GENERAL INTRODUCTION

# CHAPTER 1

# ALKANE OXIDATION BY *PSEUDOMONAS OLEOVORANS*: GENES AND PROTEINS

## INTRODUCTION

The degradation and bioconversion of aliphatic hydrocarbons has received a great deal of attention from microbiologists and biochemists for many decades (Chakrabarty, 1985; Watkinson and Morgan, 1990; Atlas and Bartha, 1992). The first half of this century, the attention of research groups, mainly located in the oil industry, was focused on the interaction of microorganisms with oil and oil components, a cause of technical as well as medical problems (Lee and Chandler, 1941; Cooney and Kula, 1970). In this context, many bacteria able to grow on oil components were isolated and studied. One of these was a strain of the bacterium *Pseudomonas oleovorans*, isolated in 1959 (Baptist *et al.*, 1963). Since then, the main reasons for studying these hydrocarbon-utilizing bacteria have changed. The impact of oil pollution on ecosystems has become a major issue, which has made biodegradation a rich field of research. In addition, enzymatic synthesis or bioconversion are now seen as alternatives for chemical synthesis routes.

This thesis deals with the molecular genetics and biochemistry of oxidation of medium chain-length alkanes by *P. oleovorans*, as part of a program to develop biotechnological processes, based on oxygenases.

## BIOLOGICAL HYDROCARBON OXIDATION

The first examples of hydrocarbon utilization by microorganisms were documented early this century, when Söhngen reported the growth of microorganisms on methane (1906) and petroleum products (1913). Starting in the 1920s, more systematic physiological and taxonomic studies were carried out, which showed that the capacity to use hydrocarbons as carbon and energy source is very common, and not restricted to any particular group. Examples have now been found in a wide range of prokaryotic and eukaryotic genera that include Gram-negative rods and cocci, Gram-positive cocci and sporing rods, coryneforms, actinomycetes, yeasts, fungi, and even some achlorophyllous algae (For reviews on hydrocarbon oxidation see e.g. Britton, 1984; Boulton and Ratledge, 1984; Bühler and Schindler, 1984).

Much of the work on the microbial utilization of petroleum hydrocarbons, carried out between 1920 and 1940, dealt with microbial contamination of oil products, such as cutting oil, which led to medical problems (Lee and Chandler, 1941). In the oil industry, microbial contamination of injection water used to force oil to the producing well caused plugging of pores in oil-bearing rock formations (Schwartz and Leathen, 1976).

Between 1940 and 1960, interest in petroleum microbiology strongly increased. The growth and survival of microorganisms in petroleum products caused clogged oil and fuel filters, the formation of sludges in fuel tanks, and corrosion of metal surfaces (Cooney and Kula, 1970), all a cause of great concern in the petroleum industry and a strong incentive for many companies to start up their own microbiology laboratories.

In the late 1950s and 1960s, research on the production of single-cell protein from petroleum fractions was initiated. This resulted in a large number of demonstration projects, and several full-scale commercial operations to produce protein for animal feed (Cooney *et al.*, 1980; Shennan, 1984). In the mid-1980s, however, the production of SCP turned out to be economically unsound, due to sharply rising oil prices. A number of dramatic oil spills, starting with the Torrey Canyon in 1969, also shifted the focus of

research to the fate of hydrocarbon pollutants in the environment, and the role and utility of microorganisms in biodegradation (Atlas and Bartha, 1992).

Starting in the 1960s, and concurrent with the developments in industry, the academic world recognized petroleum microbiology as a fruitful field for investigation as well. The enzyme systems responsible for the oxidation of hydrocarbons were seen as interesting subjects for fundamental research, the more so because of potential applications of these enzyme systems, or compounds produced in hydrocarbon fermentations. As an example, biosurfactants have an important role in biodegradation, but are also studied as a means to increase recovery of oil from exhausted oil fields by decreasing the viscosity of the oil (Chakrabarty, 1985).

*Occurrence of aliphatic hydrocarbons-* In ecological surveys, all soil samples contained hydrocarbon-oxidizing organisms, usually up to about 20 % of all culturable microorganisms (reviewed by Britton, 1984). This number is clearly higher (up to 90 %) for soil or sediment contaminated with hydrocarbons (Perry and Scheld, 1968). In fact, enrichment of hydrocarbon-utilizing microorganisms has been used to identify oil fields (Brisbane and Ladd, 1965), and it is almost always possible to isolate microorganisms that will grow on a particular hydrocarbon.

One might ask why it is so easy to find organisms that grow on hydrocarbons. It is obvious that the occurrence of microorganisms that degrade aliphatic hydrocarbons depends on the availability of such energy-rich compounds in natural systems. A major and obvious source of hydrocarbons is the earth's crust. A wide range of compounds is produced by geochemical processes from accumulated organic material. The resulting petroleum and natural gas is released into the biosphere by geological processes at an annual rate estimated at 8.000.000 metric tons, a rate to which human activities have added considerably. However, these sources of hydrocarbons cannot explain why hydrocarbon-utilizing microorganisms can be found practically anywhere, including non-contaminated soil. Other, more reliable sources of hydrocarbons are (decaying) plants, which produce ethylene, long-chain alkanes and isoprenoids (Taylor and Calvin, 1987), and microorganisms (Bird and Lynch, 1975). Methane, and to a lesser extent other short-chain hydrocarbons, are produced in large quantities by microbial decomposition of organic material in anoxic environments. Reports about the biological production of medium-chain length n-alkanes are scarce. Examples are the production of alkanes, predominantly heptane and nonane, by the fruits of the *Pittosporum resiniferum* tree (unpublished results cited in Taylor and Calvin, 1987), the production of n-hexane by the seaweed *Fucus vesiculosus* (Hlubucek *et al.*, 1970), and the production of medium-chain length alkanes in decaying plant material (Bradow and Connick, 1990), possibly to levels that would allow microorganisms to grow on these compounds.

*Degradation of aliphatic hydrocarbons-* The rate with which various hydrocarbons are degraded depends on their structure and physical properties. While linear alkanes are quite easy to degrade, usually by terminal oxidation to fatty acids, which can be metabolized in the ß-oxidation pathway, branched and cyclic alkanes are relatively recalcitrant compounds. Because of their sometimes very complex structures, a whole set of specialized enzymes may be required to convert the substrate to intermediates of the central metabolism. Some branched and alicyclic compounds, such as pristane and the hopanes, are so refractory to degradation that they are used as internal standards in studies on mixed hydrocarbon oxidation. However, for these compounds, including pristane, degradation pathways have been described (Pirnik *et al.*, 1974).

The physical properties of linear alkanes to a large extent determine their degradation rates.

**Table I**

*Gram-negative bacteria which are able to grow on n-alkanes, and which (may) contain enzyme systems similar to the P. oleovorans alkane hydroxylase system*

| Strain name | Source | Growth substrates | Properties of alkane oxidizing enzymes | References |
|---|---|---|---|---|
| *P. oleovorans* (ATCC 29347) | soil | C6-C12 | prototype three component alkane hydroxylase | 1, 2 |
| *P. aeruginosa* KSLA 473 | Y-harbour sediment | C6-C18 | one particulate, two soluble components alkane inducible, substrate range C5-C10 | 3, 4 |
| *P. aeruginosa* 196 Aa (NCIMB 9571) | fuel | C7-C16 | particulate multicomponent system, no rubredoxin detected, substrate range C7-C14 | 5, 6 |
| *P. aeruginosa* (ATCC 17423) | - | C7 | particulate and soluble fraction (more than two components) | 7 |
| *P. aeruginosa* Sol 20 (NCIMB 8704) | - | C7 | particulate, $Fe^{2+}$ and $O_2$ dependent | 8 |
| a *P. denitrificans* | - | C6, C10 | three components, including a rubredoxin, and a flavin dependent protein | 9 |
| *Pseudomonas sp.* | soil | C8, C10 | constitutive alkane oxidation | 10 |
| *Pseudomonas sp.* X2 | Thames | C10 | no data | 11 |
| *Pseudomonas sp.* | seawater | C10 | three components, $Fe^{2+}$, cyanide sensitive alkane inducible | 12 |
| *P. putida* strain | fuel | C8-C11 | no data | 13 |
| *Acinetobacter calcoaceticus* 69-V | soil | C13-C20 | particulate monooxygenase, alkane-inducible rubredoxin, rubredoxin reductase | 14, 15 |
| *Alcaligenes sp.* L.16.1 | seawater | C10-C18 | NADH-dependent alkane hydroxylase cyanide sensitive, particulate | 16 |

References: 1, Baptist *et al.* (1963); 2, Schwartz and McCoy, 1973; 3, Thysse and Van der Linden (1958); 4, Van Eyk and Bartels (1970); 5, Traxler and Bernard (1969); 6, Vandecasteele *et al.* (1983); 7, Macham and Heydeman (1974); 8, Azoulay *et al.* (1963); 9, Kusunose *et al.* (1967); 10, Robinson *et al.* (1964); 11, Bird and Molton (1967); 12, Hammer and Liemann (1976); 13, Williams *et al.* (1981); 14, Kleber *et al.* (1973); 15, Claus *et al.* (1980); 16, Bertrand *et al.* (1976).

C10 to C18 n-alkanes are degraded most readily, and support abundant growth of, for example, yeasts, *Acinetobacter* sp. and *P. aeruginosa*. Longer alkanes are progressively less soluble with increasing chain length, which results in decreasing oxidation rates. With decreasing chain-length, short-chain alkanes in the range of C5-C10 become increasingly soluble and consequently increasingly toxic (Laane *et al.*, 1987; Inoue and Horikoshi, 1991). Nevertheless, some microorganisms are able to grow on these compounds. Yeasts such as *Candida* species and *Saccharomyces carlsbergensis* are able to grow on medium-chain length alkanes when the toxicity of the growth substrate is relieved by adding a less soluble hydrocarbon, such as pristane or tetradecane (Gill and Ratledge, 1972). Some bacteria, however, are more resistant to 'soluble' hydrocarbons. Baptist *et al.* (1963) reported the isolation of a Pseudomonad able to grow directly on n-hexane. The organism was tentatively identified as a strain of *Pseudomonas oleovorans*, a bacterium isolated previously from machine shop cutting oil (Lee and Chandler, 1941). In a later taxonomic study by Stanier *et al.* (1966) the organism was classified as a *P. putida* biotype A. Only this strain has been studied extensively with respect to the genetics as well as the enzymology of (medium chain-length) alkane oxidation. This thesis describes the further analysis of genes and enzymes responsible for alkane metabolism by this strain.

Other bacteria which are able to grow on n-alkanes are listed in Table I. Most of these strains were characterized only to a very limited extent, with the exception of *P. aeruginosa* KSLA 473, *P. aeruginosa* 196 Aa, a *P. denitrificans* strain, and a marine *Pseudomonas sp.*. The available literature indicates that these strains contain alkane oxidizing enzyme systems that are similar to the *P. oleovorans* system (Van Eyk and Bartels, 1970; Vandecasteele *et al.*, 1983; Kusunose *et al.*, 1967; Hammer and Liemann, 1976).

*Oxygenases*- Mineralization of unactivated hydrocarbons is almost always initiated by an oxidation step. The incorporation of molecular oxygen in the hydrocarbon substrate is carried out by oxygenases (Hayaishi, 1974), a class of enzymes which can be divided into two categories: dioxygenases incorporate both atoms of molecular oxygen into the substrate, while monooxygenases incorporate one atom of oxygen into the substrate, the other being reduced to water. Monooxygenases are subdivided in two classes; external monooxygenases use an external source of reducing equivalents, such as NAD(P)H, while in the case of internal monooxygenases the substrate supplies reducing equivalents.

Many of the oxidation reactions catalyzed by these enzymes are highly regio- and enantioselective, and might replace complex synthesis routes in the production of fine- and perhaps mid-priced chemicals. Even though only few such processes have been developed to a commercial stage (Meijer *et al.*, 1990; Leak *et al.*, 1992), the patent literature suggests that the chemical industry continues to investigate new biochemical processes based on oxygenases.

## ENZYMOLOGY OF ALKANE OXIDATION BY *PSEUDOMONAS OLEOVORANS*

The pathway of alkane oxidation by *P. oleovorans* was characterized first by Coon and coworkers (Baptist *et al.*, 1963). They were able to show that enzyme extracts of this strain converted radioactively labeled n-octane to octanoic acid, and provided evidence that the intermediates 1-octanol and 1-octanal were produced as well. The conversion of n-octane to 1-octanol was found to be dependent on pyridine nucleotides (Gholson *et al.*, 1963). The alkane hydroxylase system, first named ω-hydroxylase because of its ability to convert fatty acids to ω-hydroxy fatty acids (Kusunose *et al.*, 1964), was charac-

terized as a external monooxygenase which follows the stoichiometry characteristic of these proteins (McKenna and Coon, 1970):

$$R\text{-}CH_3 + NADH + H^+ + O_2 \rightarrow$$

$$R\text{-}CH_2OH + NAD^+ + H_2O$$

The alkane hydroxylase consists of three components; a particulate hydroxylase and two soluble proteins, rubredoxin and rubredoxin reductase, which act as electron carriers between NADH and the hydroxylase (Peterson *et al.*, 1966) (Fig. 1). The hydroxylase was purified by Ruettinger *et al.* (1974, 1977) as a high molecular weight aggregate (Mw ~ 500 kD). Under denaturing conditions this aggregate appeared to consist of polypeptides of 42 kD. The slightly yellow preparation contained 1 atom of iron and about 20 phospholipid molecules per polypeptide chain, both of which were essential for activity of the enzyme (Ruettinger *et al.*, 1977). Cell fractionation experiments (Benson *et al.*, 1977; 1979) and immuno gold labeling (Lageveen, 1986) have shown that the enzyme is localized in the cytoplasmic membrane.

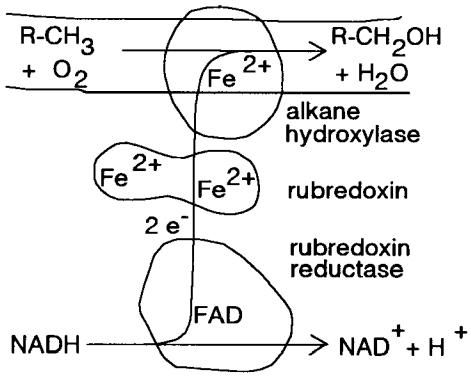


Fig. 1. **Structure of the alkane hydroxylase system.** The alkane hydroxylase system consists of three components: a membrane-bound monooxygenase, rubredoxin and rubredoxin reductase.

The UV and visible light spectra show no distinctive characteristics, and provide little information with regard to the coordination environment of the iron cofactor. The spectra change little when the iron is removed, or cyanide, substrate, hydrogen peroxide or dithionite are added. Attempts to use electron paramagnetic resonance were not very successful either. The enzyme preparation exhibited a weak signal at $g = 4.3$ which accounted for only about 16 % of the total iron present (McKenna and Coon, 1970; Ruettinger *et al.*, 1977), and which may very well have been due to non-specifically bound iron that is almost always present in membrane preparations (Meinhardt *et al.*, 1989).

Activity of the apo-enzyme can be restored by ferrous iron, not by ferric iron. However, once bound, iron can be removed efficiently from alkane hydroxylase only in the presence of dithionite, which presumably reduces the iron to the ferrous state. In conclusion, the redox state and coordination of the iron in alkane hydroxylase is not known.

The second component of the alkane hydroxylase system was characterized as a rubredoxin (Peterson *et al.*, 1967); a class of red-colored electron transfer proteins that contain one or more active centers consisting of a single iron atom coordinated by four cysteine sulfur atom ligands in a tetrahedral structure (Lovenberg and Sobel, 1965). The *P. oleovorans* rubredoxin is more than three times the size of other bacterial rubredoxins, and consists of two very similar domains connected by a linker, both of which bind one iron atom. The iron bound to the N-terminal domain is very loosely bound (Lode and Coon, 1971), and is usually lost in the isolated protein. May *et al.* (1984) showed that the carboxy-terminal CNBr fragment is active in alkane oxidation. The amino acid sequence of the *P. oleovorans* rubredoxin was determined almost completely by Benson *et al.* (1971). Sequence comparisons with other rubredoxins showed that about 45 % of all amino acids were conserved. The *P. oleovorans* rubredoxin cannot be replaced by the rubredoxins from anaerobic bacteria in alkane oxidation.

Rubredoxin reductase is a 49 kilodalton flavoprotein (Ueda *et al.*, 1972). It transfers electrons from NADH to rubredoxin, which in turn transfers the electrons to the catalytic component; alkane hydroxylase. In *in vitro* experiments, rubredoxin reductase can be replaced by the more stable spinach ferredoxin reductase. Rubredoxin reductase is inactive with spinach ferredoxin and other nonheme iron proteins such as putidaredoxin and adrenodoxin. It *is* able to transfer electrons to rubredoxins from anaerobic bacteria (Ueda and Coon, 1972).

## GENETICS OF ALKANE OXIDATION BY PSEUDOMONAS OLEOVORANS

*Analysis of the OCT-plasmid-* Chakrabarty *et al.* (1973) initiated research on the genetics of alkane oxidation by showing that the alkane hydroxylase system is encoded by the OCT-plasmid (OCT for n-octane utilization), a member of the Inc P-2 family of plasmids. Estimates of its molecular size vary from 50 to 400-500 kb (Palchaudchuri, 1977; Hansen and Olsen, 1978; Harder and Kunz, 1986; Eggink, 1987). OCT was found to be transmissible to other fluorescent Pseudomonads (with a very low frequency) due to a fertility factor designated K (Chakrabarty, 1974), and has only one other known function: mercury resistance (Harder and Kunz, 1986). The construction of a fusion plasmid between OCT and CAM, a plasmid specifying camphor degradation, allowed for easier transfer of genes specified by the OCT-plasmid (Chakrabarty, 1973).

*Genetic organization of the* alk-*genes-* In the mid-70's, Shapiro and coworkers initiated studies which substantially clarified the genetics of alkane oxidation. They found that when the OCT or CAM-OCT plasmids were introduced in wild-type *P. putida* strains, the transformed strains acquired the ability to grow on n-alkanes. In addition, the OCT plasmid complemented chromosomal alcohol dehydrogenase mutations in *P. putida alcA* strains (Grund *et al.*, 1975).

Subsequent mutagenesis of the OCT plasmid produced mutants which lack the membrane-bound hydroxylase; one of the soluble components of the alkane hydroxylase system; or the alcohol dehydrogenase. These mutants were labeled *alkB*, *alkA* and *alkC*, respectively (Benson *et al.*, 1977). In addition, pleiotropic mutants were obtained that affected expression of all three functions. These mutants were labeled *alkR* (Fennewald and Shapiro, 1977). Physical mapping of mutants, transduction mapping with phage F116L, studies on the reversion characteristics of *alk* mutants (Fennewald *et al.*, 1979), and polar effects of Tn*7* insertions (Fennewald and Shapiro, 1979), showed that the *alkB*, *alkA*, and *alkC* loci are organized as an operon: *alkBAC*.

The order of, and distance between, the *alkBAC* operon and the *alkR* locus was established using Tn*7* insertions outside the *alk*-regions as unselected markers in reciprocal three-factor crosses. These experiments indicated that *alkR* is located downstream of *alkBAC*, separated by 42 kb of DNA (Fennewald *et al.*, 1979). Interestingly, Owen (1986) found that all Tn*7S* insertions within the *alkBAC* operon are oriented in one direction, while within the *alkR* cluster they are always oriented in the opposite direction. If Tn*7S* inserts itself in one orientation only, as has been observed for other replicons, *alkBAC* and *alkR* should be transcribed in opposite directions.

Experiments in *alkB* or *alkA* mutants showed that unoxidized alkane can induce the *alkBAC* operon (Benson and Shapiro, 1975). Primary alcohols were found to be effective inducers as well, while some compounds like dicyclopropylketone and diethoxyethane are gratuitous inducers, as had been shown before for *P. aeruginosa* KSLA 473 by Van Eyk and Bartels (1968). Some alkanes (C13 and C14) apparently cannot support growth only because they do not induce alkane hydroxylase activity (Grund *et al.*, 1975). The mutants in the *alkR* locus show altered regulation of the *alkBAC* operon (Fenne-

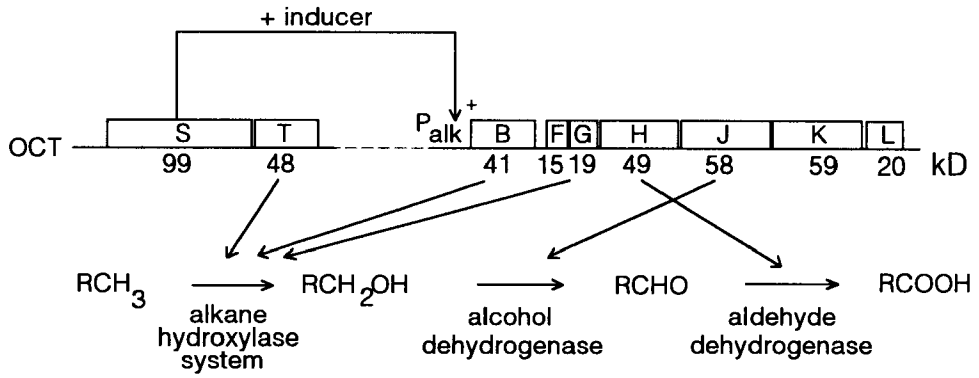


Fig. 2. **Molecular genetics and pathway of alkane oxidation by *Pseudomonas oleovorans*.** The *alk*-genes are organized in two clusters: the *alkST* and *alkBFGHJKL* operons. Arrows point from the genes to the proteins and functions they specify.

wald and Shapiro, 1977). Some show no induction, while others are constitutive, or have a more restricted range of inducers.

*Cloning of the* alk-*genes*- The *alk*::Tn*7* insertions allowed cloning of *alk*-sequences which complemented or rescued *alkBA* point mutations. These DNA fragments were subsequently used as hybridization probes to screen a gene library, which yielded plasmid pGEc29 with a 16.9 kb *Eco*RI fragment carrying the entire *alkBAC* region (Eggink *et al.*, 1984). The 16.9 kb *Eco*RI fragment by itself was sufficient to allow *P. putida* to grow on n-octane, presumably only because *alkR* encodes a transcriptional activator.

Cloning of an 18 kb *Eco*RI fragment that complemented *alkR* mutants (Eggink *et al.*, 1984) allowed the construction of a broad host-range plasmid pGEc47 containing both the 16.9 and 18 kb fragments, that enables *P. putida* to grow on octane. When pGEc47 was transferred to *E. coli*, the *alk*-genes were expressed and regulated as in the original host, allowing *E. coli* to grow well on octane, provided that the ß-oxidation pathway is expressed constitutively (Eggink *et al.*, 1987b). This was a surprise because other *Pseudomonas* degradative plasmid genes are expressed very inefficiently in *E. coli* (Frantz and Chakrabarty, 1986).

*Analysis of the* alkBAC *operon*- Using electron microscopy of R-loops, Eggink *et al.* (1987a) measured the length and determined the position of the *alkBAC* operon on the 16.9-kb *Eco*RI fragment. Subsequently, the 7.3-kb *alkBAC* operon was analyzed for translation products in minicell experiments. Starting from the promoter of the *alkBAC* operon, six polypeptides, with molecular masses of 41, 15, 49, 58, 59, and 20 kDa, were identified, and the approximate positions of the corresponding genes were determined (Fig. 2). The 41 kDa protein was identified as alkane hydroxylase by reaction with a specific antibody. Mapping, marker rescue and deletion experiments (Owen *et al.*, 1984; Eggink *et al.*, 1987a) clearly indicated that the 58 kDa protein corresponded to the dehydrogenase AlcO/AlkC described by Benson and Shapiro (1976). The genes encoding the two remaining polypeptides (59 and 20 kDa) could be deleted without abolishing growth on alkanes (Eggink *et al.*, 1987a).

*Sequence analysis of the* alk-*genes*- After DNA sequence analysis of the proximal half of the *alkBAC* operon (Kok *et al.*, 1989b), genes corresponding to the first three polypeptides were identified. An additional gene with a sequence corresponding to that expected for rubredoxin (encoding a 19 kDa peptide) was found between the genes encoding the 15 and 49 kDa polypeptides. Accordingly, the *alkBAC* operon was renamed *alkBFGHJKL*.

DNA sequence analysis confirmed that the first peptide encoded by the operon (AlkB) corresponded to the membrane-bound nonheme iron alkane monooxygenase component (Kok *et al.*, 1989a). The primary sequence of AlkB contained several stretches of hydrophobic amino acids, indicating that AlkB spans the cytoplasmic membrane up to nine times. Contrary to earlier biochemical experiments (Ruettinger *et al.*, 1977) no cysteines were found in the primary sequence.

The *alkF* gene, directly downstream of *alkB*, corresponded with the 15 kDa peptide found in minicell experiments. The amino acid translation, however, was not identical to the previously determined sequence of rubredoxin, even though it was clearly homologous. The amino acid translation of the *alkG* gene, which did not produce a peptide in the minicell experiments, *was* identical to the amino acid sequence of the rubredoxin (Benson *et al.*, 1971). The AlkG gene product probably failed to shown up in the minicell experiments because the mature protein only contains one methionine.

Deletion experiments showed that only clones which encoded the C-terminal domain of AlkG were able to restore growth on n-alkanes in *P. putida* carrying an CAM-OCT fusion plasmid with the *alkFG* genes deleted. Neither AlkF nor the N-terminal domain of AlkG were able to complement this deletion (Kok *et al.*, 1989b), which agrees with results obtained with proteolysis experiments carried out by May *et al.* (1984). Sequence comparisons indicated that AlkF was the product of a duplication event of the N-terminal, inactive part of AlkG.

The *alkH* gene was expected to encode rubredoxin reductase because it encodes a peptide of 49 kDa in minicell experiments, close enough to the 55 kDa found by Ueda *et al.* (1972) for rubredoxin reductase, and because of its location downstream of the genes for alkane hydroxylase and rubredoxin. The primary sequence however, was found to resemble several human, rat and horse aldehyde dehydrogenases. The gene complemented a *P. oleovorans* aldehyde dehydrogenase mutant, albeit weakly. The calculated amino acid composition ruled out the possibility that the *alkH* gene encoded rubredoxin reductase (Kok *et al.*, 1989b). As the *P. putida* chromosome encodes several aldehyde dehydrogenases (Grund *et al.*, 1975), AlkH is not essential for growth on n-alkanes.

*Analysis of the* alkR *region*- Genetic studies indicated that the *alkR* region encodes at least two functions: transcriptional activation of the *alkBAC* and recognition of the inducer (Fennewald and Shapiro, 1977). Owen (1984) concluded from complementation and marker rescue experiments that the *alkR* locus consists of at least three cistrons. However, subsequent molecular genetic research presented a different picture of this region. Subcloning of the *alkR* region from the 18 kb *Eco*RI fragment indicated that a 4.9 kb *Sal*I fragment carried a functional *alkR* locus. In minicell experiments the fragment was found to encode two polypeptides, subsequently named AlkS and AlkT. Only AlkS was necessary for activation of expression of the *alkBFGHJKL* operon (Eggink *et al.*, 1988). When the primary sequence of the *alkT* gene was determined it appeared that the calculated amino acid composition was identical to the amino acid composition of isolated rubredoxin reductase (Ueda *et al.*, 1972; Eggink *et al.*, 1990). AlkT has two ADP-binding folds, one for FAD at its aminoterminus, the other for NADH. The general structure of AlkT is probably very similar to that of human glutathione reductase, and other flavoprotein oxidoreductases.

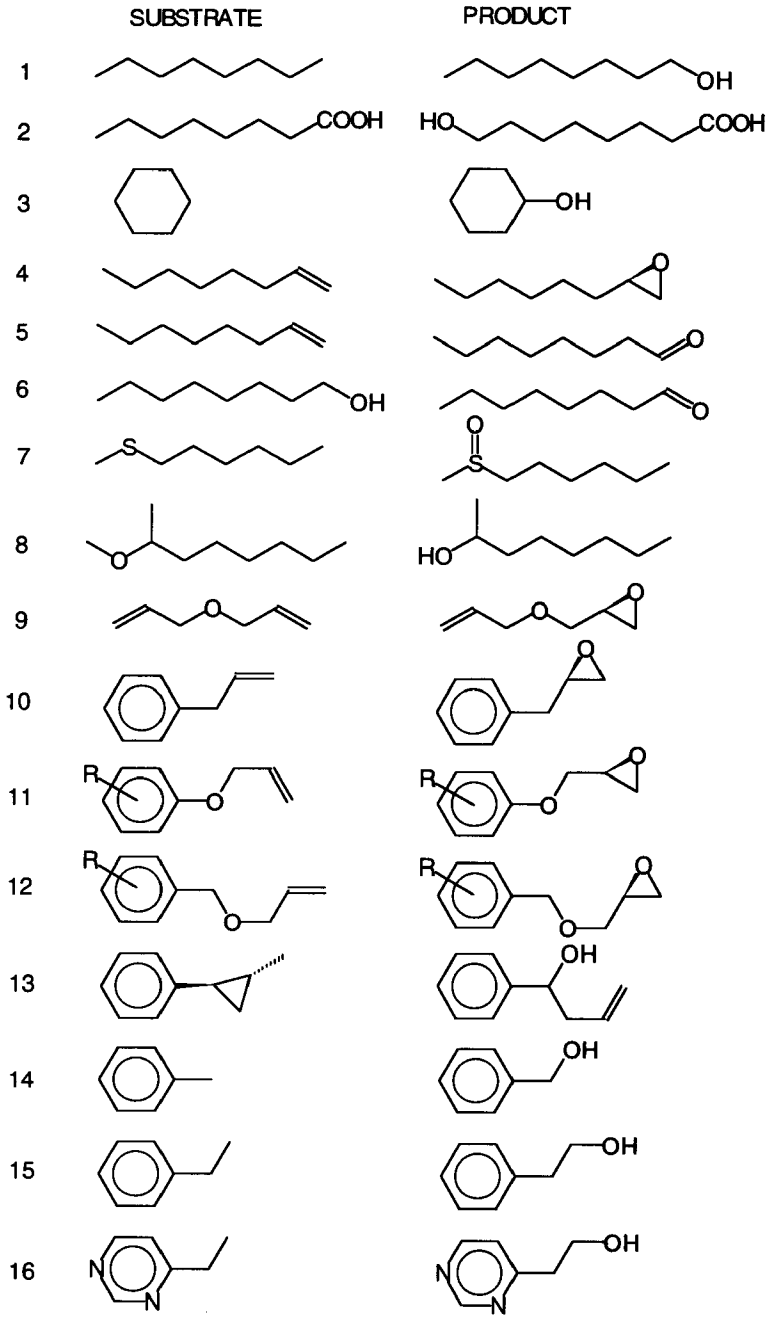
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Fig. 3. **Substrate range of the alkane hydroxylase system.** Only examples of the classes of substrates are shown. (1) C6-C14 alkanes to corresponding 1-alkanols. (2) C6-C12 fatty acids to $\omega$-hydroxy fatty acids. (3) cyclohexane to cyclohexanol. (4) C3-C12 1-alkenes to 1,2-epoxyalkanes. (5) 1-octene to 1-octanal. (6) 1-octanol to 1-octanal. (7) methyl heptyl thioether to corresponding sulfoxide (and heptylthiol). (8) branched ethers to alcohol (demethylation). (9) diallyl ether to corresponding epoxide. (10) allylbenzene to 3-phenyl-1,2-epoxypropane. (11) ring substituted allyl phenyl ethers to corresponding epoxides. (12) substituted allyl benzyl ethers to corresponding epoxides. (13) (*R,S*)-*trans*-2-phenyl-1-methylcyclopropane to 1-phenyl-3-butene-1-ol. (14) toluene to benzylalcohol. (15) ethylbenzene to 2-phenylethanol. (16) ethyl-substituted heterocyclic 5- and 6-membered aromatic rings to corresponding alcohols. 1-8, 10 and 11, references in Witholt *et al.* (1990); 9, 12-14, Fu *et al.* (1991); 15, Fukuda *et al.*, 1989; 16, European patent application EP 502524, Lonza, 1992.

*Xylene monooxygenase-* As shown in Table I, several bacteria possess enzyme systems with properties that are very similar to those of the *P. oleovorans* alkane hydroxylase. However, the only protein in the SwissProt database (Release 25) that shows sequence identity with AlkB is the catalytic component of the *P. putida* mt-2 xylene monooxygenase (Suzuki *et al.*, 1991), XylM. Like AlkB, XylM is an integral membrane protein which requires ferrous iron to reconstitute its activity (unpublished results cited in Harayama *et al.*, 1992). Xylene monooxygenase is responsible for the first step in the catabolism of toluene and xylenes: oxidation of the methyl side-chain to produce (methyl)benzyl alcohol. The second component of this enzyme system, XylA (Harayama *et al.*, 1989), is the equivalent of rubredoxin and rubredoxin reductase, but is not related to these proteins. The N-terminal region of XylA exhibits a strong similarity with chloroplast-type ferredoxins, while the remainder of XylA resembles ferredoxin-$NADP^+$ reductases (Suzuki *et al.*, 1991).

*Potential applications of the alkane hydroxylase system-* The *P. oleovorans* alkane hydroxylase system has been characterized extensively with respect to enzymology, mechanism, and genetics, because of a general interest in oxygen activation by biological systems. In addition, however, the alkane hydroxylase system has been studied because of its potential applications in organic chemistry. This was first demonstrated by May and coworkers, who found that the alkane hydroxylase system catalyzed the epoxidation of olefins (May and Abbott, 1972; 1973; May *et al.*, 1975), in addition to the previously known methyl group hydroxylation of alkanes and fatty acids (Kusunose *et al.*, 1966; McKenna and Coon, 1970). This has led to efforts to optimize the conversion of; olefins to the corresponding epoxides (De Smet *et al.*, 1981; 1983); n-octane to octanoate (Favre-Bulle *et al.*, 1991; 1992a; 1992b); n-octane to poly-(3-hydroxyalkanoates) (Preusting *et al.*, 1991, 1992), and n-alkanes to 1-alkanols (Bosetti *et al.*, 1992).

May and coworkers have shown that the alkane hydroxylase catalyzes the oxidation of alcohols to aldehydes, the O-demethylation of (branched) ethers, oxygenation of thioether substrates leading to both sulphoxide and S-demethylation products (May and Katopodis, 1986). As an extension of the olefin epoxidation, Johnstone *et al.* (1986) and Fu *et al.* (1991) showed that a wide range of allyl phenyl and allyl benzyl ethers can be epoxidized with high enantiomeric excess to the corresponding epoxides (usually *R*).

The regioselective oxidation of methyl-groups has also drawn the attention of industry. A patent application by Lonza A.G. describes the hydroxylation of ethyl-substituted heterocyclic rings, the products of which are useful in the synthesis of antibiotics. The substrate range is shown in Fig. 3. There is no reason to expect that this is the limit of the capabilities of the alkane hydroxylase system; more reaction types and substrates will certainly be identified in future research.

## SCOPE OF THE THESIS

The alkane hydroxylase system of *Pseudomonas oleovorans* is one of the best studied examples of the non-heme iron monooxygenases, and is expected to be useful in the production of synthons for the pharmaceutical and fine-chemicals industry. Efforts to optimize production processes of poly-(3-hydroxyalkanoates) and octanoic acid using cell and fermentation engineering have brought considerable improvements. At the same time, a better understanding of the enzymology and molecular genetics of alkane oxidation is necessary to solve problems that block the conversion of potential applications into commercially viable processes.

In this thesis, the molecular genetic analysis initiated by the group of Professor Shapiro in Chigaco, and continued by Gerrit Eggink and Menno Kok in the group of Professor Witholt, is further extended.

Chapter 2 describes the DNA sequence determination and analysis of the *alkJKL* genes; the 3' end of the *alkBFGHJKL* operon. The obtained DNA sequence was compared with the DNA and protein databases, and the individual genes were subjected to deletion analysis, and complementation experiments.

In chapter 3 the deletion and complementation analysis is extended to the components of the alkane hydroxylase system, AlkB, AlkG and AlkT. To be able to control the expression of the different components independent from the *alk*-promoter, we cloned the three genes in expression vectors. In addition we constructed an AlkB-AlkG fusion protein.

In chapter 4 the membrane topology of alkane hydroxylase (AlkB) is investigated using alkaline phosphatase and ß-galactosidase gene fusions.

Chapters 5 and 6 deal with the relation between the *P. oleovorans* alkane hydroxylase and the *P. putida* mt-2 xylene monooxygenase systems. In chapter 5, we tested whether rubredoxin and rubredoxin reductase can be replaced by the reductase of xylene monooxygenase; XylA, and vice versa. The subsequent chapter describes the construction of *alkB-xylM* and *xylM-alkB* gene fusions.

In chapter 7, a model is presented of the substrate binding site of alkane hydroxylase, based on the conversion rates of a range of aliphatic, alicyclic and aromatic compounds.

In the final chapter, features of the genes and proteins involved in alkane oxidation by *Pseudomonas oleovorans*, which have not been discussed in previously published papers or other chapters of this thesis, are given attention. In addition, the available knowledge on the *alkBFGHJKL* and *alkST* sequences, and the proteins they encode, is summarized.