Functional Analysis of Alkane Hydroxylases from Gram-Negative and Gram-Positive Bacteria

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We have cloned homologs of the *Pseudomonas putida* GPo1 alkane hydroxylase from *Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* CHA0, *Alcanivorax borkumensis* AP1, *Mycobacterium tuberculosis* H37Rv, and *Prauserella rugosa* NRRL B-2295. Sequence comparisons show that the level of protein sequence identity between the homologs is as low as 35%, and that the *Pseudomonas* alkane hydroxylases are as distantly related to each other as to the remaining alkane hydroxylases. Based on the observation that rubredoxin, an electron transfer component of the GPo1 alkane hydroxylase system, can be replaced by rubredoxins from other alkane hydroxylase systems, we have developed three recombinant host strains for the functional analysis of the novel alkane hydroxylase genes. Two hosts, *Escherichia coli* GEc137 and *P. putida* GPo12, were equipped with pGEc47ΔB, which encodes all proteins necessary for growth on medium-chain-length alkanes (C_6 to C_{12}), except a functional alkane hydroxylase. The third host was an *alkB* knockout derivative of *P. fluorescens* CHA0, which is no longer able to grow on C_{12} to C_{16} alkanes. All alkane hydroxylase homologs, except the *Acinetobacter* sp. ADP1 AlkM, allowed at least one of the three hosts to grow on *n*-alkanes.

Bacterial oxidation of *n*-alkanes is a very common phenomenon in soil and water (4) and is a major process in geochemical terms: the estimated amount of alkanes that is recycled per year amounts to several million tons from natural oil seepage and oil spills alone (37). Even more relevant are the alkanes (mainly waxes or paraffins) produced by plants, algae, and other organisms because they are available to bacteria throughout the biosphere. From a biotechnological perspective, alkane hydroxylases are versatile biocatalysts, which carry out a wide range of useful oxidation reactions (22, 61).

Biochemical and genetic studies have thus far focused on a very limited number of alkane hydroxylases, such as the *Pseudomonas putida* GPo1 alkane hydroxylase (33), which oxidizes C_5 to C_{12} *n*-alkanes to 1-alkanols, thereby allowing the host to grow on these compounds. Only recently has it become apparent that this enzyme is the prototype of a very diverse collection of related non-heme iron integral membrane oxygenases which includes not only the alkane hydroxylases of *Acinetobacter* sp. ADP1 (35), *Burkholderia cepacia* RR10 (29), *Acinetobacter calcoaceticus* EB104, and *P. putida* P1 (43) but also xylene monooxygenases (48), fatty acid desaturases, fatty acid monooxygenases, steroid oxygenases, and decarbonylases (41).

The *P. putida* GPo1 alkane hydroxylase system consists of three components: alkane hydroxylase (AlkB), rubredoxin (AlkG), and rubredoxin reductase (AlkT). AlkB is a non-heme iron integral membrane protein which carries out the hydroxylation reaction (26, 31, 54). Rubredoxin (33) transfers electrons from the NADH-dependent flavoprotein rubredoxin reductase (49) to AlkB. The molecular genetics of this enzyme system has been reviewed by van Beilen et al. (53, 56).

Genes that are closely related to the alkane hydroxylase gene (*alkB*) of GPo1 have been detected in a large fraction of the microbial population in oil-contaminated environments (45) and in several fluorescent pseudomonads (39, 43, 55, 58). More distantly related genes were found in many gram-positive and gram-negative strains able to grow on *n*-alkanes ranging from C_6 to C_{30} by using highly degenerate primers, which amplify internal fragments of alkane hydroxylase homologs (43) (J. B. van Beilen, unpublished data).

In earlier studies, the alkane hydroxylase genes of *P. putida* GPo1 and *Acinetobacter* sp. ADP1 have been cloned by using traditional cloning methods. In both cases, cosmids that restored growth of chemical mutants on alkanes were selected from a cosmid library (9, 35). However, this method was not successful for other alkane hydroxylase genes. For example, in classical mutagenesis experiments with *Pseudomonas aeruginosa* PG201, mutants unable to grow on alkanes usually had mutations in genes responsible for rhamnolipid biosynthesis or downstream metabolism (24). In retrospect, this can be explained by the fact that *P. aeruginosa* contains two alkane hydroxylases with overlapping substrate ranges. More recent data show that many other strains also contain multiple alkane hydroxlases (L. G. Whyte, T. H. M. Smits, D. Labbé, B. Witholt, C. W. Greer, and J. B. van Beilen, submitted for publication; J. B. van Beilen, unpublished data). In addition, although medium-chain-length alkane hydroxylases can be assayed in vitro, e.g., using the conversion of alkenes to epoxides or the cooxidation of NADH (51), such methods could not be developed for membrane-bound long-chain alkane hydroxylases, possibly because long-chain alkanes are hardly soluble in water (solubility around 10 μM), and activity is strongly limited by substrate mass transfer. Therefore, it was not possible to purify the alkane hydroxylases based on enzyme activity and use reverse genetics to clone the genes.

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In this study, we used PCR products obtained with highly degenerate primers described earlier or information from genome sequencing projects to clone a number of alkane hydroxylases from quite diverse strains. This implies that these genes are cloned based on sequence similarity, not on function. Knockout mutants of the *alkB* gene homologs could in principle be used to prove that these genes indeed encode functional alkane hydroxylases. However, many strains are not easily accessible for molecular genetic studies, as tools or methods are not (yet) available. This is the case for *Alcanivorax borkumensis* AP1, which has recently been isolated from seawater and grows almost exclusively on *n*-alkanes (62). Other strains, like *Mycobacterium tuberculosis* and *Legionella pneumophila*, are important human pathogens and are difficult to cultivate. Moreover, if a strain contains multiple *alkB* homologs, knockout mutants may not show phenotypical changes, while in vivo substrate range studies would only give information about the sum of all (induced) alkane hydroxylase activities.

Heterologous expression of novel alkane hydroxylases is complicated by the fact that all three components of the alkane hydroxylase system are necessary for enzyme activity. Fortunately, complementation experiments with the *P. putida* GPo1 alkane hydroxylase system have shown that rubredoxins from various gram-positive and gram-negative alkane degraders can replace the GPo1 rubredoxin in alkane oxidation (52). This implies that the GPo1 rubredoxin and rubredoxin reductase or equivalent proteins from other sources should be able to serve as electron donors for novel alkane hydroxylases related to the GPo1 enzyme. Based on this notion, we have constructed three different recombinant hosts that allow us to test whether the novel alkane hydroxylases oxidize *n*-alkanes.

MATERIALS AND METHODS

Strains, plasmids, and media. Strains used in this study are listed in Table 1. Luria-Bertani broth (38), E medium (57), and E2 medium (28) supplemented with carbon sources or antibiotics were used throughout. MT trace elements (28) were added to minimal media. All cultures were grown aerobically at 30°C. For growth on *n*-alkanes, petri dishes with E2 medium were incubated at 30°C with *n*-alkanes supplied through the vapor phase, in the case of *n*-octane, by placing an open Erlenmeyer flask with *n*-octane (C_8) and *n*-decane (C_{10}) in a sealed container and for *n*-dodecane (C_{12}), *n*-tetradecane (C_{14}), and *n*-hexadecane (C_{16}), by placing a Whatman 3MM filter disk with 200 μl of *n*-alkane in the lid of the petri dish. Recombinants were cultured in baffled Erlenmeyer flasks with E2 medium and 1% (vol/vol) *n*-alkanes as carbon sources at 120 rpm. Rhamnolipids (11) were added to cultures of recombinant *P. putida* GPo12 strains to a concentration of 0.01% (wt/vol) to solubilize long-chain *n*-alkanes. Alkanes that are solid at 30°C (*n*-eicosane [C_{20}], *n*-docosane [C_{22}], *n*-tetracosane [C_{24}] and *n*-octacosane [C_{28}]) were dissolved to 10% (vol/vol) in dioctylphthalate. This solution was then added to the growth medium as a second phase at 2% (vol/vol).

Escherichia coli strains and *P. putida* GPo12(pGEc47B) were transformed by electroporation according to the method of Dower et al. (7). *Pseudomonas fluorescens* CHA0 and KOB2Δ1 were transformed according to the method of Højberg et al. (20). Plasmids with an *oriT* (origin of transfer) were introduced in *P. putida* GPo12(pGEc47ΔB) by triparental matings with *E. coli* DH10B as the donor and *E. coli* CC118(RK600) as the helper strain (6). Transconjugants were selected on E medium containing the appropriate antibiotics. *E. coli* strains harboring plasmids were grown with appropriate antibiotics (tetracycline, 12.5 μl/ml; ampicillin, 100 μg/ml; gentamicin, 10 μg/ml). For *P. fluorescens* KOB2Δ1 recombinants, gentamicin was used at 100 μg/ml. For *P. putida* GPo12 recombinants, tetracycline (12.5 μg/ml) and gentamicin (100 μg/ml) were used.

DNA manipulations. Plasmid DNA was isolated with the Roche High Pure Plasmid Isolation kit or according to the method of Birnboim and Doly (2) for *Pseudomonas* recombinants. Chromosomal DNA was isolated according to the method of Sambrook et al. (38). PCRs were carried out using the Roche Expand High Fidelity Polymerase kit on a Perkin Elmer GeneAmp PCR System 9600.

The following PCR program was used: 4 min at 95°C and 25 cycles of 45 seconds at 95°C, 1 min at annealing temperature (5°C below the melting point), and 1 min at 72°C; 5 min at 72°C; and then transfer at 4°C. PCR products were purified over 1% agarose gels, cut with the respective enzymes, purified again over a 1% agarose gel, and cloned into pGEM7-Zf(+) or pKKPalk (44). All PCR fragments were checked for possible PCR artifacts by sequencing both strands of the inserts on a Li-Cor 4000L sequencer using the Amersham Thermosequenase cycle sequencing kit and IRD800-labeled −40 forward (AGGGTTTTCCCAGT CACGACGTT) and −40 reverse (GAGCGGATAACAATTTCACACAGG) primers for pGEM7-ZF(+) clones or PalkFwd (TGGCGCAAGCGTCCGATT AG), PalkFw3 (GCCAGCTCGTGTTTTTCCAGCAGACG), and pKKRev (GAGTTCGGCATGGGGTCAGGTG) for pKKPalk-derived plasmids (MWG-Biotech). Nucleotide and amino acid sequences were analyzed and compared using LASERGENE Navigator from DNASTAR. Nucleotide and amino acid sequences were compared with the EMBL, SwissProt, and GenBank databases using BLAST (1). BLAST searches were carried out at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/).

Cloning of alkane hydroxylase gene homologs and construction of expression plasmids. To clone complete alkane hydroxylase genes, easy-to-clone restriction fragments were identified by Southern blotting (38) with previously cloned DNA fragments as probes. Enriched gene banks were constructed by isolating restriction fragments of the desired size from a preparative gel. The DNA fragments were ligated between the appropriate sites of pGEM7-Zf(+) or pZErO2.1 and transformed into *E. coli* DH10B. Transformants containing the target genes were identified by colony blotting with the same probes. Both strands of the inserts were sequenced by a combination of ordered deletions, subcloning, or walking primers.

The *alkB1* and *alkB2* genes of *P. aeruginosa* PAO1 (46) were amplified by PCR from chromosomal DNA using primer combinations AlkBpaFwd (AACTGGA ATTCACGATGTTTGA) and AlkBpaRv2 (CTGCCCGAAGCTTGAGCTAT) and AlkBpaBfw (GGAGAATTCTCAGACAATCT) and AlkBpaBrv (GAGGC GAATCTAGAAAAAACTG), respectively. Primers alkMfE2 (CCGGAATTC ACTATGAATGCACCTGTA) and alkMrPS (AATAGGCCTGCAGTCACTT AGACTCTCTT) were used to amplify *alkM* of *Acinetobacter* sp. ADP1 (35). To amplify *alkB1* of *A. borkumensis* AP1, primers alkB1fw (CAAGGTGATCCAT ATGTCAGAGAAC) and alkB1rv (GCGGATCCTCAAAGTGTGAAAGC) were used. For *alkB* of *Prauserella rugosa* NRRL B-2295, primers 2295fwEco (GGAGAATTCAGATGAGCGACGACGCAC) and 2295rvBam (CGGCGAG GATCCGGTCCAGCTC) were used. The *M. tuberculosis* H37Rv *alkB* gene was amplified from cosmid pSCYB11 (34) with primers MTalkBfw2 (CGGAATTC ATATGACCACGCAAATCGGC) and MTalkBrv3 (CAGACCGGGATCCGG TAGGCGG). The *P. putida* GPo1 *alkB* gene was amplified from pGEc47 (9) with primers B5-Eco (GGAGAATTCCAAATGCTTGAG) and B3-Hind (TTT GTGAAAGCTTTCAACGCC). All PCR fragments were digested with the respective restriction enzymes (sites underlined in the primer sequences) and inserted in pCom7 or pCom8 (44). The *alkB2* (PAO1) gene was first cloned between the *Eco*RI and *Xba*I sites of pUC18Sfi and then recloned in pCom8 with *Eco*RI and *Hin*dIII. The *alkB* gene of *P. fluorescens* CHA0 was cloned directly as a 1.6-kb *Mun*I-*Sal*I fragment, including 133 bp upstream of the *alkB* gene, and inserted in the *Eco*RI-*Sal*I restriction sites of pCom5 (44).

Construction of a *P. fluorescens* CHA0 *alkB* knockout mutant. Plasmid pPF1 was digested with *Sfi*I and *Stu*I to remove a 0.5-kb internal segment of *alkB* and blunt ended with T4 DNA polymerase. Subsequently, the fragment was ligated to a blunt-ended (Klenow) 2.1-kb *res-npt-res* cassette cut from pCK217 (27) with *Hin*dIII and *Ecl*136II. After digestion of the resulting plasmid (pPF1rnrB2) with *Pvu*I and treatment with Klenow, the large fragment was ligated in *Sma*I-digested pEX18Tc (19). The final plasmid, pEXPF1rnrB2, was introduced into *P. fluorescens* CHA0 by electroporation using kanamycin selection (50 μg/ml). A Km^r Tc^s colony was obtained, which was shown by Southern blot hybridization and PCR to have a chromosomal insertion of the *res-npt-res* cassette in the *alkB* gene (data not shown). This mutant was named *P. fluorescens* KOB2. To remove the kanamycin resistance gene with its transcription terminator, the *parA* gene was supplied on pUCPParA. The latter plasmid was constructed by digesting pJMSB8 (27) with *Eco*RI and *Hin*dIII. The fragment containing the *parA* gene was isolated over a gel and ligated in pUCP24 digested with the same enzymes. Gentamicin-resistant colonies were tested for the absence of the kanamycin cassette by PCR and sensitivity to kanamycin. One positive colony, subsequently cured of pUCPParA, was designated *P. fluorescens* KOB2Δ1.

Sequences. Nucleotide sequences used in this study are available at EMBL under the following accession numbers: *Acinetobacter* sp. ADP1 *alkRM*, AJ002316; *M. tuberculosis* H37Rv *alkB* (Rv3252c), Z46863; *P. aeruginosa* PAO1 *alkB1* (PA2574), AE004685; *alkB2* (PA1525), AE004581; *P. putida* GPo1 *alkBF-GHJKL alkN alkST*, AJ245436; and *P. putida* P1 *alkBFGHJKL alkST*, AJ233397.

TABLE 1. List of strains and plasmids used in this study

Strain or plasmid	Relevant phenotype, genotype, or characteristic(s)	Source or reference
Strains		
Acinetobacter sp. ADP1	Grows on C_{12}–C_{16} alkanes	23
A. borkumensis AP1	Grows on C_8–C_{16} alkanes	62
E. coli JM101	*endA hsdR*, *supR*, *thi-I*, Δ(*lac-proAB*) F′ (*traD36*, *proAB*, *lacI*q, *lacZM15*)	63
E. coli DH10B	Cloning strain	Gibco BRL
E. coli SF800	W3110 *polA* mutant	Laboratory collection
E. coli GEc137	DH-1 *thi*, *fadR*	8
P. aeruginosa PAO1	Grows on C_{12}–C_{24} alkanes	21
P. fluorescens CHA0	Grows on C_{12}–C_{32} alkanes	47
P. fluorescens KOB2	*alkB* knockout mutant of CHA0, Km^r	This study
P. fluorescens KOB2Δ1	*alkB* knockout mutant of CHA0	This study
P. putida GPo12	GPo1 cured of the OCT plasmid	25
P. rugosa NRRL B-2295	Grows on C_{12}	22
Plasmids		
p2295	*P. rugosa alkB* fragment in pGEM7-Zf(+)	52
pAF1	*A. borkumensis* AP1 *alkG* in pZEr0-2.1	J. B. van Beilen
pAP1J1	5.0-kb *Nco*I-*Eco*RV fragment of *A. borkumensis* AP1 in pZErO2.1, *alkS alkB1*	This study
pAP1S1	7.5-kb *Bgl*II fragment of *A. borkumensis* AP1 in pZErO2.1, *alkGHJ*	This study
pAP1T1	4.6-kb *Nhe*I fragment of *A. borkumensis* AP1 in pZErO2.1, ′*alkS*	This study
pCHA0	*P. fluorescens* CHA0 *alkB1* fragment in pGEM7-Zf(+)	43
pCK217	pUC18Sfi containing *res-npt-res*	27
pCom5	Expression vector with P*alkB*, Gm^r	44
pCom7	Expression vector with P*alkB*, Gm^r *oriT*	44
pCom7B1 (PAO1)	*P. aeruginosa* PAO1 *alkB1* gene in pCom7	This study
pCom7B2 (PAO1)	*P. aeruginosa* PAO1 *alkB2* gene in pCom7	This study
pCom7B (GPo1)	*P. putida* GPo1 *alkB* gene in pCom7	This study
pCom7alkB1 (AP1)	*A. borkumensis* AP1 *alkB1* gene in pCom7	This study
pCom7M (ADP1)	*Acinetobacter* sp. ADP1 *alkM* gene in pCom7	This study
pCom8	Broad-host-range expression vector with P*alkB*, Gm^r *oriT alkS*	44
pCom8B1 (PAO1)	*P. aeruginosa* PAO1 *alkB1* gene in pCom8	This study
pCom8B2 (PAO1)	*P. aeruginosa* PAO1 *alkB2* gene in pCom8	This study
pCom8B (GPo1)	*P. putida* GPo1 *alkB* gene in pCom8	This study
pCom8M (ADP1)	*Acinetobacter* sp. ADP1 *alkM* gene in pCom8	This study
pCom8MT (H37Rv)	*M. tuberculosis* H37Rv *alkB* gene in pCom8	This study
pCom8B (2295)	*P. rugosa* NRRL B-2295 *alkB* gene in pCom8	This study
pEX18Tc	Gene replacement vector	19
pEXPF1rnrB2	pPF1rnrB *Pvu*I fragment in pEX18Tc	This study
pGEc47	*alkBFGHJKL alkST* (GPo1) in pLAFR1	9
pGEc47ΔB	pGEc47, deletion in *alkB*	54
pGEM7-Zf(+)	Cloning vector, Ap^r	Promega
pJMSB8	Ap^r, P$_{AI/04/03}$::*parA* in pJMSA8	27
pKKPalk	*E. coli* expression vector with P*alkB*, Ap^r	44
pPF1	4.5-kb *Xho*I-*Bam*HI fragment of *P. fluorescens* CHA0 in pGEM7-Zf(+), *alkB*	This study
pPF1rnrB	pPF1Δ*Sfi*I-*Stu*I, *res-npt-res* in *alkB*	This study
pPF21	*P. fluorescens* CHA0 *alkB* gene in pCom5	This study
pPru1	3.0-kb *Sac*I-*Bam*HI fragment of *P. rugosa* NRRL B-2295 in pGEM7-Zf(+), *alkB*	J. B. van Beilen
pSCYB11	Tc^r *alkB rubA rubB* (*M. tuberculosis* H37Rv)	34
pSK2	*A. borkumensis* SK2 PCR fragment in pGEM7-Zf(+)	J. B. van Beilen
pUC18Sfi	Cloning vector, Ap^r	16
pUCP24	*E. coli* and *Pseudomonas* shuttle vector	60
pUCPParA	Gm^r, *parA* as *Eco*RI-*Hin*dIII fragment in pUCP24	This study
pZErO2.1	Cloning vector, Km^r	Invitrogen

Nucleotide sequence accession numbers. Novel sequences were submitted to GenBank and are available under the following accession numbers: *A. borkumensis* Ap1, AJ295164; *P. rugosa* NRRL B-2295, AJ009587; *P. fluorescens* CHA0, AJ009579.

RESULTS

Selection of strains. For this study, we selected a number of alkane-oxidizing strains from different environments (soil, seawater, and cow rumen) and different research fields (biodegradation, biocatalysis, and pathogenesis). The alkane hydrox-ylase genes of *Acinetobacter* sp. ADP1 and *P. putida* strains GPo1 and P1, which are isolates of common soil and water organisms, have been cloned and sequenced before (35, 53). *A. borkumensis* AP1 is a marine γ-*Proteobacterium* (62) which grows almost exclusively on *n*-alkanes. The same species was isolated from seawater at many geographic locations and forms a major part of the biomass in oil-polluted marine habitats (14). *P. fluorescens* CHA0 was originally not studied for the ability to grow on *n*-alkanes but is of interest as a biocontrol strain which excretes secondary metabolites toxic to soil-borne

plant pathogens (12). *P. aeruginosa* PAO1 represents a species which is of clinical importance as the primary opportunistic pathogen among the pseudomonads (3) but is also common in soil and water and on plants. In contrast, *M. tuberculosis* is not found in the environment but is a typical and notorious example of the slow-growing pathogenic *Mycobacteria*. It is not able to grow on the paraffin-coated slides used in the so-called paraffin baiting method, unlike *P. aeruginosa* PAO1 and *Mycobacterium avium* (30, 32). *P. rugosa* NRRL B-2295 was isolated from cow rumen and was selected because it is a useful biocatalyst that converts cumene to 2-phenyl-1-propionic acid (22) if it is grown on *n*-alkanes.

Cloning of novel alkane hydroxylase genes and analysis of flanking regions. The *P. aeruginosa* PAO1 genome sequence (46) encodes two alkane hydroxylase homologs (AlkB1 and AlkB2) while the genome sequence of *M. tuberculosis* H37Rv (5) encodes one alkane hydroxylase homolog. These putative alkane hydroxylase genes were cloned as described in Materials and Methods. Several unfinished genome sequences, such as those of *L. pneumophila* strain Philadelphia-1, *Burkholderia pseudomallei* K96243 and several mycobacteria, also contain an alkane hydroxylase homolog each. These sequences were included in the phylogenetic analysis but not in the functional studies. In a separate paper, the cloning and functional analysis of the *B. cepacia* RR10 *alkB* gene have been described (29). This sequence was also included in the phylogenetic analysis. Of the above *alkB* genes or gene homologs, only the H37Rv *alkB* gene is located close to other genes that are likely to be involved in alkane oxidation: it is followed by two rubredoxin genes and a regulatory protein with a TetR signature that is similar to proteins encoded downstream of other alkane hydroxylase genes (Whyte et al., submitted).

Additional alkane hydroxylase genes were cloned by using PCR fragments obtained previously with degenerate primers, which amplify internal segments of genes that are homologous to the *P. putida* GPo1 alkane hydroxylase and rubredoxin 2 (43, 52). A 7.8-kb fragment of *P. fluorescens* CHA0 DNA containing the alkane hydroxylase was cloned by using a 550-bp CHA0 *alkB* fragment (43) as a probe in Southern and colony blots. The DNA segment downstream of the alkane hydroxylase encodes two proteins (PraA and PraB) with homology to the so-called protein activator for alkane oxidation of *P. aeruginosa* PAO1 (15) and an outer membrane protein homologous to the OmpP1 proteins of *Haemophilus influenzae*.

In the case of *A. borkumensis* AP1, we cloned a 3.5-kb *Eco*RI-*Bam*HI fragment encoding a rubredoxin similar to the *P. putida* GPo1 AlkG (52). By cloning and sequencing adjacent DNA fragments, a contiguous sequence of 11.2 kb was assembled. Sequence analysis of this region showed that the *alk* gene organization in this strain is quite similar to that of *P. putida* strains GPo1 and P1 (Fig. 1) (53). The *P. rugosa* NRRL B-2295 *alkB* gene was cloned as a 3.0-kb *Sac*I-*Bam*HI chromosomal DNA fragment by using the 559-bp *Eco*RI-*Sac*I *alkB* fragment of p2295 (43) as a probe. Sequence analysis of the 3.0-kb fragment showed that it encodes an alkane hydroxylase with a C-terminal extension that is highly homologous to rubredoxins involved in alkane hydroxylation (52). The region immediately downstream of the *alkB* gene encodes a possible regulatory protein with a TetR signature at its N terminus (pfam00440) quite homologous to the TetR protein encoded downstream of

the *M. tuberculosis* H37Rv *alkB-rubAB* genes (26% full-length sequence identity) (Fig. 1). In the related *Nocardioides* sp. strain CF8, genes encoding very similar proteins were found: a rubredoxin domain fused to an alkane hydroxylase and a TetR protein encoded upstream of the *alkB* gene (13).

Comparison of alkane hydroxylases. In a peptide alignment of the full-length alkane hydroxylases (not shown), the six membrane-spanning segments identified in the *P. putida* GPo1 alkane hydroxylase (54), three histidine motifs conserved among the hydrocarbon oxygenases and desaturases (42), and a fourth motif (NYXEHYG[L/M]) identified in the alkane hydroxylases (43) are well conserved in all sequences. Most insertions and deletions in each sequence relative to the other AlkB sequences are located in predicted cytoplasmic or periplasmic domains before the third transmembrane helix. For example, a 40-amino-acid insertion in the *P. fluorescens* CHA0 AlkB is located in the periplasmic loop between the third and fourth putative transmembrane helices while other sequences have smaller insertions in the same loop. Insertions between the conserved histidine motifs are present in the *M. tuberculosis*, *P. rugosa*, and *Nocardioides* sp. alkane hydroxylases. Especially the hydrophobic segments and the N- and C-terminal ends of the alkane hydroxylases vary strongly in sequence.

For a phylogenetic analysis of known and new alkane hydroxylases (Fig. 2) with Clustal (17), a peptide sequence alignment of the AlkB sequences corresponding to the 550-bp internal gene fragments (the sequence between the first and last histidine clusters) was generated and manually optimized. The peptide sequence identity among the alkane hydroxylases ranged from 37 to 99%. The corresponding fragment of the closest relative of the alkane hydroxylases, which does not oxidize *n*-alkanes, xylene monooxygenase (XylM), was also included in the analysis. It has 20 to 26% peptide sequence identity to the different alkane hydroxylases and is most closely related to the *L. pneumophila* Philadelphia-1 AlkB and the *P. aeruginosa* PAO1 AlkB1 and AlkB2.

Alkane hydroxylases from strains that oxidize medium-chain alkanes (*P. putida* GPo1, *P. putida* P1, *A. borkumensis* AP1, and *P. chloroaphis* RWTH529 [58]) are clustered in the phylogenetic tree (Fig. 2), whereas the putative alkane hydroxylases from long-chain alkane-degrading strains are highly divergent. Interestingly, alkane hydroxylases cloned from fluorescent pseudomonads are as divergent as the entire collection; while the *P. fluorescens* and *Burkholderia* sequences are quite closely related to gram-positive sequences, the *P. aeruginosa* sequences are quite distantly related to both the *P. putida* and *P. fluorescens* sequences.

Construction of *P. fluorescens* CHA0 *alkB* knockout mutants. An *alkB* knockout mutant of *P. fluorescens* CHA0 was constructed by inserting a kanamycin resistance gene flanked by two *res* sites (27) in the *alkB* gene. This mutant, named KOB2, did not grow on C_{12} to C_{16} *n*-alkanes, unlike the parent strain. To reduce polar effects of the kanamycin resistance gene and its terminator on the expression of the PraA and PraB proteins, a homolog of which enhances the oxidation of alkanes in *P. aeruginosa* PAO1 (15, 18), these sequences were removed using resolvase (ParA) (27) expressed from the *lac* promoter on plasmid pUCPParA. The resulting strain was named KOB2Δ1.
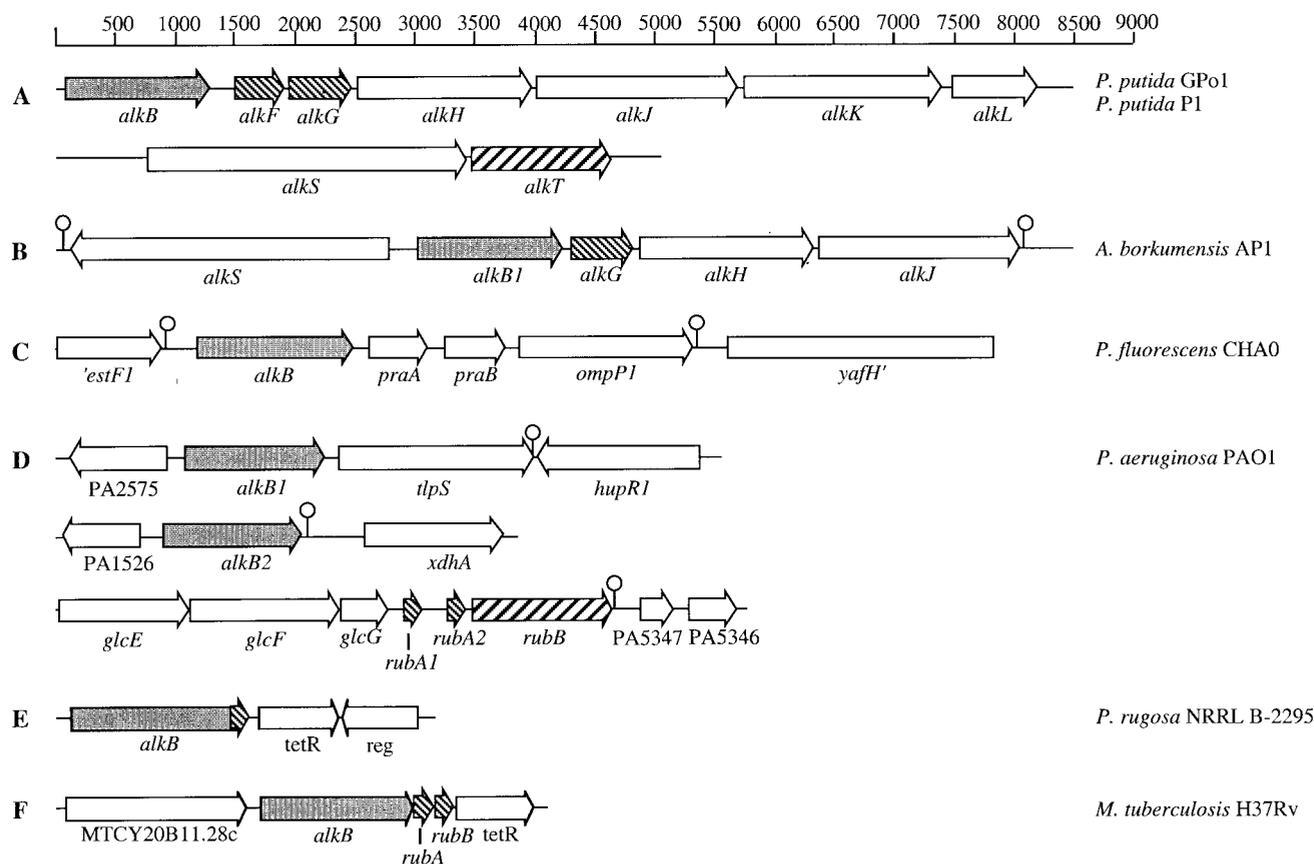
FIG. 1. Organization of *alk* genes in different organisms. Alkane hydroxylases are indicated in grey; rubredoxin and rubredoxin reductase genes are indicated by fine and coarse hatches, respectively. (A) *P. putida* GPo1 and *P. putida* P1. *alkB*, alkane hydroxylase; *alkF*, rubredoxin 1; *alkG*, rubredoxin 2; *alkH*, aldehyde dehydrogenase; *alkJ*, alcohol dehydrogenase; *alkK*, acyl coenzyme A (acyl-CoA) synthetase; *alkL*, outer membrane protein; *alkS*, regulatory protein; *alkT*, rubredoxin reductase. (B) *A. borkumensis* AP1. *alkS*, putative regulatory protein; *alkB1*, alkane hydroxylase; *alkG*, rubredoxin; *alkH*, putative aldehyde dehydrogenase; *alkJ*, putative alcohol dehydrogenase. (C) *P. fluorescens* CHA0. *'estF1*, esterase; *alkB*, alkane hydroxylase; *praA* and *praB*, protein activators of alkane oxidation; *ompP1*, outer membrane protein; *yafH'*, acyl-CoA dehydrogenase. (D) *P. aeruginosa* PAO1. PA2575, hypothetical protein; *alkB1*, alkane hydroxylase 1; *tlpS*, methyl-accepting chemotaxis protein; *hupR1*, regulatory protein of hydrogen uptake; PA1526, regulatory protein of GntR family; *alkB2*, alkane hydroxylase 2; *xdhA*, xanthine dehydrogenase homolog; *glcEFG*, genes homologous to the *glcEFG* genes of *E. coli*; *rubA1*, rubredoxin 1; *rubA2*, rubredoxin 2; *rubB*, rubredoxin reductase; PA5347 and PA5346, hypothetical proteins. (E) *P. rugosa* NRRL B-2295. *alkB*, alkane hydroxylase; tetR, putative regulatory protein of the TetR family; reg, putative regulatory protein. (F) *M. tuberculosis* H37Rv. MTCY20B11.28c, cationic transporter; *alkB*, alkane hydroxylase; *rubA*, rubredoxin 1; *rubB*, rubredoxin 2; tetR, putative regulatory protein of the TetR family.

KOB2Δ1 and KOB2 were transformed with pPF21, which encodes the *P. fluorescens* CHA0 alkane hydroxylase gene under the control of its own promoter, and compared with CHA0 with respect to growth on *n*-hexadecane. The growth rates of the three strains were similar, but the lag time of KOB2(pPF21) (around 200 h) was much longer than that of KOB2Δ1(pPF21) (around 100 h). We expect that KOB2 needs more time to produce sufficient amounts of PraAB for growth on alkanes, due to the polar effect of the kanamycin cassette. As the lag time of KOB2Δ1 is still somewhat longer than that of CHA0 (100 versus 80 h), the polar effect of the insertion in the *alkB* gene may not be eliminated completely. A 16-kDa band corresponding to PraB was detected by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the culture supernatant of CHA0 and KOB2Δ1(pPF21) at time points $t = 120$ h and $t = 168$ h after inoculation but not in KOB2(pPF21). A very weak PraB band [estimated to be 10-fold-less strong than for CHA0 and KOB2Δ1(pPF21)] was observed in KOB2(pPF21) only at $t = 216$ h and later time points (data not shown).

Characterization of *P. fluorescens* KOB2Δ1: a second alkane hydroxylase system? *P. fluorescens* KOB2Δ1 did not grow on alkanes up to 16 carbon atoms in length, unlike the parent strain CHA0. However, when KOB2Δ1 was grown on *n*-octadecane, growth was observed, with a growth rate 2 to 3 times lower than that of CHA0 (Table 2). Longer alkanes (C_{20} to C_{28}), which are solid at 30°C, supported growth of both KOB2Δ1 and CHA0 when supplied as a 10% (vol/vol) solution in dioctylphthalate (an inert high-boiling solvent that is not a growth substrate for the strains used in this study). The growth rates of CHA0 and KOB2Δ1 on C_{20} to C_{28} *n*-alkanes dissolved in dioctylphthalate were comparable for both strains (data not shown). From these data we conclude that *P. fluorescens* CHA0 possesses a second alkane oxidation system, which oxidizes *n*-alkanes from C_{18} up to at least C_{28}.
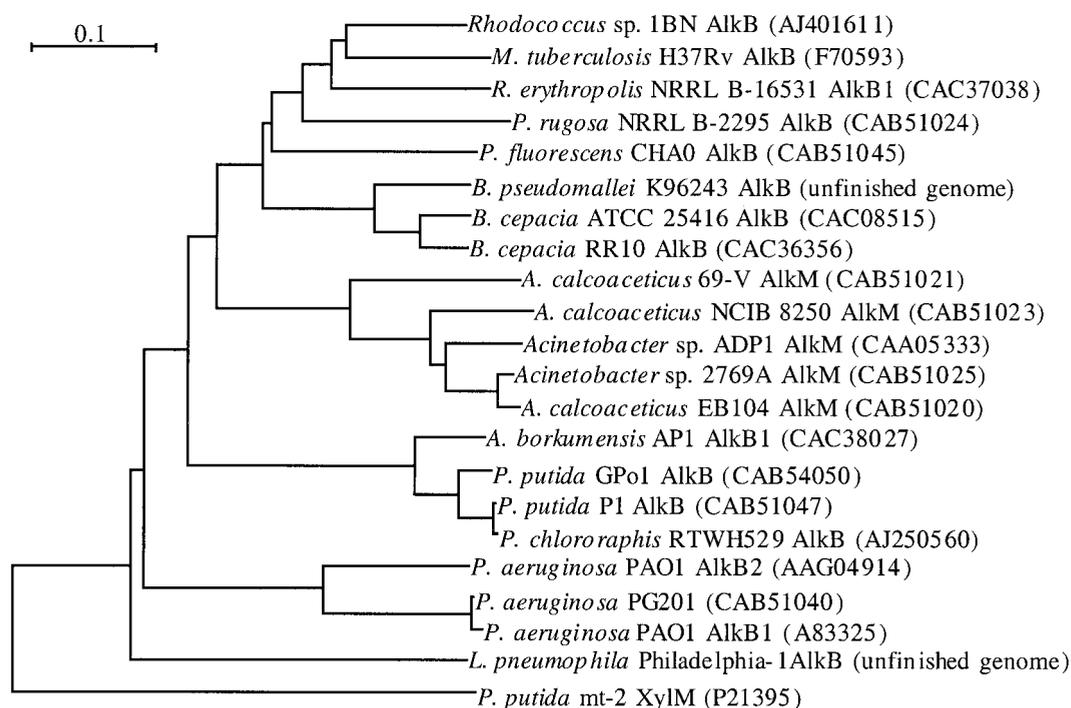
FIG. 2. Unrooted phylogenetic tree of partial sequences of published and hypothetical alkane hydroxylases. The distance tree was generated by Clustal from a manual alignment of the (putative) alkane hydroxylases. Only the segments corresponding to the 550-bp fragments obtained in PCR experiments with highly degenerate primers (based on histidine box 1 and histidine box 4) were used for the alignment. The XylM sequence was included as the out-group sequence.

Attempts to clone the second alkane hydroxylase gene by using PCR with the degenerate alkane hydroxylase primers described before (43) were not successful. In addition, Southern blots using probes based on several alkane hydroxylase genes, including the cloned CHA0 *alkB* gene, did not yield specific signals other than that corresponding to the cloned CHA0 *alkB* gene (43) (data not shown). Therefore, the second alkane hydroxylase gene must have clearly less than 70% DNA sequence identity to any of the cloned alkane hydroxylase genes, or it is a completely unrelated enzyme.

In vivo functional analysis of alkane hydroxylases. Three different host systems for the functional expression of alkane hydroxylases were developed based on the observation that the second component of the *P. putida* GPo1 alkane hydroxylase system, the electron transfer protein rubredoxin (AlkG), can be replaced by rubredoxins from other alkane hydroxylase systems (52). The broad-host-range cosmid pGEc47ΔB, a deletion derivative of pGEc47 (9) that lacks part of the alkane

hydroxylase gene *alkB* (54), expresses the *P. putida* GPo1 rubredoxin, rubredoxin reductase, and other proteins involved in the degradation of *n*-alkanes, in *E. coli* as well as in *Pseudomonas* species. Two hosts were used for this plasmid. *E. coli* GEc137 is a *fadR* mutant of *E. coli* DH-1, which grows on medium-chain-length fatty acids. The strain also grows on *n*-alkanes of the same length if it contains pGEc47 (9). *P. putida* GPo12 is a derivative of GPo1, cured of the OCT plasmid (25). Both host strains containing pGEc47ΔB did not show any detectable alkane hydroxylase activity (<0.01 U g of cells^{-1}, or less than 0.06% of full activity) (54). Plasmids carrying the alkane hydroxylase gene of *P. putida* GPo1 fully restored the alkane hydroxylase activity of both recombinant strains, based on growth rates on *n*-octane and activity assays (data not shown).

All *alkB* gene homologs were expressed from P*alkB*, the promoter of the *P. putida* GPo1 alkane hydroxylase, by using the vectors pKKPalk, pCom7, and pCom8 (44), and a gratuitous inducer of P*alkB*, dicyclopropylketone (10). The alkane hydroxylases of *P. putida* P1 and *A. borkumensis* AP1, which are quite closely related to the *P. putida* GPo1 AlkB, complement the *alkB* deletion in *E. coli* GEc137(pGEc47ΔB) and *P. putida* GPo12(pGEc47ΔB) for growth on *n*-octane (Table 3). However, similar *E. coli* recombinants containing alkane hydroxylase genes cloned from strains able to grow on C_{12} to C_{16} *n*-alkanes did not grow on these *n*-alkanes. Some *P. putida* GPo12 recombinants containing the same genes grew on *n*-dodecane and also (but only poorly) on *n*-tetradecane if rhamnolipids were added (Table 4). Therefore, the third host, *P.*

TABLE 2. Growth rates of *P. fluorescens* CHA0 and KOB2Δ1 on *n*-alkanes ranging from C_{12} to C_{20}

Strain	Growth rate (h^{-1}) on[a]:				
	C_{12}	C_{14}	C_{16}	C_{18}	C_{20} in DOPh
CHA0	0.019	0.019	0.053	0.049	0.031
KOB2Δ1	—[b]	—	—	0.015	0.028

[a] C_{12}, dodecane; C_{14}, tetradecane; C_{16}, hexadecane; C_{18}, octadecane; C_{20} in DOPh, eicosane added as a 10% solution in dioctylphthalate (DOPh).
[b] —, no growth.

TABLE 3. Functional analysis of alkane hydroxylases based on growth on alkane vapor on minimal medium plates

Alkane hydroxylase	Result[a] of functional analysis on host strain:		
	E. coli GEc137(pGEc47ΔB)	*P. putida* GPo12(pGEc47ΔB)	*P. fluorescens* KOB2Δ1
P. putida GPo1 *alkB*	++ (C_6–C_{12})	++ (C_6–C_{12})	+(C_{12})
P. putida P1 *alkB*	++ (only C_8 was tested)	NT	NT
A. borkumensis AP1 *alkB1*	++ (C_8–C_{12})	++ (C_8–C_{12})	NT
Acinetobacter sp. ADP1 *alkM*	−	−	−
P. aeruginosa PAO1 *alkB1*	−	NT	+ (C_{12}–C_{16})
P. aeruginosa PAO1 *alkB2*	−	+ (C_{12}–C_{14})	++ (C_{12}–C_{16})
P. fluorescens CHA0 *alkB*	−	+ (C_{12}–C_{14})	++ (C_{12}–C_{16})
M. tuberculosis H37Rv *alkB*	−	++ (C_{10}–C_{12})	++ (C_{12}–C_{16})
P. rugosa NRRL B-2295 *alkB*	NT	++ (C_{10}–C_{12})	++ (C_{12}–C_{16})

[a] −, no growth; +, poor growth; ++, good growth; NT, not tested. The substrates indicated in parentheses were tested and positive.

fluorescens KOB2Δ1, an *alkB* deletion mutant of *P. fluorescens* CHA0, was constructed as described above. KOB2Δ1 is not able to grow on C_{12} to C_{16} n-alkanes, unlike the parent strain, and could be complemented for growth on these n-alkanes by the native (CHA0) alkane hydroxylase gene cloned in pCom5 (Table 4). For the long-chain alkane hydroxylases, *P. fluorescens* KOB2Δ1 proved to be a much better host than *E. coli* or *P. putida*. The *P. aeruginosa* PAO1, *P. fluorescens* CHA0, *M. tuberculosis* H37Rv, and *P. rugosa* NRRL B-2295 alkane hydroxylases all restored growth of *P. fluorescens* KOB2Δ1 recombinants on at least one of the n-alkanes tested, albeit with growth rates that were sometimes clearly below that of the wild-type strain *P. fluorescens* CHA0 (Tables 3 and 4). Only the *Acinetobacter* sp. ADP1 *alkM* gene did not allow any of the three hosts to grow on any of the alkanes tested. The KOB2Δ1 recombinant containing the *P. putida* GPo1 *alkB* gene did not grow on C_8 and C_{10} (known substrates of the GPo1 alkane hydroxylase), showed weak growth on n-dodecane, and did not grow on C_{14} and C_{16}.

DISCUSSION

Functional analysis of alkane hydroxylases. To increase our understanding of enzyme systems involved in aerobic alkane degradation, we have started to clone alkane hydroxylase genes from a collection of about 200 bacteria able to grow on long- and short-chain alkanes. In this study, we used PCR products obtained with highly degenerate primers described earlier and information from genome sequencing projects to clone a number of alkane hydroxylase homologs from quite diverse strains. To be able to show that these genes encode functional alkane hydroxylases, we have developed several hosts for the expression of novel alkane hydroxylases. The best activity assay (in the absence of an in vitro assay) is growth on n-alkanes. Here, we have observed that the minimum level of activity needed for growth of a recombinant on n-octane is around 10 to 20% of the full GPo1 alkane hydroxylase activity (50). Therefore, any growth of the recombinants on n-alkanes indicates a significant level of alkane hydroxylase activity.

E. coli GEc137(pGEc47ΔB) and *P. putida* GPo12(pGEc47ΔB) were found to be suitable hosts to test medium-chain alkane hydroxylase genes (oxidizing alkanes ranging from C_6 to C_{12}) (Table 3). Some recombinants derived from GPo12 are also able to grow on n-tetradecane if rhamnolipids are added but do so quite slowly. It is likely that uptake of longer n-alkanes

requires factors (for example, porins and alkane-solubilizing compounds) that are not produced or expressed by GPo12. For this reason, we constructed an *alkB* knockout mutant of *P. fluorescens* CHA0 in which the polar effects of the *alkB* gene knockout on the alkane solubilization proteins and (putative) uptake system, encoded further downstream, are reduced. This host, KOB2Δ1, does indeed allow complementation for growth on longer alkanes by alkane hydroxylases cloned from quite diverse organisms, including two gram-positive strains (Tables 3 and 4). The *Acinetobacter* sp. ADP1 *alkM* gene could not be expressed successfully in the *E. coli* or *Pseudomonas* hosts, possibly because of the markedly different distribution of positive charges near the transmembrane stretches (data not shown): about seven positive charges are located in the three periplasmatic loops of AlkM and other *Acinetobacter* sp. AlkB homologs versus, on average, only one in AlkB homologs from other bacteria. The distribution of positive charges is important for correct folding in the cytoplasmic membrane (59), and it is conceivable that AlkM does not fold properly in an *E. coli* or *Pseudomonas* membrane. In contrast, the *Acinetobacter* sp. ADP1 rubredoxin could be expressed successfully in *E. coli* and shows exactly the same charge distribution as other rubredoxins involved in alkane hydroxylation (52).

The GPo1 *alkB* gene did not allow KOB2Δ1 to grow on

TABLE 4. Growth rates of wild-type and *alkB* recombinant strains on n-alkanes

Strain	Additional gene[c]	Growth rate (h^{-1}) on[a]:		
		C_{12}	C_{14}	C_{16}
P. aeruginosa PAO1		0.036	0.069	0.1
P. fluorescens CHA0		0.019	0.019	0.053
P. putida GPo1[b]		0.063	−	−
GPo12(pGEc47ΔB)[b]	*alkB2* (PAO1)	0.021	0.011	−
	alkB (CHA0)	0.025	0.009	−
	alkB (2295)	0.033	0.010	−
	alkB (H37Rv)	0.077	0.011	−
KOB2Δ1	*alkB* (GPo1)	0.007	−	−
	alkB1 (PAO1)	0.004	0.019	0.016
	alkB2 (PAO1)	0.033	0.032	0.046
	alkB (CHA0)	0.017	0.029	0.053
	alkB (2295)	0.014	0.050	0.049
	alkB (H37Rv)	0.043	0.041	0.038

[a] C_{12}, n-dodecane; C_{14}, n-tetradecane; C_{16}, n-hexadecane; −, no growth.
[b] Rhamnolipids were added to the GPo1 and GPo12(pGEc47ΔB) cultures.
[c] See Fig. 1 for full name of original host strain.

alkanes shorter than C_{12}, possibly because the CHA0 rubredoxin and rubredoxin reductase, which we have not cloned yet, are not induced by alkanes shorter than C_{12}. The related *P. fluorescens* strain Pf0-1, the genome of which is being sequenced, possesses a rubredoxin and a rubredoxin reductase that are closely related to proteins that were shown to function in alkane hydroxylation (52), but the Pf0-1 genome does not encode an alkane hydroxylase homolog.

Structure of membrane-bound alkane hydroxylases. The above expression and complementation systems allowed us to demonstrate that homologs of the GPo1 *alkB* gene indeed encode functional alkane hydroxylases. This allows the comparison of sequences to gain insight in the structure-function relationships of this class of enzymes. The *P. putida* GPo1 alkane hydroxylase is well studied, mainly because of its potential applications in biocatalysis (61). However, our present knowledge on the structure-function relationship of this class of integral membrane oxygenases is limited to the folding topology (54) and the likely involvement of conserved histidines in the binding of the two active-site iron atoms (40). The alkane hydroxylases that were analyzed in this study show significant sequence divergence, but the membrane folding of these alkane hydroxylases appears to follow the same pattern: the six hydrophobic stretches that are likely to span the cytoplasmic membrane (54) are conserved in all sequences. The same is true for the conserved histidines (42, 43) that are essential for enzymes related to the alkane hydroxylases and are likely to contribute to the nitrogen-rich ligand sphere of the catalytic diiron center (40). It is not yet possible to identify residues involved in other aspects of alkane hydroxylase function. Residues that are involved in the binding of rubredoxin are likely to be conserved between all alkane hydroxylases, as rubredoxins can be exchanged between alkane hydroxylase systems from all strains tested (52). However, these residues cannot be distinguished from other conserved residues. Similarly, the four medium-chain-length alkane hydroxylases are too closely related to each other to distinguish residues conserved by chance from residues conserved because of functional constraints.

Organization of genes involved in alkane degradation. The organization of genes involved in alkane oxidation varies strongly among the different alkane-degrading bacteria (Fig. 1). In most strains, genes involved in alkane degradation seem to be distributed over the genome. None of the rubredoxin reductase genes is located close to an alkane hydroxylase (except in the case of *Rhodococcus erythropolis* [Whyte et al., submitted]), perhaps because they are also involved in other pathways and require a different type of regulation. In contrast, most rubredoxin genes are located immediately downstream of the alkane hydroxylase genes. Those that are located elsewhere are encoded directly upstream of rubredoxin reductase genes. Interestingly, the *alk* gene cluster around *alkB1* of *A. borkumensis* is very similar to the *alk* gene clusters of *P. putida* strains GPo1 and P1, with respect to gene organization as well as sequence. The *P. putida alk* genes have a significantly lower G+C content than the rest of the genome, and are encoded on a putative catabolic transposon (53). These comparisons suggest that the *A. borkumensis alk* genes may have ended up in this strain by horizontal gene transfer as well. However, in this strain, the alkane hydroxylase can be considered an almost

essential enzyme, as this bacterium grows on very few other carbon sources, such as pyruvate, and is found mainly in oil-contaminated seawater. Moreover, the G+C content of these genes closely resembles that determined for the total genome of *A. borkumensis* (62).

The presence of alkane hydroxylases in the genome sequences of *L. pneumophila* Philadelphia-1, *B. pseudomallei* K96243, and *P. aeruginosa* PAO1 may reflect the double nature of these organisms as pathogens and as common soil or water organisms. Alkanes are omnipresent in the environment, and microorganisms are likely to utilize these highly reduced compounds as carbon and energy sources. This explains why it is easy to isolate alkane-degrading strains from pristine soil, aquifers recently polluted with oil (36), and oil-polluted seawater. The alkane hydroxylase of *M. tuberculosis* H37Rv may be a relic of an earlier lifestyle. Alternatively, *M. tuberculosis* still has an unrecognized reservoir in the environment where alkanes are available as C sources.

Conclusions. While the previous PCR studies (43) and other data (J. B. van Beilen, T. H. M. Smits, L. G. Whyte, S. Schorcht, M. Röthlisberger, T. Plaggemeier, K.-H. Engesser, and B. Witholt, submitted for publication) show that many alkane-degrading strains possess homologs of the *P. putida* GPo1 alkane hydroxylase, this study shows that a random selection of such homologs indeed encode functional alkane hydroxylases. The three host systems constructed in this study allowed us to show that nearly all alkane hydroxylase homologs are functional and allowed us to demonstrate that they oxidize medium-chain (C_5 to C_{11}) or long-chain (C_{12} to C_{16}) *n*-alkanes. Because CHA0 contains a second alkane hydroxylase responsible for the oxidation of C_{18} to C_{28} *n*-alkanes, the KOB2Δ1 recombinants did not yield information on the ability of cloned alkane hydroxylases to oxidize *n*-alkanes longer than C_{16}. The complementation experiments show that long-chain alkane hydroxylases show little activity with medium-chain-length alkanes and vice versa, thereby providing us with a powerful selection method to change the substrate range of the cloned alkane hydroxylases and to identify residues involved in substrate binding.

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