Role of α-Methylacyl Coenzyme A Racemase in the Degradation of Methyl-Branched Alkanes by Mycobacterium sp. Strain P101

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A new isolate, Mycobacterium sp. strain P101, is capable of growth on methyl-branched alkanes (pristane, phytane, and squalane). Among ca. 10,000 Tn5-derived mutants, we characterized 2 mutants defective in growth on pristane or n-hexadecane. A single copy of Tn5 was found to be inserted into the coding region of mcr (α-methylacyl coenzyme A [α-methylacyl-CoA] racemase gene) in mutant P1 and into the coding region of mls (malate synthase gene) in mutant H1. Mutant P1 could not grow on methyl-branched alkanes. The recombinant Mcr produced in Escherichia coli was confirmed to catalyze racemization of (R)-2-methylpentadecanoyl-CoA, with a specific activity of 0.21 μmol·min⁻¹·mg of protein⁻¹. Real-time quantitative reverse transcriptase PCR analyses indicated that mcr gene expression was enhanced by the methyl-branched alkanes pristane and squalane. Mutant P1 used (S)-2-methylbutyric acid for growth but did not use the racemic compound, and growth on n-hexadecane was not inhibited by pristane. These results suggested that the oxidation of the methyl-branched alkanoic acid is inhibited by the (R) isomer, although the (R) isomer was not toxic during growth on n-hexadecane. Based on these results, Mcr is suggested to play a critical role in β-oxidation of methyl-branched alkanes in Mycobacterium. On the other hand, mutant H1 could not grow on n-hexadecane, but it partially retained the ability to grow on pristane. The reduced growth of mutant H1 on pristane suggests that propionyl-CoA is available for cell propagation through the 2-methyl citric acid cycle, since propionyl-CoA is produced through β-oxidation of pristane.

Highly branched isoprenoid alkanes, such as pristane (2,6,10,14-tetramethylpentadecane), phytane (3,7,11,15-tetramethylhexadecane), and squalane (2,6,10,15,19,23-hexamethyltetracosane), normally occur in crude oil (3). Among the branched alkanes, the biodegradation of pristane has been most extensively studied. The oxidation pathway of pristane suggests that propionate is available for cell propagation through the 2-methyl citric acid cycle, since propionate is produced through β-oxidation of pristane.

The methyl groups of pristanic acid that are formed by α-oxidation or present in the human diet are in the (R) configuration at positions 6 and 10 (21), while the methyl group of carbon 2 occurs in both the (R) and (S) configurations (28, 37). Because only the CoA ester with the 2-methyl group in the (S)-2-methylbutyric acid configuration can be degraded by mammalian β-oxidation, (2R)-pristanoyl-CoA needs to be converted to the (2S) stereoisomer (27, 28, 35), a reaction catalyzed by α-methylacyl-CoA racemase (2, 11).

The stereochemistry of bacterial pristanic acid oxidation seems to be more complicated than that of mammalian pristanic acid oxidation. Some bacteria are capable of degrading the pristane present in crude oil, which is a mixture of a variety of stereoisomers at C-6 and C-10. Pristane is a common constituent of the lipids of many marine organisms (9), and a typical biogenic pristane, which is obtained from shark liver, has a (6R,10S)-meso configuration (9). In contrast, pristane of fossil origin is a mixture of diastereomers. M. fortuitum is known to use several configurations of pristane and preferentially oxidizes the biogenetic (6R,10S) isomer. However, previous reports (4, 8, 9) have not been concerned with the stereospecificity of β-oxidation, and both enzymological information and genetic information on bacterial pristanic acid oxidation are very limited. Recently, Bhatuk et al. found three genes, mcr, fur, and Rv3727, which encode proteins homologous to mammalian α-methylacyl-CoA racemase in the Mycobacterium tuberculosis genome (5). Also, a putative α-methylacyl-CoA racemase gene was found in a
gene cluster for isoprene metabolism in *Rhodococcus* sp. strain AD45 (34). Similar to *n*-alkane oxidation, methyl-branched alkanes are first oxidized to the corresponding carboxylic acids and then oxidized through β-oxidation. The most unusual feature of methyl-branched alkane oxidation is that the oxidized intermediates of methyl-branched alkanes are mixtures of stereoisomers. Thus, the stereospecificities of the reactions should be examined in order to understand the microbial degradation of methyl-branched alkanes. In this study, we isolated a new organism, *Mycobacterium* sp. strain P101, which is able to use several methyl-branched alkanes, including pristane, phytane, and squalane, as well as a variety of *n*-alkanes. After *Tn5* mutagenesis, we isolated two mutants that were defective for growth on pristane and *n*-hexadecane, and the disrupted genes encoded α-methylacyl-CoA racemase and malate synthase, respectively. Judging from the growth characteristics of the mutants, we suggest that α-methylacyl-CoA racemase is essential for the oxidation of methyl-branched alkanes. This paper is the first report confirming the physiological role of α-methylacyl-CoA racemase in prokaryotes and also provides evidence for stereochemical control during bacterial β-oxidation of methyl-branched fatty acids.

**MATERIALS AND METHODS**

**Enrichment and isolation of a pristane-grown bacterium.** A pristane-utilizing bacterium was isolated from a soil sample after several cycles of enrichment culturing under aerobic conditions in NA medium containing 0.5% pristane grown at 28°C for 7 days. Subsequently, 5 ml of the culture was transferred to another vessel containing fresh medium. After the enrichment culture was transferred five times, a pure culture was obtained from a single colony on an agar plate containing YT medium (10 g of Bacto Yeast Extract [Difco] per liter, 16 g of Bacto Trypton [Difco] per liter, 5 g of NaCl per liter; pH 7.0).

**Bacterial strains, culture conditions, and vectors.** Strain P101, which was isolated from a soil sample as described above, was used in this work. This strain was most closely related to members of the genus *Mycobacterium* based on 16S RNA sequence analysis, which was conducted as described by Hiraishi et al. (14, 15). The results of morphological and physiological characterization were obtained from NCIMB Japan (Shimizu, Japan). *Mycobacterium* sp. strain P101 was grown at 28°C with shaking on NA medium to which a hydrocarbon (0.5%, vol/vol) was added as the carbon source.

**Escherichia coli** DH5α (TaKaRa, Kyoto, Japan), JM109, and Rosetta(DE3) (Novagen) were used for gene cloning and expression and were usually grown on YT medium or in Luria-Bertani (LB) broth, which contained 1% Bacto Trypton, 0.5% Bacto Yeast Extract, and 0.5% NaCl (pH 7.0), in the presence of antibiotics when necessary. Plasmids and primers used in this work are listed in Table 1.

**Analytical methods.** Protein was measured with a Bio-Rad protein assay kit (Japan Bio-Rad Laboratories, Tokyo, Japan) with bovine serum albumin as the standard (6). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) was performed with a 12.5% polyacrylamide gel (19). Prestained protein markers (low range) for SDS-PAGE (Nacalai Tesque, Kyoto, Japan) were used as the molecular standards. The relative molecular masses of the native and SDS-denatured enzyme and the N-terminal amino acid sequence were determined as described previously (17). *n*-Alkanes and methyl-branched alkanes were determined by gas chromatography (GC7A; Shimadzu Co. Ltd., Kyoto, Japan) under the following conditions: column, Ultra Alloy-1(HT) capillary column (Frontier Lab, Fukushima, Japan); column temperature gradient, 170 to 350°C; and temperature of injection port and flame ionization detector, 399°C. Gas chromatography-mass spectrometry (GC6890 and MS5973N; Agilent) was performed under the following conditions: gas chromatography with a TC-FFAP column (30 m by 0.25 mm; 0.5 μm; GLC Science, Tokyo, Japan) at 110 to 240°C for 20 min; and QUAD mass spectrometry at 80 eV, 230°C, and m/z 40 to 500.

**Enzyme assays.** The activity of α-methylacyl-CoA racemase (2-methylacyl-CoA epimerase; EC 5.1.99.4) was assayed by monitoring the conversion of (R)-2-methylpentadecanoyl-CoA to the (S) isomer (35) by gas chromatographic analysis of the fatty acid released by acidic hydrolysis of the CoA ester and condensed with (R)-1-phenylethylamine (10). The malate synthase (Mbs; EC 4.1.3.2) activity was determined by a previously described method (25).

**DNA sequencing.** DNA sequencing was performed by the dideoxy chain termination method by using a Thermosequenase fluorescently labeled primer cycle.

<table>
<thead>
<tr>
<th>Plasmids or primer</th>
<th>Phenotype or sequence</th>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pSUP1014I</td>
<td>Cm' Nm' Bm' Sm'; carrying 10-kb Tn5</td>
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<tr>
<td>pBluescript II SK(+)</td>
<td>Ap'; cloning vector</td>
</tr>
<tr>
<td>pET-23a(+)</td>
<td>Ap'; used for overproduction of foreign protein in <em>E. coli</em></td>
</tr>
<tr>
<td>pTnPl</td>
<td>Ap' Nm'; carries 6.5-kb ClaI fragment from strain P1</td>
</tr>
<tr>
<td>pTnHI</td>
<td>Ap' Nm'; carries 6.6-kb ClaI fragment from strain HI</td>
</tr>
<tr>
<td>pYuR</td>
<td>Ap'; carries 4.7-kb EcoRI-KpnI fragment from strain P101 containing orf1, orf2, orf3 and orf4</td>
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<tr>
<td>pYuM</td>
<td>Ap'; carries 3.8-kb EcoRI-BamHI I fragment from strain P101 containing orf5 and orf6</td>
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<tr>
<td>pET-R</td>
<td>Ap'; carries mcr inserted into EcoRI-NdeI site</td>
</tr>
<tr>
<td>pET-M</td>
<td>Ap'; carries mls inserted into HindIII-NdeI site</td>
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<td><strong>mcr-RTTr</strong></td>
<td>5'-GCAGGGCGTACCCCTCTCGTGAAAGGTG-3'</td>
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**TABLE 1. Plasmids and primers used in this study**
sequencing kit with 7-deaza-dGTP (Amersham Bioscience K.K., Tokyo, Japan) and a DSO-1000L DNA sequencer (Shimadzu Co. Ltd.).

Transposon mutagenesis. Preparation of cells for electroporation and transposon mutagenesis was carried out principally by using the methods described previously. Cells were grown to early exponential phase to the mid-logarithmic phase. The cells were harvested, washed with buffer A and then lysed in 10% glycerol (100 μl/ml). The fractions containing DNA were digested (with EcoRI and then ligated to pET-23a(+) and transformed into Escherichia coli strain Rosetta(DE3) to obtain the productive transformants. C/s) with stepwise melting curve analyses conducted at 65 to 95°C (23°C by 0.1°C) and hybridization was carried out at 55°C (18°C by 5°C). Hybridization was performed as described previously (29). Amplicon specificity was verified by melting curve analyses conducted at 65 to 95°C (temperature transition, 0.1°C/s) with stepwise fluorescence acquisition and by ethidium bromide staining on 2% agarose gels. No fluorescence was detected by real-time quantitative PCR amplification without a template.

Purification of Mcr from E. coli Rosetta(DE3)(pET-R). To amplify the full-length open reading frame (ORF) fragment of the mcr gene, PCR-RFLP was performed with pYm as the template and primers mcr-oe-f and mcr-oe-r. The amplified fragments were digested with NdeI and HindIII and then ligated to pET-23a(+) (Novagen), yielding pET-R. E. coli Rosetta(DE3) was transformed with pET-R, and the resulting strain was grown in 2 liters of LB medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml). When the culture reached an optical density at 610 nm of 0.6, isopropyl-β-D-thiogalacto- pyranoside (IPTG) (4 mM) was added, and then the culture was incubated for an additional 6 h. The cells were harvested, washed with buffer A (50 mM potassium phosphate buffer containing 300 mM NaCl and 0.01% NaN₃, pH 8.0), disrupted by sonication for 30 min (180 W; Insonator model 210 M; Kubota, Tokyo, Japan), and centrifuged at 5,600 g for 15 min at 4°C and then at 40,000 × g for 1 h at 4°C. The resulting clear supernatant, ammonium sulfate was added to a concentration of 1.2 M, and the sample was centrifuged. The resultant supernatant was applied to a Butyl-Toyopearl 650 M column (2.3 by 20 cm; Tosoh, Tokyo, Japan) that was preequilibrated with buffer A containing 1.2 M ammonium sulfate and then eluted with a linear gradient containing decreasing ammonium sulfate concentrations (1.2 to 0 M) in buffer A. The fractions containing the 39-kDa protein, as determined by SDS-PAGE, were collected, dialyzed against buffer B (10 mM potassium phosphate buffer containing 0.01% NaN₃, pH 6.0), and then applied to a DEAE-Toyopearl 650 column (2.2 by 20 cm; Tosoh) preequilibrated with buffer B. Proteins were eluted with a linear gradient containing increasing concentrations of NaCl (0 to 0.5 M). The fractions exhibiting a single protein band at 39 kDa on an SDS-PAGE gel were collected and dialyzed against buffer B. The purified protein was used for the Mcr assay activity.

Expression of Ms protein in E. coli. To amplify the full-length ORF fragment of the mls gene, PCR-RFLP was performed with pYm as the template and primers mls-oE and mls-r. The amplified fragments were digested with NdeI and EcoRI and then ligated to pET-23a(+) (+), yielding pET-M. E. coli Rosetta(DE3) was transformed with pET-M, and the resulting strain was grown in 50 ml of LB medium containing ampicillin (50 μg/ml) and chloramphenicol (34 μg/ml).

Whole-cell reactions. Cells were grown in NA medium containing acetic acid (1 g/liter) as the carbon source for 3 days, harvested, and washed with cold water. The washed cells (12 mg [dry weight]) were inoculated into 1.0 ml of NA medium with a carbon source (0.5%, vol/vol) in the absence of N,N,N,N-tetramethyl-2,2,2-aminomethanamine as the nitrogen source. The whole-cell reactions were conducted in 50-ml anaerobic culture tubes (Bellco Glass Inc.) with butyl gum stoppers with shaking at 28°C. The volume of air in each tube was sufficient for oxidation of the amount of substrate added. A portion of each reaction mixture was removed, and the whole samples containing the cells were extracted with ethyl acetate under acidic conditions (pH less than 2.0). The extracts were subjected to gas chromatography-mass spectrometry as described above.

Nucleotide sequence accession numbers. The nucleotide sequences have been deposited in the GenBank database under the following accession numbers: 16S ribosomal DNA sequence of strain P101, AB182286; 4.7-kb EcoRI-KpnI fragment, AB182284; and EcoRI-BamHI fragment, AB182285.

RESULTS

Properties of newly isolated strain P101. The new isolate, strain P101, was able to grow on methyl-branched alkanes (pristane, phytane, and squalane) and n-alkanes (C₅ to C₁₈) as sole sources of carbon but could not grow on iso-octane, 2,2,4,4,6,8,8-heptamethylnonane, and aromatic hydrocarbons, such as benzene, toluene, p-xylene, naphthalene, and phenanthrene. Strain P101 was a high-G+C-content mycolic-acid-containing gram-positive bacterium and was most closely related to members of the genus Mycobacterium on the basis of 16S ribosomal DNA sequencing. The highest level of similarity (99.5% identity) was found with the sequence of Mycobacterium fluoranthemicum.

Figure 1 shows growth of strain P101 on NA medium containing pristane and n-hexadecane as the carbon sources. The organism completely degraded 0.5% (vol/vol) n-hexadecane within 36 h, and it took longer (60 h) to consume 0.5% (vol/vol) pristane. In the presence of both substrates, degradation of pristane followed degradation of n-hexadecane, but the degradation rates for each substrate were almost the same as those when each substrate was used as the sole carbon source. Pristane utilization occurred before n-hexadecane was com-
and n-hexadecane (B), and pristane and n-hexadecane (C). Pristane (■) and n-hexadecane (□) were added at a concentration of 0.5% (vol/vol) to NA medium. ○, growth.

Isolation of mutants deficient in the ability to grow on pristane or n-hexadecane. About 10,000 neomycin-resistant mutants were obtained by Tn5 mutagenesis by using pSUP10141. To confirm insertion of the transposon, chromosomal DNA was prepared from 83 selected transposon-containing colonies. The EcoRI-digested genomic DNA isolated from the mutants were subjected to Southern blot analyses by using a 1-kb DNA fragment amplified from the Tn5 Nmr gene sequence as the probe. These Southern blot analyses revealed that random insertion of Tn5 occurred in strain P101. Eighty of the mutants were unable to grow on both pristane and n-hexadecane but grew on NA medium containing succinate as a growth factor. The parent strain. The restriction profiles and recovered sequences from the mutants were identical for mutants P1 and P2, indicating that they were siblings (data not shown).

Determination of Tn5 insertion loci. The 6.5-kb ClaI fragment from the chromosomal DNA of mutant P1 and the 6.6-kb ClaI fragment from mutant H1 were subcloned into pBlue-scriptIII SK(+) to obtain plasmids pTnP1 and pTnH1, respectively. DNA sequence analyses of these plasmids revealed that the Tn5 insertion disrupted a gene with high sequence similarity to the α-methylacyl-CoA racemase gene in mutant P1 and the malate synthase gene (mls) in mutant H1.

Cloning and sequencing of native genomic fragments. A 4.7-kb EcoRI-KpnI fragment of plasmid pYuR, which was obtained through colony hybridization selection by using an mcr DNA fragment as the probe, contained three complete ORFs and one partial ORF (Fig. 2). The sequence of orf2 matched the sequence of the mcr gene of mutant P1, into which Tn5 was inserted. The native mcr gene was composed of 1,083 bp, corresponding to 361 amino acid residues with a predicted molecular mass of 38,867 Da. Mcr showed considerable similarity to α-methylacyl-CoA racemases from different sources. From the results of BLAST searches, the ORFs showed amino acid sequence similarity to the following known proteins: for orf1 (829 bp), 2-enoyl-CoA hydratase from Azotobacter vinelandii (accession no. AJ278756; 28% identity and 44% similarity); for orf2 (1,083 bp), α-methylacyl-CoA racemase; for orf3 (729 bp), 3-hydroxyacyl-CoA dehydrogenase from Pseudomonas putida (accession no. Q9AHY1; 52% identity and 67% similarity); and for orf4 (partial), 2-nitropropane dioxygenase from Streptomyces avermitilis (accession no. AF284037; 22% identity and 34% similarity). The product of a partial orf6 was similar to a putative protein found in M. tuberculosis (accession no. Q50597).

FIG. 1. Growth of Mycobacterium sp. strain P101 on pristane (A), n-hexadecane (B), and pristane and n-hexadecane (C). Pristane (■) and n-hexadecane (□) were added at a concentration of 0.5% (vol/vol) to NA medium. ○, growth.

FIG. 2. Gene organization and restriction maps of the fragments carrying mcr and mls. (A) 6.3-kb gene region of Mycobacterium sp. strain P101. (B) 3.8-kb gene region of Mycobacterium sp. strain P101. (C) Southern analysis of Clai-digested total DNA from the wild-type strain and mutant strain P1, with the alkaline phosphatase-labeled mcr fragment as a probe. (D) Southern analysis of Clai-digested total DNA from the wild-type strain and mutant strain H1, with the alkaline phosphatase-labeled mls fragment as a probe. Based on the results of BLAST searches, the ORFs showed amino acid sequence similarity to the following known proteins: for orf1 (829 bp), 2-enoyl-CoA hydratase from Azotobacter vinelandii (accession no. AJ278756; 28% identity and 44% similarity); for orf2 (1,083 bp), α-methylacyl-CoA racemase; for orf3 (729 bp), 3-hydroxyacyl-CoA dehydrogenase from Pseudomonas putida (accession no. Q9AHY1; 52% identity and 67% similarity); and for orf4 (partial), 2-nitropropane dioxygenase from Streptomyces avermitilis (accession no. AF284037; 22% identity and 34% similarity). The product of a partial orf6 was similar to a putative protein found in M. tuberculosis (accession no. Q50597).
which Tn5 was inserted. MLSs showed considerable similarity to malate synthases from different sources.

**Enzymatic activities of Mcr and MLSs.** Mcr protein (1.2 mg) was purified from the cell extract of *E. coli* Rosetta (DE3) (pET-R) (330 mg of protein). The N-terminal amino acid sequence was MTGPLHG, which was identical to the deduced amino acid sequence of ORF2. The relative molecular mass of the purified Mcr was estimated to be 39 kDa by SDS-PAGE, which was in good agreement with the theoretical molecular mass, as described above. The purified enzyme was confirmed to catalyze partial conversion of *(R)-2-methylpentadecanoyl-CoA* to the corresponding *(S)* isomer. The specific activity of the purified enzyme was 0.21 μmol·min⁻¹·mg of protein⁻¹.

SDS-PAGE of the cell extract of *E. coli* Rosetta(DE3) (pET-M) revealed a major protein band, whose molecular mass (80 kDa) was in close agreement with the theoretical mass of the deduced amino acid sequence of the *mls* gene product. The N-terminal amino acid sequence of the protein was MTERTTVT, which was identical to the deduced amino acid sequence. The cell extract exhibited a malate synthase activity of 6.49 μmol·min⁻¹·mg of protein⁻¹. The malate synthase activity of the control strain harboring pET-23a(+) was less than 1% of that of *E. coli* Rosetta(DE3)(pET-M).

**Transcription analysis.** To characterize the transcriptional regulation of the *mcr* gene, real-time quantitative reverse transcriptase PCR was conducted with total RNA from strain P101 cells, which were grown on NA medium containing pristane, phytane, squalane, *n*-hexadecane, or succinate. The results suggested that the *mcr* expression in the presence of pristane and squalane was induced 9.1- and 3.4-fold relative to that in the presence of succinate, respectively. Since the control reverse transcription experiment did not produce fluorescence within a reasonable number of cycles (data not shown), *mcr* was suggested to be transcribed constitutively.

**Growth characteristics of mutant strains P1 and H1.** In order to determine the physiological role of Mcr in the utilization of methyl-branched alkanes, growth of wild-type strain P101 and growth of the *mcr*-deficient mutant strain P1 on several carbon sources were compared. Although strain P1 could not grow on pristane as a sole source of carbon, it grew on NA medium containing both pristane (0 to 0.3%, wt/vol) and *n*-hexadecane (0.5%) (Fig. 3). There was no significant change in the extent of growth of strain P1, even when the concentration of pristane was increased. This extent of growth was almost the same as that observed in the absence of pristane. These results indicated that the growth of strain P1 was sustained by *n*-hexadecane and also suggested that pristane or its metabolic intermediates were not toxic for growth of the mutant strain on *n*-hexadecane. In order to determine the stereospecificity of the enzyme for 2-methyl-branched fatty acids, 2-methylbutyric acid (1.0%, wt/vol) was used as the growth substrate. Among the methyl-branched fatty acids, only the *(S)*-2-methylbutyric acid stereoisomer was commercially available. The wild-type strain was able to grow on the racemic compound, as well as the *(S)* isomer. Mutant P1 consumed the *(S)* isomer within 5 days, and the growth yield was 2.2 g (dry weight)/liter; however, mutant P1 did not grow on the racemic compound, *(R),S*-2-methylbutyric acid. These results imply that the *mcr*-deficient mutant is able to use only the *(S)* isomer of the 2-methyl-branched fatty acid and that the *(R)* isomer, or a derivative of it, inhibits utilization of the *(S)* isomer.

The *mls* mutant strain H1 had consumed about 70% of the added pristane (1.5 g/liter) after 3 days of cultivation, and the cell yield based on the amount of pristane consumed was 27%, which corresponded to 58% of the cell yield of the wild-type strain.

**Pristanic acid accumulation from pristane by mutant strain P1.** The consumption of pristane and *n*-hexadecane in whole-cell reactions with the mutant and wild-type strains was investigated. The wild-type strain completely consumed both *n*-hexadecane and pristane (1 g/liter each) during a 24-h incubation at 28°C. Mutant strain P1 consumed *n*-hexadecane at the same rate as the wild-type strain. On the other hand, about 5% of the added pristane was consumed in the reaction with the mutant strain. Only one significant peak corresponding to pristanic acid was detected on the gas chromatograph of the ethyl acetate extract, and the amount accumulated reached a maximum (0.08 mM in the reaction mixture) after 72 h of incubation (data not shown). This peak was not detected with the reaction mixture containing the wild-type strain. The mass spectrographic data for this peak matched the data for authentic pristanoic acid (m/z 43, 57, 74, 87, 99, 115, 127, and 298 [molecular ion]). Since the reaction mixture was treated under acidic conditions, pristanic acid may have been derived from pristanoyl-CoA (a possible intermediate in pristane oxidation) by acid hydrolysis.

**DISCUSSION**

A pathway for oxidation of pristane in some gram-positive bacteria has been proposed based on an analysis of key intermediates. However, the relevant enzymes have not been characterized yet. In this work, we found that *α*-methylacyl-CoA racemase plays a critical role in the complete oxidation of pristane in *Mycobacterium* sp. strain P101. Bacterial β-oxidation of branched fatty acids has been proposed to proceed through an analogous route in mammalian cells. Our findings provide corroborating evidence for the proposed pathway for
pristane oxidation and call attention to the stereospecific aspects of the β-oxidation pathway.

In mammalian cells, stereospecific enzymatic recognition occurs in the steps for desaturation of the acyl-CoA through β-oxidation, which are catalyzed by acyl-CoA oxidase in the peroxisome (35) and acyl-CoA dehydrogenase in the mitochondrial matrix (24). Since these enzymes are specific for (S)-2-methylacyl-CoA, α-methylacyl-CoA racemase is indispensable for completion of β-oxidation. In bacteria, desaturation of acyl-CoAs is catalyzed by acyl-CoA dehydrogenase (24). We suggest that this desaturation step is stereospecific for the (S) configuration and that the (R) isomer inhibits the desaturation step based on the following results: (i) the mcr-deficient mutant of strain P101 used (S)-2-methylbutyric acid but not the racemic compound, and (ii) pristanic acid, which may be derived from pristanol-CoA, was detected as a byproduct of pristane oxidation by the mutant. Judging from the evidence that n-hexadecane utilization by the mutant is not inhibited by pristane, the unmetabolized isomer, (R)-2-methylacyl-CoA, is not toxic for β-oxidation of unbranched fatty acids. This may imply that different desaturation enzymes function with 2-methylated acyl-CoAs and unmethylated acyl-CoAs.

Based on previous reports for M. fortuitum (9) and N. globulosa 432 (1), Mycobacterium sp. strain P101 was assumed to oxidize pristane through monoterminus oxidation; i.e., pristanic acid is thioesterified with CoA to form pristanoyl-CoA and then degraded by β-oxidation. The pristanic acid used in this work was derived from shark liver oil, which is composed of only the (6R,10S)-meso isomer (8). Oxidation of the isomers of pristane (compound I) is expected to produce the following four isomers of pristanic acid: (2R,6R,10R), (2S,6R,10R), (2R,6S,10S), and (2S,6S,10S) (Fig. 4). Assuming that the configurations of the CoA thioesters are the same as those of the corresponding acids, compounds III, V, VII, and IX should be substrates for Mcr. Three mcr homologues are present in the M. tuberculosis genome (5), although the physiological role of each gene product has not been elucidated. In Mycobacterium sp. strain P101, Southern blot analysis identified only one gene for the enzyme (mcr) in the entire genome (data not shown), indicating that Mcr is responsible for racemization of compounds III, V, VII, and IX. Also, the gene arrangement in the vicinity of the mcr gene was completely different in strain P101 and M. tuberculosis.

As shown in Fig. 4, three equivalents of propionyl-CoA, three equivalents of acetyl-CoA, and one equivalent of isobutyl-CoA can be produced from 1 mol of pristane. Propionyl-CoA is thought to be incorporated into cell constituents through the 2-methylcitric acid cycle that occurs in a variety of bacteria (7, 16, 20, 33). Thus, the mls-deficient mutant should obtain cell carbon through propionyl-CoA and isobutyl-CoA in an amount corresponding to 12/19 of that of the wild-type strain, which can utilize all acyl-CoAs, if the cell yield is estimated on the basis of available carbon number. When the cell yields based on pristane consumption were compared, the yield for the mutant was 58% of the yield obtained with the wild type, implying that pathways for pristane and n-alkane degradation function independently in the bacterium.

Bacterial metabolism of branched-chain fatty acids has received little attention compared with mammalian metabolism.

Further work is needed to clarify whether enzymatic discrimination between branched-chain acyl-CoAs and straight-chain acyl-CoAs occurs in the β-oxidation pathway. Some members of the order Actinomycetales, such as Rhodococcus, Nocardia, Mycobacterium, etc., are known to use a variety of hydrocarbons, including methyl-branched alkanes, and have different gene clusters for oxidation of alkanes with different chain lengths (13, 17, 31, 39). Attention should also be focused on the enzymatic discrimination between iso-alkanes and n-alkanes in their oxidation to the corresponding fatty acids.

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