Summary

Our knowledge of the biochemistry and genetics of alkane utilization by *P. oleovorans* stems from the early seventies, when Coon and coworkers purified and characterized all three components of the alkane hydroxylase complex and Gunsalus and coworkers established the (OCT-) plasmid borne nature of the alkane hydroxylase activity in *P. oleovorans* (3). The genetic structure of the alkane oxidation pathway was subsequently determined by Shapiro and coworkers who identified the plasmid coded and chromosomal cistrons involved in the conversion of alkanes into fatty acids (1,2,8).

In the meantime Schwartz and McCoy (12,13) isolated several cold stable *P. oleovorans* variants, which they reported had enhanced epoxidation activities. One of these variants was successfully used to produce considerable amounts of 1,2-epoxyoctane from 1-octene in liquid two phase fermentations (9,14).

When in the early eighties the *alk* genes were cloned from *P. putida* in Shapiro’s lab at the University of Chicago (11), and from the cold stable variant *P. oleovorans* TF4-1L by Witholt’s group in Groningen (4,7), the genetic data could be refined. We now know that all the extrachromosomal information required for growth of *P. putida* (and certain *E. coli* mutants) on intermediary chain length alkanes is present on two *EcoRI* fragments encoding the *alkST* and the *alkBFCHJKL* operons (6).

In this thesis we extend the biochemical and genetic analyses of alkane utilization. First, we have compared the performance of several *P. oleovorans* and *P. putida* strains in two liquid phase systems, second, we determined the primary structure of two of the components of the alkane hydroxylase complex from nucleotide sequence data and third, we started the analysis of aliphatic alcohol utilization by *P. oleovorans*.

In Chapters 2 and 3 of this study we have described the construction and properties of several *Pseudomonas* recombinants carrying IncP-2 plasmids related to OCT. We found that the expression of alkane hydroxylase (*alkB*) was seriously hampered in *P. oleovorans* TF4-1L recombinants in which the OCT plasmid had been replaced by either the CAM-OCT or the R-OCT-1 fusion plasmid. Although mutants could be isolated in
which both normal alkane hydroxylase expression and growth on alkanes were restored, no 1-octene utilization or production of epoxyoctane could be detected in liquid two phase fermentations.

In contrast, the expression of the Alk phenotype was normal in all other Pseudomonas recombinants, all of which could grow on 1-octene and produced 1,2-epoxyoctane. This illustrated that the cold stable variant P. oleovorans TF4-1L clearly differed from the original soil isolate. The optimal specific epoxidation rates reached with both P. oleovorans strains in two phase fermentations were however comparable, and exceeded the performance of the P. putida recombinants.

In chapter 4 we describe the expression and primary structure of the 45 kDa membrane component of alkane hydroxylase. The alk promoter, which efficiently regulates the expression of alkB in E. coli and in Pseudomonas, was identified on a 91 bp DNA fragment lacking the structural features usually found in strong E. coli promoters. The alkBFGHJKL mRNA has a 96 nucleotide 5' untranslated region preceding the alkB coding sequence.

The alkB cistron encoding the cytoplasmic membrane component of alkane hydroxylase was identified by genetic complementation, immune precipitation of the 45 kDa translation product and by comparing the nucleotide sequence with the N-terminal sequence of the purified alkane hydroxylase. AlkB has eight uncharged hydrophobic stretches large enough to span the cytoplasmic membrane, supporting previously obtained information that it is an integral membrane protein.

One of the six hydrophilic sequences is expected to interact with the soluble cytoplasmic rubredoxin, an 18 kDa iron sulfur protein encoded by the alkG gene (Chapter 5).

The translation products of the alkF and alkG genes have a 54 amino acid sequence in common with rubredoxins isolated from various Gram-positive bacteria. In AlkG this sequence, which contains one iron sulfur center (10), is located at both the amino and the carboxy terminus of the protein. We have not determined whether such an iron sulfur center is also present in the N-terminal homology region of AlkF. It could however be used to replace the corresponding region in AlkG, yielding a functional rubredoxin in vivo, suggesting that the homology domains have similar structures. The alkF coding sequence is not essential for alkane utilization in Pseudomonas.
The fourth open reading frame of the alkBFGHJKLM operon encodes an aldehyde dehydrogenase, which shares significant (up to 37%) amino acid homology with aldehyde dehydrogenases isolated from eukaryotic organisms. However, the poor expression of alkH in P. oleovorans makes its role in alkane utilization questionable, especially since a very active aldehyde dehydrogenase is encoded by the P. putida chromosome.

The codon composition of the alkBFGH genes is unusual. There is little codon bias, the preference for a G or C in the third position usually found in Pseudomonas genes is absent, and several rare codons are frequently used. Nevertheless, alkane hydroxylase (alkB) is efficiently expressed in alkane induced Pseudomonas and E. coli, challenging the generally accepted role of codon composition in gene expression. On the other hand, the two soluble components of the enzyme, rubredoxin and rubredoxin reductase, are considerably less abundant.

Alkane hydroxylase converts alkanes into primary alcohols. These aliphatic alcohols are subsequently oxidized to aldehydes either by the plasmid encoded product of the alkJKL (5,8) cistrons or by the alcohol dehydrogenase activity encoded by the Pseudomonas chromosome (1). As a first step in the characterization of the chromosomally encoded alcohol utilization pathway we identified the alcA gene of P. oleovorans (Chapter 6). The alcA cistron could be precisely positioned by combining in vitro transcription and in vivo translation data. A 56 kDa polypeptide was identified as the product of the alcA locus.

Many questions concerning alkane utilization by P. oleovorans remain to be answered. The genetic basis of the acquired cold-stability of P. oleovorans TF4-1L and its relation to the inhibition of alkane hydroxylase expression remains a mystery and the enzymological significance of the overexpression of alkB (alkane hydroxylase) relative to alkG (rubredoxin) and alkT (rubredoxin reductase) is intriguing, as is the significance of alkF, alkH, alkJ, alkK and alkL, all of which are dispensable for growth on n-octane.

These and numerous other questions cannot be answered by merely looking at the genes. We have opened the oil barrel, looked inside, and now the time has come to do something with the contents.