Biodegradation **5**: 161–174, 1994.

Genetics of alkane oxidation by *Pseudomonas oleovorans*

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Received 3 September 1993; accepted 31 January 1994

Key words: Pseudomonas oleovorans, alkane hydroxylase, rubredoxin, OCT plasmid, degradation, catabolic pathway

Abstract

Many Pseudomonads are able to use linear alkanes as sole carbon and energy source. The genetics and enzymology of alkane metabolism have been investigated in depth for *Pseudomonas oleovorans*, which is able to oxidize C5-C12 n-alkanes by virtue of two gene regions, localized on the OCT-plasmid. The so-called *alk*-genes have been cloned in pLAFR1, and were subsequent analyzed using minicell expression experiments, DNA sequencing and deletion analysis. This has led to the identification and characterization of the *alkBFGHJKL* and *alkST* genes which encode all proteins necessary to convert alkanes to the corresponding acyl-CoA derivatives. These then enter the β-oxidation-cycle, and can be utilized as carbon- and energy sources. Medium (C6-C12)- or long-chain (C13-C20) n-alkanes can be utilized by many strains, some of which have been partially characterized. The alkane-oxidizing enzymes used by some of these strains (e.g. two *P. aeruginosa* strains, a *P. denitrificans* strain and a marine *Pseudomonas* sp.) appear to be closely related to those encoded by the OCT-plasmid.

Introduction

Research on the bioconversion and degradation of oil and oil fractions has lately received a great deal of attention (Chakrabarty 1985; Watkinson & Morgan 1990; Atlas & Bartha 1992). To some extent this is due to the visibility to the general public of the oil industry and oil disasters. There are, however, more direct economic reasons; microbiological studies have shown that the interaction of microorganisms with oil and oil components causes medical and technical problems (Lee & Chandler 1941; Cooney & Kula 1970; Schwartz & Leathen 1976). In addition, microorganisms are able to use specific oil fractions as carbon and energy sources, or convert oil components to useful products (Schwartz & Leathen 1976; Chakrabarty 1985; Sariaslani 1989).

The occurrence of alkane utilizing microorganisms depends on the availability of these compounds in nature. Large amounts of alkanes are produced by geochemical processes from accumulated organic material. Other sources of hydrocarbons are plants, which produce ethylene, long-chain alkanes and isoprenoids (Taylor & Calvin 1987), and microorganisms, which have been shown to produce methane, ethylene and isoprenoids, and probably n-alkanes as well (Bird & Lynch 1974).

Systematic studies have shown that the capacity to use hydrocarbons as sole carbon and energy source is very common, and not restricted to any particular group of microorganisms. 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 Britton (1984), Boulton & Ratledge (1984) or Bühler and Schindler (1984)).

The physical properties of linear alkanes are very important for the rate with which these compounds are oxidized and metabolized. n-Alkanes ranging from C10 to C18 are degraded most readily, and support abundant growth of many different microorganisms (see above reviews). 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 to C10 become increasingly soluble and consequently increasingly toxic (Laane et al. 1987; Inoue & Horikoshi 1991). Nevertheless, some microorganisms are able to grow on these compounds. 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 & Chandler 1941). Although in a later taxonomic study by Stanier et al. (1966) the new isolate was classified as a *P. putida* biotype A, the name *P. oleovorans* continued to be used. This particular *P. oleovorans* strain was subsequently studied extensively with respect to both the enzymology as well as the genetics of alkane oxidation.

P. oleovorans was initially shown to contain an ω-fatty acid hydroxylase activity, and the corresponding enzyme system was dissected exhaustively by Coon and his coworkers in the 60's and 70's. Their work on the three protein components of the ω-hydroxylase system, and on the substrate range of this enzyme, showed it to be a non-heme iron monooxygenase capable of introducing oxygen into a wide range of intermediate chain length alkanes and related compounds. This work was extended by McCoy, May and others who showed that alkane monooxygenase also catalyzes epoxidation, sulfoxidation and demethylation reactions (see below). In the mid 70's, Shapiro and coworkers initiated studies which substantially clarified the genetics of alkane oxidation, followed by our own work in the past decade on the detailed structure of the *alk*-genes.

During the same period, other bacteria which were able to grow on n-alkanes were isolated and described (see Table 1). Most of these strains, mainly Pseudomonads, 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 hydroxylase systems that are very similar to the *P. oleovorans* system (Van Eyk & Bartels 1970; Vandecasteele et al. 1983; Kusunose et al. 1967; Hammer & Liemann 1976). In addition, the two *P. aeruginosa* strains also appear to possess alkane inducible, soluble cofactor-independent alcohol dehydrogenases (Azoulay et al. 1963; Bonamy et al. 1983), similar to that found in *P. oleovorans* (Benson & Shapiro 1976).

Since alkanes and related compounds are rather difficult to activate regio- and/or stereoselectively using chemical agents, the attack on these compounds by microorganisms represents an interesting biochemical process which might replace complex synthesis routes in the production of fine- and perhaps medium priced-chemicals. The substrate range of the alkane-oxidizing enzyme systems of the strains discussed above is in fact very wide, as is illustrated by the stereoselective epoxidation of allylphenyl ethers and related compounds by *P. oleovorans*, and the *P. aeruginosa* strains KSLA 473, Sol 20, 196 Aa (Johnstone et al. 1986; Fu et al. 1991). The *P. aeruginosa* KSLA 473 paraffin hydroxylase was earlier shown to hydroxylate a wide range of alkanes, alkylbenzenes and (alkyl)cyclohexanes (Van Ravenswaay Claasen & Van der Linden 1971), while, in addition to the previously known methyl group hydroxylation (Kusunose et al. 1964; McKenna & Coon 1970), the *P. oleovorans* alkane hydroxylase system was found to catalyze the epoxidation of olefins (May & Abbott 1972, 1973; May et al. 1975; Schwartz & McCoy 1973), aliphatic sulfoxidation, and ether demethylation (Katopodis et al. 1988), as reviewed in Witholt et al. (1990). The other alkane-oxidizing enzyme systems have not been characterized in detail with respect to substrate range, but can be expected to catalyze similar reactions.

The alkane hydroxylase system of *Pseudomonas oleovorans*

The pathway of alkane oxidation by *P. oleovorans* was characterized by Coon & coworkers (Baptist et al. 1963), who showed that enzyme extracts of this strain converted radioactively labelled n-octane to octanoic acid via the intermediates 1-octanol and 1-octanal. Subsequently, the enzyme system responsible for the conversion of n-octane to 1-octanol was isolated. It was originally named ω-hydroxylase by Peterson et al. (1966), based on the ω-oxidation of fatty acids by this enzyme system (Kusunose et al. 1964a, b), but in subsequent studies the name 'alkane hydroxylase system' has been preferred, since the system is believed to have evolved for the utilization of n-alkanes as carbon and energy sources (Nieder & Shapiro 1975). It is conceivable that new data on the origin of the *alk*-genes might lead to a revision of this latter view (see below). For now however, we will continue referring to this enzyme system as the 'alkane hydroxylase system'.

The alkane hydroxylase system consists of three components; a particulate hydroxylase and two soluble proteins, rubredoxin and rubredoxin reductase, which act as electron carriers between NADH and

Table 1. Gram-negative bacteria able to grow on n-alkanes.

Strain name	Source	Growth substrates	Properties of alkane oxidation enzymes	References
P. oleovorans TF4-1L (ATCC 29347)	soil	C6-C12	prototype three component alkane hydroxylase	Baptist et al. 1963 Schwartz & McCoy 1973
P. aeruginosa KSLA 473	Y-harbour sediment	C6–C18	one particulate, two soluble components alkane inducible, substrate range C5–C10	Thysse & Van der Linden 1958 Van Eyk & Bartels 1970
P. aeruginosa 196 Aa (NCIMB 9571)	fuel	C7–C16	particulate multicomponent system, no rubredoxin detected, substrate range C7–C14	Traxler & Bernard 1969 Vandecasteele et al. 1983
P. aeruginosa (ATCC 17423)	–	C7	particulate and soluble fractions (more than two components)	Macham & Heydeman 1974
P. aeruginosa Sol 20 (NCIMB 8704)	–	C7	particulate, Fe^{2+} and O_2 dependent	Azoulay et al. 1963
P. denitrificans strain	–	C6, C10	three components, including a rubredoxin, and a flavin dependent protein	Kusunose et al. 1967
Pseudomonas sp.	soil	C8, C10	constitutive alkane oxidation	Robinson et al. 1964
*Pseudomonas sp.*X2	Thames	C10	no data	Bird & Molton 1967
Pseudomonas sp.	seawater	C10	three components, Fe^{2+}, cyanide sensitive alkane inducible	Hammer & Liemann 1976
P. putida strain	fuel	C8–C11	no data	Williams et al. 1981
Acinetobacter calcoaceticus 69V	soil	C13–C20	particulate monooxygenase, alkane-inducible rubredoxin, rubredoxin reductase	Kleber et al. 1973 Claus et al. 1980
Alcaligenes sp. L.16.1	seawater	C10–C18	NADH-dependent alkane hydroxylase cyanide sensitive, particulate	Bertrand et al. 1976

the hydroxylase (Peterson et al. 1966) (Fig. 1). The enzyme system follows the stoichiometry of external monooxygenases (McKenna & Coon 1970):

$$R - CH_3 + NADH + H^+ + O_2 \rightarrow$$
$$R - CH_2OH + NAD^+ + H_2O$$

Partially purified hydroxylase (alkane-1-monooxygenase, EC 1.14.15.3) was found not to be inhibited by carbon monooxide nor were spectral changes observed when oxygen was replaced by carbon monoxide, indicating that the hydroxylase does not contain heme (Peterson et al. 1966). The hydroxylase was purified by Ruettinger et al. (1974, 1977) as a high molecular weight aggregate (Mw $\approx$ 500,000). Under denaturing conditions this aggregate appeared to consist of polypeptides of 42,000 Daltons. The slightly yellow preparation contained about 20 phospholipid molecules, 1 atom of iron, but only traces of heme per polypeptide chain. The iron and phospholipids were essential for activity of the enzyme (Ruettinger et al. 1977). Cell fractionation experiments (Benson et al. 1977, 1979) and immuno gold labelling (Lageveen

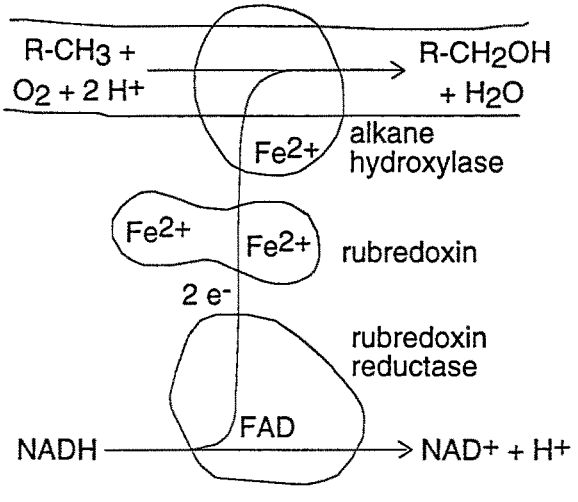

Fig. 1. Structure of the alkane hydroxylase system. The alkane hydroxylase system consists of three components: a membrane-bound monooxygenase, rubredoxin and rubredoxin reductase. The monooxygenase contains one atom of ferrous iron and requires phospholipids for its activity, rubredoxin contains two iron atoms, and rubredoxin reductase contains FAD.

1986) have shown that the enzyme is localized in the cytoplasmic membrane.

The second component of the alkane hydroxylase system was characterized as a rubredoxin (Peterson et al. 1967; Peterson & Coon 1968); a class of electron transfer proteins that contain one or more active centers consisting of a single iron atom with four cysteine sulfur atoms serving as the ligands in a tetrahedral structure, and have a red color (Lovenberg & Sobel 1965). The amino acid sequence of the *P. oleovorans* rubredoxin was determined almost completely by Benson et al. (1971). The *P. oleovorans* rubredoxin is almost three times the size of other bacterial rubredoxins, and consists of two very similar domains (connected by an 80 amino acid linker) which show about 45% sequence identity with other bacterial rubredoxins. Both domains bind one iron atom. The iron in the N-terminal domain is very loosely bound (Lode & Coon 1971), and is usually lost in the isolated protein. May et al. (1984) showed that the carboxy-terminal CNBr fragment suffices for alkane hydroxylase activity.

The third component, rubredoxin reductase, is a 55 kilodalton flavoprotein (Ueda et al. 1972; Ueda & Coon 1972). It transfers electrons from NADH to rubredoxin, which in turn transfers the electrons to the catalytic component; alkane hydroxylase. Both rubredoxin and rubredoxin reductase are soluble proteins.

As shown in Table 1, and discussed above, several strains may possess enzyme systems related to the *P. oleovorans* alkane hydroxylase system. We have recently found that antibodies directed against the three components of the *P. oleovorans* alkane hydroxylase (alk. hyd.) system also react with the three components of the *P. aeruginosa* KSLA 473 paraffin hydroxylase (Van Beilen, unpubl.).

Genetics of alkane oxidation by *Pseudomonas oleovorans*

Research on the genetics of alkane oxidation by *P. oleovorans* was initiated by Chakrabarty et al. (1973) who showed 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. 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 is now known to encode two other functions: mercury resistance (Harder & Kunz 1986) and D-lysine catabolism (Cao et al. 1993). Estimates of its molecular size have not been in agreement but tend towards a value of 350–400 kb (Palchaudchuri 1977: 45 kb; Hansen & Olsen 1978: 280-360 kb; Harder & Kunz 1986: 345 kb; Eggink 1987: 400–500 kb). 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).

The knowledge that the genes responsible for alkane oxidation are plasmid localized facilitated the classical genetic analysis that was undertaken by Shapiro and coworkers. When the OCT or CAM-OCT plasmids were introduced in a wild-type *P. putida* strain, the transformed strains acquired the ability to grow on n-alkanes. In addition, it was found that OCT 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 labelled *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 labelled *alkR* (Fennewald & 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 & Shapiro 1979), showed that the *alkB, alkA* and *alkC* loci are organized as an operon: *alkBAC*.

Experiments in which Tn*7* insertions outside the *alk* regions were used as unselected markers in reciprocal three-factor crosses indicated that *alkR* is located about 42 kb downstream of *alkBAC* (Fennewald et al. 1979). Interestingly, Tn*7S* (a slightly different transposon) was found to insert itself in the *alkBAC* cluster in only one orientation relative to the direction of transcription, while copies of Tn*7S* inserted within the *alkR* cluster were always oriented in the opposite direction. If Tn*7S* inserts itself into the CAM-OCT plasmid in one orientation only, as has been observed for other replicons, *alkBAC* and *alkR* should be transcribed in opposite directions (Owen et al. 1984; Owen 1986) (see Fig. 2).

Experiments in *alkB* or *alkA* mutants showed that unoxidized alkanes can induce the *alkBAC* operon (Benson & Shapiro 1975). Induction tests showed that primary alcohols are effective inducers as well, while some compounds such as dicyclopropylketone

and diethoxyethane are gratuitous inducers, as had been shown before for similar compounds in the case of *P. aeruginosa* KSLA 473 (Van Eyk & Bartels 1968). Some alkanes (C13 and C14) apparently cannot support growth only because they fail to induce alkane hydroxylase activity (Grund et al. 1975). The mutants in the *alkR* locus showed altered regulation of the *alkBAC* operon (Fennewald & Shapiro 1977): some mutants showed no induction, while others were constitutive, or had a more restricted range of inducers. Owen (1986) mapped and subdivided the mutants into substrate recognition and expression activation mutants, and assigned these to three different cistrons in the *alkR* locus.

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 not sufficient to enable *P. putida* to grow on alkanes. This was presumably because *alkR*, which encodes a transcriptional activator, was missing. 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 containing both the 16.9 and 18 kb fragments (Eggink et al. 1987b). This plasmid, pGEc47, enabled *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 β-oxidation pathway was expressed constitutively. This was a surprise because other *Pseudomonas* degradative plasmid genes seemed to be expressed inefficiently in *E. coli* (Frantz & Chakrabarty 1986). However, several promoters from catabolic plasmids such as TOL and NAH have now been shown to be functional in *E. coli* as well (De Lorenzo et al. 1993).

Analysis of the *alkBFGHJKL* 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, while the 58 kDa protein was identified as the NAD-independent alcohol dehydrogenase described by Benson & Shapiro (1976).

After DNA sequence analysis of the proximal half of the *alkBAC* operon (Kok et al. 1989a, 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 kD peptide) was found between the genes encoding the 15 and 49 kD polypeptides. Accordingly, the *alkBAC* operon was renamed *alkBFGHJKL*, based on the identification and location of the genes for the 6 polypeptides found in the minicell experiments, and on the identification of the additional open-reading-frame (*alkG*) (see below). The DNA sequence of the *alkJKL* region, and its function in alkane oxidation, were determined by Van Beilen et al. (1992b). Table 2 and Fig. 3 summarize the sequence data and show properties of the proteins encoded by the *alkBFGHJKL* operon. The DNA sequence was deposited in the EMBL nucleotide sequence database under accession number X65936.

AlkB

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). Contrary to earlier biochemical experiments (Ruettinger et al. 1977) no cysteines were found in the primary sequence. The primary sequence of alkB contained several stretches of hydrophobic amino acids, indicating that AlkB spans the cytoplasmic membrane up to nine times. This model has been tested by determining the alkaline phosphatase and β-galactosidase activities of a set of AlkB'-PhoA and AlkB'-LacZ fusion proteins with fusions at specific sites near the N- or C-terminal ends of presumed membrane spanning amino acid sequences. The properties of the fusion proteins showed that AlkB spans the membrane six times, exposing only three very short loops to the periplasm (Van Beilen et al. 1992a).

The only protein in the Swiss-Prot database (release 25) to show clear sequence homology with AlkB is XylM, the membrane component of xylene monooxygenase (Suzuki et al. 1991). An interesting feature

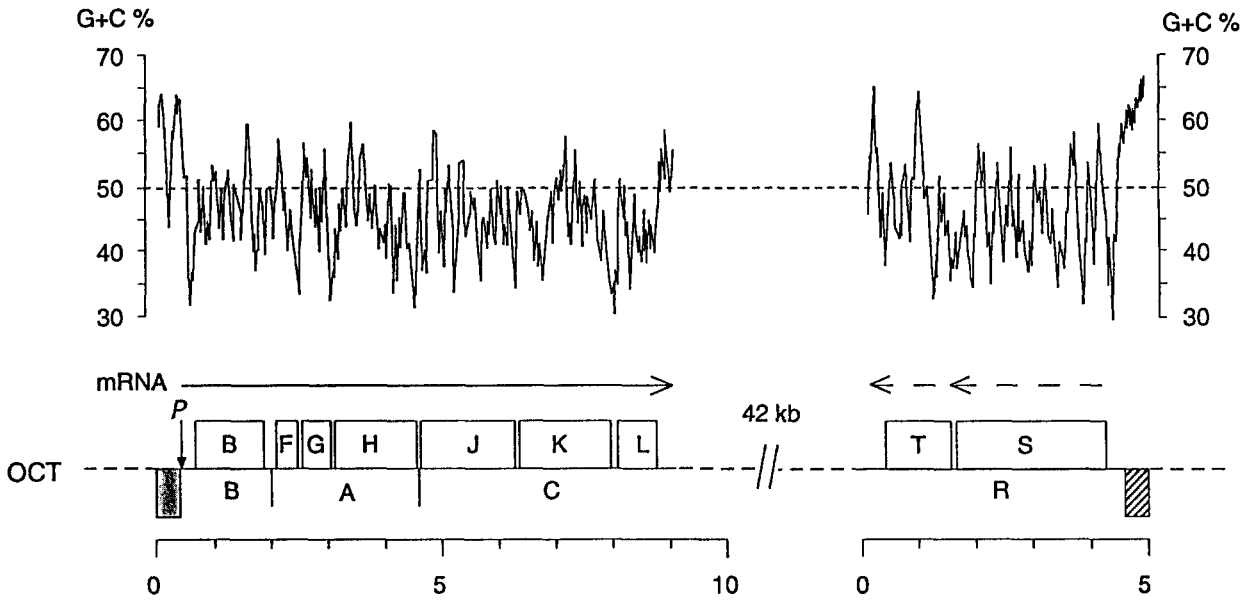

Fig. 2. G+C-content and relative position of the *alkBFGHJKL* and *alkST* gene regions. The two *alk* regions are located back-to-back on the OCT-plasmid. Length and direction of the *alkBFGHJKL* mRNA are from Eggink et al. (1987a). The *alkST* genes are transcribed from right to left, but the length of the mRNA(s) is not known (Eggink et al. 1988). The G+C-content was calculated using a sliding window of 100 bases. The grey box upstream of *alkB* denotes 87% identity with IS*53*. The hatched box upstream of *alkS* denotes 64% identity with IS*52*.

of the alignment is the conservation of four HXXXH sequence motifs. This sequence has been shown to bind divalent metals when it occurs in an α-helix (Arnold & Haymore 1991). Two of these HXXXH sequences might form a structure akin to the planar heme tetrapyrrole ring and bind iron.

AlkF and AlkG

The minicell experiments carried out by Eggink et al. (1987a) showed that the region directly downstream of *alkB* produced a peptide, presumably rubredoxin, while a small region further downstream appeared to be non-coding. DNA sequencing (Kok et al. 1989b) showed that this same region contained two open-reading-frames, the first (*alkF*) corresponding to the peptide found in the minicell experiments. However, the amino acid translation of the second ORF, *alkG*, was identical to the amino acid sequence of rubredoxin (Benson et al. 1971). AlkG probably did not shown up in [^{35}S]-methionine labeled minicells because the protein, as isolated, contains only one methionine (Kok et al. 1989b).

Previous amino acid sequencing of the *P. oleovorans* rubredoxin (Benson et al. 1971) had shown that it consists of two domains homologous to other rubredoxins (AlkG1 and AlkG2), separated by a linker. Deletion experiments showed that all clones which encoded the C-terminal domain of AlkG (AlkG2) were able to restore growth on n-alkanes in *P. putida* carrying a CAM-OCT *alkA*-plasmid, confirming the conclusion reached by May et al. (1984) on the basis of proteolysis experiments. Neither the N-terminal domain of AlkG (AlkG1) nor AlkF were able to complement this deletion (Kok et al. 1989b). The primary sequence of AlkF consists of a rubredoxin-like N-terminal domain (AlkF1), and an 80 amino acid C-terminal extension which shows only limited homology with AlkG.

Sequence comparisons showed that AlkF is the product of a duplication event of the N-terminal, inactive part of AlkG; AlkF1 and AlkG1 are more closely related to each other than to AlkG2. In a tree of identity generated using the program CLUSTAL (PC/GENE, IntelliGenetics, Geel, Belgium) of the three rubredoxin domains; AlkF1, AlkG1, AlkG2, with other rubredoxins in the SwissProt database release 25, AlkF1 and AlkG1 were placed outside a cluster containing AlkG2 and all other rubredoxins. Interestingly, although AlkF1 and AlkG1 appear to be non-functional, none of the cysteines that bind iron have

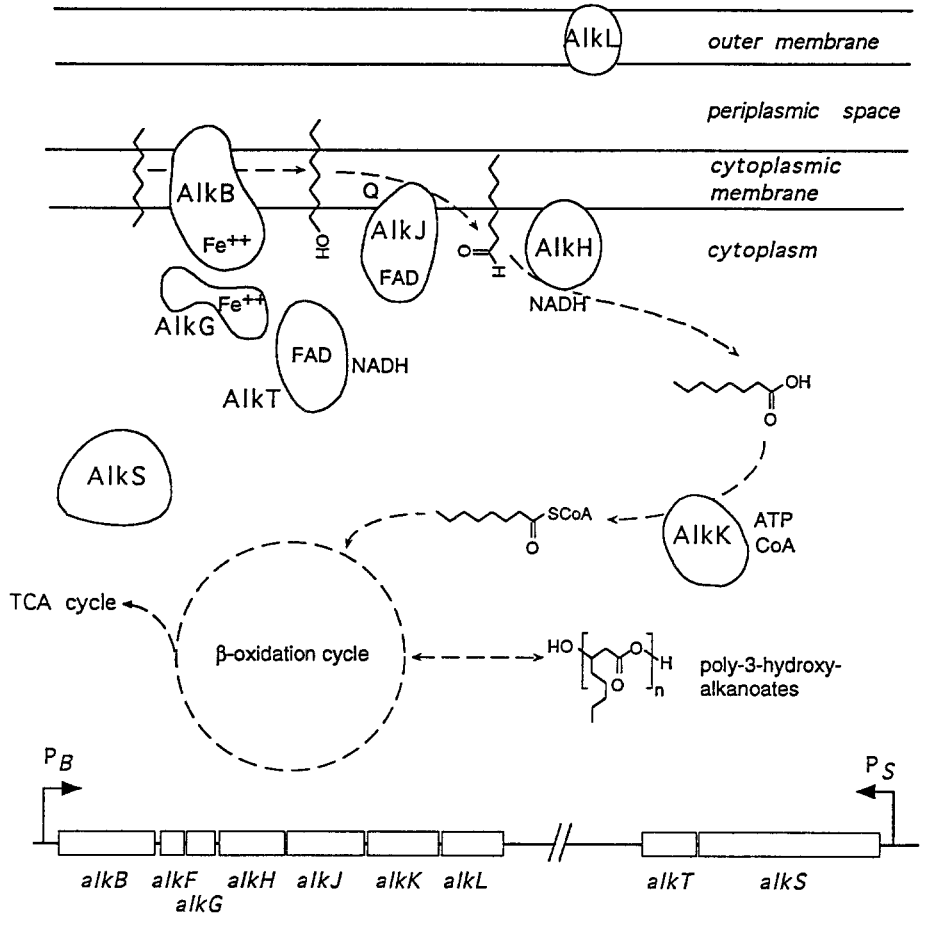

Fig. 3. Molecular genetics and pathway of alkane oxidation by *Pseudomonas oleovorans*. The *alk*-genes are organized in two clusters: the *alkST* locus and the *alkBFGHJKL* operon, both located on the OCT plasmid. Proteins are identified by their gene name (see Table 2 for full name and function).

changed. In contrast, the glycine residue that follows the second iron binding cysteine in all active rubredoxins (sequence motif: Cys-X-X-Cys-Gly) is changed in AlkF1 and AlkG1, which may explain why these rubredoxin domains are not functional.

AlkH

The *alkH* gene (part of the former *alkA* locus) was originally expected to encode rubredoxin reductase, as it produced a peptide of 49 kD in minicell experiments (Eggink et al. 1987a), close enough to the 55 kD found by Ueda et al. (1972) for rubredoxin reductase. The primary sequence, however, was found to resemble that of several mammalian aldehyde dehydrogenases, and the *alkH* gene indeed complemented a *P. oleovorans* aldehyde dehydrogenase mutant, albeit weakly. In addition, the calculated amino acid composition ruled out the possibility that the *alkH* gene encoded rubredoxin reductase (Kok et al. 1989b).

In a comparison of 11 ADH superfamilies, Horn et al. (1991) found that AlkH is most closely related to a rat aldehyde dehydrogenase. The rat-ADH is $NADP^+$-dependent, and is specific for aromatic aldehydes. In this respect it is interesting to note that the alkane hydroxylase system allows *P. oleovorans* to grow on ethylbenzene as well as on linear alkanes (Fukuda et al. 1989). As the *P. putida* chromosome encodes several aldehyde dehydrogenases (Grund et al. 1975), AlkH is not essential for growth on n-alkanes.

Table 2. Properties of the gene products of the *alkBFGHJKL* operon.

	Nucleotide Position	Ribosomal binding site	Size (amino acids; calculated; minicells)	Protein or function	Localization[1]	pI[2]	Purified	N-terminus[3]	Reference
AlkB	622-1825	UGGAGA(7)AUG	401, 45.7 kDa, 41 kDa	alkane hydroxylase	CM	7.24	YES	YES	Kok et al. 1989a
AlkF	2044-2439	AGGAGA(8)AUG	132, 14.6 kDa, 14 kDa	rubredoxin 1 (inactive)	cytoplasm?	4.90	NO	NO	Kok et al. 1989b
AlkG	2490-3008	UGGUGA(5)AUG	172, 18.7 kDa, ?	rubredoxin 2	cytoplasm	4.03	YES	YES	Kok et al. 1989b
AlkH	3058-4506	AGGACA(8)AUG	483, 52.7 kDa, 49 kDa	aldehyde dehydrogenase	CM?	10.05	NO	NO	Kok et al. 1989b
AlkJ	4548-6221	CGAGAA(6)AUG	558, 60.9 kDa, 58 kDa	alcohol dehydrogenase	CM	7.39	NO	YES[4]	Van Beilen et al. 1992b
AlkK	6284-7921	UGAGGC(7)AUG	546, 59.3 kDa, 59 kDa	acyl-CoA synthetase	cytoplasm	5.72	NO	NO	Van Beilen et al. 1992b
AlkL	8026-8715	CGAGGG(7)AUG	230, 25.0 kDa, 20 kDa	unknown	OM	4.32	NO	NO	Van Beilen et al. 1992b
AlkS	628-3273	CGAGAA(7)AUG	882, 99.8 kDa, 99 kDa	regulation of P_{alk}	unknown	9.93	NO	YES[4]	Wubbolts et al. submitted
AlkT	3326-4476	GGAGAG(6)AUG	385, 41.0 kDa, 48 kDa	rubredoxin reductase	cytoplasm	6.01	YES	YES	Eggink et al. 1990

[1] CM: cytoplasmic membrane; OM: outer membrane. [2] The pI was calculated from the primary sequence using the PC/GENE software package (IntelliGenetics, Geel, Belgium).
[3] YES: N-terminal sequence was determined, and agreed with the amino acid sequence deduced from the nucleotide sequence; NO: N-terminal sequence has not been determined.
[4] N-terminal sequence has been determined from protein blotted on PVDF-membrane.

AlkJ

In addition to the alkane hydroxylase system, the OCT-plasmid encodes a soluble cofactor-independent, membrane-bound alcohol dehydrogenase AlcO/AlkC (Benson & Shapiro 1976; Benson et al. 1977), which converts aliphatic medium chain length alcohols into aldehydes. Marker rescue (Owen et al. 1986) and complementation experiments (Eggink et al. 1987b) mapped this alcohol dehydrogenase function to a cistron corresponding to a 58 kDa protein in minicell experiments (Eggink et al. 1987a), which was subsequently designated *alkJ* (Kok et al. 1989b).

Sequence analysis of the *alkJ* gene showed it to encode a protein of 59 kDa, which shows significant homology with four flavin proteins; choline dehydrogenase, a glucose dehydrogenase and two oxidases (Van Beilen et al. 1992b), all of which are independent of soluble cofactors. The primary sequence of these proteins, including AlkJ, contains a characteristic fingerprint for ADP binding, at or close to the amino termini.

The enzymatic properties of AlkJ and the above sequence comparisons suggest that AlkJ is linked to the electron transfer chain. As is the case for AlkH, the *P. putida* chromosome encodes an equivalent for AlkJ (Chakrabarty et al. 1973); AlkJ is not essential for alkane utilization in wild-type strains.

AlkK

Eggink et al. (1987a) showed that the region downstream of *alkJ* encoded two more peptides; a 59 kDa and a 25 kDa protein, later designated AlkK and AlkL, respectively (Kok et al. 1989b). Neither had an apparent function in alkane oxidation: none of the *alk*-mutations obtained by classical mutagenesis mapped to this region, and both the *alkK* and the *alkL* gene could be deleted without affecting growth on n-alkanes (Eggink et al. 1987a). In addition, the other Alk-proteins already constituted a complete pathway from alkane to fatty acid.

DNA sequence analysis (Van Beilen et al. 1992b) showed that AlkK is homologous to a range of proteins which act by an ATP-dependent covalent binding of AMP to their substrates (Babbitt et al. 1992; Turgay et al. 1991). This list of proteins includes a number of acyl-CoA synthetases, which catalyze the first step in the metabolism of fatty acids. Subsequent complementation tests showed that the *alkK* gene does indeed complement a *fadD* (fatty acid CoA ligase) mutation in *Escherichia coli*. Cell fractionation experiments showed that, unlike FadD, AlkK is located in the cytoplasm (Van Beilen et al. 1992b).

AlkL

Like *alkK*, the *alkL* gene could be deleted without affecting growth on n-alkanes (Eggink et al. 1987a). Comparisons of the primary sequence (Van Beilen et al. 1992b) showed that AlkL is homologous to OmpW, a *Vibrio cholerae* outer membrane protein of unknown function, and a hypothetical polypeptide encoded by *ytt4* in *E. coli*. AlkL, OmpW and Ytt4 all have a signal peptide and end with a sequence characteristic of outer membrane proteins (Struyvé et al. 1991). The *alkL* gene product was found in the outer membrane of *E. coli* W3110 containing the *alk*-genes. This suggests that AlkL might be involved in transport of substrates. However, deletion of *alkL* did not affect the substrate range of whole cells containing the *alk*-genes. For the moment its function remains unknown.

Analysis of the alkST region

Genetic studies indicated that the *alkR* region encodes at least two functions: transcriptional activation of the *alkBFGHJKL* (*alkBAC*) operon and recognition of the inducer (Fennewald & 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 analysis 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 found to be necessary for activation of expression of the *alkBFGHJKL* operon (Eggink et al. 1988). The complete nucleotide sequence of *alkST* has been determined (Eggink et al. 1990; Wubbolts et al. submitted) and has been deposited in the EMBL database under accession number X73044. Table 2 and Fig. 3 summarize the available knowledge on AlkS and AlkT.

AlkS

The *alkS* gene sequence was recently determined by Wubbolts et al. (submitted). An open reading frame of 2646 nucleotides, corresponding to the AlkS polypep-

tide with a molecular weight of 99,833, was identified (Table 2). The *alkS* open reading frame was confirmed by amino-terminal sequencing of an AlkS-β-galactosidase fusion polypeptide. The molecular weight of the putative polypeptide agrees with the apparent molecular weight of AlkS (99 kDa) found in minicells (Eggink et al. 1988).

The carboxy-terminal 52 residues of the AlkS polypeptide are homologous to a helix-turn-helix (HTH) domain found at a similar position in a number of regulatory proteins belonging to the LuxR/UhpA family (Henikoff et al. 1990; Gross et al. 1989; Bairoch 1992: *PROSITE release 9.1* in EMBL database release 33.0). AlkS resembles MalT, the regulator of the maltose regulon (Schwartz 1987), along the complete sequence. Both regulators contain an ATP/GTP binding motif (Linder et al. 1989) near the amino-terminus. ATP binding to MalT is required for induction of the maltose regulon in *E. coli* (Richet & Raibaud 1989), suggesting that this might also be necessary for the AlkS dependent induction of the *alkBFGHJKL* operon.

AlkT

As discussed above (*alkH*), the third component of the alkane hydroxylase system; rubredoxin reductase, was previously expected to be encoded by the *alkA* region (*alkFGH*). However, analysis of the primary sequence of the *alkH* gene ruled this out (Kok et al. 1989b). The amino acid composition of the *alkT* gene product was found to be identical to the amino acid composition of isolated rubredoxin reductase (Ueda et al. 1972; Eggink et al. 1990). Using alignments between *AlkT* and a range of reductases, it was possible to identify the binding sites for NADH and FAD. The relative spatial positions of these binding regions were modelled, based on the 3-D structure of glutathione reductase; a protein that shows weak but significant sequence identity with AlkT (Eggink et al. 1990).

In in vitro experiments, AlkT can be replaced by ferredoxin reductase (Peterson et al. 1966). Apparently, AlkT can be replaced by other reductases in vivo as well, as Eggink (1987) found that it was possible to obtain mutants able to grow on n-octane from a *P. putida* recombinant containing only the *alkBFGHJKL* operon.

Table 3. Nucleotide composition of the *alkBFGHJKL* and *alkST* gene regions.

DNA region	Position	G + C
5' of *alkB*	1– 522	56%
5' of *alkB*	523– 621	38%
alkBFGHJKL coding regions	622–8715	46%
alkBFGHJKL non-coding regions	622–8715	38%
3' of *alkL*	8716–9092	52%
5' of *alkS*	1– 527	59%
5' of *alkS*	528– 627	42%
alkST coding regions	628–4476	45%
alkST non-coding region	628–4476	31%
3' of *alkT*	4477–4719	55%

The nucleotide sequences of the *alkBFGHJKL* and *alkST* genes

Table 3 and Fig. 2 show the G+C-content of the coding regions, the non-coding regions and the regions up- and downstream of the *alk*-operons. The average G+C-content of the coding regions is 46%, which is in marked contrast with the 62–67% of the rest of the OCT-plasmid (Fennewald et al. 1978) and the *P. oleovorans* chromosome (Mandel 1966). The non-coding regions separating the coding regions have an even lower average G+C-content (38%), like the 100 nucleotide region directly upstream of the *alkB* open-reading frame. The low G+C-content is expected to result in minimal secondary structure in these regions, which could promote initiation of translation.

The regions upstream of the *alkB* and *alkS* genes show a G+C-content of 56 and 59%, respectively, while the regions downstream of the *alkL* and *alkT* cistron show a G+C-content of about 55%, all much closer to that of the OCT-plasmid. Thus, the *alkBFGHJKL* operon and *alkST* region differ from the flanking DNA in this respect, which suggests they may not have evolved in *P. oleovorans*. This hypothesis is supported by the observation that the regions upstream of *alkS* and *alkB* contain sequences that are homologous to mobile genetic elements. The region upstream of *alkS* is highly homologous to IS*52* from *P. syringae* pv. *savastanoi* (Yamada et al. 1986) and IS*5* from *E. coli* (Schoner & Kahn 1981) (65 and 62% identity, respectively). The sequence preceeding *alkB* is highly homologous (87% identity) to IS*53*, also from *P. syringae* pv. *savastanoi* (Soby et al. 1993). The same sequence also resembles a

segment of the *Agrobacterium tumefaciens* Ti-plasmid that borders the T-region (58% identity).

Expression of the *alk*-genes in *P. putida* and *E. coli* hosts

Upon induction of the alk system, either by an n-alkane or by a gratuitous inducer such as dicyclopropylketone (Grund et al. 1975), synthesis of the components of the alkane hydroxylase system is initiated (Benson et al. 1979; Eggink et al. 1987b). Studies of the induction kinetics of the alkane hydroxylase membrane component AlkB in several hosts, such as the parent strain *P. oleovorans* or *E. coli* DH1, have shown that the level of AlkB increases rapidly for about 2 h after induction, and reaches 1.5% of the cellular protein. This corresponds to about 35000 copies per cell, or 25–30% of the total cytoplasmic membrane protein (Lageveen 1986; Eggink et al. 1987b).

The expression of the *alk*-genes in other host strains varies considerably. In one strain; *E. coli* W3110 (pGEc47), AlkB is expressed to 10–15% of total cellular protein, which has major effects on cell function and morphology (Nieboer et al. 1993). Addition of n-octane to steady state continuous cultures of this recombinant caused instability of the plasmid harbouring the *alk*-genes. This effect was not observed in E. coli HB101 (pGEc47) which showed a much lower but stable expression of alkane oxidation activity (Favre-Bulle et al. 1993).

The expression of the other Alk-proteins has not been studied in such detail. Like AlkB, rubredoxin (AlkG) is alkane inducible. In *P. oleovorans* it is expressed to 0.1–0.2% of total cellular protein (Kok et al. 1989b). The level of rubredoxin reductase (AlkT) was estimated to be about 0.05% of total cellular protein from the yield in a purification procedure (Ueda et al. 1972). AlkJ, AlkK and AlkL are expressed to very different levels, even though they are encoded by consecutive cistrons in the *alkBFGHJKL* operon (Van Beilen et al. 1992b). AlkF, AlkH, and AlkS are expressed to very low levels, and have not yet been identified on SDS-PAA gels.

Origin and physiological role of the *alk*-genes

Data obtained by Fennewald et al. (1979), and Owen (1986) indicate that the *alkBFGHJKL operon* and the *alkST* region are located back-to-back on the OCT-plasmid, separated by 42 kb of DNA, and flanked by DNA with a base composition which is clearly different from that of the *alk*-genes. The *alk*-DNA may therefore well be part of a single or composite 57 kb transposable element (Fig. 2), which may be responsible for the horizontal transfer of the *alk*-genes between (some of) the bacteria listed in Table 1. The same has been observed for several catabolic operons, which has led to the conclusion that large transposable elements are common in microorganisms that carry catabolic plasmids (reviewed in Van der Meer et al. 1992).

At this moment we can only speculate on the origin of the *alk*-genes. The homology of DNA flanking the *alk*-genes with DNA sequences from plant pathogens suggests a plant-pathogen or perhaps the plant itself as the origin of the *alk*-genes, as has been proposed for the T-DNA (Martinez-Zapater et al. 1993). As for the physiological function of the *alk*-genes, Nieder & Shapiro (1975) have shown that *P. putida* PpG6 (derived from *P. oleovorans*) uses the *alk*-genes to grow on n-alkanes. However, Fukuda et al. (1989) found that the same *alk*-genes also enables *P. oleovorans* to grow on ethylbenzene. In soil organisms, the *alk*-genes might allow growth on volatile compounds, including n-alkanes, released by plant residues (Bradow & Connick 1990), while in plants the same genes might have a function in the metabolism of the volatile compounds or of other compounds which have been shown to be substrates of the alkane hydroxylase system, such as fatty acids.

Conclusions and perspectives

The cloning and molecular genetic analysis of the *alk*-genes has made it possible to produce recombinant strains which may be useful in the production of fine-chemicals and synthons, such as alcohols, fatty acids and epoxides (Bosetti et al. 1992; Favre-Bulle 1992; Favre-Bulle et al. 1993). In addition, it has provided data on a unique class of non-heme iron monooxygenases, constituted only by the alkane hydroxylase and the TOL-plasmid encoded xylene monooxygenase XylM. Detailed analysis of the *alk*-genes has shown that the OCT-plasmid encodes all of the functions necessary to convert alkanes into the corresponding acyl-CoAs.

Several questions remain to be answered, however. Transcription and regulation of the *alkST* region has not yet been studied, while the functions of AlkF (rubredoxin 1) and AlkL (outer membrane protein) are still unknown. The genetic analysis of alkane oxida-

tion has also provided a number a new questions, related to the origin of the *alk*-genes. Are the *alk*-genes located on a transposable element which has inserted itself in the OCT-plasmid? Are *alk*-genes present in other organisms? Since many functions encoded by the *alk*-regions have chromosomal equivalents, does DNA downstream of *alkL* and *alkT* encode other functions related to alkane oxidation, but not essential for growth on alkanes in *P. putida* and *E. coli*? Cloning and sequencing of *alk*-genes from other bacteria listed in Table 1, and of DNA surrounding the *alkBFGHJKL* and *alkST* regions in the OCT-plasmid will undoubtedly answer many of these questions.

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