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Cloning and functional analysis of bacterial genes involved in alkane oxidation

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Summary

Many bacteria can use linear *n*-alkanes as sole carbon and energy source for growth. In *Pseudomonas putida* (*oleovorans*) GPo1, the first step of alkane oxidation is catalyzed by an integral-membrane non-heme iron alkane hydroxylase (AlkB). The aim of this study was to clone new alkane hydroxylases for structure-function analysis. For this purpose, highly degenerate oligonucleotides were developed to amplify internal fragments of genes homologous to the GPo1 alkane hydroxylase. Many bacteria able to grow on medium (C6-C11) or long-chain (C12-C16) alkanes yielded PCR fragments encoding peptides with 37.1-100 % sequence identity to the corresponding fragment of the GPo1 AlkB. Using the PCR fragments as probes, we have cloned the *alkB* homologs from several Gram-negative and Gram-positive strains. Four recombinant host strains expressing all proteins necessary for growth on alkanes except an alkane hydroxylase and alkane-responsive expression vectors were developed for the functional analysis of the alkane hydroxylase homologs. All newly cloned alkane hydroxylases enable at least one of the hosts to grow on alkanes. Sequence comparisons and *in vivo* mutagenesis of the GPo1 *alkB* give first indications as to which amino acid residues are important for substrate binding and catalysis.

Zusammenfassung

Viele Bakterien wachsen auf *n*-Alkanen. Der erste Schritt des Alkanmetabolismus wird in *Pseudomonas putida* (*oleovorans*) GPo1 von einer membrangebundenen nicht-Häm-Eisen Alkan-Hydroxylase (AlkB) katalysiert. Das Ziel dieser Studie war es, neue Alkan-Hydroxylasen zu klonieren beziehungsweise Struktur-Funktionsbeziehungen zu analysieren. Dazu wurden, mittels hoch degenerierter Oligonucleotiden, interne Genfragmenten amplifiziert, welche der GPo1 Alkan-Hydroxylase Gensequenz ähnlich sind. Aus einer Vielzahl von Gram-negativen und Gram-positiven Bakterien die auf mittellangen (C6-C11) oder langkettigen (C12-C16) Alkanen wachsen, konnten solche PCR-Fragmenten, deren Proteinsequenzen 37.1-100 % Identität zu dem korrespondierenden Teil der GPo1 Alkan-Hydroxylase haben, erhalten werden. Mittels PCR-Fragmenten als Sonden für das Southern und Colony Blotten wurden homologe *alkB* Gene aus fünf Gram-negativen und zwei Gram-positiven Bakterien kloniert. Zur Bestätigung, dass die homologe Alkan-Hydroxylasen auch wirklich Alkanen oxidieren, wurden vier Wirt-Stämme entwickelt, welche mit Ausnahme der Alkan-Hydroxylase alle für das Wachstum auf Alkanen notwendig Proteine exprimieren. Die Funktion der neu klonierten Alkan-Hydroxylasen wurde in den vier Wirt-Stämme erfolgreich getestet. Sequenzanalysen und *in vivo* Mutagenese des GPo1 *alkBs* ergaben zudem erste Hinweise auf die für Substratbindung und Katalyse wichtige Aminosäuren.

Abbreviations

Ap	ampicillin
bp	base pair(s)
C5	pentane
C6	hexane
C8	octane
C10	decane
C11	undecane
C12	dodecane
C14	tetradecane
C16	hexadecane
C18	octadecane
C20	eicosane
C24	tetracosane
C28	octacosane
C30	triacontane
C32	dotriacontane
Cm	chloramphenicol
(k)Da	(kilo)dalton
DCPK	dicyclopropylketone
DMMZ	Department of Medical Microbiology Zürich
DOPh	dioctylphthalate
EMBL	European Molecular Biology Laboratory
dNTP	deoxynucleotide triphosphate
FAD	flavin adenine mononucleotide
G+C	guanine-plus-cytosine
Gm	gentamycin
HPLC-MS	high-performance liquid chromatography-mass spectroscopy
IPTG	isopropyl-ß-D-thiogalactopyranoside

kb	kilobasepairs
Km	kanamycin
LB	Luria-Bertani broth
MALDI-TOF	matrix-assisted laser desorption/ionisation, time-of-flight mass spectrometry
MCP	methyl-accepting chemotaxis protein
MT	metal traces
NAD(H)	nicotinamide adenine dinucleotide (reduced form)
NCBI	National Centre for Biotechnology Information
OMP	outer membrane protein
ORF	open-reading-frame
PCR	polymerase chain reaction
SDS-PAA	sodium dodecyl sulfate-polyacrylamide
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Tc	tetracycline
v/v	volume per volume
w/v	weigth per volume

x

Chapter 1

INTRODUCTION

Theo H. M. Smits

1. INTRODUCTION

1.1 Origin of hydrocarbons in the environment

Hydrocarbons are ubiquitous organic compounds consisting of carbon and hydrogen atoms. Many hydrocarbons like alkanes, terpenes and aromatic compounds are produced by biological or geochemical processes, while others are produced by synthetic organic chemistry processes.

Plants produce *n*-alkanes as natural constituents of waxes, which also contain very-long-chain fatty acids, alcohols, ketones, esters, triterpenes, sterols and flavonoids (212). The amount of *n*-alkanes present in plant waxes varies from trace amounts to being the major constitutent of the wax. Plant *n*-alkanes are mainly very-long odd-chain length *n*-alkanes, varying between 25 and 37 carbon atoms, though even-chain alkanes are as well found (54). As each plant wax has a different composition, this feature can be used as a marker to determine the species composition of complete plant communities (54).

The greenhouse gas methane is produced in large quantities by microbial decomposition of organic material under anoxic conditions, e.g. in landfills, marshlands and oceans. It also occurs in many coal formations and is a major constituent of natural-gas deposits. Largely due to human activities over the last 200 years, the atmospheric concentration of methane has doubled (166).

A large amount of hydrocarbons ends up in nature by human activities. Annually, around 35 million metric tons of petroleum enters the sea. The major part of this oil is not released by large tanker accidents, which constitute 'only' one million metric tons per annum, but comes from municipal and industrial wastes and runoffs, leaks in pipelines and storage tanks and discharge of dirty ballast and bilge waters. Oil input to the sea from natural sources, principally seepages, only accounts for 0.5 million tons annually (218). The composition of mineral oil is dependent on the oil well (206). Mineral oil generally consists of paraffins, cycloalkanes (naphthenenes), aromatics and other compounds (e.g. resins and asphaltenes) (101).

1.2 Biodegradation of hydrocarbons

Many petroleum hydrocarbons are readily degraded by microorganisms. However, the oil components are degraded differentially depending on structure and molecular weight. Aliphatic paraffins are more readily degraded than aromatic hydrocarbons. Saturated compounds are degraded more readily than unsaturated compounds, and branched chains are decomposed less readily than straight chain compounds (37).

The residence time of individual oil compounds reflects the biodegradability. Particularly the polycyclic aromatic hydrocarbon (PAH) fraction of petroleum is not easily degraded. Its recalcitrance is directly proportional to the molecular weight of the compound, mainly due to the low solubility and availability for biological uptake (9). Microbial degradation rates are determined by the balance of the substrate uptake and substrate transfer to the cells. The substrate transfer in turn is limited by the desorption of the substrate from the soil matrix. As degradation rates of PAH are low, halflife times of individual compounds vary between less than one year and infinite (105).

1.2.1 Microbiology of oil spills

The presence of hydrocarbons in the environment frequently brings about an *in situ* selective enrichment for hydrocarbon-utilizing microorganisms. The relatively low level of hydrocarbon degraders in pristine soil can increase by three orders of magnitude directly after an oil spill, and then decreases slowly (218). In nature, biodegradation of hydrocarbons predominantly occurs by mixed populations and in the presence of other organic compounds. However, studies on mixed cultures are rare. One example is a recent study, which showed that alkane-degrading yeasts can overgrow bacteria in sandy soil (225). Numerous hydrocarbon-degrading species belonging to the bacteria, yeasts, fungi have been isolated in the past (37). Enrichments of microorganisms from pristine and polluted soil yield isolates with varying substrate specificities (217). These isolates are usually Gram-positives or pseudomonads, while members of other bacterial species are found less frequently.

1.2.1.1 Genetic approaches to assess degradation potential

Several authors have tried to find a relation between biodegradation capacity and the presence of genes coding for aromatic and aliphatic degradation pathways using known gene fragments as probes for Southern or colony blotting (98, 243, 247). It was found that the *xylE* gene, encoding the aromatic ring-cleaving enzyme catechol-2,3-dioxygenase, occurs in large populations of oil-degrading bacteria isolated from contaminated aquifers (98, 243). Other gene probes also give specific signals, but at much lower levels. One such probe is the alkane hydroxylase gene *alkB* of *P. putida* (*oleovorans*) GPo1 (see section 1.3.4.1), which was found in up to two third of the culturable organisms after enrichment on crude oil (243). The results indicated that hexadecane mineralization is highly correlated to the presence of *alkB*. However, other authors found that hybridization only occurred when strains were able to grow on decane (247, 273).

1.2.2 Anaerobic degradation of hydrocarbons

It has long been assumed that degradation of hydrocarbons can not take place under anaerobic conditions. However, over the last ten years several microorganisms have been isolated that are able to degrade aromatic and aliphatic hydrocarbons in the absence of oxygen. Most of these organisms are denitrifying or sulphate reducing and belong to the ß- and ∂-subclasses of proteobacteria (reviewed in (108)).

The initial attack of the anaerobic degradation of aromatic compounds does not involve an oxygen-dependent step, but is achieved by coupling a cellular metabolite (for toluene succinate) to the hydrocarbon. Genes encoding the initial enzyme activity for toluene degradation have been cloned and sequenced. This mechanism is general for all anaerobic toluene degraders (108).

The anaerobic degradation of *n*-alkanes has been reported for alkanes in the range between hexane and eicosane, although only a few pure cultures of anaerobic alkane degrading bacteria were obtained (2, 71, 242), all of which belong to the ∂-subclass of proteobacteria. The biochemical basis of anaerobic alkane metabolism is still poorly understood. Dehydrogenation of the alkane to a 1-alkene and hydratation to an 1-alcohol as described for *P. aeruginosa* 196Aa (205) most probably was an artefact (11). Based on

the fatty acid pattern of strain Hxd3, which shows formation of odd-chain fatty acids from even-chain alkanes, a carboxylation or carbonylation reaction has been proposed (2). This would then correspond to a reversed mechanism of the alkane biosynthesis pathway in plants (212). A different mechanism found in the sulfate reducing strain AK-01 involves the coupling of an unknown component to the alkane resulting in a fatty acid, which can then be found back in the cellular fatty acids or is degraded via the ß-oxidation (241). Recently, it was shown for a sulfate-reducing, dodecane-grown bacterial enrichment culture, that fumarate was coupled to the dodecane molecule, forming dodecylsuccinnic acid (146).

1.2.3 Degradation of aromatic hydrocarbons

Several pathways are known for the aerobic degradation of aromatic compounds. These pathways have been well characterized both on the biochemical and the genetic level. The TOL plasmid encodes the *xyl*-genes which oxidize toluene and its derivates. These genes are divided in the upper and lower pathway (38). The upper pathway encodes the xylene monooxygenase XylMA, a benzylalcohol dehydrogenase XylB and the benzaldehyde dehydrogenase XylC (102, 251). The lower pathway genes *xylXYZLTEGFJQKIH* encode the enzymes of the meta-pathway, responsible for the subsequent degradation of benzoic acid, the end product of the upper pathway (38). Here, the ring is oxidized to form a catechol, which is subsequently opened to give intermediates that enter the central metabolism. A second pathway for the degradation of benzoate is the ortho-pathway or ß-ketoadipate pathway, which in the case of *P. putida* mt-2 is chromosomally located (182).

A large number of aromatic compounds is initially oxidized to a dihydrodiol by dioxygenases. These enzymes form a large gene family of multicomponent Rieske non-heme iron oxygenases (39, 93), of which toluene dioxygenase (encoded by *todABC1C2*)(94), benzene dioxygenase (encoded by *bedABC1C2*)(252) and naphthalene dioxygenase (encoded by *nahAaAbAcAd*)(237) can be considered as prototype enzyme systems. The dihydrodiol is subsequently converted to a catechol, after which a ring opening takes place similar to that of the xylene lower pathway.

1.2.4 Degradation of alicyclic hydrocarbons

Although cyclic alkanes are a major component of petroleum, few bacteria have been isolated that can degrade these components (217). The failure to enrich strains able to grow on cyclic alkanes led to the conclusion that in nature these substrates are utilized via cooxidation and commensalism (101). However, several pure cultures have been isolated over the past years (206, 227)(van Beilen, unpublished results).

The initial reaction of cyclic alkane degradation usually involves an oxidation of the ring-system, yielding a cyclic alcohol. A dehydrogenation step yields the keton, which is subsequently oxidized by a Baeyer-Villiger monooxygenase. The resulting lactone is hydrolysed spontaneously or by an esterase, yielding an ω-hydroxy fatty acid, which can be further metabolized (48, 206).

The two key steps in cycloalkane oxidation are the ring-oxidation and the Baeyer-Villiger reaction. The initial oxidation of the ring is done by a monooxygenase. Several alkane-oxidizing strains can oxidize cyclic compounds when the alkane oxidation system is induced (259, 264), although these strains do not grow on cyclic compounds, because downstream metabolizing enzymes are not present.

The Baeyer-Villiger monooxygenase is an oxygen-dependent monooxygenase which breaks a carbon-carbon bond and inserts one atom of oxygen. Baeyer-Villiger monooxygenases have been isolated from several species belonging to the bacteria and fungi (48, 254, 279). Recently, several Baeyer-Villiger monooxygenase genes were cloned from organisms able to grow on cycloalkanes (36, 47)(J. B. van Beilen, unpublished results).

1.3 Aerobic degradation of linear aliphatics

The degradation and bioconversion of aliphatic hydrocarbons has received a great deal of attention from microbiologists and biochemists for many decades. The capacity of microorganisms to use aliphatic hydrocarbons (alkanes) as carbon and energy source is very common, and a large number of species beloning to bacteria, yeasts and fungi have been described to utilize alkanes. Some excellent reviews on this field have been published in the past (31, 37).

1.3.1 Pathways for alkane oxidation

The degradation pathway of n-alkanes generally starts with oxidation of the alkane to an alcohol. In most cases, this takes place at the terminal carbon atom, but subterminal oxidation of alkanes has also been described (section 1.3.5). Several enzyme systems can perform this initial attack. In yeasts, but also in some bacteria, cytochrome P450s perform the initial monooxygenation (section 1.3.2), while for some bacteria integral-membrane and soluble non-heme iron alkane hydroxylases have been described (sections 1.3.3 and 1.3.4).

The terminal alcohol, the product of the first monooxygenase reaction, is converted to a fatty acid via an aldehyde. The fatty acid is subsequently activated with Co-enzyme A (CoA) by an acyl-CoA synthetase, and enters the ß-oxidation cycle. The end product of the ß-oxidation cycle is acetyl-CoA, which can enter the Krebs cycle to yield carbon dioxide and energy (23). Alternatively, the fatty acids are directly incorporated into the phospholipids, where they reflect the chain-length on which the strain is grown: odd-chain-length alkanes will yield more odd-chain fatty acid moieties in the phospholipids, while even-chain-length alkanes yield a higher amount of even-chain fatty acid moieties. The ratio between saturated and unsaturated fatty acid moieties in the phospholipids shifts more towards saturated fatty acids when n-alkanes are used as carbon source (63).

1.3.2 Cytochrome P450 systems

Cytochrome P450 enzymes are ubiquitous in nature (180). While broad substrate spectrum cytochrome P450s in mammals oxidize many xenobiotics and steroids, cytochrome P450s in microorganisms can have a more narrow substrate specifity (130). Bacterial P450s often occur in catabolic pathways, catalysing the initial hydroxylation reaction of the growth substrate (180).

Alkane-oxidizing cytochrome P450s are nearly exclusively found in yeasts, but some reports describe the presence of alkane-oxidizing cytochrome P450s in bacteria. In the yeasts *Candida rugosa* and *Yarrowia lipolytica*, several orthologs have been found, but only few of those are proven to be involved in alkane oxidation (122, 196). The *Candida maltosa* cytochrome P450 52A3, which is involved in the initial oxidation of n-alkanes to

alcohols, can also catalyze more than one step, yielding also aldehydes, fatty acids, α-ω-diols, ω-hydroxy-fatty acids and α-ω-dicarboxylic acids (221). Bacterial cytochrome P450s involved in alkane oxidation are identified in *Rhodococcus rhodochrous* ATCC 19067 (44) and in *Acinetobacter calcoaceticus* EB104 (179).

1.3.3 Non-heme iron oxygenases

The large group of non-heme iron oxygenases can be divided in two subgroups: the short-chain non-heme iron monooxygenase systems and the integral-membrane non-heme iron oxygenases.

1.3.3.1 Short-chain non-heme iron monooxygenase systems

Several short-chain hydrocarbon oxidation systems have been characterized in great detail. Several enzyme systems can be distinguised: soluble and particulate methane monooxygenases and alkene monooxygenases.

Methane is oxidized to methanol by either a soluble or a particulate methane monooxygenase (MMO). While virtually all methanotrophs studied to date possess a particulate MMO, soluble MMOs are less widespread (181).

The two most extensively characterized soluble MMO enzymes are those from *Methylococcus capsulatus* (Bath) and *Methylosinus trichosporium* OB3b (reviewed in (160)). Soluble MMO is a non-heme iron-containing enzyme complex consisting of three components: a hydroxylase, the so-called Protein B, which serves an "effector" or regulatory role, and a reductase. The hydroxylase consists of three subunits arranged in an $\alpha_2\beta_2\gamma_2$ configuration. The α-subunit contains a non-heme bis-μ-hydroxo-bridged binuclear iron centre where methane an oxygen interact to form methanol at the active site of the enzyme (181). The diiron centre in soluble MMO is coordinated by a conserved amino acid sequence motif including conserved glutamate or aspartate and histidine residues ($EX_{\sim 40}$-(D/E)EXRH-$X_{\sim}100$-$EX_{\sim 40}$-(D/E)EXRH)(33). This enzyme system is expressed only when the copper-to-biomass ratio is low. Soluble MMO has an extremely broad substrate specificity and can oxidize a wide range of non-growth substrates such as alkanes, alkenes and aromatic compounds.

Particular MMO is mainly expressed in methanotrophs under conditions where the copper-to-biomass ratio is high. The membrane-bound enzyme system consists of three subunits, and contains two iron and approximately 15 copper atoms per mol. Copper-binding compounds are likely to be involved in the stabilization of the enzyme. Particular MMO has a relatively narrow substrate range, oxidizing alkanes and alkenes of up to 5 carbons but not aromatic compounds (181).

Xanthobacter sp. Py2 is able to oxidize short-chain alkenes such as propene to epoxides, but is not able to oxidize similar chain-length alkanes (267). The enzyme system consists of four components (238): a monomeric reductase, a ferredoxin, an $\alpha_2\beta_2\gamma_2$ structured epoxygenase and a coupling protein are required for full activity.

The alkene monooxygenase system of *Rhodococcus rhodochrous* (formerly *Nocardia corallina*) B-276 consists of a two-component epoxygenase, a coupling protein and a reductase component (178). Whole cells of *R. rhodochrous* B-276 can enantioselectively epoxidize a variety of alkenes, but can not hydroxylate alkanes (89).

Both epoxygenases mentioned above have a binuclear non-heme iron center similar to that of methane monooxygenases (90, 291) with iron coordination via glutamate/aspartate and histidine. Based on the homology of the monoxygenase component of these enzyme systems and the coordination of the non-heme iron center, a large family of soluble non-heme iron oxygenases can be defined which also includes the benzene- (*bmo*), toluene-4- (*tmo*), toluene/benzene- (*tbu*) and phenol (*phl*) monooxygenases and soluble acyl-ACP desaturases (232, 292).

1.3.3.2 Non-heme iron integral membrane oxygenases

All members of the non-heme iron integral membrane oxygenases oxidize relatively hydrophobic substrates using one atom oxygen originating from molecular oxygen. The other oxygen atom is reduced to water using two electrons from NAD(P)H. This group includes a large number of structurally similar enzyme families (232): alkane monooxygenases (143, 207, 213), xylene monooxygenase and related enzymes (66, 124, 251), fatty acid modifying enzymes such as double- and triple-bond forming desaturases, fatty acid monooxygenases and epoxidases (35, 156, 233), steroid oxygenases (19, 162),

ß-carotene hydroxylases and ketolases (120, 128, 176, 177) and aldehyde decarbonylases (1).

The electrons necessary for the monooxygenation reaction come from NADH or NADPH. These are transferred to the monooxygenase by either ferredoxin and ferredoxin reductase or cytochrome $b5$ in the case of fatty acid desaturases (233), rubredoxin and rubredoxin reductase in the case of alkane hydroxylases (91, 147) or a ferredoxin-ferredoxin reductase fusion protein for xylene monooxygenases (66, 124, 251).

Three, four or six membrane spanning elements can be identified in all primary amino acid sequences of proteins belonging to the membrane-bound non-heme iron oxygenases, with varying numbers of amino acids between each transmembrane helix (233, 261). All members of this family also contain eight highly conserved histidine residues which are most probably involved in iron coordination. They are grouped in three sequence motifs ($HX_{3-4}H$, $HX_{2-3}HH$, $HX_{2-3}HH$) and are essential for the function of these enzymes (233)(M. W. Wubbolts, personal communication). For the *Pseudomonas putida* mt-2 xylene monooxygenase XylM, a ninth essential histidine residue was identified by site-directed mutagenesis (M. W. Wubbolts, personal communication). This histidine residue is also conserved in all proteins that can be classified as integral-membrane non-heme iron monooxygenases.

It is not known which residues are involved in the substrate specificity of the membrane-bound non-heme iron enzymes. For the soluble fatty acid desaturases, the mutagenesis of five amino acids was sufficient to change the position at which the double bond was formed (40). Replacement of seven amino acids in one of the membrane-bound enzymes converted a strict desaturase to a bifunctional desaturase-hydroxylase comparable in activity to a unmodified hydroxylase (35).

The *Pseudomonas putida* (*oleovorans*) GPo1 alkane hydroxylase (207) is a prototype of the integral-membrane non-heme iron monooxygenases. The enzyme contains six membrane spanning segments (261). Mössbauer studies have shown that an exchange-coupled dinuclear iron cluster is present in the protein of the type present in soluble non-heme iron proteins (231). It was proposed that all integral-membrane non-heme iron proteins contain these diiron clusters.

1.3.4 Non-heme iron integral membrane alkane monooxygenases

1.3.4.1 The *P. putida* (*oleovorans*) GPo1 alkane hydroxylase system

The most thoroughly described microorganism growing on *n*-alkanes is *Pseudomonas putida* (*oleovorans*) GPo1, previously named *P. oleovorans* GPo1 (228, 260). This organism is able to oxidize linear alkanes ranging from pentane to dodecane by virtue of the alkane hydroxylase system (18). In addition to the hydroxylation of aliphatic and alicyclic compounds (158, 173, 259), the alkane hydroxylase system has been shown to catalyse: the oxidation of terminal alcohols to the corresponding aldehydes; demethylation of branched methyl ethers; sulfoxidation of thioethers, and epoxidation of terminal olefins (131, 132, 171, 172). Although the substrate range of the *P. putida* (*oleovorans*) GPo1 (TF4-1L) alkane hydroxylase system has been investigated in detail, it is not clear to what extent it is determined by factors such as host strain, substrate solubility, uptake and regulation.
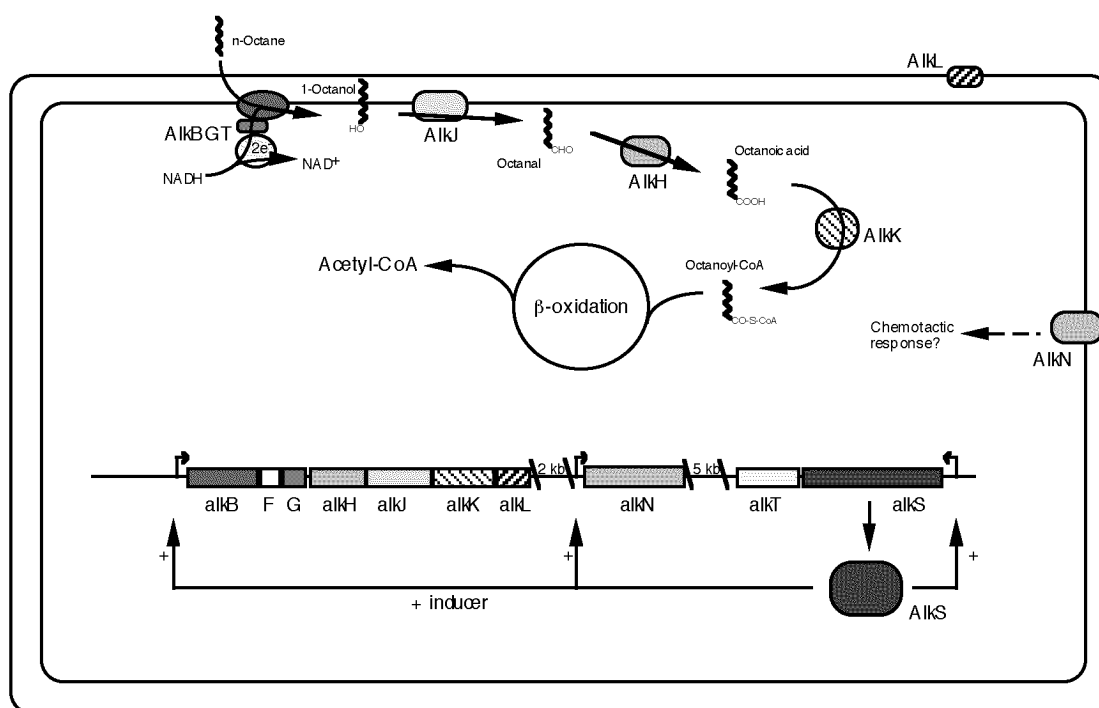

Figure 1.1: alkane degradation by *Pseudomonas putida* (*oleovorans*) GPo1.

All genes involved in the conversion of alkanes to CoA-activated fatty acids are encoded by two gene clusters (figure 1.1), located on the OCT-plasmid (20, 45, 73). The gene clusters were cloned in pLAFR1 as 18 kb and 16.9 kb *Eco*RI fragments, to create pGEc47 (69, 260). Minicell experiments and partial sequencing revealed that the 18 kb fragment contains an operon named *alkBFGHJKL*, which encodes two components of the alkane hydroxylase system (the alkane hydroxylase AlkB and the rubredoxin AlkG) and enzymes involved in further metabolic steps (70, 142, 143, 258). The 16.9 kb fragment encodes the third component of the alkane hydroxylase system (the rubredoxin reductase AlkT) and AlkS, which regulates expression of the *alkBFGHJKL* operon (68, 202). The start of the *alkBFGHJKL* operon was mapped by S1 nuclease protection studies (42, 143), while the *alkS* gene itself is under control of a σ^s-dependent promoter, which allows low levels of transcription during the exponential phase, and considerably increased levels in the stationary phase (43). Recently, an overlapping AlkS-dependent promoter, which positively controls the expression of the *alkS* gene was identified as well (42). Interestingly, the G+C content of the *alk* genes of *P. putida* (*oleovorans*) GPo1 is only 45 %, which is clearly lower than the G+C content of DNA around the *alk* genes and the OCT-plasmid as a whole (74, 143, 202). This suggests that the *alk* genes originate from a low G+C host, and entered the OCT-plasmid as a transposon. Evidence for this hypothesis was found in the flanking regions of the *P. putida* (*oleovorans*) GPo1 and *P. putida* P1 *alk* genes, the latter strain containing *alk* genes closely related to those of *P. putida* (*oleovorans*) GPo1 (260).

1.3.4.2 Alkane hydroxylase systems related to the *P. putida* (*oleovorans*) GPo1 *alk*-system

Biochemical studies on *P. aeruginosa* strains isolated in the 60's and 70's describe enzyme systems that are similar to those from *P. putida* (*oleovorans*) GPo1 (reviewed in (269)). These strains are able to grow on medium-chain alkanes, but also grow on long-chain alkanes. Chemical mutants of *P. aeruginosa* ATCC 17423 were no longer able to grow either on medium-chain alkanes or on long-chain alkanes (186). It was therefore concluded that *P. aeruginosa* ATCC 17423 contains more than one alkane hydroxylase

system. More recently, the medium-chain alkane-degrading *P. aeruginosa* strains were shown to contain *alk* systems (nearly) identical to that of *P. putida* (*oleovorans*) GPo1 (262). The GPo1 *alkB* gene probe did not detect any specific signal in Southern blots with chromosomal DNA from the strictly long-chain alkane degrader *P. aeruginosa* PAO1 (262)(chapter 6).

Several other strains able to degrade long-chain alkanes were reported to contain enzyme systems similar to that of *P. putida* (*oleovorans*) GPo1 (reviewed in (263)), such as *Acinetobacter calcoaceticus* 69-V (49, 138), where a particulate monooxygenase, an alkane-inducible rubredoxin and a rubredoxin reductase were found. The alkane hydroxylase system of *Acinetobacter* sp. ADP1 was cloned (91, 213). The alkane hydroxylase, called AlkM, has 41 % sequence identity on amino acid level to the *P. putida* (*oleovorans*) GPo1 AlkB (213), and is regulated by AlkR, the positive regulator of *alkM* (214), which is encoded directly upstream of *alkM* (213). It was shown that, although the regulator AlkR is inducible by a broad range of alkanes, the substrate range of the strain was determined by the substrate range of the alkane hydroxylase (214). The rubredoxin *rubA* and rubredoxin reductase *rubB* genes are not encoded in close proximity to the alkane hydroxylase gene (91), and form an operon together with the two downstream genes *estB* and *oxyR* (92). A subunit of the general secretory pathway, XcpR, was found to be necessary for the degradation of dodecane of *Acinetobacter* sp. ADP1, as deletion impaired growth on hexadecane and abolished growth on dodecane (204). This protein was shown to be necessary for general protein secretion, as lipase and esterase were no longer secreted. The *rubAB* and *xcpR* genes are constitutively expressed (91, 204).

1.3.4.3 Catalytic model of the alkane hydroxylase

Three classes of diiron clusters have been recognized: class I as is present in hemerythrin, class II as in ribonuclease reductase R2, methane monooxygenase and soluble desaturases, and class III as in membrane-bound non-heme iron proteins (33). The iron molecules in the latter class are most probably coordinated by the eight histidine residues,

as concluded from the isomer shifts of reduced alkane hydroxylase in Mössbauer studies (231).

For the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1, a radical mechanism has been proposed (12, 85, 86) (figure 1.2). First, a high-valence Fe^{IV}-oxo intermediate is generated (figure 2). This activated oxygen attacks a carbon atom and extracts a H•, leaving a carbon radical. This radical subsequently attacks the Fe^{III}-O bond, to form the C-OH bond. The second electron of the Fe^{III}-O bond shifts to the neighbouring iron, to form back the initial situation. During the oxidation of terminal olefins, the activated oxygen attacks the substrate from the *re* face, to give mainly the *R* enantiomers with enantiomeric excesses ranging from 70 to 100 % (85, 86).
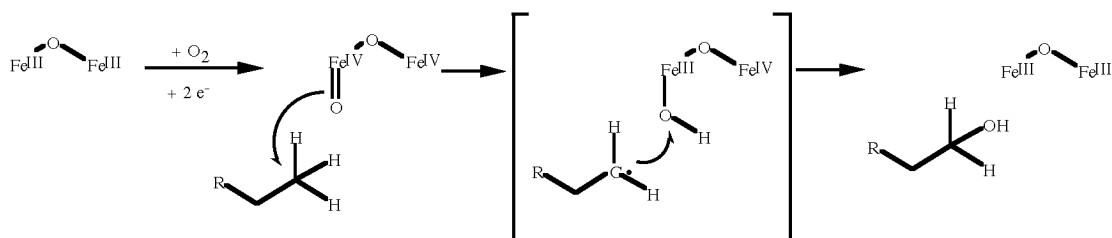
Figure 1.2: catalytic model for alkane hydroxylases.

1.3.5 Subterminal oxidation of alkanes

Most enzymes oxidizing *n*-alkanes catalyze the reaction at the 1-position of the *n*-alkane (see above). However, subterminal oxidation of *n*-alkanes also takes place. It is conjecturable what is the overall importance of subterminal oxidation compared to terminal oxidation (169).

The subterminal oxidation of alkanes yields secondary alcohols and ketones as products (84, 169). *P. aeruginosa* NCIB 9904 produces significant amounts of subterminal products in addition to products of terminal oxidation (84). The pathway for degradation of tridecane by *Burkholderia cepacia* and *P. aeruginosa* Sol 20 included a primary

oxidation of the alkane to the 2-alcohol, which subsequently was dehydrogenated to 2-tridecanone (82). This in turn is oxidized by a Baeyer-Villiger monooxygenase to 1-undecyl acetate (32, 81, 83), which is the substrate for the undecyl acetate esterase, yielding 1-undecanol and acetate (234, 235). Undecanol is further degraded to a fatty acid, which enters the ß-oxidation cycle.

1.4 Alkane oxidation and biocatalysis

Alkane hydroxylases are of interest for biocatalysis, because they perform the regio- and stereospecific oxidation of non-functionalized hydrocarbons (281). The products are valuble compounds e.g. as precursors for the manufacturing of complex organic molecules like pharmaceuticals or other other fine chemicals (89).

The alkane hydroxylase of *P. putida* (*oleovorans*) GPo1 has a broad substrate specificity (259). Due to this versatility, many compounds can be produced. The enzymatic oxidation of methyl groups on aromatic heterocycles has been patented by Lonza AG (137). Li *et al.* (158) described the production of optically active *N*-benzyl-3-hydroxypyrrolidine using *P. putida* (*oleovorans*) GPo1 and other alkane-oxidizing strains. One of these strains, *Sphingomonas* sp. HXN-200, can be used to produce alicyclic alcohols with a very high regio- and stereospecificity when the strain is pregrown on octane (46, 158).

Other alkane-degrading strains used in commercial production of oxidized products are *Rhodococcus erythropolis* NRRL B-16531 for the production of 2-phenyl-1-propionic acid, an intermediate of pharmaceuticals as ibuprofen, and *Rhodococcus rhodochrous* NCIMB12566 for the production of phenoxy propanoic acids, which are intermediates for the production of herbicides (119).

Several problems limit the use of alkane hydroxylases for biocatalysis. First, most of the substrates are only poorly water soluble. This problem could be solved by the addition of (bio)surfactants (see section 1.5; chapter 8) or two-liquid phase systems (29, 222, 268). As most alkane hydroxylases are membrane-bound multi-component enzyme systems requiring the cofactor NADH for catalysis, the use of purified enzymes in enzyme reactors will be limited (222). This would make it necessary to use whole-cell systems for

the production of target compounds. Here, a suitable host is required to avoid degradation of the product by downstream metabolism.

1.5 Additional factors involved in alkane oxidation

As described above, the oxidation of alkanes takes place at or in the cytoplasmic membrane. One of the limiting factors in growth on alkanes is the solubilization and uptake of n-alkanes. The solubility of n-alkanes decreases with the chain-length of the alkanes. Medium-chain n-alkanes are soluble to such an extent that the aqueous phase concentration is sufficient that the alkanes can simply traverse the outer membrane of a cell (245). However, this may not be true for long-chain alkanes.

Generally, two modes of uptake have been considered: interfacial accession and biosurfactant-mediated hydrocarbon uptake (28). The interfacial accession requires a cell to have a hydrophobic cell surface, allows direct contact to substrate droplets. Culture supernatant fluids have a high surface tension. This mechanism is widespread e.g. among the rhodococci and *Acinetobacter* spp., although in some cases it is combined with biosurfactant production (biosurfactant-enhanced interfacial uptake). Here, cells have a high or medium hydrophobicity, but culture fluids have a low to medium surface tension. For biosurfactant-mediated hydrocarbon uptake, the microorganism has contact with so-called accommodated or solubilized hydrocarbons. In this case, the surface tension of culture supernatant fluids is low, and cells remain hydrophilic (28). This mechanism is generally used by *Pseudomonas* spp., but also by other genera.

1.5.1. Biosurfactants

Surfactants are amphiphatic molecules with both hydrophilic and hydrophobic (generally hydrocarbon) moieties that partition preferentially at the interphase between fluid phases with different degrees of polarity and hydrogen bonding such as oil/water or air/water interfaces. Biosurfactants are a structurally diverse group of surface-active molecules synthesized by microorganisms (77). A biosurfactant may have one of the following structures: mycolic acid, glycolipids, polysaccharide-lipid complex, lipoprotein or lipopeptide, phospholipid or the microbial cell surface itself (129). These molecules

reduce surface and interfacial tensions in both aqueous solutions and hydrocarbon mixtures. Biosurfactants have several advantages over chemical surfactants, such as lower toxicity, higher biodegradability, better environmental compatibility, stronger foaming, high selectivity and specific activity at extreme temperatures, pH and salinity (57). Potential applications of biosurfactants include enhanced oil recovery, crude oil drilling, lubricants, surfactant-aided bioremediation of water-insoluble pollutants, health care and food processing (17).

Several recent reviews cover the genetics, biochemistry, production and possible applications of biosurfactants (17, 77, 129, 165). Here, two specific biosurfactants that play a role in this thesis are discussed in more detail.

1.5.1.1 Rhamnolipids

The rhamnose-containing glycolipid biosurfactant rhamnolipids are produced by *Pseudomonas aeruginosa* strains (59, 193, 265). Two major glycolipids are produced in liquid cultures: L-rhamnosyl-L-rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate and L-rhamnosyl-ß-hydroxydecanoyl-ß-hydroxydecanoate, referred to as rhamnolipids 1 and 2 (Rhl 1 and Rhl 2) respectively. Two more products having one ß-hydroxydecanoyl moiety have also been described, although these may represent degradation products derived from rhamnolipids 1 and 2 (193). Rhamnolipids have two modes of action by which they enhance the degradation of hydrocarbons. First, they increase the solubility of hydrocarbons, thereby increasing the bioavailability (290). The second mode is by interacting with the bacterial cell, causing the cell surface to become more hydrophobic and to associate more easily with hydrophobic substrates (289). The mechanism of this interaction is by extracting LPS from the outer membrane, increasing the relative hydrophobicity of the cell (3). The second mode of action may be more important for *in situ* bioremediation of hydrocarbons, as, in contrast to solubilization, only low rhamnolipid concentrations are required to alter the cell surface (165).

The biosynthesis of rhamnolipids has been described in detail (41, 192, 194, 195). In short, precursors are withdrawn from the ß-oxidation by RhlG (41) and coupled to two rhamnosyl units from thymidine-diphospho-L-rhamnose by the rhamnosyltransferases 1

and 2, encoded by *rhlAB* and *rhlC*, respectively (165, 192). Rhamnolipid biosynthesis is regulated by RhlR, a LuxR-type regulator (194), which in turn is activated by the autoinducer synthetase RhlI (195). The RhlR/RhlI system is part of a very complex regulatory network also regulating the expression of virulence-associated traits (30, 165).

1.5.1.2 "Protein activator of alkane oxidation"

In the case of *Pseudomonas aeruginosa*, the so-called "protein activator of alkane oxidation", PA, plays a role. This protein was isolated first from *P. aeruginosa* S_7B_1, as activator of the alkane hydroxylation activity in oxygen-uptake studies (114). The protein, which has a weak emulsifying power, is exported in large amounts (between 50 an 120 μg/ml) to the culture liquid. It has a role especially in the initial growth phase on hexadecane (113). The gene encoding PA was cloned from *P. aeruginosa* PG201 and a knockout mutant was made (103). This mutant grew only slightly slower on hexadecane than the wildtype, which proves that it has a less dominating role in hexadecane assimilation as rhamnolipids.

1.5.2 Uptake

A major barrier for uptake of the hydrophobic substances is the cell envelope, which consists of a cell wall and one or two lipid membranes. Gram-negative bacteria have, in addition to the cytoplasmic membrane, an outer membrane that consists of phospholipids and lipopolysaccharides (LPS). The two membranes are separated by a thin peptidoglycan layer (190). Due to the presence of the lipophilic LPS, the outer membrane shows a very low permeability toward hydrophobic compounds. Low specificity porins in the outer membrane form water-filled channels which allow small polar solutes with a molecular mass smaller than 600 Da to diffuse passively across the outer membrane (189). The cytoplasmic membrane shows a low permeability for polar and charged molecules, but apolar compounds can easily penetrate the lipid bilayer (236). Specific transport processes could play a role in the mineralization of low concentrations of hydrocarbons, but the transport activities may not easily be revealed due to passive diffusion of the compounds. The general opinion is thus that uptake of hydrocarbons is a

passive process, i.e. they dissolve in the cell membrane. The cell membrane of bacteria represents a hydrophobic phase which accumulates nonpolar compounds from the surrounding aqueous phase (236).

Recent studies have shown the presence of genes encoding outer membrane proteins (OMPs) in hydrocarbon catabolic operons (125, 127, 274). Some of these OMPs are homologous to the *E. coli* FadL protein, which allows specific transport of long-chain fatty acids across the outer membrane (22). By analysing the expression levels of *tbuX*, involved in toluene utilization by *Ralstonia pickettii* PKO1, it was speculated that the protein is always present at low levels, and that the protein levels increase significantly upon exposure to toluene (127). Although deletion of the *tbuX* gene resulted in a significantly impaired growth of the mutant on toluene, the exact role of the protein has not been shown. In the *alk*-system of *P. putida* (*oleovorans*) GPo1, an outer membrane protein (AlkL), which is homologous to OmpW of *Vibrio cholerae*, was found. However, *alkL* negative mutants did not show phenotypic changes (258).

1.6 Aim and scope of this thesis

The initial aim of the project was to clone new members of the non-heme iron alkane monooxygenase family. Studies about the structural and catalytic properties of an enzyme are most effective when sequences from a broader range of organisms are known. All aspects of growth on alkanes are considered.

Chapter 2 describes the use of highly degenerate PCR primers to clone internal gene fragments of alkane hydroxylase gene homologs from a broad range of Gram-positive and Gram-negative strains.

In Chapter 3, we describe the construction of alkane-responsive expression vectors for heterologous expression of proteins in both *E. coli* and *Pseudomonas* species.

In Chapter 4, the cloning of novel alkane hydroxylase homologs and their flanking regions is reported. Functional expression of alkane hydroxylases was achieved by constructing alkane hydroxylase negative hosts, that could be complemented for growth on *n*-alkanes by alkane hydroxylases from Gram-negative and Gram-positive strains.

Chapter 5 describes the functional analysis of rubredoxin reductases in recombinant *E. coli* strains containing all *alk*-genes of *P. putida* (*oleovorans*) GPo1 except for the rubredoxin reductase gene.

The Chapters 6 and 7 give a more detailed description of the alkane oxidation genes of two Gram-negative strains. For *P. aeruginosa* PAO1 (Chapter 6) a detailed analysis of flanking regions is shown, and environmental and clinical isolates are tested for their ability to grow on alkanes and for the presence of alkane hydroxylase genes. For *P. fluorescens* CHA0 (Chapter 7), characterization of the knockout mutants KOB2 and KOB2Δ1 is described, while characterization of culture supernatant and overexpression of PraA and PraB shows that *P. fluorescens* CHA0 produces only PraB when grown on hexadecane. In the Appendix to chapter 7, some initial data about the effect of the *alkB* knockout on biocontrol activity are shown.

In Chapter 8, we describe additional intrinsic and extrinsic factors including the effect of rhamnolipids and the host strain that influence the apparent substrate range of the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1. We also describe mutations in the AlkB which allows *P. fluorescens* KOB2Δ1 recombinants to grow on hexadecane.

Finally, in Chapter 9, we summarize all knowledge about *alk*-systems in Gram-positive and Gram-negative strains collected over the last years.

Chapter 2

MOLECULAR SCREENING FOR ALKANE HYDROXYLASE GENES IN GRAM-NEGATIVE AND GRAM-POSITIVE STRAINS

Theo H.M. Smits, Martina Röthlisberger, Bernard Witholt and Jan B. van Beilen

SUMMARY

We have developed highly degenerate oligonucleotides to amplify internal segments of genes related to the *Pseudomonas putida* (*oleovorans*) GPo1 and *Acinetobacter* sp. ADP1 alkane hydroxylases.

Many Gram-negative and Gram-positive strains able to grow on medium (C6-C11) or long chain *n*-alkanes (C12-C16), yielded PCR products of the expected size. The PCR fragments were cloned and sequenced and found to encode peptides with 37.1-100 % sequence identity to the corresponding fragment of the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase. Strains that were unable to grow on *n*-alkanes did not yield PCR products with homology to alkane hydroxylase genes. Several alkane degraders, predominantly those isolated on short-chain alkanes, did not yield PCR fragments either. The phylogenetic analysis shows that all *Acinetobacter* sequences are clustered in one group of the phylogenetic tree, like the sequences from Gram-negative strains able to grow on medium-chain alkanes. In contrast, sequences obtained from the fluorescent pseudomonads or sequences found in a single Gram-negative or Gram-positive strain are as divergent as the complete collection.

The alkane hydroxylase genes of *Acinetobacter calcoaceticus* EB104 and *P. putida* P1 were cloned using the PCR products as probes. The two genes allow an alkane hydroxylase negative mutant of *Acinetobacter* sp. ADP1 and an *E. coli* recombinant containing all *P. putida* (*oleovorans*) *alk*-genes except *alkB*, respectively, to grow on *n*-alkanes, showing that the cloned genes do indeed encode alkane hydroxylases.

INTRODUCTION

Many bacterial strains are able grow on medium or long chain-length *n*-alkanes. Of these strains, *P. putida* (*oleovorans*) GPo1 (TF4-1L), which can grow on alkanes ranging from pentane to dodecane (228), has been studied in detail with respect to both the enzymology (173, 208, 255, 256) and the genetics of *n*-alkane metabolism (142, 143,

263), mainly because the strain has proven to be an interesting and versatile biocatalyst (85, 136, 210, 259). In addition, the alkane hydroxylase AlkB is the prototype of a class of non-heme iron integral membrane monooxygenases which includes the *P. putida* mt-2 xylene monooxygenase (251) and the alkane hydroxylase of *Acinetobacter* sp. ADP1 (213).

Genes with high sequence identity to the alkane hydroxylase gene (*alkB*) occur in a large fraction of the microbial population in oil-contaminated environments as demonstrated by colony blots (243), while genes almost identical to *alkB* have been cloned from *P. aeruginosa*, *P. putida*, *P. aureofaciens* and *P. mendocina* (226, 262, 273). On the other hand, DNA of other alkane-degrading strains does not hybridize with an *alkB*-gene probe, suggesting that they contain alkane oxidation enzyme systems that are unrelated or distantly related to *alkB*. These enzyme systems could be useful as biocatalysts to convert inexpensive compounds into valuable intermediates for the synthesis of pharmaceuticals or other fine-chemicals (89). Examples include *Rhodococcus rhodochrous* NCIMB12566, which, when pregrown on *n*-alkanes, is able to stereoselectively oxidize substituted phenoxy propane to phenoxy propanoic acids, compounds that are used for the production of herbicides, or *R. erythropolis* NRRL B-16531, which carries out similar stereoselective oxidation reactions, such as the oxidation of cumene to 2-phenyl-1-propionic acid (119), which can be used to produce pharmaceuticals (e.g. ibuprofen) or other high value fine-chemicals. None of the enzyme systems involved have been characterized.

As only few enzymes that oxidize medium- or long-chain *n*-alkanes are well characterized, a comparison with related enzyme systems in other bacteria will help us to understand their function, evolution and structure, and provide us with new biocatalysts, hopefully with superior properties. In addition, the role of alkane hydroxylases in oil degradation or soil-ecology can be studied in greater detail when more members of this class of enzymes are known, or convenient methods are available to isolate their genes. One method that can be used to isolate distantly related genes is PCR with degenerate primers, based on conserved sequence elements. A comparison between the alkane hydroxylases of *P. putida* (*oleovorans*) GPo1 (70) and *Acinetobacter* sp. ADP1 (213)

allowed us to identify such regions, basically centered around two histidine clusters that were previously shown to be essential for the function of the structurally related desaturases (233). Using this method we could show that a large proportion of bacteria able to grow on n-alkanes used in this study possess genes that are related to the $P.$ $putida$ ($oleovorans$) gene $alkB$, which encodes the catalytic component of the alkane hydroxylase system, while such genes can not be amplified from strains that are unable to grow on n-alkanes. In addition, we cloned the alkane hydroxylase genes of $P.$ $putida$ P1 and $A.$ $calcoaceticus$ EB104 and showed by complementation that these indeed encode functional alkane hydroxylases.

Table 1: strains and plasmids used in this study

Strain	Growth on alkanes[a]			PCR product[b]	Plasmid derived	Reference
	C8	C12	C16			
Pseudomonas putida (*oleovorans*) GPo1	+	+	-	550 bp[c,d,e] -	(228)	
Pseudomonas putida (*oleovorans*) GPo12	-	-	-	-	-	(141)
Pseudomonas oleovorans ATCC 8062	-	-	-	289 bp[c]	-	(155)
Pseudomonas putida PpG1	-	-	-	-	-	(216)
Pseudomonas putida KT2442	-	-	-	≈ 800 bp[c,g]	-	(14)
Pseudomonas putida P1	+	-	-	550 bp[c]	pP1	This study
Pseudomonas fluorescens CHA0	-	+	+	550 bp[c]	pCHA0	(250)
Pseudomonas aeruginosa PAO1	-	+	+	550 bp[c]	pTS4	(118)
Pseudomonas aeruginosa PG201	-	+	+	550 bp[c]	pTS2	(97)
Pseudomonas aeruginosa KSLA473	+	+	+	not tested	-	(253)
Burkholderia cepacia ATCC 25416	-	+	+	550 bp[c]	p25416	(198)
Burkholderia cepacia RR10	-	+	+	550 bp[c]	pRR10	(288)
Comamonas testosteroni DSM 50244	-	-	-	350 bp[c]	-	(246)
Stenotrophomonas maltophilia DSM 50170	-	-	-	-	-	(246)
Ralstonia eutropha JM134 (pJP4)	-	-	-	-	-	(62)
Acinetobacter sp. ADP1	-	+	+	not tested	-	(126)
Acinetobacter sp. WH405	-	-	-	not tested	-	(213)
Acinetobacter calcoaceticus 69-V	-	+	+	550 bp[d]	p69-V	(139)
Acinetobacter calcoaceticus EB102	-	-	-	≈ 600 bp[f,g]	-	(139)
Acinetobacter calcoaceticus EB104	-	+	+	550 bp[c]	pEB104	(139)
Acinetobacter calcoaceticus EB114	-	-	-	≈ 600 bp[f,g]	-	(139)
Acinetobacter calcoaceticus EB6	-	-	-	-	-	(139)
Acinetobacter calcoaceticus NCIB 8250	-	+	+	550 bp[c]	p8250	(76)
Acinetobacter calcoaceticus CCM 2355	-	-	-	≈ 600 bp[f,g]	-	(139)
Acinetobacter sp. 2796A	-	+	-	550 bp[c]	p2769A	This study
Alcanivorax borkumensis SK2	+	+	+	550 bp[c]	pSK2	(284)
Xanthobacter flavus strain 5	+	+	+	550 bp[c,h]	pXf5 (484 bp)	This study
Rhodococcus rhodochrous NCIMB 12566	+	+	-	289 bp[c]	-	(50)
Rhodococcus erythropolis NRRL B-16531	+	+	+	550 bp[c]	p16531	(119)
Prauserella rugosa NRRL B-2295	-	+	-	559 bp[c]	p2295	(119)

Rhodococcus sp. 1BN	+	n.t.	+	550 bp[c]	p1BN	(6)
R. erythropolis 23-D	+	+	+	550 bp[c]	p23-D1, p23D-2,	
					p23-D3	(226)
R. erythropolis 35-O	+	+	+	550 bp[c]	p35-O	(226)
Gram-positive 42-O	+	+	+	550 bp[c]	p42-O	(226)
R. erythropolis 50-V	+	+	+	550 bp[c]	p50-V	(226)
R. erythropolis 62-O	+	+	+	550 bp[c]	p62-O	(226)
R. fascians 115-H	+	+	+	550 bp[c, h]	p115-H (240 bp)	(226)
R. fascians 154-S	+	+	+	550 bp[c]	p154-S	(226)
HXN 100 (CNM group)	+	+	+	-	-	(211)
HXN 200 (*Sphingomonas* sp.)	+	+	+	-	-	(211)
HXN 300 (Gram-negative)	+	+	+	-	-	(211)
HXN 400 (*P. aeruginosa*)	+	+	+	550 bp[c]	pHXN400	(211)
HXN 500 (*Mycobacterium*?)	+	+	+	-	-	(211)
HXN 600 (*Mycobacterium* sp.)	+	+	+	550 bp[c]	pHXN600	(211)
HXN 1000 (CNM group)	+	+	+	550 bp[c]	pHXN1000	(211)
HXN 1100 (*P. mendocina* lineage)	+	-	-	550 bp[c]	pHXN1100	(211)
HXN 1200 (*Rhodococcus* sp.)	+	+	+	-	-	(211)
HXN 1300 (not identified)	+	+	+	-	-	(211)
HXN 1400 (*Comamonas*?)	+	+	-	-	-	(211)
HXN 1500 (*Mycobacterium* sp.)	+	+	+	-	-	(211)
HXN 1600 (*Ochrobactrum*?)	+	+	+	-	-	(211)
HXN 1700 (*Alcaligenes*?)	+	+	+	-	-	(211)
HXN 1800 (not identified)	+	+	+	-	-	(211)
HXN 1900 (*Rhodococcus* sp.)	+	+	+	-	-	(211)
HXN 2000 (CNM group)	+	+	+	550 bp[c]	pHXN2000	(211)

Plasmids:	Genotype[a]:	Reference
pGEc47	Tc, *alkBFGHJKL/alkST* (*P. putida* (*oleovorans*) GPo1) in pLAFR1	(69)
pGEc47ΔB	Tc, *alkFGHJKL/alkST* (*P. putida* (*oleovorans*) GPo1) in pLAFR1,	
	deletion in *alkB*	(261)
pGEc48	Ap, ColE1, *alkBFGH* (*P. putida* (*oleovorans*) GPo1) in pBR322	(70)
pSCYB11	Tc, *alkB* (*Mycobacterium tuberculosis* H37Rv)	(209)
pGEM7-Zf(+)	Cloning vector, Ap	Promega
pEB1	Ap, 3.0 kb *Eco*RI-*Hind*III insert in pGEM7-Zf(+),	
	alkRM (*A. calcoaceticus* EB104)	This study
pEB7	pEB1 Δ*Eco*RV-*Eco*RI, 1.7 kb insert, *alkM* (*A. calcoaceticus* EB104)	This study
pWH1274	Ap, Tc, *oriA*, ColE1, shuttle vector for *Acinetobacter*	(121)
pWH968	Ap, *oriA*, ColE1, *alkRM* (*Acinetobacter* sp. ADP1), *rubAB*	(214)
pWHEB7	Ap, *oriA*, ColE1, 1.7 kb insert of pEB7 in pWH1274,	
	alkM (*A. calcoaceticus* EB104)	This study
pBR322	Cloning vector, Ap, Tc, ColE1	(25)
pP1EH	Ap, ColE1, *alkBFGHJ* (*P. putida* P1) in pBR322	This study

Footnotes: [a] Abbreviations: C8: octane, C12: dodecane, C16: hexadecane, bp: basepairs, Ap: Ampicillin, Tc: tetracycline, n.t.: not tested. [b] - : no PCR fragment; Fragment size: PCR fragment observed. Primer combinations used are: [c] TS2S/Deg1RE; [d] TS2Smod/Deg1RE; [e] TS2Smod/(Deg1RE/Deg1RE2, 1:1; mix); [f] TS2Smod2/Deg1RE. [g] PCR fragment was observed, but not cloned. [h] smaller size fragment cloned (length in parentheses behind plasmid name)

MATERIAL AND METHODS

Strains, growth media and materials. Strains and plasmids used or constructed in this study are listed in Table 1. *E. coli* JM101 (*endA*, *hsdR*, *supR*, *thi-1*, Δ(*lac-proAB*) F'(*traD36*, *proAB*, *lacI^a*, *lacZM15*) (285) and DH10B (Gibco BRL) were used for cloning and the production of plasmid DNA for sequencing. All other strains are able to grow on *n*-alkanes or were used as negative controls in the PCR reactions. LB (Luria Broth) (220) and E2 medium (151), supplemented with carbon sources or antibiotics were used throughout. For growth on *n*-octane, *n*-dodecane or *n*-hexadecane, Petri dishes with E2 medium were incubated at 30°C with *n*-alkanes supplied through the vapor phase (for *n*-octane by placing an open erlenmeyer with *n*-octane in a sealed container, for *n*-dodecane and *n*-hexadecane by placing a Whatman 3MM filter disc with 200 μl *n*-alkane in the lid of the Petri dish. All cultures were grown aerobically at 30°C or 37°C. *E. coli* strains were transformed by electroporation (64). To select *E. coli* transformants, ampicillin was used at 100 μg/ml. Transformation of *Acinetobacter* was done as previously described (199). Selection of transformants was done on LB with 300 μg/ml ampicilline. Growth of *Acinetobacter* sp. ADP1, and mutants thereof, on *n*-dodecane was tested on *Acinetobacter* minimal medium supplemented with metal solution 44 (204).

Molecular biology. Restriction enzymes, T4-DNA ligase and dideoxynucleotides were from Boehringer Mannheim and used as specified by the supplier. Oligonucleotides were synthesized by Microsynth, Switzerland. In the oligonucleotides Y = T + C, R = A + G and I = Inosine, basepairing with all four nucleotides. Taq DNA polymerase was obtained from Promega. PCR products were purified over a 1% agarose gel in TBE-buffer, cut out from the gel, and isolated by electroelution (220). The PCR fragments were cut with *Sac*I and *Eco*RI, purified again over a 1% agarose gel, isolated from gel by electroelution, and cloned between the *Sac*I and *Eco*RI sites of pGEM7-Zf(+) (Promega). Plasmid DNA was isolated according to Birnboim and Doly (21), or using the Roche High Pure Plasmid Isolation Kit if sequencing grade DNA was required. Both strands of the inserts were sequenced on a Li-Cor 4000L sequencer using IRD41- (IRD800-) labelled -40 forward

(agggttttcccagtcacgacgtt) and -40 reverse (gagcggataacaatttcacacagg) primers (MWG-Biotech) and the Amersham Thermosequenase cycle sequencing kit.

Chromosomal DNA was isolated from Gram-positive and Gram-negative strains according to Desomer *et al.* (58). If strains failed to lyse, the EDTA concentration in the first step was increased to 10 mM, the end concentration of SDS in the second step was doubled to 0.2%, and/or cells were incubated at 60°C for 30 minutes.

To clone intact and functional alkane hydroxylase genes, easy-to-clone restriction fragments were identified by Southern blotting. To construct an enriched genebank, all restriction fragments in the range around the desired size were cut out from a preparative gel, isolated by electroelution, ligated between the appropriate sites of pGEM7-Zf(+) (Promega) or pBR322 (25) and transformed into *E. coli* DH10B (Gibco BRL). Transformants containing the desired genes were identified by colony blotting. To sequence the inserts, deletion and subclones were made.

In the case of *A. calcoaceticus* EB104, a 3.0 kb *Eco*RI-*Hind*III fragment was cloned, resulting in plasmid pEB1. Plasmid pEB7 was constructed from pEB1 by digestion with *Eco*RI and *Eco*RV, made blunt using Klenow, and religated. The insert of pEB7 was cut out with *Xho*I and *Bam*HI, purified over gel and ligated in *Sal*I-*Bam*HI digested pWH1274 (121) , a shuttle vector for *Acinetobacter*, resulting in pWHEB7. For *P. putida* P1 a 7.5 kb *Eco*RI-*Hind*III fragment was cloned, resulting in pP1EH.

The nucleotide and amino acid sequences were analyzed and compared using LASERGENE Navigator from DNASTAR (Madison, Wisconsin, USA). Nucleotide and amino acid sequences were compared with the EMBL, SwissProt and GenBank databases using BLAST (5). BLAST searches were carried out at ISREC (Swiss Foundation for Cancer Research).

PCR. PCR reactions were carried out as described in Innis *et al.* (123) using a Perkin Elmer GeneAmp PCR System 9600. Degenerate oligonucleotides were designed based on amino acid sequences conserved between the *P. putida* (*oleovorans*) GPo1 and the *Acinetobacter* sp. ADP1 alkane hydroxylases (Figure 1). The forward primers TS2S, TS2Smod and TS2Smod2 contain a *Sac*I site, and four additional bases to allow reliable

cleavage of the site, while the reverse primers Deg1RE and Deg1RE2 contain an *Eco*RI site and three additional bases. For PCR using these primers, the following program was used: 4' 95°C, 25x(45'' 95°C, 1' 40°C, 1' 72°C), 5' 72°C, ∞ 4°C. After a first round of PCR, 1 μl (out of 20 μl) of product was used in a second round of PCR using the same primers.

16S rDNA sequencing was done according to Karlson *et al.* (130). The primers used for amplification were 16F27 and 16R1525; primers used for sequencing were 16F355 and 16R1488 (107).

Southern and colony blots. Chromosomal DNAs were digested to completion with appropriate restriction enzymes and loaded on a 1% agarose gel in TAE-buffer (Tris-Acetate-EDTA) (167). After washing the gel for 10 minutes in 0.25 N HCl, and three 15 minutes washes with 0.5 N NaOH, the DNA was blotted to positively charged nylon membrane (Roche Diagnostics). After blotting, the filters were baked at 120°C for 30 min. For colony hybridisation, colonies were transferred from LB plates with about 250 - 500 transformants onto a Nylon Membrane for Colony and Plaque Hybridisation (Roche Diagnostics) according to the manufacturer. Colonies positive in the colony blots were picked from the original plates. Detection of Southern and colony blots was carried out using the Roche DIG-kit according to the manufacturer. Hybridizations were carried out using standard hybridization buffer without formamide at 60°C (non-stringent, only Southern blot with *M. tuberculosis* H37Rv *alkB*) or at 68°C (stringent, all other blots). Alkaline phosphatase coupled to Anti-DIG antibodies were used with CSPD as the chemiluminescent substrate.

Probes were prepared as follows: *alkB* (*P. putida* (*oleovorans*) GPo1) was amplified from pGEc48 (70) using primers B5/1 (gatgtcgagagtgagagtg) and B3R/EcoRI (gcactctttgtgagagaattcaac), the *alkM* fragment from chromosomal DNA of *Acinetobacter* sp. ADP1 using primers alkMfE2 (ccggaattcactatgaatgcacctgta) and alkMrPS (aataggcctgcagtcacttagactctctt), the *alkB* gene homolog of *M. tuberculosis* H37Rv from cosmid pSCYB11 (209) using primers MTalkBfw2 (cggaattcatatgaccacgcaaatcggc) and MTalkBrv (gtagaagcttcccgggcaccggtagg). Other fragments were obtained from the

plasmids pTS2 (*P. aeruginosa* PG201), pCHA0 (*P. fluorescens* CHA0) and p16531 (*R. erythropolis* NRRL B-16531) by excising the cloned 550 bp PCR fragment with *Eco*RI and *Sac*I. All DNA fragments were purified over a 1% agarose gel, electroeluted and labeled by the random priming method using the DIG labeling kit (Roche Diagnostics).

Sequences. Sequences used in this study for comparisons are available in EMBL under accession numbers X65936 (*alkB, P. putida* (*oleovorans*) GPo1), AJ002316 (*alkM, Acinetobacter* sp. ADP1), Z95121 (*alkB, M. tuberculosis* H37Rv).

The sequences of the degenerate PCR products determined in this study are deposited in GenBank under the following accession numbers: AJ009579 (*P. fluorescens* CHA0); AJ009580 (*P. aeruginosa* PAO1); AJ009581 (*P. aeruginosa* PG201); AJ009582 (*A. calcoaceticus* 69-V); AJ009584 (*A. calcoaceticus* NCIB 8250); AJ009585 (*Acinetobacter* sp. 2769A); AJ009586 (*R. erythropolis* NRRL B-16531), AJ009587 (*Pr. rugosa* NRRL B-2295) and AJ293344 (*B. cepacia* ATCC 25416). The sequence of the *A. calcoaceticus* EB104 *alkRM* genes is deposited under accession number AJ233398. The sequence of the *P. putida* P1 *alkBFGHJ* genes is deposited under accession number AJ233397.

The 16S rDNA sequences determined in this study are available under accession numbers AJ009588 (*A. calcoaceticus* 69-V); AJ009589 (*A. calcoaceticus* EB104); AJ009590 (*Acinetobacter* sp. 2769A) and AJ009591 (*R. erythropolis* NRRL B-16531). As the 16S rDNA sequences of strains *Acinetobacter* sp. ADP1 and *A. calcoaceticus* NCIB 8250 were identical to the previously determined sequences, these were not submitted.

RESULTS

Selection of strains. We tested 55 strains, listed in Table 1, for growth on *n*-octane (C8), *n*-dodecane (C12) and *n*-hexadecane (C16). Twenty-six strains were obtained from strain collections, five were isolated by enrichment with *n*-octane as the C-source, seven were isolated from oil-contaminated soil near a gas station in Bremen (226), and seventeen were isolated from a hexane air filter in Stuttgart (211). Several strains reported to be *n*-

alkane degraders were not able to grow on *n*-alkanes in our hands (*P. oleovorans* ATTC 8062 and *A. calcoaceticus* strains EB102, EB114, EB6, and CCM 2355). Other strains belong to species of which specific strains were previously found to be able to grow on *n*-alkanes.

Design of degenerate PCR primers. Three sequences of integral membrane non-heme iron alkane hydroxylases have been deposited in the sequence databases: AlkB (*P. putida* (*oleovorans*) GPo1) (70), AlkM (*Acinetobacter* sp. ADP1) (213) and AlkBpm (*Stenotrophomonas* (*Pseudomonas*) *maltophilia* N246) (157). In addition, an alkane hydroxylase gene homolog is present on the chromosome of *Mycobacterium tuberculosis* H37Rv. As the functionality of the *M. tuberculosis* H37Rv gene was not confirmed prior to this study, its sequence was not considered in the primer design, but does not conflict with it either. The *S. maltophilia* N246 alkane hydroxylase sequence was not considered in the primer development nor in the phylogenetic analysis, because both the sequence and properties of this enzyme are significantly different from those of other alkane hydroxylases.

Sequence alignments of the amino acid sequences of the *P. putida* (*oleovorans*) GPo1 and the *Acinetobacter* sp. ADP1 alkane hydroxylases show that several regions are well conserved. We chose two regions around amino acid 145 (HEL(G/S)HK) and amino acid 320 (HSDHH) to develop the degenerate oligonucleotides shown in Figure 1.

The forward primer TS2S is based mainly on the AlkB sequence, and ignores the AAA lysine codon, three glycine codons, two leucine codons and the serine codons of the AlkM sequence. TS2Smod includes the AAA lysine codon, and additional glycine and leucine codons. In TS2Smod2, the TCI serine codons corresponding to position 153 of AlkM replace the glycine codons. TS2S has a degree of degeneracy of 64, whereas TS2Smod and TS2Smod2 have a degree of degeneracy of 1064. The reverse primer Deg1RE contains the TCI serine codons, while Deg1RE2 contains the AGY serine codons. Deg1RE and Deg1RE2 have a degree of degeneracy of 64 and 32, respectively. The length of the expected PCR product including the primers (based on the *alkB* and *alkM* sequences) is 557 basepairs (bp).

A: Forward primers

```
alkB      400   5' ctcaatacaggacacgaactcggtcacaagaaggagact 3'   438
AlkB      134      .L..N..T..G..H..E..L..G..H..K..K..E..T.        146

alkM      436   5' gtgaataccgcgcatgaattgagccataaagcagatcga 3'   474
AlkM      146      .V..N..T..A..H..E..L..S..H..K..A..D..R.        158

TS2S            5' ...aayagagctcaygarytrggtcayaag......... 3'

TS2Smod         5' ...aayagagctcaygaritiggicayaar......... 3'

TS2Smod2        5' ...aayagagctcaygarititcicayaar......... 3'
```

B: Reverse primers

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alkB      925   5' cttcagcggcactcggatcaccacgcgcatccaacacgt 3'   963
AlkB      309      .L..Q..R..H..S..D..H..H..A..H..P..T..R.        321

alkM      961   5' ttacaacgacattcagatcatcacgcttatccgacgcgt 3'   999
AlkM      321      .L..Q..R..H..S..D..H..H..A..Y..P..T..R.        333

deg1RE          3' .........gtragictrgtrgtrcgcttaaggtg.... 5'

deg1RE2         3' .........gtrtcrctrgtrgtrcgcttaaggtg.... 5'
```

Figure 1: conserved regions of AlkB (*P. putida* (*oleovorans*) GPo1) and AlkM (*Acinetobacter* sp. ADP1) that were used to design the degenerate primers. The amino acids are shown below the second nucleotide of the codon. Nucleotides coding for restriction sites are shown in italic print. The differences between TS2S, TS2Smod and TS2Smod2, and Deg1RE and Deg1RE2 are underlined. Primer TS2S does not include 2 leucine, 3 glycine, 6 serine and 1 lysine codons, whereas primer TS2Smod includes all codons except 6 serine codons. Primer TS2S includes 4 serine codons, and does not include 4 glycine codons. Primer Deg1RE does not include 3 alanine and 2 serine codons, whereas Deg1RE2 does not include 3 alanine and 4 serine codons. A: forward primers; B: reverse primers.

Using the PCR program described in the material and methods section, we obtained 29 sequences from 27 strains that were able to grow on *n*-alkanes, in most cases using primer combination TS2S/Deg1RE (see Table 1). In several cases, PCR products of clearly different sizes were obtained as well. From the eleven strains that were unable to grow on *n*-alkanes and 12 hexane air filter strains, we did not obtain PCR products of the expected length. Another 8 *alkB* gene homologs were obtained from 6 strains using Southern blots (chapter 4; J. B. van Beilen, unpublished results)(168, 273) and genome sequences. These sequences are also included in the phylogenetic analysis.

Figure 2: alignment of alkane hydroxylase sequences, generated with CLUSTAL and manually optimized. The three histidine-boxes are underlined and marked by roman capitals I, II and III. The hydrophobic stretches are marked by solid bars. Peptide numbering refers to the position in the cloned PCR fragment rather than to that of the full-length enzyme.

DNA sequence determination and comparisons. The PCR fragments were cloned in pGEM7-Zf(+), and sequenced. Most of the PCR products were of the same length (557 bp including primers), while one PCR product was 9 bp longer. In two cases, the PCR fragments contained an internal *Sac*I or *Eco*RI site, respectively, and only part of the fragment was cloned and sequenced. In several cases, more than one PCR fragment cloned from a particular strain were sequenced, and in two cases, this yielded more than one unique sequence. BlastX alignments (5) showed that all of cloned PCR fragments encode peptides that have a high level of sequence identity to the corresponding region of the *P. putida* (*oleovorans*) AlkB and *Acinetobacter* sp. AlkM proteins (see below). In several cases, different size fragments were obtained. Three of these, ranging from 289 to 350 bp, were cloned and sequenced, and found to be unrelated to the alkane hydroxylase genes. Other fragments were the product of only one of the primers (data not shown) and were not cloned. In one case, a 350 bp fragment that appeared in addition to the 550 bp fragment was cloned and found to be due to binding of the forward primer to a region encoding a similar histidine motif (EHXXGHH) at position 167 in AlkB. In further work we ignored this fragment when the 550 bp fragment was obtained as well.

A 550 bp PCR fragment, obtained with *B. cepacia* RR10 chromosomal DNA as template, was cloned and sequenced. The PCR fragment was subsequently used as a probe to clone the complete alkane hydroxylase from this strain, but the sequence of the complete *alkB* gene did not correspond to that of the cloned PCR fragment (168), even though the homology was high enough for use as a probe in Southern and colony blots. Primers based on the PCR fragment sequence did not yield the expected fragment with chromosomal DNA from *B. cepacia* RR10 (data not shown). The PCR fragment most probably originates from a contaminating Gram-positive strain, which could not be identified among our strain collection.

For DNA sequence alignments and phylogenetic analysis, the sequences corresponding to the primer annealing sites were removed before comparing the sequences, because they

```
        10        20        30        40        50        60        70        80        90       100
1  GHELGHKKETFDRWMAKIVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGESIYKFSIREIPGAFIRAWGLEEQRLSRRGQSVWS----FDNEILQPM   P. putida (oleovorans) GPo1
1  AHELSHKADRLDHILSHLALVPTGYNHFRIEHPYGHHKRAATPEDPASSQMGETFYEFWPRTVFGSLKSAIEIETHRLKRKGKKFWS----KDNELLQGW   Acinetobacter sp. ADP1
1  AHEMGHKKDSLERWLSKITLLAQTCYGHFYIEHNRGHHVRVSTPEDPASARFGETLWEFLPRSVIGGLRSAVHLEAQRLRRLGVSPWNPMTYLRNDVLNAW  M. tuberculosis H37Rv
1  AHELGHKHDRIDHILSHLALVPTGYNHFRIEHPYGHHKRAATPEDPASSRMGETFYEFWPRTVIGSFKSAIEIEKNRLKRKGKEFWS----LDNELLQGW   A. calcoaceticus EB104
1  GHELGHKKEAFDRWMAKIVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGENIYKFSTREIPGAFRRAWGLEEQRLSRRGQSVWS----FDNEILQPM   P. putida P1
1  AHELIHKDSALEQAAGGILLAAVCYAGFKVEHVRGHHVHVSTPEDASSARFGQSVYQFLPHAYKYNFLSAWRLEAVRLRKKGLPVFG----WQNELIWWY   P. aeruginosa PAO1 AlkB1
1  SHELIHKDPQLEQNAGGLLAAVCYAGFKVEHVRGHHVHVSTPEDASSSRYGQSLYSFLPHAYKHNFLNAWRLEAERLKRKGLPALH----WRNELIWWY    P. aeruginosa PAO1 AlkB2
1  AHELGHKNRGMAKFLAKLALASTFYGHFFVEHNRGHHVRVATPEDPASSRLGESFWSFLPRTVWFSLSSAWHLESQRLEKLGLPTLH----WKNGVLSAW   P. fluorescens CHAO
1  AHELGHKTNRFERWLAKITLLAPVAYGHFFVEHNRGHHVRVATAEDPASARYGESFWAFLPRTVVGSVRSAWRLERTRLARLGRSPWT----WRNEVLHAW   B. cepacia RR10
1  AHELGHKKESHERWLSKIALAQSFYGHFYIEHNRGHHVRVSTPEDPASSRVGESFYRFWPRTVVGSLRSAWRLERKRYARRDRHPFR----IGNDVLNAW   Pr. rugosa NRRL B-2295
1  AHELGHKKESVERWLSKIVLAQSAYGHFYLEHNRGHHVRVSTPEDPATSRFGETLYGFWPRSVGGGLKSAWHLEKTRFERLGTTHWS----IKNDVLNAW   R. erythropolis NRRL B-16531 AlkB1
1  AHELGHKKDDLERWLSKITLAQSFYGHFYIEHNRGHHVRVATPEDPASSRFGESFWTFLPRSVWGSLRSSWSLEKARLDRLGKKPWT----IRNDVLHSW   R. erythropolis NRRL B-16531 AlkB2
1  AHELGHKSSRLERRLAKIVLAQSFYGHFYIEHNYGHHVNVATPRDPASARFGESFWLFLPRSVFGGLKSGWRIESARLRRQGSPALS----PRNNIVQAW   R. erythropolis NRRL B-16531 AlkB3
1  AHELGHKSEKLEKWLAKVALAQSFYGHFYVEHNHGHHVRVATPEDPASSRYKENFWFFLPRSVIGGLRSGWRIESARLRRQGRRTWS----YRNNILNAW   R. erythropolis NRRL B-16531 AlkB4
1  AHELGHKKTELERWLAKITLLAQTFYGHFYIEHNRGHHVRVATPEDPASSRFGESFWTFLPRSVWGSLKSSWELEKTRMQRLGKSTWS----IHNDVLNAW   Rhodococcus sp. 1BN
   .HE..HK.........L....Y..F..EH..GHH....T..D.............F.................E..R.............N......      Consensus
     I               II
```

```
       110       120       130       140       150       160       170       180
97  IITVILYAVLLALFGPKMLVFLPIQMAFGWWQLTSANYIEHYGLLRQKMEDG---RYEHQKPHHSWNSNHIVSNLVLFHLQRHSDHHAH   P. putida (oleovorans) GPo1
97  GMSAAFHSSIIAIFGKGTIPYLVTQAFYGISLFEIINYIEHYGLLKRQKRADG---NYERTMPEHSWNNNNIVTNLFIYLQLQRHSDHHAY   Acinetobacter sp. ADP1
101 LMSVVLWGGLIAVFGPALIPFVIQAVFGSLLEAVNYLEHYGLLRQKSANG---RYERCAPVHSWNSDHIVTNLFLYHLQRHSDHHAN   M. tuberculosis H37Rv
97  GMSAAFHASMVGLFGKGTIPYLATQAFYGISLFEIINYIEHYGLLKKENG---QYERTMPEHSWNNNNVVTNLFLYLQLQRHSDHHAN   A. calcoaceticus EB104
97  VITVVLYTLLLAFFGPKMLVFLPIQMAFGWWQLTSANYIEHYGLLREKMADG---RYEHQKPHHSWNSNHIVSNLVLFHLQRHSDHHAF   P. putida P1
97  LLSLALLVGFGWAFGWLGMVFFLGQAFVAVTLLEIINYVEHYGLLHRRKGEDG---RYERTNHTHSWNSNFVFTNLVLFHLQRHSDHHAF   P. aeruginosa PAO1 AlkB1
97  AISALFLLGFSLAFGWLGAIFFLGQSVMAFTLLEIVNYVEHYGLLHRRRLDNG---RYERTTPEHSWNSNFLLTNLFLFHLQRHSDHHAY   P. aeruginosa PAO1 AlkB2
97  LYNVVLWGVLIAWLGAAVIPFLVIQGIYGFSLLEVVNYVEHYGLLRQKQPNG---RYERCSPRHSWNSNRIVTNIFLFQLQRHADHHAN   P. fluorescens CHAO
97  AMTVVVWGIAIAIGGVVVIPFLVIQAVYGASLLEVVNYVEHYGLLGRRLPDG---RYERCTPQHSWNSNHVVTNLFLYLQLQRHADHHAN   B. cepacia RR10
97  LMSAVLWSVMIAWLGIGVLPYLLIQAVVGFSLLEIVNYMEHYGMLRQKRGSPERRYERVDPSHSWNSNNIATNVLLYHLQRHSDHHAN   Pr. rugosa NRRL B-2295
97  LMSVVLFGVALAVFGIGIAPYLVIQAFIGFSLLEAINYLEHYGLLRQKTASG---RYERCTPAHSWNSDRICTNVFLYHLQRHSDHHAN   R. erythropolis NRRL B-16531 AlkB1
97  LMSVVLFGVLVAVFGLSVLPFLVLQAVFGFCLLETVNYLEHYGLKRRRLDSG---RYERAAPEHSWNSDHICTNLFLYLQRHSDHHAN   R. erythropolis NRRL B-16531 AlkB2
97  SLSAALFGTLITLFGWQILPWLLQTLAGITFLEAANYLEHYGLLRVRRQDG---SFVKARPEDSWNSDHLVSNLFLYLQLQRHSDHHAN   R. erythropolis NRRL B-16531 AlkB3
97  AMSVVLFGSLIAAFGWEIAPWLAVQAIAGFTFLETANYLEHYGLLRAKRPDG---SYVRCSPEDSWNSDHVVSNLFLYLQLQRHSDHHAN   R. erythropolis NRRL B-16531 AlkB4
97  LMSVVLFGALIAIFGPVVIPFLIIQAVYGFSLLETVNYLEHYGLLMRQKTASG---RYERCAPAHSWNSDHIVTNIFLYLQRHSDHHAN   Rhodococcus sp. 1BN
    .G...........Q...........NY.EHYG..R........SWN.......N..L..LQRH.DHHA.                          Consensus
             III
```

differ from the original chromosomal sequence. The nucleotide alignment was based on the peptide alignment (see below), because in many cases pairwise alignment of PCR fragments using the Wilbur-Lipman method (278) was not possible due to the low level of sequence identity. The resulting levels of DNA sequence identity range from 45.0 to 100 %.

In a number of cases, three PCR clones from the same organism were sequenced. This showed that on average less than one nucleotide change per PCR fragment had occurred due to the PCR. Possible errors introduced by the PCR will not change the phylogenetic analysis significantly, except for closely related genes. Since we intend to clone full length genes from several selected microorganisms, we did not attempt to resolve PCR artefacts at this point by sequencing additional clones.
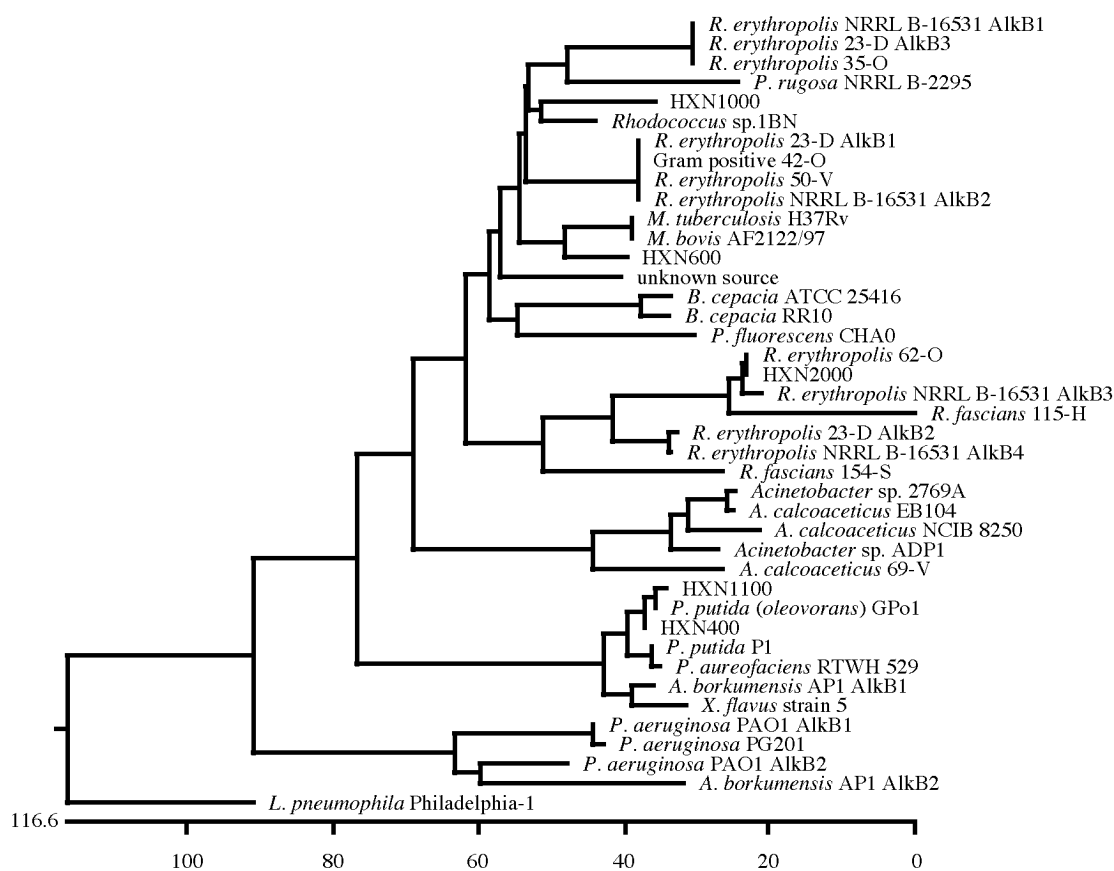

Figure 3: phylogenetic tree generated by CLUSTAL from the alignment of peptide sequences obtained in this study and published sequences. The primer encoded sequences are excluded from the alignment.

For a comparison of the peptides encoded by the PCR fragments, the nucleotide sequences were translated. In all cases when a 550 bp (559 bp) fragment was cloned, a contiguous open-reading-frame was obtained. The resulting peptide sequences were compared with the known alkane hydroxylase sequences (AlkB, AlkM), and with the putative alkane hydroxylase from *M. tuberculosis* H37Rv. An alignment produced using CLUSTAL (111), and manually optimized, is shown in Figure 2. For the alignment, we selected a series of representative sequences from all branches of the phylogenetic tree based on the manual alignment of all sequences, excluding the amino acids encoded by the primers, shown in figure 3.

Southern blotting. We selected six DNA fragments to use as gene probes in Southern blots (see Table 2). The six sequences are on different branches of the phylogenetic tree shown in Figure 3, and are chosen such that they should detect signals in Southern blotting in the remaining strains. Table 2 summarizes the result of these experiments. The probe derived from *P. aeruginosa* PG201 is selective for the *P. aeruginosa* strains and detects both alkane hydroxylase gene homologs (chapter 6), while the probe derived from *P. putida* (*oleovorans*) GPo1 detects related genes in *P. putida* P1 and in *P. aeruginosa* KSLA 473. The latter strain is, unlike other *P. aeruginosa* strains, able to grow on *n*-octane in addition to *n*-dodecane and *n*-hexadecane, and was previously shown to contain a gene identical to the *P. putida* (*oleovorans*) *alkB* gene (262), in addition to the genes detected by the probe derived from *P. aeruginosa* PG201. The probe derived from *P. fluorescens* CHA0 only detects the source strain. Using the *alkM* probe weak signals were detected in the other *Acinetobacter* strains, which can be explained by the presence of related genes with levels of DNA sequence identity ranging from 68 to 80 % to the *alkM* sequence, enough to detect a signal in Southern blotting. Related genes could not be detected in other strains. A probe derived from the *A. calcoaceticus* 69-V PCR fragment gave similar results (data not shown). The *R. erythropolis* NRRL B-16531 and *M. tuberculosis* H37Rv probes detect fragments in the *R. erythropolis* strain and in *P. rugosa* NRRL B-2295. These two probes also gave signals with several high G+C, mycolic acid containing actinomycetes (J. B. van Beilen, unpublished results).

Table 2: summary of Southern blots.

Strain	Growth on n-alkanes[1]	Probe[2]					
		GPo1[3]	ADP1[3]	CHAO[3]	PG201[3]	H37Rv[3]	16531[3]
P. putida (*oleovorans*) GPo1	C5-C12	+++	-	-	-	-	-
P. putida (*oleovorans*) GPo12	no	-	-	-	-	*	*
P. oleovorans ATCC 8062	no	-	-	-	-	-	-
P. putida PpG1	no	-	-	-	-	-	-
P. putida KT2442	no	-	-	-	-	-	-
P. putida P1	C8	+++	-	-	-	-	-
P. fluorescens CHAO	C12, C16	-	-	+++	-	-	-
P. aeruginosa PAO1	C12-C16	-	-	-	+++	*	-
P. aeruginosa KSLA 473	C8-C16	+++	-	-	+++	*	-
A. calcoaceticus 69-V	C12-C16	-	+	-	-	*	*
Acinetobacter sp. ADP1	C12-C16	-	++	-	-	*	*
Acinetobacter sp. 2769A	C12, C16	-	+	-	-	-	*
B. cepacia ATCC 25416	C10-C16	-	-	-	-	*	*
C. testosteroni DSM 50244	no	-	-	-	-	*	-
S. maltophilia DSM 50170	no	-	-	-	-	*	-
R. eutropha JM134 (pJP4)	no	-	-	-	-	-	-
R. rhodochrous NCIMB 12566	C8, C12	-	-	-	-	-	-
R. erythropolis NRRL B-16531	C8, C12, C16	-	-	-	-	+	++
Pr. rugosa NRRL B-2295	C12	-	-	-	-	++	+

Footnotes: [1]: Abbreviations: C5: pentane, C8: octane, C10: decane, C12: dodecane, C16: hexadecane; [2]: +++ very strong signal, ++ strong signal, + signal, *, aspecific signal, - no signal; [3]: probes derived from: GPo1: *P. putida* (*oleovorans*) GPo1; ADP1: *Acinetobacter* sp. ADP1; CHAO: *P. fluorescens* CHAO; PG201: *P. aeruginosa* PG201; H37Rv: *M. tuberculosis* cosmid pSCYB11; 16531: *R. erythropolis* NRRL B-16531.

Cloning and functional analysis of the *A. calcoaceticus* EB104 and *P. putida* P1 alkane hydroxylase gene homologs. A 3.0 kb *Eco*RI -*Hind*III fragment, containing the alkane hydroxylase gene homolog of *A. calcoaceticus* EB104 was identified by Southern hybridisation, cloned from an enriched genebank containing all 2.5-3.5 kb *Eco*RI-*Hind*III restriction fragments by colony blotting, and sequenced. Sequence analysis revealed two open-reading-frames that have 59.6 % and 72.6 % DNA sequence identity to *Acinetobacter* sp. ADP1 *alkR* and *alkM*, respectively (213). The ORFs were labelled *alkR* and *alkM*, respectively. To establish the function of *alkM*, we transformed an *alkM*

negative mutant of *Acinetobacter* sp. ADP1 (WH405) (213) with pWHEB7, which contains only *alkM* (*A. calcoaceticus* EB104). As a positive control, plasmid pWH968, containing the *Acinetobacter* sp. ADP1 *alkRM* and *rubAB* operons, was used (214). Both plasmids complemented WH405 for growth on dodecane, proving that AlkM (*A. calcoaceticus* EB104) encodes an alkane hydroxylase.

In the same way, a 7.5 kb *Eco*RI -*Hind*III fragment of *P. putida* P1 was identified, cloned from an enriched genebank with *Eco*RI-*Hind*III restriction fragments ranging from 6.5-8.5 kb, and sequenced. This fragment has on average 80 % DNA sequence identity to the *alkBFGHJ* genes of *P. putida* (*oleovorans*) GPo1, and complemented an *E. coli* recombinant, containing all *P. putida* (*oleovorans*) GPo1 *alk*-genes except *alkB* (GEc137[pGEc47ΔB]) (261) for growth on octane.

16S rDNA sequencing. *Nocardia globerula* NRRL B-2769 was obtained from the NRRL culture collection. On plating two types of colonies appeared, only one of which could grow on *n*-alkanes after purification. The PCR fragment obtained from this strain (2769A) has a G+C-content of 37.1 % (*Nocardia* strains: 60 % G+C) and is more closely related to the *Acinetobacter* sp. ADP1 *alkM* gene than the PCR fragment obtained from *A. calcoaceticus* strain 69-V. Therefore, we determined a partial 16S rDNA sequence of strain 2769A, and found that it had highest sequence identity to *Acinetobacter haemolyticus* ATCC 17922 (99.9 %, Acc. nr. Z93436), indicating that 2769A is an *Acinetobacter* species.

Partial 16S rDNA sequences were obtained from *Acinetobacter* strains *Acinetobacter* sp. ADP1 (100 % to *Acinetobacter* sp. DSM587 (=ADP1, Acc. nr. X89709)), 69-V (100 % to *Acinetobacter* sp. 10090 (Acc. nr. Z93449)), EB104 (99.8 % to *A. baumannii* DSM 30007 (Acc. nr. X81660)), NCIB 8250 (100 % to *Acinetobacter* sp. DSM590 (=NCIB 8250, Acc. nr. X81659) and *R. erythropolis* NRRL B-16531 (100 % to *Rhodococcus* sp. DN-22 (Acc. nr. X89240)) to confirm their classification.

DISCUSSION

We have developed degenerate oligonucleotides to amplify internal segments of genes related to the *P. putida* (*oleovorans*) GPo1 *alkB* and the *Acinetobacter* sp. ADP1 *alkM* genes, which encode homologous membrane-bound non-heme iron alkane hydroxylases. We found that most Gram-negative and Gram-positive bacteria able to grow on *n*-alkanes contain one or more genes related to *alkB* and *alkM*. Strains unable to grow on *n*-alkanes, including those that were reported to grow on *n*-alkanes but failed to do so in our hands, did not yield PCR fragments related to *alkB* or *alkM*.

Several strains able to grow on *n*-alkanes yielded neither the expected 550 bp PCR fragment nor a positive signal in Southern blots. This may be due to the fact that specific subsets of the degenerate oligonucleotides may have properties that interfere with the PCR. Primers that could in principle be effective in amplifying a certain gene may be selected out of the pool because of primer dimer or hairpin formation. Alternatively, strains that were negative in the PCR may contain alkane hydroxylase gene homologs in which the regions that were used to develop the primers are not so well conserved. Finally, the strain may contain an alkane hydroxylase belonging to a different enzyme class: a cytochrome P450 as was proposed for *R. rhodochrous* ATCC 19067 and *A. calcoaceticus* EB104 (44, 179), dioxygenases as in *Acinetobacter* sp. M1 (164), or other soluble alkane hydroxylases. Nevertheless, the data presented here show that many strains able to grow on *n*-alkanes, including several high G+C, mycolic acid containing actinomycetes, contain homologous but distantly related enzymes belonging to the class of integral-membrane non-heme iron monooxygenases.

The peptide alignment shows that certain regions are well conserved in all sequences. As the primers TS2S and Deg1RE allowed amplification of the PCR fragments described in this paper, the first and the third histidine boxes, on which these primers are based, must be well conserved. The second histidine-box (EHXXGHH), starting at position 167 in the AlkB sequence, is also highly conserved in all sequences, as is the motif NYXEHYG(L/M) starting at position 269 in AlkB. The histidine residue of the latter motif is conserved in all proteins that can be classified as integral-membrane non-heme

iron proteins and contain the three histidine motifs defined by Shanklin *et al.* (233). In all cases it is preceded by a stretch of hydrophobic residues (data not shown), which, in the case of AlkB, has been shown to traverse the membrane twice (261) (figure 2, solid bars). Site-directed mutagenesis of this histidine residue in the *P. putida mt*-2 xylene monooxygenase leads to the complete inactivation of the enzyme (M.G. Wubbolts, personal communication), which could mean that the corresponding residues are essential for the function of the related alkane hydroxylases as well.

The phylogenetic tree based on the DNA sequences, generated with CLUSTAL (111), shows that all *Acinetobacter* sequences cluster in one group (figure 3). This could be due to the low G+C content of these fragments, which is around 40 %, while nearly all other sequences are from high G+C organisms. However, sequences within the genus *Pseudomonas* are as divergent as sequences from two different genera. In the case of the *P. putida* (*oleovorans*) GPo1 and *P. putida* P1 *alk*-genes, horizontal gene transfer clearly plays a role. The *alk*-genes from these strains have a G+C content that is much lower than the G+C content of the *P. putida* (*oleovorans*) chromosome and the OCT-plasmid, and may constitute a transposon which originates from a low G+C host (260, 263). The sequences of the *P. fluorescens* CHA0 and *B. cepacia alkB* gene fragments have a high G+C content, and appear to have higher homology to sequences from Gram-positive strains, which are only slightly more related to each other than to some of the sequences from Gram-negative strains.

Sequences found in different strains often show higher homology than sequences found in the same strain, as observed for some Gram-positive strains. *P. aeruginosa* strains able to grow on medium-chain *n*-alkanes and *A. borkumensis* AP1 contain genes that are identical or closely related to the *P. putida* (*oleovorans*) GPo1 *alkB* gene, in addition to genes that are identical or closely related to the *P. aeruginosa* PAO1 *alkB1* and *alkB2* genes (262)(chapter 6).

To evaluate the use of the cloned PCR fragments in detecting genes involved in alkane metabolism, we carried out Southern blots using several different PCR fragments or gene fragments as probes. We found that probes derived from a particular gene only give

information about the presence or absence of the original host or closely related strains, as the cloned alkane hydroxylase gene homologs do not show clear DNA sequence homology to each other. Therefore, the probes cannot be used to screen environmental isolates able to grow on n-alkanes, as each strain probably contains alkane hydroxylase genes that are too divergent to be detected by the probes used. An exception is the *P. putida* (*oleovorans*) GPo1 *alkB* gene, which has been found in several pseudomonads, probably due to the fact that it is located on a plasmid.

A limitation of the PCR approach is that this method does not show the presence of two or more related genes in one strain, as is the case for several Gram-negative and Gram-positive strains (J. B. van Beilen, unpublished results; chapter 6), unless a (significant) number of cloned PCR fragments from a single strain is sequenced. Even then, it is possible that only one gene is amplified efficiently. For example, in *A. borkumensis* AP1 only *alkB2* was obtained by PCR with the *alkB* degenerate primers. Nevertheless, the oligonucleotides described in this study are very useful to clone *alkB* gene homologs. When strains are able to grow on either medium- or long-chain n-alkanes, gene fragments related to *alkB* and *alkM* could be amplified from many Gram-positive and Gram-negative strains. However, this does not prove that these gene fragments are part of alkane hydroxylase genes. Therefore, we cloned the alkane hydroxylase genes of *A. calcoaceticus* EB104 and *P. putida* P1, and showed by complementation experiments that the cloned genes did indeed encode functional alkane hydroxylases. We are currently cloning additional genes from a number of strains and constructing knock-out mutants of these strains, to establish that these genes also encode alkane hydroxylases. For this purpose we set up several *in vivo* complementation systems in which the alkane hydroxylase gene homologs can be functionally expressed (chapter 4). This allowed us to demonstrate that the alkane hydroxylase homologs from *A. borkumensis* AP1 (AlkB1), *M. tuberculosis* H37Rv, *P. rugosa* NRRL B-2295 (chapter 4), *P. aeruginosa* PAO1 (AlkB1 and AlkB2) (chapter 6) and *P. fluorescens* CHA0 (chapter 7) do indeed oxidize n-alkanes.

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Chapter 3

NEW ALKANE-RESPONSIVE EXPRESSION VECTORS FOR *E. COLI* AND *PSEUDOMONAS*

Theo H. M. Smits, Markus A. Seeger, Bernard Witholt and Jan B. van Beilen

SUMMARY

We have developed *Escherichia coli* and *Pseudomonas* expression vectors based on the alkane-responsive *Pseudomonas putida* (*oleovorans*) GPo1 promoter P*alkB*. The expression vectors were tested in several *E. coli* strains, *P. putida* GPo12 and *P. fluorescens* KOB2Δ1 with catechol-2,3-dioxygenase (XylE). Induction factors ranged between 100 and 2700 for pKKPalk in *E. coli* and pCom8 in *Pseudomonas* strains, but were clearly lower for pCom8, pCom9 and pCom10 in *E. coli*. XylE expression levels of more than 10 % of total cell protein were obtained for *E. coli* as well as for *Pseudomonas* strains.

INTRODUCTION

Escherichia coli is the most frequently used prokaryotic host for high-level production of heterologous proteins. However, it can not be used for all proteins due to the formation of inclusion bodies, or incomplete processing and export of extracellular proteins (100, 116). In addition, the different codon usage and G+C content of target genes may lead to low expression levels. *Pseudomonas* strains are suitable alternatives as they have a high G+C content and export extracellular proteins in large quantities (79).

Unfortunately, only a few *Pseudomonas* expression vectors are available. Examples are the *nahR* regulated expression vector pKMY319 (286), the *lac*-based pVLT series (55), and the *tac* -based vectors pHA10, pHA12, pKT240 and pMMB series (8, 15), all based on the low-copy number vector RSF1010. Higher copy number vectors like the pUCP series (53, 229, 277) depend on additional plasmids containing a *lacI* gene or specially constructed host strains.

The *Pseudomonas putida* (*oleovorans*) GPo1 *alkB* promoter (P*alkB*) (143) controls expression of the *alkBFGHJKL* operon, which encodes proteins involved in alkane degradation (reviewed in (263)). In *E. coli* W3110, alkane hydroxylase could be expressed in a correctly folded form from P*alkB* (its own promoter) to 10-15 % of total cell protein, which is extremely high for an integral membrane protein (185). In *E. coli* and *P. putida* (*oleovorans*), the PHA polymerase PhaC1 could be expressed

from the same promoter to 15 and 10 % of total cell protein, respectively (Q. Ren, G. de Roo, B. Witholt and B. Kessler, unpublished results)(144). Xylene monooxygenase (XylMA), which could not be expressed to high levels from other promoters, and styrene monooxygenase (StyAB) were also expressed from P*alkB* in *E. coli* in an active form (202, 203).

The positive regulator of P*alkB*, AlkS (67, 202), is activated by C7-C12 *n*-alkanes, alkenes, and gratuitous inducers such as haloalkanes, ethylacetate, ethylether and dicyclopropylketone (DCPK) (96, 283). AlkS is a member of the LuxR-UhpA family of regulatory proteins, and is most closely related to MalT, the regulator of the maltose regulon (202).

Expression from P*alkB* is negatively influenced by carbon catabolite repression in the wild type strain *P. putida* (*oleovorans*) GPo1, especially in LB-medium (244, 287). In *E. coli*, no catabolite repression was observed with the carbon sources glucose, lactate, glycerol and pyruvate (244).

Several vectors based on P*alkB* were constructed previously, such as an *E. coli* vector, which can be used for the production of VSV-G tagged proteins to allow easy detection and purification (184). Another P*alkB* vector system was developed for integration of the expression cassette in the chromosome of *E. coli* and *Pseudomonas* strains (201, 202). Because these vectors could not be used for complementation studies in which components of various alkane hydroxylase systems are replaced by their counterparts from other enzyme systems (chapter 4), easy-to-use *Pseudomonas* and *E. coli* expression vectors based on the *P. putida* (*oleovorans*) GPo1 *alkB* promoter were constructed. These plasmids are useful as general expression vectors for *E. coli* as well as for *Pseudomonas*, as the P*alkB* function in both species is well-characterized (69, 143, 287) and yields high induction and expression levels of both cytoplasmic and membrane proteins (184, 202).

MATERIALS AND METHODS

Strains, plasmids and media. Strains and plasmids used in this study are listed in table 1. E2 medium supplemented with metal traces (151) and glycerol (0.3 % w/v), glucose (0.5 % w/v) or citrate (0.2 % w/v) and LB (220) were used throughout this

study. 0.1 % Yeast extract was added to the *E. coli* cultures. Antibiotics were used at the following concentrations: ampicillin (200 μg/ml), kanamycin (50 μg/ml), tetracycline (12.5 μg/ml) or gentamicin (10 μg/ml for *E. coli*, 100 μg/ml for *P. putida* (*oleovorans*) and *P. fluorescens*). *E. coli* and *P. fluorescens* KOB2Δ1 (chapter 4) were transformed by electroporation (64, 117), while plasmids were introduced in *P. putida* (*oleovorans*) GPo12 by three parental mating using RK600 as the helper plasmid (61).

Table 1: strains and plasmids used in this study.

Strain:	Genotype:	Reference:
E. coli strains:		
DH10B	cloning strain	Gibco BRL
GEc137	TG1 *fadR, thi*	(69)
W3110	prototroph	Lab collection
Pseudomonas strains:		
P. putida (*oleovorans*) GPo12	GPo1 cured of OCT plasmid	(141)
P. fluorescens KOB2Δ1	CHA0, *alkB* gene deleted	chapter 4

Plasmid:	Characteristics:	Reference:
pBG11EΔN	pBG11 with *Eco*RI site and mutated *Nde*I site, *alkS*	(202)
pSPZ2E	pSPZ2 harbouring *xylE*	(201)
pEX100T	Knockout plasmid, *oriT*	(230)
pEX18Tc	Knockout plasmid, TcR	(115)
pCK217	Knockout plasmid, Ap^R, Km^R	(145)
pGEc47	*alkBFGHJKL, alkST* in pLAFR1	(69)
pGEc74	*alkST* in pLAFR1	(69)
pKK223-3	Expression vector, P*trc*, pMB1 *ori*	(34)
pUCP25	*E. coli-Pseudomonas* shuttle vector	(277)
RK600	Helper plasmid, *tra$^+$, mob$^+$*	(78)
pKKPalk	pKK223-3 derivative with P*alkB*, Ap^R	this study
pKKPalk-XylE	pKKPalk, *xylE* in *Nde*I-*Pst*I sites	this study
pCom5	pKKPalk, *rep* and *aacC1* from pUCP25, Gm^R	this study
pCom7	pCom5, *oriT* from pEX100T	this study
pCom8	pCom7, *alkS* from pBG11EΔN	this study
pCom8-XylE	pCom8, *xylE* in *Nde*I-*Hind*III sites	this study
pCom9	pCom8; Tc^R instead of Gm^R	this study
pCom9-XylE	pCom9, *xylE* in *Nde*I-*Hind*III sites	this study
pCom10	pCom8; Km^R instead of Gm^R	this study
pCom10-XylE	pCom10, *xylE* in *Nde*I-*Hind*III sites	this study

DNA methods. Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), T4 DNA polymerase and dideoxynucleotides were from Roche Molecular

Biochemicals (Rotkreuz, Switzerland). Oligonucleotides were synthesized by Microsynth (Balgach, Switzerland). PCR-products were purified over a 1% agarose gel, cut with appropriate restriction enzymes, purified again over a 1% agarose gel, and cloned into a suitable vector. Plasmid DNA was isolated with the Roche High Pure Plasmid Isolation Kit.

PCR reactions were carried out with the Roche Expand High Fidelity Polymerase kit using a Perkin Elmer GeneAmp PCR System 9600. For PCR, the following program was used (4' 95°C, 25x (45'' 95°C, 1' annealing temperature (usually 5°C below Tm), 1' 72°C), 5' 72°C, ∞ 4°C). All cloned PCR fragments were sequenced on a Li-Cor 4000L sequencer using the Amersham Thermosequenase cycle sequencing kit (Amersham, Rainham, UK) and IRD800-labelled PalkFw2 (ccctcggtcccccagatagcc), PalkFw3 (gccagctcgtgtttttccagcagacg) and pKKRev (gagttcggcatggggtcaggtg) for pKKPalk derived plasmids (MWG-Biotech, Ebersberg, Germany). Sequences were analyzed using LASERGENE Navigator (DNASTAR, Madison, Wisconsin, USA).

Construction of pKKPalk, pCom7, pCom8, pCom9 and pCom10. Plasmid pKKPalk was constructed as follows: plasmid pKK223-3 (34) was digested with *Nde*I and *Sma*I, made blunt with Klenow DNA polymerase, and ligated. The resulting plasmid was linearized with *Bam*HI and ligated to a PCR fragment containing P*alkB* (271 bp upstream of the ATG of *alkB*), generated with primers PalkRBS (cgggatcccgggcgcgccaagcatatggaattctcc, *Bam*HI, *Sma*I, *Asc*I, *Nde*I, *Eco*RI sites) and Palk5 (gggttttggagatctccaatcgtg, *Bgl*II site) with pGEc47 (69) as template, and cut with *Bam*HI and *Bgl*II. Plasmids containing the inserted P*alkB* fragment were checked for the right orientation of the insert, and sequenced using primer pKKRev. To construct a pKKPalk derivative able to replicate in pseudomonads, the plasmid was digested with *Fsp*I and *Ssp*I, to remove most of the ampicillin resistance gene. The remaining part of the plasmid was purified by gel electrophoresis. Plasmid pUCP25 (277) was digested with *Fsp*I and *Sac*II, and the 2.8 kb fragment, which contains the *Pseudomonas* origin of replication (*rep*) and the *aacC1* gene, was isolated, made blunt with T4 DNA polymerase and ligated in the large fragment of pKKPalk, resulting in pCom5. The orientation of *rep* and *aacC1* was determined by restriction analysis.

A 566 bp fragment containing the origin of transfer (*oriT*, intergenic region of *traJ* and *traK* (88)) was amplified from pEX100T (230) with primers oriTfw3 (cattgat<u>gcatgc</u>caggtaccgctcgagctcatagtccac, *Sph*I, *Asp*718I, *Xho*I, *Sac*I sites) and oriTrv2 (ctttggg<u>atcc</u>tctctcgcct, *Bam*HI site), digested with *Bam*HI and *Sph*I and cloned in the *Sph*I-*Bcl*I sites of pCom5, resulting in pCom7. The *oriT* was then sequenced with the primers pKKRev and PalkFw2. The regulatory gene *alkS*, mutagenized to remove an internal *Nde*I site, was amplified from plasmid pBG11EΔN (202) with primers alkSfw3 (ggaaat<u>gggtacc</u>gcgagctact, *Asp*718I site) and alkSrv (cgggatc<u>ctcgag</u>tatacttttcactatatc, *Bam*HI, *Xho*I sites). This PCR fragment, containing the *alkS* gene and 248 bp of its own promoter, was digested with *Asp*718I and *Xho*I and cloned in pCom7, digested with the same enzymes. The resulting plasmid was called pCom8. A part of *alkS* was sequenced with the primers pKKrev and PalkFw2. Plasmids pCom9 and pCom10 were constructed as follows: most of pCom8, except the *aacC1* gene, was amplified with primers pCom8fw (cgctt<u>atcgat</u>tggatgcccgaggc, *Cla*I site) and pCom8rv (tgatt<u>atcgat</u>tggtaactgtcagacc, *Cla*I site). The tetracycline resistance gene was amplified from pEX18Tc (115) with primers pEX18Tcfw (atac<u>atcgat</u>atatgtatccgc, *Cla*I site) and pEX18Tcrv (gattggc<u>atcgat</u>tcttggagt, *Cla*I site). The PCR fagments were digested with *Cla*I and ligated. The resulting plasmid was called pCom9. The orientation of the *tet* gene was determined by restriction analysis. In the same manner, the pCom8 PCR fragment was ligated to a *Cla*I digested PCR fragment containing the kanamycin resistance gene *npt* of pCK217 (145), amplified with primers pCK217fw (ctggt<u>atcgat</u>gggaagccctgc, *Cla*I site) and pCK217rv (gttgggc<u>atcgat</u>tggtcggtca, *Cla*I site), which resulted in pCom10. The orientation of the *npt* gene was also determined by restriction analysis.

To construct pKKPalk-XylE, the *xylE* gene was cut from pSPZ2E (201) with *Nde*I and *Pst*I and cloned in pKKPalk. To construct plasmid pCom8-XylE, pCom9-XylE and pCom10-XylE, the *xylE* gene was cut from pKKPalk-XylE with *Nde*I and *Hind*III and cloned in the respective expression vector, digested with the same enzymes.

Catechol-2,3-dioxygenase assays. Strains harbouring plasmids pKKPalk-XylE, pCom8, pCom8-XylE, pCom9-XylE and pCom10-XylE were grown in E2 minimal medium containing the appropriate antibiotics to the mid-logarithmic phase (OD_{450} ~ 0.5 - 1.0) and induced with 0.05 % DCPK. After 2 and 5 hours, 30 ml samples were

harvested, washed once with 10 mM $MgSO_4$ and resuspended in 0.6 ml 50 mM phosphate buffer (pH 7.5). The cells were disrupted in a bead-mill and the crude cell extract was recovered by centrifugation (10 minutes in a cooled microfuge). The protein concentration was determined using Bradford reagent (Biorad Laboratory, München, Germany). Catechol-2,3-dioxygenase activity was measured according to Nozaki (191). One unit (U) of activity corresponds to 1 μmol catechol converted per minute.

SDS-Polyacrylamide gel electrophoresis was carried out using 12 % running and 6 % stacking gels. About 10 μg protein was loaded per lane. Gels were stained with Coomassie Brilliant Blue R-250 (104).

Sequences. The sequences of pKKPalk, pCom7, pCom8, pCom9 and pCom10 were deposited in GenBank, and received the accession numbers AJ299425, AJ299426, AJ299427, AJ302086 and AJ302087, respectively.

RESULTS AND DISCUSSION

Construction of pKKPalk, pCom7, pCom8, pCom9 and pCom10. Plasmid pKKPalk (figure 1a) is a 2.7 kb pKK223-3 derivative (34) which confers ampicillin resistance, and can be used in *E. coli* strains harbouring the regulator gene *alkS in trans*, either inserted in the chromosome (201) or on a second compatible plasmid, such as pGEc74 (69). The multiple cloning site (figure 1b) includes an *Eco*RI site at the ribosome binding site and an *Nde*I site overlapping with the start codon. The *rrnB* terminator of pKK223-3, located downstream of the multiple cloning site, prevents readthrough from the P*alkB* into regions necessary for replication of the plasmid. Due to the deletion of the *rop* gene during construction of the plasmid, pKKPalk has a copy number between those of pBR322 and the pUC series, based on plasmid amounts isolated with standard procedures (data not shown).

Plasmid pCom7 (figure 1a) is an *E. coli - Pseudomonas* shuttle vector which confers gentamicin resistance and replicates in *Pseudomonas* strains, such as *P. aeruginosa*, *P. fluorescens* and *P. putida* (197, 277), by virtue of the pUCP25 (pRO1600)(197, 277) origin of replication. The origin of transfer (*oriT*) of plasmid RP4 (78, 88) enables transfer of plasmid pCom7 from *E. coli* to *Pseudomonas* by three parental
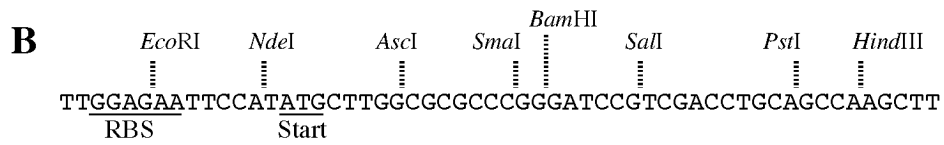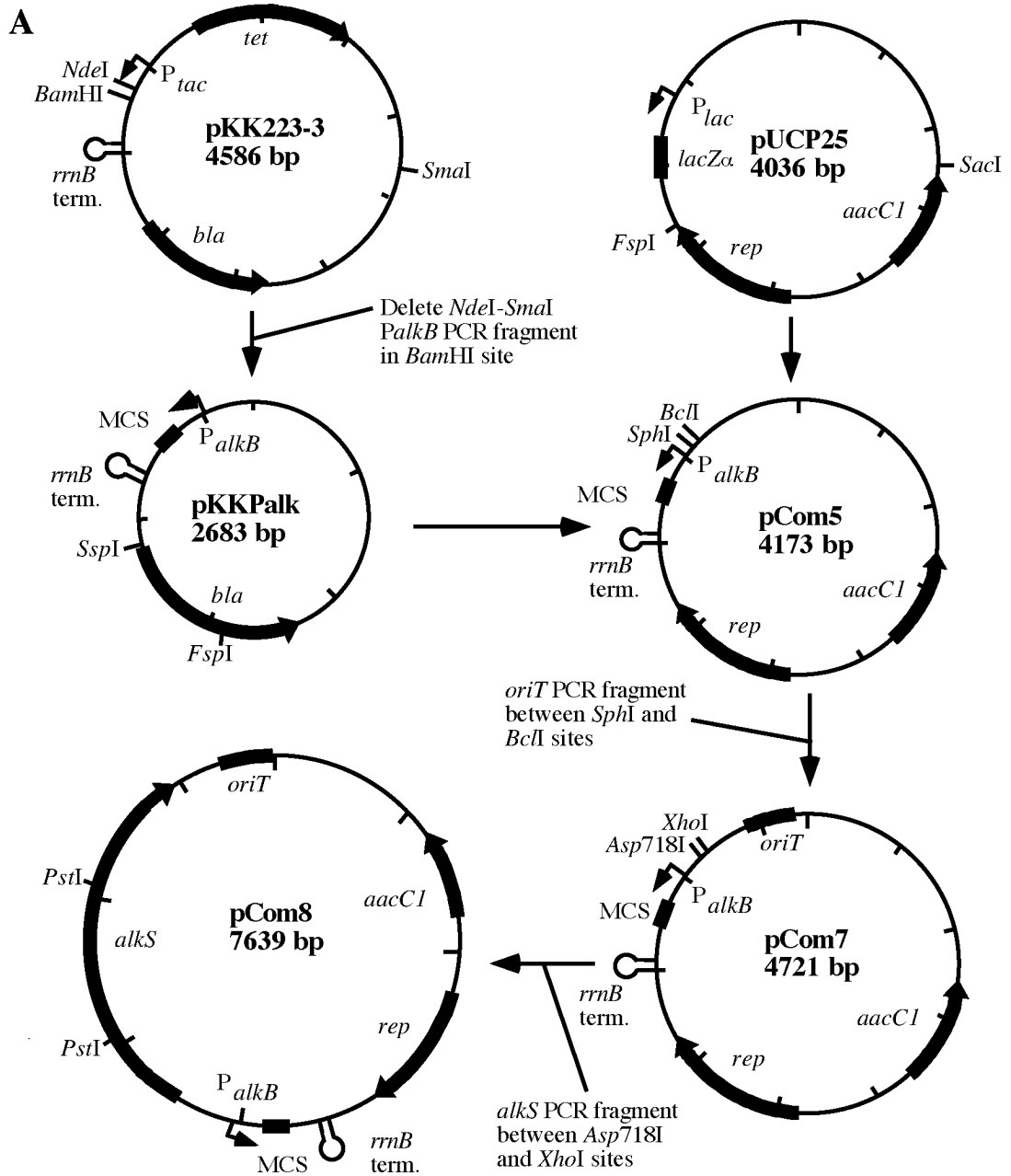
A

pKK223-3 4586 bp — *tet*, *Ndel*, *Bam*HI, P*tac*, *rrnB* term., *bla*, *Sma*I

Delete *Nde*I-*Sma*I
P*alkB* PCR fragment
in *Bam*HI site

pKKPalk 2683 bp — MCS, P*alkB*, *rrnB* term., *SspI*, *bla*, *FspI*

pUCP25 4036 bp — P*lac*, *lacZα*, *FspI*, *rep*, *aacC1*, *Sac*I

pCom5 4173 bp — *BcII*, *SphI*, P*alkB*, MCS, *rrnB* term., *rep*, *aacC1*

oriT PCR fragment
between *Sph*I and
*Bcl*I sites

pCom7 4721 bp — *XhoI*, *Asp*718I, *oriT*, MCS, P*alkB*, *rrnB* term., *rep*, *aacC1*

alkS PCR fragment
between *Asp*718I
and *Xho*I sites

pCom8 7639 bp — *oriT*, *PstI*, *aacC1*, *alkS*, *rep*, *PstI*, P*alkB*, MCS, *rrnB* term.

B

EcoRI   NdeI   AscI   SmaI   BamHI   SalI   PstI   HindIII

TTGGAGAATTCCATATGCTTGGCGCGCCCGGGATCCGTCGACCTGCAGCCAAGCTT

RBS   Start

Figure 1. A: construction of pKKPalk, pCom7 and pCom8. B: multiple cloning site of the vectors. The ribosomal binding site (RBS) and start codon are underlined and marked below the sequence. Two additional *PstI* sites are present in *alkS*.

mating using helper plasmids containing the *tra* and *mob* genes, like RK600 or RK2013 (78).

Frequencies of transfer of pCom7 are higher than those of pLAFR1 with the same helper plasmids. To use this plasmid, host strains must also contain a copy of the *alkS* gene, located either on the chromosome or on a plasmid such as OCT (*P. putida* (*oleovorans*) GPo1) or pGEc74 (69). As it may not always be possible to use a strain containing *alkS in trans*, *alkS* was inserted in pCom7, resulting in pCom8 (figure 1a). Two derivatives of pCom8 that confer tetracycline (pCom9; 8121 bp) or kanamycin (pCom10; 7675 bp) resistance, were constructed because the gentamycin resistance reduces growth rates and final cell densities.

Catechol-2,3-dioxygenase activity assays. To test expression levels and induction factors, *xylE* was cloned in pKKPalk, pCom8, pCom9 and pCom10. Recombinant strains containing the resulting plasmids and a number of control strains were tested for catechol-2,3-dioxygenase (XylE) activity (table 2). Recombinants containing pKKPalk or pCom8 without the *xylE* gene showed no measurable XylE activity. *E. coli* strain GEc137[pGEc74, pKKPalk-XylE] showed very low XylE activities under non-induced circumstances, while activities of 0.34 and 0.75 U/mg protein for glucose- and glycerol-grown cells, respectively, were measured 2 hours after induction. Because of this result, glycerol was used for all further *E. coli* cultures. Citrate was used as a carbon-source for *Pseudomonas* recombinants as it does not cause catabolite repression of P*alkB* (244).

Plasmid pCom8-XylE was tested in the *E. coli* strains DH10B, GEc137 and W3110, and in the pseudomonads *P. putida* (*oleovorans*) GPo12 and *P. fluorescens* KOB2Δ1. *E. coli* strains containing pCom8-XylE showed high XylE activities under non-induced circumstances, with values of up to 4.2 U/mg protein. 5 Hours after induction, specific activities of around 10 U/mg protein or 10 to 25 % of total cell protein were found, with induction factors ranging from 2.3 to 16 (table 2, figure 2).

Table 2: overview of results from the XylE induction experiments.

Strain	Carbon source	Time after induction (h)	Specific activities (U/mg total cell protein)		Induction factor	Percent XylE[a] (% of total cell protein)	
			Non-induced	Induced		Non-induced	Induced
E. coli GEc137[pGEc74, pKKPalk-XylE]	Glucose	2	0.0004	0.34	850	n.d.[b]	n.d.
E. coli DH10B[pCom8-XylE]	Glycerol	2	0.0005	0.75	1500	n.d.	n.d.
E. coli DH10B[pCom8-XylE]	Glycerol	2	1.1	4.7	4.3	~5	~20
		5	1.1	9.1	8.3	~5	>25
E. coli GEc137[pCom8-XylE]	Glycerol	2	0.25	1.9	7.6	2-5	~20
		5	0.63	10.3	16	~5	>25
E. coli W3110[pCom8-XylE]	Glycerol	2	1.9	6.0	3.1	2-5	~10
		5	4.2	9.7	2.3	~5	>25
E. coli DH10B[pCom9-XylE]	Glycerol	2	0.08	1.2	15	<1	2-5
		5	0.08	2.8	35	<1	~10
E. coli DH10B[pCom10-XylE]	Glycerol	2	0.51	3.6	7.0	2-5	~10
		5	0.51	13.9	27.3	2-5	~20
P. putida (oleovorans)GPo12[pCom8-XylE]	Citrate	2	0.001	2.7	2700	<0.1	>5
		5	0.003	4.7	1566	<0.1	>10
P. fluorescens KOB2A1[pCom8-XylE]	Citrate	2	0.018	2.0	111	<0.1	>5
		5	0.067	3.8	56.7	<0.1	>10

Footnotes: [a] estimated from SDS-PAGE; [b] n.d.: not determined

Similar activities but slightly higher induction factors ranging from 7.0 to 35 were obtained with pCom9-XylE and pCom10-XylE in *E. coli* DH10B (table 2). When calculated with the specific activities of highly purified catechol-2,3-dioxygenase (227 U/mg protein), the measured activity corresponds to approximately 4-6 % of total cell protein. The difference with the expression level estimated from SDS-PAGE may be due to the formation of inclusion bodies.
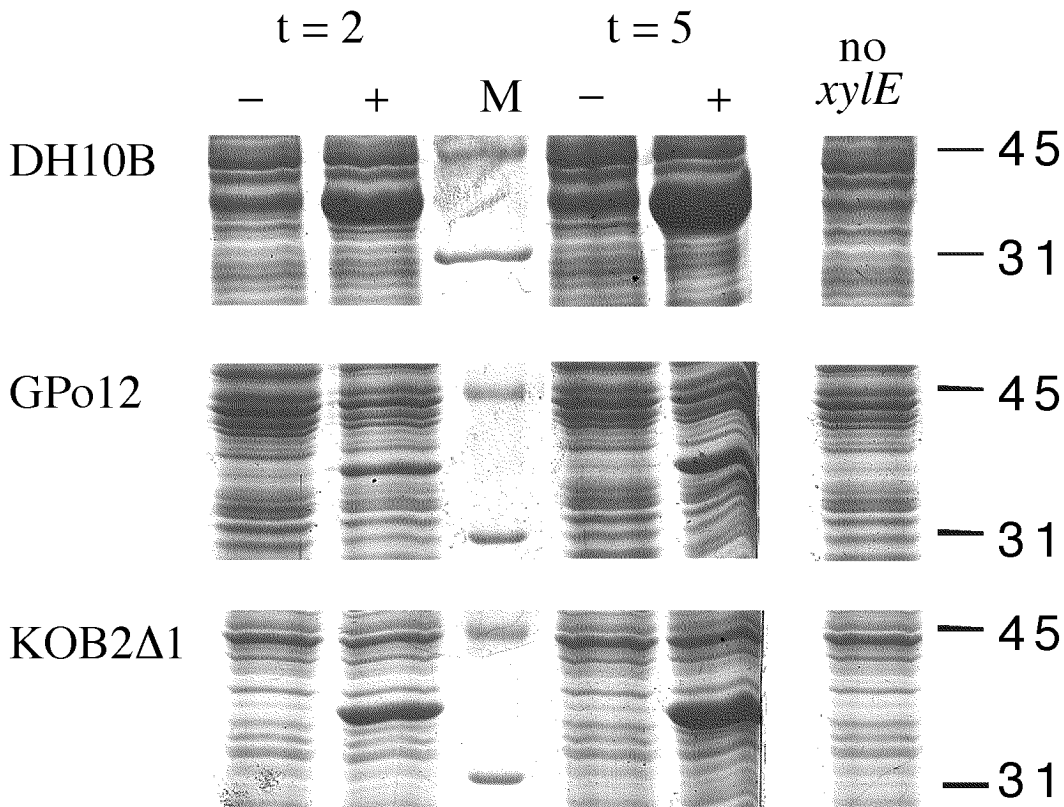

Figure 2: protein gels of the pCom8-XylE recombinants.
Non-induced (-) and induced (+) samples, 2 and 5 hours after induction, were loaded. M: marker. No *xylE*: strain containing pCom8 without insert, after 5 hours induction with DCPK.

The high background activities, and consequently low induction factors of pCom8-XylE in *E. coli* strains may be due to the high copy number of the positive regulator *alkS*. Previously constructed expression vectors containing P*alkB* were based on the medium-copy number plasmid pBR322 (202), or were dependent on *alkS* encoded on the

chromosome or on a separate low-copy number plasmid, such as the pLAFR1-derived pGEc74 (184). These vectors showed tight control of P*alkB*, as was also observed in this study for pKKPalk-XylE.

In pseudomonads, where pUCP25 derivatives have a lower copy number (229), the induction factors were clearly higher (up to 110 for *P. fluorescens* KOB2Δ1, and up to 2700 for *P. putida* (*oleovorans*) GPo12). The absolute expression levels reached 4.7 U/mg protein. In non-induced cultures of GPo12[pCom8-XylE] and KOB2Δ1[pCom8-XylE], XylE is not visible as a band on the SDS-PAA gels, in accordance with the activity data, while a clear XylE band accounting for up to 10 % of total cell protein is visible in induced cultures (figure 2). Previously described *Pseudomonas* expression systems only reached induction values of around 12 to 40, although the absolute XylE activities for *Pseudomonas* were comparable (8, 175, 286).

In conclusion, plasmids pKKPalk, pCom7, pCom8, pCom9 and pCom10 constitute a useful alternative expression system for heterologous expression in *E. coli* and *Pseudomonas*. The plasmids have convenient restriction sites, are fully sequenced, have a medium to high copy number in *E. coli* and *Pseudomonas*, and yield expression levels as high as 25 % with induction levels as high as 2700. The vectors are compatible with pLAFR-derivatives (RK2), and most probably also with RSF1010 based *Pseudomonas* expression vectors. Induction of P*alkB* with *n*-alkanes, ethylacetate or DCPK can be an alternative to expensive inducers such as IPTG, or to methods that are cumbersome on a large scale, such as heat shock activation. For low background activities and high induction factors in *E. coli*, pKKPalk is well suited, while pCom8, pCom9 or pCom10 are useful for expression in pseudomonads.

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Chapter 4

CLONING AND FUNCTIONAL ANALYSIS OF ALKANE HYDROXYLASES FROM GRAM-NEGATIVE AND GRAM-POSITIVE ORGANISMS

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SUMMARY

We have cloned homologs of the *Pseudomonas putida* (*oleovorans*) GPo1 alkane hydroxylase from *Pseudomonas aeruginosa* PAO1, *Pseudomonas fluorescens* CHA0, *Alcanivorax borkumensis* AP1, *Mycobacterium tuberculosis* H37Rv and *Prauserella rugosa* NRRL B-2295. Sequence analysis and comparisons show that the three *Pseudomonas* alkane hydroxylases are as distantly related to each other as to the remaining alkane hydroxylases. On the other hand, the *A. borkumensis* enzyme is closely related to the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase, suggesting that in this case horizontal gene transfer has taken place.

For the functional analysis of the (putative) alkane hydroxylase genes, we have developed three recombinant host strains, based on the observation that one of the electron transfer components of the GPo1 alkane hydroxylase system, rubredoxin, can be replaced by rubredoxins from other alkane hydroxylase systems. The three host strains express all proteins necessary for growth on alkanes except an alkane hydroxylase. All alkane hydroxylases enable at least one of the hosts to grow on alkanes, using electrons supplied by the rubredoxin and rubredoxin reductase of the host strain.

INTRODUCTION

Bacterial oxidation of *n*-alkanes is a very common phenomenon in soil and water (37), 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 (218). Perhaps even more significant are the alkanes (mainly waxes or paraffins) produced by plants, algae and other organisms, because they are available to bacteria in the entire biosphere. Apart from their important role in the carbon cycle, alkane hydroxylases are versatile biocatalysts which carry out a wide range of useful oxidation reactions (119, 281).

Biochemical and genetic studies have thus far focussed on a very limited number of alkane hydroxylases, such as the *Pseudomonas putida* (*oleovorans*) GPo1 alkane

hydroxylase (207), which oxidizes C5-C12 *n*-alkanes to 1-alkanols, thereby allowing the host to grow on these compounds. It has become apparent only recently that this enzyme is the prototype of a very diverse collection of related non-heme iron integral membrane oxygenases which includes the alkane hydroxylases of *Acinetobacter* sp. ADP1 (213), *Acinetobacter calcoaceticus* EB104 and *P. putida* P1 (239), but also xylene monooxygenases (251), fatty acid desaturases, fatty acid monooxygenases, steroid oxygenases and decarbonylases (232).

The *P. putida* (*oleovorans*) 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 (143, 173, 261). AlkG (207) transfers electrons from AlkT to AlkB, and AlkT is an NADH-dependent flavoprotein (256). The genetics of this enzyme system has been reviewed by van Beilen *et al.* (263). 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 (243) and in several fluorescent pseudomonads (226, 239, 262, 273). More distantly related genes were found in many Gram-positive and Gram-negative strains able to grow on *n*-alkanes ranging from C6 to C30 (239)(chapter 2) using highly degenerate primers which amplify internal fragments of alkane hydroxylase homologs. In this study, we used these PCR fragments and genome sequencing data to clone a number of alkane hydroxylases from a diverse group of bacteria. Based on the observation that rubredoxins from Gram-negative and Gram-positive alkane degrading strains can replace the *P. putida* (*oleovorans*) GPo1 rubredoxin in alkane oxidation (J. B. van Beilen, unpublished results), we have constructed hosts and expression vectors that allow us to test whether the novel alkane hydroxylases oxidize *n*-alkanes.

MATERIAL AND METHODS

Strains, plasmids and media. Strains used in this study are listed in table 1. LB (Luria Bertani broth) (220), E-medium (272) and E2-medium (151), supplemented with carbon sources or antibiotics were used throughout. MT trace elements (151) were added to

minimal media. All cultures were grown aerobically at 30°C or 37°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 with *n*-octane (C8) and *n*-decane (C10) in a sealed container, for *n*-dodecane (C12), *n*-tetradecane (C14) and *n*-hexadecane (C16) by placing a Whatman 3MM filter disc with 200 μl *n*-alkane in the lid of the Petri dish. Recombinants were cultured in baffled Erlenmeyer flasks with E2 medium and 1 % (v/v) *n*-alkanes as carbon source. Rhamnolipids (97) were added to a concentration of 0.1 % (w/v) to solubilize long-chain *n*-alkanes.

Table 1: list of strains and plasmids used in this study

Strain	Relevant phenotype	Reference
P. aeruginosa PAO1	alk^+	(118)
P. putida (*oleovorans*) GPo12	GPo1 cured of the OCT plasmid	(141)
P. fluorescens CHA0	alk^+	(250)
P. fluorescens KOB2Δ1	*alkB* knockout mutant of CHA0	This study
Acinetobacter sp. ADP1	alk^+	(126)
Alcanivorax borkumensis AP1	alk^+	(284)
Prauserella rugosa NRRL B-2295	alk^+	(119)
E. coli JM101	*endA, hsdR, supR, thi-1,* Δ(*lac-proAB*)	
	F'(*traD36, proAB, lacI^q, lacZM15*)	(285)
E. coli DH10B	cloning strain	Gibco BRL
E. coli SF800	W3110 *polA^-*	Laboratory collection
E. coli GEc137	*thi, fadR*	(67)

Plasmid	Characteristics	Reference
pGEM7-Zf(+)	Cloning vector, Ap^R	Promega
pUC18Sfi	Cloning vector, Ap^R	(110)
pZero2.1	Cloning vector, Km^R	Invitrogen
pKKPalk	Expression vector using P*alkB*, Ap^R	(240)
pCom5	Expression vector using P*alkB*, Gm^R	(240)
pCom7	Expression vector using P*alkB*, Gm^R, *oriT*	(240)
pCom8	Expression vector using P*alkB*, Gm^R, *oriT, alkS*	(240)
pCK217	pUC18Sfi containing *res-npt-res*	(145)
pEX18Tc	gene replacement vector	(115)
pUCP24	*E. coli-Pseudomonas* shuttle vector	(277)
pJMSB8	Ap^R, $P_{A1/04/03}$::*parA* in pJMSA8	(145)
pUCP24ParA	Gm^R, *parA* as *Eco*RI-*Hind*III fragment in pUCP24	This study
pCHA0	CHA0 PCR fragment	(239)
pPF1	4.5 kb *Xho*I-*Bam*HI fragment of *P. fluorescens* CHA0 in pGEM7-Zf(+), *alkB*	This study

pPF1mrB	pPF1Δ*Sfi*I-*Stu*I, *res-npt-res* in *alkB*	This study
pEXPF1mrB2	pPF1mrB *Pvu*I fragment in pEX18Tc	This study
pAF1	Plasmid containing *alkG* (AP1)	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 BglII fragment of *A. borkumensis* AP1 in pZero2.1, *alkGHJ*	This study
pAP1T1	4.6 kb NheI fragment of *A. borkumensis* AP1 in pZero2.1, '*alkS*	This study
pSCYB11	Tc^R, *alkB, rubA, rubB* (*M. tuberculosis* H37Rv)	(209)
p2295	*P. rugosa* NRRL B-2295 PCR fragment	(239)
pPru1	3.0 kb *Sac*I-*Bam*HI fragment of *P. rugosa* NRRL B-2295 in pGEM7-Zf(+), *alkB*	J. B. van Beilen
pGEc47	*alkBFGHJKL*/*alkST* (GPo1) in pLAFR1	(69)
pGEc47ΔB	pGEc47, deletion in *alkB*	(261)
pPF21	*alkB* gene (CHA0) with own promoter in pCom5	This study
pCom7B1 (PAO1)	*alkB1* gene (PAO1) in pCom7	This study
pCom7B2 (PAO1)	*alkB2* gene (PAO1) in pCom7	This study
pCom7B (GPo1)	*alkB* gene (GPo1) in pCom7	This study
pCom7alkB1 (AP1)	*alkB1* gene (AP1) in pCom7	This study
pCom7M (ADP1)	*alkM* gene (ADP1) in pCom7	This study
pCom8B1 (PAO1)	*alkB1* gene (PAO1) in pCom8	This study
pCom8B2 (PAO1)	*alkB2* gene (PAO1) in pCom8	This study
pCom8B (GPo1)	*alkB* gene (GPo1) in pCom8	This study
pCom8M (ADP1)	*alkM* gene (ADP1) in pCom8	This study
pCom8MT (H37Rv)	*alkB* gene (H37Rv) in pCom8	This study
pCom8B (2295)	*alkB* gene (2295) in pCom8	This study

E. coli strains and *P. putida* (*oleovorans*) GPo12[pGEc47B] were transformed by electroporation according to Dower (64). *P. fluorescens* CHA0 and KOB2Δ1 were transformed according to Højberg (117). Plasmids pCom7B2 (PAO1), pCom7alkB1 (AP1) and pCom7M (ADP1) were transferred to *P. putida* (*oleovorans*) GPo12[pGEc47ΔB] by three parental matings with *E. coli* DH10B as the donor and *E. coli* CC118[RK600] as the helper strain (61). Transconjugants were selected on E-medium containing the appropriate antibiotics. *E. coli* strains harbouring plasmids were grown with appropriate antibiotics (tetracycline (Tc) 12.5 μl/ml; ampicillin (Ap) 100 μg/ml; gentamycin (Gm) 10 μg/ml). For *P. fluorescens* KOB2Δ1 recombinants, gentamycin was used at 100 μg/ml. For *P. putida* (*oleovorans*) GPo12 recombinants, tetracycline (12.5 μg/ml) and gentamycin (100 μg/ml) were used.

DNA manipulations. Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), T4 DNA polymerase and dideoxynucleotides were from Roche Diagnostics

(Rotkreuz, Switzerland) and used as specified by the supplier. Oligonucleotides were synthesized by Microsynth, Balgach, Switzerland.

Plasmid DNA was isolated with the Roche High Pure Plasmid Isolation Kit or according to Birnboim-Doly (21) for *Pseudomonas* recombinants. Chromosomal DNA was isolated according to Sambrook *et al.* (220). PCR reactions 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' 95°C, 25x (45'' 95°C, 1' annealing temperature (5°C below Tm), 1' 72°C), 5' 72°C, ∞ 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(+) (Promega, Madison, USA) or pKKPalk (240). All PCR fragments were checked for possible PCR artefacts by sequencing both strands of the inserts on a Li-Cor 4000L sequencer using the Amersham Thermosequenase cycle sequencing kit and IRD800-labelled -40 forward (agggttttcccagtcacgacgtt) and -40 reverse (gagcggataacaatttcacacagg) primers for pGEM7-ZF(+) clones or PalkFwd (tggcgcaagcgtccgattag), PalkFw3 (gccagctcgtgtttttccagcagacg) and pKKRev (gagttcggcatgggggtcaggtg) for pKKPalk-derived plasmids (MWG-Biotech, Ebersberg, Germany).

Nucleotide and amino acid sequences were analyzed and compared using LASERGENE Navigator from DNASTAR (Madison, Wisconsin, USA). Nucleotide and amino acid sequences were compared with the EMBL, SwissProt and GenBank databases using BLAST (5). BLAST searches were carried out at NCBI.

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 (220) using 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(+) (Promega, Madison, USA) or pZero2.1 (Invitrogen, Leek, The Netherlands) and transformed into *E. coli* DH10B (Gibco BRL). Transformants containing the target genes were identified by colony blotting using the same probes. Both strands of the inserts were sequenced.

The *alkB1* and *alkB2* genes of *P. aeruginosa* PAO1 (249) were amplified by PCR from chromosomal DNA using primer combinations AlkBpaFwd (aactggaattcacgatgtttga) and AlkBpaRv2 (ctgcccgaagcttgagctat) and AlkBpaBfw (ggagaattctcagacaatct) and AlkBpaBrv (gaggcgaatctagaaaaaactg) respectively. Primers alkMfE2 (ccggaattcactatgaatgcacctgta) and alkMrPS (aataggcctgcagtcacttagactctctt) were used to amplify *alkM* of *Acinetobacter* sp. ADP1 (213). To amplify *alkB1* of *A. borkumensis* AP1, primers alkB1fw (caaggtgatccatatgtcagagaac) and alkB1rv (gcggatcctcaaagtgtgaaagc) were used. For *alkB* of *P. rugosa* NRRL B-2295 primers 2295fwEco (ggagaattcagatgagcgacgacgcac) and 2295rvBam (cggcgaggatccggtccagctc) were used. The *M. tuberculosis* H37Rv *alkB* gene was amplified from cosmid pSCYB11 (209) using primers MTalkBfw2 (cggaattcatatgaccacgcaaatcggc) and MTalkBrv3 (cagaccgggatccggtaggcgg). The *P. putida* (*oleovorans*) GPo1 *alkB* gene was amplified from pGEc47 (69) using primers B5-Eco (ggagaattccaaatgcttgag) and B3-Hind (tttgtgaaagctttcaacgcc). All PCR fragments were digested with the respective restriction enzymes and inserted in pCom7 or pCom8 (240); *alkB2* (PAO1) was cloned first in the *Eco*RI and *Xba*I sites of pUC18Sfi, and recloned in pCom8 using *Eco*RI and *Hind*III. 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 (240).

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 (145) with *Hind*III 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 (115). The final plasmid, pEXPF1rnrB2, was introduced into *P. fluorescens* CHA0 by electroporation using Km selection. A Km-resistant, Tc-sensitive colony was obtained, which was shown by Southern blot hybridisation 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 transcriptional terminator, the *parA* gene was supplied on pUCPParA. The latter plasmid was constructed by digesting pJMSB8 (145) with *Eco*RI and *Hind*III. The fragment containing the *parA* gene was isolated over gel, and ligated in pUCP24 digested with the same enzymes. Gentamycin resistant colonies were tested for absence of the Km-cassette by PCR and sensitivity to Km. One positive colony, subsequently cured of pUCPParA, was designated *P. fluorescens* KOB2Δ1.

Sequences. Unfinished genome searches were done at NCBI (http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html). Nucleotide sequences used in this study are available at EMBL under the following accession numbers: *Acinetobacter* sp. ADP1: *alkRM*: AJ002316; *Alcanivorax borkumensis* AP1: AJ295164; *Mycobacterium tuberculosis* H37Rv: *alkB* (Rv3252c): Z46863; *Prauserella rugosa* NRRL B-2295: AJ009587; *Pseudomonas aeruginosa* PAO1: *alkB1* (PA2574): AE004685; *alkB2* (PA1525): AE004581; *Pseudomonas fluorescens* CHA0: AJ009579; *Pseudomonas putida* (*oleovorans*) GPo1: *alkBFGHJKL, alkN, alkST*: AJ245436; *Pseudomonas putida* P1: *alkBFGHJKL, alkST*: AJ233397

RESULTS

Selection of strains. For this study, we have selected a number of alkane-oxidizing strains from different environments (soil, seawater, cow-rumen), and different research fields (biodegradation, biocatalysis, pathogenicity). In the case of *Acinetobacter* sp. ADP1 and *P. putida* strains GPo1 and P1, which are isolates of common soil and water organisms, the alkane hydroxylase genes have been cloned and sequenced before (213, 260). *Alcanivorax borkumensis* AP1 is a marine γ-*Proteobacterium* (284), which grows almost exclusively on *n*-alkanes. The same bacterium was isolated from sea water at many geographic locations, and forms a major part of the biomass in oil-polluted marine habitats (101). *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 soilborne plant pathogens (99). *P. aeruginosa* PAO1 represents a species which is of clinical importance as the primary opportunistic pathogen among the pseudomonads (27), but is also common in soil, 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*. *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 (119) if it is grown on *n*-alkanes.

Cloning of novel alkane hydroxylase genes, and analysis of the flanking regions. The *P. aeruginosa* PAO1 genome sequence (249) encodes two alkane hydroxylase (*alkB1* and *alkB2*) gene homologs, while the genome sequence of *M. tuberculosis* H37Rv (51) encodes one alkane hydroxylase homolog. These putative alkane hydroxylase genes were cloned as described in the Materials and Methods section. Alkane hydroxylase homologs in the genome sequences of other *Mycobacterium* strains were not further investigated because they are (nearly) identical to the *M. tuberculosis* H37Rv *alkB*. The unfinished genome sequences of *Legionella pneumophila* Philadelphia-1 and *Burkholderia pseudomallei* K96243 also contain an alkane hydroxylase homolog each. These sequences were included in the phylogenetic analysis, but not in the functional studies. Of these genes, 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 other proteins encoded downstream of other alkane hydroxylase genes.

Other alkane hydroxylase genes were cloned 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 (239)(J. B. van Beilen, unpublished results). A 7.8 kb DNA segment containing the alkane hydroxylase of *P. fluorescens* CHA0 was cloned using a 550 bp CHA0 *alkB* fragment (239) as a probe in Southern and colony blots. Downstream of the alkane hydroxylase, the cloned DNA encodes two proteins (PraA and PraB) with homology to the so-called "protein activator for alkane oxidation" of *P. aeruginosa* PAO1 (103), and an outer membrane protein homologous to the OmpP1 proteins of *Haemophilus influenzae*.

Figure 1: organization of *alk*-genes in different organisms. The scale is in basepairs.

A: *P. putida* (*oleovorans*) GPo1 and *P. putida* P1. *alkB*: alkane hydroxylase, *alkF*: rubredoxin 1, *alkG*: rubredoxin 2, *alkH*: aldehyde dehydrogenase, *alkJ*: alcohol dehydrogenase, *alkK*: 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, 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, PA5346: hypothetical proteins.

E: *P. rugosa* NRRL B-2295. *alkB*: alkane hydroxylase, *tetR*: putative regulatory protein of 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 TetR family.

In the case of *A. borkumensis* AP1, we previously cloned a 3.5 kb *Eco*RI-*Bam*HI fragment encoding an AlkG-like rubredoxin (J. B. van Beilen, unpublished results). 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 (figure 1)(260). However, the rubredoxin gene *alkF* (142)(J. B. van Beilen, unpublished results) as well as the acyl-CoA-synthetase gene *alkK* and the *alkL* gene, encoding an outer membrane protein of unknown function (258), are not present in AP1. More surprisingly, *alkS* is located immediately upstream of *alkB1*. It is transcribed in the opposite direction, and is not followed by the rubredoxin reductase gene *alkT*, unlike in *P. putida* strains GPo1 and P1. Instead, a possible transcriptional terminator (a strong inverted repeat) is located directly downstream of *alkS*, while further downstream DNA has homology with a *Vibrio cholerae* Na^+/H^+ antiporter.

The *Prauserella rugosa* NRRL B-2295 *alkB* gene was cloned as a 3.0 kb *Sac*I-*Bam*HI chromosomal DNA fragment using the 559 bp *Eco*RI-*Sac*I *alkB* fragment of p2295 (239) as a probe. Sequence analysis of the 3 kb fragment showed that it encodes an alkane hydroxylase with a C-terminal extension that is highly homologous to rubredoxins (J. B. van Beilen, unpublished results). The region immediately downstream of the *alkB* gene encodes a possible regulatory protein with a TetR signature at its N-terminus
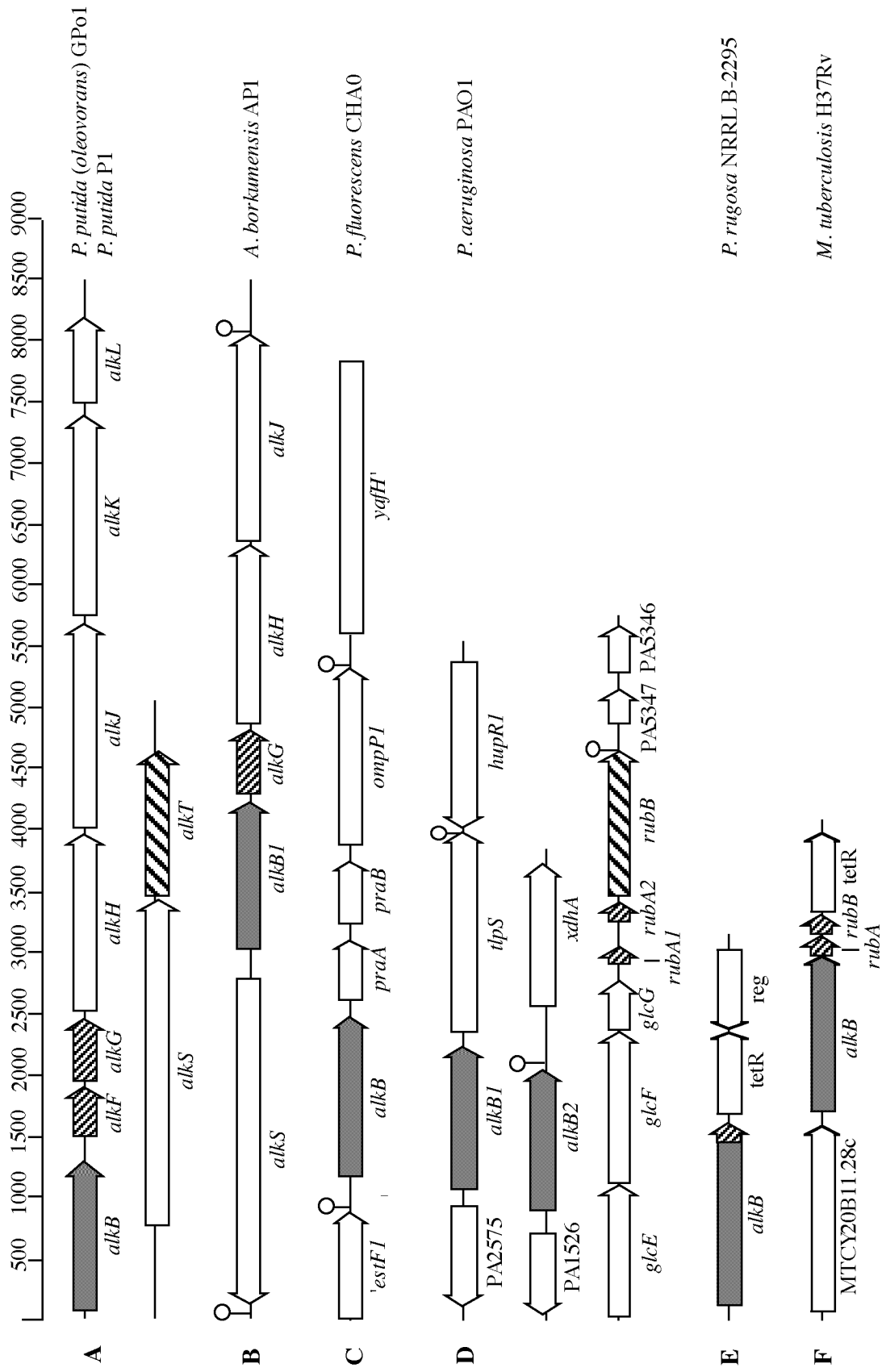
(pfam00440), quite homologous to the TetR-protein encoded downstream of the *M. tuberculosis* H37Rv *alkB-rubAB* genes (26% sequence full-length sequence identity) (figure 1).

Comparison of alkane hydroxylases. For a phylogenetic analysis of known and new alkane hydroxylases (figure 2), a peptide sequence alignment of the full length alkane hydroxylases was generated with Clustal (111) and manually optimized. The peptide sequence identity among the alkane hydroxylases ranged from 37 % to 99 %. The closest relative of the alkane hydroxylases that does not oxidize *n*-alkanes, xylene monooxygenase (XylM), was also included in the analysis. It has 20 % to 26 % peptide sequence identity to the alkane hydroxylases, and is most closely related to the *L. pneumophila* Philadelphia-1 and the *P. aeruginosa* PAO1 enzymes.
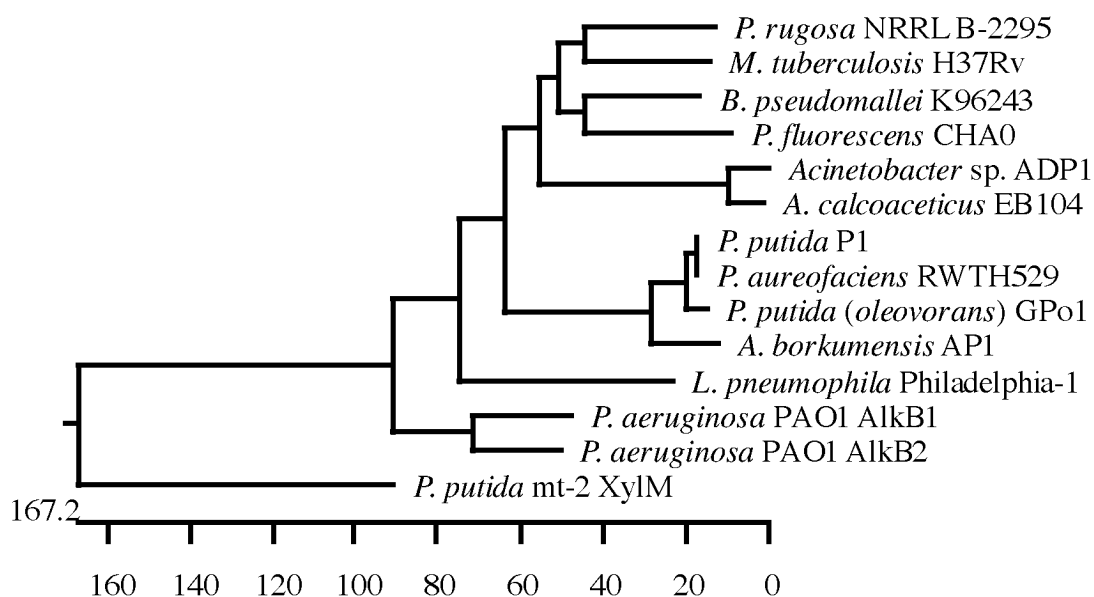

Figure 2: phylogenetic tree of published and hypothetical alkane hydroxylases, generated by Clustal from the manual alignment of the (putative) alkane hydroxylases. XylM is included as outgroup.

The alignment shows that the six membrane spanning segments identified in the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase (figure 3)(261), three histidine motifs conserved

among the hydrocarbon oxygenases and desaturases (233), and the additional

NYXEHYG(L/M) motif identified in the alkane hydroxylases (239) are well conserved in

all sequences. Most insertions and deletions of each sequence, relative to the other AlkB-

sequences, are located in predicted cytoplasmic or periplasmic domains before the third

transmembrane helix. Only in the *M. tuberculosis* H37Rv and *P. rugosa* NRRL B-2295

alkane hydroxylases insertions are located between the conserved histidine motifs. A 40

amino acid insertion in the *P. fluorescens* CHA0 AlkB is probably located in the

periplasmic domain between the third and fourth putative transmembrane helices. The C-

terminal end of the alkane hydroxylases also varies strongly in length and composition.
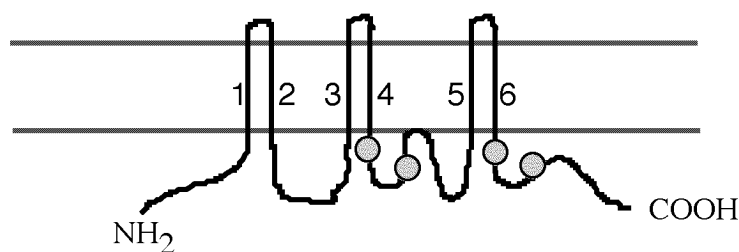

Figure 3: topology model of the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase. Grey dots indicate the localization of the histidine motifs. Numbers indicate the membrane spanning segments (based on *lacZ* and *phoA* fusion protein studies (261)).

Alkane hydroxylases from strains that oxidize medium-chain alkanes (*P. putida*

(*oleovorans*) GPo1, *P. putida* P1, *A. borkumensis* AP1 and *P. aureofaciens* RWTH529

(273)) are clustered in the phylogenetic tree (figure 2), while the putative alkane

hydroxylases from long-chain alkane degrading strains are highly divergent.

Interestingly, alkane hydroxylase genes cloned from fluorescent pseudomonads are as

divergent as the entire collection, while the *A. borkumensis* AP1 alkane hydroxylase is

quite closely related to the *P. putida* alkane hydroxylases.

In vivo **functional analysis of alkane hydroxylases.** Three different systems for the

functional expression of alkane hydroxylases were developed, based on the observation

that the second component of the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase

system, the electron transfer protein rubredoxin (AlkG), can be replaced by rubredoxins from other alkane hydroxylase systems (J. B. van Beilen, unpublished results). The broad-host range cosmid pGEc47ΔB, a deletion derivative of pGEc47 (69) that lacks part of the alkane hydroxylase gene *alkB* (261), expresses the *P. putida* (*oleovorans*) GPo1 rubredoxin and rubredoxin reductase, and other proteins involved in the degradation of alkanes, in *E. coli* as well as in *Pseudomonas* species. Two hosts were used in this study. *E. coli* GEc137 is a *fadR*-mutant of *E. coli* DH-1, which allows it to grow on medium chain-length fatty acids and *n*-alkanes of the same length, if the strain contains pGEc47. *P. putida* (*oleovorans*) GPo12 is a derivative of GPo1, cured of the OCT-plasmid. Both host strains containing pGEc47ΔB did not show any detectable alkane hydroxylase activity (< 0.01 U g^{-1} cells, or less than 0.06% of full activity)(261). Plasmids encoding the alkane hydroxylase gene of *P. putida* (*oleovorans*) GPo1 could fully restore alkane hydroxylase activity of both recombinant strains, based on growth rates on *n*-octane and activity assays (data not shown).

Table 2: functional analysis of alkane hydroxylases.

Alkane hydroxylase	System for functional analysis [a]		
	E. coli GEc137 [pGEc47ΔB]	*P. putida* (*oleovorans*) GPo12[pGEc47ΔB]	*P. fluorescens* KOB2Δ1
P. putida (*oleovorans*) GPo1 *alkB*	++ (C8 - C12)	++ (C8 - C12)	+ (C10 - C16)
P. putida P1 *alkB*	++ (C8)	n.t.	n.t.
A. borkumensis AP1 *alkB1*	++ (C8-C12)	++ (C8 - C12)	n.t.
Acinetobacter sp. ADP1 *alkM*	-	-	-
P. aeruginosa PAO1 *alkB1*	-	n.t.	++ (C12 - C20)
P. aeruginosa PAO1 *alkB2*	-	+ (C12 - C16)	++ (C12 - C20)
P. fluorescens CHA0 *alkB*	-	+ (C12 - C16)	++ (C12 - C20)
M. tuberculosis H37Rv *alkB*	-	n.t.	++ (C12 - C14)
P. rugosa NRRL B-2295 *alkB*	n.t.	n.t.	++ (C12)

Footnote: [a] - : no growth; + : poor growth; ++ : good growth; n.t.: not tested. The substrates indicated between brackets were tested and positive.

All *alkB* gene homologs were expressed from P*alkB*, the promoter of the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase, using the vectors pKKPalk, pCom7 and pCom8 (240), and a gratuitous inducer of P*alkB*, dicyclopropylketone (DCPK)(96). The medium-

chain length alkane hydroxylases of *P. putida* P1 and *A. borkumensis* AP1 complement

the *alkB* deletion in *E. coli* GEc137[pGEc47ΔB] and *P. putida* (*oleovorans*)

GPo12[pGEc47ΔB] for growth on *n*-octane (table 2). However, similar *E. coli*

recombinants containing alkane hydroxylase genes cloned from strains able to grow on

long-chain *n*-alkanes, including C12-C16 *n*-alkanes, did not grow on C12-C16 *n*-alkanes.

P. putida (*oleovorans*) GPo12 recombinants containing the same genes only grew poorly

on these alkanes, even if rhamnolipids were added (table 3). Therefore, a third host, *P.*

fluorescens KOB2Δ1, an *alkB* deletion mutant of *P. fluorescens* CHA0, was constructed

as described in the Material and Methods section. KOB2Δ1 is not able to grow on C10-

C16 *n*-alkanes, unlike the wild-type, but could be complemented for growth on these *n*-

alkanes by the native (CHA0) alkane hydroxylase gene, cloned in pCom5 (table 3). For

the long-chain alkane hydroxylases, *P. fluorescens* KOB2Δ1 proved to be a much better

host than *E. coli* or *P. putida* strains: the *P. aeruginosa* PAO1, *P. fluorescens* CHA0, *M.*

tuberculosis H37Rv and *P. rugosa* NRRL B-2295 alkane hydroxylases all allowed

growth of *P. fluorescens* KOB2Δ1 recombinants on at least one of the *n*-alkanes tested,

albeit often with growth rates clearly below that of the wild-type strain *P. fluorescens*

CHA0 (table 2, table 3). The KOB2Δ1 recombinant containing the *P. putida* (*oleovorans*)

GPo1 *alkB* gene did not grow on *n*-octane, but showed weak growth on C10 and C12.

Table 3: doubling times of wild-type and *alkB* recombinant strains on *n*-alkanes

Strain	Additional gene	Doubling times (t_d) (hours)		
		C12[a]	C14	C16
PAO1	-	19	-[b]	19
CHA0	-	20	31	10
GPo12[pGEc47ΔB]	*alkB2* (PAO1)	33	63	> 200
	alkB (CHA0)	28	78	> 200
KOB2Δ1	*alkB1* (PAO1)	176	37	44
	alkB2 (PAO1)	21	22	15
	alkB (CHA0)	42	24	13
	alkB (2295)	72	n.g.	n.g.
	alkB (H37Rv)	80	176	n.g.

Footnotes: [a] C12: dodecane; C14: tetradecane; C16: hexadecane. [b] -: not tested; n.g.: no growth

However, short-chain alkane hydroxylases did not allow KOB2Δ1 recombinants to grow on *n*-octane. Possibly, the CHA0 rubredoxin and rubredoxin reductase, which we have not cloned yet, are not induced under these conditions. The related *P. fluorescens* strain Pf0-1, the genome of which has been sequenced, possesses such genes, but does not encode an alkane hydroxylase homolog. As the Pf0-1 rubredoxin and rubredoxin reductase are closely related to proteins that were shown to function in alkane hydroxylation (J. B. van Beilen, unpublished results), this strain may have lost its alkane hydroxylase.

DISCUSSION

Functional analysis of alkane hydroxylases. The alkane hydroxylase genes of *P. putida* (*oleovorans*) GPo1 and *Acinetobacter* sp. ADP1 have been cloned using traditional cloning methods. In both cases, cosmids that restored growth of chemical mutants on alkanes were selected from a cosmid library (69, 213). Two main reasons necessitated the development of a PCR method to clone additional long-chain alkane hydroxylase genes. Firstly, some strains were found to contain more than one alkane hydroxylase with overlapping substrate ranges, which made the classical genetic approach difficult. Secondly, although medium-chain length alkane hydroxylases can be assayed *in vitro*, e.g. using the conversion of alkenes to epoxides or the co-oxidation of NADH (259), such methods could not be developed for membrane-bound long-chain alkane hydroxylases, possibly because long-chain substrates are poorly soluble in water, 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.

Here, we used PCR products obtained with highly degenerate primers described earlier, and information from genome sequencing projects to clone a number of alkane hydroxylases from a quite diverse collection of strains. This implies that all of these genes were 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 *A. borkumensis* AP1, which has recently been isolated from sea water, and grows almost exclusively on *n*-alkanes (284). Other strains, like *M. tuberculosis* and *L. pneumophila* are important human pathogens, and are difficult to cultivate. In addition, several strains contain multiple (at least up to five) *alkB* homologs (chapter 2; J. B. van Beilen, unpublished results). Here, knockout mutants, if they can be generated, 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 the novel alkane hydroxylases is complicated by the fact that only in a few cases all three components of the alkane hydroxylase systems were cloned, or could be identified unequivocally from genome sequence data. Fortunately, earlier complementation experiments with the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase system have shown that rubredoxins from various Gram-positive and Gram-negative alkane degraders can replace the *P. putida* (*oleovorans*) GPo1 rubredoxin in alkane oxidation (J. B. van Beilen, unpublished results). 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 these considerations, we have developed several hosts for the expression of novel alkane hydroxylases. The best 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 10-20 % of the full GPo1 alkane hydroxylase activity (257). Therefore, any growth of the recombinants on *n*-alkanes indicates a significant level of alkane hydroxylase activity.

E. coli GEc137[pGEc47ΔB] and *P. putida* (*oleovorans*) GPo12[pGEc47ΔB] are suitable hosts to test medium-chain alkane hydroxylase genes (table 2). Recombinants derived from GPo12 are also able to grow on alkanes ranging from C12 to C16 if rhamnolipids are added, but do so quite slowly. It is likely that uptake of longer *n*-alkanes requires factors (porins or similar proteins) that are not present in GPo12. For this reason, we

constructed a non-polar *alkB* knockout mutant of *P. fluorescens* CHA0, in which the alkane solubilization and (putative) uptake system is left intact. This host does indeed allow complementation for growth on longer alkanes by alkane hydroxylases cloned from quite diverse organisms, including Gram-positive strains (table 2, table 3).

In summary, the expression and complementation systems allow us to demonstrate that homologs of the GPo1 *alkB* gene indeed encode functional alkane hydroxylases. This makes it possible to compare sequences to gain insight in the structure-function relationship of this class of enzymes. Alkane hydroxylases of the *P. putida* (*oleovorans*) GPo1 type are well-studied, mainly because of their potential applications in biocatalysis. However, our present knowledge on the structure-function relationship of this class of integral membrane oxygenases is limited to the folding topology (261) and the likely involvement of conserved histidines in binding of the two active-site iron atoms (231). The alkane hydroxylases that are 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 (261) are highly conserved in all sequences. The same is true for the four histidine boxes (233, 239), which are essential for enzymes related to the alkane hydroxylases, and are likely to contribute to the nitrogen-rich ligand sphere of the catalytic di-iron center (231). It is not yet possible to identify residues involved in other aspects of alkane hydroxylase function. Residues that are involved in 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. However, these residues cannot easily be distinguished from other conserved residues. Similarly, the four medium chain-length alkanes hydroxylases are too closely related to each other to distinguish residues conserved by chance from residues conserved because of functional constraints.

The organization of genes involved in alkane oxidation varies strongly among the different alkane degrading bacteria (figure 1). In most strains, genes involved in alkane degradation seem to be distributed over the genome. None of the rubredoxin reductases is

located close to an alkane hydroxylase, 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*-genes of *A. borkumensis* are very similar to the *alk*-genes 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 (260). 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.

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 human 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 source. This explains why it is easy to isolate alkane-degrading strains from pristine soil, aquifers recently polluted with oil (217) and oil-polluted sea water. The alkane hydroxylase of *M. tuberculosis* 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.

While the previous PCR-studies (239), and later results (chapter 2; J. B. van Beilen, unpublished results) show that many alkane degrading strains possess homologs of the *P. putida* (*oleovorans*) 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 allow us to demonstrate that they oxidize medium-chain (C5-C11) or long-chain (C12-C16) *n*-alkanes. These experiments also show that long-chain alkane

hydroxylases show little activity with medium-chain length alkanes, and vice versa, and thereby provide us with a powerful selection method to change the substrate range of the cloned alkane hydroxylases, and identify residues involved in substrate binding.

ACKNOWLEDGEMENTS

Chapter 5

FUNCTIONAL ANALYSIS OF RUBREDOXIN REDUCTASES INVOLVED IN ALKANE OXIDATION

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SUMMARY

The rubredoxin reductase AlkT of *Pseudomonas putida* (*oleovorans*) GPo1 is an essential component of the alkane hydroxylase system. Homologs of AlkT are present in several alkane degrading strains, but also occur in other bacteria. *In vivo* complementation assays show that rubredoxin reductase homologs cloned from *Pseudomonas aeruginosa* PAO1 and *Acinetobacter* sp. ADP1 transfer electrons to the rubredoxin component of the GPo1 alkane hydroxylase system. However, the flavorubredoxin reductase from *E. coli* K-12 and the ferredoxin reductase subunit of the chlorobenzene dioxygenase of *Pseudomonas* sp. P51, which are as closely related to the GPo1 rubredoxin reductase as the PAO1 and ADP1 reductases, cannot replace AlkT *in vivo*.

INTRODUCTION

The *Pseudomonas putida* (*oleovorans*) GPo1 rubredoxin reductase AlkT, which transfers electrons from NADH to rubredoxin (68, 256), is an essential component of the alkane hydroxylase system. The other two components are a non-heme iron integral membrane protein (AlkB) (143, 173, 261) and rubredoxin (AlkG) (207), a small red protein which transfers electrons from AlkT to AlkB.The enzyme system oxidizes C5-C12 *n*-alkanes to 1-alkanols, thereby allowing the host to grow on these compounds.

Spinach ferredoxin and ferredoxin reductase can replace the rubredoxin and rubredoxin reductase of *P. putida* (*oleovorans*) GPo1 as electron transfer proteins in *in vitro* assays (20, 207). In *in vivo* activity assays with an AlkT deletion derivative of pGEc47, pGEc47ΔT, which contains all *alk*-genes necessary for growth on alkanes except the rubredoxin reductase, considerable background activity was found in *E. coli* and *P. putida* recombinants, this activity was even sufficient to allow growth of the recombinant on octane (257). In addition, rubredoxins from Gram-negative and Gram-positive alkane degrading strains can replace the *P. putida* (*oleovorans*) GPo1 rubredoxin in alkane oxidation, even though they show as little as 43 % sequence identity (J. B. van Beilen, unpublised results). This suggests that reductases cloned from other organisms might also

be able to replace the rubredoxin reductase of *P. putida* (*oleovorans*) GPo1. Therefore, we investigated alkane degrading strains and genome sequences for the presence of rubredoxin reductase gene homologs, and tested whether they can replace the GPo1 rubredoxin reductase.

Rubredoxin reductases involved in alkane oxidation.

Several rubredoxin reductases have now been cloned from alkane degrading strains (68, 91, 260)(J. B. van Beilen, unpublished results). The rubredoxin reductase genes of *P. putida* strains GPo1 and P1 are located directly downstream of the *alkS* gene encoding the positive regulator of alkane degradation (260). In *Acinetobacter* sp. ADP1, the rubredoxin reductase gene is in an operon structure with rubredoxin genes (91)(chapter 6). The rubredoxin reductase gene *rubB* of *Rhodococcus erythropolis* NRRL B-16531) is located immediately downstream of an alkane hydroxylase (*alkB1*) and two rubredoxin (*rubA1* and *rubA2*) genes (J. B. van Beilen, unpublished results).

We also searched relevant genome sequences for rubredoxin reductase homologs (at NCBI: http://www.ncbi.nlm.nih.gov/Microb_blast/unfinishedgenome.html).The rubredoxin reductase of *Pseudomonas aeruginosa* PAO1 is encoded directly downstream of two rubredoxin genes (*rubA1* and *rubA2*). These genes, organized in a putative operon structure, are not located in the vicinity of either of the two alkane hydroxylases (249)(chapter 4, 6). Two genes encoding hypothetical proteins (Rv0688 and Rv1869c) in the genome sequence of *Mycobacterium tuberculosis* H37Rv (51) have significant homology (36.1 and 35.8 % identity, respectively) to the rubredoxin reductase of *R. erythropolis* NRRL B-16531 (figure 1). These proteins are not encoded in the proximity of the alkane hydroxylase and the rubredoxins.

Interestingly, the genomes of several organisms that are not able to grow on n-alkanes, or do not possess alkane hydroxylase homologs, do contain rubredoxin reductase homologs. The genome sequence of *E. coli* K-12 (24) encodes a rubredoxin reductase gene homolog, named flavorubredoxin reductase (*orf377*). The start codon of *orf377* overlaps with the stop-codon of flavorubredoxin (*orf479*), which has a rubredoxin-like domain at its C-terminus (95, 275). This protein is possibly involved in the response of *E. coli* to oxygen stress during anaerobic growth or as regeneration system for NADH during
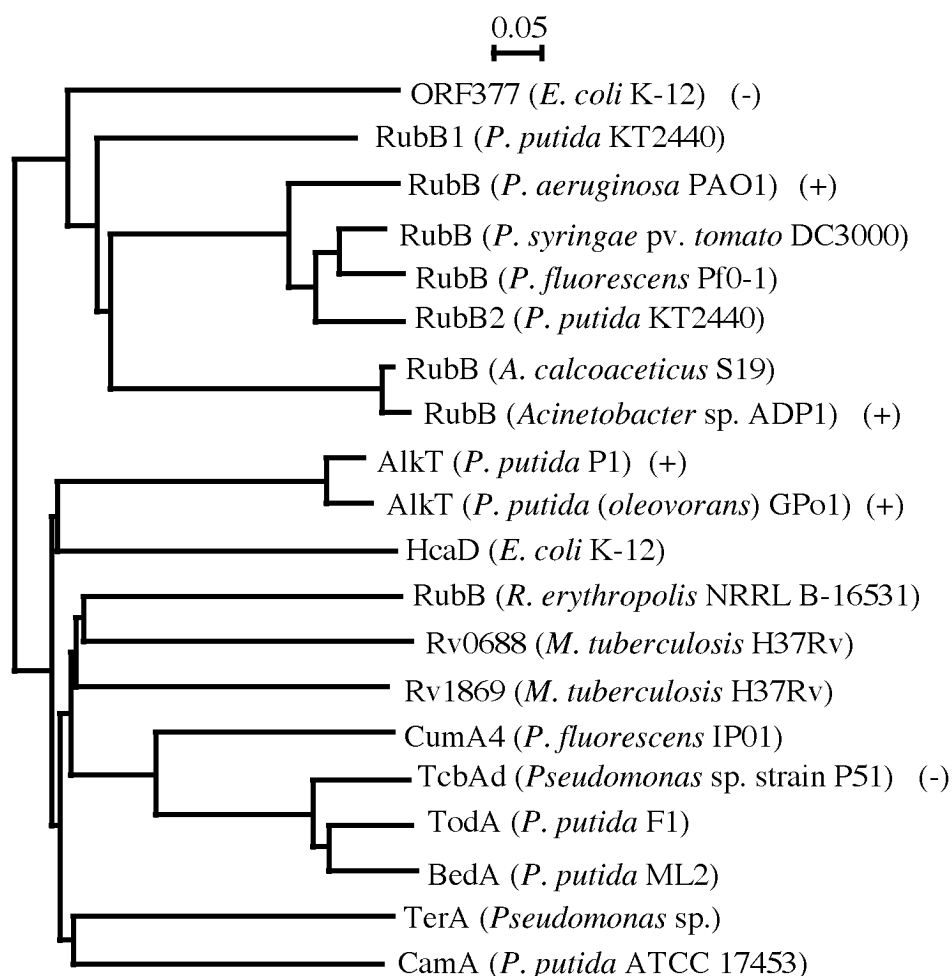
Figure 1: unrooted phylogenetic tree of FAD-containing reductases, generated with ClustalX from the manual alignment of the rubredoxin reductases with homologous proteins. Not all known peptide sequences of ferredoxin reductase homologs have been included in the phylogenetic tree. The results of the complementation assays with *E. coli* GEc137[pGEc47ΔT] is indicated by (-) and (+).

Names and descriptions of the proteins (accession numbers):

TerA: terpredoxin reductase (P450-TERP) of *Pseudomonas* sp. (P33009); CamA: putidaredoxin reductase (P450-CAM) of *P. putida* ATTC 17453 (P16640); CumA4: cumene dioxygenase, ferredoxin reductase subunit of *P. fluorescens* IP01 (BAA07078); TodA: toluene 1,2-dioxygenase, ferredoxin reductase subunit of *P. putida* F1 (P13452); BedA: benzene 1,2-dioxygenase, ferredoxin reductase subunit of *P. putida* ML2 (Q07946); TcbAd: chlorobenzene dioxygenase, ferredoxin reductase subunit of *Pseudomonas* sp. P51 (U15298); Rv0688: hypothetical protein of *M. tuberculosis* H37Rv (C70640); Rv1869c: hypothetical protein of *M. tuberculosis* H37Rv (E70667); RubB: rubredoxin reductase of *R. erythropolis* NRRL B-16531 (AJ009586); HcaD: 3-phenylpropionate dioxygenase, ferredoxin reductase subunit of *E. coli* K-12 (P77650); AlkT: rubredoxin reductase of *P. putida* P1 (CAB69078.1); AlkT: rubredoxin reductase of *P. putida* (*oleovorans*) GPo1 (CAB54063.1); ORF377: flavorubredoxin reductase of *E. coli* K-12; RubB1: putative rubredoxin reductase 1 of *P. putida* KT2440; RubB2: putative rubredoxin reductase 2 of *P. putida* KT2440; RubB: putative rubredoxin reductase of *P. syringae* pv. *tomato* DC3000; RubB: putative rubredoxin reductase of *P. fluorescens* Pf0-1; RubB: rubredoxin reductase of *P. aeruginosa* PAO1 (AAG08734.1); RubB: rubredoxin reductase of *Acinetobacter calcoaceticus* S19 (CAA75809); RubB: rubredoxin reductase of *Acinetobacter* sp. ADP1 (CAA86926.1).

aerobiosis (95). Another *E. coli* AlkT-homolog, HcaD, transfers electrons from NADH via a ferredoxin to the 3-phenylpropionate dioxygenase (60) (figure 1). We identified two putative rubredoxin reductases in the unfinished genome sequence of *P. putida* KT2440, one of which is fused to a rubredoxin domain. Rubredoxin and rubredoxin reductase gene homologs were also found in the genomes of *Pseudomonas syringae* pv. *tomato* DC3000 and *Pseudomonas fluorescens* Pf0-1. These proteins are quite closely related to the *P. aeruginosa* PAO1 rubredoxin and rubredoxin reductase. As the genome sequences of *P. syringae* pv. *tomato* DC3000, *P. fluorescens* Pf0-1 and *P. putida* KT2440 do not contain alkane hydroxylase homologs, the function of these proteins is unknown.

These findings indicate that rubredoxin reductase homologs have diverse physiological roles in addition to alkane degradation. Possibly, most rubredoxin reductases are not located close to alkane hydroxylases because they are also involved in other pathways, or because the expression levels need not be very high (245). The *P. putida* (*oleovorans*) GPo1 rubredoxin reductase AlkT was also detected in non-induced cultures, but its synthesis rate increased 4-fold after induction (245). In *Acinetobacter* sp. ADP1, the expression of rubredoxin and rubredoxin reductase is constitutive and transcriptionally linked to two genes, *estB* and *oxyR*, that are not necessary for alkane degradation (92).

Phylogenetic analysis. A phylogenetic analysis of the rubredoxin reductases and related proteins (> 20 % sequence identity to at least one of the rubredoxin reductases known to play a role in alkane oxidation) showed that the rubredoxin reductases can be divided in two groups which have low sequence identities with one another (ranging between 18 and 45 %).Within each of the two groups, the levels of sequence homology are much higher (typically over 30 %). One group contains the chromosomal encoded (flavo)rubredoxin reductases of Gram-negative bacteria, including the PAO1 and ADP1 rubredoxin reductases, while the second group includes the rubredoxin reductase of *R. erythropolis* NRRL B-16531 (J.B. van Beilen, unpublished results) and the *alk*-transposon encoded rubredoxin reductases of *P. putida* strains GPo1 and P1 (260). The second group has highest (up to 37 %) but all the same low sequence identities to FAD-

containing reductases, which transfer electrons to other oxygenases like cytochrome P450s and class II aromatic ring dioxygenases (39)(figure 1).

Functional analysis of rubredoxin reductases. Strains and plasmids used are described in table 1. For the functional analysis of rubredoxin reductases, an *alkT* deletion derivative of pGEc47, which lacks a 355 basepair fragment corresponding to the translation start site and amino acids 1-113 of the rubredoxin reductase (AlkT) of *P. putida* (*oleovorans*) GPo1, was introduced in *E. coli* GEc137 (257). The alkane hydroxylase activity of the resulting recombinant *E. coli* GEc137[pGEc47ΔT] was quite significant at 10% of the activity measured with *E. coli* GEc137[pGEc47], The remaining activity can be attributed to unknown reductases encoded by the *E. coli* chromosome (257).

The rubredoxin reductase genes of *P. aeruginosa* PAO1 (*rubB*) (249) and of *Acinetobacter* sp. ADP1 (*rubB*) (91) were amplified by PCR from chromosomal DNA using the primer combinations RubCfwd (caatcacagaggaacgcatatgagc) and RubCrev (ctgcgcaagcttcgtccgacaa) and rubBfE (ctacggagaattctaatgcacc) and rubBrB (aaaaaggatcctcagatgaatg), respectively and cloned in pKKPalk (240). *E. coli* GEc137[pGEc47ΔT] recombinants containing a plasmid encoding *rubB* of *Acinetobacter* sp. ADP1 or *rubB* of *P. aeruginosa* PAO1 showed growth on E2 minimal medium plates supplemented with MT trace elements (151) and exposed to *n*-octane vapor after three days.

These results show that these two rubredoxin reductases can complement the *alkT* deletion, even though these proteins are as distantly related to the *P. putida* (*oleovorans*) GPo1 AlkT as to other FAD-containing reductases (figure 1).

As the flavorubredoxin reductase of *E. coli* K-12 has high homology to rubredoxin reductases (figure 1), we amplified the gene from chromosomal DNA of the K-12 derived strain GEc137 with primers Ecoli-AlkT-FW-Eco (ggcatcgaattcaaaatgagtaacgg) and Ecoli-AlkT-RV-Hin (gtcgcatccgaaagcttaggcacag) and cloned it in pKKPalk (240). However, when expressed from the P*alkB* promoter, the flavorubredoxin reductase did not complement the AlkT deletion although the protein can transfer electrons from

NADH to the rubredoxin of *Desulfovibrio gigas* (95). Based on these results, it is still not clear what causes the background activity in *E. coli*. Possible candidates for this role are HcaD, the ferredoxin reductase subunit of the 3-phenylpropionate dioxygenase (60) or other reductases, which have even lower sequence identities to the rubredoxin reductases involved in alkane oxidation.

Table 1: list of strains and plasmids used in this study

Strain name	Relevant phenotype	Reference
P. aeruginosa PAO1	*alk⁺*	(118)
Acinetobacter sp. ADP1	*alk⁺*	(126)
P. putida (*oleovorans*) GPo12	GPo1 cured of the OCT plasmid	(141)
E. coli DH10B	cloning strain	Gibco BRL
E. coli GEc137	*thi, fadR*	(67)

Plasmid name	Characteristics	Reference
pKKPalk	Expression vector using P*alkB*, Ap^R	(240)
pGEc47	*alkBFGHJKL/alkST* (GPo1) in pLAFR1	(69)
pGEc47ΔG	pGEc47, deletion in *alkG*	(257)
pGEc47ΔT	pGEc47, deletion in *alkT*	(257)
pTCB144	*tcbAaAbAcAd* genes in pUC19	(276)
pRubB (PAO1)	*rubB* gene (PAO1) in pKKPalk	This study
pKKRubB (ADP1)	*rubB* gene (ADP1) in pKKPalk	This study
pKKEcRubB (K-12)	*orf377* gene (K-12) in pKKPalk	This study

Exchange of electron transfer proteins from more distantly related systems. Because spinach ferredoxin and ferredoxin reductase can replace AlkG and AlkT *in vitro* (20, 207), we tested whether the ferredoxin (TcbAc) and ferredoxin reductase (TcbAd) from the chlorobenzene dioxygenase system of *Pseudomonas* sp. strain P51 (276) can replace AlkG or AlkT in the *in vivo* complementation assay. The chlorobenzene dioxygenase enzyme system is highly homologous to the benzene and toluene dioxygenases, which are classified as class IIB dioxygenases (39). TcbAd has 30.4 % protein sequence identity to the *P. putida* (*oleovorans*) GPo1 rubredoxin reductase, which is similar to the homology between AlkT and RubB (PAO1)(31.7 %) or RubB (ADP1)(29.0 %) (figure 1). We introduced a plasmid containing the *tcbAaAbAcAd* genes, which can be expressed from the *lacZ* promoter (pTCB144)(276), in the *E. coli* strains GEc137[pGEc47ΔG] and

GEc137[pGEc47ΔT]. *E. coli* recombinants containing pTCB144 produced indigo when cultivated on LB, which indicates that the dioxygenase system is functionally intact. However, the electron transfer proteins of the chlorobenzene dioxygenase did not complement the deletion in either *alkG* or in *alkT* to the extent necessary for growth on *n*-octane. The results show that alkane hydroxylase and chlorobenzene dioxygenase components are not able to interact *in vivo*, possibly because structural features prevent transfer of electrons to the alkane hydroxylase. The same conclusion was reached for AlkG1-type rubredoxins (J. B. van Beilen, unpublished results) and for XylA, the electron transfer component of the *P. putida* mt-2 xylene monooxygenase, which also cannot replace AlkG/AlkT *in vivo* (257).

Conclusions

Rubredoxin reductases can not be replaced by structural homologs from other electron transfer systems in *in vivo* experiments, although the protein sequences are homologous. It thus seems that both rubredoxins and rubredoxin reductases, that are part of the alkane hydroxylase system, differ significantly from homologs present in nature in the docking geometry so that these homologs cannot interact with either rubredoxin or rubredoxin reductase of the alkane hydroxylase system. The fact that rubredoxins involved in alkane degradation are more related to each other than to other rubredoxins in the phylogenetic tree (J. B. van Beilen, unpublished results), could also be an indication for this phenomenon. Therefore, crystallization studies on the alkane hydroxylase system and mutagenesis experiments are necessary to investigate these interactions in more detail.

ACKNOWLEDGEMENTS

Chapter 6

FUNCTIONAL CHARACTERIZATION OF GENES INVOLVED IN ALKANE OXIDATION BY *PSEUDOMONAS AERUGINOSA*

Theo H. M. Smits, Bernard Witholt and Jan B. van Beilen

SUMMARY

Many *Pseudomonas aeruginosa* strains grow on medium- and long-chain *n*-alkanes. Heterologous expression shows that two alkane hydroxylase (*alkB1* and *alkB2*) homologs of *P. aeruginosa* PAO1 indeed do oxidize long-chain alkanes, while two rubredoxin (*rubA1* and *rubA2*) and a rubredoxin reductase (*rubB*) homologs can replace their *P. putida* (*oleovorans*) GPo1 counterparts in *n*-octane oxidation. The two long-chain alkane hydroxylase genes are present in all environmental and clinical isolates of *P. aeruginosa* strains tested in this study.

INTRODUCTION

Pseudomonas aeruginosa is of clinical importance as the primary opportunistic pathogen among the pseudomonads, but is also a common organism in soil, water and on plants (27). Environmental and pathogenic isolates are indistinguishable by chemotaxonomic and molecular techniques (80, 112). In both contexts, alkane oxidation is a relevant property. Enrichments from soil or water with *n*-alkanes as the carbon source often yield *P. aeruginosa* strains. Conversely, the ability of *P. aeruginosa* to use paraffins as sole carbon source is used to identify clinical isolates, in combination with other methods like fluorescence and oxidase assays (170). Nevertheless, not much is known about *n*-alkane degradation by *P. aeruginosa*. In fact, only compounds which solubilize *n*-alkanes and make them available for uptake, such as rhamnolipids (140, 290) and the so-called "protein activator of alkane oxidation" PraA (103, 114) have been studied in some detail. Most reports dealing with the biochemistry of alkane degradation by *P. aeruginosa* concern strains able to grow on C6-C10 *n*-alkanes (269). Recently, it was shown that these *P. aeruginosa* strains contain alkane hydroxylases that are virtually identical to the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase (262). This enzyme system, however, is probably not involved in growth of these strains on long-chain alkanes, as the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase only oxidizes alkanes with a chain length from

hexane (C6) up to dodecane (C12) (259). *P. aeruginosa* long-chain alkane hydroxylases have not been characterized biochemically or genetically.

Growth of *Pseudomonas aeruginosa* strains on alkanes. We tested the environmental and clinical *P. aeruginosa* isolates listed in table 1 for the ability to grow on medium- and long-chain alkanes. *P. aeruginosa* PAO1 and most of the clinical isolates grew on E2 minimal medium plates (151) with *n*-dodecane, *n*-tetradecane and *n*-hexadecane as carbon and energy source, which is consistent with previous data (170). None of the clinical isolates grew with *n*-octane and *n*-decane vapor. In contrast, the environmental isolates described earlier (13, 163, 266, 269) grew on medium-chain *n*-alkanes from *n*-hexane to *n*-decane as well as on the longer *n*-alkanes. Strain PG201 was an exception in that it showed weak growth with octane and decane.

Cloning of genes involved in alkane oxidation from *P. aeruginosa* PAO1. Highly degenerate primers based on conserved regions of alkane hydroxylase gene homologs earlier yielded almost identical PCR fragments with *P. aeruginosa* PAO1 and PG201 (239). The PG201 PCR fragment was used as a probe in Southern blots, which showed that the *P. aeruginosa* PAO1 genome contains two related *alkB* gene homologs. Two independent cosmids were isolated by screening a PAO1 genebank (271) with the same probe. Cosmid pTS200 contains an *alkB* homolog, corresponding to the PG201 degenerate PCR fragment, which was designated *alkB1*. Cosmid pTS100 contains a second *alkB* homolog , designated *alkB2*, with only 65 % overall DNA sequence identity to *alkB1* but significantly higher homology in the internal gene segment that was used as probe, explaining the cross-reactivity in the Southern blot. Both genes show no significant DNA sequence identity with the *P. putida* (*oleovorans*) GPo1 *alkB* gene in a pairwise alignment by the Wilbur-Lipman method (278). AlkB1 and AlkB2 show 37.4 % amino acid sequence identity to the GPo1 alkane hydroxylase and 67.7 % to each other. The two genes are also present in the *Pseudomonas aeruginosa* Genome Project (PAGP) sequence (249). No additional *alkB* homologs were found (figure 1a and 1b).

The *P. putida* (*oleovorans*) GPo1 alkane hydroxylase requires two electron transfer components for activity: a rubredoxin and a rubredoxin reductase. We inspected the

PAGP database for the presence of corresponding homologs, and found a possible operon

consisting of two rubredoxin (*rubA1* and *rubA2*) and one rubredoxin reductase (*rubB*)

gene homologs (figure 1c). The two rubredoxins are most closely related to RubA of

Acinetobacter sp. ADP1 (70-72 % protein sequence identity) (91) and show between 50 -

65 % sequence identity with other rubredoxins involved in alkane oxidation. All extant

rubredoxin sequences are more distantly related. The putative rubredoxin reductase is

most closely related to the rubredoxin reductase (RubB) of *Acinetobacter* sp. ADP1

(40.1% protein sequence identity) (91) and the rubredoxin reductase (AlkT) of *P. putida*

(*oleovorans*) GPo1 (37.1 %) (68).

Table 1: list of *Pseudomonas aeruginosa* strains and growth behavior on E2 minimal medium with 0.2 % citrate or *n*-alkane vapor as carbon source

| Labname | Official name[a] | Isolated from | Growth on E2 medium with[b] | | | | | | Reference |
			Citr.	C8	C10	C12	C14	C16	
PAO1	ATCC 15692	Infected wound	++[c]	-	+	++	+++	+++	(118)
PG201	DSM 2659	Soil	++	+	+	++	+++	+++	(97)
KSLA 473	KSLA 473	Y-harbor, Amsterdam	++	+++	++	++	+++	+++	(266)
Sol 20	NCIMB 8704	Soil	++	+++	++	++	+++	+++	(13)
196Aa	NCIMB 9571	Soil	++	+++	++	++	+++	+++	(269)
ATCC 17423	ATCC 17423	Soil	++	+++	++	++	+++	+++	(163)
CPA1[d]	DMMZ V10 18600	Urine	++	-	-	+	++	++	This study
CPA2	DMMZ V07 19924-1	Ethmoid tissue, CF	++	-	-	-	-	-	This study
CPA3	DMMZ V07 19924-4	Ethmoid tissue, CF	-	-	-	-	-	-	This study
CPA4	DMMZ V07 19925-2	Ethmoid tissue, CF	++	-	-	-	-	-	This study
CPA5	DMMZ V07 19939	Urine	++	-	+	+	++	++	This study
CPA6	DMMZ V07 19941	Urine	++	-	-	+	++	++	This study
CPA7	DMMZ V07 19965	Pleural fluid	++	-	-	+	+	+	This study
CPA8	DMMZ V09 20207	Urine	++	-	-	+	++	+	This study
CPA9	DMMZ V09 20227-1	Bronchial secretion	++	-	-	+	+	+	This study
CPA10	DMMZ V05 20348-2	Urine	++	-	-	+	+	+	This study
CPA11	DMMZ V05 20391-2	Tracheal aspirate	++	-	-	+	++	++	This study
CPA12	DMMZ V07 21517	Tracheal aspirate	++	-	-	+	+	++	This study

[a] DMMZ: Department of Medical Microbiology Zürich; CF: cystic fibrosis
[b] Citr.: 0.2 % citrate; C8: octane; C10: decane; C12: dodecane; C14: tetradecane; C16: hexadecane
[c] Growth was analyzed after 90 h. incubation. +++ good growth, ++ average growth, + weak growth, - no growth
[d] Clinical *P. aeruginosa* strains are identified according to (112) at DMMZ, Zürich

Functional analysis. Based on the observation that the electron transfer components of

alkane hydroxylase systems can be exchanged (J. B. van Beilen, unpublished results), we

have developed recombinant hosts that express two of the three alkane hydroxylase components (chapters 4 and 5; J. B. van Beilen, unpublished results). These strains allow us to test novel alkane hydroxylase, rubredoxin and rubredoxin reductase gene homologs for the ability to hydroxylate *n*-alkanes, or transfer electrons from NADH to rubredoxin, or from rubredoxin reductase to an alkane hydroxylase. The alkane hydroxylase genes *alkB1* and *alkB2* were cloned in the *Eco*RI and *Hind*III sites of pCom8, a *Pseudomonas-E. coli* expression vector (240). The resulting plasmids pCom8B1 (PAO1) and pCom8B2 (PAO1) were introduced in KOB2Δ1, an alkane hydroxylase negative mutant of *P. fluorescens* CHA0 (chapter 4) as described before (117). Both recombinants were plated on E2 minimal medium plates with 0.5 % α-cyclodextrin and 0.05 % *n*-alkanes ranging

Table 2: other strains and plasmids oligonucleotides used in this study

Strains

Name	Phenotype	Reference
E. coli DH10B	cloning strain	Gibco BRL
E. coli GEc137	*thi, fadR*	(69)
P. fluorescens KOB2Δ1	*alkB⁻*	chapter 4

Plasmids

Name	Characteristics	Reference
pGEM7-Zf(+)	Cloning vector, Ap^r	Promega
pKKPalk	Expression vector using *alk*-promoter, Ap^r	(240)
pCom8	pCom7 with *alkS* from pBG11EΔN	(240)
pGEc47	*alkBFGHJKL/alkST* in pLAFR1	(69)
pGEc47ΔG	pGEc47, deletion in *alkG*	(263)
pGEc47ΔT	pGEc47, deletion in *alkT*	(263)
pGEc48	*alkBFGH'* in pBR322	(70)
pTS2	PG201 PCR fragment in pGEM7-Zf(+)	(239)
pTS100	Cosmid harboring *alkB2* gene	This study
pTS200	Cosmid harboring *alkB1* gene	This study
pRubA1 (PAO1)	*rubA1* gene in pKKPalk	This study
pRubA2 (PAO1)	*rubA2* gene in pKKPalk	This study
pRubB (PAO1)	*rubB* gene in pKKPalk	This study
pCom8B1 (PAO1)	*alkB1* gene in pCom8	chapter 4
pCom8B2 (PAO1)	*alkB2* gene in pCom8	chapter 4

from C12 to C24 under dicyclopropylketone vapor, a gratuitous inducer of the *alkB* promoter(96). The recombinant strain KOB2Δ1[pCom8B1 (PAO1)] was able to grow on *n*-alkanes ranging from C16 to C24, while KOB2Δ1[pCom8B2 (PAO1)] grew on C12 -

C20 *n*-alkanes. Thus, the two alkane hydroxylases appear to have overlapping substrate specificities. The AlkB2 recombinants consistently showed stronger growth on plates. This trend was also obvious in liquid cultures, where KOB2Δ1[pCom8B2 (PAO1)] grew about twice as fast as KOB2Δ1[pCom8B1 (PAO1)] on all substrates tested (table 3). The doubling times were in the same range as those found with the wild-type *P. aeruginosa* PAO1 or *P. fluorescens* CHA0.

To analyse whether the *P. aeruginosa* PAO1 rubredoxin (*rubA1*, *rubA2*) and rubredoxin reductase (*rubB*) are able to function as electron transfer proteins in alkane oxidation, the three proteins were tested for their ability to replace the corresponding components of the alkane hydroxylase system of *P. putida* (*oleovorans*) GPo1. For this purpose, we used derivatives of pGEc47 having a deletion in the rubredoxin gene *alkG* (pGEc47ΔG) or the rubredoxin reductase gene *alkT* (pGEc47ΔT), respectively (J. B. van Beilen, unpublished results; chapter 5). The *rubA1*, *rubA2* and *rubB* genes were cloned separately into the *Eco*RI and *Asc*I, the *Eco*RI and *Pst*I and the *Nde*I and *Hind*III sites of pKKPalk, respectively (240) and transferred to *E. coli* GEc137[pGEc47ΔG] (*rubA1* and *rubA2*) or *E. coli* GEc137[pGEc47ΔT] (*rubB*). The resulting recombinants were plated on E2 minimal medium with *n*-octane supplied through the gas phase. As a positive control, *E. coli* GEc137[pGEc47] was used, while the negative controls were GEc137[pGEc47ΔG] and GEc137[pGEc47ΔT]. Growth on *n*-octane was observed after three days with all constructs and the positive control, while very slight growth was observed with the negative controls.

Table 3: doubling and lag times of *P. aeruginosa* PAO1 and *P. fluorescens* recombinants on alkanes.

| Strain | Additional gene | Doubling time (t_d) and lag time (in parenthesis) in hours | | | | |
		C12[a]	C14	C16	C18 (10%) in DOPh	C20 (10%) in DOPh
PAO1	-	19.1 (150)	-[b]	19.5 (240)	35.2 (80)	10.6 (40)
KOB2Δ1	*alkB1* (PAO1)	176.2 (40)	36.6 (180)	43.7 (20)	18.8 (100)	19.2 (100)
	alkB2 (PAO1)	20.6 (8)	21.6 (8)	15.4 (30)	12.9 (45)	11.9 (20)

Footnotes: [a] C12, dodecane; C14, tetradecane; C16, hexadecane; C18 (10%) in DOPh, 10% octadecane in dioctylphthalate; C20 (10%) in DOPh, 10% eicosane in dioctylphthalate; [b] -, not tested.

Gene organization and flanking genes. Genes that are directly or indirectly involved in the initial alkane oxidation step (*alkB1*, *alkB2*, *rubA1A2B*, *praA*, *rhlABRI*) are dispersed over the chromosome of *P. aeruginosa* PAO1 (249). Analysis of regions flanking these genes indicates that these have no obvious relation to alkane degradation (figure 1), with one possible exception, *tlpS*, a gene coding for a methyl-accepting chemotaxis protein (MCP) (282) (figure 1a). The intergenic region between *alkB1* and *tlpS* is only 111 basepairs, and no clear inverted repeats, which could point towards a *rho*-dependent terminator, could be found between the two ORFs. Directly downstream of the MCP, two
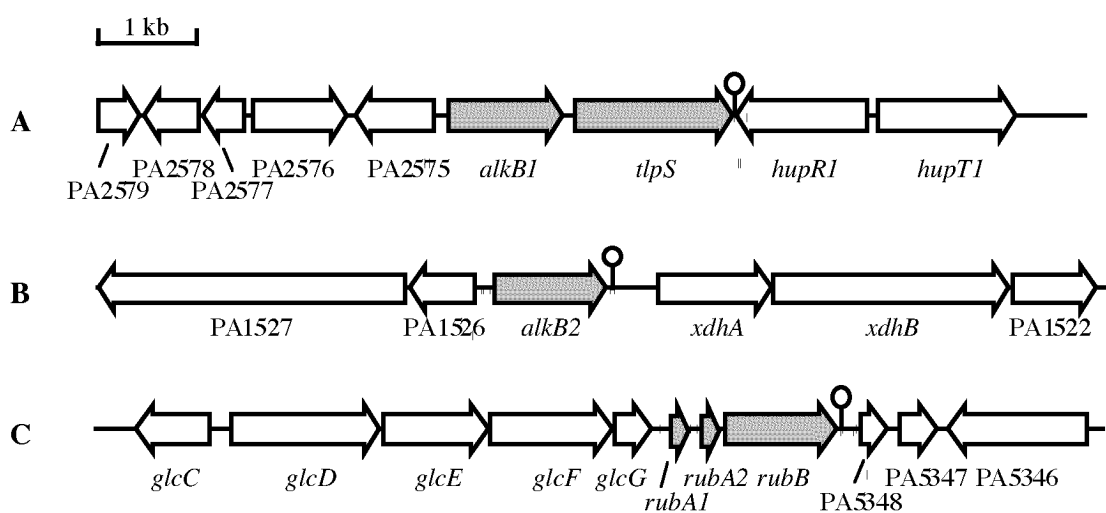

Figure 1: analysis of the open reading frames surrounding the *P. aeruginosa* PAO1 genes involved in alkane degradation. The data were obtained from the *P. aeruginosa* genome sequence (www.pseudomonas.com) (249).

A: Alkane hydroxylase 1 (*alkB1*; PA2574; from 2911876 to 2910728) and flanking region. Other genes: *tlpS*: methyl-accepting chemotaxis protein; *hupR1*: two-component regulatory system involved in the regulation of the [NiFe] hydrogenase activity; *hupT1*: sensor protein involved in repression of hydrogenase synthesis. PA2577: putative transcriptional regulator of the AsnC family; PA2579: homologous to human tryptophan-2,3-dioxygenase; PA2578, PA2576, PA2575: hypothetical proteins.

B: Alkane hydroxylase 2 (*alkB2*; PA1525, from 1660546 to 1659413) and flanking region. Other genes: PA1527: homologous to yeast chromosome separation protein SMC; PA1526: putative transcriptional regulator of the GntR family; *xdhA*: homologous to the N-terminal domain of eukaryotic xanthine dehydrogenases (XDH); *xdhB*: homologous to internal fragments of eukaryotic XDHs and total XDH of *Rhodobacter capsulatus*; PA1522: homologous to the N-terminal domain of *R. capsulatus* XDH.

C: The *rubA1A2B* gene cluster (*rubA1*: PA5351; from 6019347 to 6019180; *rubA2*: PA5350; from 6018996 to 6018829; *rubB*: PA5349; from 6018777 to 6017623) and flanking region. Other genes: *glcCDEFG*: genes homologous to the *glcRDEFG* genes of *E. coli* involved in glycolate oxidation; PA5348: homologous to histone-like protein HU from *P. aeruginosa*; PA5347, PA5346: hypothetical proteins.

clear inverted repeats are located at the end of the *hupR1* gene, which is transcribed in the opposite orientation. TlpS could be involved in chemotaxis towards long-chain *n*-alkanes, as *P. aeruginosa* PAO1 and other *P. aeruginosa* strains show strong chemotaxis towards hexadecane (J. B. van Beilen, unpublished results).

Genes involved in the regulation of the *P. aeruginosa* PAO1 *alkB1*, *alkB2* or *rubA1A2B* could not be identified, although the *alkB2* gene is preceded by a putative regulatory gene, transcribed in the opposite direction.

Southern blot detection of *P. aeruginosa* PAO1 *alkB1/alkB2* and *P. putida* (*oleovorans*) GPo1 *alkB* in environmental and clinical *P. aeruginosa* strains. To study the presence of *alkB* genes in environmental and clinical isolates of *P. aeruginosa*, we carried out Southern blots using the *P. aeruginosa* PG201 *alkB1* probe and a *P. putida* (*oleovorans*) GPo1 *alkBFGH'* probe. Chromosomal DNA was isolated according to standard procedures, digested with *Bam*HI, and blotted onto positively charged nylon membranes (Roche Diagnostics, Rotkreuz, Switzerland). With the GPo1 *alkBFGH'*-probe, bands were detected only in the four environmental strains able to grow on *n*-octane (data not shown), in accordance with previous results (262). PAO1, PG201, and the clinical strains did not show a band with this probe. The *alkB1* probe hybridized with two fragments in all *P. aeruginosa* strains, including the strains that did not grow on long-chain alkanes (figure 2, table 1).

Most clinical *P. aeruginosa* isolates are able to grow on long-chain *n*-alkanes, in accordance with literature data (4). However, the clinical strains did not grow as well on long-chain alkanes as the environmental strains and had a longer lag-time, perhaps because they are not adapted to grow on these substrates (170). All other strains, except PAO1, which is a clinical isolate as well, were isolated on *n*-alkanes, and thus were preselected for their ability to grow well on these substrates. Even though some of the clinical strains did not grow on alkanes, they do contain both alkane hydroxylase genes (figure 2). It is possible that these strains have mutations in the *alkB* genes or in other genes that are directly or indirectly involved in the initial alkane oxidation. For example, mutations in the rhamnolipid biosynthesis pathway can lead to reduced or abolished

growth on n-alkanes (140, 192). One strain (CPA3) did not grow on E2 medium with 0.2 % citrate, and may have an auxotrophy.
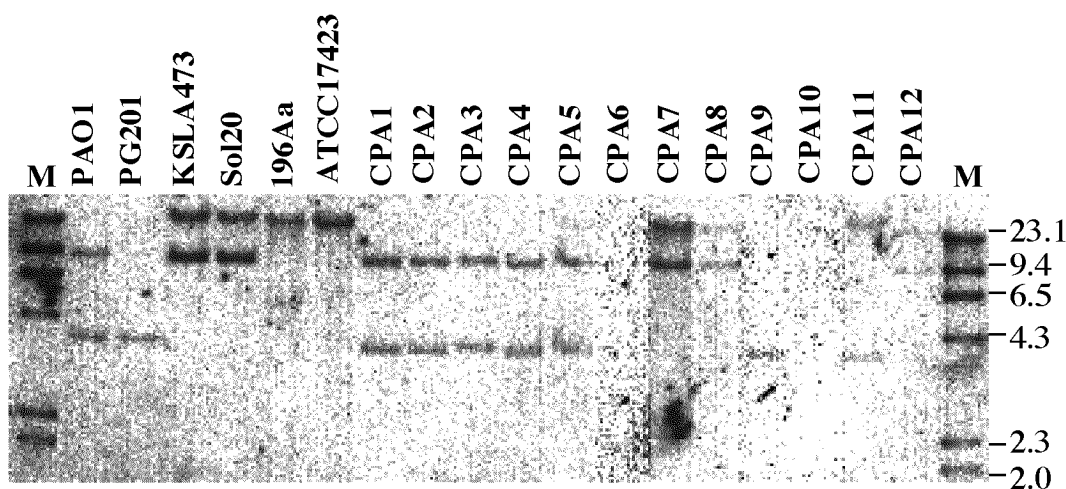

Figure 2: Southern blot of chromosomal DNA of environmental and clinical isolates of *P. aeruginosa* digested with *Bam*HI. As a probe, the 550 bp internal segment of *alkB1* from *P. aeruginosa* PG201 was used. The marker (M) is digoxigenin-labeled lambda DNA digested with *HindIII*; marker sizes are indicated in kb. For strains PG201, 196Aa and ATCC 17423, weak bands of around 0.7, 0.8 and 0.9 kb, respectively, were observed as well (not shown). The signal for the large fragment (±23 kb) of strains CPA6 and CPA9 was visible on the film, but could not be enhanced for the figure.

Concluding remarks. Based on the results of this study, it might be concluded that the ability of *P. aeruginosa* strains to grow on long-chain alkanes might have a larger physiological role than only degradation of oil pollutants or naturally occurring n-alkanes. The genome sequences of the clinical microorganisms *Burkholderia pseudomallei* K96243 and *Legionella pneumophilia* Philadelphia-1, which are also common soil or water organisms, do also contain alkane hydroxylase homologs (chapter 4), while AlkB homologs are not found in the genome sequences of anaerobic or strictly parasitic clinically important microorganisms, such as *Clostridium*. In contrast, the ability to grow on medium chain-length n-alkanes is clearly a property of environmental *P. aeruginosa* isolates: strains isolated from gasoline spills with hexane or octane as sole carbon source, are often *P. aeruginosa* strains that contain *alk*-genes (nearly) identical to

the *P. putida* (*oleovorans*) GPo1 *alk*-system (243, 262). The relevance of alkane degradation in clinical and natural environments has therefore to be studied in more detail.

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Chapter 7

CLONING AND CHARACTERIZATION OF GENES INVOLVED IN LONG-CHAIN ALKANE OXIDATION BY *PSEUDOMONAS FLUORESCENS* CHA0

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SUMMARY

Pseudomonas fluorescens CHA0 is an *n*-alkane degrading biocontrol strain. A 7.8 kb chromosomal DNA fragment containing a homolog of the *P.putida* GPo1 alkane hydroxylase gene was cloned and analysed. In addition to the alkane hydroxylase gene (*alkB*), it contains two "protein activator of alkane oxidation" genes (*praA* and *praB*), an outer membrane protein gene (*ompP1*), and two incomplete genes. A *P. fluorescens* CHA0 *alkB* knockout mutant was found to have lost the ability to grow on C12-C16 *n*-alkanes, but could be complemented for growth on these *n*-alkanes by plasmids containing the *P. fluorescens* CHA0 *alkB* gene.

A 16 kDa protein in the supernatant of hexadecane-grown *P. fluorescens* cells was identified as PraB. PraA was not detectable. When inoculated in the presence of spent supernatant containing PraB, *P. fluorescens* CHA0 cultures showed a shorter lag-phase. However, on SDS-PAA gels PraB was detectable only in the stationary phase.

In the presence of Triton X-100, the *alkB* knockout mutant showed weak growth on hexadecane, while the addition of dioctylphthalate allowed the wild-type *P. fluorescens* as well as the *alkB* knockout strain to grow on very-long-chain *n*-alkanes (C18-C28). This indicates that CHA0 possesses a second alkane hydroxylase.

INTRODUCTION

While the biochemistry and genetics of medium-chain length alkane degradation is well studied in strains such as *Pseudomonas putida* (*oleovorans*) GPo1 (263), little is known about membrane-bound long-chain alkane hydroxylases, with the exception of the *Acinetobacter* sp. ADP1 alkane hydroxylase (213). In this study, we focus on long-chain alkane hydroxylation by *Pseudomonas fluorescens* CHA0, a strain which is of interest because it inhibits growth of fungal pathogens in the plant rhizosphere (99, 250). CHA0 grows on *n*-alkanes ranging from dodecane to hexadecane (239).

PCR with degenerate primers based on the sequences of the alkane hydroxylase (*alkB*) genes of *P. putida* (*oleovorans*) GPo1 and *Acinetobacter* sp. ADP1, yielded PCR

fragments of *alkB* homologs from several Gram-positive and Gram-negative strains able to grow on long-chain alkanes (239). As functional expression of these genes could not be accomplished in *E. coli* and *P. putida*, presumably due to alkane uptake problems, an alkane hydroxylase knockout mutant of a strain able to grow on long-chain alkanes was required (chapter 4). We chose CHA0 for this purpose, as it is accessible for genetic engineering and produces all factors necessary for growth on long-chain alkanes. Such alkanes are practically insoluble in water due to their hydrophobicity. Therefore, microorganisms must use direct interfacial contact or biosurfactant-mediated solubilization to be able to use long-chain alkanes as their carbon source (28). Several *Pseudomonas* species, including *P. fluorescens*, use the latter mechanism. In the case of *P. fluorescens*, two lipopeptides with antibiotic function (viscosin and viscosinamide) were reported to have surfactant properties as well (183, 188). A surfactant called AP-6, containing 16 % carbohydrate, 34 % protein and 40 % lipids was isolated from the *P. fluorescens* isolate PG-1 (215), but the molecular structure of this surfactant was not elucidated. *Pseudomonas aeruginosa* strains produce rhamnolipids to solubilize alkanes (77, 193), but also excrete the so-called "protein activator of alkane oxidation" PraA, a small extracellular protein which stimulates hexadecane oxidation in respiratory experiments (103, 114).

In this study, a CHA0 *alkB* gene fragment (239) was used to clone the CHA0 alkane hydroxylase gene *alkB* and flanking DNA, which encode two homologs (PraA and PraB) of the *P. aeruginosa* PG201 PraA (103), and an outer membrane protein (OmpP1). Gene knockouts and complementation show that the CHA0 *alkB* encodes a functional alkane hydroxylase, while the alkane-solubilizing effect of both PraA and PraB was shown after overexpression of the proteins in *E. coli*.

MATERIAL AND METHODS

Strains, plasmids and media. Strains and plasmids used in this study are listed in table 1. LB (Luria Bertani broth) (220), E-medium (272) and E2-medium (151), supplemented with carbon sources or antibiotics were used throughout this study. MT trace elements

Table 1: strains and plasmids used in this study

Strain :	Properties:	Reference:
P. fluorescens CHA0	*alk*⁺	(250)
P. fluorescens KOB2	*alkB*⁻, Km^R	This study
P. fluorescens KOB2Δ1	*alkB*⁻	Chapter 4
P. fluorescens KOT1	*alkB*⁻ *praAB*⁻ *ompP1*⁻	This study
P. fluorescens KOT2	*alkB*⁻ *praAB*⁻ *ompP1*⁻	This study
E. coli DH10B	cloning strain	Gibco
E. coli GM48	*thr, leu, thi, lacY, galK, galT, ara,*	
	tonA, tsx, dam, dcm, supE44	J. Davison
E. coli JM101::alkS-Km	JM101, *alkS* integrated on chromosome, Km^R	(200)

Plasmids:	Relevant genotype:	Reference:
pGEM7Zf(+)	Cloning vector, Ap^R	Promega
pZeRO1	Cloning vector, Zeocin^R	Invitrogen
pKKPalk	Expression vector, Ap^R	(240)
pCom5	Expression vector, Gm^R	(240)
pEX18Tc	Knockout vector, Tc^R	(115)
pCK217	*res-npt-res* cassette in pUC18Sfi	(145)
pPF1	4.5 kb *Xho*I-*Bam*HI fragment of *P. fluorescens* CHA0 in pGEM7Zf(+), '*estF1-alkB-praAB-ompP1*'	This study
pPF7	*alkB* gene in pGEM7, deletion clone of pPF1	This study
pPF8	*ompP1* fragment in pGEM7, deletion clone of pPF1	This study
pPF110	4 kb *Pst*I fragment of *P. fluorescens* CHA0 in pZeRO1, *ompP1-yafH*'	This study
pPF20	7.8 kb fragment of *P. fluorescens* CHA0 in pGEM7Zf(+), '*estF1-alkB-praAB-ompP1-yafH*'	This study
pPF21	1.8 kb *Mun*I-*Sal*I fragment in pCom5, *alkB*	This study
pPF22	2.8 kb *Mun*I-*Pst*I fragment in pCom5, *alkB-praAB*	This study
pPF23	4.0 kb *Mun*I-*Xho*I fragment in pCom5, *alkB-praAB-ompP1*	This study
pPFrnrB	*res-npt-res* cassette in *Sfi*I-*Stu*I sites of pPF1	This study
pEXPFrnrB2	pEX18TC with *Pvu*I fragment of pPFrnrB	This study
pPF31	*Sal*I deletion clone of pPF20	This study
pPF32	*Asp*718-*Eco*RV deletion clone of pPF31	This study
pPF32npt2	*npt*-gene of pCK217 as *Xba*I fragment in pPF32	This study
pPF105	*praA* in pKKPalk	This study
pPF106	*praB* in pKKPalk	This study
pPF107	*praAB* in pKKPalk	This study

(151) were added to all minimal media. All cultures were grown aerobically at 30°C or 37°C. For growth on *n*-alkanes, Petri dishes with E2 medium were incubated at 30°C with *n*-alkanes supplied through the vapour phase (for *n*-octane by placing an open erlenmeyer with *n*-octane (C8) in a sealed container, for *n*-decane (C10), *n*-dodecane (C12), *n*-tetradecane (C14) and *n*-hexadecane (C16) by placing a Whatman 3MM filter disc with 200 μl *n*-alkane in the lid of the Petri dish.

P. fluorescens CHA0 and recombinants derived from this strain were grown in liquid E2 medium. Liquid *n*-alkanes were added to the medium to a concentration of 1 % (v/v). Very-long-chain alkanes (C18 and longer) were dissolved to 10% (v/v) in dioctylphthalate (DOPh) and added to a concentration of 0.2 % (v/v) to the medium. In some cases, rhamnolipids (97) or Triton X-100 were added to a concentration of 0.1 % (w/v) to improve the uptake of long-chain alkanes.

E. coli strains were transformed by electroporation according to Dower (64). *E. coli* strains harbouring plasmids were grown with appropriate antibiotics (kanamycin (Km) 50 µg/ml), tetracycline (Tc) 12.5 µl/ml; ampicillin (Ap) 100 µg/ml; gentamycin (Gm) 10 µg/ml). *P. fluorescens* CHA0 was transformed according to Højberg (117). To select transformants, kanamycin (50 µg/ml), gentamycin (100 µg/ml) and tetracycline (100 µg/ml) were added to the medium.

DNA manipulations. Restriction enzymes, T4 DNA ligase, DNA polymerase (Klenow fragment), T4 DNA polymerase and dideoxynucleotides were from Roche Molecular Diagnostics (Rotkreuz, Switzerland) and used as specified by the supplier. Oligonucleotides were synthesized by Microsynth, Balgach, Switzerland. PCR-products were purified over 1% agarose gels, cut with the appropriate enzymes, purified again over a 1% agarose gel, and cloned into pGEM7-Zf(+) (Promega) or pKKPalk (240)(chapter 3). Chromosomal DNA was isolated according to Desomer (58). Plasmid DNA was isolated according to Birnboim and Doly (21), or with the Roche High Pure Plasmid Isolation Kit to obtain sequencing grade DNA. Both strands of the inserts were sequenced on a Li-Cor 4000L sequencer using the Amersham Thermosequenase cycle sequencing kit and IRD41- (IRD800-) labelled -40 forward (agggttttcccagtcacgacgtt) and -40 reverse (gagcggataacaatttcacacagg) primers for pGEM7-Zf(+) clones or PalkFwd (tggcgcaagcgtccgattag) and pKKRev (gagttcggcatggggtcaggtg) for pKKPalk derived plasmids (MWG-Biotech). Nucleotide and amino acid sequences were analyzed and compared using LASERGENE Navigator from DNASTAR (Madison, Wisconsin, USA). Nucleotide and amino acid sequences were compared with the EMBL, SwissProt and GenBank databases using BLAST (5). BLAST searches were carried out at NCBI.

PCR. PCR reactions were carried out as described in (123) using a Perkin Elmer GeneAmp PCR System 9600. The following program was used (4' 95°C, 25x (45'' 95°C, 1' annealing temperature (5°C below Tm), 1' 72°C), 5' 72°C, ∞ 4°C). When no product was visible after a first round of PCR, 1 μl of product was used as the template in a second round of PCR. To amplify complete genes for expression studies the Roche Expand High Fidelity Polymerase kit was used. The sequence of cloned PCR fragments was verified by DNA sequencing.

Cloning of the *alkB* gene and flanking regions. To clone the complete *P. fluorescens* CHA0 alkane hydroxylase gene and flanking regions, easy-to-clone restriction fragments were identified by Southern blotting (220). 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(+) (Promega, Madison, USA) or pZeRO1 (Invitrogen, Leek, The Netherlands) and transformed into *E. coli* DH10B (Gibco BRL). Transformants containing the target genes were identified by colony blotting using the same probes. Both strands of the inserts were sequenced.

A 4.5 kb *Xho*I-*Bam*HI chromosomal DNA fragment was identified and cloned using the PCR fragment of pCHA0 (239) as a probe. An overlapping 4.0 kb *Pst*I fragment was cloned using the insert of pPF8 as a probe, resulting in plasmid, pPF110.

To construct plasmid pPF20, pPF1 was digested with *Bam*HI and *Nsi*I, and ligated with a 3.3 kb *Bam*HI-*Pst*I fragment, cut from pPF110 and purified over gel.

Construction of an *alkB-praAB-ompP1* knockout mutant. Plasmid pPF20 was digested with *Sal*I and religated. The resulting plasmid was digested with *Asp*718 and *Eco*RV, treated with Klenow and religated. This plasmid, digested with *Xba*I, was ligated to the small *Xba*I fragment of pCK217, containing the kanamycin resistance. The resulting plasmid, called pPF32npt2 was introduced in *P. fluorescens* CHA0 by electroporation, and integrants were selected with kanamycin. A kanamycin resistant clone was cultured on LB until the kanamycin resistance was lost, resulting in *P. fluorescens* KOT1 and KOT2. The extent of the deletion in these mutants was confirmed

by PCR with primers Hindfw (tcgaagcttcaagagcaagttgag) and mutCrv
(ccgccgtactgcttgggaatgat).

Construction of expression vectors. To construct *P. fluorescens* expression plasmids
containing *alkB* (pPF21), *alkB-praAB* (pPF22) or *alkB-praAB-ompP1* (pPF23), plasmid
pPF20 was cut with *Mun*I and *Sal*I, *Mun*I and *Pst*I, or *Mun*I and *Xho*I, respectively.
Fragments of the right size were isolated from agarose gels, and ligated in pCom5
(240)(chapter 3), digested with *Eco*RI and *Sal*I for the *alkB* and *alkB-praAB-ompP1*
containing fragments, and with *Eco*RI and *Pst*I for the *alkB-praAB* fragment.
E. coli expression vectors for *praA*, *praB* and *praAB* were constructed as follows: *praA*
was amplified from pPF1 using primers PraAfw and PraArv; *praB* using primers PraBfw
and PraBrv, and *praAB* with primers praAFw and PraBrv. The PCR fragments were
purified, digested with the appropriate restriction enzymes and cloned in pKKPalk
(240)(chapter 3), digested with the same enzymes. The resulting plasmids were called
pPF105, pPF106 and pPF107 encoding *praA*, *praB* and *praAB* respectively.

Southern and colony blots. Chromosomal DNAs were digested to completion with
restriction enzymes and loaded on an 1 % agarose gel in TAE buffer. DNA was
transferred onto positively charged nylon membrane (Roche Molecular Diagnostics,
Rotkreuz, Switzerland) by alkaline transfer as described previously (220). The 550 bp
PCR fragment from pCHA0 (239) and the inserts of pPF7 (*alkB*) and pPF8 (*ompP1*) were
used as probes for Southern hybridisation. All DNA fragments were purified over a 1%
agarose gel, electroeluted and labelled by the random priming method with digoxigenin-
dUTP using the DIG labelling and chemiluminescence detection kit (Roche Molecular
Diagnostics, Rotkreuz, Switzerland). Hybridizations were carried out using standard
hybridization buffer without formamide at 65°C. Detection of Southern and colony blots
was carried out using the Roche DIG-kit according to the manufacturer. Alkaline
phosphatase coupled to Anti-DIG antibodies was used with CSPD as the
chemiluminescent substrate.

Protein analysis. SDS-PAA gel electrophoresis was done according to Laemmli (149). About 10 μg protein was loaded per lane. To concentrate supernatant samples, 5 μl StrataClean resin (Stratagene, La Jolla, USA) was added to 0.5 ml culture supernatant, and incubated at 37°C for 30 minutes. The resin was collected by centrifugation, resuspended in loading buffer and loaded on 15 % SDS-PAA gels.

Electrophoresed proteins were stained with Coomassie Brilliant Blue R-250 or transferred to a PVDF filter membrane for subsequent N-terminal amino acid sequence analysis.

For MALDI-TOF analysis, Coomassie stained bands containing PraA and/or PraB were cut from the gel, washed with 100 mM ammonium acetate, then with acetonitrile, and with 50 mM ammonium acetate in a 50 % acetonitrile solution, and subsequently dried. The proteins were subjected to *in-gel* trypsin digestion for three hours at 37°C, and the resulting mixture was subjected to mass spectrometry analysis (MALDI-TOF) using 2,5-dihydroxybenzoate as matrix.

Sequence. The sequence of the 7.8 kb *Xho*I-*Pst*I fragment of *P. fluorescens* CHA0 determined in this study was deposited in GenBank and received the accession number AJ009579.

RESULTS AND DISCUSSION

Cloning and sequence analysis of a 7.8 kb chromosomal DNA fragment of *P. fluorescens* CHA0 encoding the *alkB*, *praAB* and *ompP1* genes. A 550 bp PCR fragment, obtained earlier from *P. fluorescens* CHA0 using degenerate primers which amplify internal fragments of *alkB* homologs (239), was used as a probe to identify and clone a 4.5 kb *Xho*I-*Bam*HI fragment. To complete an ORF encoding an outer membrane protein, an overlapping 4.0 kb *Pst*I fragment was cloned as well. The complete 7.8 kb sequence was found to contain six open reading frames (ORFs)(figure 1). The first ORF named *estF1* is incomplete due to the *Xho*I cloning site, and has 69.7 % DNA sequence identity to the lactone-specific esterase (*estF1*) of *P. fluorescens* DSM50106 (135). The

alignment suggests that the N-terminal 40 amino acids of the *P. fluorescens* CHA0 EstF1 are missing.
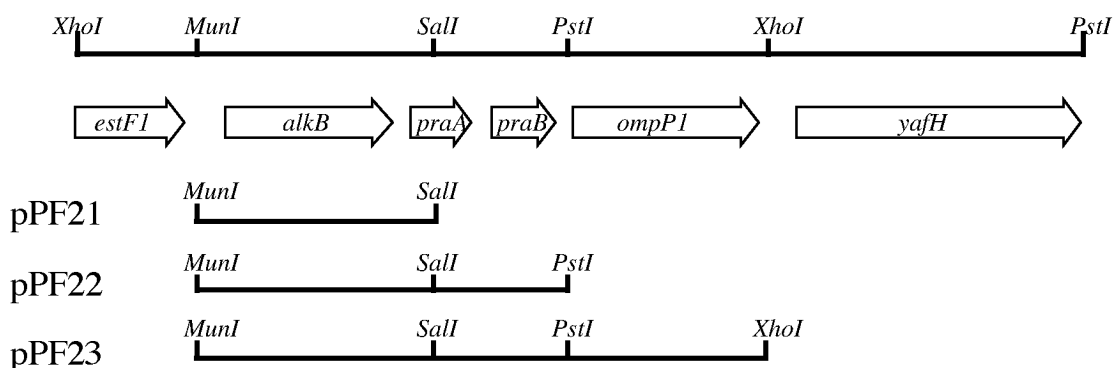
Figure 1: organisation of the 7.8 kb *XhoI-PstI* fragment encoding the *alkB* gene and extent of the inserts of the complementation plasmids pPF21, pPF22 and pPF23.

The alkane hydroxylase gene *alkB* is located 295 bp downstream of *estF1*, and has 64.0 % protein sequence identity to the partial sequence of a putative *alkB* gene of *P. fluorescens* DSM50106 (135) and 40-60 % protein sequence identity to other alkane hydroxylases (chapter 4). Like its homologs, the CHA0 AlkB sequence contains the conserved histidines, which are likely ligands for the two non-heme iron atoms in the active site (233, 239). Six transmembrane helices could be identified and suggest that the CHA0 AlkB has the same topology as the *P. putida* (*oleovorans*) GPo1 AlkB (261). Comparison with other alkane hydroxylases (chapters 4 and 9) showed that the *P. fluorescens* CHA0 alkane hydroxylase has a large insertion between the third and fourth transmembrane helices.

Two ORFs located directly downstream of the alkane hydroxylase encode proteins with 30.7 and 44.8 % sequence identity to the "protein activator of alkane oxidation" protein PraA of *P. aeruginosa* PG201 (103) (figure 2). Therefore, these ORFs were named *praA* and *praB*. The three Pra proteins have no other homologs in the sequence databases, including the unfinished genome sequences. PraB of CHA0 is clearly more closely related to PraA of *P. aeruginosa* PG201 than the CHA0 PraA. The N-termini of both PraA and PraB strongly resemble signal peptides (SIGNALP: http://www.cbs.dtu.dk)

(187). The predicted cleavage site is indicated with an arrow in figure 2. The mature *P. aeruginosa* PG201 PraA and *P. fluorescens* CHA0 PraB are rich in small amino acids (55 % GSTAP) and contain very few charged residues (7 and 4 % DEKR, respectively)(table 2). The CHA0 PraA contains significantly more charged amino acid residues (18 % DEKR).

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                 10             20             30             40
                  |              |              |              |
1   M K S I K S L P S F A A L A L C L S V S S M A S A↓A T I T - - - - P V N S A F T  PraA (PG201)
1   M - S I S P L F T A Q L F V V L V G I S L M G A A E A↓A R I E - - P A N T E F T  PraA (CHA0)
1   M K G I K T L V S A T A I V A C L G A A S M A S A↓A S I A I D G A N P D G P F T  PraB (CHA0)

    M . . I . . L . . . . . . . . . . . . . . . M . . . A . . . . . . . . . . . . F T  Consensus

                 50             60             70             80
                  |              |              |              |
37  A P G - T I S V S S P A S L N L P V T - - - C N I T F K G K T A A D G S Y A S I  PraA (PG201)
38  A K G - - - - - - - P I S F A K S I I N A D C T I Q V S G K V S P D G S F A S V  PraA (CHA0)
41  T P G G T I T V R S P S S F N Q P V T - - - C N I N F S G N I A - - G G V A S I  PraB (CHA0)

    . . G . . . . . . . P . S . . . . . . . . . . C . I . . . G . . . . . G . . A S .  Consensus

                 90            100            110            120
                  |              |              |              |
73  D S V T V S G S N T L C S V P Q M T G L P W K L T V S S T T A G K V D G V G F K  PraA (PG201)
71  E K V D F S G G L K - C G Q V E A T H L P W K L I A K D E T S G A M S G I Q V T  PraA (CHA0)
76  T G A T V S G S N A L C N L P K I T G L P W T L S A S S T T A G T V T N V G Y T  PraB (CHA0)

    . . . . . S G . . . . C . . . . . T . L P W . L . . . . . T . G . . . . . . . .  Consensus

                130            140            150            160
                  |              |              |              |
113 I L - - - - S S T C G P S T V N G S W S N A T N T L S - A S N Q S L A G N C K I  PraA (PG201)
110 V H A P L V G G D C G P S T A E G S W N N S T G K L E - A A Q V S L D G G C T I  PraA (CHA0)
116 I S F - F P A T N C G P T T I N V A W S N V T H S L S L S T P Q T L S G G C S V  PraB (CHA0)

    . . . . . . . . . C G P . T . . . . W . N . T . . L . . . . . . . . L . G . C . .  Consensus

                170
                  |
148 N S L S V K P T P A F V V N P        PraA (PG201)
149 K T V S I Q M P P T F R V A P        PraA (CHA0)
155 D V L N A K P S P Q V S V I          PraB (CHA0)

    . . . . . . . . P . . . V . .        Consensus
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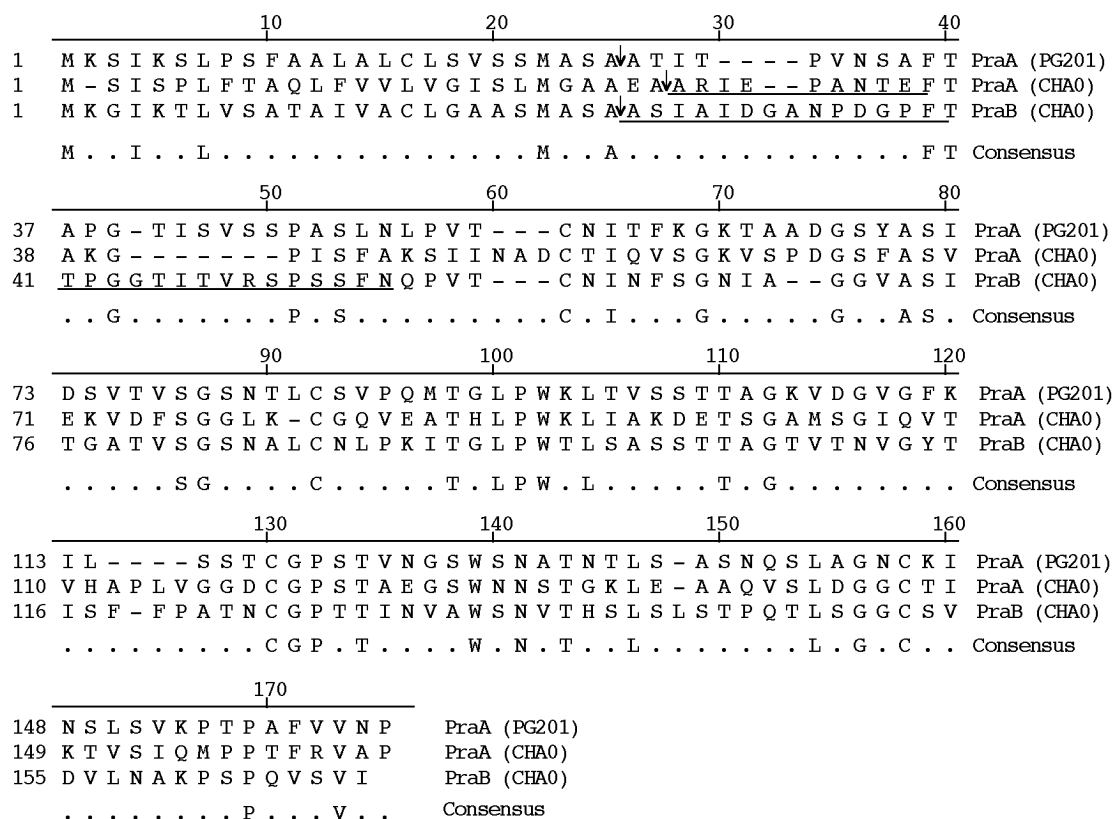
Figure 2: alignment of the *P. aeruginosa* PG201 PraA with the *P. fluorescens* CHA0 PraA and PraB. Arrows indicate the predicted cleavage site of the prepeptide. The underlined sequences are the N-terminal sequences determined in this study.

The fifth ORF, designated *ompP1*, encodes a peptide which has 23-25 % sequence identity to several *Haemophilus influenzae* outer membrane proteins of the OmpP1 family. A highly homologous hypothetical protein (68 % peptide sequence identity) is encoded immediately downstream of the *pra* gene in *P. aeruginosa* PAO1 (249). OmpP1 has weaker sequence homology to FadL (21 % sequence identity), the long-chain fatty

acid uptake protein of *E. coli* (22), and to a set of hypothetical proteins encoded by aromatic catabolic operons, which include TodX (21 % sequence identity), TbuX and SalD (125, 127, 274). TbuX was shown to be involved in uptake of toluene into the cell (127).

Table 2: amino acid composition of the mature peptides of PraA (*P. aeruginosa* PG201), PraA (*P. fluorescens* CHA0) and PraB (*P. fluorescens* CHA0).

	Basic		Acidic			Polar						Apolar								
	K	R	D	E	H	N	S	T	Y	Q	C	G	A	V	L	I	M	F	W	P
PraA (PG201)	7	0	3	0	0	11	23	18	1	2	4	11	12	13	9	6	1	4	2	10
PraA (CHA0)	9	2	6	7	2	4	14	11	0	5	4	15	14	11	6	9	2	5	2	9
PraB (CHA0)	2	1	3	0	1	12	20	20	1	3	4	15	12	12	8	10	0	5	2	12

The outer membrane of Gram-negative bacteria consists mainly of phospholipids and LPS, and constitutes a barrier for hydrophobic compounds (190). As the *P. fluorescens* CHA0 OmpP1 has homology to FadL, which transports fatty acids over the outer membrane (22), OmpP1 may have a similar function in the transport of long-chain alkanes over the outer membrane.

The translation product of the sixth, incomplete, ORF directly downstream of *ompP1*, has homology to the hypothetical protein YafH of *E. coli* (24) and to several mammalian very-long-chain acyl-CoA dehydrogenases involved in the metabolism of very-long-chain fatty acids (7, 52).

Strong inverted repeats are located directly downstream of *estF1* and *ompP1* but are not present downstream of *alkB*, *praA*, and *praB*. Therefore, *alkB*, *praA*, *praB* and *ompP1* may be transcribed from a single transcription site upstream of *alkB*.

We did not succeed in cloning the *P. fluorescens* CHA0 rubredoxin and rubredoxin reductase genes, the electron transfer components of the alkane hydroxylase system using PCR with highly degenerate primers for rubredoxins (T. H. M. Smits and J. B. van Beilen, unpublished results). However, the genome sequence of another *P. fluorescens* strain (Pf0-1) does contain a rubredoxin and a rubredoxin reductase gene homolog, even though it does not possess an alkane hydroxylase homolog. These genes are highly homologous (around 80 %) to genes encoded by the *P. aeruginosa* PAO1 genome(249),

which complement deletions of the *P. putida* (*oleovorans*) GPo1 rubredoxin and

rubredoxin reductase genes (chapter 6).

Alkane-solubilizing compounds in the supernatant of *P. fluorescens* CHA0 cultures.

P. fluorescens CHA0 possesses two genes related to the so-called "protein activator of

alkane oxidation" PraA of *P. aeruginosa* PG201. This protein is an extracellular protein,

which is found in large amounts in the culture supernatant of hexadecane-grown *P.*

aeruginosa strains (113). To determine whether *P. fluorescens* CHA0 also excretes Pra

proteins, supernatant samples were concentrated and analysed for the presence of these

proteins. A 16 kDa band was visible in supernatants of cultures grown on *n*-alkanes

(figure 3A), but was not detected when CHA0 was grown on citrate or dodecanol (data

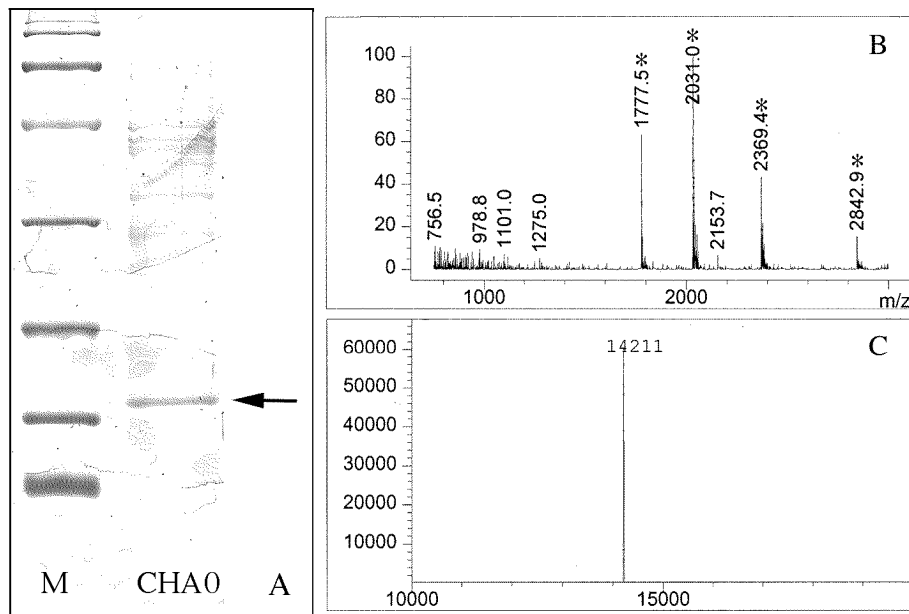not shown). Addition of 1 ml culture supernatant of a filter sterilized hexadecane-grown

Figure 3: Analysis of supernatant from hexadecane-grown *P. fluorescens* CHA0.
A: 15 % SDS-PAGE of extracellular proteins from hexadecane-grown *P. fluorescens* CHA0. M: marker, CHA0: sample CHA0 grown on hexadecane. The arrow indicates the 16 kDa band.
B: HPLC-MS analysis of the 16 kDa protein. Asterisks indicate the peaks used to determine the molecular mass of the protein (panel C).
C: Molecular mass determined from the LC-MS spectrum. The peak at 14211 Da corresponds to the calculated molecular weight of PraB after removing the signal sequence (14213 Da).

culture *P. fluorescens* CHA0 to a fresh 50 ml culture resulted in a clearly reduced lag-time and an increased growth rate (8 vs. 32 hrs. lag-time , 7 vs. 10 hrs. doubling time). The addition of culture supernatant from citrate- or dodecanol-grown *P. fluorescens* CHA0 cultures did not have this effect.

The N-terminal sequence of the 16 kDa band corresponds to amino acids 26 to 55 of the PraB sequence, exactly after the predicted signal peptidase recognition site (underlined in figure 2). The N-terminal sequence data did not show evidence of an underlying PraA sequence. To investigate whether the N-terminal sequence of PraA is blocked or whether PraA is just absent, culture supernatant samples were analysed by MALDI-TOF and HPLC-MS. PraA was not detectable by these methods either (figure 3B and 3D). The molecular weight (14211 Da) of PraB measured by HPLC-MS corresponds well to the calculated molecular weight of the mature protein (14213 Da).

Heterologous expression of PraA and PraB in *E. coli* JM101::alkS-Km. To determine whether PraA and/or PraB solubilize *n*-alkanes, we constructed *praA*, *praB* and *praAB* expression plasmids based on pKKPalk (240)(chapter 3). Introduction of these plasmids in *E. coli* JM101::alkS-Km (200) allows expression from the *P. putida* (*oleovorans*) GPo1 *alkB* promoter after induction with DCPK. All three strains were grown on minimal medium containing glycerol as the carbon source.

Upon induction with 0.05 % DCPK, *E. coli* JM101::alkS-Km[pPF106] expressing only PraB showed clearly lower growth rates. The growth rate of the other two recombinants was not influenced by the addition of DCPK. SDS-PAGE analysis of the culture supernatants of all recombinants showed that in cultures expressing the *praA* gene and the *praAB* genes, a 16 kDa protein was prominent (up to 10 % of supernatant proteins). The N-terminal sequence of this protein confirmed the cleavage of a signal sequence in PraA at the predicted position (sequence underlined in figure 2). In contrast to the *P. fluorescens* CHA0 supernatant, many additional proteins were observed in the *E. coli* culture supernatants, indicating lysis of induced cells.
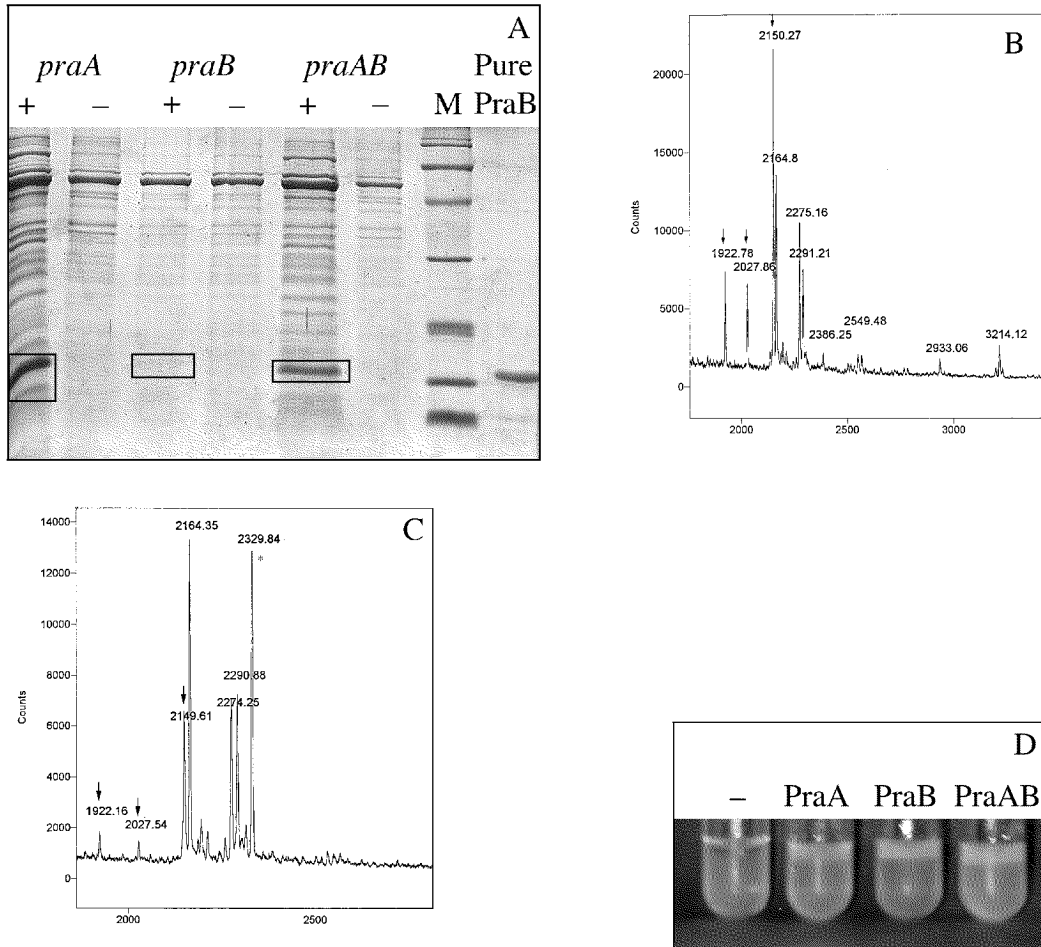
Figure 4: expression of PraA, PraB and PraAB in *E. coli* JM101::alkS-Km
A: 15 % SDS-PAGE gel of supernatant samples. Induced (+) and not-induced (-) samples were applied. Boxes indicate PraA, PraB and PraAB respectively. M: marker
B: MALDI-TOF analysis of PraA after trypsin treatment. Peaks indicated by an arrow correspond to fragments expected from PraA.
C: MALDI-TOF analysis of PraAB after trypsin treatment. Peaks indicated by an arrow correspond to fragments expected from PraA. The peak indicated by an asterisk correspond to a fragment expected from PraB.
D: Emulsification test of supernatants containing PraA, PraB and PraAB. Negative control is a supernatant of non-induced cells.

In supernatant of cultures expressing PraB only a faint band could be seen (figure 4A), but N-terminal sequencing and MALDI-TOF confirmed the identity of this protein. MALDI-TOF analysis (figure 4C) showed that PraA and PraB were expressed simultaneously in *E. coli* JM101::*alkS*-Km[pPF107]. However, the relative amounts

could not be determined. These results show that *E. coli* processes and exports PraA as well as PraB, as was found for PraA of *P. aeruginosa* PG201 (103).

In emulsification experiments (59), the emulsification ability of the protein solutions was determined. Supernatants containing either PraA, PraB or both PraA and PraB all showed stable emulsification of hexadecane, while negative controls (non-induced samples) showed a fast phase separation (figure 4D).

Construction of *alkB* knockout mutants. A knockout mutant of *P. fluorescens* CHA0 (chapter 4) was constructed by inserting a kanamycin resistance gene, flanked by two *res* sites (145), in the *alkB* gene. This mutant, named KOB2, did not grow on C12-C16 *n*-alkanes.
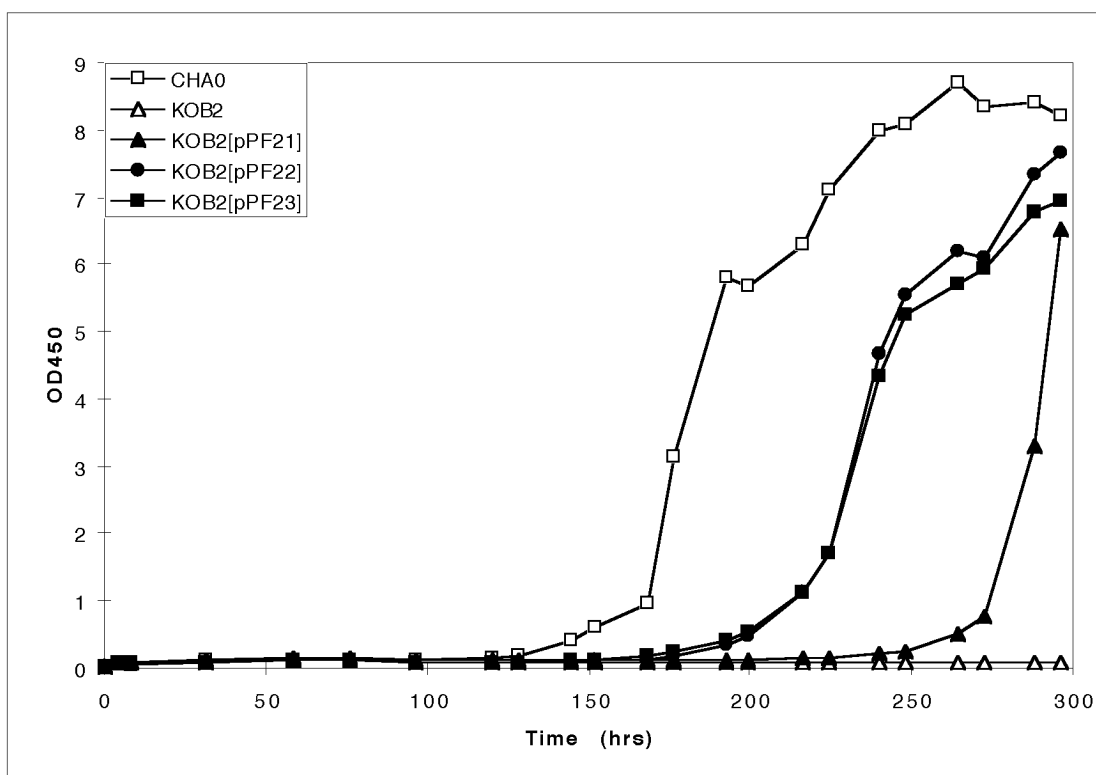

Figure 5: growth curves of *P. fluorescens* CHA0, KOB2 and recombinants KOB2[pPF21], KOB2[pPF22] and KOB2[pPF23].

To test whether the knockout mutant could be complemented for growth on *n*-alkanes, KOB2 was equipped with the pCom5 derived plasmids pPF21 (encoding AlkB), pPF22 (encoding AlkB and PraAB) and pPF23 (encoding AlkB, PraAB and OmpP1). All three plasmids restored growth on *n*-alkanes. However, the lag-time of the recombinant containing pPF21 was significantly longer than that of the recombinants containing pPF22 or pPF23 (figure 5). This is probably due to a polar effect of the transcriptional terminator in the *res-npt-res* cassette (145) on the expression of the downstream encoded *praAB* genes, which suggests that the *praAB* genes are part of an *alkB-parAB* operon. The observation that KOB2 recombinants containing pPF22 and pPF23 have almost identical growth curves suggests that the *ompP1* gene does not play a role in alkane degradation, at least under these circumstances, and for the tested alkane, hexadecane. The fact that the lag-time, but not the growth rates are affected by the reduced expression of the *pra*-genes is in accordance with the results of Hardegger *et al.* (103). The difference between the wild-type CHA0 and KOB2[pPF22] or KOB2[pPF23] could be due to limited expression of *alkB-praAB* from the pCom5-derived plasmids, as only 132 bp upstream of the *alkB* gene were cloned. The promoter region may therefore be incomplete.

To reduce the polar effect on the expression of the *praAB* genes for complementation experiments with other alkane hydroxylases (chapter 4), the kanamycin resistance gene and its terminator were removed using the resolvase gene (145), expressed from the *lac*-promoter on plasmid pUCPParA.

The resulting *alkB* deletion mutant KOB2Δ1 was transformed with pPF21 (chapter 4), and compared with CHA0 and KOB2[pPF21]. The growth rates of the three strains were similar, but the lag-time of KOB2Δ1[pPF21] (around 100 h) was much shorter than that of KOB2[pPF21] (around 200 h). As the lag-time still is longer than that of CHA0 (around 60-100 hours), the polar effect may not be eliminated completely. PraB was detected in the culture supernatant of CHA0 and KOB2Δ1[pPF21] at time points t = 120 and t = 168 after inoculation, but not in KOB2[pPF21]. A very weak PraB band (estimated to be tenfold less strong than for CHA0 and KOB2Δ1[pPF21]) was observed in KOB2[pPF21] only at t = 216 and later timepoints (data not shown).
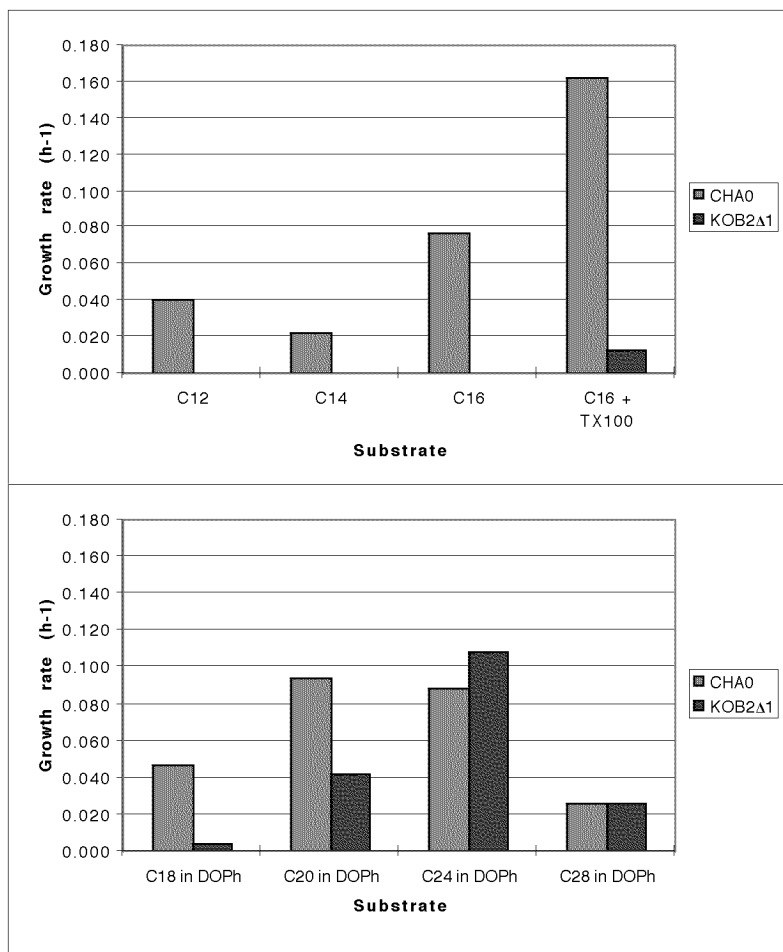
Figure 6: growth rates of *P. fluorescens* CHA0 and KOB2Δ1 on alkanes.
Upper panel: growth rates on long-chain alkanes. C12: dodecane; C16: hexadecane; TX100: Triton X-100.
Lower panel: growth rates on very-long-chain alkanes, added as 10 % solution in dioctylphthalate (DOPh).
C18: octadecane; C20: eicosane; C24: tetracosane; C28: octacosane.

Characterization of *P. fluorescens* KOB2Δ1: a second alkane hydroxylase system? In the absence of additives to improve growth on *n*-alkanes, *P. fluorescens* KOB2Δ1 does not grow on any of the alkanes tested, However, when KOB2Δ1 was grown on hexadecane in the presence of 0.1 % Triton X-100, some growth was observed, with a growth rate ten times lower than that of CHA0 (figure 6). Longer alkanes (C18-C28), which are solid at 30°C, do not support growth of CHA0. However, these alkanes supported growth of both KOB2Δ1 and CHA0 when they were dissolved to 10 % in dioctylphthalate. The difference in growth rates between CHA0 and KOB2Δ1 decreased with increasing chain length, and disappeared for C24 and C28 (figure 6). From these data we conclude that *P. fluorescens* CHA0 possesses a second alkane oxidation system,

which oxidizes very-long-chain alkanes. These alkanes can only be used as C-sources if they are made accessible to CHA0, for example, by dissolving the alkanes in an inert organic phase. In soil, they may be accessible because they occur together with shorter alkanes, or because CHA0 excretes different solubilizing compounds.

Attempts to clone the second alkane hydroxylase gene using PCR with the degenerate alkane hydroxylase primers described before (239) were not successful. As no specific signals other than that corresponding to the cloned CHA0 *alkB* gene were found during Southern blot analysis (239), the second alkane hydroxylase gene must have less than 70 % sequence identity to the alkane hydroxylase genes used as probes, including the cloned CHA0 *alkB* gene (data not shown).

ACKNOWLEDGEMENTS

Appendix to chapter 7:

Influence of *alkB* knockout mutations on disease suppression by *Pseudomonas fluorescens* CHA0: an initial study

Theo H.M. Smits, Stefanie B. Balada, Monika Maurhofer, Geneviève Défago, Bernard Witholt and Jan B. van Beilen

INTRODUCTION

Biocontrol is the suppression of fungal pathogens causing plant root diseases by certain rhizobacteria which effectively colonize roots and produce extracellular antifungal compounds (99). Soils containing such rhizobacteria are called suppressive soils. For disease protection, the biocontrol strain must be present on the roots at the right location (at the root tips), at the right time (before the pathogen causes too much damage) and in sufficient numbers (10^5-10^6 colony forming units per gram soil).

One of the best characterized biocontrol strains is *Pseudomonas fluorescens* CHA0 (250). This strain, isolated from suppressive soil in Morens (Switzerland), is an effective root colonizer and produces a wide range of secondary metabolites with antifungal properties, such as 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, hydrogen cyanide, and phenazines (e.g. phenazine-1-carboxylate)((99) and references therein), but many other extracellular metabolites have an antibiotic function as well (183, 188).

Many fluorescent pseudomonads are able to grow on *n*-alkanes. We isolated and characterized the alkane hydroxylase gene (*alkB*) and flanking regions from *P. fluorescens* CHA0 (chapter 4, 7). Alkane hydroxylase knockout mutants were generated, which lost the ability to grow on alkanes ranging from dodecane (C12) to hexadecane (C16), while they were still able to grow on very-long-chain alkanes (octadecane and longer). The proteins encoded by *praAB* and *ompP1*, which are located downstream of

alkB, could play a role in solubilization and uptake of alkanes, although their role has not been proven unequivocally (chapter 7).

Alkanes are ubiquitous in natural environments, as they are natural components of plant waxes (212). These alkanes could be a possible carbon source for rhizosphere bacteria involved in biocontrol. To test this hypothesis, we assayed *P. fluorescens* strains CHA0 and the alkane hydroxylase negative mutants KOB2 and KOB2Δ1 for their biocontrol ability. Here we report first results, which indicate that inactivation of the alkane hydroxylase gene has a significant effect on the biocontrol ability of *P. fluorescens* CHA0.

MATERIAL AND METHODS

P. fluorescens strains CHA0, KOB2 and KOB2Δ1 were described before (250)(Chapter 7). Tobacco and wheat plants were grown for three weeks in a gnotobiotic system containing artificial soil composed of quartz sand and vermiculite (134) in the presence or absence of biocontrol strains. Tobacco plants were inoculated with the pathogen *Thielaviopsis basicola*, a fungus which causes black root rot (134) and wheat plants were inoculated with the pathogens *Gaeumannomyces graminis* var. *tritici*, which causes take-all desease in wheat (133). After harvesting, the plants were assayed for fresh weight, percentage of root infection and bacterial root colonization. Root colonization was measured as the average amount of colony forming units per gram soil per experiment. A modified disease rating for wheat and *G. graminis* var. *tritici* as described in Keel *et al.* (133) has been used for scoring the Ggt disease index: 0 = No symptoms; 1 = < 10% of roots infected; 2 = 10-25% of roots infected; 3 = 25-50% or roots black and at least one main root infected; 4 = > 50% of roots black, or all main roots infected; 5 = Lesion on stem base; 6 = Lesions all around stem and starting chlorosis of leaves; 7 = Plant strongly chlorotic and severely stunted; 8 = Plant dead or nearly dead.

Table 1: effect of *alkB* knockout on biocontrol ability by *P. fluorescens* against tobacco pathogen *T. basicola* .

Microorganisms added		Plant fresh weight	Root infection	Bacterial root colonization
P. fluorescens	*T. basicola*	(mg)	(%)	(x 10^8 cfu/g soil)
none	-	537 a	0	0
none	+	126 d	89 a	0
CHA0	+	391 b	17 b	4.3 a
KOB2	+	215 c	53 c	11.5 a
KOB2Δ1	+	253 c	46 c	4.9 a

Data of two experiments were pooled. The means of twenty repetitions (ten repetions per experiment, one plant per repetition) are presented. Means in the same column followed by the same letter are not significantly different at $P = 0.05$ (Fisher's protected LSD test, Systat).

RESULTS AND DISCUSSION

Two gnotobiotic test systems were used to assay the biocontrol ability of the *P. fluorescens* strains CHA0, KOB2 and KOB2Δ1 (133, 134): black root rot of tobacco caused by the pathogen *Thielaviopsis basicola* and take-all disease of wheat caused by *Geaumannomyces graminis* var. *tritici.*

The effectiveness of *P. fluorescens* biocontrol on black root rot of tobacco is shown in table 1. The wild-type strain CHA0 is clearly able to reduce the amount of infected roots and the effect of infection on the plant fresh weight, while both *alkB* knockout mutants show significantly reduced protection. Root colonization seems not to be affected by the knockout mutation, which would point to a role of the *alkB* gene in biocontrol, but it has to be noted that the root colonization measurements are based on a single point per experiment, by pooling the soil of 10 plants, and is not measured for each plant separately. As this results in only two data points in total, the standard error of this average is quite large.

In table 2, the results of the *P. fluorescens* biocontrol experiments on take-all disease of wheat are shown. Here, similar results can be observed as with the tobacco biocontrol assay: both mutants have a lower biocontrol ability than the wildtype. Interestingly, the polar mutant KOB2 has a significant lower biocontrol ability than the non-polar mutant KOB2Δ1.

Table 2: effect of *alkB* knockout on biocontrol ability against the wheat pathogen *G. graminis* var. *tritici* by *P. fluorescens*.

Microorganisms added		Plant fresh weight	Disease index (see M&M)	Bacterial root colonization
P. fluorescens	*G. graminis* var. *tritici*	(mg)		(x 10^8 cfu/g soil)
none	-	2550 a	0	0
none	+	1930 d	4.2 a	0
CHA0	+	2435 ab	3.2 d	1.7 a
KOB2	+	2103 cd	3.9 b	2.0 a
KOB2Δ1	+	2259 bc	3.6 c	2.6 a

Data of three experiments were pooled. The means of 12 repetitions (4 repetitions per experiment, 5 plants per repetition) are presented. Means in the same column followed by the same letter are not significantly different at $P = 0.05$ (Fisher's protected LSD test, Systat).

The behaviour of the *P. fluorescens* strains in the biocontrol assays can be explained by several hypotheses:

- Plants excrete a number of compounds, known as root exudates (174). These root exudates can be divided in water-soluble and water-insoluble compounds. Wheat, for example, excretes around 20 % of its total assimilated carbon, 75 % of which is water-extractable, and 25 % water-insoluble. The water-extractable fraction contains carbohydrates (sugars), organic acids and amino acids (174), but the insoluble fraction was not characterized. In the gnotobiotic system, the only carbon sources for the bacteria during root colonization are root exudates, as no extra carbon sources are added. Possibly, the insoluble fraction of the root exudate contains *n*-alkanes, that are degraded by the bacteria. However, plant wax *n*-alkanes are mainly very-long-chain alkanes in the range between C25 and C37 (212), while the *alkB* knockout mutants only lost the ability to grow on C12-C16 n-alkanes (chapter 7). Furthermore, the colonization of the roots, which is described as an important factor for biocontrol (161), seems not to be affected by the *alkB* deletion, as the numbers of colony forming units per gram soil for the wild-type and both knockout mutants are within the same order of magnitude.

- The alkane hydroxylase could have a role in the biosynthesis of (some of) the antifungal secondary metabolites. However, the biosynthetic pathway for the most important secondary metabolites has been elucidated ((99) and references therein), and a function for the alkane hydroxylase in these pathways is unlikely.

- Degradation products of alkane oxidation could be necessary as precursors for the production of (some of) the antifungal secondary metabolites, such as 2,4-diacetylphloroglucinol (75, 133). Nearly all other antifungal compounds derive from other precursors ((99) and references therein). It has been described that a block in one of the biosynthetic pathways for production of the secondary metabolites can cause a partial (often insignificant) loss of the biocontrol ability (generally up to 30 %)(99). We observed similar and even larger losses in biocontrol ability by the two *alkB* mutants. However, the production levels of the antifungal compound pyoluteorin by the *alkB* knockout mutants are not affected in laboratory experiments, and are even enhanced (M. Maurhofer, unpublished results).

- The mutant strains could have an additional mutation in a pathway or regulatory cascade which affects the biocontrol ability. A general trait of *P. fluorescens* strains cultivated for long time on rich medium is the instability of the *gacA/gacS* regulatory system (65). GacA and GacS are important as global regulators for the production of the antifungal secondary metabolites (154). Laboratory experiments showed that both KOB2 and KOB2Δ1 still produce antibiotics that are under control of the *gacA/gacS* regulatory system (M. Maurhofer, unpublished results), so that a *gacA/gacS* mutation can be excluded. However, the regulatory cascades of *P. fluorescens* CHA0 are highly complex and not fully understood.

- A significant difference in the effect of the two *alkB* knockout mutants in the wheat test system is correlated with the presence of the terminator in the *res-npt-res* cassette inserted in the *alkB* gene. This terminator affects the expression of genes located downstream of *alkB*, such as the *praAB-ompP1* genes, which are possibly involved in the solubilization and uptake of long-chain alkanes (chapter 7). Even the expression of the putative very-long-chain acyl-coA dehydrogenase, or other genes located further downstream, could be affected by the inactivation of *alkB*.

CONCLUSION

Based on the results of this study, we can not yet explain why the mutation in *alkB* influences the biocontrol ability of *P. fluorescens* CHA0 to the observed extent. The chemical composition of plant root exudates needs to be determined, and similar measurements would have to be made for plants grown in the presence of either *P. fluorescens* and/or fungal pathogen. A role for PraAB or OmpP1 can not be excluded at this stage, and knockout mutants will be useful to show the effect of these deletions on biocontrol. As a second alkane hydroxylase system is present in *P. fluorescens* CHA0, it will be necessary to clone the gene and construct knockout mutants as well, both in the wild-type CHA0 as in the mutant KOB2Δ1. These experiments may in the end give a clearer view on the influence of inactivation of the alkane hydroxylase genes on the biocontrol ability of *P. fluorescens* CHA0.

Chapter 8

FACTORS INFLUENCING THE SUBSTRATE RANGE AND ACTIVITY OF THE ALKANE HYDROXYLASE OF *PSEUDOMONAS PUTIDA* (*OLEOVORANS*) GPo1

Theo H.M. Smits, Bernard Witholt and Jan van Beilen

SUMMARY

Intrinsic and extrinsic factors such as regulation, uptake, host strain, substrate solubility and downstream metabolism influence the apparent substrate range of the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1. Biosurfactants, such as rhamnolipids, enhance growth of all recombinant strains on *n*-alkanes, and also enable induction of the *alk*-system by *n*-dodecane. In addition, rhamnolipids have a positive effect on product accumulation rates of the *alk*-system in *in vivo* activity assays with high logP substrates. However, rhamnolipids do not allow *P. putida* (*oleovorans*) GPo1 and an *E. coli* recombinant in which the GPo1 alkane hydroxylase genes are expressed, to grow on *n*-tetradecane or *n*-hexadecane. Therefore, we tested a recombinant *P. fluorescens* strain containing the GPo1 *alkB* gene. It also does not grow on these substrates, but mutants able to grow on C14 and C16 can be selected. In five independent mutants, a single point mutation changed the tryptophan residue at position 55 to a serine (TGG → TCG) or cysteine (TGG → TGC or TGT) residue.

INTRODUCTION

The substrate range of the *P. putida* (*oleovorans*) GPo1 (TF4-1L) alkane hydroxylase system has been investigated in detail, but it is not clear to what extent it is determined by the dimensions of the substrate binding site or by factors depending on the host strain, substrate solubility, uptake and regulation. Besides the hydroxylation of aliphatic and alicyclic compounds (173, 259), the alkane hydroxylase system has been shown to catalyse: the oxidation of terminal alcohols to the corresponding aldehydes; demethylation of branched methyl ethers; sulfoxidation of thioethers, and epoxidation of terminal olefins (131, 132, 171, 172).

Some of these compounds cannot be utilised as carbon sources by the wild-type *P. putida* (*oleovorans*) GPo1, because they do not induce the *alk*-genes. For example, dodecane, a reasonably good substrate for the alkane hydroxylase (150), is not a growth substrate for *P. putida* (*oleovorans*) GPo1 because this compound does not induce the *alk*-genes efficiently (248, 283). The same could also be true for tetradecane, and even hexadecane (173), which were oxidised at around 22 and 3 %,

respectively, of the activity with *n*-octane, based on the disappearance of NADPH. In other cases, the downstream metabolism cannot handle the products of the initial oxidation reactions. For instance, in contrast to ethylbenzene, which is a growth substrate, substituted ethylbenzenes and isopropylbenzene are oxidised, but do not allow growth (87).

A third factor is the solubilisation and uptake of alkanes. *P. putida* (*oleovorans*) GPo1 and *E. coli* recombinants equipped with the *P. putida* (*oleovorans*) GPo1 *alk*-system (69) do not produce surfactants (223), but grow well on medium-chain alkanes, probably because medium-chain alkanes simply traverse the outer membrane and diffuse into the cytoplasmic membrane (245). However, this is not true for long-chain alkanes, which must be solubilised by (bio)surfactants (28). The most thoroughly studied biosurfactants are the rhamnolipids, produced by *Pseudomonas aeruginosa* strains (reviewed by (193)). Addition of rhamnolipids above the critical micelle concentration (around 40 mg/l) enhances growth of *P. aeruginosa* on *n*-alkanes (109, 290).

In this study, we test the influence of rhamnolipids on the growth rates of the wild-type *P. putida* (*oleovorans*) GPo1 and the recombinant host strains *E. coli* GEc137 and *P. fluorescens* KOB2Δ1 expressing the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1, to determine the effect of the extrinsic factors mentioned above on the apparent substrate range of the alkane hydroxylases. In addition, we obtained AlkB mutants with an expanded substrate range by selection on longer *n*-alkanes.

MATERIAL AND METHODS

Bacteria and growth conditions. *P. putida* (*oleovorans*) GPo1 (228), *E. coli* GEc137[pGEc47] (69) and *P. fluorescens* KOB2Δ1[pCom8B (GPo1)] (chapter 4) were used in this study. LB with appropriate antibiotics was used for precultures (220). To select for pGEc47 in *E. coli*, tetracycline was added to a concentration of 12.5 μg/ml. For *P. fluorescens* KOB2Δ1, gentamycin was used at 100 μg/ml. Recombinants were grown in 50 ml baffled Erlenmeyer flasks with either M9 medium (220) supplemented with 10 μM $CaCl_2$ and 10 μM $FeSO_4$ or E2 medium (151). In the case of *E. coli* GEc137, thiamine was added to a concentration of 0.001 % (w/v). As

carbon sources, 1 % (v/v) octane (C8), decane (C10), dodecane (C12) tetradecane (C14) or hexadecane (C16) were used, supplied via the gas phase or directly added to the medium. Rhamnolipids were added to the medium in an end concentration of 0.1 % w/v. For optical density measurements, 1 ml culture liquid was spun down at 15000 rpm in a microfuge, and 0.5 ml supernatant including the alkane and alkane-surfactant micelles were removed. After addition of 0.5 ml water, the cell pellet was resuspended and the OD_{450} was measured (280).

DNA manipulations. Methods for plasmid isolation and DNA sequencing were described before (chapter 4). Primers used for sequencing pCom8B (GPo1) were PalkFw3 and pKKrev (240).

Resting cell experiments. For *in vivo* alkane hydroxylase activity measurements, *E. coli* strains GEc137 and K19 were grown in M9 medium. 200 ml M9 medium (220) supplemented with 10 μM $CaCl_2$ and 10 μM $FeSO_4$ was inoculated with 2 ml of an overnight preculture of the strains in LB with tetracycline, and incubated at 30°C in a rotary shaker for 8 hours. As carbon source, 0.5 % glucose was used. Subsequently, the *alk*-system was induced with 0.05 % DCPK. Cells were harvested three hours after induction and resuspended in 50 mM Na-phosphate buffer, pH 7.0, 10 mM $MgSO_4$, 0.1 % glucose. Portions of 1 ml were incubated in 10 ml Pyrex tubes with 1 % (v/v) substrate at 30°C, 200 rpm. Rhamnolipids were added to a concentration of 0.01 % (w/v). Tubes were taken out respectively after 1, 2, 4 and 21 hours and frozen until the samples were extracted.

The cell suspension was acidified to pH $\approx$ 2, and products were extracted with 1 ml hexane containing 0.01 % 2-octanol as internal standard. Samples were analysed on a Fisons HRGC MEGA 2 series capillary gaschromatograph equipped with a fused capillary column CP-Wax CP52 (Chrompack, Middelburg, The Netherlands). To separate the extracted compounds, a temperature gradient from 60°C to 200°C with an increment of 10°C per minute, after 2 minutes 60°C, was used. Products were analysed using Gas Chromatography-Mass Spectroscopy (GC-MS) under the same conditions.

Chemicals. All linear alkanes, alkenes, cumene and *p*-cymene were obtained from Fluka. DCPK was from Aldrich. Rhamnolipids were a gift from Dr. Urs Ochsner. The extraction procedure from *P. aeruginosa* PG201 was described before (97).

RESULTS AND DISCUSSION

Influence of rhamnolipids on the growth of *P. putida* (*oleovorans*) GPo1 on alkanes. Rhamnolipids are biosurfactants which facilitate the growth of *Pseudomonas aeruginosa* on alkanes ranging from C16 to C20 (109, 289, 290). To study whether rhamnolipids promote growth of a medium-chain length alkane oxidising strain on *n*-alkanes, *P. putida* (*oleovorans*) GPo1 was grown on C8, C10 and C12 supplied via the vapour phase, and C12, C14 and C16 directly added to the medium (table 1) in the presence or absence of the biosurfactant. In these experiments, the *alk*-genes were induced by the addition of dicyclopropylketone (DCPK), a gratuitous inducer of the *alk*-system (96) to guarantee that the *alk*-genes are expressed to the same level in all experiments.

Growth of *P. putida* (*oleovorans*) GPo1 on C8-C12 vapour and C12 added directly to the medium was significantly faster in the presence of rhamnolipids than in its absence. However, the biosurfactant did not allow *P. putida* (*oleovorans*) GPo1 to grow on C14 and C16. As C12-C16 1-alkanols are growth substrates for this strain, this may be due to either an uptake problem or the substrate range of AlkB.

The growth rates of *P. putida* (*oleovorans*) GPo1 on both C8 and C10 vapour were somewhat lower than the growth rates found with C8 and C10 as a second phase (C8: 1.5 h., C10: 2.2-2.5 h. (150, 224)) suggesting that mass transfer of C8 and C10 is a limiting factor for growth on alkane vapour. Apparently, rhamnolipids facilitate transfer of alkanes from the vapour phase to the liquid phase and/or the cells, resulting in a higher growth rate.

Effect of rhamnolipids on the induction of the *alk*-system in the presence or absence of an inducer. Dodecane is a substrate of the alkane hydroxylase (150, 259), but it cannot induce the *alk*-genes efficiently (96, 283). This could be due to the geometry of the inducer binding site of AlkS, but could also be uptake-related: C12

Table 1: doubling and lag-times (hours) of *P. putida* (*oleovorans*) GPo1, *E. coli* GEc137[pGEc47] and *P. fluorescens* KOB2Δ1[pCom8B (GPo1)] during growth on *n*-alkanes.

Substrate [a]	*P. putida* (*oleovorans*) GPo1 [b]				*E. coli* GEc137[pGEc47] [b]		*P. fluorescens* KOB2Δ1[pCom8B (GPo1)] [b]		
	-DCPK, -Rhl	-DCPK, +Rhl	+DCPK, -Rhl	+DCPK, +Rhl	+DCPK, -Rhl	+DCPK, +Rhl	+DCPK, -Rhl	+DCPK, +Rhl	+DCPK, +Rhl [c]
C8 vapour	-	-	3.3 (2)	1.6 (2)	n.g.	1.6 (2)	-	n.g.	
C10 vapour	-	-	2.7 (2)	1.8 (2)	16 (32)	2.8 (2)	28 (290)	10 (16)	
C12 vapour	107 (8)	21 (8)	17 (8)	11 (8)	22 (4)	11 (4)	-	-	
C12 liquid	n.g.	n.g.	43 (4)	11 (4)	19 (24)	14 (32)	-	88 (170)	17 (90)
C14 liquid	-	-	n.g.	n.g.	n.g.	n.g.	158 (700)	101 (400)	17 (90)
C16 liquid	-	-	n.g.	n.g.	n.g.	n.g.	138 (700)	46 (500)	32 (180), 9.3 (8)[d]

Footnotes: [a] : C8 : octane; C10 : decane; C12 : dodecane; C14 : tetradecane; C16 : hexadecane; [b] : n.g. : no growth; - : not tested; values are doubling times in hours, values in parenthesis are lag-times in hours; [c] : these values are growth rates of cultures after one round of adaptation; [d] : growth rate after multiple rounds of adaptation

may not reach AlkS. Therefore, we grew *P. putida* (*oleovorans*) GPo1 on C12 vapour, with and without rhamnolipids and DCPK. In the absence of DCPK, rhamnolipids allow a growth rate on C12 that is five times faster than that obtained without rhamnolipids. The addition of DCPK only doubles the growth rate (table 1). This shows that C12, if solubilised, can induce the *alk*-genes to a significant extent, and thus is available to the cells and AlkS. *P. putida* (*oleovorans*) GPo1 did not grow on C12 directly added to the medium in the absence of DCPK.

Growth of a recombinant *E. coli* containing the *P. putida* (*oleovorans*) GPo1 *alk*-system on alkanes. Introduction of the *alk*-genes on cosmid pGEc47 in *E. coli* GEc137, a *fadR* mutant strain of DH1, enables this strain to grow on minimal medium plates with *n*-alkanes ranging from C6 - C12 supplied via the vapour phase (69). We also tested this recombinant strain for growth on *n*-alkanes in liquid cultures, with and without rhamnolipids.

Generally, in the presence of rhamnolipids, *E. coli* GEc137[pGEc47] grew only slightly slower on *n*-alkanes than *P. putida* (*oleovorans*) GPo1. However, in the absence of the biosurfactant, GEc137[pGEc47] did not grow in liquid cultures on C8 vapour and very poorly on C10 vapour. *E. coli* strains containing the *alk*-system of *P. putida* (*oleovorans*) GPo1 are highly sensitive to *n*-octane, when supplied as a second phase during growth on glucose, especially in the stationary phase (72). Possibly, C8 is toxic for *E. coli* GEc137[pGEc47] in liquid cultures, while this effect is less significant for longer alkanes. The toxic effect may be neutralised by rhamnolipids, which form micelles containing the *n*-alkane.

The simplest interpretation of the above results is that medium-chain length alkanes simply traverse the cell membranes of *E. coli* by diffusion (nearly) as easily as for *Pseudomonas* and that rhamnolipids have a positive influence on the diffusion rates. Therefore, the main causes for a difference in growth rates between *E. coli* and *Pseudomonas* may be the low specific activity of the alkane hydroxylase system in *E. coli* (245) and the toxicity of medium-chain length alkanes to the *E. coli* recombinants (236).

The effect of rhamnolipids on growth on alkanes of a recombinant *P. fluorescens* containing the *P. putida* (*oleovorans*) GPo1 *alkB*-gene. Neither *P. putida* (*oleovorans*) GPo1 nor *E. coli* GEc137[pGEc47] grow on tetradecane and hexadecane. This may be due to an unknown barrier for uptake of these alkanes or to the substrate range of the alkane hydroxylase. The ideal host to test these alternatives is the alkane hydroxylase negative mutant *P. fluorescens* KOB2Δ1, which is derived from a strain able to grow on long-chain *n*-alkanes (chapter 4) We tested *P. fluorescens* KOB2Δ1 containing a pCom8 derivative encoding the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase gene (pCom8B (GPo1)) for growth on *n*-alkanes ranging from C8 to C16. No growth was observed on C8 with rhamnolipids. As the parent strain of *P. fluorescens* KOB2Δ1, CHA0, does not grow on C8 to C10 alkanes either, the rubredoxin and rubredoxin reductase genes, which encode the other two components of the alkane hydroxylase system necessary for activity, may not be efficiently induced. Initially, growth on C14 and C16 started only after a long lag-time, and was slow. However, further cultivation of all cultures on the same substrate resulted in significantly higher growth rates with shorter lag-times (table 1), presumably by an adaptation process.

To check whether the sequence of the GPo1 alkane hydroxylase was changed to allow growth on hexadecane, the *alkB* gene of pCom8B (GPo1) was sequenced. Indeed, the sequence of the *alkB* gene isolated from the adapted KOB2Δ1[pCom8B (GPo1) showed a single point mutation at position 165. This mutation changed a tryptophan codon (TGG) into a cysteine codon (TGT), thus changing a large hydrophobic amino acid into a small residue. In independent cultures, the same codon changed twice to a TCG codon, encoding serine (W55S) and twice to a TGC codon (cysteine). In all five cases, no other changes were found in the *alkB*-sequence. Other possible one-basepair changes in the W55 codon were not found (possible amino acids by a one-basepair change are CSGRL). Amino acids present in other AlkBs at the corresponding position are AVLI (chapter 9), suggesting that additional mutations might include TGG → TTG (W55L).

When the plasmid containing the W55C mutation was transformed back into KOB2Δ1, growth on hexadecane in presence of rhamnolipids started after a lag-time of only 48 hours with a doubling time of 56 hours, similar to the growth rate observed after the long lag-phase in the initial cultures. Apparently, the mutation enables the *P.*

putida (*oleovorans*) GPo1 alkane hydroxylase to oxidize hexadecane with a rate that is sufficient to allow the *P. fluorescens* recombinant to grow on this substrate.

Prediction of residues involved in substrate binding. Based on the topology model for the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1 (261), the tryptophan residue at position 55 is located in the second membrane helix.

Table 1 shows that cultures grown on tetradecane need the same very long lagtime as hexadecane, while cultures start to grow on dodecane after a short lagtime. This suggests that dodecane fits in the substrate binding pocket, while tetradecane and hexadecane are too large, and need the mutation of W to C or S to be able to bind. Supposing that linear alkanes bind in a fully extended conformation, the length of a dodecane molecule is about 17 Å. Also assuming that the six transmembrane segments of AlkB fold as perfect α-helices, rising 1.5 Å per residue, a dodecane molecule corresponds to 11 amino acids in an α-helix. Based on comparisons of the transmembrane stretches, the W is located about 10 residues from the periplasmic end of the hydrophobic core sequence. Counting from the periplasmic end of the hydrophobic core sequence of α-helices 4 and 6, the essential histidines are at position 20 and 24 for helix 4, and position 24 for helix 6, or 10 and 14 residues from a position corresponding to W55, the correct distance to position the activated oxygen at the end of an extended dodecane molecule.

Transmembrane (TM) helices often form well-packed bundles. TM helices adjacent in sequence tend to be neighbours in the structure (153), and are consequently antiparallel. In the alkane hydroxylases, all three proposed periplasmic loops are quite short, which suggests that the integral membrane domain of the AlkBs consist of three antiparallel TM helix pairs. All six TM helices have approximately the same length, as far as can be estimated from the multiple alignments. This implies that the angle of the TM helices relative to the membrane surface must be similar and is probably close to 90°. The W55C and W55S mutants show that the enzyme accommodates the highly apolar substrates in a hydrophobic pocket inside the membrane-domain of the alkane hydroxylases, which is formed by residues on helix 2 and other helices. In the distantly related desaturases, the segment corresponding to helices 1 and 2 is not present. Recent studies with desaturase chimeras of the Δ^6 fatty acid desaturase and Δ^8 sphingolipid desaturase from *Borago officinalis* indicate that TM helices 1 and 2

(corresponding to TM helices 3 and 4 in the alkane hydroxylases) are involved in forming the substrate binding site of these enzymes (159). Therefore, helices 3 and 4 in the alkane hydroxylases may play the same role. It is possible that the desaturases are active as dimers, and the active site is located at the interface of the two subunits, while in the alkane hydroxylases, the substrate binding pocket is located in one protein unit.

The only cofactors in the alkane hydroxylases are the two irons that are bound by the histidine clusters located downstream of TM helices 4 and 6. As the histidine motifs and W55 delineate the vertical boundaries of the substrate binding pocket in the GPo1 AlkB, other residues involved in substrate binding may be located on the same side of helix 2 as W55 (residues 58, 59, 62, 66) , the first 10 amino acids of helix 3 (residues 89-98), and the last 10 amino acids of helix 4 (residues 128-137).

Influence of substrate solubility on *in vivo* activity of the alkane hydroxylase. In previous *in vivo* activity assays the effect of substrate solubility was reduced by the addition of 0.05 % Triton X-100 without knowing why this is effective (245, 259). In this study, we investigated the link between the hydrophobicity of alkane hydroxylase substrates, expressed in logP values (148, 270), and the *in vivo* activity of the alkane hydroxylase in *E. coli* GEc137[pGEc47], in the presence or absence of rhamnolipids. The observed activity of the alkane hydroxylase towards C8 and C10, in the presence of rhamnolipids (table 2), was in the range obtained before in similar experiments with Triton X-100 (245, 259). With some of the substrates, more than one product was formed due to further oxidation of the primary products (table 2).

The accumulation rates in the absence of rhamnolipids were strongly dependent on the logP value of the substrate. The low logP substrates cumene and cymene were converted with high rates, while no activity could be observed for substrates with a logP value higher than that of octane, which was converted at a low rate. Rhamnolipids showed a negative effect on accumulation rates with the low logP substrate cumene, perhaps by increasing the level of cumene in the membrane to toxic amounts (236). High logP substrates gave a lower activity, probable as the alkane hydroxylase oxidises these substrates with lower efficiency (259) or because uptake is less efficient. It has to be noted that the ß-oxidation cycle in the strain is still fully

Table 2: *in vivo* activities of the alkane hydroxylase in GEc137[pGEc47] in presence (+ Rhl) or absence (- Rhl) of rhamnolipids.

Substrate	Substrate logP	Accumulation rate of total products (U/g CDW)		Products	Accumulation rate per product (U/g CDW)		Effects of Rhl[a]
		+ Rhl	- Rhl		+ Rhl	- Rhl	
Cumene	3.4[b]	3.9	6.4	2-phenyl-1-propanol	0.5	0.8	-
				2-phenyl-1-propanoic acid	3.5	5.6	
Cymene	4.1	7.8	8.1	2-(4-methylphenyl)-1-propanol	0.4	0.2	x
				2-(4-methylphenyl)-1-propanoic acid	7.3	7.9	
Octane	4.5	12.1	2.6	Octanol	1.6	-[d]	+
				Octanoic acid	10.5	2.6	
Decane	5.6	10.3	< 0.01[c]	Decanoic acid	10.3	-	+
Dodecene	6.2	7.0	< 0.01	1,2-epoxydodecane	2.5	-	+
				11-dodecenoic acid	4.3	-	
				11,12-epoxy-dodecenoic acid	0.2	-	
Dodecane	6.6	5.3	< 0.01	Dodecanoic acid	5.3	-	+
Tetradecene	6.7	0.4	< 0.01	1,2-epoxytetradecene	0.3	-	+
				13-tetradecenoic acid	0.1	-	
Tetradecane	7.6	< 0.01[c]	n.d.[e]	No products	-	-	n.d.

Footnotes: [a] : -: negative effect, x: no effect, +: positive effect. [b] : estimated value, no literature value. [c] : below detection limit. [d] : no products. [e] : not determined.

active, and fatty acids can be oxidised further, which may affect the obtained activity. In addition, epoxides might be oxidised at the ω-position.

These results show that rhamnolipids extend the substrate range of the alkane hydroxylase by making hydrophobic compounds more soluble and thereby more accessible for the host strain.

CONCLUDING REMARKS

Rhamnolipids significantly influence the availability of hydrophobic substrates for the *P. putida (oleovorans)* GPo1 alkane hydroxylase system, as shown by the growth experiments as well as the *in vivo* activity assays. Expression of the GPo1 alkane

hydroxylase in *P. fluorescens* KOB2Δ1 allowed the selection of *alkB* mutants that are able to oxidize hexadecane, while repeated cultivation of one of these mutants on hexadecane resulted in growth rates that are comparable with that of the wildtype *P. fluorescens* CHA0 (chapter 7).

As the host strain and substrate solubility have a large effect on the apparent substrate range of an enzyme, mapping and modification of the substrate range of enzymes, such as the alkane hydroxylase, should be accompanied by studies on the uptake of the target substrates by the host strain.

ACKNOWLEDGEMENTS

Chapter 9

SUMMARY AND CONCLUSIONS

Theo H. M. Smits

Although the degradation of medium- and long-chain alkanes has received much attention from researchers in the fields of microbiology, molecular genetics, biochemistry, bioremediation, and biocatalysis, most of these studies have concentrated on a relatively small number of strains. The most extensively described alkane oxidation system is that of *Pseudomonas putida* (*oleovorans*) GPo1 (reviewed in (263)).

Highly degenerate PCR primers based on the alkane hydroxylases of *P. putida* (*oleovorans*) GPo1 (143) and *Acinetobacter* sp. ADP1 (213), enabled us to show that many strains able to grow on *n*-alkanes contain at least one, and in some cases up to five non-heme iron alkane hydroxylase homologs (239)(chapter 2; J. B. van Beilen, unpublished results). Using some of the PCR fragments as probes in Southern and colony hybridizations, a total of 17 full-length alkane hydroxylase gene sequences and flanking regions were obtained from several Gram-positive and Gram-negative strains (168)(chapter 4; J. B. van Beilen, unpublished results). Functional analysis by heterologous expression showed that all tested alkane hydroxylase homologs indeed hydroxylate alkanes.
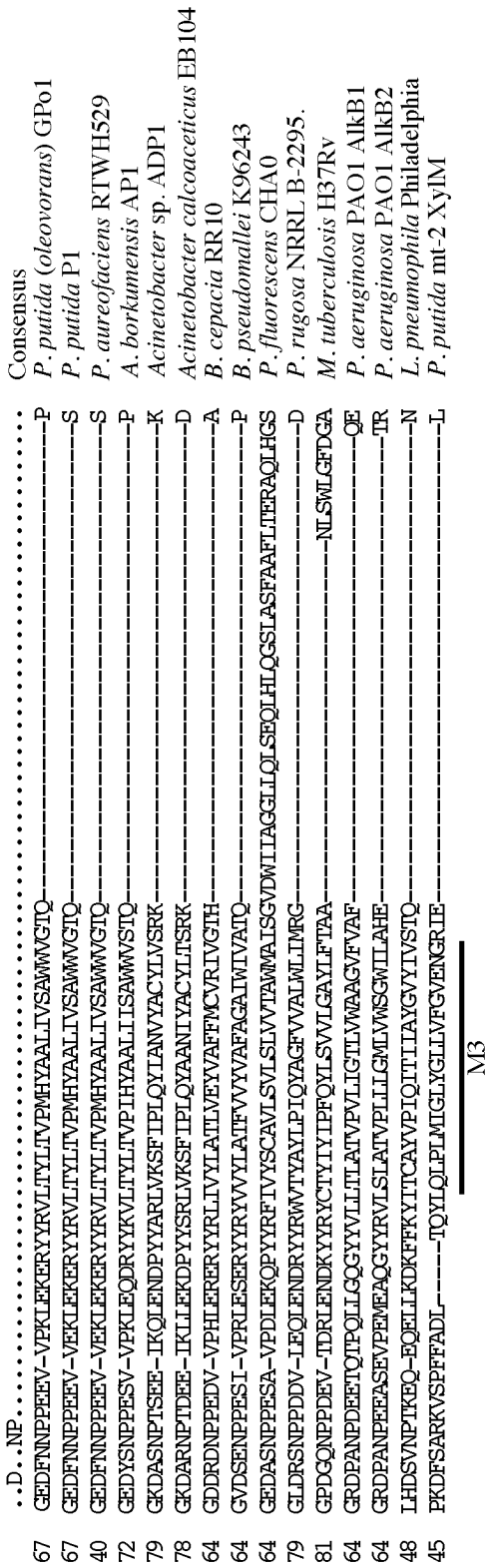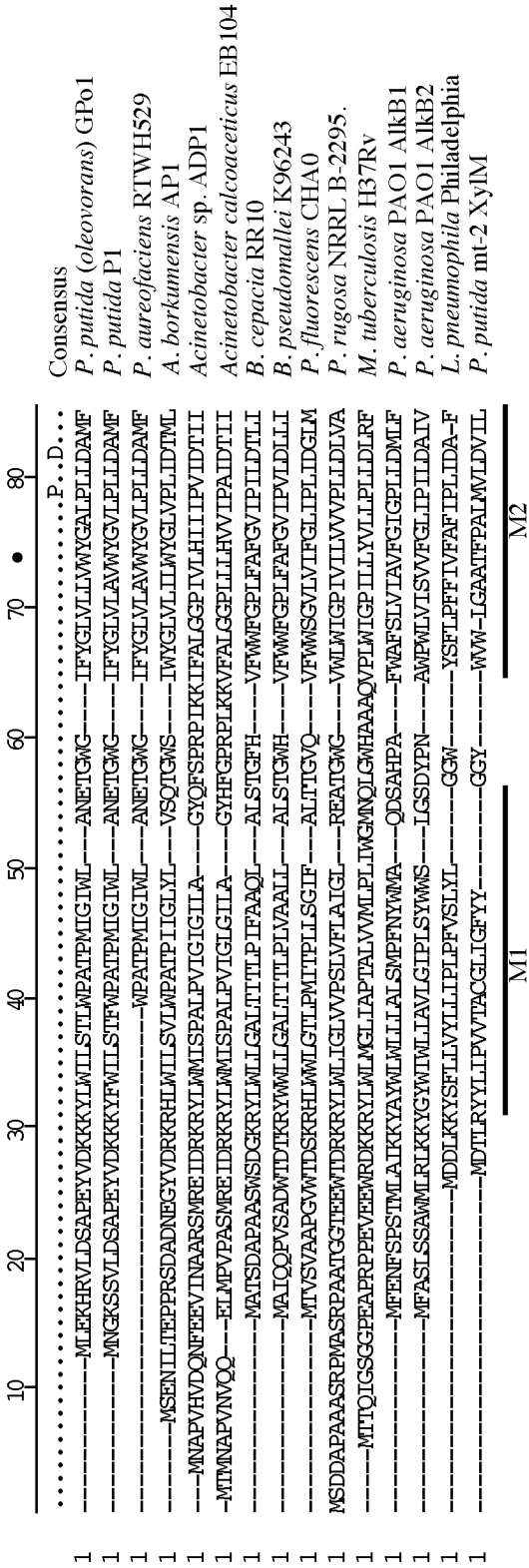
Structural features of alkane hydroxylases and other integral-membrane non-heme iron oxygenases. Comparison of the alkane hydroxylase sequences (chapter 4) shows that the integral-membrane non-heme iron alkane hydroxylases have significant sequence divergence, with protein sequence identities as low as 35 %. The closest relatives of the alkane hydroxylases that oxidize other substrates, the *P. putida* mt-2 xylene monooxygenase (251) and related enzymes, only have around 25 % protein sequence identity, while the other related integral-membrane non-heme iron oxygenases, such as the fatty acid desaturases, have even lower levels of sequence identity. Based on homology, alignments and the function of these enzymes, we could define thirteen different subclasses of the integral-membrane non-heme iron oxygenases (table 1, figure 2), some structural features of which will be discussed below.

The alignment of the complete alkane hydroxylase sequences shown in figure 1 was manually optimized based on the topology model for the *P. putida* (*oleovorans*) GPo1 alkane hydroxylase (261). In all AlkB sequences, the six hydrophobic membrane spanning segments could be identified easily. Most hydrophobic residues are not well

Table 1: sequence motifs of integral-membrane non-heme iron oxygenases

Enzyme subclass	No. seqs[a]	Length	Histidine box no.			
			H1[b]	H2	H3	H4
Alkane hydroxylases	16	355-490	HE(L/m)XHK	EHXXGHH	NYXEHYG(L/M)	LQRH(S/A)DHH
Xylene monooxygenases	5	369-377	HELXH	H(I/V)XXHH	NYXQHYG(L/Q)	EITNHX(N/D)HH
Bacterial carotene hydroxylases	6	162-176	H(R/K)(W/Y)IMHG	HXSHH	(L/V)HDG(L/V)VH	AHR(L/M)HHAV
Plant carotene hydroxylases	5	308-322	H(R/K)A(I/L)WH	HXSHH	YMFVHDGLVH	AHXLHH
Carotene ketolases	5	242-320	HDCMHG	HXXHH	FGT(W/Y)LPH(K/R)P	H(H/W)EHH
Decarbonylases, CER-like	3	567-622	H(R/K)X(L/F)H	YHS(H/F)HHSS	DFMNNMGHCNFEL	YTPS(F/Y)HSLHH
Decarbonylases, Glossy-like	2	524/566	H(R/K)X(L/F)H	YHS(H/F)HHSS	DF(L/M)RCLGH(C/S)NVEI	YTPSYHSLHH
C4 steroid oxygenases	8	253-406	HR(L/G)(L/F)H	YK(Y/kn)(I/v)H(K/s)XHH	H(S/C)GY	HDXHH
C5 steroid oxygenases	9	271-365	H(R/m)XLH	H(A/K)XHH	(I/d)H(D/t)	H(T/ad)XHH
Cholesterol hydroxylases	2	272/298	HLLHH	HKVHH	EDHSGY	HHD(L/M)HHS
Δ12/Ω-6 desaturases	9	374-443	H(E/D)C(G/a)H(H/k)	H(R/d)(R/n)HH	TX(L/v)(Q/H)HT	(T/v)HV(V/i)XHH(L/i)(F/s)
Δ9 desaturases	7	355-510	HR(L/m)W(S/A)HR	HR(I/A)HH(R/K)	NS(A/L)AHXXG	GEG(F/Y)HN(Y/F)HH
Ω-3 desaturases	6	359-453	GDHCGHG	H(R/k)THH	VT(Y/f)LHH	HV(I/a)HH(L/i)F

Footnotes: [a]: No seqs: number of sequences used to determine the consensus sequences of the 4 histidine boxes. The sequences used to determine the consensi are representatives of each subclass, and it is well possible that of a certain subclass many more sequences are known. [b]: capitalized letters: absolutely conserved residues; in parentheses: residues not absolutely conserved, capitals: predominant residues, small letters: minor residues.

```
            10        20        30        40        50        60        70 •     80
                                                                          P..D...
Consensus   ..................................................................P..D...          Consensus
1 --------MLEKHRVLDSAPEYVDKKKYLWILSTLWPATPMIGIWL----ANETGWG-----IFYGLVLIWWYGALPLLDAMF     P. putida (oleovorans) GPo1
1 --------MNGKSSVLDSAPEYVDKKKYFWILSTFWPATPMIGIWL----ANETGWG-----IFYGLVLAWWYGVLPLLDAMF     P. putida P1
1 -----------------------WPATPMIGIWL----ANETGWG-----IFYGLVLAWWYGVLPLLDAMF                P. aureofaciens RTWH529
1 ----MSENILTEPPRSDADNEGYVDRKRHLWILSVLWPATPIIGLYL----VSQTGWS-----IWYGLVLIIWYGLVPLLIDTML  A. borkumensis AP1
1 ---MNAPVHVDQNFEEVINAARSMREIDRKRYLWMISPALPVIGIGIIA-----GYQFSPRPIKKTFALGGPIVLHIIIPVIDYII Acinetobacter sp. ADP1
1 -MTMNAPVNVQQ----ELMPVPASMREIDRKRYLWMISPALPVIGIGIIA-----GYHFGPRPLKKVFALGGPLLLHVVIPAIDYII Acinetobacter calcoaceticus EB104
1 --------MATSDAPAASWSDGKRYLWLLGALITILPIFAAQI-----ALSTGFH-----VFWWFGPLFAFGVIPILDYILI     B. cepacia RR10
1 --------MAIQQPVSADWIDYKRYWWLLGALITILPIVAALL-----ALSTGWH-----VFWWFGPLFAFGVIPVLDLLI      B. pseudomallei K96243
1 --------MTVSVAAPGVWTDSKRHLWWLGTLPMITPLLSGIF-----ALTTGVQ-----VFWWSGVIVIFGLIPLIDGLM      P. fluorescens CHA0
1 MSDDAPAAASRPMASRPAATGGTEEWTDRKRYLWLIGLWPSIVFLAIGL----REATGWG-----VWLWIGPIVILVVVPLLDIVA P. rugosa NRRL B-2295.
1 --------MFENFSPSTMLAIKKYAYWLWILIALSMPFNYWMA-----QDSAHPA-----FWAFSLVIAVFGIGPLLDMLF      M. tuberculosis H37Rv
1 --------MFASLSSAWMLRLKKYGYWIWLIAVLGIPLSYWWS----LGSDYPN-----AWPMLVISVVFGLIPILDAIV       P. aeruginosa PAO1 AlkB1
1 ----------MDDIKKYSFLLVYILIPPLPFVSLYL-----GGW------YSFLPFFIVFAFIPLIDA-F                P. aeruginosa PAO1 AlkB2
1 ------------MDYILRYLLIPVVTAGLIGFYY-----GGY------WWW-LGAATFPAIMVLDVIL                   L. pneumophila Philadelphia
1                                                                                        P. putida mt-2 XylM
                        M1                                  M2
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            90       100       110       120       130       140       150       160
Consensus   ..D..NP...........................................................                P
67 GEDFNNPPEEV-VPKLEKERYYRVLITYLIVPMHYAALIVSAWWGTQ----------------------------------P     P. putida (oleovorans) GPo1
67 GEDFNNPPEEV-VEKLEKERYYRVLITYLITVPMHYAALIVSAWWGTQ---------------------------------S     P. putida P1
40 GEDFNNPPEEV-VEKLEKERYYRVLITYLITVPMHYAALIVSAWWGTQ---------------------------------S     P. aureofaciens RTWH529
72 GEDYSNPPESV-VPKLEQDRYYKVLITYLITVPIHYAALIISAWWVSTQ--------------------------------P     A. borkumensis AP1
79 GKDASNPTSEE-IKQLENDPYYARLVKSFIPLQYIANVYACYLVSRK---------------------------------K     Acinetobacter sp. ADP1
78 GKDARNPTDEE-IKLLEKDPYYSRLVKSFIPLQYAANIYACYLTSRK---------------------------------D     Acinetobacter calcoaceticus EB104
64 GDDRDNPPEDV-VPHLERERYRRLIVYLATILVEVVAFFMCVRIVGTH--------------------------------A     B. cepacia RR10
64 GVDSENPPESI-VPRLESERYRYRVVYLAIFVVYVAFAGAIWIVAIQ---------------------------------P     B. pseudomallei K96243
64 GEDASNPPESA-VPDLEKQPYYRFIVYSCAVLSVLSLVVTAWMAISGVDWIIAGGLLQLSEQIHLQGSLASFAAFLTERAQLHGS  P. fluorescens CHA0
79 GLDRSNPPDDV-LEQLENDRYXRWMTYAYLPIQYAGFVVALWLIMRG--------------------------------D     P. rugosa NRRL B-2295.
81 GPDGQNPPDEV-TDRLENDKYYRYCTYIYIPFQYLSVVLGAYLFTAA-----------------------NLSWLGFDGA     M. tuberculosis H37Rv
64 GRDPANPDEFTQTPQLLGQGYYVLLITLAIVPVLIGTLVWAAGVFVAF-------------------------------QE     P. aeruginosa PAO1 AlkB1
64 GRDPANPEFASEVPEMEAQGYYRVLSLAIVPLLLGMLVWSGWILAHE-------------------------------TR     P. aeruginosa PAO1 AlkB2
48 LHDSVNPTKEQ-EQELLKDKFFKYITCAVVPIQTTIIAYGYIVSTQ--------------------------------N     L. pneumophila Philadelphia
45 PKDFSARKVSPFFADL-----TQYLQLPLMIGLYGLLVFGVENGRIE-------------------------------L     P. putida mt-2 XylM
                                      M3
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                                                                                                                    Consensus
          180       190       200       210       220       230       240       250
          |         |         |         |         |         |         |         |
          .........G....G.......HE..HK...........L....Y..F..EH..GHH....T..D......G.....F...        Consensus
114  MSWLEIGALALSLGIVNG—LALNIGHELGHKKETFDRWMAKIVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGESIYKFSIR        P. putida (oleovorans) GPo1
114  MSWFEIVALALSLGIVNG—LALNIGHELGHKKEAFDRWMAKIVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGENIYKFSIR        P. putida P1
87   MSWFEIGALALSLGIVNG—LALNIGHELGHKKEAFDRWMAKIVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGENIYKFSIR        P. aureofaciens RTWH529
119  IGVFEFLALALSLGIVNG—LALNIGHELGHKKETFDRWMAKLVLAVVGYGHFFIEHNKGHHRDVATPMDPATSRMGESIYTFSLR        A. borkumensis AP1
126  TSFIDKILLGISMGAING—IAVNTAHEISHKADRLDHILSHLAIVPTGYNHFRIEHPYGHHKRAAITPEDPASSQMGEIFYEFWPR        Acinetobacter sp. ADP1
125  TSFIDKILLGISMGAING—IAINTAHEISHKHDRIDHILSHLAIVPTGYNHFRIEHPYGHHKRAAITPEDPASSRMGEIFYEFWPR        Acinetobacter calcoaceticus EB104
111  LAWYDYVGFALSLGAATG—ISINTAHEIGHKTNRFERWLAKITIAPVAYGHFFVEHNRGHHVRVAITAEDPASARYGESFWAFLPR        B. cepacia RR10
111  LAWYDYLGFAFSLGAATG—ISINTAHEIGHKTSPLERWLAKITIAPVAYGHFFVEHNRGHHVRVAITPDDPASARYGESFWAFLPR        B. pseudomallei K96243
148  IGMFTYLGMAMSTGAATG—IAINLAHEIGHKNRGMAKFLAKIALASTFYGHFYIEHNRGHHVRVAITPEDPASSRIGESFWSFLPR        P. fluorescens CHA0
126  LSTVDKVGLAITVGCVGG—IGINTAHEIGHKKESHERWLSKIAIAQSFYGHFYIEHNRGHHVRVSTPEDPASARFGETLMEFLPR        P. rugosa NRRL B-2295.
137  LSWAGKLGVALSVGVLGG—VGINTAHEMGHKKDSLERWLSKITLAQTCYGHFYIEHNRGHHVRVSTPEDPASARFGETLMEFLPR        M. tuberculosis H37Rv
113  WGMLGRLGMILSMGTVMGAVEIVVAHELIHKDSALEQAAGIIAAVCYAGFKVEHVRGHHVHVSTPEDASSARFGQSVYQFLPH        P. aeruginosa PAO1 AlkB1
113  WDWVGQLGMILSVGTVMGAIGITVSHELIHKDPQIEQNAGIIAAVCYAGFKVEHVRGHHVHVSTPEDASSSRYGQSLYSFLPH        P. aeruginosa PAO1 AlkB2
95   LVWNEMLGFTMSLGLISGGVGINFAHEMMHKNSKLQQIMSKILLVTVCYGHFIEHVKGHHVKVATPDDPATARIGENLYQFLPK        L. pneumophila Philadelphia
88   SEPLQVAGCILSLAWLSGVPTLPVSHEIMHRRHWLPRKMAQLIAMFYGDPNRDIAHVNTHHLYLDTPLDSDTPYRGQTIYSFVIS        P. putida mt-2 XylM
                   ———               ——                                          ——
                   M4                H1                                          H2
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```
                                                                                                                    Consensus
          260       270       280       290       300       310       320       330       340
          |         |         |         |         |         |         |         |         |
          .......A...E..R..............N..............G............Q..........G........NY.EHYG..R        Consensus
198  EIPGAFTRAWGLEEQRISRRGQSWMS----FDNEILQPMITVTLYAVVLALFGEPKMIVFLPIQMAFGWMQLTSANYIEHYGLHR        P. putida (oleovorans) GPo1
198  EIPGAFRRAWGLEEQRISRRGQSWMS----FDNEILQPMVITVVLYTLLAFFGEPKMIVFLPIQMAFGWMQLTSANYIEHYGLHR        P. putida P1
171  EIPGAFRRAWGLEEQRISRRGQSWMS----FDNEILQPMVITVILYTLLAFFGEPKMIVFLPIQMAFGWMQLTSANYIEHYGLHR        P. aureofaciens RTWH529
203  EIPGAFKRAWGLEEQRISRCGKSWMS----LDNEVLQPMIITVVLYAALLAFFGPLMLIFLPIQMAFGWMQLTSANYIEHYGLHR        A. borkumensis AP1
210  TVFGSLKSALEIETHRIKRKGKKFWS----KDNEILQGWGMSAAFHSSIIAIFGKGTIPYIVTQAFYGISLFEIINYIEHYGLKR        Acinetobacter sp. ADP1
209  TVIGSFKSALEIEKNRIKRKGKEFWS----LDNEILQGWGMSAAFHASMVGIFGKGTIPYIATQAFYGISLFEIINYIEHYGLHR        Acinetobacter calcoaceticus EB104
195  TVVGSVRSAVRLERTRIARLGRSPWT----WRNEVLHAWAMTVVVWGIAIAIGGVVVIPFFVIQAVYGASLEVVNYVEHYGLGR        B. cepacia RR10
195  TVIGSTASAWRLEKARLARSGRSAWS----WRNEVLQAWAMTVALWSAAIAFGGAKVVPFFLIQAAYGASLEVVNYVEHYGLGR        B. pseudomallei K96243
232  TVWFSLSSAWHLESQRLEKLGLPTLH----WKNGVLSAWLYSVVLWGVLIAWLGAAVIPFFVIQGIYGFSLLEVVNYVEHYGLHR        P. fluorescens CHA0
210  TVVGSLRSAWRLERKRYARDRHEPFR----IGNDVLNAWLMSAVIWSVMIAWLGIGVLPYLLQAVVGFSLLEIVNYMEHYGMLR        P. rugosa NRRL B-2295.
221  SVIGGLRSAVHLEAQRLRRLGVSPMNPMTYLRNDVLNAWLMSVVLWGLLIAVFGPALLPFVIQAVFGFSLLEAVNYLEHYGLHR        M. tuberculosis H37Rv
198  AYKYNFTNAWRLEAVRLRKKGLPVFG----WQNELIWWYLLSLALLVGFGWAFGWLGMVFFLGQAFVAVTLEIINYVEHYGLHR        P. aeruginosa PAO1 AlkB1
198  AYKHNFINAWRLEAERLKRKGLPALH----WRNELIWWYAISALFLLGFSLARGWLGAIFFLGQSVMAFTLEIVNYVEHYGLHR        P. aeruginosa PAO1 AlkB2
180  TLLGSFQSALIEEKRLSHKNYSLWS----IHHNFWVLLTVPLMICIVCFIFGGLIALSFFLLQSFTAILIEIVNYIEHYGLER        L. pneumophila Philadelphia
173  ATVGSVKDAIKIEAFTLRRKGQSPW------NLSNKTYQYVALLLALPGLVSYLGGFPALGLVTIASMIIARGLVEGFNYFQHYGL—        P. putida mt-2 XylM
           ————————————————            ————————————————            ————————————————      ———
           M5                          M6                          M5 / M6             H3
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          350       360       370       380       390       400       410       420
    .........YE.....HSWN......N..L..LQRH.DHHA......Q.LR.....P..P.GY........M.....          Consensus
279 QKMEDG----RYEHQKPHHSWMNSNHIVSNLVLFHLQRHSDHHAHPTRSYQSLRDFPGLPALPTGYPGAFLMAMIPQWFRSVM-DPK  P. putida (oleovorans) GPo1
279 EKMADG----RYEHQKPHHSWMNSNHIVSNLVLFHLQRHSDHHAHPTRSYQSLRDFPGLPALPTGYPGAFLMAMIPQWFRSVM-DPK  P. putida P1
252 EKMADG----RYEHQKPHHSWMNSNHIVSNLVLFHLQRHSDHHAHPTRSYQSLRDFPGLPALPTGYPGAFLMAMIPQWFRSVM-DPK  P. aureofaciens RTWH529
284 EKLPNG----RYEHQKPHHSWMNSNHVMSNLILFHLQRHSDHHA                                              A. borkumensis AP1
291 QKRADG----NYERITMPEHSWMNNNIVTNLFLYQLQRHSDHHAYPTRPFQALRHFDEAPELPSGYASMLPAMIPPLWFKMM-DKR   Acinetobacter sp. ADP1
290 QKKENG----QYERITMPEHSWMNNNVVTNLFLYQLQRHSDHHAYPTRPFQALRHFDEAPELPSGYASMLPAMIPSWWFKMM-DKR   Acinetobacter calcoaceticus EB104
276 RRLPDG----RYERCTPQHSWMNSNHVVTNLFLYQLQRHADHHANPTRSYQALRHFDDAPQLPAGYATMLLAYVPPLWYRVM-NPR   B. cepacia RR10
276 RQLPTG----RYERCTPQHSWMNSNHVVTNLFLYQLQRHADHHANPTRSYQALRHFDDSPQLPAGYAAMLLAYVPPLWFRVM-NPR   B. pseudomallei K96243
313 QKQPNG----RYERCSPRHSWMNSNRIVTNLFFLFQLQRHSDHHANPTRAYQALRHFDESPQLPYGYATMIVWAYVPALWRRLM-DHR  P. fluorescens CHA0
291 QKRGSPERRRYERVRDPSHSWMNSNIATNVLIYHLQRHSDHHANPVRRYQTLRDFFPESPVLPTGYAGMIVLALIPPLWRRVM-DPR  P. rugosa NRRL B-2295.
306 QKSANG----RYERCAPVHSWMNSDHIVTNLFIYHLQRHSDHHANPTRRYQTLRSMAGAPNLPSGYASMISLTYFPPLWRKVM-DHR  M. tuberculosis H37Rv
279 RKGEDG----RYERINHTHSWMNSNFVFTNLVIFHLQRHSDHHAFAKRPYQVLRHYDDSPQMPSGYAGWVLALIPPLWRAVM-DPK   P. aeruginosa PAO1 AlkB1
279 RRLDNG----RYERTTPEHSWMNSNFLLITNLFTNLFHLQRHSDHHAYAKRRYQVLRHYDSSPQLPNGYAGMIVLALFPPLWRAVM-DPK  P. aeruginosa PAO1 AlkB2
261 KMLSNG----QYEKVNPNHSWMNANHWLSNQLLIHLQRHSDHHAHGARPYQILRHMENSPQLPSGYLGMIIALIPPLWHYIM-DKR   L. pneumophila Philadelphia
252 VRDLDQ-------PTILHHAWNHMGTTVRPLGCETTNHINHHIDGYTRFYELRPEKEAPQMPSLFVCF-LLGLIPPLWFALIAKPK   P. putida mt-2 XylM
                                                                    ____
                                                                    H4
```

```
    430       440       450       460       470       480       490       500       510
    ...........................................................................................          Consensus
                                                                  ┌─────────────────────────┐
                                                                  │  Rubredoxin domain (57 a.a.) │
                                                                  └─────────────────────────┘
    v.......                                                                                             Consensus
360 VVDWAGGDLNKIQIDDSMRETYLKKFGTS--SAGHSSSTSAVAS                                                         P. putida (oleovorans) GPo1
360 VVNWANGDLSKIQIEDSMRAEYIKKFTHNVGADDKRGATAVAS                                                          P. putida P1
290                                                                                                      P. aureofaciens RTWH529
365 VMEWAHGDINKIQIQPGMREFYEQKFGVK----GSESPDTTVAK                                                         A. borkumensis AP1
372 VFEHYKEDLTKANIYPKRRAKILAKFGLTDPNIENGK                                                                Acinetobacter sp. ADP1
371 VFDHYKGDINKANISPKRRAKIFKKFGVGG                                                                       Acinetobacter calcoaceticus EB104
357 VVAHYRGNMAQSNIKPSIRDKVLAQYPAPA                                                                       B. cepacia RR10
357 VVAHYGADMAQSNIKPSIRQKVLAELAIANAP                                                                     B. pseudomallei K96243
394 VLAHYSGDVRLANMHPAKRDELTLKRYGFSNTDSDTAL                                                               P. fluorescens CHA0
375 VLAHFDGDITRANTQPGKREKILARYGRPDSGSGADSGAEKGAAGVGAVVGEDTRGDA                                           P. rugosa NRRL B-2295.
387 VLEHYGGDITRVNLHPRVREKALARYGASA                                                                       M. tuberculosis H37Rv
360 VRAYYAGVEFQLTAEQSERPAAS                                                                              P. aeruginosa PAO1 AlkB1
360 VRAYYAGEEYQLTDTQRI                                                                                   P. aeruginosa PAO1 AlkB2
342 VLAYRIQLRQLEQL                                                                                       L. pneumophila Philadelphia
330 LRDWDQRYATPGERELAMAANKKAGWPLWCESELGRVASI                                                             P. putida mt-2 XylM
```

conserved, but are replaced by similar residues, while a number of other residues in the transmembrane helices, often glycine and proline, are well conserved. The other integral-membrane non-heme iron oxygenases contain 3, 4, 5 or 6 membrane helices, two of which are located between the histidine boxes H2 and H3 (figure 2 and explanation below). A third membrane helix is always located directly upstream of the first histidine box.

All AlkB sequences contain the eight previously identified highly conserved histidine residues in three histidine boxes that are essential for catalytic activity (233), but also contain a ninth histidine residue in a conserved NYXEHYG(L/M) sequence motif (239). The corresponding histidine residue of the *P. putida* mt-2 xylene monooxygenase was shown to be essential for the activity of this enzyme (M. W. Wubbolts, personal communication) and has a pendant in all integral-membrane non-heme iron oxygenases (table 1). In the topology model for the alkane hydroxylases (261) this residue is located immediately after transmembrane helix 6 at the membrane surface, a position equivalent to the first histidine box, which is located immediately after transmembrane helix 4. This supports the hypothesis that this residue plays a role in the catalytic centre by coordinating the iron atoms in the active site. The peptide sequences directly flanking the histidine boxes are well conserved in each of the subgroups (table 1), and can be used as signature sequences in the functional analysis of newly sequenced genomes.

The length of the N-terminus, C-terminus, and the regions between the histidine boxes and membrane helices also helps classification into subclasses. For example, the bacterial carotene hydroxylases (CrtZ) can be distinguished from the plant carotene hydroxylases, as the latter typically have very long N-terminal extensions. Similarly, the Δ9 desaturases have far fewer residues between histidine box H2 and the third transmembrane helix than the other desaturases. However, large variations in length of these regions can also occur within each subclass, like the fusion to the rubredoxin domain at the C-terminus of the *P. rugosa* NRRL B-2295 alkane hydroxylase.

The alignment of the alkane hydroxylases does not allow the identification of long- or medium-chain alkane hydroxylating enzymes solely from the sequence, as the peptide sequence identities between long-chain hydroxylases are as low as between a long-chain and a medium-chain alkane hydroxylase. In addition, only one point mutation (W55S or W55C) is required to allow the medium-chain alkane hydroxylase from GPo1 to hydroxylate longer alkanes such as tetradecane or hexadecane (chapter 8). As W55 is located in the middle of the second membrane helix (figure 1), we conclude that the hydrophobic transmembrane helices are involved in the substrate binding. This is supported by the findings of Libisch *et al.* (159) who found indications that transmembrane helix 1 and 2 of desaturases are involved in substrate binding. Further research is necessary to map the substrate binding site of the alkane hydroxylases in more detail.

Structural features of the integral-membrane oxygenases, such as the presence of transmembrane helices, and the localization of the catalytic centre close to the membrane are clearly connected to the role of these enzymes in oxidizing relatively hydrophobic substrates, which are soluble in the membrane bilayer, but hardly dissolve in water (236). In contrast, oxidation of the more water-soluble short-chain alkanes and short-chain alkenes can also be catalysed by soluble non-heme iron monooxygenases (160, 178, 267).
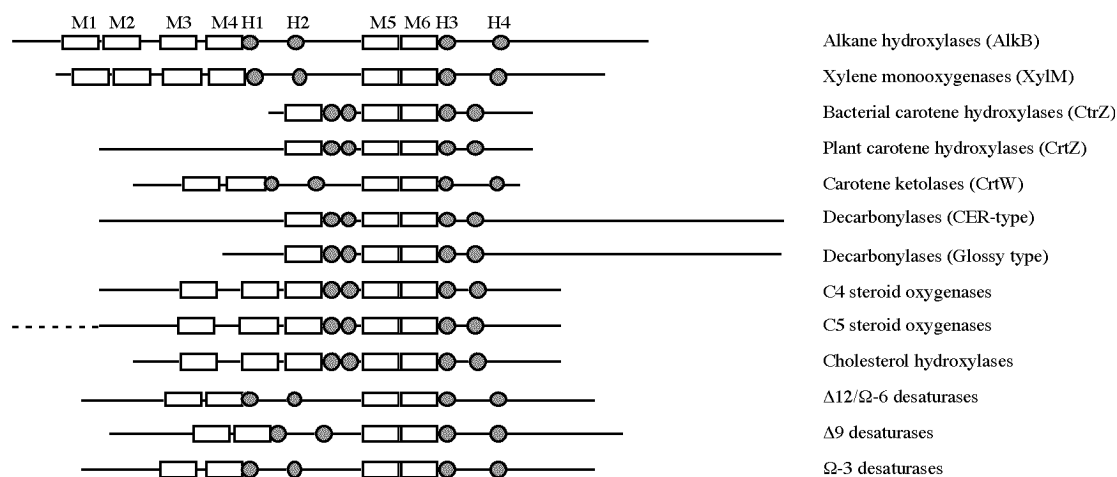

Figure 2: linear representation of integral-membrane non-heme iron oxygenases. White bars represent the membrane helices, while grey circles represent histidine boxes. Drawing not to scale.

Role of substrate uptake in alkane oxidation. The growth rates of bacteria on long-chain alkanes is largely dependent on the mode of alkane uptake. All *Pseudomonas* strains yielded growth rates on hexadecane of maximally 0.06 h^{-1} (t_d = 5 h) in the presence of exogenous surfactants. Without surfactants, growth rates did not exceed 0.03 h^{-1} (t_d = 10 h)(chapters 4, 6, 7, 8). However, literature values of growth rates by *Acinetobacter* strains on hexadecane are in the order of 0.3-0.4 h^{-1} (t_d = 1-1.5 h)(10, 16, 214). The main difference between both genera is the mode of uptake. *Acinetobacter* strains take up alkanes via surfactant-mediated direct interfacial uptake, while many *Pseudomonas* strains produce biosurfactants to enable solubilization of the alkanes (28). Both the production of biosurfactants and cell surface hydrophobicity may therefore limit growth rates. The addition of exogenous surfactants to *Pseudomonas* cultures cannot compensate for the effect of a rather hydrophilic cell surface; the growth rates seem to be limited by diffusion of the alkane over the outer membrane. In addition, the production of vast amounts of biosurfactants puts a metabolic burden on the bacteria.

In contrast, growth on medium-chain *n*-alkanes seems not to be limited by substrate uptake or cell surface hydrophobicity. Apparently, these alkanes simply traverse the membrane by diffusion (245). Growth rates of *P. putida* (*oleovorans*) GPo1 on octane as second phase are in the order of 0.4 h^{-1} (t_d = 1.5 h)(151, 223), which is close to the maximum growth rate of *Pseudomonas* strains on glucose or citrate (223).

Environmental relevance of non-heme iron integral membrane alkane hydroxylases. Using the degenerate PCR approach, we were able to show that many bacteria contain alkane hydroxylases related to the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1 (chapter 2). Although these organisms were isolated from a large variety of environments, the relative importance of strains harboring integral-membrane non-heme iron alkane hydroxylases in these environments has not been determined yet.

A few other enzyme systems are known to oxidize *n*-alkanes. Of these, the alkane oxidizing cytochrome P450 enzymes were described in some detail. These enzyme systems are most often found in yeasts (122, 196), but seem to have their counterparts in bacteria as well (44, 179). In a sandy soil microcosm, alkane assimilating yeasts were

able to overgrow bacteria such as rhodococci and pseudomonads in the oxidation of a C10/C14 mixture (225). Nevertheless, the results of such experiments are highly dependent on the physicochemical properties of the soil used for these studies. Soil composition, water content, pore size and bioavailability of the contaminant are important factors influencing the biodegradation by soil organisms (106, 152).

The use of molecular probes to detect specific genes or gene families is a commonly used technique in microbial ecology studies. To elucidate the role of non-heme iron alkane hydroxylases in the degradation of *n*-alkanes in the environment probes can be developed based on the available sequences. However, a probe based on the alkane hydroxylase of *P. putida* (*oleovorans*) GPo1 did not yield a correlation between biodegradation ability of alkanes and presence of this particular alkane hydroxylase in the environment (98, 243, 247). The results described in chapter 2 (239) show that due to the low sequence identities of the individual alkane hydroxylase sequences, a molecular probe derived from a single alkane hydroxylase only detects alkane hydroxylases of that specific branch in the phylogenetic tree. An attempt to create a single oligonucleotide probe for all alkane hydroxylases failed on the low specificity to detect some of the alkane hydroxylases (T. H. M. Smits and J. B. van Beilen, unpublished results). However, the degenerate primers (239) can be used to amplify internal fragments of the alkane hydroxylases, and molecular techniques such as restriction analysis, fluorescence *in situ* hybridisation (FISH) or denaturating gradient gel electrophoresis (DGGE) would reveal the diversity of alkane hydroxylases in pristine and contaminated soil or water samples.

Alkane hydroxylases as biocatalysts: a perspective. The *P. putida* (*oleovorans*) GPo1 alkane hydroxylase has a broad substrate spectrum and can oxidize hydrocarbons regio- and stereoselectively (259). This makes this class of enzymes interesting for the biocatalytic production of fine-chemicals (281). Alkane oxidizing strains have already been used for the production of fine-chemicals such as octanoic acid (219), epoxyalkanes (56, 89), phenylpropanol and phenylpropanoic acid (119) and phenoxy propanoic acids (50). A strain collection of around 200 alkane degrading strains in microtitre plate format, put together in our laboratory (J. B. van Beilen, W. Duetz, personal communication), has

been screened successfully for the regio- and stereoselective oxidation of *N*-benzylpyrrolidines to produce optically active *N*-benzyl-3-hydroxypyrrolidine (158). One of the most promising strains in this study, *Sphingomonas* sp. HXN-200, was subsequently used for the oxidation of *N*-substituted pyrrolidones (46). This organism contains a soluble alkane hydroxylase that is not related to the GPo1 alkane hydroxylase (D. Chang, Z. Li and J. B. van Beilen, unpublished results).

A potential limitation of the use of wild-type strains in the strain collection is that some of these strains contain more than one alkane hydroxylase (chapter 2). The alkane-inducible activity measured with a single wild-type strains thus reflects the sum of all alkane hydroxylase activities which are present under the applied conditions. This could preclude the detection of enzymes with superior catalytic properties or enantioselectivity, because the enzymes may have the opposite stereo-selectivity or a different regio-selectivity for the desired reaction. For this reason, a clone library of recombinant strains for the expression of single alkane hydroxylase genes would be useful. The setup of such a clone library would require the construction of suitable hosts, in which the oxidized product is not further metabolized. This principle has been proven effective to test the substrate range of the GPo1 alkane hydroxylase, where a medium-chain alcohol dehydrogenase negative strain has been used as a host (26, 259). The recombinant hosts used for the complementation experiments (chapter 4) would not be suitable for the oxidation of *n*-alkanes to 1-alcohols, as alcohol dehydrogenase activity is present. Nevertheless, they can be used to convert non-metabolizable compounds into high-value fine-chemicals.

CONCLUSION

In the last few years, a large body of information has been gathered on the genetics of alkane oxidation, and many alkane hydroxylase genes have been cloned and expressed. These experiments illustrate the essential roles of electron transfer, alkane solubilization and uptake, and regulation in alkane degradation. Mutagenesis experiments have given us

a first insight in the location of the substrate binding pocket. However, only a crystal structure of an alkane hydroxylase would allow us to identify residues important for substrate binding, catalysis, electron transfer and interaction with the membrane and other proteins. This could also lead to the rational design of alkane hydroxylases with better catalytic properties.

This study demonstrates the diversity of the class of integral membrane alkane hydroxylase, which has an impact on the environmental research in that more (complex) probes are necessary to study the microbial communities in pristine and contaminated environments.

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