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 monoxygenase genes (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 the rhodococcal alkB2 were able to mineralize and grow on C12 to C16 n-alkanes. All rhodococcal 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 the rhodococcal alkB2 were able to mineralize and grow on C12 to C16 n-alkanes.

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 GP01, which degrades C5 to C12 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 monoxygenase (alkB) and two soluble proteins, rubredoxin (alkG) and rubredoxin reductase (alkT) (47). The P. putida alk genes are located in two different loci (alkBFGHIKL and alkST) on the OCT plasmid, separated by 10 kb of DNA (44). Five chromosomal genes (alkM, rubA4, rubB, alkR, and xcrP) in at least three different loci are required for degradation of C12 to C18 alkanes in Acinetobacter sp. strain ADP1 (12, 30, 31). Similar to P. putida GP01, the initial terminal alkane oxidation is also catalyzed by a three-component alkane hydroxylase system, which comprises an alkane monoxygenase (alkM), rubredoxin (RubA), and rubredoxin reductase (RubB). More recently, Acinetobacter sp. strain M-1 was shown to possess two alkane monoxygenase 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 monoxygenase 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 Pseudomonas CHA0 and in P. putida GP012 (pGEC472B) and were shown to oxidize alkanes ranging from C10 to C16 (36).

Rhodococcus and other closely related high-G+C, mycolic acid-containing actinomycetes, such as Mycobacterium, Corynebacterium, Gordonia, 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 monoxygenase 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),
isolated from petroleum-contaminated soil in Japan. *Rhodococcus* sp. strain Q15 deg... 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_{18} n-alkanes 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 in this study are listed in Table 1. *Rhodococcus* strains Q15 and Q15 NP (plasmid cured) were grown on tryptase 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

<table>
<thead>
<tr>
<th>Gene</th>
<th>No. of amino acid residues</th>
<th>% DNA identitya</th>
<th>% amino acid identityb</th>
<th>Best database match</th>
</tr>
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<tr>
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</tr>
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<tr>
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<tr>
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<td>96.7</td>
<td>Nocardia ferredoxin reductase (36/410)</td>
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<tr>
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<td>M. reg.7 (38/192)</td>
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<td>100</td>
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<td>386</td>
<td>99.7</td>
<td>99.7</td>
<td>Mt AlkB (49/386)</td>
</tr>
</tbody>
</table>

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 pGEc7 (pGEc47AB [45], pGEc47AG [43], and pGEc47AT [42]) cannot grow on n-octane unless the deletant gene, or an equivalent gene from another organism, is supplied in trans on additional plasmids. *P. fluorescens* KO2B1Δ1 is an alkB1 deletion derivative of *P. fluorescens* CHA0 which no longer grows on C16 to C18 n-alkanes. KO2B1Δ1 can be complemented for growth on these alkanes by pCom8 derivatives containing alkB8 genes from other bacteria (36). Plasmid pCom8 is a broad-host-range vector based on pUCP25 and the *P. putida* GPO1 alkB 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 hadR supR thi-1 Δ(lac-proAB) F' [traD36 proAB lacIq lacZΔM15] (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 (JM101Δdam, 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 digestions, ligations, and *E. coli* transformations were performed using standard molecular techniques (3). PCR amplifications were performed using Taq DNA polymerase (Amersham Pharmacia Biotech, Piscataway, N.J.) or DNA polymerase (Amer sham Pharmacia Europe GmbH, Freiburg, Germany). Plasmid DNA was isolated using the High Pure plasmid isolation kit (Roche Diagnostics). Both strains were completely sequenced on a Li-Cor 4000L sequencer using IRD800-labeled PCR fragment (Q15 alkB1 probe).

Appropriately sized Q15 alkB1 probe-positive EcoRI fragments were gel purified and used to construct an enriched DNA library in pBluescript II KS(−/−) (Strategene). *E. coli* DH10B clones were sequenced by colony hybridization using the Q15 alkB1 probe. The recombinant plasmid, designated pKSL, was purified from an alkB1′ clone, and the complete nucleotide sequence (6,389 bp) of the EcoRI insert was determined both on strands by primer walking with a T7 DNA-sequencing kit (Applied Biosystems, Foster City, Calif.). The primers Q15alkB1-2L (CAGCTGGAACAGTGATCGCATCTG; position 884 on Q15) and Q15alkB1-5L (GACCTTCTCGCGACGGCCGAGTCG; position 1315 on alkB1) were used in an 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 4,435-bp BglII 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 amplifications using primers from within the NRRL B-16531 alkB3 sequence (alkB3F2, GGTGTTGACGCCTCCCTGCCA TGCC, and Q15 alkB3-R2, CGCGTGGTGGATAGAGCTCG) and from the NRRL B-16531 alkB4 sequence (alkB4FW, CGAGATTCACAGACGACC TCCGCG, and alkB4RVH, GTCGTGACTAAAGCTAGTCCGCG). 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, \( \text{rubA}, \text{rubB} \), and \( \text{rubC} \) genes in *P. fluorescence* and *E. coli*. C. *erythropsis* NRRL B-16531 alkB2, alkB3, and alkB4 were amplified and primers alkB2FWE (GGAGGAATTCCATGTCGACGCACG), alkB3RVEH (GGTGCGAAGCTTGCTAGCAAG), and alkB4RVEH (GGTGCATGAAATGCTGATCCG), respectively. As an EcoRI site is located immediately upstream of the ATG start codon of the \( \text{algB} \) gene, this gene was cloned as an EcoRI-EcoRI fragment from the \( \text{Bang} \) genomic clone. All genes were inserted into pCom8 (38), using the EcoRI and HindIII sites introduced by the primers (underlined in the sequences) in the case of alkB2-A. 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* KO231 according to the method of Hirogami et al. (14). E. coli and P. *fluorescens* KO231 recombinants harboring pCom8 derivatives were selected with 10 and 100 \( \mu \text{g} \) of gentamicin/ml, respectively. PCR amplification and cloning of the NRRL B-16531 \( \text{rubA1}, \text{rubA2}, \text{rubA3}, \text{and rubA4} \) genes in P. *KKPAk* have been described elsewhere (43). Q15 \( \text{rubA1}, \text{rubA2}, \text{rubA3}, \text{and rubA4} \) were also cloned in pKKPAk, and the recombinant plasmids were transformed into E. coli GE137 containing pGEc4720 for \( \text{rubA4} \) plasmids or pGEc4725 for \( \text{rubA} \) plasmids (42). E. coli transformants were selected for on LB supplemented with tetracycline (12.5 \( \mu \text{g} \) and carbenicillin (50 \( \mu \text{g} \)) or ampicillin (200 \( \mu \text{g} \)).

To measure in vivo alkane hydroxylase activity, the \( \text{algB}, \text{rubA}, \text{rubB} \), and \( \text{rubC} \) recombinants were assayed for the ability to mineralize \( 1^4\text{C}\)-radiolabeled alkane (C12, C14, and C16) (51) in minimal salts medium (MSM) supplemented with 100 \( \mu \text{g} \) of unlabeled alkane/liter, 50 \( \mu \text{g} \) of yeast extract/liter, the indicated \( 1^4\text{C} \)-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 alkane at 30°C. For *E. coli* strains, growth on M9 agar plates supplemented with 0.001% thiamine was monitored; \( n \)-alkanes (C16, C18, and C20) were provided as vapor in a closed system in the sole C source. The growth of *P. fluorescens* KO231 recombinants on alkane was monitored at 30°C and 200 rpm in 250-ml baffled Erlemeyer flasks containing 50 ml of MSM (22) supplemented with 1% (vol/vol) \( n \)-alkanes (C10, C12, C14, and C16). 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 *C. erythropsis* NRRL B-16531 alkane hydroxylase genes and flanking DNA have been submitted to GenBank and received the following accession numbers: alkB1, AJ009586; alkB2, A2597269; alkB3, AJ031876; and alkB4, AJ031877. For Rhodococcus sp. strain Q15, the accession numbers are as follows: alkB1, AF308181; alkB2, AF308182; alkB3, AF308179; and alkB4, AF308180.

**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-nucleotide 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 alkB 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). Hence, 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 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) and, to a lesser degree (41 to 50%), to other alkane monooxygenases. In addition to the high full-length homology, the four rhodococcal alkB1 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][X]X; Hist2, EHXGHH; 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 monoxygenases. 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 monoxygenases, such as three xylene monoxygenases (XylM), a nitrotoluene monoxygenase (NtnMa), and two cyrene monoxygenases (CymAa and NYXQHYG[L][Q]). Therefore, the Hist3 motif and the HYG motif can be used as apparent signature motifs specific for bacterial alkane monoxygenases. The positions and lengths of the six transmembrane helices initially reported in *P. putida* Gp01 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
rubredoxins contained the PS00202 rubredoxin signature (PROSITE database) and were single-domain proteins, like the \textit{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 \textit{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 \textit{alkB1} and \textit{rubA1} genes and the \textit{rubA2} and \textit{rubB} genes had 3'-end–5'-end ATGA and TGATG sequence overlaps, respectively. The \textit{rubA1} and \textit{rubA2} genes did not overlap.
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. P43494; thiocarbamate homolog (36) (up- and downstream, respectively) that is located immediately downstream of 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 genes have the same gene organization and close to 70% DNA sequence identity over their entire lengths, including the putative transporter 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 identity to a hypothetical transcriptional 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 C12 to C16 n-alkanes (Table 3) and mineralize 14C-radiolabeled n-dodecane and n-hexadecane (Table 4). Mineralization of 14C-radiolabeled n-octacosane was observed in both the alkB2 clones and the alkB2 knockout mutant of *P. fluorescens* CHA0, due to an additional long-chain
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 C₅ to C₇ 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.

### 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-

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

<table>
<thead>
<tr>
<th>Straina</th>
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<td>+/–</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>pCom8 + NRRL B-16531 alkB3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/–</td>
<td>++/+</td>
</tr>
<tr>
<td>pCom8 + NRRL B-16531 alkB4</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/–</td>
<td>++/+</td>
</tr>
<tr>
<td>pCom8 + Q15 alkB1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>+/–</td>
<td>++/+</td>
</tr>
<tr>
<td>pCom8 + Q15 alkB2</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>E. coli</em> GEc137</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEc47: alk</td>
<td>++</td>
<td>++</td>
<td>+/–</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>ΔT</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔT: pKKPalk Q15 rubB</td>
<td>++</td>
<td>++</td>
<td>+/–</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>ΔG</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk NRRL B-16531 rubB</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk NRRL B-16531 rubA1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk NRRL B-16531 rubA2</td>
<td>++</td>
<td>++</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk NRRL B-16531 rubA3</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk Q15 rubA1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk Q15 rubA2</td>
<td>–</td>
<td>+/–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSM control</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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).

c Values represent average percentages of 14C recovered as CO₂ from duplicate samples after 2 weeks of incubation at 28°C. The values for the control represent the background radioactivity.

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

<table>
<thead>
<tr>
<th>Straina</th>
<th>Mineralization of alkaneb</th>
<th>C₁₂</th>
<th>C₁₆</th>
<th>C₂₈</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. fluorescens</em> KOB2Δ1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCom8</td>
<td>1.2</td>
<td>1.9</td>
<td>18.5</td>
<td></td>
</tr>
<tr>
<td>pCom8 + Q15 alkB1</td>
<td>2</td>
<td>2</td>
<td>13.8</td>
<td></td>
</tr>
<tr>
<td>pCom8 + Q15 alkB2</td>
<td>16</td>
<td>32</td>
<td>16.2</td>
<td></td>
</tr>
<tr>
<td>pCom8 + NRRL B-16531 alkB2</td>
<td>17</td>
<td>25</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><em>E. coli</em> GEc137</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pGEc47: Alk</td>
<td>39</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔT</td>
<td>&lt;1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔT: pKKPalk Q15 rubB</td>
<td>27</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG</td>
<td>&lt;1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ΔG: pKKPalk NRRL B-16531 rubA2</td>
<td>13</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MSM control (not inoculated)</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td>&lt;1</td>
<td></td>
</tr>
</tbody>
</table>

a ΔG, pGEc47ΔG; ΔT, pGEc47ΔT.

b - Values represent average percentages of 14C recovered as CO₂ from duplicate samples after 2 weeks of incubation at 28°C. The values for the control represent the background radioactivity.
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 GPl0 alkB promoter as shown by mineralization and growth assays, confirming their respective functions in n-alkane degradation. The mineralization and growth assays of Pseudomonas strains containing the alkB2 gene indicate that AlkB2 is at least partly responsible for the initial oxidation of C12 to C16 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 monoxygenase (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 C6 and C12 for E. coli and P. putida or between C12 and C16 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 C20) (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 GPl0 counterpart (43). (iv) The AlkB proteins could produce secondary alcohols by subterminal alkane oxidation. However, P. fluorescens KOB2a1 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 C12 to C16 alkanes. (v) It is possible that the three AlkB homologs are not alkane monoxygenases. However, all three have >50% full-length protein sequence identity to functional alkane monoxygenases from gram-positive bacteria even if several residues which are well conserved in all other alkane monoxygenase sequences are not conserved in AlkB3 and AlkB4 (Fig. 2). The three homologs also have >45% sequence identity to 13 functional alkane monoxygenases from gram-negative bacteria; the alkane monoxygenase sequences from gram-positive bacteria constitute just one branch of a more deeply rooted alkane monoxygenase tree (46). The closest relatives of the AlkB homologs are the xylene and cymene monoxygenases, with only 25 to 30% sequence identity to the alkane monoxygenases (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 monoxygenases 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 GPl0 alkF-GHKL operon (47). This operon also encodes two rubredoxins, AlkF and AlkG (21). As in GPl0, 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 GPl0 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 monoxygenase 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 actinomyce-
tes 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 hybrid-
ization 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 monoxygenase 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 character-
ized alkane hydroxylase systems; (ii) the conservation in the
Rhodococcus alkane monoxygenases of the eight-histidine motif,
including the apparent alkane monoxygenase signature
motifs, and hydrophobic membrane-spanning regions found in
all known alkane monoxygenases; and (iii) functional hetero-
logous expression of some of these genes in E. coli and
Pseudomonas alk expression systems. The most likely explana-
tion for the presence of four alkane monoxygenases in one strain
(assuming that all four oxidize alkanes) is that each
alkane monoxygenase is specific for a certain range of
alkanes. For example, P. putida GP01 AlkB does not act on
alkanes longer than C₁₈, while the M. tuberculosis and P. rugosa
AlkBs do not efficiently oxidize alkanes shorter than C₁₃ (36).
As the Rhodococcus strains studied here oxidize alkanes up to
C₃₂ to C₈₀, AlkB1, AlkB3, and AlkB4 may each cover a part of the
C₁₈ to C₈₀ range. Unfortunately, it is not yet possible to
link sequence features, for example, specific amino acid resi-
dues or sequences within the hydrophobic transmembrane
regions, with an alkane oxidation range. To make this possible,
future 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 pos-
sess multiple, but not always the same, alkane monoxygenase
homologs (46) may help to answer this and other questions related
to horizontal gene transfer and the evolution of alkane
monoxygenase genes in actinomycetes.

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