

Propane Monooxygenase and NAD⁺-Dependent Secondary Alcohol Dehydrogenase in Propane Metabolism by *Gordonia* sp. Strain TY-5

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A new isolate, *Gordonia* sp. strain TY-5, is capable of growth on propane and *n*-alkanes with C₁₃ to C₂₂ carbon chains as the sole source of carbon. In whole-cell reactions, significant propane oxidation to 2-propanol was detected. A gene cluster designated *prmABCD*, which encodes the components of a putative dinuclear-iron-containing multicomponent monooxygenase, including the large and small subunits of the hydroxylase, an NADH-dependent acceptor oxidoreductase, and a coupling protein, was cloned and sequenced. A mutant with *prmB* disrupted (*prmB::Kan^r*) lost the ability to grow on propane, and Northern blot analysis revealed that polycistronic transcription of the *prm* genes was induced during its growth on propane. These results indicate that the *prmABCD* gene products play an essential role in propane oxidation by the bacterium. Downstream of the *prm* genes, an open reading frame (*adh1*) encoding an NAD⁺-dependent secondary alcohol dehydrogenase was identified, and the protein was purified and characterized. The Northern blot analysis results and growth properties of a disrupted mutant (*adh1::Kan^r*) indicate that Adh1 plays a major role in propane metabolism. Two additional NAD⁺-dependent secondary alcohol dehydrogenases (Adh2 and Adh3) were also found to be involved in 2-propanol oxidation. On the basis of these results, we conclude that *Gordonia* sp. strain TY-5 oxidizes propane by monooxygenase-mediated subterminal oxidation via 2-propanol.

Gaseous *n*-alkanes ranging from C₂ to C₅ are recognized as components of nonmethane hydrocarbons, and the increased concentrations of these gases in the atmosphere threaten to destabilize ecosystems through a variety of mechanisms (48). Although these gases are produced as natural intermediates of bacterial, plant, and mammalian metabolism, the main sources of pollution are natural oil seepage and oil spills (42). From a biotechnological perspective, gaseous alkanes are inexpensive carbon sources for microbial cultivation, and the enzymes participating in the oxidation pathway promise to be versatile biocatalysts.

A number of microorganisms have been isolated for their ability to use gaseous *n*-alkanes as a sole carbon source. In the case of bacteria, these abilities have been found in some *Pseudomonas* strains (57) and many strains belonging to the order *Actinomycetales*, such as those of the genera *Rhodococcus*, *Mycobacterium*, *Corynebacterium*, *Nocardia*, and *Pseudonocardia* (3, 15). Some of the bacteria are known to degrade various environmental pollutants (trichloroethylene, chloroform, methyl ethers, etc.) through cometabolism with gaseous alkanes (13, 52).

The pathways for the oxidation of gaseous alkanes have received little attention compared with those for the microbial oxidation of methane (34) and liquid *n*-alkanes (24). Recently, the terminal oxidation pathway of butane (butane → 1-butanol → butyraldehyde → butyrate) by “*Pseudomonas butanovora*” has been confirmed through enzymological and genetic ap-

proaches (2, 14). The first reaction is catalyzed by a soluble butane monooxygenase (sBMO) similar to soluble methane monooxygenase (sMMO) (50). In considering propane oxidation, several possibilities have been proposed (3). Propane could be oxidized by monooxygenase-mediated terminal oxidation via 1-propanol or subterminal oxidation via 2-propanol. As a third possibility, a mixture of 1-propanol and 2-propanol could result from propane oxidation. Since biochemical and genetic findings on propane monooxygenase have been limited, the propane oxidation pathways present in individual isolates have been considered mainly through the properties of alcohol dehydrogenases and other enzymes participating in the metabolism of intermediates such as propanal, acetone, acetol, and so on (3, 28). Published studies indicate that pathways for gaseous alkane oxidation vary depending on the microbial strains and the substrates, but the metabolic reactions in each pathway remain ambiguous. In order to assess each pathway, more extensive enzymological and genetic approaches are required.

We have recently isolated a gram-positive bacterium, *Gordonia* sp. strain TY-5, that is able to use propane but no other gaseous alkanes. We have cloned the genes for propane monooxygenase and for three types of NAD⁺-dependent secondary alcohol dehydrogenases. From the results of gene expression and growth characteristics of several mutant strains carrying inactivated genes, we conclude that propane is oxidized through subterminal oxidation in *Gordonia* sp. strain TY-5. This is the first report confirming that bacterial propane oxidation is catalyzed by an NADH-dependent multicomponent enzyme system belonging to the family of dinuclear-iron oxygenases.

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MATERIALS AND METHODS

Enrichment and isolation of a propane-grown bacterium. A bacterial strain that uses propane as the sole source of carbon and energy was enriched in a 25-ml sealed culture vessel containing 5 ml of AY medium under a gas mixture containing propane, O₂, and CO₂ (30:65:5, vol/vol/vol). AY medium contained (per liter) 4 g of K₂HPO₄, 2 g of KH₂PO₄, 2 g of NH₄Cl, 0.2 g of MgSO₄ · 7H₂O, 4 mg of CaCl₂ · 2H₂O, 5 mg of H₃BO₃, 0.4 mg of CuSO₄ · 5H₂O, 1 mg of KI, 2 mg of FeSO₄ · 7H₂O, 4 mg of MnSO₄ · 4~7H₂O, 4 mg of ZnSO₄ · 7H₂O, and 1 mg of Na₂MoO₄ · 2H₂O, pH 7.0. The vessel was shaken at 30°C for 4 to 6 days, and then 50 µl of the culture was transferred to another vessel containing fresh medium. After the enrichment culture had been transferred five times, an aliquot of the culture was plated onto AY medium containing 1.5% Bacto Agar, which was placed in a desiccator under the same gas mixture described above at 30°C. Finally, a pure culture was obtained from a single colony, which was grown on yeast-tryptone (2× YT) medium (pH 7.0) containing (per liter) 10 g of Bacto Yeast Extract, 16 g of Bacto Tryptone, and 5 g of NaCl.

Bacterial strains, culture conditions, and vectors. Strain TY-5, which was isolated from a soil sample as described above, was used in this work. The strain was most closely related to members of the genus *Gordonia* on the basis of 16S rRNA gene sequencing analysis, which was conducted as described by Hiraishi et al. (17, 18). The morphological and physiological characteristics were obtained from NCIMB Japan (Shimizu, Japan). *Gordonia* sp. strain TY-5 was grown on AY medium, to which a carbon source (1.0%, wt/vol) was added, at 30°C under shaking conditions. When propane was used as the carbon source, a 500-ml shaking flask containing 100 ml of AY medium under a propane-air mixture (ratio, 3:7) and sealed with a butyl rubber stopper was shaken at 30°C. A large-scale culture (10 liters) was grown in a 15-liter jar fermentor at 30°C with stirring at 300 rpm and aeration at 10 liters/ml.

Escherichia coli DH5α (TaKaRa, Kyoto, Japan) was used for gene cloning and was usually grown on 2×YT medium in the presence of ampicillin (50 mg/liter) or kanamycin (25 µg/liter) when necessary. pBluescript II SK⁺ (Toyobo, Osaka, Japan), pUC118 (TaKaRa), and pGEM-T Easy (Promega, Madison, Wis.) were used as cloning vectors.

Whole-cell reactions. Whole-cell reactions with several carbon sources were conducted principally as described by Arp (2). Cells were grown on propane as described above for 3 days, harvested, and suspended in medium with no carbon source. Five milliliters of the cell suspension in a 25-ml sealed culture vessel under a gas mixture containing gaseous alkane and air (3:7, vol/vol) was shaken at 30°C. In order to inhibit further oxidation of the alcohol produced and furnish NADH, excess amounts of 1-butanol and 2-butanol (each at 5 mM) were added to the reaction mixture. A portion of the medium was sampled through a syringe and used for determination of alcohols formed by gas chromatography (45).

Cell extract. Cells grown on 2-propanol to late exponential phase (optical density at 610 nm of 3.0) were harvested by centrifugation (28,000 × g for 10 min at 4°C) and washed twice with ice-cold AY medium containing no carbon source. The cells were suspended in 20 mM Tris-Cl buffer, pH 7.8; disrupted by six passages through a high pressure laboratory homogenizer (MINI-LAB, type 8.3 H; Rannie a/s, Copenhagen, Denmark) at 80 MPa; and centrifuged at 5,600 × g for 15 min at 4°C and then at 150,000 × g for 1 h at 4°C. The resultant clear supernatant was used as the cell extract.

Analytical methods. Gaseous alkanes and alcohols were determined by gas chromatography as previously described (45). 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-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with a 12.5% polyacrylamide gel by the method of Laemmli (29). Prestained protein markers (low range) for SDS-PAGE (Nacalai Tesque, Kyoto, Japan) were used as molecular size standards. The relative molecular mass of a native enzyme was determined by gel filtration with a fast protein liquid chromatography system (Amersham Bioscience, Piscataway, N.J.) with a Superose 12HR 10/30 column equilibrated with 50 mM Tris-Cl buffer (pH 7.5) containing 0.1 M KCl. The standard proteins used were from the Oriental Yeast Co., Ltd. (Tokyo, Japan). In order to determine the N-terminal amino acid sequence, a single band of each purified enzyme on SDS-PAGE was electroblotted onto a P⁹⁰PVDF membrane (Millipore Corp. Bedford, Mass.) at 15 V for 40 min with a transfer buffer containing 75 mM Tris base and 580 mM glycine in 20% (vol/vol) methanol. The amino acid sequence was determined by Edman's method with a Perkin-Elmer protein sequencer (model 476A).

Enzyme assays. NAD⁺-dependent alcohol dehydrogenase was assayed in a reaction mixture (1 ml) containing 20 mM sodium carbonate buffer (pH 9.5), 1.0 mM NAD⁺, 200 mM (NH₄)₂SO₄, 100 mM alcohol, and an appropriate amount of enzyme. The reverse reaction was assayed in a reaction mixture (1 ml) con-

TABLE 1. Primers used in this study

Primer	Sequence
Primer N	5'-ATGGAAGCGGAGAAGGA-3'
Primer C	5'-CTGGCCGATGAGGGTCTTGC-3'
Primer 3'	5'-ACTGTTTCCGCTGGATGTTGCGCTGGTTGG-3'
Primer 5'	5'-CCTGTACTGGATCGCGAACGACCAAGATCCT-3'
Adh2-N	5'-ATGAARGCNCTSGTNTTYCAYGGSCC-3'
Adh2-I	5'-TSCCSACSGCCTCGATSACSACRCTCNGCNCC-3'
Adh2-inv-1	5'-ATGTCGACGGCGATGACCCG-3'
Adh2-inv-2	5'-TGGCGATGACCGACGGACTC-3'
Adh3-N	5'-ATGACSGTSTACGCSAAGCCSGGNACNGA-3'
Adh3-I	5'-TASCCSCCGAASGCSGCGTGNGCNGGRTA-3'
Adh3-inv-1	5'-AGAGGGTGTGCTGGCGATGTGGATGGCGT-3'
Adh3-inv-2	5'-GGCCGTGTGATCGCGAACGACCTACCGAC-3'
prmA-f	5'-ATGCGGTCTGACTTCCCGATGGAAGAGGAG-3'
prmA-r	5'-TCAGACCGTGACGGCCCGTCTTGTGGC-3'
prmB-f	5'-ATGGCCGACACCCACAAGATCAGCTTCAAGAA-3'
prmB-r	5'-TCAGGAGTCGAATGCGGGCTGGTGAACCTT-3'
prmC-f	5'-ATGTCCGCACCAGCGCAACCCCGGGAACGC-3'
prmC-r	5'-TCACTTGTTCAACTCCTCTGGGAT ATCGAC-3'
prmD-f	5'-ATGCAATTCGGAGCCGACACCGAGTTCTCC-3'
prmD-r	5'-CTAGGAGGCGGTGAGTCGAAGCCGATGAA-3'
adh1-f	5'-ATGAGAGCAGTACAGGTGGTTCGATACAC-3'
adh1-r	5'-TCAGGGGATCAGGATGGCGCGCCCTGAC-3'
adh2-f	5'-ATGAAGGCATCGTTTCCACGGTCCCGC-3'
adh2-r	5'-TCAGGCGCTCATGATCACCTTGGCGCCTT-3'
adh3-f	5'-ATGACTGTTTACGCGAAGCCGGGTACCGAG-3'
adh3-r	5'-TTAGAAGAATCCCTTCGCACTCTGAGCGTA-3'
kan-f-Sph	5'-GCATGCGGACCAGTTGGTGATTTTGAACCT-3'
kan-r-Sph	5'-GCATGCTTAGAAAACTCATCGAGCATCAA-3'
kan-f-Bam	5'-GGATCCGACAGTTGGTGATTTTGAACCT-3'
kan-r-Bam	5'-GGATCCTTAGAAAACTCATCGAGCATCAA-3'
adh1-500r	5'-CAATCCGCCTGCGCCGATCAGCAGCAGCT-3'
adh2-500r	5'-CCCGATCACCCGCGACCACATCACCAGGTTT-3'
adh3-500r	5'-GAGCGCGGGCGCGAGCTTCCACACGGCCAT-3'

taining 20 mM sodium acetate buffer (pH 4.0), 0.25 mM NADH, 200 mM (NH₄)₂SO₄, 100 mM ketone, and an appropriate amount of enzyme. Activities of the forward and reverse reactions were assayed by measuring the increase and decrease in absorbance at 340 nm, respectively, with a Shimadzu spectrophotometer (UV-160) with a cuvette with a 1-cm light path. As a reference, a reaction mixture with no substrate for the forward or reverse reaction was used. One unit of enzyme activity was defined as the amount of enzyme that catalyzed the reduction or oxidation of 1.0 µmol of NAD⁺ and 1.0 µmol of NADH, respectively, per min at 30°C. Initial velocity studies on the forward reaction of a secondary alcohol dehydrogenase were conducted under standard conditions at 30°C, with respect to NAD⁺ at fixed concentrations of 2-propanol. The *K_m* for a substrate under an infinite concentration of another substrate was obtained by replottting the slopes and intercepts versus the reciprocal concentration (27).

Cloning and nucleotide sequencing of a gene cluster encoding propane monoxygenase. The primers used in this work are listed in Table 1. To amplify the DNA fragment carrying the gene cluster encoding propane monoxygenase (*prm*) from chromosomal DNA of strain TY-5, primers N and C were designed on the basis of the conserved regions between the epoxidase large subunit of alkene monoxygenase (AMO) from *Nocardia corallina* B-276, which is now commonly referred to as *Rhodococcus rhodochrous* B-276 (44) (residues 52 to 57 and 467 to 473), and the hydroxylase α subunit of sMMO from *Methylococcus capsulatus* (56) (residues 70 to 75 and 472 to 478). Chromosomal DNA extracted from *Gordonia* sp. strain TY-5 with an AquaPure Genomic DNA Isolation Kit (Bio-Rad Laboratories) was used as a template for amplification of a portion of the *prm* gene cluster by PCR. *Ex Taq* polymerase (TaKaRa) was used for PCR in accordance with the manufacturer's instructions. The amplified 1.3-kb fragment was gel purified and then ligated with pGEM-T Easy. The propagated recombinant plasmid was digested with *Eco*RI, and the resulting 1.3-kb insert fragment was gel purified and used as the hybridization probe.

Genomic Southern analysis was done as described previously (58). Southern analysis showed that a 3.4-kb *Pst*I fragment hybridized to the probe. To construct a *Pst*I library, 3.4-kb *Pst*I fragments of strain TY-5 genomic DNA were gel purified and ligated into the *Pst*I site of pBluescript II SK⁺. *E. coli* DH5α cells were transformed with the resulting ligation mixtures. The colony hybridization

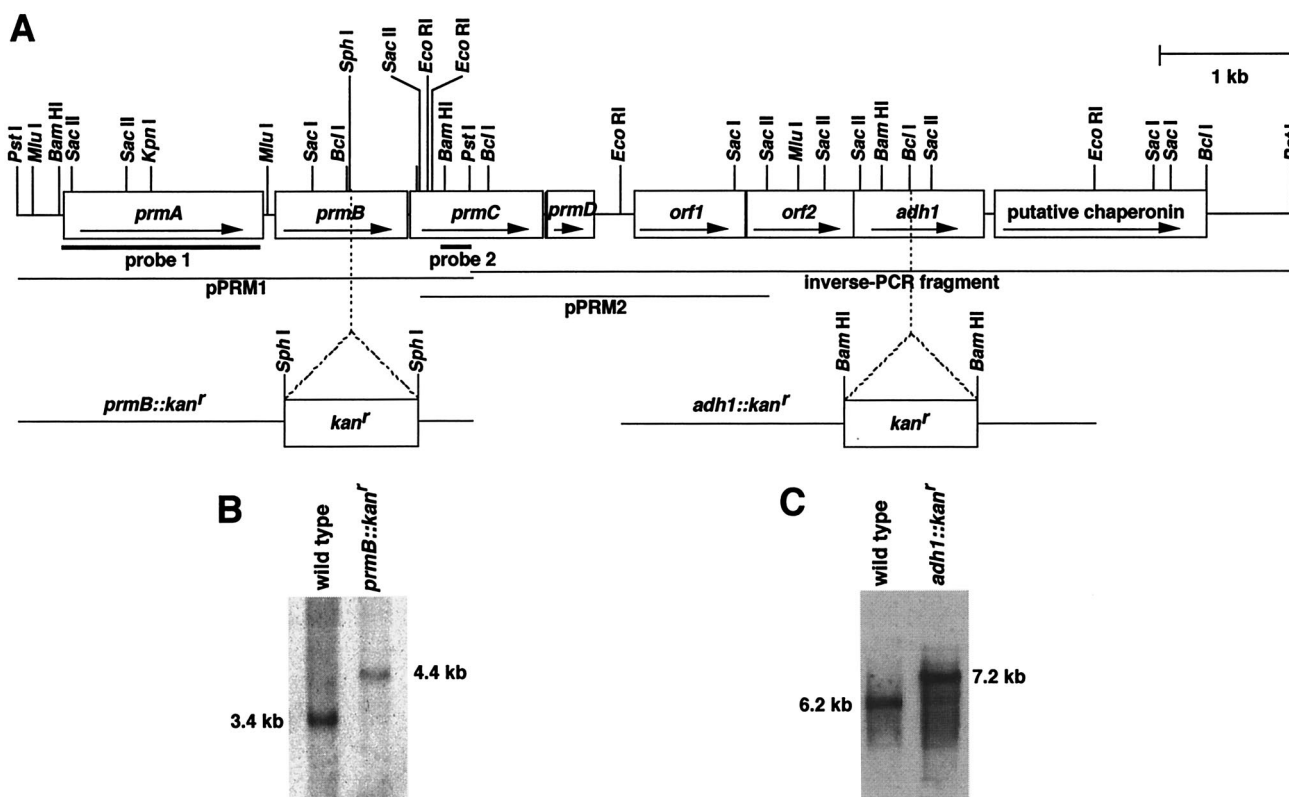


FIG. 1. The 9.2-kb gene region of *Gordonia* sp. strain TY-5. (A) Genetic organization of the gene cluster and restriction map. The orientation of the identified genes is indicated by arrows. A *kan* gene (1,033 bp) was inserted into the *Sph*I site within the *prmB* gene and into the *Bcl*I site within the *adh1* gene. The overlapping inserts of the two plasmids, pPRM1 and pPRM2, and the inverse-PCR product representing the gene region analyzed are indicated by lines. (B) Genomic Southern analysis of *Pst*I-digested total DNAs (5.0 μ g of each) from the wild-type strain and the mutant strain with *prmB* disrupted, with the 32 P-labeled *prmB* fragment as a probe. (C) Genomic Southern analysis of *Pst*I-digested total DNAs (5.0 μ g of each) from the wild-type strain and the mutant strain with *prmB* disrupted, with the 32 P-labeled *adh1* fragment as a probe.

experiment was performed as described previously (58). Clones that showed strong signals were picked from the original plates and used for further studies (pPRM1, Fig. 1). DNA sequencing was performed by the dideoxy chain termination method with the ThermoSequenase fluorescently labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Biosciences K.K., Tokyo, Japan) and a DSQ-1000L DNA sequencer (Shimadzu Co. Ltd., Kyoto, Japan). The 400-bp *Bam*HI-*Pst*I-digested fragment of pPRM1 was used as a probe to clone the region downstream of the *prm* gene cluster. Genomic Southern analysis showed that a 2.5-kb *Sac*II fragment hybridized to the probe, and this fragment was cloned in a similar colony hybridization experiment (pPRM2, Fig. 1). By an inverse-PCR procedure (38), the region downstream of the *prm* gene cluster was amplified. Chromosomal DNA from strain TY-5 was digested with *Pst*I and then self-ligated. The ligation mixture was then used as the template for PCR amplification with *LA Taq* polymerase (TaKaRa) with primer 3' and primer 5'. The amplified fragment was subcloned into pGEM-T Easy and sequenced (Fig. 1).

Cloning of Adh2- and Adh3-encoding genes. To amplify DNA fragments encoding Adh2 and Adh3 from strain TY-5 chromosomal DNA by PCR, the upstream primers for *adh2* and *adh3* were designed from the N termini of the purified enzymes (Adh2-N and Adh-3, respectively), and the downstream primers were designed on the basis of multiple aligned sequences of NAD⁺-dependent alcohol and aldehyde dehydrogenases. Cloning of the *adh2* and *adh3* genes was conducted by essentially the same procedures. Chromosomal DNA extracted from strain TY-5 as mentioned above was used as the template for amplification. To clone the full-length *adh2* gene, strain TY-5 chromosomal DNA was digested with *Sac*II, religated, and used as a template for an inverse PCR with primers Adh2-inv-1 and Adh2-inv-2. This inverse-PCR amplification product was cloned and sequenced. In the case of *adh3*, the chromosomal DNA was digested with *Bgl*II, religated, and used as a template for an inverse PCR with primers Adh3-inv-1 and Adh3-inv-2.

Northern blot analysis. *Gordonia* sp. strain TY-5 was cultured at 30°C on AY medium containing a carbon source to exponential phase (optical density at 610 nm of 0.8 to 0.9) and harvested. In order to examine the transcriptional induction by gaseous alkanes that did not support growth, succinate-grown cells were harvested and washed with AY medium containing no carbon source. The resultant cells were shaken in AY medium containing a gaseous alkane for 4 h at 30°C and harvested. Total cellular RNA was extracted with an RNeasy Mini Kit (QIAGEN, Hilden, Germany) in accordance with the manufacturer's protocol, electrophoresed on a 0.8% agarose gel in 20 mM morpholinepropanesulfonic acid (MOPS) buffer containing 1.0 mM EDTA and 2.2 M formaldehyde, and then transferred to a nylon membrane filter (GeneScreen Plus; NEN Life Science Products, Boston, Mass.) in 20 \times SSC (1 \times SSC is 0.15 M NaCl plus 0.015 M sodium citrate). Hybridization was carried out as described previously (33). DNA probes specific for individual genes were generated by PCR with the following primer combinations: *prmA*-f and *prmA*-r for *prmA*, *prmB*-f and *prmB*-r for *prmB*, *prmC*-f and *prmC*-r for *prmC*, and *prmD*-f and *prmD*-r for *prmD*.

Construction of mutants with disrupted genes. The kanamycin resistance gene (*kan*), including its own promoter, was amplified by PCR with pKT231 as the template. For inactivation of *prmB*, primers flanking the *Sph*I site, *kan*-f-*Sph* and *kan*-r-*Sph*, were used. The amplified *kan* gene was inserted into the *Sph*I site within the *prmB* gene of pPRM1 that had been linearized and dephosphorylated. The resulting plasmid, pDisPrmB, was digested with *Pst*I and then introduced into *Gordonia* sp. strain TY-5 by double-crossover homologous recombination. For inactivation of *adh1*, primers flanking the *Bam*HI site, *kan*-f-*Bam* and *kan*-r-*Bam*, were used. The amplified *kan* gene was inserted into the *Bcl*I site within the *adh1* gene in the *Eco*RI fragment (Fig. 1), yielding pDisAdh1. The plasmid was digested with *Eco*RI and then introduced into *Gordonia* sp. strain TY-5 as

described above. Kanamycin-resistant transformants were selected, and each gene disruption was confirmed by Southern analysis (Fig. 1).

Purification of enzymes catalyzing NAD⁺-dependent 2-propanol dehydrogenation. Purification was performed at 4°C. The cell extract, which contained 1,650 mg of protein, was used for enzyme purification. A precipitate obtained by ammonium sulfate fractionation (1.6 to 3.2 M) was dialyzed against 20 mM Tris-Cl buffer, pH 8.5 (buffer A), containing 1.2 M ammonium sulfate. The dialyzed solution was applied to a Butyl-Toyopearl 650 M column (2.3 by 20 cm; Tosoh, Tokyo, Japan) that was preequilibrated with the dialyzing buffer and then eluted with a linear gradient containing decreasing ammonium sulfate concentrations (1.2 to 0 M) in buffer A. The active fractions were collected, dialyzed against buffer A, and then applied to a DEAE-Toyopearl 650 column (2.2 by 20 cm; Tosoh) preequilibrated with buffer A. Two active fractions, I and II, were separated by elution with a linear gradient containing increasing KCl concentrations (0.2 to 0.5 M). The activity peaks of fractions I and II were eluted with buffers containing approximately 0.33 and 0.42 M KCl, respectively. After dialysis against buffer A, each fraction was chromatographed on a MonoQ HR 5/5 column (0.5 by 5 cm; Amersham Biosciences) preequilibrated with buffer A with a linear gradient containing increasing concentrations of KCl (0.1 to 0.5 M). During the chromatography process, fraction I was divided into two active fractions, Ia and Ib, whose activity peaks were found in the eluates containing 0.18 and 0.3 M KCl. Fractions Ia and Ib were heated to 60 and 70°C, respectively, for 20 min and centrifuged at 15,000 × g for 15 min. The heat-treated preparations of fractions Ia and Ib and the MonoQ preparation of fraction II were homogeneous on SDS-PAGE and were stored at 0°C until use. Fractions Ia, Ib, and II are designated Adh2, Adh3, and Adh1, respectively, for consistency with the gene-cloning experiments described above.

Nucleotide sequence accession numbers. The sequences determined in this study have been submitted to the GenBank database and assigned the following accession numbers: 16S rRNA gene sequence of strain TY-5, AB112923; 9.2-kb *Pst*I fragment, AB112920; *adh2*, AB112921; *adh3*, AB112922.

RESULTS

Properties of newly isolated strain TY-5. The new isolate, strain TY-5, was able to grow on propane and *n*-alkanes with C₁₃ to C₂₂ carbon chains as the sole source of carbon but was incapable of growth on methane, ethane, *n*-alkanes with C₄ to C₁₂ carbon chains, and *n*-alkenes with C₂ to C₅ carbon chains. Strain TY-5 was a high-G+C-containing and mycolic-acid-containing, gram-positive bacterium and was most closely related to members of the genus *Gordonia* on the basis of 16S rRNA gene sequencing analysis, the highest similarities (99.4% identity) being found for the sequence of *G. polyisopenivorans*.

Whole-cell reactions with propane. Significant propane oxidation activity (157 nmol of 2-propanol formed · mg of protein⁻¹ · h⁻¹) was detected in reaction mixtures with whole cells of *Gordonia* sp. strain TY-5 when 1-butanol and 2-butanol (each at 5 mM) were added to the reaction mixture to prevent further oxidation of any alcohols formed as reaction products. No production of the terminal oxidation product, 1-propanol, was detected under the same conditions. The reaction rate was comparable to that of butane oxidation to 1-butanol by whole cells of *P. butanovora* (2). These results imply that propane metabolism in *Gordonia* sp. strain TY-5 is initiated by a monooxygenase that catalyzes subterminal oxidation.

Sequence analysis of the genes encoding Prm and Adh1. The complete nucleotide sequence of a 9.2-kb chromosomal DNA fragment from *Gordonia* sp. strain TY-5 represented by the overlapping inserts of recombinant plasmids pPRM1 and pPRM2 and inverse-PCR fragments was determined on both strands (Fig. 1). Analysis of the sequence revealed eight putative open reading frames (ORFs) encoded on the same strand in the same direction. The upstream four complete ORFs in this region were closely spaced with respect to each other and

designated (in the order of transcription) *prmA*, *prmB*, *prmC*, and *prmD*. Each ORF has its own putative ribosomal binding site, but there were no significant similarities to conserved sequences of bacterial promoters in the upstream region. In the sequence downstream of *prmD*, a G+C-rich region of dyad symmetry not followed by a series of thymidine residues was identified, which corresponds to a rho-dependent transcription terminator. These observations suggest the possibility that the gene cluster *prmABCD* functions as a polycistronic transcriptional unit.

A BLAST search against the available sequence databases suggested that the *prmABCD* gene products comprise an enzyme belonging to a family of enzymes containing nonheme carboxylate-bridged dinuclear-iron sites (61). The enzyme encoded by *prmABCD* has significant sequence similarity to several three- or four-component monooxygenases (Table 2). The highest overall similarities were found for the proteins of tetrahydrofuran monooxygenase of *Pseudonocardia* sp. strain K1 (59), which are encoded in the same order as those of the *prm* gene cluster. The second highest sequence similarities were found with the AMO of *R. rhodochrous* B-276 (44). PrmA was similar to the hydroxylase large subunits of tetrahydrofuran monooxygenase, the epoxidase of AMO, and the α subunit of sMMO. The existence of a pair of conserved amino acid sequences (Glu-X-X-His) in the putative sequence of *prmA* is consistent with the assignment of several monooxygenases in the family of dinuclear-iron oxygenases (12, 30, 43, 53), suggesting that this protein could catalyze the hydroxylation of propane. From the similarity analysis, the *prm* gene cluster most likely encodes the propane monooxygenase (Prm), of which *prmA*, *prmB*, *prmC*, and *prmD* encode the large subunit of the hydroxylase, the NADH-dependent acceptor oxidoreductase (reductase), the small subunit of hydroxylase, and the coupling protein, respectively.

Four ORFs were identified downstream of the *prm* gene cluster (Fig. 1). From the results of BLAST searches, ORF1 and ORF2 encode hypothetical proteins and ORF4 showed high similarity to GroEL from *Bacillus subtilis* (47). The putative polypeptide predicted from ORF3 (*adh1*) was similar to NAD⁺-dependent and zinc-containing alcohol dehydrogenases from several organisms (Table 3). The ORF for *adh1* consists of 1,026 bp, and the deduced amino acid sequence includes 341 amino acid residues with a theoretical molecular mass of 35,532 Da. In the putative amino acid sequence, an alcohol dehydrogenase motif, GHENAGWVEAIGDAV (residues 62 to 77), an NAD⁺-binding motif, GLGHIG (residues 80 to 85), and ligands of a catalytic zinc atom (Cys39 and His64) (41) were identified. As described below, the theoretical molecular mass was close to the relative molecular mass of purified Adh1 and the deduced N-terminal amino acid sequence was obtained from the purified enzyme.

Transcript analysis. To determine the transcriptional regulation of the *prm* gene cluster, Northern blot analysis of total RNA from propane-grown *Gordonia* sp. strain TY-5 cells was conducted with probes for *prmA*, *prmB*, *prmC*, and *prmD*. The sizes of the hybridizing bands obtained with all four probes were identical and corresponded to the entire length of the transcription product of *prmABCD* (4.2 kb) (Fig. 2A). Next, Northern blot analysis was conducted on total RNA from cells that had been grown on succinate and then incubated with a

TABLE 2. Sequence similarity between propane monoxygenase and multicomponent monoxygenase proteins

Gene	Size (aa) ^a	Identity (%)	Similarity (%)	Organism	Accession no.	Reference
<i>prmA</i>	498					
<i>thmA</i>	545	39.16	68.47	<i>Pseudonocardia</i> sp. strain K1	AJ296087	59
<i>amoC</i>	501	34.74	63.25	<i>Rhodococcus rhodochrous</i> B-276	D37875	44
<i>bmoX</i>	530	29.12	59.04	<i>Pseudomonas butanovora</i>	AY093933	50
<i>mmoX</i>	526	27.71	58.23	<i>Methylocystis</i> sp. strain M	U81594	31
<i>mnoX</i>	527	27.11	58.43	<i>Methylococcus capsulatus</i> (Bath)	JQ0702	56
<i>mnoX</i>	527	27.11	58.23	<i>Methylomonas</i> sp. strain KSP III	AB025021	49
<i>prmB</i>	346					
<i>thmD</i>	360	42.20	75.14	<i>Pseudonocardia</i> sp. strain K1	AJ296087	59
<i>amoD</i>	342	34.68	70.76	<i>Rhodococcus rhodochrous</i> B-276	D37875	44
<i>tomA5</i>	354	31.21	69.08	<i>Burkholderia cepacia</i> G4	AF319657	
<i>phkF</i>	358	30.92	70.23	<i>Burkholderia kururiensis</i>	AB063252	
<i>tbc1F</i>	354	30.92	69.94	<i>Burkholderia cepacia</i> JS150	AF282897	26
<i>mnoC</i>	348	30.35	67.63	<i>Methylococcus capsulatus</i> (Bath)	P2868	56
<i>prmC</i>	368					
<i>thmB</i>	346	27.72	65.22	<i>Pseudonocardia</i> sp. strain K1	AJ296087	59
<i>amoA</i>	343	24.46	61.41	<i>Rhodococcus rhodochrous</i> B-276	D37875	44
<i>mnoY</i>	389	22.83	59.24	<i>Methylococcus capsulatus</i> (Bath)	M90050	56
<i>bmoY</i>	391	21.74	58.97	<i>Pseudomonas butanovora</i>	AY093933	50
<i>mnoY</i>	395	19.29	58.15	<i>Methylocystis</i> sp. strain M	U81594	31
<i>mnoY</i>	394	19.02	57.61	<i>Methylosinus trichosporium</i> OB3b	P27354	9
<i>prmD</i>	111					
<i>thmC</i>	117	30.63	65.77	<i>Pseudonocardia</i> sp. strain K1	AJ296087	59
<i>amoB</i>	117	23.42	57.66	<i>Rhodococcus rhodochrous</i> B-276	D37875	44

^a aa, amino acids.

carbon source (methane, ethane, propane, butane, or succinate). The probe for *prmC* hybridized against the total RNAs of cells induced weakly with ethane and butane, with the band sizes corresponding to the entire length of the operon product (Fig. 2B). Judging from the results and the gene organization described above, expression of the *prm* gene cluster is regu-

lated at the mRNA level and the *prm* gene cluster is transcribed as a polycistronic operon that is induced by propane during growth. The organism did not grow on ethane and butane but was able to grow on the corresponding alcohols, ethanol and 1- or 2-butanol, respectively (data not shown). These results imply that the *prm* gene product is only active in

TABLE 3. Sequence similarity between Adh1, Adh2, and Adh3 and NAD⁺-dependent dehydrogenases

Protein ^a	Size (aa) ^b	Identity (%)	Similarity (%)	Organism	Accession no.	Reference
Adh1	341					
ADH	347	35.48	69.21	<i>Sulfolobus solfataricus</i>	P39462	1
ADH	347	34.90	69.21	<i>Sulfolobus</i> sp. strain RC3	P50381	7
ADHHT	339	32.55	68.14	<i>Bacillus stearothermophilus</i>	P42328	8
SADH	346	32.26	67.74	<i>Rhodococcus ruber</i>	Q8KLT9	
ADHT	337	31.38	68.62	<i>Bacillus stearothermophilus</i>	P12311	46
Adh2	351					
ADH	366	35.04	69.52	<i>Alcaligenes eutrophus</i>	P14940	25
FDH	424	33.05	66.38	<i>Methylobacter marinus</i>	P47734	55
ADH	362	29.91	66.10	<i>Pseudomonas putida</i>	L35343	22
AACR	349	29.63	63.82	<i>Bacillus cereus</i>	Q93R65	20
XDH	363	27.64	62.96	<i>Trichoderma reesei</i>	AAO42466	
Adh3	506					
THCA	506	79.45	96.05	<i>Rhodococcus erythropolis</i>	P46369	35
HDALD	506	71.94	90.12	<i>Oleomonas sagaranensis</i>	Q9Z918	
ACOD	506	71.74	89.72	<i>Alcaligenes eutrophus</i>	P46368	40
ALD1	503	71.34	90.51	<i>Acinetobacter</i> sp. M-1	Q9FDS1	23
ALDB	506	70.75	89.13	<i>Xanthobacter autotrophicus</i>	O50206	5

^a Abbreviations: ADH, alcohol dehydrogenase; ADHHT, higher thermostable alcohol dehydrogenase; SADH, secondary alcohol dehydrogenase; ADHT, thermally stable alcohol dehydrogenase; FDH, formaldehyde dehydrogenase; AACR, acetylacetoin reductase; XDH, xylitol dehydrogenase; THCA, thiocarbamate-inducible aldehyde dehydrogenase; HDALD, aldehyde dehydrogenase from a petroleum-degrading bacterium, strain HD-1; ACOD, acetaldehyde dehydrogenase; ALD1, aldehyde dehydrogenase 1; ALDB, chloroacetaldehyde dehydrogenase.

^b aa, amino acids.

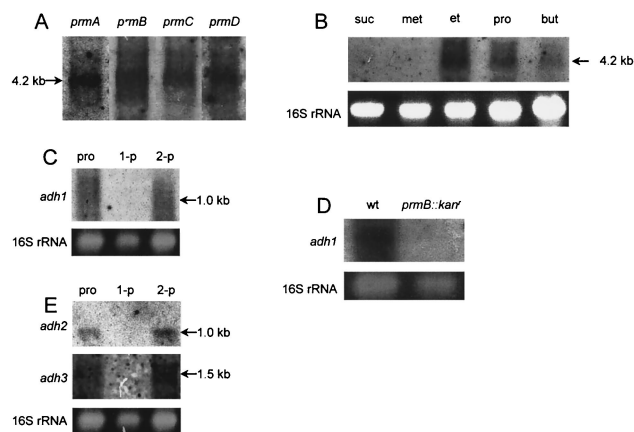


FIG. 2. (A) Northern blot analysis of the *prmABCD* gene cluster. A 20- μ g portion of total RNA was loaded into each lane, and *prmABCD* transcription was detected by hybridization with 32 P-labeled *prmA*, *prmB*, *prmC*, and *prmD* fragments as probes. Total RNA was prepared from *Gordonia* sp. strain TY-5 cells grown on propane. (B) *prmABCD* transcription was detected by hybridization with a 32 P-labeled *prmA* fragment as the probe. Total RNAs were prepared from cells induced with methane (met), ethane (et), propane (pro), and butane (but) as described in Materials and Methods and from cells grown on succinate (suc). (C) *adh1* transcription was detected by hybridization with a 32 P-labeled *adh1* fragment as the probe. Total RNAs were prepared from cells grown on propane, 1-propanol (1-p), and 2-propanol (2-p). (D) *adh1* transcription was detected by hybridization with a 32 P-labeled *adh1* fragment as the probe. Total RNA was prepared from cells with *prmB* disrupted that were induced with 2-propanol. wt, wild type. (E) Transcription of *adh2* and *adh3* was detected by hybridization with 32 P-labeled *adh2* and *adh3* fragments, respectively, as probes. Total RNAs were prepared from wild-type cells grown on propane, 1-propanol, and 2-propanol.

response to propane, although the *prm* operon is induced by ethane, propane, and butane. The enzymological properties of Prm have yet to be elucidated.

To examine the expression and regulation of *adh1*, Northern blot analysis was carried out with total RNA from cells grown on propane, 1-propanol, and 2-propanol (Fig. 2C). Each *prm* gene and the *adh1* gene were used as probes. One intensively hybridizing band was detected with the probe for *adh1* with the mRNA from propane- and 2-propanol-grown cells but not 1-propanol- or succinate-grown cells. The sizes of all hybridizing bands were identical and corresponded to the entire length of the transcription product of *adh1* (1.0 kb). No hybridization band appeared when the probes for the *prm* genes were used, suggesting that the *prm* gene cluster and the *adh1* gene are in distinct transcriptional units. These results suggest that *adh1* is inducibly expressed in response to propane and 2-propanol and that the enzyme plays a significant role in propane oxidation via 2-propanol but does not participate in growth on 1-propanol.

Inactivation of the *prmB* and *adh1* genes. In order to determine the physiological roles of the products of the *prm* and *adh1* genes, *prmB* and *adh1* were inactivated by homologous recombination with gene disruption plasmids as described in Materials and Methods. Results of studies of the growth of the wild-type strain and the mutants on propane, 2-propanol, and 1-propanol are shown in Fig. 3. Neither the *prmB* (*prmB::Kan^r*) mutant nor the *adh1* mutant (*adh1::Kan^r*) was able to grow on

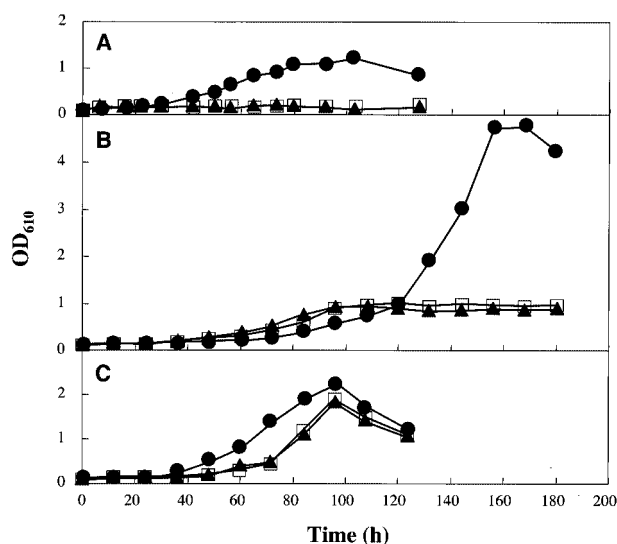


FIG. 3. Growth of the wild-type strain (circles), a mutant strain with *prmB* disrupted (*prmB::Kan^r*) (triangles), and a mutant strain with *adh1* disrupted (*adh1::Kan^r*) (squares) on propane (A), 2-propanol (B), and 1-propanol (C). OD₆₁₀, optical density at 610 nm.

propane (Fig. 3A), indicating that the products of the *prmB* operon and the *adh1* gene are required for propane metabolism. Curiously, growth of the *prmB* mutant on 2-propanol was depressed similarly to that of the *adh1* disruption mutant (Fig. 3B), despite the fact that *prmB* and *adh1* are distinct transcriptional units. In order to clarify this phenomenon, the expression of the *adh1* gene in the *prmB* mutant was investigated through Northern blot analysis (Fig. 2D). The results indicated that *adh1* was not transcribed in the 2-propanol-grown mutant with *prmB* disrupted. Therefore, the insertion of Kan^r into *prmB* inhibited transcription of the downstream gene, *adh1*.

Notably, both mutant strains still grew to some extent on 2-propanol (Fig. 3B). This suggested that another enzyme(s) might participate in 2-propanol oxidation. Indeed, the other two genes for NAD⁺-dependent secondary alcohol dehydrogenases, *adh2* and *adh3*, were found on the chromosomal DNA as described below and were transcribed in the cells grown on propane and 2-propanol (Fig. 2E). Inducible activities of the three genes were compared by real-time quantitative PCR with the same total RNA as used for Northern blot analysis with the following primers: *adh1*-f and *adh1*-500r for *adh1*, *adh2*-f and *adh2*-500r for *adh2*, and *adh3*-f and *adh3*-500r for *adh3* (data not shown). When total RNA preparations from propane- and 2-propanol-grown cells were used, the three genes were transcribed, consistent with the results of Northern blot analyses. Under these conditions, transcription of *adh1* was eight and four times higher than that of *adh2* and *adh3*, respectively. This quantitative gene transcription analysis suggests that Adh1 is the primary dehydrogenase involved in 2-propanol oxidation.

Both the *prmB* and *adh1* mutants grew to the same maximum level as the wild-type strain on 1-propanol, although the growth of the mutant strains was somewhat delayed compared with that of the wild-type strain (Fig. 3C). The NAD⁺-dependent alcohol dehydrogenases Adh1, Adh2, and Adh3 were not

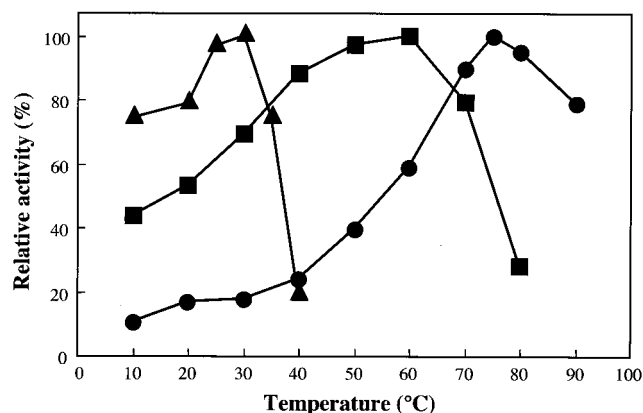


FIG. 4. Effect of temperature on the 2-propanol-oxidizing activities of Adh1 (triangles), Adh2 (circles), and Adh3 (squares).

induced with 1-propanol, indicating that another enzyme participates in 1-propanol oxidation.

Comparison of properties of three NAD⁺-dependent alcohol dehydrogenases. As described in Materials and Methods, three NAD⁺-dependent alcohol dehydrogenases were found in cells of 2-propanol-grown *Gordonia* sp. strain TY-5. Three enzymes, Adh1, Adh2, and Adh3, were purified 19.9-, 145-, and 17.2-fold, respectively, from the extract of 2-propane-grown cells. The relative molecular masses of Adh1, Adh2, and Adh3 were estimated to be 38, 42, and 50 kDa, respectively, by SDS-PAGE and 67, 72, and 100 kDa, respectively, by gel filtration, indicating that these enzymes are dimeric. Maximum activities of all three enzymes were found at pH 10 for the forward reactions. For the reverse reaction (acetone reduction to 2-propanol), Adh1, Adh2, and Adh3 were most active at pHs 6.0, 4.0, and 5.5, respectively. The three enzymes had different temperature profiles. The optimum temperatures for Adh1, Adh2, and Adh3 were 30, 60 and 75°C, respectively (Fig. 4). The K_m and k_{cat} values of the three enzymes for 2-propanol were as follows: Adh1, 4.4 mM and 2.7 s⁻¹; Adh2, 0.024 mM and 21.6 s⁻¹; Adh3, 4.3 mM and 2.6 s⁻¹. The K_m s for NAD⁺ were 0.071 mM for Adh1, 0.088 mM for Adh2, and 0.14 mM for Adh3. NAD⁺ could not be replaced by NADP⁺ (at concentrations of up to 5 mM) for 2-propanol oxidation by the three enzymes. The activities of the three enzymes with a variety of alcohols and ketones are listed in Table 4. Adh1 was active toward primary alcohols with C₂ to C₅ carbon chains and secondary alcohols with C₃ to C₆ carbon chains. Adh2 was active toward only ethanol and 1-propanol among the primary alcohols tested. Adh3 was specific for the secondary alcohols, with only negligible activity for primary alcohols. On the basis of their substrate specificities, the three enzymes were classified as NAD⁺-dependent secondary alcohol dehydrogenases.

Cloning and sequence comparison with Adh2 and Adh3. Two genes for alcohol dehydrogenases, *adh2* and *adh3*, which were located at different loci on the chromosomal DNA, were cloned and sequenced. The calculated molecular masses and deduced N-terminal amino acid sequences were in good agreement with those obtained from the purified enzymes. A BLAST search against the available sequence databases suggested that the deduced amino acid sequences of Adh2 and

TABLE 4. Substrate specificities of purified Adh1, Adh2, and Adh3

Activity and substrate	Relative activity ^a (%)		
	Adh1	Adh2	Adh3
Primary alcohol oxidation			
Methanol	ND ^b	ND	ND
Ethanol	25.6	22.9	ND
1-Propanol	21.4	32.6	ND
1-Butanol	50.3	ND	ND
1-Pentanol	14.8	ND	ND
1-Hexanol	ND	ND	ND
1-Heptanol	ND	ND	ND
1-Octanol	ND	ND	ND
Secondary alcohol oxidation			
2-Propanol	100	100	100
2-Butanol	119	82.8	62.9
(R)-(-)-2-Butanol	13.7	165	44.6
(S)-(+)-2-Butanol	178	125	78.7
2-Pentanol	129	40.0	63.5
2-Hexanol	19.2	12.7	78.1
2-Heptanol	ND	ND	109
2-Octanol	ND	ND	112
Ketone reduction			
Acetone	36.9	54.9	34.9
2-Butanone	65.1	64.9	30.6

^a 100% relative activity was defined as the activity with 2-propanol as the substrate.

^b ND, activity of <5%.

Adh3 are homologous to those of several NAD⁺-dependent dehydrogenases (Table 3). Adh2 has an NAD⁺-binding motif, GTGPVG (between residues 180 and 185), an alcohol dehydrogenase motif, GHEGVGTITEVGDV (between residues 63 and 77), and ligands typical of a reactive zinc atom, Cys38 and His60, indicating that Adh2 is a typical zinc-containing, NAD⁺-dependent alcohol dehydrogenase. Adh3 has the alternative NAD⁺-binding domain GFGVEAG (between residues 219 and 225), which has been found in several aldehyde dehydrogenases capable of catalyzing irreversible reactions (16, 35). The catalytic-site sequences containing Glu and Cys residues of the *S*-ethyl dipropylcarbamothionate-inducible aldehyde dehydrogenase (*thcA*) from *Rhodococcus* sp. strain NI86/21 (35) were completely conserved in Adh3: LELGGKSP (between residues 261 to 268) for Glu and FALNQGEVCTAPS (between residues 293 to 305) for Cys. Judging from the deduced sequence, Adh3 is an unusual alcohol dehydrogenase that is highly homologous in primary structure to aldehyde dehydrogenases, although it was not active with any of the aldehydes tested.

DISCUSSION

Gordonia sp. strain TY-5 was able to grow with only propane among the gaseous alkanes (C₂ to C₅) as its sole source of carbon and energy, and it was not capable of growth on methane and alkenes. Propane oxidation to 2-propanol was detected in whole-cell reactions. The *prmABCD* gene cluster located on the chromosome of TY-5 was cloned and sequenced. Significant function of the *prm* genes in *n*-propane metabolism has been demonstrated by the following results: (i) the mutant with *prmB* disrupted lost the ability to grow on

propane, (ii) the *prmABCD* genes were transcribed polycistronically and transcribed in response to propane, and (iii) the deduced proteins from the *prmABCD* gene cluster were characteristic of a dinuclear-iron-containing multicomponent monooxygenase.

Soluble dinuclear-iron-containing monooxygenases are classified into two groups based on the subunit structures (59, 65). In one group are the monooxygenases composed of three components, a hydroxylase, a reductase, and a coupling protein (11, 19, 34, 36, 37, 44, 50, 59). Representatives include AMO and tetrahydrofuran monooxygenase (Thm), which have hydroxylases composed of two subunits and other hydroxylases consisting of three subunits. The second group consists of monooxygenases composed of four components, including an additional ferredoxin (51, 60, 64, 65). The Prm of *Gordonia* sp. strain TY-5 belongs to the former class, and its hydroxylase is composed of two subunits. Detailed analysis of *prmABCD* based on the functional studies of AMO (44), sMMO (61), and Thm (59) predicts the following model for Prm. Hydroxylation of propane occurs at a dinuclear iron of the large subunit (PrmA) accompanied by the small subunit (PrmC), and electrons needed for O₂ activation are provided by an NADH reductase (PrmB). The third component, the regulatory protein PrmD, may influence the reaction rate or product distribution.

Several polypeptides participating in the oxidation of gaseous *n*-alkanes have been described (15, 39, 63). Since very little is known about the genetics of these systems, we were unable to determine whether any of the reported polypeptides are related to the gene products of *prmABCD*. Recently, the genes encoding the sBMO from *P. butanovora* were cloned. The enzyme is composed of three components, a dinuclear-iron-site-containing hydroxylase, a reductase, and a third component (an effector or regulator) like sMMO (50). Among the components of Prm, the putative large and small subunits of the hydroxylase show a relatively higher sequence similarity to those of sBMO, while sequences of the other components show lower similarities. There have been no reports that described propane-oxidizing activity in Thm and sBMO (50, 59), and AMO is incapable of hydroxylation of any *n*-alkanes (32). The monooxygenase encoded by the *prm* operon of *Gordonia* sp. strain TY-5 is the first bacterial enzyme that has been genetically confirmed to participate in propane oxidation.

Some strains belonging to the order *Actinomycetales*, such as strains of the genera *Rhodococcus* (54, 62) and *Nocardioides* (15), are known to degrade *n*-alkanes with a wide range of carbon chain lengths. *Gordonia* sp. strain TY-5 can utilize liquid alkanes with longer carbon chains (C₁₃ to C₂₂), as well as propane. Since the *prmB* mutant strain could still grow on the liquid alkanes, the organism should possess another monooxygenase(s) for the oxidation of liquid alkanes.

We have concluded that propane is oxidized by monooxygenase-mediated subterminal oxidation via 2-propanol from the following results: (i) whole cells of *Gordonia* sp. strain TY-5 produced 2-propanol and not 1-propanol from propane, (ii) *adh1* was transcribed in response to propane and 2-propanol and not in response to 1-propanol, (iii) disruption of *adh1* prevented the organism from growing on propane and 2-propanol but did not affect its ability to utilize 1-propanol, and (iv) strain TY-5 could grow on acetone, acetol, and methyl acetate,

which are intermediates in the subterminal oxidation pathway (data not shown).

There are several possible pathways for the oxidation of propane (3). Among them, the terminal oxidation pathway of *Mycobacterium vaccae* JOB-5 (10), both the terminal and subterminal oxidation pathways of *P. fluorescens* NRRL-B-1244 (21), and the subterminal oxidation pathway of *R. rhodochrous* PNKb1 (4) have been proposed mainly on the basis of the properties of the alcohol dehydrogenases that participate in 1- or 2-propanol oxidation. Three different secondary alcohol dehydrogenases, Adh1, Adh2, and Adh3, were found in *Gordonia* sp. strain TY-5, purified, and characterized. All three genes were transcribed in response to propane and 2-propanol but not in response to 1-propanol. Among them, Adh1 appears to be an important dehydrogenase in the propane oxidation pathway. Interestingly, Adh2 and Adh3 had somewhat higher activity for 2-propanol and showed higher specificity for secondary alcohols than did Adh1, but the mutant with *adh1* disrupted, in which *adh2* and *adh3* were expressed, was able to grow only partially on 2-propanol and was incapable of growth with propane. This implies that the data on catalytic properties and induction profiles are not enough to confirm the physiological role of an enzyme.

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