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Two Distinct Monooxygenases for Alkane Oxidation in *Nocardioides* sp. Strain CF8

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Alkane monooxygenases in *Nocardioides* sp. strain CF8 were examined at the physiological and genetic levels. Strain CF8 can utilize alkanes ranging in chain length from C_2 to C_{16}. Butane degradation by butane-grown cells was strongly inhibited by allylthiourea, a copper-selective chelator, while hexane-, octane-, and decane-grown cells showed detectable butane degradation activity in the presence of allylthiourea. Growth on butane and hexane was strongly inhibited by 1-hexyne, while 1-hexyne did not affect growth on octane or decane. A specific 30-kDa acetylene-binding polypeptide was observed for butane-, hexane-, octane-, and decane-grown cells but was absent from cells grown with octane or decane in the presence of 1-hexyne. These results suggest the presence of two monooxygenases in strain CF8. Degenerate primers designed for PCR amplification of genes related to the binuclear-iron-containing alkane hydroxylase from *Pseudomonas oleovorans* were used to clone a related gene from strain CF8. Reverse transcription-PCR and Northern blot analysis showed that this gene encoding a binuclear-iron-containing alkane hydroxylase was expressed in cells grown on alkanes above C_6. These results indicate the presence of two distinct monooxygenases for alkane oxidation in *Nocardioides* sp. strain CF8.

A number of bacteria have been isolated for their ability to utilize gaseous or liquid alkanes as growth substrates (5, 7, 44). It is convenient to recognize three classes of alkane-utilizing bacteria, namely, those that grow on methane, those that grow optimally on gaseous alkanes, and those that grow optimally on liquid alkanes. The methane utilizers (methanotrophs) have been well characterized. Methane metabolism is initiated by the oxidation of methane to methanol in a reaction catalyzed by methane monooxygenase (MMO). Two forms of MMO have been characterized, a particulate MMO (pMMO) that contains copper, and a soluble MMO (sMMO) that contains a binuclear iron cluster at the catalytic site (27, 50). Both pMMO and sMMO can catalyze the oxidation of several alkanes in addition to methane but cannot grow on any of these compounds (10, 11). Long-chain, liquid alkanes are utilized by a wide variety of bacterial species, including both gram-negative and gram-positive bacteria (8, 47). Among these bacteria, the alkane hydroxylase system in *Pseudomonas oleovorans* has been characterized most thoroughly. *P. oleovorans* can grow on *n*-alkanes ranging from C_6 to C_{12} (5). It harbors a large plasmid (OCT plasmid) encoding the enzymes required to oxidize *n*-alkanes to the corresponding terminal acyl coenzyme A derivatives, which then enter the β-oxidation cycle (46). Alkane hydroxylase consists of three polypeptides: a hydroxylase (AlkB; 41 kDa), a rubredoxin (AlkG; 19 kDa), and a rubredoxin reductase (AlkT; 48 kDa) (13–15). The alkane hydroxylase component contains a binuclear iron cluster, which seems to be a common motif among bacteria that harvest alkanes, alkenes, and aromatic hydrocarbons as growth substrates (40). Several different long-chain alkane oxidation pathways have been described for strains of *Acinetobacter* spp.: alkane dioxygenase is involved in degradation of alkanes ranging from C_{13} to C_{44} in *Acinetobacter* sp. strain M-1 (28, 37), some strains utilize a P-450 monooxygenase system (3), and an alkane hydroxylase homologous to that of *P. oleovorans* has been found in *Acinetobacter* sp. strain ADP1 (34). Gaseous alkanes have not been shown to serve as growth substrates for these bacteria.

Although a wide variety of microorganisms can utilize long-chain, liquid alkanes, the ability to utilize short-chain, gaseous alkanes is mostly restricted to the *Corynebacterium-Nocardia-Mycobacterium-Rhodococcus* group of gram-positive bacteria (2, 29). In addition, some gram-negative *Pseudomonas* spp. were reported to grow on short-chain alkanes other than methane (25, 44). There have been neither descriptions of the purification to homogeneity of a monooxygenase from short-chain-alkane-utilizing bacteria nor any isolations of genes that code for this group of monooxygenases. We have recently isolated a butane-utilizing bacterium, *Nocardioides* sp. strain CF8, and characterized a butane monooxygenase in this organism at the physiological level (22, 24). Butane degradation by strain CF8 was strongly inhibited by allylthiourea (ATU) and inactivated by light and acetylene. Both ATU and light are known inhibitors and inactivators of copper-containing monooxygenases such as pMMO and ammonia monooxygenase (AMO) (6). Acetylene serves as an inactivator of butane monooxygenase in strain CF8, and incubation with [^{14}C]acetylene results in the covalent binding of ^{14}C label to a specific polypeptide with a molecular mass of 30 kDa (24). This result supports the similarity between butane monooxygenase of strain CF8 and the copper-containing monooxygenases, pMMO and AMO, which contain an acetylene-binding protein with a similar molecular mass (ca. 27 kDa) (26, 33). Therefore, it was suggested that butane monooxygenase in strain CF8 is a third example of the copper-containing monooxygenases, which include pMMO and AMO.

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Bacteria that grow on gaseous alkanes often include some liquid alkanes in their range of growth substrates. This observation also extends to *Nocardioides* sp. strain CF8 (22). In this work, we extended the characterization of the alkane monooxygenase system in *Nocardioides* sp. strain CF8 to include longer-chain alkanes (C_6 to C_{10}). The possibility of two distinct monooxygenases for alkane degradation in *Nocardioides* sp. strain CF8 was examined.

MATERIALS AND METHODS

Growth conditions. Cells of *Nocardioides* sp. strain CF8 were grown as previously described (23). Strain CF8 was grown in the *Xanthobacter* Py2 medium (48) except that NH_4Cl replaced $NaNO_3$, yeast extract was removed, and the pH was adjusted to 7.5. Cells were incubated in 150-ml sealed vials containing 50 ml of medium and alkanes as growth substrates. Gaseous alkanes (50 ml) were added as an overpressure to the gas phase. Liquid alkanes were added to a total amount of 500 μmol. All cultures were incubated at 30°C with constant shaking. Cell growth was monitored by removing a portion of the cultures (1 ml) and measuring the optical density at 600 nm (OD_{600}). To examine the effect of ATU and 1-hexyne on growth, ATU (200 μM) and 1-hexyne (4.5 μmol) were added at the beginning of growth assays. The extent of growth of each culture was determined by measuring the OD_{600} at selected times (4 days for C_4-, C_6-, and C_8-grown cells; 5 days for C_{10}-grown cells). No significant increase in OD_{600} was observed in any of the experimental setups after 5 days. Experiments were repeated at least three times.

Butane degradation assay. Cells were harvested from cultures by centrifugation (6,000 × *g* for 10 min), washed twice with the same phosphate buffer as the growth medium, and resuspended to a constant cell density (based on OD). Butane degradation assays were conducted as previously described (24). For ATU inhibition assays, butane degradation was monitored with 200 μM ATU in the reaction vials. Experiments were repeated at least three times.

[^{14}C]acetylene labeling assays. Cells of C_4-, C_6-, C_8-, and C_{10}-grown strain CF8 were radiolabeled with [^{14}C]acetylene as described previously (24). C_8- and C_{10}-grown cells were also grown in the presence of 1-hexyne (4.5 μmol) and subjected to [^{14}C]acetylene labeling. The labeled cells were disrupted with a Mini-beadbeater (Biospec Products, Bartlesville, Okla.). Protein samples (50 μg) were then separated on a sodium dodecyl sulfate–12% polyacrylamide gel at a constant current of 15 mA. The gel was stained with Coomassie blue, dried onto filter paper, and radioactive polypeptides were visualized by exposure to phosphor screens (Molecular Dynamics, Sunnyvale, Calif.) for 5 days.

Protein determinations. Protein content was determined by using the biuret assay (19) after cells were solubilized in 3 N NaOH for 30 min at 65°C. Bovine serum albumin was used as the standard.

PCR, sequence analysis, and library screening to characterize *alkB* in strain CF8. Chromosomal DNA was isolated from strain CF8 by the CTAB (hexadecyltrimethylammonium bromide) method described previously (4). PCRs were performed with degenerate oligonucleotide primers TS2S (5′-AAYAGAGCTC AYGARYTRGG TCAYAAG-3′) and deg1RE (5′-GTGGAATTCG CRTGRT GRTC IGARTG-3′) (42), and the program (4 min at 95°C; 25 cycles of 45 s at 95°C, 1 min at 40°C, and 1 min at 72°C; 5 min at 72°C; indefinitely at 4°C) described by Smits et al. (42) was used. The PCR products were cloned into the pGEM-T Easy vector (Promega, Madison, Wis.) following the directions of the manufacturer.

A genomic library was constructed in Lambda FIX II (Stratagene, La Jolla, Calif.) using Gigapack III XL-11 packaging extract (Stratagene) following the direction of the manufacturer. The genomic library was screened as described (38) using a DNA probe generated by PCR with TS2S and deg1RE primers. DNA probes were labeled by random priming using a Prime-a-gene kit (Promega) and [α-^{32}P]dCTP (3,000 Ci/mmol; DuPont NEN products, Wilmington, Del.). Lambda phage DNA isolation, restriction digests, agarose gel electrophoresis, Southern hybridization, and cloning were performed as standard procedures (38). DNA fragments (0.6, 1, and 2.7 kb) of a *Xho*I digest were cloned into pBluescript II KS (Stratagene) and sequenced. A total of 3.2 kb, including the complete *alkB*, was sequenced in both directions at least three times by a combination of primer walking with custom oligonucleotides and primers complementary to cloning vectors.

DNA sequencing and oligonucleotide synthesis were performed at the Center for Gene Research and Biotechnology at Oregon State University (Corvallis, Oreg.). The nucleotide and the predicted amino acid sequences were compared with the EMBL, SWISSPROT, and GenBank databases using BLAST (1). DNA sequencing data was analyzed and assembled using the Genetics Computer Group (Madison, Wis.) software package (Wisconsin Package, version 10.0).

TABLE 1. Effect of allylthiourea on butane degradation by strain CF8 grown on various alkanes

Growth substrate[a]	Butane degradation rate[b] (nmol min^{-1} mg of protein^{-1})	
	No additions	+ATU (200 μM)
C_4	30.8 ± 2.1	<1.5
C_6	28.2 ± 5.1	3.0 ± 3.8
C_8	30.9 ± 4.9	6.4 ± 3.4
C_{10}	23.9 ± 1.9	7.5 ± 2.4

[a] Butane was added as 50% (vol/vol [gas phase]) in the gas phase as an overpressure. Liquid alkanes were added to a total amount of 500 μmol.
[b] Data are expressed as experimental means ± standard deviations.

Reverse transcription (RT)-PCR and Northern blotting. Cells of strain CF8 were grown on *n*-alkanes ranging from C_4 to C_8 in 50 ml of medium as described earlier. Cells were grown to late exponential phase (OD_{600} of 0.5 to 0.6), and 25 ml of each culture was harvested by centrifugation (6,000 × *g* for 10 min) in a rotor chilled to 4°C. Total cellular RNA was isolated as described by Brzostowicz et al. (9) with slight modifications. The pellets were resuspended in 0.7 ml of an ice-cold lysis solution (1% sodium dodecyl sulfate, 100 mM sodium acetate at pH 5) and transferred to a 2-ml conical screw-cap Microtube (Biospec Products) containing 0.7 ml of aqueous phenol (pH 5) and 0.3 ml of 0.5-mm-diameter zirconia beads (Biospec Products). The tubes were placed in a Mini-beadbeater (Biospec Products) and disrupted at 4,600 beats/min for 2 min. The liquid phase was transferred to microcentrifuge tubes and centrifuged for 3 min at 14,000 × *g*. The supernatant was extracted twice with phenol (pH 5) and twice with phenol-chloroform solution (pH 7.5). Nucleic acids were precipitated from the aqueous phase with ethanol, resuspended in 200 μl of diethyl pyrocarbonate-treated water, and treated with 5 U of RNase-free DNase (Promega Co.) at 37°C for 2 h. Subsequently, the solution was extracted twice with phenol-chloroform solution (pH 7.5). RNA was recovered by ethanol precipitation and resuspended in 200 μl of diethyl pyrocarbonate-treated water.

The extracted RNA was subjected to RT and subsequent PCR amplification. Primers, revalk (5′-AGTGTCGCTG CAGGTGGTA-3′) and cfalkF (5′-AGAA GGAGAG CCACGAACG-3′), were designed based on the nucleotide sequence of the PCR product amplified with TS2S and deg1RE primers. The RT reaction mixtures (20 μl) contained 200 U of Moloney murine leukemia virus (M-MLV) reverse transcriptase (Promega Co.), 1 mM each deoxynucleoside triphosphate, 1 U of RNase inhibitor (Promega Co.), 30 pmol of primer revalk, and 100 ng of extracted RNA in 1× M-MLV buffer provided by the manufacturer. Control reactions were performed without addition of M-MLV reverse transcriptase to verify the absence of DNA in the RNA preparations. RT was carried out at 42°C for 15 min, 95°C for 1 min, and 5°C for 5 min. Aliquots from each RT reaction (5 μl) were used as templates in subsequent PCR mixtures (25 μl) containing deoxynucleoside triphosphates (0.2 mM each), $MgCl_2$ (2 mM), revalk and cfalkF primers (30 pmol each), and 2.5 U of *Taq* polymerase (Promega Co.). The following temperature profile was used: 4 min at 94°C and 35 cycles of 1 min at 94°C, 1 min at 62°C, and 1 min at 72°C. The PCR products (10 μl) were separated by electrophoresis on a 1.2% (wt/vol) agarose gel in Tris-borate-EDTA buffer and visualized with ethidium bromide staining.

Northern blot analysis was carried out as described (39). DNA probes specific for *alkB* and 16S rRNA were generated by PCR with revalk and cfalkF primers and a set of primers described by Giovannoni (18), respectively. The hybridization signals were visualized using a PhosphorImager (Molecular Dynamics).

Nucleotide sequence accession number. The nucleotide sequence reported in this study has been deposited in the GenBank database under accession number AF350429.

RESULTS

Nocardioides sp. strain CF8 was grown on *n*-alkanes of various chain lengths and tested for butane degradation activity. Cells grown on butane, hexane, octane, and decane (C_4, C_6, C_8, and C_{10} chain length, respectively) readily degraded butane at similar rates (Table 1). In our previous study, it was shown that butane degradation by butane-grown strain CF8 was strongly

inhibited by ATU, a copper-selective chelator (24). Along with other lines of evidence, it was suggested that butane-grown strain CF8 contains a copper-containing butane monooxygenase that is responsible for butane oxidation in this organism (22). We have now examined the effect of ATU on butane degradation by cells grown on longer alkanes. As previously shown, 200 μM ATU inhibited butane degradation by C_4-grown cells to below detectable levels (1.5 nmol min^{-1} mg of $protein^{-1}$). In contrast, C_6-, C_8-, and C_{10}-grown cells of strain CF8 showed detectable levels of butane degradation (10 to 30% relative to the absence of ATU) in the presence of the same concentration of ATU (Table 1). These low levels of activity could be due to an incomplete inhibition of the copper-containing butane monooxygenase by ATU, although this explanation would require different inhibitory effects of ATU on cells grown on various alkanes. Alternatively, it is possible that in addition to copper-containing butane monooxygenase, a second monooxygenase which degrades butane and is not inhibited by ATU is expressed when cells are grown on C_6, C_8, and C_{10} but not C_4 alkanes.

To further investigate the latter possibility, we examined the inhibitory effects of ATU and 1-hexyne on the growth of strain CF8 with C_4, C_6, C_8, and C_{10} alkanes as the growth substrate (data not shown). As expected, growth on C_4 was greatly inhibited (~80%) by 200 μM ATU. However, ATU (200 μM) did not inhibit growth on C_6, C_8, and C_{10} alkanes. In the presence of 1-hexyne (4.5 μmol), growth was not observed on C_4 and C_6 alkanes. Growth on C_8 and C_{10} was unaffected by the presence of 1-hexyne (4.5 μmol) (data not shown). These results are consistent with the presence of a second monooxygenase that is not inhibited by ATU and is less sensitive to 1-hexyne inhibition (inactivation). The different response of C_6-grown cells to 1-hexyne inhibition compared to those of C_8- and C_{10}-grown cells may reflect the higher affinities of the second monooxygenase toward longer-chain alkanes. Because ATU is a reversible inhibitor, butane-oxidizing activity of the copper-containing butane-monooxygenase can be recovered in cells grown in the presence of ATU. Indeed, cells grown on C_6, C_8, and C_{10} in the presence of ATU (200 μM) and subsequently washed free of ATU had similar butane degradation rates to those grown in the absence of ATU (data not shown). In contrast, the cells grown on C_8 and C_{10} in the presence of 1-hexyne (4.5 μmol) and washed as above showed much lower butane degradation rates (5.2 ± 2.7 and 4.3 ± 3.1 nmol min^{-1} mg of $protein^{-1}$, respectively [means ± standard deviations]) than those grown in the absence of 1-hexyne (Table 1). These results support the ideas that 1-hexyne irreversibly inactivates the copper-containing monooxygenase and that the residual butane degradation activity of the cells grown on C_8 and C_{10} in the presence of 1-hexyne is due to the second monooxygenase.

We have previously shown that the incubation of strain CF8 with [^{14}C]acetylene results in the covalent binding of ^{14}C label to a specific polypeptide with a molecular mass of 30 kDa and that the incorporation of ^{14}C label correlates with butane monooxygenase activity (24). Thus, cells grown on various chain length alkanes either with or without 1-hexyne (4.5 μmol) were treated with [^{14}C]acetylene (Fig 1). The ^{14}C-labeled 30-kDa polypeptide was present in C_4-, C_6-, C_8-, and C_{10}-grown cells without 1-hexyne. In contrast, ^{14}C label was not incorporated into cellular polypeptides of cells grown on C_8 and C_{10} in the presence of 1-hexyne. Since labeling of the 30-kDa polypeptide with ^{14}C from acetylene requires active butane monooxygenase, the 30-kDa polypeptide of the copper-containing butane monooxygenase is most likely being produced and then irreversibly inactivated by the 1-hexyne in the growth medium. These results further confirm that the cells grown in the presence of 1-hexyne do not possess an active copper-containing butane-monooxygenase. Again, production of a second monooxygenase would support growth under these conditions. Interestingly, there were no additional labeled polypeptides observed other than the 30-kDa polypeptide under any of the growth conditions tested. This result indicates that acetylene might act either as a conventional reversible inhibitor or as an unusually weak mechanism-based inactivator for the second monooxygenase.

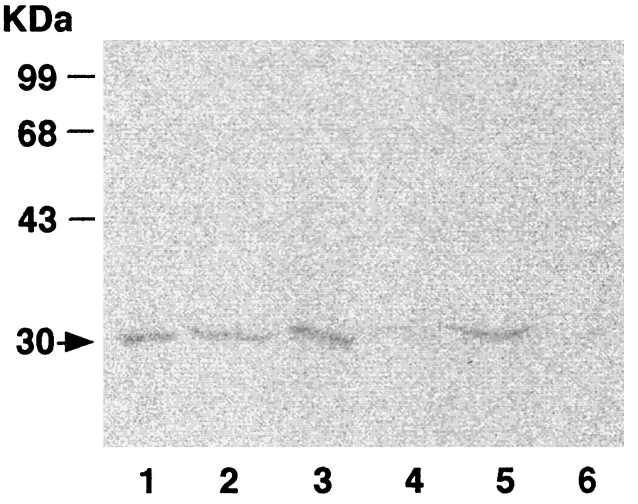

FIG. 1. Incorporation of ^{14}C from [^{14}C]acetylene into cellular proteins of strain CF8 grown on C_4, C_6, C_8, and C_{10} alkanes (lanes 1, 2, 3, and 5, respectively) and cells grown with C_8 or C_{10} in the presence of 1-hexyne (lanes 4 and 6, respectively). Incorporation of ^{14}C into polypeptides was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and visualized with a phosphorimager as described in Materials and Methods. The sizes of molecular mass markers and the apparent molecular mass of the labeled polypeptide (arrow) are shown on the left side of the gel. Each gel lane contains approximately 50 μg of cell extract protein.

Among the long-chain-alkane-oxidizing enzymes, alkane hydroxylase in *P. oleovorans* has been characterized most thoroughly (13–15). Recent study has shown that a large proportion of bacteria able to grow on long-chain alkanes possess genes related to the alkane hydroxylase gene in *P. oleovorans* (42). Based on biochemical analyses and sequence comparisons, alkane hydroxylase from *P. oleovorans* belongs to a family of integral-membrane, binuclear-iron hydrocarbon oxygenases including alkane hydroxylase from *Acinetobacter* sp. strain ADP1 and xylene monooxygenase from *Pseudomonas putida* (34, 40, 41). These enzymes all contain an eight-histidine motif as iron-binding ligand (41). This motif is also conserved among the soluble, binuclear-iron hydrocarbon oxygenases, such as sMMO and toluene 2-monooxygenase from *Burkholderia cepacia* G4 (17). It was shown that acetylene is a weak inactivator of toluene 2-monooxygenase, while longer-chain alkynes are more effective inactivators (49). These previous studies drew

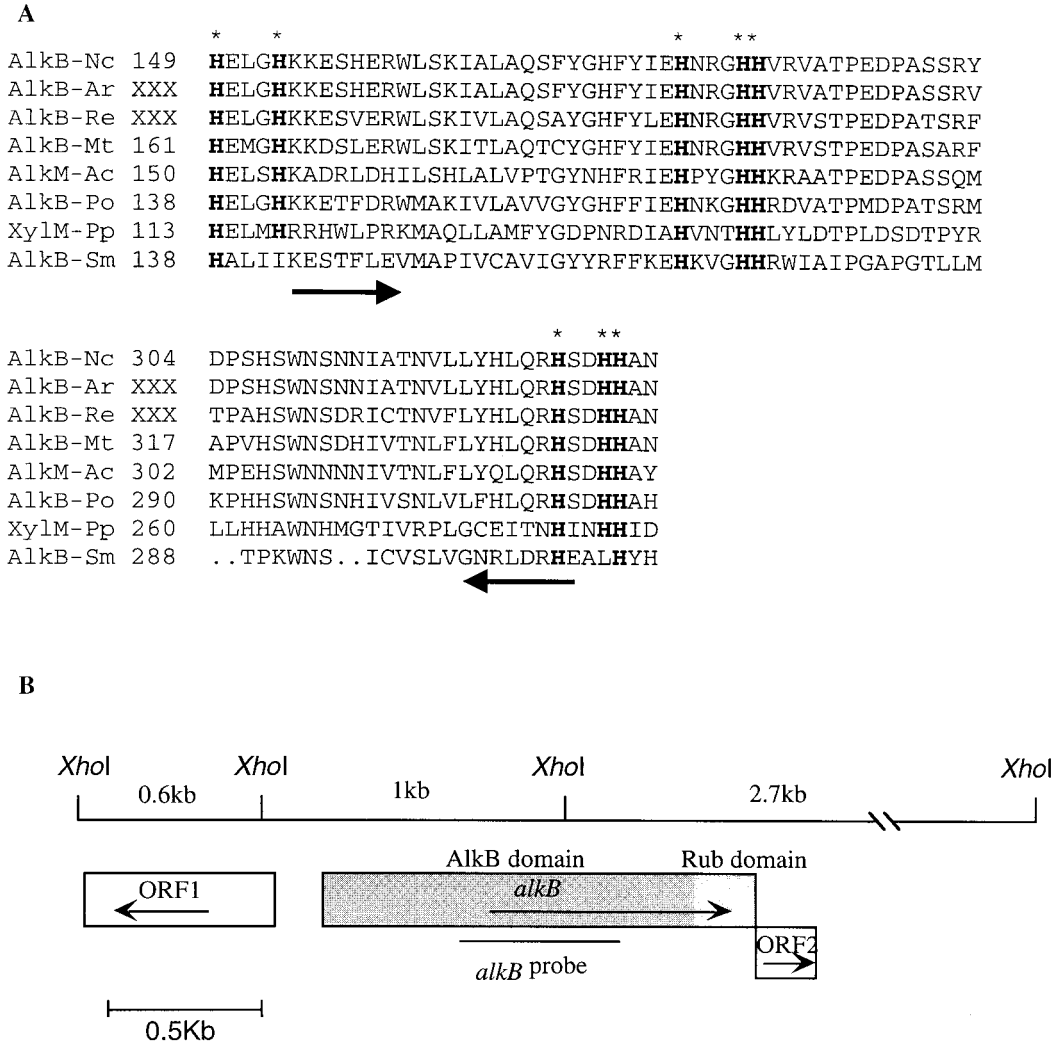

FIG. 2. (A) Part of a multiple sequence alignment of AlkB from strain CF8 with other known integral-membrane, binuclear-iron hydrocarbon oxygenases. Eight conserved His residues are indicated by letters in boldface type and marked by asterisks. The positions of the PCR primers used for RT-PCR are shown by arrows. Peptide numbering refers to the position in the full-length enzyme (XXX is shown for the sequences whose full length sequences are not available). Abbreviations: Nc, *Nocardioides* sp. strain CF8; Ar, *A. rugosa*; Re, *R. erythropolis*; Mt, *M. tuberculosis*; Ac, *Acinetobacter* sp. strain ADP1; Po, *P. oleovorans*; Pp, *P. putida*; Sm, *Stenotrophomonas maltophilia.* (B) Gene organization and restriction map of the cloned regions including complete AlkB gene. Location of the PCR fragment used as a probe for *alkB* is shown. Each box indicates an ORF, and the arrows indicate the orientation of each gene.

our attention to the binuclear-iron monooxygenases as potential candidates for the second monooxygenase in strain CF8.

In order to examine the possible presence of a binuclear-iron monooxygenase gene in strain CF8, we employed the PCR method developed recently by Smits et al. (42) which uses degenerate primers based on the sequence alignment of the conserved histidine motif (42). By using this PCR method, a PCR product of the expected size (557 bp) was obtained from strain CF8. The fragment was cloned and sequenced. The peptide sequence encoded by the PCR product was compared with the known alkane hydroxylase sequences, including some of the PCR fragments obtained by Smits et al. (42), and the sequence of xylene monooxygenase (Fig. 2A). The sequence alignment showed that the regions around the eight-histidine motif were well conserved in the sequence from strain CF8. The deduced peptide fragment (186 amino acids) from strain CF8 showed high sequence identities to putative AlkB fragments from other gram-positive bacteria, including *Amycolatopsis rugosa* (86%) and *Rhodococcus erythropolis* (69%), and an alkane hydroxylase homologue present on the chromosome of *Mycobacterium tuberculosis* H37Rv (63%). Lower sequence identities were observed to the corresponding sequences from gram-negative bacteria, including AlkM from *Acinetobacter* sp. strain ADP1 (54%), AlkB from *P. oleovorans* (49%), and XylM from *P. putida* (27%).

The complete AlkB gene (*alkB*) in strain CF8 was isolated by screening a genomic library with the cloned PCR fragment as a probe. The nucleotide sequences of three contiguous *Xho*I restriction fragments totaling 3.2 kb and including *alkB* were determined, and sequence analysis revealed three open reading frames (ORFs) (Fig. 2B). *alkB* was located in an ORF that continuously contains a domain similar to rubredoxin (de-

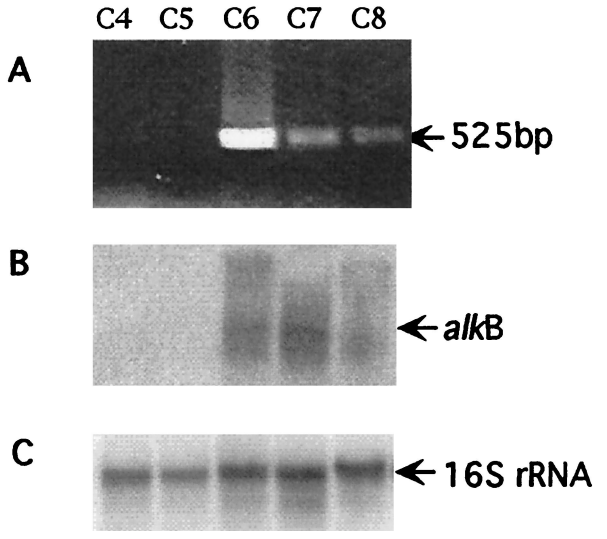

FIG. 3. RT-PCR (A) and Northern blot analysis (B and C) of *alkB* expression in strain CF8 grown on alkanes of various chain length. Total RNA was prepared from cells grown on C_4 to C_8 alkanes. RT-PCR was performed using primers shown in Fig. 2A, and the expected product size (525 bp) is indicated (A). Northern blot analysis was performed with the *alkB* probe shown in Fig. 2B, and the hybridizing band (about 2 kb) is indicated (B). The same blot was stripped and hybridized to the 16S rRNA probe (C).

duced amino acid sequence showed 55% identity to rubredoxin 2 in *P. oleovorans*, 52% identity to rubredoxin in *Clostridium butyricum*, and 51% identity to rubredoxin 2 of *P. putida*). The entire ORF consists of 1,452 bp and potentially encodes a unique alkane hydroxylase fused to rubredoxin, although further biochemical characterization of this enzyme is required to confirm this idea. The stop codon of the *alkB-rub* ORF overlaps with the start codon of a second ORF (ORF2) which consists of 192 bp. The deduced amino acid sequence of ORF2 shows 30 to 40% identity to the ferredoxins of *Rhodococcus fascians*, *Thermotoga maritima*, and *Archaeoglobus fulgidus*. ORF1 is located 150 bp upstream of the *alkB-rub* ORF in the reverse orientation. The putative product of this gene, encoding 208 amino acid residues, shows ~30% identity to the TetR-family of transcriptional regulators and contains the *tetR* DNA-binding helix-turn-helix motif.

We investigated whether this *alkB* encodes the second monooxygenase that was proposed to be expressed when cells of strain CF8 were grown on alkanes above C_6 in chain length. The expression of *alkB* in strain CF8 was examined by RT-PCR using primers designed from the cloned 550 bp PCR fragment, and by Northern hybridization using the same PCR fragment as a probe. Total RNA was prepared from cells grown on alkanes ranging from C_4 to C_8 as growth substrates. RT-PCR showed that a single product of the expected size (525 bp) was amplified from RNA prepared from cells grown on alkanes above C_6 in chain length, while no products were detected from RNA prepared from C_4 and C_5-grown cells (Fig. 3A). The absence of *alkB* transcripts from C_4 and C_5-grown cells was also confirmed by the Northern blot (Fig. 3B). The probe hybridized to one major transcript of approximately 2 kb. These transcripts were absent from RNA from C_4 and C_5-grown cells. Hybridization with the 16S rRNA gene probe showed that all lanes contained similar amounts of RNA (Fig. 3C). These results indicate that *alkB* in strain CF8 is expressed when cells are growing on long-chain alkanes (C_6) but not on the short-chain alkanes. Therefore, it seems likely that expression of *alkB* leads to production of the second monooxygenase in strain CF8.

DISCUSSION

Our results indicate that *Nocardioides* sp. strain CF8 can produce two distinct monooxygenases for oxidation of alkanes: a copper-containing monooxygenase and an integral-membrane, binuclear-iron monooxygenase. In this study, we took advantage of the sensitivity of the copper-containing monooxygenase to ATU and to alkynes in order to reveal the presence of the binuclear iron monooxygenase which is not inhibited by ATU or inactivated by alkynes. Expression of the copper-containing monooxygenase occurred on all the alkanes tested, while the binuclear iron monooxygenase was observed only in cells grown on alkanes C_6 and above.

The ability of alkane-grown cells to express more than one monooxygenase is not without precedent. Several methanotrophs can produce two methane monooxygenases (pMMO and sMMO). The two situations are similar in that the copper-containing butane monooxygenase from strain CF8 is similar to pMMO. Like sMMO, the second alkane monooxygenase in strain CF8 apparently contains a binuclear-iron cluster. A distinct difference between this system and that of the methanotrophs is that in strain CF8, both monooxygenases can be expressed simultaneously, whereas, in methanotrophs, expression is limited to one monooxygenase at a time (32, 43). Another difference is in the apparent protein composition and cellular location of the binuclear-iron monooxygenases. While sMMO is a soluble enzyme and the hydroxylase component contains three polypeptides (16), the hydroxylase component of the binuclear-iron monooxygenase from strain CF8 is expected to be membrane-associated and to contain only a single polypeptide based on the similarity of the deduced amino acid sequence to that of the alkane hydroxylase (13, 46). Recently, the presence of two alkane hydroxylases in long-chain alkane-utilizing *Acinetobacter* sp. strain M-1 was reported (45). In this case, two alkane hydroxylases are both integral-membrane, binuclear-iron enzymes with high sequence similarity to the sequence of alkane hydroxylase (*alkM*) of *Acinetobacter* sp. strain ADP1. The expression of two alkane hydroxylase-encoding genes is regulated by the chain length of alkanes: *alkMa* expression is induced by solid alkanes ($>C_{22}$), while *alkMb* expression is preferentially induced by liquid alkanes (C_{16} to C_{22}) (45).

Sequence analysis of the complete *alkB* in strain CF8 revealed putative AlkB and rubredoxin domains in one continuous ORF that potentially encodes a fusion protein. The presence of a monooxygenase as a fused polypeptide is not without precedent. The cytochrome P-450 fatty acid monooxygenase from *Bacillus megaterium* was shown to consist of hydroxylase and reductase components on a single polypeptide encoded by a single continuous ORF (31, 36). This enzyme can be cleaved by trypsin into the respective domains (30). Further biochemical characterization of the alkane hydroxylase in strain CF8 is required to elucidate the function of the fused polypeptide and

the possible involvement of the adjacent ferredoxin gene product.

The genetic organization of *alkB* in strain CF8 showed that genes encoding the enzymes in alkane metabolism are not clustered with *alkB*. This organization is unlike the *alk* genes in *P. oleovorans*, where the genes encoding alkane hydroxylase, two rubredoxins, an aldehyde dehydrogenase, an alcohol dehydrogenase, an acyl coenzyme A synthetase, and an outer membrane protein constitute a single operon (*alkBFGHJKL*) on the OCT plasmid (46). In the case of *alk* genes in *Acinetobacter* sp. strain ADP 1, the essential genes for alkane degradation are separately located on the chromosome where *alkM* and *alkR* are located about 369 kb from the *rubA* and *rubB* genes, encoding rubredoxin and rubredoxin reductase, respectively (20). The genetic organization of *alkB* in strain CF8 is rather similar to that of *M. tuberculosis*, in which the putative *alkB* is followed by the putative rubredoxin A and B, and no other genes encoding enzymes of alkane metabolism are nearby (12).

Our results suggest that the chain length of the alkane growth substrate plays a major role in the regulation of the expression of the binuclear iron monooxygenase in *Nocardioides* sp. strain CF8. In strain CF8, expression of the binuclear-iron monooxygenase was only observed in cells grown on alkanes C_6 and above. In contrast, the substrate range of the enzyme appeared to extend to the gaseous alkane, butane. This response is reminiscent of *alkB* regulation in *P. oleovorans*, where the alkane specificity of the transcriptional regulator is more restrictive than the range of alkanes oxidized by the monooxygenase. In the case of AlkB in *P. oleovorans*, expression of the alkane hydroxylase operon (*alkBFGHJKL*) is regulated by a LuxR-UhpA-like transcriptional regulator, AlkS (15). AlkS induces the expression of the *alkBFGHJKL* operon in the presence of alkanes that are used as growth substrates (21). In contrast to AlkB, AlkM, the alkane hydroxylase from *Acinetobacter* sp. strain ADP1, can be induced by a variety of alkanes, including non-growth-supporting alkanes (35). AlkR, an AraC-XylS-like transcriptional regulator, induces the expression of *alkM* in the presence of alkanes ranging from C_7 to C_{11}, which do not support growth of ADP1, as well as alkanes ranging from C_{12} to C_{18}, which are used as growth substrate (35). Approximately 150 bp upstream of the putative *alkB* sequence in strain CF8, there is an ORF that shows low similarity to TetR-like transcriptional regulators. This ORF may be a transcriptional regulator for *alkB* in strain CF8.

In conclusion, *Nocardioides* sp. strain CF8 possesses two distinct monooxygenases for alkane oxidation. To our knowledge, this is the first example of an alkane-utilizing bacterium that contains both copper- and binuclear-iron-containing monooxygenases specific for different chain length alkanes. The expression of the binuclear iron monooxygenase is influenced by the alkane chain length.

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