction (23). Thus, it is valid to expect Mno production when an alcohol other than methanol is present in the culture medium, even though Mno is required primarily for methanol utilization *in vivo* (23).

We further cloned the ~200-bp region upstream from the translation start site of *MSMEG_6239* in pSD5b (42) ahead of the *lacZ* reporter gene to generate pSD6239. We next monitored the expression of *lacZ* from both the *mno* and *MSMEG_6239* promoter regions by carrying out β-galactosidase assays in the absence and presence of methanol and 1,3-propanediol. We observed higher β-galactosidase activities from both promoter regions when the culture medium contained either methanol or 1,3-propanediol, which immediately suggested that both gene promoters respond to all of the alcohols (Fig. 6B and C); very low β-galactosidase activity was observed in the absence of alcohols, which corroborates our Western blotting data. Thus, a significantly higher amount of promoter activity in the presence of either methanol or 1,3-propanediol suggests that MnoSR allows for cross-induction of both

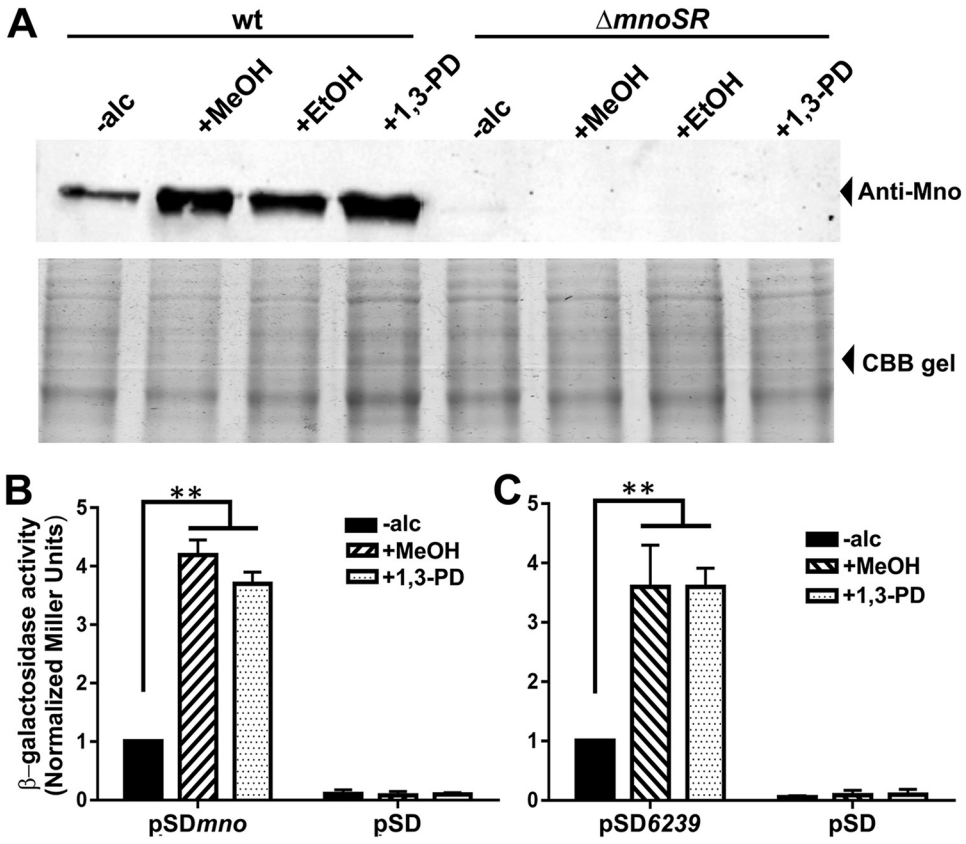

FIG 6 MnoSR functions as a bona fide alcohol-sensing two-component system that allows cross-induction of gene expression. (A) Expression of Mno monitored in *M. smegmatis* wild-type (wt) and Δ*mnoSR* cells in the absence (-alc) and presence of various alcohols, such as methanol (MeOH), ethanol (EtOH), and 1,3-propanediol (1,3-PD), by Western blotting using anti-Mno antibodies. Mno production occurs in the presence of all of the alcohols examined in this study and is observed only in the wild-type cells and not in the Δ*mnoSR* cells. CBB gel represents the Coomassie blue-stained SDS-PAGE gel showing the equal amounts of proteins in the samples processed for Western blotting. Promoter assays by measuring the β-galactosidase activity were carried out with an ~200-bp promoter region of both *mno* (pSD*mno* [B]) and *MSMEG_6239* (pSD*6239* [C]) fused with *lacZ* in an *E. coli-Mycobacterium* promoterless shuttle plasmid, pSD5b, in the absence (-alc) and the presence of methanol (+MeOH) or 1,3-propanediol (+1,3-PD). The enzyme activity is presented as normalized Miller units with respect to -alc. The expression from both *mno* and *MSMEG_6239* promoter regions is observed in the presence of either of the alcohols in the culture medium. The promoterless empty plasmid (pSD) acted as a negative control. The assays were performed at least thrice. Error bars depict the standard deviation. **, P = very significant.

mno and *MSMEG_6239* genes by the addition of either of the alcohols in the culture medium. This further confirms the involvement of similar regulatory mechanisms for the expression of the two genes.

DISCUSSION

Gene expression regulation in *M. smegmatis*, a soil-dwelling microbe, is complex and deals with an abundance of regulatory factors, which extends from TFTRs to two-component systems (24, 26, 43). TCSs are of special interest due to their involvement in the survival of *Mycobacterium tuberculosis* and in establishing a successful infection (26). Various studied mycobacterial TCSs to date justify their presence in both pathogenic and non-pathogenic mycobacterial species and perform a wide range of functions from virulence, hypoxia, stress and survival during infection, nutrient sensing and uptake to development of antibiotic resistance (25–27, 40). Although several *Mycobacterium* species are able to utilize methanol as the sole source of carbon and energy (44), it is intriguing that they employ different and unique pathways for carbon assimilation during methylotrophic metabolism (23, 44, 45). A previous report suggests that the methanol dehydrogenase in *Mycobacterium* JC1 is under positive regulation of MdoR, a TFTR (19). Nevertheless, the

mechanisms involved in the regulation of methanol metabolism are only poorly understood and have not been explored in detail in the mycobacterial methylotrophs.

Here, we have identified and characterized a two-component system, MnoSR, that is required for the establishment of the methylotrophic metabolism in *M. smegmatis*. We show that MnoSR not only regulates expression of methanol dehydrogenase gene (*mno*) but is also required for the expression of *MSMEG_6239*, which codes for a putative 1,3-propanediol dehydrogenase. Thus, MnoSR is essential for the growth of *M. smegmatis* on alcohols, such as methanol and 1,3-propanediol, as the sole carbon sources. Additionally, the unaffected growth of the Δ*mnoSR* strain in the presence of formaldehyde, a key intermediate of methanol metabolism (3), suggests that the regulation of methanol oxidation and formaldehyde detoxification in *M. smegmatis* differ from these processes in other mycobacterial species and require further exploration.

Although the loss of Mno production in the Δ*mnoSR* strain in the presence of any of the alcohols suggests that the MnoSR TCS responds to the presence of alcohols in the culture medium, we have previously shown that *mno* overexpression is observed in the presence of both methanol and formaldehyde (23). Hence, the regulation of Mno in *M. smegmatis* involving factors other than the MnoSR TCS cannot be ruled out, and it remains to be elucidated if an alcohol is the primary and/or a direct inducer for *mno* expression. Additionally, it may be hypothesized here that the GAF domain present in the N terminus of MnoS that is similar to DevS is involved in sensing and responding to small secondary messenger molecules, such as cGMP, cAMP, or cyclic-di-GMP (38, 46). Although the GAF domain is present in a number of sensory proteins (39, 46), it is not always necessary that it binds or responds to the cyclic nucleotides (47). Thus, it remains to be seen if global regulation of methylotrophy involves cyclic nucleotides as specific signals. Taken together, we believe that additional factors are involved in the regulation of methylotrophic metabolism as a whole in mycobacteria and that MnoSR forms a dedicated regulatory system for the initial step of methanol metabolism by acting as a sensor for methanol and other alcohols.

Our study presents a comprehensive analysis of the regulation of the utilization of methanol and other alcohols by involving MnoSR as the regulatory proteins. The observation that MnoSR also regulates the gene coding for a putative C_3 alcohol dehydrogenase (i.e., 1,3-propanediol) suggests that MnoSR has far-reaching effects on mycobacterial physiology and is likely involved in functions beyond the regulation of methylotrophic metabolism. It is interesting to note that although methanol dehydrogenases from *Mycobacterium* JC1 and *M. smegmatis* share high homology, their modes of regulation of expression differ (19, 23). Thus, our current and previous findings together strongly suggest that the regulation of methylotrophic metabolism in *M. smegmatis* differs from that in other mycobacterial species and appears to be unique among the known methylotrophs (19, 23, 44, 45). We believe that our work on methylotrophy in *M. smegmatis* will help us to obtain a broader understanding of the gene expression regulation mechanisms functional in mycobacteria.

MATERIALS AND METHODS

Bacterial strain, media, and growth conditions. *Escherichia coli* strain XL1-Blue was used for all of the cloning experiments, whereas production of recombinant proteins was performed in the BL21(DE3) strain. Both of the strains were grown in LB broth (Difco) at 37°C with constant shaking at 200 rpm. *M. smegmatis* strain mc^2155 and its derivatives generated here were grown in MB7H9 broth (Difco) containing 2% glucose (as required) or 2% (vol/vol) of the desired alcohols, such as methanol, ethanol, or 1,3-propanediol, as carbon sources, wherever required, along with 0.05% Tween 80. Appropriate antibiotics were added in the media for all of the plasmid-bearing cultures at the concentrations reported elsewhere (23). When required, *M. smegmatis* cells were induced with 2% acetamide to monitor the expression of recombinant proteins.

Construction of genetic knockouts. Construction and confirmation of *mnoSR* and *MSMEG_6244* genetic knockouts were carried out by employing the strategy as described previously (23, 32). Upstream and downstream fragments of both of the genes were PCR amplified from *M. smegmatis* genomic DNA, whereas the Hyg^r cassette was PCR amplified from the pVV16 vector (obtained through BEI Resources, NIAID, NIH; Naked Plasmid pVV16 for expression in *Mycobacterium smegmatis*, catalog number NR-13402). Allelic exchange substrate (AES) was then constructed using the primers listed in Table 1. Linear AES DNA fragments were then electroporated in *M. smegmatis* cells containing pJV53 (kind gift from

TABLE 1 List of oligonucleotides used in the present study[a]

Oligonucleotide	Sequence (5′–3′)	Purpose
MnoSFor	ATGGCCGAAGCGGCCCGCACC	Cloning of *mnoS* in pMS-QS-CHS and pSS4
MnoSRev	GCAGAATGTCGTTGAGACGGTTGAGC	
MnoRFor	ATGACCGTCACGACGCGCGAG	Cloning of *mnoS* in pMS-QS-CHS and pSS4
MnoRRev	GATCAACCCGCGCTTGCTC	
MnoSUpFor	CAGGTCGGGGGCCTGCTCGACC	Amplification of *mnoS* upstream fragment
MnoSUpRev	CCACGTACATCACCACAAGCACCCACTCGGCGTCGAGG	
MnoSHygFor	CGAGTGGGTGCTTGTGGTGATGTACGTGGCGAACTCC	Amplification of Hyg^r cassette
MnoSHygRev	CGTACACGGCCTGATCCGGGGGGCGTCAGG	
MnoSDownFor	CCTGACGCCCCCCGGATCAGGCCGTGTACGCGGCGAGC	Amplification of *mnoS* downstream fragment
MnoSDownRev	GGTGTTCACCGGCGTCGTGCGTTCC	
MnoSLongFor	CTGGCGTTCACCAACGCGATCC	PCR confirmation of Δ*mnoS*
MnoSLongRev	CGATCTGACCCCTGACGAACTGTCC	
MnoRD60AFor	CGACGTGGTGCTGCTGGCCCTCAAGCTCTCGGCCGGATC	Site directed mutation for generating MnoR^{D60A}
6244Upfor	AGAACTCTAGAGGTGGGCGAGGGTGC	Amplification of *MSMEG_6244* upstream fragment
6244UpRev	CGTACATCACCACGCTCGCGTGCCGCGTC	
6244HygFor	GCACGCGAGCGTGGTGATGTACGTGGCGAAC	Amplification of Hyg^r cassette
6244HygRev	CTAACCGCCTGAGGGATCCGGGGGGCGTC	
6244DownFor	GACGCCCCCCGGATCCCTCAGGCGGTTAG	Amplification of *MSMEG_6244* downstream fragment
6244DownRev	GCTGCTGTTCGGGTTTGGGTCGTTC	
6244LongFor	CAGGTCCGGGCAGCTGACACCACGG	PCR confirmation of Δ*MSMEG_6244*
6244LongRev	GCTGTTGTCGGTATCGCCACAGCATTACC	
rpoBRTFor	TCGATGTCACTGTCCTTCTCGGATC	RT-qPCR for *rpoB* expression
rpoBRTRev	GACCGTCTGGCTCTTGATCTC	
mnoRTFor	TCTGCTTGTTGGTGGACTTG	RT-qPCR for *mno* expression
mnoRTRev	GTCGAACCCCAAGGACTACA	
6239RTFor	GAAATCGTGTTCGGCATCGATTCG	RT-qPCR for *MSMEG_6239* expression
6239RTRev	CTCCAGACCTGCGGGGTCACG	
*mno*pSDRev	GCATGCCAATGGCCATTGGTTCACTCCTCGCTG	Cloning of 200-bp *mno* fragment upstream from start codon in pSD5b
*mno*250pSDFor	ACGACCATCTAGAGCCTGAGCGATC	
NobiotinpSD	CCACTGCAGTGCATATGGAAGTGATTCC	PCR amplification of probe for EMSA
biotinpSD	Biotin-CCACTGCAGTGCATATGGAAGTGATTCC	
6239pSDrev	ACCGCATGCGGCGCGGACTCCACCTGC	Cloning of 200-bp *MSMEG_6239* fragment upstream from start codon in pSD5b
6239200pSDFor	GTATGCGACAAGGTGGTCGTCG	

[a]Sequence of each oligonucleotide from 5′ to 3′ is given. The purpose of each oligonucleotide in the present study is also mentioned for easy reference.

Graham Hatfull, University of Pittsburgh, USA; Addgene plasmid number 26904). Plasmid curing of pJV53 was performed essentially as described previously (48). Plasmid-cured cells were further used for complementation.

Cloning of *mnoS* and *mnoR*. Sequences of *mnoS* (*MSMEG_6238*) and *mnoR* (*MSMEG_6236*) were obtained from Mycobrowser knowledge base (28). *M. smegmatis* genomic DNA was used as a template for PCR amplification of the genes using primers given in Table 1. PCR products of the genes *mnoS* and *mnoR* were cloned in *E. coli* expression vector pMS-QS-CHS (49) to generate pADt7MnoS and pADt7MnoR, respectively. Plasmids pMV261 (carrying the *hsp60* promoter [50]) and pMVAcet (carrying a mycobacterial acetamide-inducible promoter system [33, 34]) were further modified to yield pSS1 (23) and pSS4, respectively, which helped in blunt-end cloning experiments. MnoS and MnoR were cloned in pSS4 to obtain pADatMnoS and pADatMnoR, respectively. The plasmids were subsequently used to express proteins in mycobacteria. Additionally, coexpression of *mnoS* and *mnoR* in the mycobacterial expression vector was achieved by cloning *mnoS* and *mnoR* in tandem in pSS4 to generate pADatMnoSR. Colony PCR was performed to screen for positive clones that were further confirmed by DNA sequencing. The MnoR^{D60A} mutant was constructed by performing site-directed mutagenesis as per the described protocol (51) using the primer listed in Table 1 to generate pADt7MnoR^{D60A}.

Real-time PCR for the relative mRNA expression. To monitor the relative expression of *MSMEG_6239* in both the wild type and the Δ*mnoSR* strain, RNA isolation, cDNA synthesis, and quantitative PCR (qPCR) were performed from the log-phase cells (optical density at 600 nm [OD_{600}] of ~0.8) essentially as described previously (23). Relative expression level was normalized against the expression of the internal control gene, *rpoB*. Primers used in the RT-qPCR are listed in Table 1. *P* values generated from a two-tailed Student's *t* test were considered to calculate the level of significance within the experiments performed as three independent sets.

Protein expression and purification. *E. coli* BL21(DE3) cells were transformed with pADt7MnoS, pADt7MnoR, and pADt7MnoR^{D60A} for the expression and purification of MnoS, MnoR, and MnoR^{D60A}, respectively. For the expression of recombinant proteins, *E. coli* BL21(DE3) cells were induced at an OD_{600} of ~0.6 by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the protein induction was allowed to take place at 22°C for 12 h. Standard Ni-nitrilotriacetic acid (NTA) column chromatography was performed for the purification of MnoR and MnoR^{D60A} as discussed previously for other proteins (33). MnoS purification was carried out as described elsewhere (52). Both of the eluted proteins were assessed by SDS-PAGE for purity. Purified proteins were dialyzed against buffer containing 40 mM Tris-Cl (pH 8.0), 150 mM

NaCl, 1 mM dithiothreitol (DTT), and 40% glycerol, and stored at −20°C until further use. Quantification of proteins was carried out by Bradford assay (Bio-Rad) as per the manufacturer's instructions.

***In vitro* kinase and phosphotransferase activities of MnoS.** Purified MnoS and MnoR at a concentration of 10 μM were subjected to autokinase activity by following the method described previously (40). Briefly, the proteins were incubated with 10 μCi of [γ-^{32}P]ATP (Brit, India) in a buffer containing 50 mM Tris-Cl (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, and 50 μM ATP) at 25°C for 60 min. To assess the phosphotransferase activity, MnoR or $MnoR^{D60A}$ at a concentration of 10 μM was incubated with MnoS in the reaction mixture mentioned above for specified times. In all of the cases, the reaction mixtures were subsequently mixed with SDS loading dye containing 6 M urea, loaded on an SDS-PAGE gel without boiling, and electrophoresed. The gel was further dried and exposed to a phosphor screen, and the autoradiogram was recorded on a Typhoon FLA 9000 phosphorimager (GE Healthcare).

Analysis of expressed proteins by immunoblotting. To monitor the expression of various proteins, *M. smegmatis* cells harboring required plasmids were induced at log phase with 2% acetamide, wherever required, and harvested after 5 h. Assessment of the protein expression by immunoblotting was carried out essentially as described before (23). Immunoblotting of Mno was carried out using anti-Mno antibody raised in rabbit (Bioneeds India Pvt. Ltd.). For proteins carrying the hexa-histidine tag, anti-His antibody raised in mouse (Sigma-Aldrich) was used. Blots were further probed with either anti-mouse IgG DyLight 680-conjugated secondary antibody or anti-rabbit IgG DyLight 800-conjugated secondary antibody, as required. Blots were developed on an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE, USA). The protein amounts in all of the samples were quantified by Bradford assay, and equal amounts were loaded on the gel for the analysis of protein expression.

Generation of promoter-reporter constructs and β-galactosidase assay. To assess *mno* and *MSMEG_6239* promoter activity in the presence and absence of methanol and 1,3-propanediol, putative promoter regions of *mno* and *MSMEG_6239* were cloned in a promoterless *E. coli-Mycobacterium* shuttle plasmid, pSD5b (42). Approximately 150- and 200-bp regions upstream from the translation start site of *mno* and a 200-bp region upstream from the translation start site of *MSMEG_6239* were PCR amplified using the primers mentioned in Table 1 and cloned in the pSD5b vector between the SphI and XbaI sites to generate pSD*mno*, pSD150mno, and pSD*6239*, respectively. Positive clones were verified by DNA sequencing. For the β-galactosidase assay, *M. smegmatis* cells carrying the required plasmid were grown in the presence or absence of the desired alcohol; log-phase cells were used to carry out the assay by following the method as described before (53, 54).

Interaction of MnoR and *mno* promoter by electrophoretic mobility shift assay. EMSA was performed using the biotin-labeled DNA fragment containing the putative *mno* promoter region. The DNA fragment was PCR amplified from pSDmno and using the primers listed in Table 1; the reverse primers NobiotinpSD and biotinpSD were used to obtain nonbiotinylated or biotinylated DNA fragments, respectively. Purified MnoR and the biotinylated DNA probe were incubated in the binding buffer (40 mM Tris-acetate [pH 8.3], 10 mM magnesium acetate, 1 mM EDTA, 150 mM NaCl, 5% glycerol, and 5 mM 2-mercaptoethanol) for 30 min at 25°C. The DNA-MnoR complex was separated on a 7% nondenaturing acrylamide gel at 10 V/cm for 70 min at 4°C and was further detected by using the LightShift chemiluminescent EMSA kit (Thermo Fisher Scientific) following the manufacturer's instructions. For the specific competition experiments, nonbiotinylated DNA was added to the reaction mixture before PAGE separation.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at https://doi.org/10.1128/AEM.00535-19.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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We declare no conflicts of interest.

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