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Gene Cloning and Characterization of Multiple Alkane Hydroxylase Systems in *Rhodococcus* Strains Q15 and NRRL B-16531

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The alkane hydroxylase systems of two *Rhodococcus* strains (NRRL B-16531 and Q15, isolated from different geographical locations) were characterized. Both organisms contained at least four alkane monooxygenase gene homologs (*alkB1*, *alkB2*, *alkB3*, and *alkB4*). In both strains, the *alkB1* and *alkB2* homologs were part of *alk* gene clusters, each encoding two rubredoxins (*rubA1* and *rubA2*; *rubA3* and *rubA4*), a putative TetR transcriptional regulatory protein (*alkU1*; *alkU2*), and, in the *alkB1* cluster, a rubredoxin reductase (*rubB*). The *alkB3* and *alkB4* homologs were found as separate genes which were not part of *alk* gene clusters. Functional heterologous expression of some of the rhodococcal *alk* genes (*alkB2*, *rubA2*, and *rubA4* [NRRL B-16531]; *alkB2* and *rubB* [Q15]) was achieved in *Escherichia coli* and *Pseudomonas* expression systems. *Pseudomonas* recombinants containing rhodococcal *alkB2* were able to mineralize and grow on C_{12} to C_{16} *n*-alkanes. All rhodococcal alkane monooxygenases possessed the highly conserved eight-histidine motif, including two apparent alkane monooxygenase signature motifs (LQRH[S/A]DHH and NYXEHYG[L/M]), and the six hydrophobic membrane-spanning regions found in all alkane monooxygenases related to the *Pseudomonas putida* GPo1 alkane monooxygenase. The presence of multiple alkane hydroxylases in the two rhodococcal strains is reminiscent of other multiple-degradative-enzyme systems reported in *Rhodococcus*.

Although many microorganisms are capable of degrading aliphatic hydrocarbons and are readily isolated from contaminated and noncontaminated sites, relatively little is known about the genetic characteristics of their alkane-degradative systems. Indeed, until recently, only the alkane-degradative genes of a small number of gram-negative bacteria, namely, *Pseudomonas* and *Acinetobacter*, have been described in detail. Of these, the *alk* system found in *Pseudomonas putida* GPo1, which degrades C_5 to C_{12} *n*-alkanes, remains the most extensively characterized alkane hydroxylase system (44, 47). The initial terminal oxidation of the alkane substrate to a 1-alkanol is catalyzed by a three-component alkane hydroxylase complex consisting of a particulate nonheme integral-membrane alkane monooxygenase (AlkB) and two soluble proteins, rubredoxin (AlkG) and rubredoxin reductase (AlkT) (47). The *P. putida alk* genes are located in two different loci (*alkBFGHJKL* and *alkST*) on the OCT plasmid, separated by 10 kb of DNA (44). Five chromosomal genes (*alkM*, *rubA*, *rubB*, *alkR*, and *xcpR*) in at least three different loci are required for degradation of C_{12} to C_{18} alkanes in *Acinetobacter* sp. strain ADP1 (12, 30, 31). Similar to *P. putida* GPo1, the initial terminal alkane oxidation is also catalyzed by a three-component alkane hydroxylase system, which comprises an alkane monooxygenase (AlkM), rubredoxin (RubA), and rubredoxin reductase (RubB). More recently, *Acinetobacter* sp. strain M-1 was shown to possess two alkane monooxygenase genes (*alkMa* and *alkMb*), as well as single copies of *rubA* and *rubB*, located in three different loci (40).

Much less is known about the alkane-degradative systems of gram-positive bacteria. A putative alkane monooxygenase gene has been identified in the finished genome sequence of *Mycobacterium tuberculosis* H37Rv (6), while other *alkB* homologs were amplified from *Rhodococcus erythropolis* NRRL B-16531 and *Prauserella rugosa* NRRL B-2295 DNAs using highly degenerate primers (37). Using the same primers, a C_6 to C_8 alkane-inducible *alkB*-homolog was cloned from *Nocardioides* sp. strain CF8 (13). The *M. tuberculosis* and *P. rugosa alkB* homologs could be functionally expressed in an *alkB* knockout derivative of *Pseudomonas fluorescens* CHA0 and in *P. putida* GPo12(pGEc47ΔB) and were shown to oxidize alkanes ranging from C_{10} to C_{16} (36).

Rhodococcus and other closely related high-G+C, mycolic acid-containing actinomycetes, such as *Mycobacterium*, *Corynebacterium*, *Gordona*, and *Nocardia*, are increasingly recognized as ideal candidates for the biodegradation of hydrocarbons because of their ability to degrade a wide range of organic compounds (4), their hydrophobic cell surfaces, their production of biosurfactants, and their ubiquity and robustness in the environment (23, 48). Considerable interest is being devoted to using bacterial alkane oxidation systems as biocatalysts for the production of fine chemicals and pharmaceuticals (15, 18, 20, 23–25, 29).

In the present study, we describe the isolation and characterization of multiple alkane monooxygenase genes found in two rhodococci from different geographical locations, *Rhodococcus* sp. strain Q15 (51, 53), isolated from Lake Ontario sediment, and *R. erythropolis* strain NRRL B-16531 (ATCC 15960; formerly *Corynebacterium hydrocarboclastus* p-9) (17),

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics (genotype)	Reference or source
Strains		
P. fluorescens KOB2Δ1	*alkB1* deletion; C_{12}–C_{16} Alk^-; C_{18}–C_{28} Alk^+	36
R. erythropolis NRRL B-16531	Wild type; C_6–C_{36} Alk^+	15, 17, 46
Rhodococcus sp. strain Q15	Wild type; C_8–C_{32} Alk^+	51, 53
Rhodococcus sp. strain Q15 NP	Plasmid cured; C_8–C_{32} Alk^+	51
P. putida GPo12(pGEc47ΔB)	Alk^- (*alkB Bam*HI deletion); Tet^r	36
E. coli GEc137(pGEc47)	C_6–C_{12} Alk^+ (*P. putida alkBFGHJKL alkST*); Tet^r	10
E. coli GEc137(pGEc47ΔB)	Alk^- (*alkB Bam*HI deletion); Tet^r	45
E. coli GEc137(pGEc47ΔT)	Alk^- (*alkT Hpa*I-*Acc*I deletion); Tet^r	47
E. coli GEc137(pGEc47ΔG)	Alk^- (*alkG Hin*dIII deletion); Tet^r	43
Cloning and expression vectors		
pBluescript II KS(+/−)	Ap^r	Stratagene
pGEM7-Zf(+)	Cloning vector; Ap^r	Promega
pZErO-2.1	Cloning vector; Km^r; *ccdB*	Invitrogen
pKKPalk	Expression vector; *alkB* promoter; Ap^r	38
pCom8	Expression vector; *alkB* promoter; gentamicin^r; broad host range	38
Plasmids containing DNA from *Rhodococcus sp.* strain Q15		
pKS1	6,389-kb *Eco*RI-*Eco*RI Q15 NP $alkB1^+$ genomic DNA fragment cloned in pBluescript II KS(+/−) *Eco*RI-*Eco*RI	This study
pKS2	4.1-kb *Bgl*II-*Bgl*II Q15 NP $alkB2^+$ genomic DNA fragment cloned in pBluescript II KS(+/−) *Bam*HI-*Bam*HI	This study
pCom8Q15alkB1	1,179-bp *Eco*RI-*Sma*I fragment from pTQ15alkB1 cloned in pCom8/*Eco*RI/*Sma*I	This study
pCom8Q15alkB2	1,269-bp *Eco*RI-*Hin*dIII PCR fragment cloned in pCom8/*Eco*RI/*Hin*dIII	This study
pKKPalkQ15rubA1	203-bp *Eco*RI-*Bam*HI PCR fragment cloned in pKKPalk/*Eco*RI/*Bam*HI	This study
pKKPalkQ15rubA2	235-bp *Eco*RI-*Bam*HI PCR fragment cloned in pKKPalk/*Eco*RI/*Bam*HI	This study
pKKPalkQ15rubB	1,287-bp *Eco*RI-*Hin*dIII PCR fragment cloned in pKKPalk/*Eco*RI/*Hin*dIII	This study
Plasmids containing DNA from *R. erythropolis* NRRL B-16531		
p16531	pGEM7-Zf(+) with 550-bp NRRL B-16531 *alkB1* fragment	37
p23-D1	pGEM7-Zf(+) with 550-bp NRRL B-16531 *alkB2* fragment	46
p62-O	pGEM7-Zf(+) with 550-bp NRRL B-16531 *alkB3* fragment	46
p23-D2	pGEM7-Zf(+) with 550-bp NRRL B-16531 *alkB4* fragment	46
pAlkB1	3,040-bp *Bam*HI fragment cloned in pZErO2.1	This study
pRubB	4,560-bp *Pst*I fragment cloned in pZErO2.1	This study
pAlkB2	3,392-bp *Eco*RI fragment cloned in pZErO2.1	This study
pAlkB3	1,967-bp *Bam*HI fragment cloned in pZErO2.1	This study
palkB3 (Sau3)	1.2-kb *Sau*3A partial fragment cloned in pZErO2.1	This study
pAlkB4 (Sau1-8)	8 different *Sau*3A partial fragments in pZErO2.1	This study
pCom8-alkB1 (Rer)	pCom8 with *R. erythropolis* NRRL B-16531 *alkB1*	This study
pCom8-alkB2 (Rer)	pCom8 with *R. erythropolis* NRRL B-16531 *alkB2*	This study
pCom8-alkB3 (Rer)	pCom8 with *R. erythropolis* NRRL B-16531 *alkB3*	This study
pCom8-alkB4 (Rer)	pCom8 with *R. erythropolis* NRRL B-16531 *alkB4*	This study
pKKPalk-rubA1 (Rer)	pKKPalk with *R. erythropolis* NRRL B-16531 *rubA1*	43
pKKPalk-rubA2 (Rer)	pKKPalk with *R. erythropolis* NRRL B-16531 *rubA2*	43
pKKPalk-rubA3 (Rer)	pKKPalk with *R. erythropolis* NRRL B-16531 *rubA3*	43
pKKPalk-rubA4 (Rer)	pKKPalk with *R. erythropolis* NRRL B-16531 *rubA4*	43
pKKPalk-rubB (Rer)	pKKPalk with *R. erythropolis* NRRL B-16531 *rubB*	This study

isolated from petroleum-contaminated soil in Japan. *Rhodococcus* sp. strain Q15 degrades a broad range of aliphatics (C_8 to C_{32} *n*-alkanes, branched alkanes, and a substituted cyclohexane) at temperatures ranging from 0 to 30°C and oxidizes alkanes by both the terminal and the subterminal oxidation pathways (51). *R. erythropolis* NRRL B-16531 degrades C_6 to C_{36} *n*-alkanes (46) and was one of eight strains able to stereospecifically oxidize the alkyl side chain of cumene in a collection of 1,229 bacteria, yeasts, and fungi (15).

In both bacteria, four alkane hydroxylase gene homologs were found, two of which are parts of gene clusters containing rubredoxin and rubredoxin reductase genes. Functional heterologous expression of some of these genes was achieved. The *alkB* gene clusters of NRRL B-16531 and Q15 were initially cloned and characterized independently by a Swiss and a Canadian laboratory, respectively. Subsequent communication between the two groups revealed the similarity of their results, and consequently, the groups continued this research as a collaborative effort.

MATERIALS AND METHODS

Bacterial strains, plasmids, and general methods. The bacterial strains and plasmids used or constructed in this study are listed in Table 1. *Rhodococcus* strains Q15 and Q15 NP (plasmid cured) were grown on trypticase soy agar at 28°C and maintained at 4°C. *R. erythropolis* NRRL B-16531 was grown on Luria broth (LB) medium at 30°C and maintained at 4°C. Plasmid pGEc47 carries all

TABLE 2. Comparison of Q15 and NRRL B-16531 *alk* systems

Gene	No. of amino acid residues		% DNA identity[a]	% amino acid identity[b]	Best database match (% amino acid identity/no. of amino acids)[c]
	Q15	NRRL B-16531			
alkB1 cluster					
alkB1	391	391	97.4	99.7	Mt AlkB (62/396-aa overlap)
rubA1	56	56	94.7	98.2	Mt RubA (74/50)
rubA2	63	63	98.0	98.0	Mt RubB (83/56)
rubB	418	418	96.2	96.7	*Nocardia* ferredoxin reductase (36/410)
alkU1	205	208	96.0	98.5	Mt reg.? (38/192)
alkB2 cluster					
alkB2	408	408	99.3	99.3	Mt AlkB (65/415)
rubA3	61	61	98.9	100	Mt RubA (76/51)
rubA4	60	60	100	100	Mt RubB (84/59)
alkU2	218	162[d]	99.4	98.8	Mt reg.? (63/191)
alkB3	383	383	98	97	Mt AlkB (47/380)
alkB4	386	386	99.7	99.7	Mt AlkB (49/386)

[a] DNA sequence identity between Q15 and NRRL B-16531 *alk* genes.
[b] Amino acid sequence identity between Q15 and NRRL B-16531 Alk proteins.
[c] reg.?, hypothetical protein Rv3249c, a TetR family protein; Mt, *M. tuberculosis* H37Rv.
[d] Incomplete ORF.

of the genes necessary to convert *n*-alkanes into the corresponding fatty acids (10). *Escherichia coli* and *Pseudomonas* strains containing deletion derivatives of pGEc47 (pGEc47ΔB [45], pGEc47ΔG [43], and pGEc47ΔT [42]) cannot grow on *n*-octane unless the deleted gene, or an equivalent gene from another organism, is supplied in *trans* on additional plasmids. *P. fluorescens* KOB2Δ1 is an *alkB1* deletion derivative of *P. fluorescens* CHA0 which no longer grows on C_{12} to C_{16} *n*-alkanes. KOB2Δ1 can be complemented for growth on these alkanes by pCom8 derivatives containing *alkB* genes from other bacteria (36). Plasmid pCom8 is a broad-host-range vector based on pUCP25 and the *P. putida* GPo1 *alkBp* promoter (38). Plasmid pKKPalk is an *E. coli* expression vector with the same promoter (38).

For the NRRL B-16531 experiments, *E. coli* JM101 [*endA hsdR supR thi-1* Δ(*lac-proAB*) F′(*traD36 proAB lacI*^{q} *lacZM15*)] (55) and DH10B (Gibco BRL) were used for cloning and the production of plasmid DNA for sequencing. LB (32) and E2 medium (22) supplemented with carbon sources and/or antibiotics were used throughout. To culture NRRL B-16531 on *n*-octane, petri dishes with E2 medium were incubated in a sealed container together with an open Erlenmeyer flask containing *n*-octane. *n*-dodecane, *n*-tetradecane, and *n*-hexadecane were supplied by placing a Whatman 3MM filter disk with 200 μl of *n*-alkane in the lid of the petri dish. All cultures were grown aerobically at 30 or 37°C.

For the Q15 experiments, *E. coli* DH10B or JM110 (JM101*dam*, *dcm*) was used as a host for recombinant plasmids. The *E. coli* strains were routinely cultured in LB at 37°C. When necessary, the LB medium was supplemented with ampicillin (50 μg/ml). Plasmid and chromosomal DNA purifications, enzymatic digests, ligations, and *E. coli* transformations were performed using standard molecular techniques (3). PCRs were performed with *Taq* DNA polymerase (Amersham Pharmacia Biotech, Piscataway, N.J.) or *Pfu* DNA polymerase (Stratagene, La Jolla, Calif.) when PCR products were cloned. Nonradioactive DNA probe labeling and Southern and colony hybridizations (DIG System; Roche Molecular Biochemicals, Rotkreuz, Switzerland) were performed according to the manufacturer's instructions.

Cloning and sequence analysis of NRRL B-16531 and Q15 *alk* genes. Chromosomal DNA was isolated from NRRL B-16531 according to the method of Desomer et al. (8). To clone the four full-length NRRL B-16531 alkane hydroxylase genes, suitable restriction fragments in the range of 2 to 5 kb were selected by Southern blotting using probes obtained from PCR fragments cloned into plasmids p16531 (*alkB1*) (37), p62-O (*alkB2*), p23-D1 (*alkB3*), and p23-D2 (*alkB4*) (46). Chromosomal restriction fragments around the desired size were cut out from a preparative agarose gel, isolated by electroelution, ligated between the appropriate sites of pGEM7-Zf(+) (Promega, Madison, Wis.) or pZErO-2.1 (Invitrogen, Basel, Switzerland), and transformed into *E. coli* DH10B (Invitrogen) by electroporation (9). *E. coli* transformants were selected with ampicillin (200 μg/ml) or kanamycin (50 μg/ml). The transformants containing the desired genes were identified by colony blotting using the probes described above. The 16531-*alkB1* probe was used to clone a 2.75-kb *Bam*HI fragment. As this fragment included the start of a rubredoxin reductase gene, in addition to two rubredoxin genes and the complete *alkB1* gene, we also cloned an overlapping 3.9-kb *Pst*I fragment containing the complete rubredoxin reductase gene. In the same way, the 23-D1-*alkB2* and 62-O-*alkB3* probes were used to clone a 3.4-kb *Eco*RI and a 1.6-kb *Bam*HI fragment, respectively. The *Eco*RI fragment contained an *alkB* homolog and two rubredoxin genes, while the *Bam*HI fragment contained an incomplete *alkB* homolog, the N-terminal 50 amino acids of which were missing. An overlapping 1.2-kb *Sau*3A fragment yielded the missing part of *alkB3*. The 23-D2-*alkB4* probe was used to select several overlapping *Sau*3A clones. The resulting sequence contained the complete *alkB* homolog (*alkB4*). Plasmid DNA was isolated using the High Pure plasmid isolation kit (Roche Diagnostics). Both strands of the inserts were completely sequenced on a Li-Cor 4000L sequencer using IRD800-labeled −40 forward (AGGGTTTTC CCAGTCACGACGTT) and −40 reverse (GAGCGGATAACAATTTCACAC AGG) primers and the Amersham Thermosequenase cycle-sequencing kit (Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany).

For the Q15 experiments, the PCR primer Mt-alkB-F1 (AACACCGCCCAC GAAATGGGGC) and the reverse primer Mt-alkB-R1 (GGCGTGGTGATCG CTGTGTCGCTG), derived from the corresponding DNA sequences from the first and third highly conserved histidine motif boxes in *M. tuberculosis* H37Rv *alkB* (6), yielded a PCR fragment of the expected size (548 bp) from Q15. The fragment, designated *alkB1*, was cloned and sequenced as previously described (49). In order to clone the complete Q15 *alkB1* gene, Southern analysis was done on total DNA from the Q15 NP strain restricted with different enzymes and probed with the 548-bp digoxigenin-dUTP-labeled PCR fragment (Q15 *alkB1* probe). Appropriately sized Q15 *alkB1* probe-positive *Eco*RI fragments were gel purified and used to construct an enriched DNA library in pBluescript II KS(+/−) (Stratagene). *E. coli* DH10B clones were screened by colony hybridization using the Q15 *alkB1* probe. The recombinant plasmid, designated pKS1, was purified from an *alkB1*$^+$ clone, and the complete nucleotide sequence (6,389 bp) of the *Eco*RI insert was determined on both strands by primer walking with a T7 DNA-sequencing kit (Applied Biosystems, Foster City, Calif.). The primers Q15alkB1-2L (CAGCTGGAACAGTGATCGCATCTG; position 884 on *alkB1*) and Q15alkB1-5L (GACCTTCTCGCGGACGCCGCAGTC; position 1315 on *alkB1*) resulted in the amplification of an unexpected ~430-bp PCR fragment that was subsequently gel purified and sequenced; it was homologous, but not identical, to Q15 *alkB1*. The 430-bp PCR fragment, designated *alkB2*, was used as a probe to clone a 4,145-bp *Bgl*II fragment from Q15 NP genomic DNA as described above for Q15 *alkB1*. Genes homologous to NRRL B-16531 *alkB3* and *alkB4* in Q15 were detected by PCR analyses using primers from within the NRRL B-16531 *alkB3* sequence (Q15 alkB3-F2, GGTGTCGACGCTCCTGCA TGGC, and Q15 alkB3-R2, CGCCTTGGTGTGAATGAGCTCG) and from the NRRL B-16531 *alkB4* sequence (alkB4FWE, CGGAATTCACATGACGACC TTCGCGG, and alkB4RVH, GGTCGTACTAAAGCTTAGTCCGGC). A 1,282-nucleotide (nt) PCR amplification product for Q15 *alkB3* and a 1,217-nt

Q15 *alkB4* amplification product were purified, cloned, and sequenced. DNA sequencing was performed with the 373 automated fluorescence sequencer (Applied Biosystems).

Nucleotide and amino acid sequences were compared with the EMBL, SwissProt, and GenBank databases using BLASTN and BLASTX at the National Center for Biotechnology Information (1). DNA and protein sequences were further analyzed using GeneWorks II software (Intelligenetics, Mountain View, Calif.) and LASERGENE Navigator from DNASTAR (Madison, Wis.).

Functional expression of rhodococcal *alkB*, *rubA*, and *rubB* genes in *P. fluorescens* and *E. coli*. *R. erythropolis* NRRL B-16531 *alkB2*, *alkB3*, and *alkB4* were amplified using primers alkB2FWE (GGAGGAATTCCATGTCGACGCACG), alkB2RVH (GGCGCGAAGCTTCTTTCTGCGGC), alkB3FWE (GCTCGAGAATTCTCGATGACAG), alkB3RVH (GGTGAAGCTTGCATGAGTCGGG), alkB4FWE (CGGAATTCACATGACGACCTTCGCGG), and alkB4RVH (GGTCGTACTAAAGCTTAGTCCGGC), respectively. As an *Eco*RI site is located immediately upstream of the ATG start codon of the a*lkB1* gene, this gene was cloned as an *Eco*RI-*Bam*HI fragment from the *Bam*HI genomic clone. All genes were inserted into pCom8 (38), using the *Eco*RI and *Hin*dIII sites introduced by the primers (underlined in the sequences) in the case of *alkB2-4*. For Q15, *alkB1* and *alkB2* were cloned into pCom8 like the corresponding NRRL B-16531 genes and transformed into *E. coli.* The pCom8 derivatives (Table 1) were isolated and then transformed into *P. fluorescens* KOB2Δ1 according to the method of Højberg et al. (14). *E. coli* and *P. fluorescens* KOB2Δ1 recombinants harboring pCom8 derivatives were selected with 10 and 100 μg of gentamicin/ml, respectively. PCR amplification and cloning of the NRRL B-16531 *rubA1*, *rubA2*, *rubA3*, and *rubA4* genes in pKKPalk have been described elsewhere (43). Q15 *rubA1*, *rubA2*, and *rubB* and NRRL B-16531 *rubB* were also cloned in pKKPalk, and the recombinant plasmids were transformed into *E. coli* GEc137 containing pGEc47ΔG for *rubA* plasmids or pGEc47ΔT for *rubB* plasmids (42). *E. coli* transformants were selected for on LB supplemented with tetracycline (12.5 μg/ml) and carbenicillin (50 μg/ml) or ampicillin (200 μg/ml).

To measure in vivo alkane hydroxylase activity, the *alkB*, *rubA*, and *rubB* recombinants were assayed for the ability to mineralize ^{14}C-radiolabeled alkanes (C_{12}, C_{16}, or C_{28}) (51) in minimal salts medium (MSM) supplemented with 100 mg of unlabeled alkane/liter, 50 mg of yeast extract/liter, the indicated ^{14}C-labeled alkane substrate, and 0.01% rhamnolipid surfactant for the *Pseudomonas* strains or 0.1% Triton X-100 surfactant for the *E. coli* strains. The recombinants were also monitored for growth on various alkanes at 30°C. For *E. coli* strains, growth on M9 agar plates supplemented with 0.001% thiamine was monitored; *n*-alkanes (C_8, C_{10}, and C_{12}) were provided as vapor in a closed system as the sole C and energy source. The growth of *P. fluorescens* KOB2Δ1 recombinants on alkanes was monitored at 30°C and 200 rpm in 250-ml baffled Erlenmeyer flasks containing 50 ml of MSM (22) supplemented with 1% (vol/vol) *n*-alkanes (C_8, C_{10}, C_{12}, C_{14}, and C_{16}). For optical density measurements, culture liquid (1 ml) was spun down in an Eppendorf 5415 C microcentrifuge (15,000 rpm), and 0.5 ml of supernatant containing the alkane droplets was removed. After the addition of 0.5 ml of water, the cell pellet was resuspended and the optical density was measured at 450 nm.

Nucleotide sequence accession numbers. The sequences of the four *R. erythropolis* NRRL B-16531 alkane hydroxylase genes and flanking DNA have been submitted to GenBank and received the following accession numbers: *alkB1*, AJ009586; *alkB2*, AJ297269; *alkB3*, AJ301876; and *alkB4*, AJ301877. For *Rhodococcus* sp. strain Q15, the accession numbers are as follows: *alkB1*, AF388181; *alkB2*, AF388182; *alkB3*, AF388179; and *alkB4*, AF388180.

RESULTS

Identification of *Rhodococcus* strains NRRL B-16531 and Q15. Comparison of the 16S ribosomal DNA sequences from Q15 (EMBL accession no. AF046885) and NRRL B-16531 (EMBL accession no. AJ009591) revealed 99% DNA sequence identity on an 809-nt overlap, indicating that the two rhodococci are closely related but not identical. The NRRL B-16531 16S sequence was identical to that of the *R. erythropolis* type strain, ATCC 4277. Strain NRRL B-16531 possesses a plasmid similar in size to the larger of the two large plasmids (~90 and ~115 kb) found in Q15 (51) and also possesses a smaller (~3.5-kb) plasmid not found in Q15 (data not shown).

Cloning and sequence analyses of the NRRL B-16531 and Q15 *alk* genes. Comparison of the regions cloned from Q15 and NRRL B-16531 revealed that their *alk* genes, including the spacer regions, are almost identical, with 94.7 to 100% DNA sequence identity. The derived amino acid sequences have 97 to 100% amino acid sequence identity and generally the same length (Table 2). Therefore, sequence comparisons for the *alk* genes and encoded proteins in the two rhodococcal strains are described together.

Sequence analysis of the DNA and deduced amino acid sequences of both the NRRL B-16531 and Q15 *alkB1* gene regions revealed five consecutive open reading frames (ORFs) (Fig. 1) whose products possessed the greatest amino acid sequence identities with the complete sequences of alkane hydroxylase components and a putative regulatory protein. We have designated the five genes *alkB1* (alkane monooxygenase), *rubA1* (rubredoxin), *rubA2* (rubredoxin), *rubB* (rubredoxin reductase), and *alkU1* (putative TetR-related regulatory protein). The *alkB2* gene regions in both strains contained four ORFs (Fig. 1) designated *alkB2* (alkane monooxygenase), *rubA3* (rubredoxin), *rubA4* (rubredoxin), and *alkU2* (putative TetR-related regulatory protein). The NRRL B-16531 *alkB3* and *alkB4* gene regions both contained an ORF encoding an alkane monooxygenase homolog. These genes were not flanked by rubredoxin or rubredoxin reductase genes. The flanking regions of Q15 *alkB3* and *alkB4* were not investigated.

The four rhodococcal *alkB* genes encoded proteins similar in size and with the greatest amino acid sequence identities (47 to 65%) to the alkane monooxygenases of *M. tuberculosis* and *P. rugosa* and, to a lesser degree (41 to 50%), to other alkane monooxygenases. In addition to the high full-length homology, the four rhodococcal AlkB proteins possessed eight histidines which are highly conserved in nonheme iron integral membrane alkane hydroxylases and desaturases and which are believed to be required for catalytic activity by these enzymes (33, 34, 37). Sequences corresponding to the three histidine boxes (Hist1, HE[L/M]XHK; Hist2, EHXXGHH; and Hist3, LQRH[S/A]DHHA) are highly conserved in all bacterial alkane monooxygenases. The Hist3 box is the longest almost perfectly conserved stretch in all alkane hydroxylases but is not well conserved in other closely related hydrocarbon monooxygenases. An additional well-conserved histidine box (NYXEHYG[L/M], designated the HYG motif [Fig. 2]) is located about 30 amino acids [aa] upstream of the Hist3 box (37). This HYG motif is also quite well, but not perfectly, conserved in related hydrocarbon monooxygenases, such as three xylene monooxygenases (XylM), a nitrotoluene monooxygenase (NtnMa), and two cymene monooxygenases (CymAa) (NYXQHYG[L/Q]). Therefore, the Hist3 motif and the HYG motif can be used as apparent signature motifs specific for bacterial alkane monooxygenases. The positions and lengths of the six transmembrane helices initially reported in *P. putida* GPo1 AlkB (45) were also conserved in all rhodococcal AlkBs and all other alkane hydroxylases. However, there was relatively little amino acid homology within these hydrophobic stretches (Fig. 2, bar graph).

The four rubredoxin genes found immediately downstream of the *alkB1* and *alkB2* genes encoded proteins with the greatest amino acid sequence identities to RubA and RubB of the alkane hydroxylase system in *M. tuberculosis* and to other bacterial rubredoxins involved in alkane oxidation (43). All four

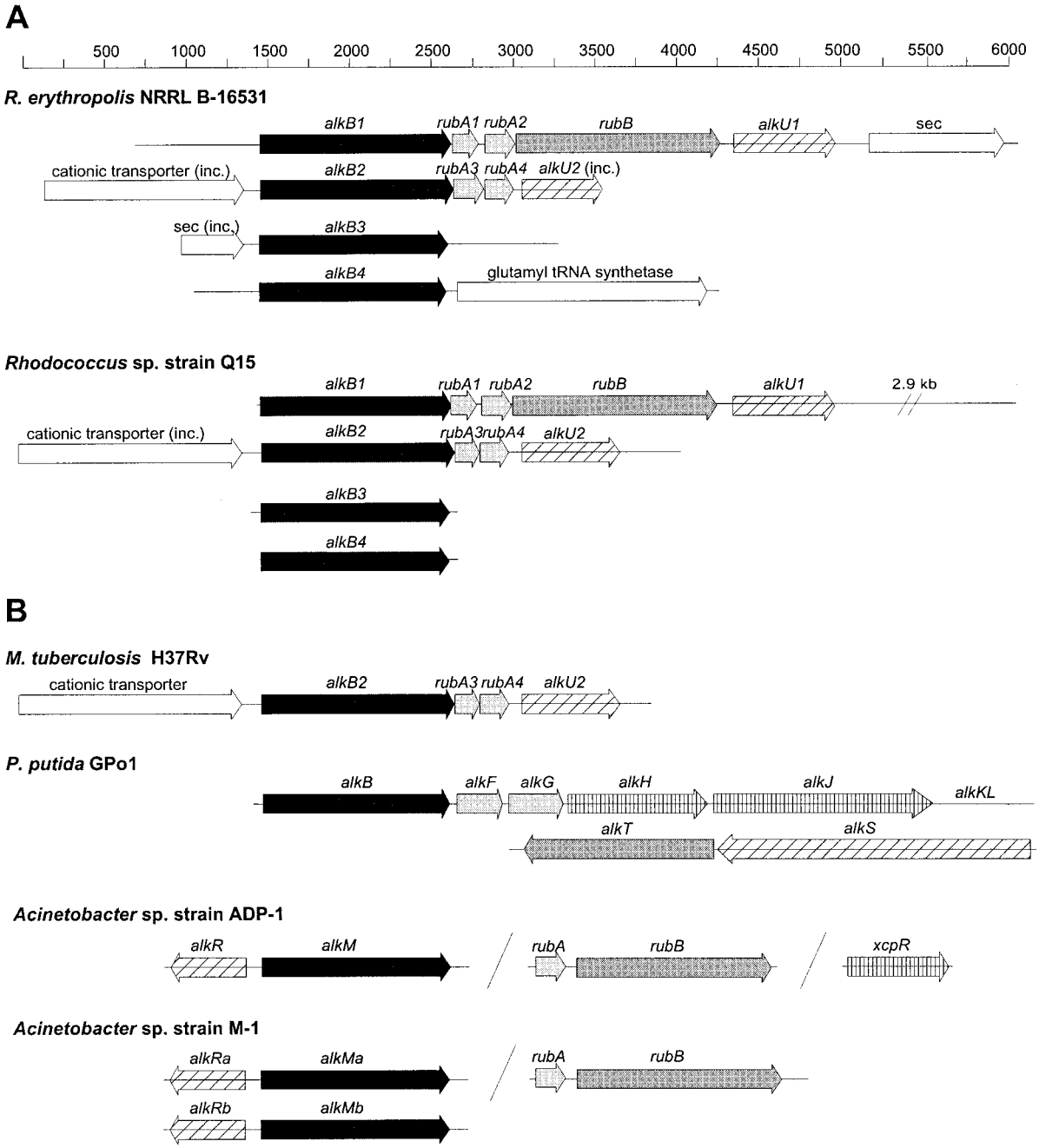

FIG. 1. (A) Cloned *alk* gene fragments from *Rhodococcus* strains NRRL B-16531 and Q15. (B) For comparison, the gene organizations of other bacterial alkane-degradative systems are shown (taken from references 36 and 40). Similar shading patterns of the arrows represent similar functions; solid, alkane monoxygenases; light shading, rubredoxins; dark shading, rubredoxin reductases; hatched, transcriptional regulatory proteins; vertically striped, other *alk* genes. The open arrows indicate genes that are not part of *alk* gene clusters. The directions of the arrows indicate the directions of transcription. inc., incomplete ORF. The 2.9-kb sequence downstream of *alkU1* in Q15 does not encode proteins with detectable sequence identity to any sequence in GenBank.

rubredoxins contained the PS00202 rubredoxin signature (PROSITE database) and were single-domain proteins, like the *Acinetobacter* sp. strain ADP1 rubredoxin (11). In a phylogenetic analysis, RubA2 and RubA4 of NRRL B-16531 and Q15 were more closely related to the ADP1 rubredoxin and the C-terminal domain of GPo1 AlkG than to rhodococcal RubA1 and RubA3. The last two proteins are more closely related to the N-terminal domains of *P. putida* GPo1 AlkG and AlkF (AlkG1 and AlkF1), which are not able to transfer electrons from the GPo1 rubredoxin reductase to the GPo1 alkane hydroxylase (21, 43). In both the NRRL B-16531 and Q15 strains, the *alkB1* and *rubA1* genes and the *rubA2* and *rubB* genes had 3′-end–5′-end ATGA and TGATG sequence overlaps, respectively. The *rubA1* and *rubA2* genes did not overlap.

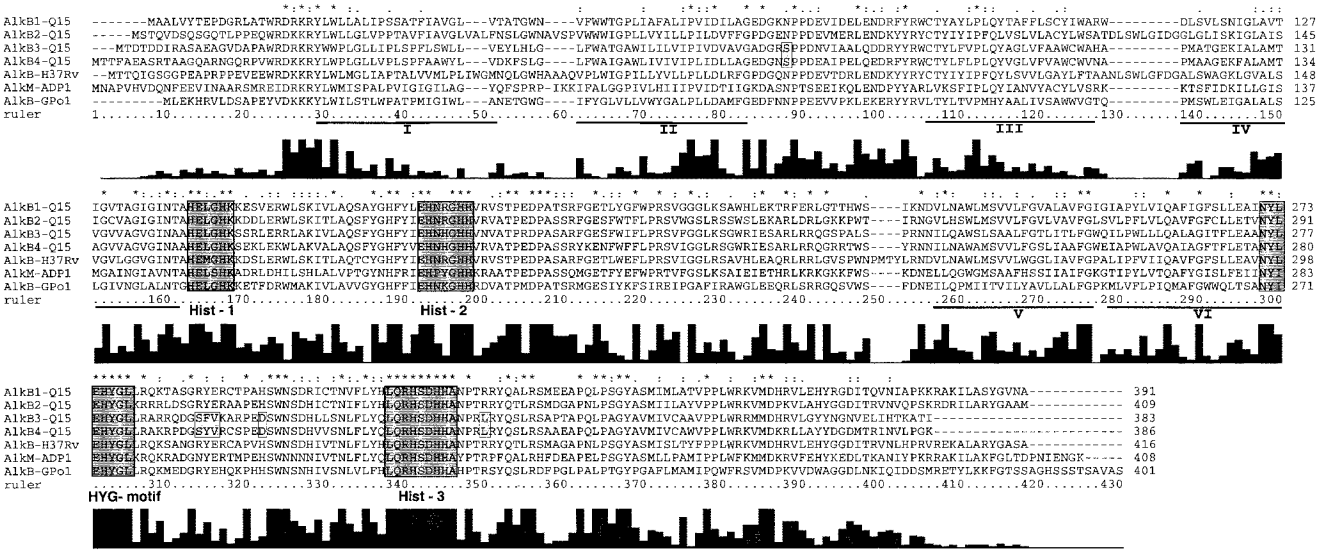

FIG. 2. Manual alignment of predicted amino acid sequences corresponding to alkane monooxygenase genes from various bacteria. The three conserved histidine boxes of the eight-histidine motif (34) (Hist-1, Hist-2, and Hist-3) and the additional histidine motif NYXEHYGL (HYG-motif) are boxed and darkly shaded. The locations of the six putative transmembrane helices in the *P. putida* GPo1 AlkB sequence are underlined and marked by roman numerals. Amino acid residues conserved in all alkane monooxygenases are marked by asterisks above the alignment. The degree of conservation of each position is indicated by the bar graph below the alignment, created by Clustal X. Six positions in AlkB3-Q15 and AlkB4-Q15 that deviate from all other alkane monooxygenase are boxed and lightly shaded. AlkB-H37Rv, alkane hydroxylase from *M. tuberculosis* H37Rv (accession number CAB08323.1); Alkm-ADP-1, *Acinetobacter* sp. strain ADP1 AlkM (AJ002316); AlkB-GPo1, *P. putida* GPo1 AlkB (AJ245436).

For the *alkB2* gene cluster, the NRRL B-16531 and Q15 *alkB2* and *rubA3* genes contained 3′-end–5′-end ATGA and TGATG sequence overlaps, respectively. Both the NRRL B-16531 and Q15 *rubA3* and *rubA4* genes contained 3′-end–5′-end ATGA sequence overlaps.

The next large ORF in the *alkB1* cluster, *rubB*, encoded a large protein exhibiting significant full-length sequence identity to a variety of bacterial reductase subunits of hydroxylase systems, including the *P. putida* and *Acinetobacter* alkane hydroxylase systems (Table 2), dioxygenase systems, and cytochrome P-450 systems involved in hydrocarbon degradation. The greatest amino acid homologies were found with a *Nocardia* sp. ferredoxin reductase (39% amino acid identity; 408-aa overlap) (EMBL accession no. AB017795; phenanthrene degradation), *R. erythropolis* ThcD (38% amino acid identity; 395-aa overlap) (EMBL accession no. P43494; thiocarbamate degradation), and several hypothetical ferredoxin reductases found in *M. tuberculosis.*

The ORFs designated *alkU1* and *alkU2*, which immediately follow the *alkB1* and *alkB2* gene clusters, respectively, encode proteins which have the greatest amino acid sequence identities to a hypothetical transcription regulatory protein (accession number CAB08350.1) (38 and 63% amino acid identity, respectively) that is located immediately downstream of the *alkB-rubA-rubB* gene cluster in *M. tuberculosis* H37Rv. Related peptides are also encoded immediately adjacent to the *P. rugosa* alkane hydroxylase gene (36) and the *Nocardioides* sp. strain CF8 *alkB* homolog (13) (up- and downstream, respectively). AlkU1 and AlkU2 possessed helix-turn-helix DNA binding motifs near the N terminus that are also present in known transcriptional regulatory proteins of the TetR/ArcR family (PFAM 00440; TetR; bacterial regulatory proteins).

The remaining parts of the Q15 and NRRL B-16531 *alkB1*, *-2*, *-3*, and *-4* fragments did not encode proteins with similarity to known alkane alcohol dehydrogenase or aldehyde dehydrogenase genes. The ORFs downstream of NRRL B-16531 *alkB1* and upstream of NRRL B-16531 *alkB3* show weak sequence identity to (putative) exported proteins (Fig. 1). Interestingly, the corresponding DNA regions almost immediately downstream from the Q15 and NRRL B-16531 *alkB1* gene clusters show relatively little DNA homology (data not shown) and have markedly different G+C contents: 60.2% in NRRL B-16531 versus 53.3% in Q15. The Q15 and NRRL B-16531 *alkB2* and *M. tuberculosis alkB* gene clusters have the same gene organization and close to 70% DNA sequence identity over their entire lengths, including the putative transporter and *alkU2* genes, indicating that these clusters have a common origin. The level of sequence identity is similar to the level of DNA sequence identity between *alkB1* and *alkB2* and between *alkB3* and *alkB4* but clearly higher than the DNA sequence identity between *alkB1* or *-2* and *alkB3* or *-4* (63%).

Heterologous expression of the rhodococcal *alk* genes in *E. coli* and *P. fluorescens*. Efficient expression vectors and recombinant *E. coli* and *Pseudomonas* hosts that allowed the functional expression of the *M. tuberculosis* and *P. rugosa* AlkB homologs (36) and rubredoxins and rubredoxin reductases from several microorganisms were used to heterologously express the rhodococcal *alk* genes (Tables 3 and 4). The four NRRL B-16531 *alkB* genes and Q15 *alkB1* and *alkB2* were cloned in pCom8 (38). Only NRRL B-16531 and Q15 *alkB2* allowed an *alkB* knockout mutant of *P. fluorescens* CHA0, KOB2Δ1, to grow on C_{12} to C_{16} *n*-alkanes (Table 3) and mineralize ^{14}C-radiolabeled *n*-dodecane and *n*-hexadecane (Table 4). Mineralization of ^{14}C-radiolabeled *n*-octacosane was observed in both the *alkB2* clones and the *alkB* knockout mutant of *P. fluorescens* CHA0, due to an additional long-chain

TABLE 3. Heterologous expression of the rhodococcal *alk* genes in *E. coli* and *P. fluorescens* as measured by growth on *n*-alkanes

Strain[a]	Growth on alkane[b]					
	C_8	C_{10}	C_{12}	C_{14}	C_{16}	C_{18}
P. fluorescens KOB2Δ1						
pCom8	−	−	−	−	+/−	++++
pCom8 + NRRL B-16531 *alkB1*	−	−	−	−	+/−	++++
pCom8 + NRRL B-16531 *alkB2*	−	+	++	+++	+++	++++
pCom8 + NRRL B-16531 *alkB3*	−	−	−	−	+/−	++++
pCom8 + NRRL B-16531 *alkB4*	−	−	−	−	+/−	++++
pCom8 + Q15 *alkB1*	−	−	−	−	+/−	++++
pCom8 + Q15 *alkB2*	−	+	++	+++	+++	++++
E. coli GEc137						
pGEc47: *alk*$^+$	+++	+++	+/−			
ΔT	−	−	−			
ΔT; pKKPalk Q15 *rubB*	++	+++	+/−			
ΔT; pKKPalk NRRL B-16531 *rubB*	−	−	−			
ΔG	−	−	−			
ΔG; pKKPalk NRRL B-16531 *rubA1*	−	−	−			
ΔG; pKKPalk NRRL B-16531 *rubA2*	++	++	−			
ΔG; pKKPalk NRRL B-16531 *rubA3*	−	−	−			
ΔG; pKKPalk NRRL B-16531 *rubA4*	++	++	−			
ΔG; pKKPalk Q15 *rubA1*	−	−	−			
ΔG; pKKPalk Q15 *rubA2*	−	+/−	−			
MSM control	−	−	−			

[a] ΔG, pGEc47ΔG; ΔT, pGEc47ΔT.
[b] −, (no growth); +/− (very slight growth) to ++++ (heavy growth) as determined by an increase in turbidity in liquid medium or growth on solid medium relative to the corresponding controls (not inoculated or not containing an alkane).

alkane hydroxylase system in this strain (36). None of the NRRL B-16531 *alkB* genes allowed *E. coli* GEc137 or *P. putida* GPo12 containing an *alkB* deletion derivative of pGEc47 to grow on C_6 to C_{12} alkanes. Q15 *alkB1* also could not be functionally expressed in the *E. coli* host.

We tested whether the rhodococcal rubredoxin and rubredoxin reductase genes complement deletions of the corresponding GPo1 proteins (AlkG and AlkT, respectively) in *E. coli*, using pKKPalk (38). Here, we found that NRRL B-16531 *rubA2* and *rubA4*, but not the *rubA1* and *rubA3* genes, complemented an *E. coli* recombinant containing pGEc47ΔG for growth on *n*-octane vapor (43). We were unable to obtain functional expression of the Q15 rubredoxin genes. The Q15 rubredoxin reductase gene (*rubB*), but not the NRRL B-16531 *rubB*, complemented an *alkT* deletion, as determined by growth on *n*-octane, *n*-decane, and *n*-dodecane (Table 3) and mineralization of *n*-dodecane (Table 4). Much greater levels of mineralization were observed when the media were supplemented with the appropriate surfactant (rhamnolipid or Triton X-100 for the *Pseudomonas* system; Triton X-100 for the *E. coli* system). Mineralization in the appropriate controls was minimal.

TABLE 4. Heterologous expression of the rhodococcal *alk* genes in *E. coli* and *P. fluorescens* as measured by mineralization of various ^{14}C-labeled *n*-alkanes

Strain[a]	Mineralization of alkane[b]		
	C_{12}	C_{16}	C_{28}
P. fluorescens KOB2Δ1			
pCom8	1.2	1.9	18.5
pCom8 + Q15 *alkB1*	2	2	13.8
pCom8 + Q15 *alkB2*	16	32	16.2
pCom8 + NRRL B-16531 *alkB2*	17	25	12
E. coli GEc137			
pGEc47: Alk$^+$	39		
ΔT	<1		
ΔT + pKKPalk Q15 *rubB*	27		
ΔG	<1		
ΔG + pKKPalk NRRL B-16531 *rubA2*	13		
MSM control (not inoculated)	<1	<1	<1

[a] ΔG, pGEc47ΔG; ΔT, pGEc47ΔT.
[b] Values represent average percentages of ^{14}C recovered as CO_2 from duplicate samples after 2 weeks of incubation at 28°C. The values for the control represent the background radioactivity.

DISCUSSION

Multiple alkane monooxygenases and rubredoxins and a rubredoxin reductase in two similar rhodococcal strains isolated in Japan and Canada were cloned and characterized. This is the first report of a detailed genetic characterization of alkane hydroxylase systems in *Rhodococcus*, a genus considered to be an important component of hydrocarbon-containing microbial communities present in contaminated soils and sediments (39, 48). The presence of multiple alkane hydroxylases in *Rhodococcus* strains NRRL B-16531 and Q15 may be a common feature of *Rhodococcus* strains, as three to five *alkB* homologs were also found in eight other *Rhodococcus* strains (46). It is also reminiscent of the two separate *alkM* genes found in *Acinetobacter* sp. strain M-1 (40) and of other multiple degradative enzyme systems reported in *Rhodococcus* responsible for polychlorobiphenyl degradation (2) and indene bio-

conversion (41). In yeasts, multiple cytochrome P450 alkane hydroxylases with overlapping substrate ranges have been reported as well (16, 54).

Heterologous expression of some of the rhodococcal *alk* genes (NRRL B-16531 *alkB2*, *rubA2*, and *rubA4* and Q15 *alkB2* and *rubB*) was achieved in *E. coli* and *Pseudomonas* expression systems based on the *P. putida* GPo1 *alkB* promoter as shown by mineralization and growth assays, confirming their respective functions in *n*-alkane degradation. The mineralization and growth assays of *Pseudomonas* clones containing the *alkB2* gene indicate that AlkB2 is at least partly responsible for the initial oxidation of C_{12} to C_{16} *n*-alkanes by the two rhodococcal strains, while RubA2, RubA4, and RubB are able to function as electron transfer components.

We were unable to show functional heterologous expression of rhodococcal AlkB1, AlkB3, and AlkB4 in the *Pseudomonas* or *E. coli* expression system. Several explanations can be put forward. (i) Functional expression in *E. coli* or *Pseudomonas* requires proper synthesis, correct folding, and proper assembly, which is not always ensured for rhodococcal and other heterologous proteins (5, 7, 19, 26–28). For example, AlkM, the *Acinetobacter* sp. strain ADP-1 alkane monooxygenase (30), could also not be functionally expressed with the same *Pseudomonas* or *E. coli* expression system (36). (ii) The three AlkB proteins may have substrate ranges that lie outside the range that can be tested in our recombinant host strains (between C_6 and C_{12} for *E. coli* and *P. putida* or between C_{12} and C_{16} for *P. fluorescens*). Here, it should be noted that AlkB3 and AlkB4 homologs occur more frequently in gram-positive strains able to grow on very long chain alkanes (over C_{20}) (46). (iii) AlkB1, AlkB3, and AlkB4 may not accept electrons from the rubredoxins in the host strains. However, this is unlikely, at least in the case of AlkB1, as this protein is encoded in an operon-like arrangement with a rubredoxin (RubA2) that could replace its *P. putida* GPo1 counterpart (43). (iv) The AlkB proteins could produce secondary alcohols by subterminal alkane oxidation. However, *P. fluorescens* KOB2Δ1 is able to grow well on secondary alcohols and ketones (data not shown). Therefore, complementation should be possible if AlkB1, AlkB3, and AlkB4 produce secondary alcohols from C_{12} to C_{16} alkanes. (v) It is possible that the three AlkB homologs are not alkane monooxygenases. However, all three have >50% full-length protein sequence identity to functional alkane monooxygenases from gram-positive bacteria even if several residues which are well conserved in all other alkane monooxygenase sequences are not conserved in AlkB3 and AlkB4 (Fig. 2). The three homologs also have >45% sequence identity to 13 functional alkane monooxygenases from gram-negative bacteria; the alkane monooxygenase sequences from gram-positive bacteria constitute just one branch of a more deeply rooted alkane monooxygenase tree (46). The closest relatives of the AlkBs, with a different but still closely related function, are the xylene and cymene monooxygenases, with only 25 to 30% sequence identity to the alkane monooxygenases (or homologs) described in this paper, followed by the desaturases with <20% sequence identity.

In conclusion, the first two explanations, that the three AlkB homologs are alkane monooxygenases that cannot be expressed in *E. coli* and *Pseudomonas* or have substrate ranges that lie outside the range that can be tested with these hosts, are the most likely and should be explored in more detail, e.g., by developing other expression hosts for rhodococcal proteins, such as *Streptomyces lividans* (35) or, ideally, alkane-negative *Rhodococcus* strains or mutants.

The genetic organization of the *alk* genes of various bacterial alkane hydroxylase systems is summarized in Fig. 1. The head-to-tail organization of the rhodococcal *alkB1* and *alkB2* gene clusters suggests that they may be transcribed as an operon. Moreover, several ORFs in these gene clusters have overlapping stop and start codons. This phenomenon is indicative of translational coupling and is thought to ensure the production of stoichiometric amounts of the involved proteins. Translational coupling has been observed in several rhodococcal operon-like structures from aromatic degradation pathways (23). The rhodococcal *alkB1* gene clusters are the only bacterial alkane hydroxylase gene clusters identified to date that encode all three components of an alkane hydroxylase system in a single operon-like structure. The *alkB1* and *alkB2* gene organization is also reminiscent of the *P. putida* GPo1 *alkBFGHJKL* operon (47). This operon also encodes two rubredoxins, AlkF and AlkG (21). As in GPo1, only the second rubredoxins of NRRL B-16531 (RubA2 and RubA4) but not the first rubredoxins (RubA1 and RubA3) in each gene cluster are functional electron transfer components. In addition, RubA2 and RubA4 possess relatively greater amino acid sequence identity to rubredoxins known to be required for alkane utilization than RubA1 and RubA3. The functions of RubA1 and RubA3, and of other closely related rubredoxins in gram-negative and gram-positive alkane-degrading strains, remain unknown (43).

The *alkB3* and *alkB4* genes are not accompanied by rubredoxin or rubredoxin reductase genes. Probably, the rubredoxins and rubredoxin reductase encoded in the *alkB1* and *alkB2* gene clusters also serve as electron transfer components for AlkB3 and AlkB4. In this respect, the rhodococcal *alk* gene organization would be similar to that reported for *Acinetobacter* sp. strain M-1, where a single constitutively expressed rubredoxin and a rubredoxin reductase serve as electron transfer proteins for two differentially regulated alkane hydroxylases (40). For the rhodococcal *alk* genes, this implies that the *alkB1* gene cluster may have to be expressed constitutively. The two putative TetR-type transcription regulation genes, found in the cloned *alkB1* and *alkB2* gene regions, and similar genes found adjacent to *alk* genes in other similar actinomycetes do not resemble previously identified *alk* gene regulatory proteins and thus may constitute a new class of regulatory proteins involved in alkane degradation by these bacteria. Neither of the *alkB1* or *alkB2* gene clusters contains alcohol dehydrogenase or aldehyde dehydrogenase genes, unlike the GPo1 *alk* system but similar to the situation in most other alkane-degrading bacteria.

Due to the low DNA sequence identity of the four rhodococcal *alkB* genes to the alkane monooxygenase genes of most of the known gram-negative bacteria, DNA probes based on the rhodococcal genes may be used for detecting and monitoring similar alkane-degradative rhodococci and other closely related high-G+C, mycolic acid-containing actinomycetes in hydrocarbon-contaminated sites. Screening by PCR and colony hybridization has already provided evidence that DNAs with high sequence identity to rhodococcal *alkB1* and *alkB2* exist in

a variety of hydrocarbon-contaminated soils (52), as well as in previously isolated psychrotrophic alkane-degradative actinomycete strains (50) (data not shown). This indicates that these genotypes are widespread in nature and may be important components in hydrocarbon degradation at contaminated sites. However, the high-G+C, mycolic acid-containing actinomycetes contain several additional, highly divergent *alkB* genes that cannot be detected using probes based on the NRRL B-16531 and Q15 *alkB* genes (46). Therefore, a combination of hybridization experiments and PCR with highly degenerate primers based on the third histidine box and the highly conserved HYG box is likely to give a more comprehensive overview of the occurrence of *alkB* homologs in nature.

In summary, four alkane monooxygenase homologs (two part of alkane gene clusters and two occurring as separate genes) were identified in two closely related *Rhodococcus* spp. based on (i) the significant full-length amino acid sequence identity of their components with other genetically characterized alkane hydroxylase systems; (ii) the conservation in the *Rhodococcus* alkane monooxygenases of the eight-histidine motif, including the apparent alkane monooxygenase signature motifs, and hydrophobic membrane-spanning regions found in all known alkane monooxygenases; and (iii) functional heterologous expression of some of these genes in *E. coli* and *Pseudomonas alk* expression systems. The most likely explanation for the presence of four alkane monooxygenases in one strain (assuming that all four oxidize alkanes) is that each alkane monooxygenase is specific for a certain range of alkanes. For example, *P. putida* GPo1 AlkB does not act on alkanes longer than C_{12}, while the *M. tuberculosis* and *P. rugosa* AlkBs do not efficiently oxidize alkanes shorter than C_{12} (36). As the *Rhodococcus* strains studied here oxidize alkanes up to C_{32} to C_{36}, AlkB1, AlkB3, and AlkB4 may each cover a part of the C_{18} to C_{36} range. Unfortunately, it is not yet possible to link sequence features, for example, specific amino acid residues or sequences within the hydrophobic transmembrane regions, with an alkane oxidation range. To make this possible, further studies will focus on the specific role of each of the rhodococcal *alk* genes in the degradation of alkane or other compounds. The observation that other rhodococci also possess multiple, but not always the same, alkane monooxygenase homologs (46) may help to answer this and other questions related to horizontal gene transfer and the evolution of alkane monooxygenase genes in actinomycetes.

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