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Identification of the Monooxygenase Gene Clusters Responsible for the Regioselective Oxidation of Phenol to Hydroquinone in Mycobacteria▽

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Mycobacterium goodii **strain 12523 is an actinomycete that is able to oxidize phenol regioselectively at the *para* position to produce hydroquinone. In this study, we investigated the genes responsible for this unique regioselective oxidation. On the basis of the fact that the oxidation activity of *M. goodii* strain 12523 toward phenol is induced in the presence of acetone, we first identified acetone-induced proteins in this microorganism by two-dimensional electrophoretic analysis. The N-terminal amino acid sequence of one of these acetone-induced proteins shares 100% identity with that of the protein encoded by the open reading frame Msmeg_1971 in *Mycobacterium smegmatis* strain mc^{2}155, whose genome sequence has been determined. Since Msmeg_1971, Msmeg_1972, Msmeg_1973, and Msmeg_1974 constitute a putative binuclear iron monooxygenase gene cluster, we cloned this gene cluster of *M. smegmatis* strain mc^{2}155 and its homologous gene cluster found in *M. goodii* strain 12523. Sequence analysis of these binuclear iron monooxygenase gene clusters revealed the presence of four genes designated *mimABCD*, which encode an oxygenase large subunit, a reductase, an oxygenase small subunit, and a coupling protein, respectively. When the *mimA* gene (Msmeg_1971) of *M. smegmatis* strain mc^{2}155, which was also found to be able to oxidize phenol to hydroquinone, was deleted, this mutant lost the oxidation ability. This ability was restored by introduction of the *mimA* gene of *M. smegmatis* strain mc^{2}155 or of *M. goodii* strain 12523 into this mutant. Interestingly, we found that these gene clusters also play essential roles in propane and acetone metabolism in these mycobacteria.**

Mycobacterium goodii strain 12523 is a unique actinomycete that is able to oxidize phenol regioselectively at the *para* position to produce hydroquinone (20). This microorganism was discovered for application in biocatalysis: chemical synthesis of hydroquinone is accompanied by side reactions leading to undesired by-products such as catechol, whereas *M. goodii* strain 12523 enabled gram-scale production of hydroquinone without by-products (21). Although *M. goodii* strain 12523 cannot utilize phenol, this microorganism can use acetone and methylethylketone as sources of carbon and energy. Interestingly, the oxidation activity of *M. goodii* strain 12523 toward phenol is induced in the presence of acetone and methylethylketone (20). To date, there have been only a few reports concerning monooxygenases that convert phenol to hydroquinone. P450BM-3, a cytochrome P450 monooxygenase from *Bacillus megaterium*, exhibits such an activity, although this activity is very low (23). A P450BM-3 mutant was then created, which exhibited 16.5 times higher activity than the wild type (23). A strain with a mutation of toluene-*o*-xylene monooxygenase, a binuclear iron monooxygenase from *Pseudomonas stutzeri*, also converts phenol to hydroquinone and catechol in a proportion of 80:20, although the wild type produces only catechol (27).

In this study, we identified a monooxygenase gene cluster that is responsible for the conversion of phenol to hydroquinone in *M. goodii* strain 12523. On the basis of the fact that the oxidation activity of *M. goodii* strain 12523 toward phenol is induced in the presence of acetone, we first identified acetone-induced proteins in this microorganism by two-dimensional electrophoretic analysis. N-terminal amino acid sequencing of these acetone-induced proteins, combined with analysis of the genome sequence of *Mycobacterium smegmatis* strain mc^{2}155, subsequently led to identification of the monooxygenase gene cluster by deletion and complementation analyses. We found that this gene cluster encodes a multicomponent monooxygenase that belongs to the binuclear iron monooxygenase family and that its homologous gene cluster found in *M. smegmatis* strain mc^{2}155 also encodes monooxygenase activity toward phenol. Furthermore, we found that these gene clusters were also involved in propane and acetone metabolism in these mycobacteria.

MATERIALS AND METHODS

Bacterial strains, plasmids, and cultivation media. The bacterial strains and plasmids that were used or constructed in this study are listed in Table 1. Strain 12523, which had previously been identified as a *Mycobacterium* sp. (20), was recently identified as *M. goodii* based on 16S rRNA gene sequence analysis (1). The bacteria were grown in Luria-Bertani (LB) medium, which contained (per liter) Bacto tryptone (10 g), Bacto yeast extract (5 g), and NaCl (10 g) (pH 7.0), or in KG medium, which contained (per liter) $(NH_4)_2SO_4$ (3 g), KH_2PO_4 (1.4 g), Na_2HPO_4 (2.1 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), $FeCl_2 \cdot 5H_2O$ (10.6 mg), $CaCl_2 \cdot 2H_2O$ (8 mg), $ZnSO_4 \cdot 7H_2O$ (4 mg), $MnCl_2 \cdot 4H_2O$ (2 mg), $CuSO_4 \cdot 5H_2O$ (0.02 mg), KI (0.2 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.2 mg), $CoCl_2 \cdot 6H_2O$ (0.2 mg), H_3BO_3 (0.4 mg), and NaCl (10 mg) (pH 7.2).

Two-dimensional electrophoretic analysis. *M. goodii* strain 12523 cells were cultivated for 6 days in LB medium (2 ml) at 30°C. After centrifugation at

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Characteristics	Reference or source
Strains		
M. goodii 12523	Wild type	20
M. smegmatis mc^{2}155	Wild type, ATCC 700084	ATCC
M. smegmatis mc^{2}155 Δ*mimA*	*M. smegmatis* strain mc^{2}155 with *mimA* deletion	This study
Gordonia sp. strain TY-5	Wild type	7
Gordonia sp. strain TY-5 *prmB*::Kan^r	*Gordonia* sp. strain TY-5 with *prmB* disruption	7
E. coli DH5α	Host used for cloning	Takara Bio
Plasmids		
pET21a	Vector used for cloning	Novagen
pETmimABCD$_{go}$	pET21a containing *mimABCD*$_{go}$ of *M. goodii* 12523	This study
pETmimABCD$_{sm}$	pET21a containing *mimABCD*$_{sm}$ of *M. smegmatis* mc^{2}155	This study
pK18mobsacB	Vector used for deletion mutagenesis, *aph sacB*	NBRP (NIG, Japan)
pKΔ*mimA*	pK18mobsacB containing a deleted *mimA* gene of *M. smegmatis* mc^{2}155	This study
pRHK1	*Rhodococcus* (*Mycobacterium*)-*E. coli* shuttle vector, Kan^r	6
pUCkap1	pUC18 containing the kap1 promoter region from *R. erythropolis*	13
pRHKkap1	pRHK1 containing the kap1 promoter region	This study
pRHKkap1mimA$_{go}$	pRHKkap1 containing the *mimA*$_{go}$ gene under the control of the kap1 promoter	This study
pRHKkap1mimA$_{sm}$	pRHKkap1 containing the *mimA*$_{sm}$ gene under the control of the kap1 promoter	This study

10,000 × *g* for 10 min at 4°C, the cells were suspended in KG medium (2 ml) with or without acetone (1%, vol/vol), and were incubated for 24 h at 30°C. *M. goodii* strain 12523 cells with and without acetone induction were harvested by centrifugation, washed with potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10%, vol/vol), and stored at −80°C until use.

The frozen cells were suspended in a solution containing Tris (60 mM), urea (5 M), thiourea (1 M), Complete Mini EDTA-free (one tablet per 10 ml; Roche, Mannheim, Germany), 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid (CHAPS) (10 g/liter), Triton X-100 (1% vol/vol), and dithiothreitol (10 g/liter) (pH 8.8 to 9.0) and were disrupted with an ultraoscillator. After centrifugation at 100,000 × *g* for 20 min at 4°C, the resulting supernatant was supplemented with acrylamide (0.1 M) and was subjected to two-dimensional electrophoretic analysis. Isoelectric focusing was performed for the first dimension. The supernatant (75 μg protein) was loaded onto an agar gel (pH 3 to 10; ATTO, Tokyo, Japan) and was analyzed using the AE-6540 electrophoresis unit (ATTO). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed for the second dimension. The agar gel after isoelectric focusing was loaded onto a polyacrylamide gel (12.5%) and was analyzed using the AE-6541 electrophoresis unit (ATTO). Proteins in the gel after SDS-PAGE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane, and their N-terminal amino acid sequences were determined by APRO Life Science (Tokushima, Japan) using Edman degradation.

Cloning and sequence analysis. The genomic DNAs of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 were prepared using a DNeasy tissue kit (Qiagen, Germantown, MD). Two oligonucleotide primers, mimABCD-F and mimABCD-R (Table 2), were designed to amplify the binuclear iron monooxygenase gene clusters of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155, based on the N-terminal amino acid sequences of two acetone-induced proteins from *M. goodii* strain 12523 and the genome sequence of *M. smegmatis* strain mc^{2}155 (GenBank accession number, NC_008596). The region between the two oligonucleotide primers was amplified from the genomic DNAs of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 using PCR. These amplified DNA fragments were digested with NdeI and EcoRI and were inserted into the pET21a vector (Table 1). The resulting plasmids, pETmimABCD$_{go}$ for *M. goodii* strain 12523 and pETmimABCD$_{sm}$ for *M. smegmatis* strain mc^{2}155, were amplified in *Escherichia coli* DH5α cells, and the inserts were sequenced using an ABI PRISM 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA).

Deletion of the *mimA* gene in *M. smegmatis* strain mc^{2}155. The *mimA* gene in *M. smegmatis* strain mc^{2}155 was deleted in frame using the pK18mobsacB vector (19) (Table 1). Two oligonucleotide primers, mimA5′-F and mimA5′-R (Table 2), were designed to amplify the 5′-terminal region of the *mimA* gene based on the genome sequence of *M. smegmatis* strain mc^{2}155. The region between the two oligonucleotide primers was amplified from the genomic DNA of *M. smegmatis* strain mc^{2}155 using PCR. These amplified DNA fragments were digested with HindIII and XbaI and were inserted into the pK18mobsacB vector. Two other oligonucleotide primers, mimA3′-F and mimA3′-R (Table 2), were designed to amplify the 3′-terminal region of the *mimA* gene. These amplified DNA fragments were digested with XbaI and EcoRI and were subsequently inserted into the pK18mobsacB vector containing the 5′-terminal region of the *mimA* gene. The resulting plasmid, pKΔ*mimA*, contained a deleted *mimA* gene encoding a 42-amino-acid protein instead of a full-length *mimA* gene encoding a 542-amino-acid protein.

A two-step recombination was performed to delete the *mimA* gene from the chromosome of *M. smegmatis* strain mc^{2}155 as described previously (11, 22, 26). The pKΔ*mimA* plasmid was introduced into *M. smegmatis* strain mc^{2}155 cells by electroporation. Single-crossover mutants, into which the plasmid was integrated, were selected on an LB plate containing kanamycin (50 μg/ml). Kanamycin-resistant strains were then subjected to repeated cultivation in LB medium without kanamycin. Finally, double-crossover mutants, which had lost the vector backbone and were sensitive to kanamycin, were selected on LB plates with or without kanamycin. Deletion of the *mimA* gene was confirmed by PCR using the

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Restriction site
mimABCD-F	GAATTCCATATGAGCAGACA AAGCCTGACCAAG	NdeI
mimABCD-R	CCGGAATTCCTGTTGGCCGT CTTTTTCGTACAT	EcoRI
mimA5′-F	CCCAAGCTTGTGATCCAGCA GCACCGTCTGATC	HindIII
mimA5′-R	GCTCTAGACGGCTCCCACGT GAGTTCGCTTATC	XbaI
mimA3′-F	GCTCTAGAATGACCGACGAC GAGCGCGAC	XbaI
mimA3′-R	CGGAATTCTCTTTGATTCCCT TGCCCAGTTCG	EcoRI
kap1-F	AACTGCAGGCCGCGACGTGG CTGTTGCTGA	PstI
kap1-R	GCTCTAGAACGAATTGATCT TCTGGCGCGT	XbaI
mimA-F	GCTCTAGACCAACTATCAGG AGGCTCACGTTG	XbaI
mimA-R	CCGGAATTCTCAGGCCGGGA CCCCGCCGGCGCG	EcoRI

[a] Restriction sites are underlined.

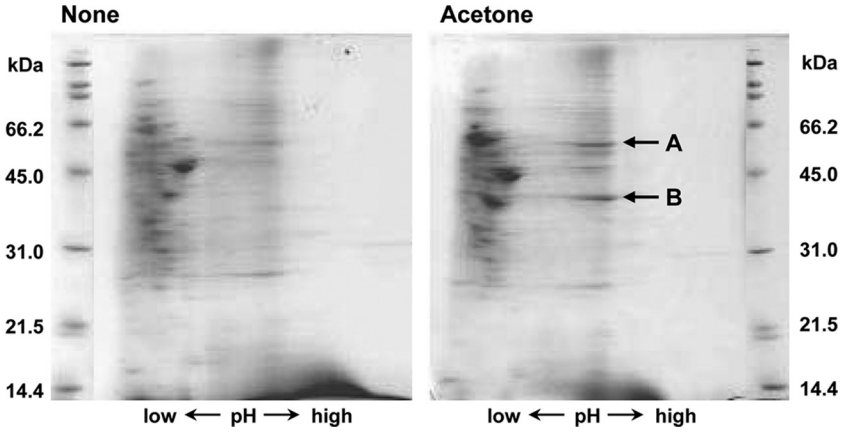

FIG. 1. Identification of acetone-induced proteins. Two-dimensional electrophoretic protein profiles of extracts from *M. goodii* strain 12523 cells in the absence and presence of acetone (left and right panels, respectively) are shown. The two acetone-induced proteins whose N-terminal sequences were determined (A and B) are indicated by arrows.

two oligonucleotide primers mimA5′-F and mimA3′-R (Table 2). This procedure resulted in the deletion mutant *M. smegmatis* mc^{2}155 Δ*mimA* (Table 1).

Construction of the *mimA* expression plasmids. The plasmids used for expression of the *mimA* genes of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 were constructed using the pRHK1 vector (6) (Table 1) and the kap1 promoter (13) (GenBank accession number, AB083090) from *Rhodococcus erythropolis*. Two oligonucleotide primers, kap1-F and kap1-R (Table 2), were designed to amplify the kap1 promoter region. The region between the two oligonucleotide primers was amplified from the pUCkap1 plasmid (13) (Table 1) using PCR. These amplified DNA fragments were digested with PstI and XbaI and were inserted into the pRHK1 vector to construct pRHKkap1. Two other oligonucleotide primers, mimA-F and mimA-R (Table 2), were designed to amplify the *mimA* genes and their putative ribosome binding sites, based on the determined sequences of these genes and the sequence upstream from the *mimA* gene in the genome sequence of *M. smegmatis* strain mc^{2}155. The region between the two oligonucleotide primers was amplified from the genomic DNAs of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 using PCR. These amplified DNA fragments were digested with XbaI and EcoRI and were subsequently inserted into the pRHKkap1 vector. The resulting plasmids, pRHKkap1mimA$_{go}$, for *M. goodii* strain 12523 and pRHKkap1mimA$_{sm}$ for *M. smegmatis* strain mc^{2}155, were introduced into *M. smegmatis* strain mc^{2}155 Δ*mimA* cells by electroporation.

Assays of monooxygenase activity and growth substrates. Monooxygenase activities of mycobacterial strains toward phenol were examined using growing cells. Wild-type and mutant strains were inoculated into KG medium (2 ml) containing Tween 80 (0.05%, vol/vol) and pyruvate (1 g/liter), which was used as a source of carbon and energy, in glass vials (14 ml) sealed with screw caps. The headspace volume was sufficient to prevent any oxygen limitation during growth and reaction. The strains were cultivated at 37°C with reciprocal shaking at a speed of 240 strokes per min. After cultivation for 24 h, acetone (1%, vol/vol) and phenol (500 μM) were added to the medium as an inducer and a substrate, respectively, and the growing cells were reacted with phenol for 5 days. Following the reaction, high-pressure liquid chromatography (HPLC) analysis was performed using an HPLC system (1100 series; Agilent, Palo Alto, CA) with a Cosmosil 5C18-PAQ column (4.6 mm by 250 mm; particle size, 5 μm; Nacalai Tesque, Kyoto, Japan). The reaction mixture was acidified by the addition of HCl (pH 2 to 3) and was then extracted with ethyl acetate (2 ml). The extract (1 ml) was evaporated, and the resulting residue was dissolved in a water-methanol mixture at a ratio of 50:50 (200 μl). After filtration through a 0.20-μm-pore-size polytetrafluoroethylene membrane (Advantec, Tokyo, Japan), the sample (10 μl) was injected into the HPLC system. Mobile phases A and B were composed of potassium phosphate buffer (10 mM, pH 6.9) and methanol, respectively. The samples were eluted with a linear gradient of 0% to 20% B for 10 min, followed by a linear gradient of 20% to 80% B for 5 min at a flow rate of 1 ml/min. Hydroquinone produced from phenol was spectrophotometrically detected at 290 nm.

We also examined the monooxygenase activities of *Gordonia* sp. strain TY-5 and its mutant strain (7) (Table 1) toward phenol using resting cells. The strains were cultivated for 2 days in LB medium (2 ml) at 30°C. After centrifugation at 10,000 × *g* for 10 min at 4°C, the cells were suspended in KG medium (2 ml) with acetone (1%, vol/vol) and were incubated for 24 h at 30°C. The cells of *Gordonia* sp. strain TY-5 and its mutant strain with acetone induction were harvested by centrifugation, washed with potassium phosphate buffer (50 mM, pH 7.5) containing glycerol (10%, vol/vol), and suspended in the same buffer (200 μl). The resting cells were reacted with phenol (2 mM) at 30°C with vigorous shaking for 5 h. Following the reaction, HPLC analysis was performed as described above.

Propane, 1-propanol, 2-propanol, acetone, methylethylketone, and acetol were examined as potential growth substrates of mycobacterial strains. Wild-type and mutant strains were inoculated into KG medium (2 ml) containing Tween 80 (0.05%, vol/vol) and individual compounds as a source of carbon and energy in glass vials (14 ml) sealed with screw caps. The strains were cultivated at 37°C with reciprocal shaking at a speed of 240 strokes per min. Propane was added to the headspace at a concentration of 20% (vol/vol). 1-Propanol, acetone, methylethylketone, and acetol were added to the medium at a concentration of 1% (vol/vol), whereas 2-propanol was added at a concentration of 0.5% (vol/vol). Cell growth was measured turbidimetrically at 660 nm and is expressed as cell dry weight.

Nucleotide sequence accession number. The nucleotide sequence of the monooxygenase gene cluster of *M. goodii* strain 12523 has been submitted to GenBank, and the assigned accession number is AB568291.

RESULTS

Identification of acetone-induced proteins by two-dimensional electrophoretic analysis. On the basis of the fact that the oxidation activity of *M. goodii* strain 12523 toward phenol is induced in the presence of acetone (20), we first examined acetone-induced proteins in this microorganism by two-dimensional electrophoretic analysis. Comparison of the protein profiles of extracts from *M. goodii* strain 12523 cells with and without acetone induction revealed the presence of several spots that appeared only with acetone induction (Fig. 1). We chose two major spots corresponding to proteins A (ca. 57 kDa) and B (ca. 43 kDa) on the gel shown in Fig. 1 and determined the N-terminal amino acid sequences of these proteins. The N-terminal sequences of proteins A and B were SRQSLTKAHAKISELTWE and MYEKDGQQYFIV DSHVHL, respectively.

These N-terminal sequences were analyzed using the BLAST program at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence of protein A shares 100% identity with that of the protein encoded by the open reading frame (ORF) Msmeg_1971 in *M. smegmatis* strain mc^{2}155, whose genome sequence has been determined.

Similarly, the sequence of protein B shares 100% identity with that of the protein encoded by Msmeg_1975. Although the functions of the Msmeg_1971 and Msmeg_1975 proteins have not been experimentally determined, these proteins exhibit amino acid similarities with oxygenase large subunits of binuclear iron monooxygenases and amidohydrolases, respectively. Furthermore, the Msmeg_1972, Msmeg_1973, and Msmeg_1974 proteins, which are encoded by the ORFs between Msmeg_1971 and Msmeg_1975, exhibit amino acid similarities with reductases, oxygenase small subunits, and coupling proteins, respectively, of binuclear iron monooxygenases.

Cloning and sequence analysis of monooxygenase gene clusters in *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155. Assuming that the binuclear iron monooxygenase gene cluster consisting of Msmeg_1971, Msmeg_1972, Msmeg_1973, and Msmeg_1974 in *M. smegmatis* strain mc^{2}155 and its homologous gene cluster in *M. goodii* strain 12523 are involved in the conversion of phenol to hydroquinone, we cloned these gene clusters. Two oligonucleotide primers were designed based on the N-terminal amino acid sequences of the two acetone-induced proteins from *M. goodii* strain 12523 and the genome sequence of *M. smegmatis* strain mc^{2}155. The region between the two oligonucleotide primers was amplified from the genomic DNAs of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 using PCR and was cloned into the pET21a vector. Sequence analysis revealed the presence of four ORFs, Msmeg_1971, Msmeg_1972, Msmeg_1973, and Msmeg_1974, in the PCR product from *M. smegmatis* strain mc^{2}155 and their homologous four ORFs in that from *M. goodii* strain 12523. These four ORFs were carried on the same strand and were designated *mimABCD* for the mycobacterial binuclear iron monooxygenase gene clusters. For convenience, we named the gene cluster from *M. goodii* strain 12523 *mimABCD*$_{go}$ and that from *M. smegmatis* strain mc^{2}155 *mimABCD*$_{sm}$.

We confirmed that the nucleotide sequence of *mimABCD*$_{sm}$ (4,238 bp) from *M. smegmatis* strain mc^{2}155 coincided with that determined by the genome sequencing project and further sequence analysis (3). The nucleotide sequence of *mimABCD*$_{go}$ (4,231 bp) from *M. goodii* strain 12523 shares 92% overall identity with that of *mimABCD*$_{sm}$ from *M. smegmatis* strain mc^{2}155 based on a BLAST search. Intergenic distances between the stop codons and the start codons were 91 bp for *mimA*$_{sm}$ and *mimB*$_{sm}$ and 23 bp for *mimB*$_{sm}$ and *mimC*$_{sm}$, whereas the 3′-terminal nucleotide of *mimC*$_{sm}$ and the 5′-terminal nucleotide of *mimD*$_{sm}$ overlapped by 4 bp. Similarly, intergenic distances between the stop codons and the start codons were 83 bp for *mimA*$_{go}$ and *mimB*$_{go}$ and 24 bp for *mimB*$_{go}$ and *mimC*$_{go}$, whereas the 3′-terminal nucleotide of *mimC*$_{go}$ and the 5′-terminal nucleotide of *mimD*$_{go}$ overlapped by 4 bp. Putative ribosome binding sites were found in the upstream regions of *mimB*$_{sm}$, *mimC*$_{sm}$, and *mimD*$_{sm}$ and *mimB*$_{go}$, *mimC*$_{go}$, and *mimD*$_{go}$. We also confirmed that a putative ribosome binding site and a putative promoter exist in the upstream region of the *mimA*$_{sm}$ gene in the genome sequence of *M. smegmatis* strain mc^{2}155. These observations suggest that the gene clusters are transcribed polycistronically.

Unexpectedly, we found that the multicomponent monooxygenases encoded by the *mimABCD* gene clusters exhibit high amino acid similarities with two propane monooxygenases (Prm) from *Rhodococcus* sp. strain RHA1 (22) and *Gordonia* sp. strain TY-5 (7) (Table 3). Furthermore, these monooxygenases also exhibit appreciable amino acid similarities with a tetrahydrofuran monooxygenase (Thm) from *Pseudonocardia* sp. strain K1 (25) and a propene monooxygenase (Pmo) from *Mycobacterium* sp. strain M156 (2) (Table 3). The order of the *mimABCD* gene clusters is an oxygenase large subunit, a reductase, an oxygenase small subunit, and a coupling protein, which is identical to that of the *prm* and *thm* gene clusters but is different from that of the *pmo* gene cluster. The *mimA*$_{go}$ and *mimA*$_{sm}$ genes each encode 542-amino-acid proteins. The MimA$_{go}$ protein of strain 12523 shares 99, 94, and 93% amino acid identity with MimA$_{sm}$ of strain mc^{2}155, PrmA of strain RHA1, and PrmA of strain TY-5, respectively (Table 3). We confirmed that a pair of motif sequences (Glu-X-X-His) that coordinate to the binuclear iron center at the active site (16) are conserved in MimA$_{go}$ and MimA$_{sm}$. Similarly, the MimB$_{go}$, MimC$_{go}$, and MimD$_{go}$ proteins of strain 12523 exhibit amino acid similarities with their corresponding components of strains mc^{2}155, RHA1, and TY-5 (Table 3). Compared with MimA$_{go}$ and MimD$_{go}$, MimB$_{go}$ and MimC$_{go}$ exhibit relatively low similarities (64 to 81%) with the propane monooxygenase components.

Deletion and complementation analyses of the monooxygenase gene clusters. To confirm that the binuclear iron monooxygenase gene clusters in *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 are involved in the conversion of phenol to hydroquinone, we performed deletion and complementation analyses of the *mimA* genes. We first confirmed that *M. smegmatis* strain mc^{2}155 was also able to oxidize phenol to hydroquinone (Fig. 2). *M. smegmatis* strain mc^{2}155 was used for construction of the deletion mutant because this strain can be easily transformed, whereas attempts to introduce heterologous genes into *M. goodii* strain 12523 cells were unsuccessful. We examined the monooxygenase activities of the wild-type and mutant *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains toward phenol using growing cells in the presence of pyruvate and acetone, which were used as a source of carbon and energy and an inducer, respectively. *M. goodii* strain 12523, *M. smegmatis* strain mc^{2}155, and the deletion mutant mc^{2}155 Δ*mimA* were all able to grow on a pyruvate carbon source. The growing cells of *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 produced 114 μM and 104 μM hydroquinone, respectively, from 500 μM phenol in 5 days, whereas the deletion mutant mc^{2}155 Δ*mimA* did not produce any hydroquinone (Fig. 2). To confirm that the loss of monooxygenase activity toward phenol in this mutant was due only to deletion of the *mimA* gene, we determined if complementation of this gene in the deletion mutant mc^{2}155 Δ*mimA* would restore hydroquinone production. The *mimA*$_{sm}$ gene was constitutively expressed under the control of the kap1 promoter derived from *R. erythropolis* using the pRHK1 vector (Table 1). The growing cells of *M. smegmatis* strain mc^{2}155 Δ*mimA* that was complemented with the *mimA*$_{sm}$ gene produced 61.1 μM hydroquinone from phenol (Fig. 2). Furthermore, when we complemented the deletion mutant mc^{2}155 Δ*mimA* with the *mimA*$_{go}$ gene from *M. goodii* strain 12523, the growing cells produced 63.9 μM hydroquinone from phenol (Fig. 2). These results indicate that the *mimA* genes are essential for the oxidation of phenol to hydroquinone.

We also examined the monooxygenase activity of *Gordonia*

TABLE 3. Amino acid identity between *mimABCD*$_{go}$-encoded proteins and other binuclear iron monooxygenases

mimABCD$_{go}$ gene and comparison gene	No. of amino acid residues	% Identity (no. of identical amino acids/total no. of amino acids)[a]	Organism	Accession no.
mimA$_{go}$	542		*M. goodii* 12523	AB568291
mimA$_{sm}$	542	99 (537/542)	*M. smegmatis* mc^{2}155	YP_886336
prmA	544	94 (516/544)	*Rhodococcus* sp. strain RHA1	YP_700435
prmA	545	93 (501/537)	*Gordonia* sp. strain TY-5	BAD03956
thmA	545	41 (217/520)	*Pseudonocardia* sp. strain K1	CAC10506
pmoC	501	38 (202/524)	*Mycobacterium* sp. strain M156	AAS19484
mimB$_{go}$	348		*M. goodii* 12523	AB568291
mimB$_{sm}$	348	93 (327/348)	*M. smegmatis* mc^{2}155	YP_886337
prmB	370	76 (267/347)	*Rhodococcus* sp. strain RHA1	YP_700436
prmB	346	64 (229/355)	*Gordonia* sp. strain TY-5	BAD03957
thmD	360	47 (164/347)	*Pseudonocardia* sp. strain K1	CAC10508
pmoD	340	36 (123/341)	*Mycobacterium* sp. strain M156	AAS19485
mimC$_{go}$	368		*M. goodii* 12523	AB568291
mimC$_{sm}$	368	94 (346/368)	*M. smegmatis* mc^{2}155	ABJ96310
prmC	368	81 (299/368)	*Rhodococcus* sp. strain RHA1	YP_700437
prmC	368	74 (273/368)	*Gordonia* sp. strain TY-5	BAD03958
thmB	346	32 (96/296)	*Pseudonocardia* sp. strain K1	CAC10509
pmoA	346	29 (90/304)	*Mycobacterium* sp. strain M156	AAS19482
mimD$_{go}$	114		*M. goodii* 12523	AB568291
mimD$_{sm}$	114	96 (110/114)	*M. smegmatis* mc^{2}155	YP_886338
prmD	113	91 (102/112)	*Rhodococcus* sp. strain RHA1	YP_700438
prmD	111	90 (100/110)	*Gordonia* sp. strain TY-5	BAD03959
thmC	117	30 (32/106)	*Pseudonocardia* sp. strain K1	CAC10510
pmoB	111	28 (29/102)	*Mycobacterium* sp. strain M156	AAS19483

[a] Amino acid identity was determined using the BLAST program.

sp. strain TY-5 and its mutant strain with a *prmB* disruption in the *prmABCD* propane monooxygenase gene cluster (7) (Table 1) using resting cells with acetone induction. The resting cells of *Gordonia* sp. strain TY-5 regioselectively produced 1.1 mM hydroquinone from 2 mM phenol in 5 h, whereas the disruption mutant TY-5 *prmB*::Kan^r did not produce any hydroquinone (data not shown).

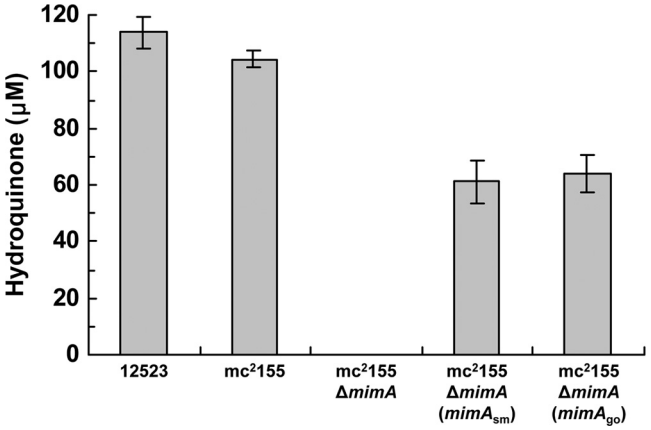

FIG. 2. Monooxygenase activities of wild-type and mutant strains toward phenol. Growing cells of wild-type 12523 and mc^{2}155, the deletion mutant mc^{2}155 Δ*mimA*, and the complemented strains mc^{2}155 Δ*mimA* (*mimA*$_{sm}$) and mc^{2}155 Δ*mimA* (*mimA*$_{go}$), which were induced with acetone, were reacted with phenol, and the monooxygenation product hydroquinone was quantified using HPLC. Bars represent the averages from three independent experiments, and error bars represent the standard deviations from the means.

Growth on propane and acetone. Since the multicomponent monooxygenases encoded by the *mimABCD* gene clusters exhibit high amino acid similarities with propane monooxygenases from *Rhodococcus* sp. strain RHA1 (22) and *Gordonia* sp. strain TY-5 (7), we examined the growth of the wild-type and mutant *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains on propane. We first confirmed that the wild-type *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 were able to grow on propane as a source of carbon and energy (Fig. 3). We then found that the deletion mutant mc^{2}155 Δ*mimA* had lost the ability to grow on propane, whereas this ability was restored by introduction of the *mimA*$_{sm}$ gene or the *mimA*$_{go}$ gene into this mutant (Fig. 3). These results suggest that the gene clusters encode monooxygenase activity toward propane as well as phenol. We also confirmed that the wild-type *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains were able to grow on 1-propanol and 2-propanol (Fig. 3). Furthermore, we found that growth on 2-propanol was dependent on the presence of the *mimA*$_{sm}$ gene or the *mimA*$_{go}$ gene, whereas growth on 1-propanol was not (Fig. 3).

We next examined the growth of the wild-type and mutant *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains on acetone and methylethylketone, since the monooxygenase activity of these strains toward phenol is known to be induced in the presence of acetone and methylethylketone (20). The wild-type *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains were able to grow on acetone and methylethylketone as a source of carbon

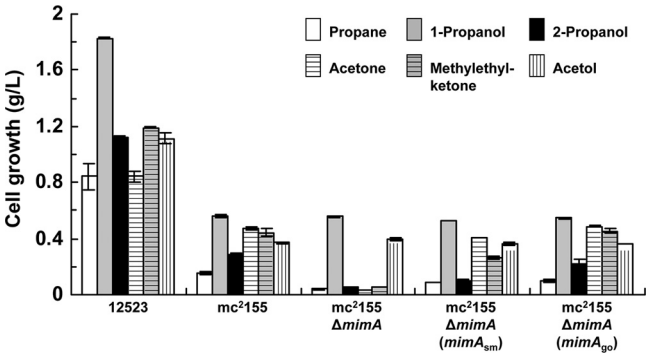

FIG. 3. Growth of wild-type and mutant strains on propane and related compounds. Cells of wild-type 12523 and mc^{2}155, the deletion mutant mc^{2}155 Δ*mimA*, and the complemented strains mc^{2}155 Δ*mimA* (*mimA*$_{sm}$) and mc^{2}155 Δ*mimA* (*mimA*$_{go}$) were cultivated on individual compounds as a source of carbon and energy for 8 days. Cell growth is expressed as cell dry weight per liter of medium. Bars represent the averages from two independent experiments, and error bars represent the standard deviations from the means.

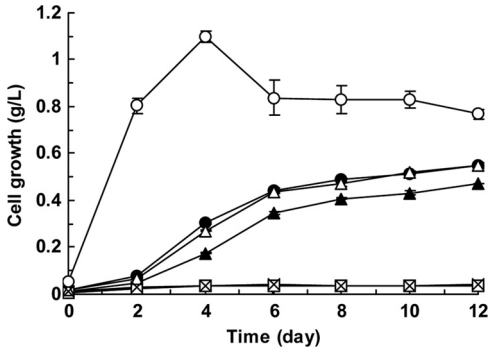

FIG. 4. Growth profiles of wild-type and mutant strains on acetone. Cells of wild-type 12523 (white circles) and mc^{2}155 (white triangles), the deletion mutant mc^{2}155 Δ*mimA* (squares), and the complemented strains mc^{2}155 Δ*mimA* (*mimA*$_{sm}$) (black triangles) and mc^{2}155 Δ*mimA* (*mimA*$_{go}$) (black circles) were cultivated on acetone as a source of carbon and energy. The growth of the deletion mutant mc^{2}155 Δ*mimA* in the absence of acetone is indicated by crosses. Cell growth is expressed as cell dry weight per liter of medium. Plots represent the averages from two independent experiments, and error bars represent the standard deviations from the means.

and energy (Fig. 3 and 4). Surprisingly, the deletion mutant mc^{2}155 Δ*mimA* had lost the ability to grow on acetone and methylethylketone, whereas this ability was restored by introduction of the *mimA*$_{sm}$ gene or the *mimA*$_{go}$ gene into this mutant (Fig. 3 and 4). These results indicate that the monooxygenase gene clusters are involved in acetone and methylethylketone metabolism. Although the wild-type *M. goodii* 12523 and *M. smegmatis* mc^{2}155 strains were able to grow on acetol, the deletion mutant mc^{2}155 Δ*mimA* did not lose the ability to grow on this substrate (Fig. 3).

DISCUSSION

Binuclear iron monooxygenases are a family of proteins that contain a binuclear iron center at the active site and introduce one oxygen atom derived from molecular oxygen into organic molecules, including alkanes, alkenes, and aromatics (9). These monooxygenases are widely distributed throughout prokaryotes such as actinomycetes, methanotrophs, and pseudomonads. The monooxygenases in actinomycetes have only recently been reported and constitute a new subfamily. The first monooxygenase to be reported was the alkene monooxygenase (Amo) of *Rhodococcus corallines* strain B-276 (18). This enzyme was able to produce optically active epoxides from their corresponding alkenes. A second alkene monooxygenase, propene monooxygenase (Pmo), was discovered in *Mycobacterium* sp. strain M156 (2). Propane monooxygenase (Prm) gene clusters were identified in *Gordonia* sp. strain TY-5 (7) and *Rhodococcus* sp. strain RHA1 (22) by deletion analysis, although enzymatic characterization or heterologous expression of these monooxygenases has not been reported, probably due to technical difficulties. The report regarding *Gordonia* sp. strain TY-5 suggested that this monooxygenase oxidized propane to 2-propanol (7). All of the reported monooxygenase gene clusters of this actinomycete subfamily consist of four genes that encode an oxygenase large subunit, an oxygenase small subunit, a reductase, and a coupling protein. The oxygenase component, consisting of the catalytic large subunit and the structural small subunit, activates molecular oxygen using electrons, which are transferred from NAD(P)H by the reductase component. The coupling protein is believed to be involved in electron transfer and substrate oxidation (9).

In this study, we succeeded in identifying binuclear iron monooxygenase gene clusters in *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155. The gene clusters consist of four genes, *mimA*, *mimB*, *mimC*, and *mimD*, which encode an oxygenase large subunit, a reductase, an oxygenase small subunit, and a coupling protein, respectively. We demonstrated that the *mimABCD* gene clusters were responsible for the conversion of phenol to hydroquinone (Fig. 2). These are the first binuclear iron monooxygenases that have been shown to oxidize phenol regioselectively at the *para* position, although several monooxygenases in this family have been reported to convert phenol to catechol (4, 12, 14), and many mutants of binuclear iron monooxygenases have been created for their application in biocatalysis (15, 17, 27). Furthermore, we confirmed that the *prmABCD* propane monooxygenase gene cluster in *Gordonia* sp. strain TY-5, which exhibits high sequence similarity with *mimABCD*, also encodes the same regioselective oxidation activity toward phenol. It will be of interest to study the factors that control the unique regioselectivity.

We found that *M. smegmatis* strain mc^{2}155, which is a typical strain of mycobacteria that has been well studied, was able to not only oxidize phenol to hydroquinone but also grow on propane and acetone as a source of carbon and energy. *M. smegmatis* and *M. goodii* are closely related phylogenetically, and the 16S rRNA gene sequence of *M. smegmatis* strain mc^{2}155 shows 99% identity with that of *M. goodii* strain 12523. We demonstrated that the *mimABCD* gene clusters in *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155 were also responsible for propane oxidation (Fig. 3). Although *M. smegmatis* strain mc^{2}155 has often been used as a surrogate for *Mycobacterium tuberculosis*, this human pathogen does not have a homolog of the *mimABCD* gene cluster. In contrast, homologs of this gene cluster were found in the sequenced genomes of several *Rhodococcus* and *Gordonia* strains in ad-

dition to *Rhodococcus* sp. strain RHA1 and *Gordonia* sp. strain TY-5 (data not shown). It is conceivable that the *mim* and *prm* gene clusters and their homologs might play important roles in hydrocarbon degradation by actinomycetes in polluted environments. Recently, it was reported that the *prm* gene cluster in *Rhodococcus* sp. strain RHA1 was also responsible for *N*-nitrosodimethylamine degradation (22).

We also showed that the deletion mutant mc^{2}155 Δ*mimA* had lost the ability to grow on acetone as a source of carbon and energy and that this ability was restored by introduction of the *mimA* gene of *M. smegmatis* strain mc^{2}155 or of *M. goodii* strain 12523 into this mutant. Although acetone metabolism in bacteria has been well studied (5), this study led to the first identification of binuclear iron monooxygenases that are involved in acetone metabolism. Several Gram-positive bacteria, including mycobacteria, have been reported to metabolize acetone via acetol (10, 24, 28). We confirmed that the deletion mutant mc^{2}155 Δ*mimA* did not lose the ability to grow on acetol as a source of carbon and energy. These results suggest that the *mimABCD* gene clusters might be responsible for the conversion of acetone to acetol. Elucidation of the catalytic function in acetone metabolism awaits detailed characterization. *Gordonia* sp. strain TY-5 was reported to oxidize acetone to methyl acetate by a Baeyer-Villiger monooxygenase (AcmA) during acetone metabolism (8). We confirmed that no homolog of the *acmA* gene exists in the genome sequence of *M. smegmatis* strain mc^{2}155 (data not shown). There is a possibility that *M. smegmatis* strain mc^{2}155 metabolizes acetone through a pathway that is different from that in *Gordonia* sp. strain TY-5.

In conclusion, we identified binuclear iron monooxygenase gene clusters that are responsible for the regioselective oxidation of phenol to hydroquinone in *M. goodii* strain 12523 and *M. smegmatis* strain mc^{2}155. Furthermore, this study fortunately provided new insights into propane and acetone metabolism in mycobacteria. In other words, it would be reasonable to conclude that the acetone-induced monooxygenases that play physiologically essential roles in propane and acetone metabolism fortuitously have the ability to oxidize phenol regioselectively at the *para* position. This unique fortuitous reaction is of biochemical interest and biotechnological importance. Further investigations will focus on heterologous expression of the monooxygenases for their enzymatic characterization and also for their practical application in biocatalysis.

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