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Physiology of aliphatic hydrocarbon-degrading microorganisms

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Abstract

This paper reviews aspects of the physiology and biochemistry of the microbial biodegradation of alkanes larger than methane, alkenes and alkynes with particular emphasis upon recent developments. Subject areas discussed include: substrate uptake; metabolic pathways for alkenes and straight and branched-chain alkanes; the genetics and regulation of pathways; co-oxidation of aliphatic hydrocarbons; the potential for anaerobic aliphatic hydrocarbon degradation; the potential deployment of aliphatic hydrocarbon-degrading microorganisms in biotechnology.

Introduction

Aliphatic hydrocarbons represent a wide range of potential substrates for microorganisms. They may be saturated (alkanes) or unsaturated (alkenes and alkynes). They range from gases, such as methane and ethane, through liquids to long-chain molecules of 40 or more carbon atoms that are solid at physiological temperatures. They may be straight-chain compounds, simple branched compounds or highly branched. However, they are all insoluble, hydrophobic molecules composed entirely of carbon-carbon and carbon-hydrogen linkages.

A wide variety of bacteria, filamentous fungi and yeasts can metabolize aliphatic hydrocarbon substrates. Although the identities assigned to organisms in older papers may be of questionable validity to the modern taxonomist, it is important to be aware of the diversity of microorganisms capable of degrading alkanes and alkenes. Table 1 is a partial list of genera of microorganisms that have been shown to metabolize aliphatic hydrocarbons. The physiology of microbial aliphatic hydrocarbon degradation has been extensively studied. From a fundamental viewpoint there has been particular interest in substrate uptake mechanisms and the metabolic processes responsible for initiating catabolism. There is also extensive interest in the application of aliphatic hydrocarbon-degraders in biotechnology. In a review such as this it is only possible to consider selected examples of physiology in order to illustrate the fundamental properties of microbial utilisation of these substrates. It is the aim of this paper to review recent developments in our understanding of the physiology and biochemistry of aliphatic hydrocarbon degradation and to relate fundamental knowledge to the potential applications of such organisms. A specific exception to the coverage will be the degradation of methane since the metabolism of this compound appears to represent a relatively specialised physiology confined to a distinct group of microorganisms (de Vries et al. 1990).

Microbial uptake of aliphatic hydrocarbons

Physicochemical properties of aliphatic hydrocarbons

Aliphatic hydrocarbons pose a variety of challenges to degradative microorganisms due to their fundamental physicochemical properties. Table 2 lists some basic properties of a few selected examples of n-alkanes, branched alkanes and alkenes. The physical state of the compounds at physiological temperatures may be gaseous, liquid or solid. It is generally true to state that the gaseous and liquid compounds are the most readily degraded but liquids of lower molecular weight may prove to be inhibitory to microorganisms by virtue of their solvent effect (Atlas 1981; Pfaender & Buckley 1984). However, the most significant property of aliphatic hydrocarbons with respect to their utilisation as metabolic substrates is their extremely limited solubility in water. As can be seen from Table 2, the solubility of aliphatic compounds rapidly decreases with increasing molecular weight. From a microbiological viewpoint, the solubility of aliphatic hydrocarbons can be considered as insignificant except for the compounds of very low molecular weight. As a consequence, microorganisms have had to develop a variety of specific adaptations in order to be able to utilise the majority of potential hydrocarbon substrates. These will be briefly discussed below and are, of course, equally relevant for the utilisation of other poorly soluble substrates.

Microbial adaptations for hydrocarbon uptake

The challenges to substrate uptake presented by the insolubility and hydrophobicity of aliphatic hydrocarbons may be met by microorganisms in a variety of ways. There are three possible routes for hydrocarbon uptake: soluble materials only; *via* microdroplets (i.e. droplets much smaller than the microbial cell); *via* macrodroplets. The uptake of aliphatic hydrocarbons following their dissolution in water is only feasible for the very low molecular weight compounds since the heavier compounds exhibit both negligible solubility and slow dissolution. This problem was elegantly modelled by Miller & Bartha (1989) who demonstrated uptake-limitation of the degradation of n-hexatricontane (nC_{36}) in aqueous cultures by comparing degradation rates before and after microencapsulation of the hydrocarbon substrate.

The uptake of hydrocarbon in droplet form is very common and frequently involves the production of biological surfactant molecules as emulsifying agents to produce microdroplets of hydrocarbon. In such cases the hydrocarbon droplets may be encapsulated within a surfactant micelle. Biological surfactants are described in detail elsewhere in this issue (see paper by Hommel). Droplets of any size or free-phase hydrocarbon material must then be taken up by the microorganisms and there exist a variety of adaptations to facilitate this. This has been extensively reviewed by Finnerty & Singer (1985). Many hydrocarbon-degrading microorganisms have highly hydrophobic cell surfaces and may frequently associate with hydrocarbon droplets or pass into the organic phase during growth. It has been widely demonstrated that extensive changes in membrane lipid composition occur during growth on alkanes (Singer & Finnerty

Table 1. Some genera of microorganisms that have been shown to metabolize aliphatic hydrocarbons other than methane.

Bacteria	Yeasts	Filamentous fungi
Acetobacter	Candida	Aspergillus
Acinetobacter	Cryptococcus	Cladosporium
Actinomyces	Debaryomyces	Corollaspora
Alcaligenes	Hansenula	Dendryphiella
Bacillus	Pichia	Gliocladium
Beneckea	Rhodotorula	Lulworthia
Corynebacterium	Sporobolomyces	Penicillium
Flavobacterium	Torulopsis	Varicospora
Mycobacterium	Trichosporon	
Nocardia		
Pseudomonas		
Rhodococcus		
Xanthomonas		

Selected information from Britton (1984); van Ginkel & de Bont (1986); Hommel & Kleber (1984); Kirk & Gordon (1988); Lindley et al. (1986); Nakajima et al. (1985); Wood & Murrell (1989).

1984; Ratledge 1978). In some cases this may represent an adaptation for cell association with the hydrocarbon phase. Ng & Hu (1989) showed that the production of biosurfactants by *Acinetobacter calcoaceticus* did not affect the association of the cells with the hydrocarbon phase. Microscopic studies of yeasts give evidence for pores in the cell wall that permit the penetration of hydrocarbons to the surface of the cell membrane (Scott & Finnerty 1976a). Transport across the membrane has generally been thought to be a passive process (Ratledge 1978) but there is evidence for an energy requirement for uptake in some yeasts (Scott & Finnerty 1976b; Bassel & Mortimer 1985). In either case intracellular hydrocarbon droplets can then be observed microscopically (Scott & Finnerty 1976a,b).

A particularly interesting bacterial system has been described by Kappeli & Finnerty (1979) for *Acinetobacter* HO1-N growing on n-hexadecane. This organism solubilises the hydrocarbon by encapsulating it in membrane microvesicles which are then taken into the cell by an active process (Singer & Finnerty 1984a). Intracellular structures have been observed in other organisms growing on hydrocarbons. For example, Watkinson (1980) described intracellular vesicles and tubules in a *Nocardia* which were suggested to play a role in hydrocarbon uptake. It should be noted, however, that intracellular inclusions produced during growth on hydrocarbons may be a consequence of hydrocarbon utilisation rather than an adaptation for uptake. Such structures are exemplified by microbodies in alkane-grown yeasts which are structures rich in oxidative enzymes (Fukui & Tanaka 1979). Indeed, Hommel & Ratledge (1990) showed that the fatty alcohol oxidases involved in n-alkane metabolism by *Candida bombicola* were solely present in the microsomal fraction. Whilst the details of hydrocarbon uptake systems in many yeasts and bacteria have yet to be elucidated, even less information is available concerning hydrocarbon uptake by filamentous fungi. Kirk & Gordon (1988) have demonstrated that some marine, alkane-degrading, filamentous fungi produce emulsifying agents which cause the production of hydrocarbon droplets. These are then surrounded and penetrated by hyphae.

Metabolic pathways for aliphatic hydrocarbons

The metabolic pathways responsible for the degradation of a wide variety of hydrocarbons have been reported in the literature. Generally, degradation can only be initiated under aerobic conditions since oxygenase reactions appear to be necessary for the initial metabolic activation of alkane molecules. Some reports of anaerobic degradation have been made and these will be critically discussed subsequently. Earlier literature tended to emphasise

Table 2. Physical properties of selected aliphatic hydrocarbons.

Compound	C atoms	Mol. wt.	m.p. (°C)	b.p. (°C)	Solubility (mg l^{-1})
Ethane	2	30.1	−172.0	−88.6	63.7
n-hexane	6	86.2	−94.3	68.7	12.3
n-decane	10	128.3	−31.0	174.0	0.05
n-hexadecane	16	226.4	19.0	287.0	5.2×10^{-5}
n-eicosane	20	282.6	36.7	343.0	3.1×10^{-7}
n-hexacosane	26	366.7	56.4	412.2	1.3×10^{-10}
2-methylpentane	6	86.2	−154.0	60.3	13.8
2,2,4-trimethylpentane	8	114.2	−107.2	127.0	2.4
4-methyloctane	9	128.3	–	142.0	0.12
1-hexene	6	84.2	−139.8	63.5	50.0
trans-2-heptene	7	98.2	−109.5	98.0	15.0
1-octene	8	112.2	−121.3	121.0	2.7

Data selected from Eastcott et al. (1988). m.p. is the melting point and b.p. the boiling point at normal temperature and pressure.

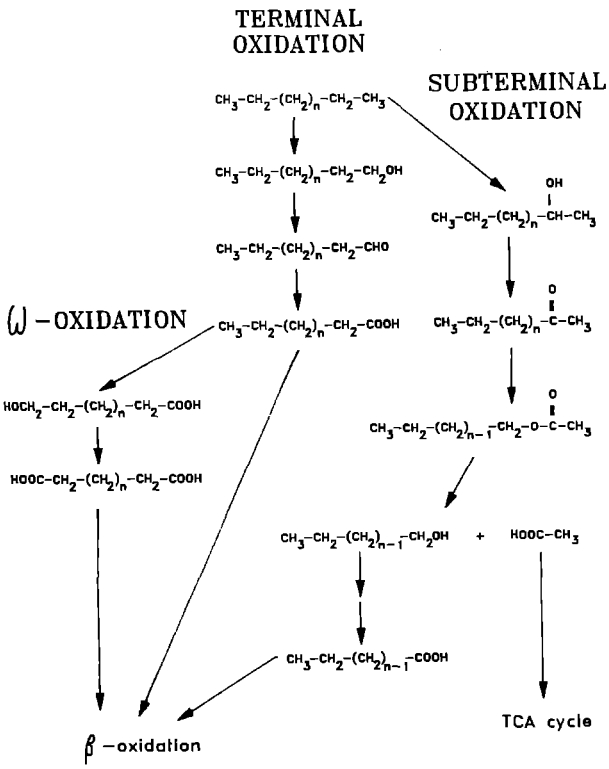

Fig. 1. Basic metabolic pathways for the degradation of n-alkanes. Illustrated are the three main metabolic routes documented for microorganisms: terminal oxidation; terminal oxidation followed by ω-oxidation; subterminal oxidation.

degradation of n-alkanes and, to a lesser extent, simple branched alkanes. This material has been extensively reviewed, for example by Ratledge (1978), Britton (1984) and Singer & Finnerty (1984a). Consequently, it is only necessary for the purposes of this review to reiterate briefly the basic principles of these metabolic pathways. Research described in recent years has resulted in a greater understanding in the metabolism of more complex branched alkanes, of alkenes and of co-metabolism of aliphatic compounds.

Metabolism of n-alkanes

Of the aliphatic hydrocarbons, it is the n-alkanes that are claimed to be the most rapidly degraded components in both laboratory culture and the natural environment (Wakeham et al. 1986; Kennicutt 1986; Oudot et al. 1989). The fundamental details of n-alkane degradation have been well documented (Britton 1984; Singer & Finnerty 1984a). Most microorganisms convert n-alkanes to the corresponding alkan-1-ol by means of a hydroxylase (monooxygenase) system:

$$R\text{-}CH_3 + O_2 + NAD(P)H + H^+ \rightarrow R\text{-}CH_2OH + NAD(P)^+ + H_2O$$

Hydroxylation reactions of this type may be linked to a number of types of electron carrier system. Systems linked to rubredoxin (e.g. *Pseudomonas putida*) and cytochrome P-450 (e.g. *Candida* spp.) have been the most thoroughly investigated. Dioxygenase systems have also been reported but these are less common. In these systems the n-alkanes are transformed into the corresponding hydroperoxides and subsequently reduced to the corresponding alkan-1-ol:

$$R\text{-}CH_3 + O_2 \rightarrow R\text{-}CH_2OOH + NAD(P)H + H^+ \rightarrow R\text{-}CH_2OH + NAD(P)^+ + H_2O$$

Subterminal oxidations of n-alkanes to secondary alcohols may also occur but this is rarer. For example, Rehm & Reiff (1982) investigated the initial mode of attack on C_8 to C_{18} n-alkanes by a variety of bacteria and fungi. Most organisms brought about terminal oxidation only but with certain *Aspergillus, Fusarium* and *Bacillus* spp. subterminal oxidation was detected. This resulted primarily in the production of 4-, 5-, or 6-substituted products with lesser amounts of 2- and 3- substituted compounds being produced.

Subsequent metabolism of the alcohol may follow a number of pathways as illustrated in Fig. 1. Following terminal oxidation, the produced alcohol is normally oxidised to the corresponding aldehyde and fatty acid by means of pyridine nucleotide-linked dehydrogenases. In some *Candida* spp., alcohol oxidases have been shown to be present in place of alcohol dehydrogenases. This has been reported, for example, by Blasig et al. (1988) for *Candida maltosa*, Kemp et al. (1988) for *C. tropicalis* and Hommel & Ratledge (1990) for *C. bombicola*. As an alternative to monoterminal oxidation, ω-oxidation may occur resulting in the pro-

duction of either or both α,ω-dioic acids and ω-hydroxy fatty acids. Rehm et al. (1983) and Blasig et al. (1988, 1989) have recently described the action of both mono- and diterminal oxidation systems in *Mortierella isabellina* and *Candida* spp., respectively. Woods & Murrell (1989) have reported that the propane-oxidising bacterium *Rhodococcus rhodochrous* can produce monoterminal and diterminal oxidation of C_2–C_8 n-alkanes *via* a system that is not linked to cytochrome P-450. In a survey of n-alkane oxidation in a number of microbial species, Rehm & Reiff (1982) observed diterminal oxidation products only rarely. All of these products may be further metabolized by means of the β-oxidation pathway for fatty acids. Subterminal alcohols are oxidised to the corresponding ester and hydrolytically cleaved to produce an acid and an alcohol. Following oxidation of the alcohol, the fatty acids produced may be metabolized via normal cellular pathways.

There is also some weak evidence for n-alkane metabolism via alkenes produced by the action of a NAD(P)-linked dehydrogenase. The alkene is purported to be hydroxylated across the double bond and further metabolized as described above. The existence of this pathway appears to be relatively dubious and even when reported is described as being relatively slow (Ratledge 1978; Singer & Finnerty 1984a). However, such a pathway does represent a means of alkane degradation independent of oxygenase activity and may therefore represent a potential route for anaerobic attack upon alkanes. This possibility is considered in more detail below. Recent observations of aromatic hydrocarbon degradation under anaerobic conditions also make one wary of discounting the possibility of other pathways for aliphatic hydrocarbon breakdown under anaerobic conditions.

Metabolism of branched-chain alkanes

Branched-chain alkanes tend to be less readily degraded than n-alkanes and in hydrocarbon mixtures degradation of branched compounds is generally repressed by the presence of straight-chain substrates (Pirnik et al. 1984). It is possible to make very general assertions about the relationship between the structure of branched alkanes and their degradability. Highly branched compounds are more recalcitrant to biodegradation than simpler compounds. Particularly recalcitrant are β-branched (anteiso-) and quaternary branched compounds due to steric hindrance of oxidation enzymes (Britton 1984). However, detailed correlation of structure and biodegradability is not possible (Singer & Finnerty 1984a) and even simple generalisations such as those given above do not hold true in all cases. For example, quaternary compound degradation has been described as in the case of the conversion of 2,2-dimethylheptane to 2,2-dimethylpropionate by '*Achromobacter*' sp. as cited by Singer & Finnerty (1984a).

It is becoming evident that degradation of a diverse range of branched alkanes can occur and that much of the reported recalcitrance of such compounds is due to the absence of suitable experimentation. The isoprenoid hydrocarbon pristane (2,6,10,14-tetramethylpentadecane) is commonly used as an internal marker in environmental hydrocarbon analysis since it is viewed as being highly persistent during the degradation of crude oil and petroleum products. However, its degradation has been widely studied and clearly elucidated in '*Brevibacterium*' sp. (Pirnik et al. 1974), *Corynebacterium* sp. (McKenna & Kallio 1971) and *Rhodococcus* sp. (Nakajima & Sato 1983). The metabolic pathways involved have been reviewed by Pirnik (1977) and degradation may occur by β- or ω-oxidation as illustrated in Fig. 2. Other complex branched alkanes have also been shown to be metabolized. Nakajima et al. (1985) described a *Rhodococcus* sp. capable of degrading phytane (2,6,10,14-tetramethylhexadecane), norpristane (2,6,10-trimethylpentadecane) and farnesane (2,6,10-trimethyldodecane) as sole sources of carbon and energy. In all cases isopropyl units on the molecules were oxidised to terminal alcohols and thence to the corresponding acids. Cox et al. (1976) reported a *Mycobacterium* sp. that could degrade phytane, norpristane, 2,6,10-trimethyltetradecane and 2,6,10,14-tetramethylheptadecane. Unlike the report of Nakajima & Sato (1983), initial attack upon the molecules did not occur only at isopropyl

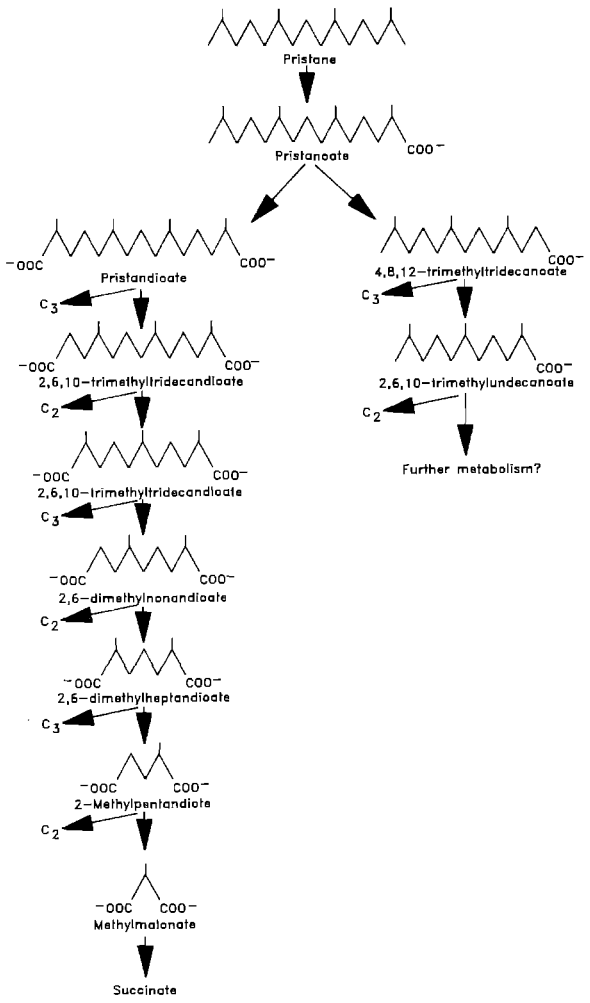

Fig. 2. Metabolic routes for pristane (2,6,10,14-tetramethylpentadecane) by microorganisms. This pathway serves as a model for the degradation of isoprenoid aliphatic hydrocarbons in bacteria. From Britton (1984), reprinted by courtesy of Marcel Dekker Inc.

termini but the products of initial oxidation were always terminal alcohols. In an exciting development, Rontani & Giusti (1986) have reported the utilisation of 2,2,4,4,6,8,8-heptamethylnonane as a sole carbon and energy source by a mixed marine microbial population. Degradation was relatively rapid and the only metabolic intermediates detected were straight-chain fatty acids. The metabolic pathway involved oxidation at the β-position to give the corresponding ketone. This was then oxidised to an ester which was hydrolysed prior to β-oxidation. A range of other highly-branched al-

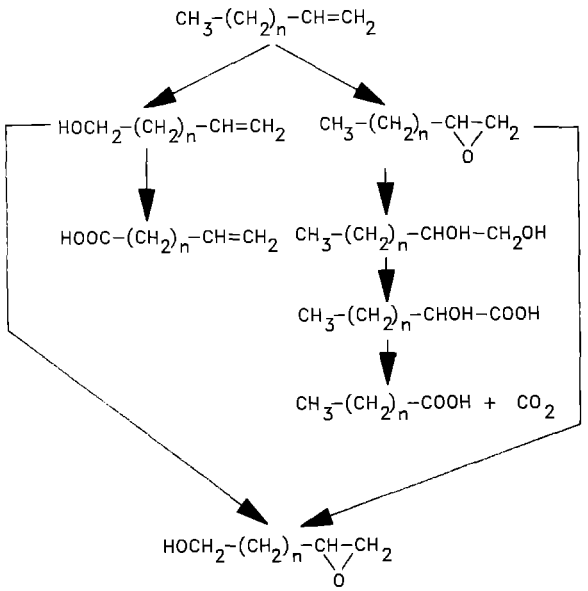

Fig. 3. Basic metabolic pathways for the degradation of alkenes in microorganisms. Initial attack on the molecule may be across the double bond to produce a subterminal epoxide or diol or may be analogous to n-alkane oxidation and leave the double bond initially intact.

kanes could be utilised provided that there was a free β-carbon available for oxidation.

Metabolism of alkenes

The metabolism of alkenes may be initiated either by attack upon the double bond or by an oxidation reaction elsewhere in the molecule as occurs for n-alkanes. Britton (1984) recognised four major patterns of initial attack: oxygenase attack upon a terminal methyl group to the corresponding unsaturated alcohols and acids; subterminal oxygenase attack to the corresponding alcohols and acids; oxidation across the double bond to the corresponding epoxide; oxidation across the double bond to the corresponding diol. A basic outline of the processes of initial metabolism is given in Fig. 3 but it is important to note that organisms may exhibit more than one of these pathways.

Recent studies have concentrated primarily on the metabolism of short-chain (hexene and below) alkenes and this work has been thoroughly re-

viewed by Hartmans et al. (1989). The basic pathways involved in the metabolism of short-chain compounds are as described above but several interesting observations have been made. van Ginkel & de Bont (1986) and van Ginkel et al. (1987) obtained *Mycobacterium, Nocardia* and *Xanthobacter* strains growing on short-chain alkenes which were unable to metabolize the corresponding alkanes since the only metabolic pathway present involved epoxidation of the double bond. This possession of a single pathway is very common in microorganisms capable of utilising short-chain alkenes but rare in those utilising longer-chain compounds. Particular interest has been focused upon the degradation of propene since this compound presents special difficulties to the degradative organisms. Oxidation reactions may result in the production of acrylate and then the non-degradable acrylate-CoA which thereby depletes the cellular CoA pool. As a means of overcoming this, strain PL-1 reported by Cerniglia et al. (1976) splits propene across the double bond into C_1 and C_2 units. The propene-utilising *Mycobacterium* and *Xanthobacter* strains described by de Bont et al. (1980) and van Ginkel and de Bont (1986) perform the same process *via* 1,2-epoxypropane.

Note that the aerobic degradation of alkynes can also occur, probably by means of hydratase activity (Hartmans et al. 1989).

Anaerobic degradation of aliphatic hydrocarbons

The anaerobic degradation of hydrocarbons has been a topic of intense scientific debate for many years. Recently, reliable evidence has been obtained demonstrating the degradation of aromatic hydrocarbons in anoxic environments (see later review by Smith). The anaerobic degradation of alkanes is a subject that still arouses diverse opinions. Bertrand et al. (1989) appear to be optimistic concerning the possibility of anaerobic alkane degradation despite the absence of convincing evidence that this is a real phenomenon. In contrast, Schink (1989) remains unconvinced of the possibility of anaerobic alkane degradation. Schink (1985a) has calculated that the anaerobic metabolism of alkanes to produce methane is an energetically favourable process but remains sceptical of the possibility that such processes occur since suitable enzyme systems to bring about such reactions have not been observed to date despite extensive research.

The most convincing evidence to date for the anaerobic degradation of aliphatic hydrocarbons has come from Schink (1985a). He added a large number of hydrocarbons to methanogenic enrichment cultures and monitored increased methane production due to their degradation. Of the substrates tested, including n-alkanes, branched alkanes and alkenes, only two, 1-hexadecene and squalene, increased methanogenesis. Repeated sub-culturing to ensure the complete elimination of alternative carbon sources confirmed the apparent degradation. For hexadecene, the extra methane detected accounted for 78–91% of the theoretical methane producible for this substrate. For squalene growth was much poorer and methanogenesis stopped after a period. It was calculated that the $\Delta G_0'$ values of such reactions were favourable for microbial growth and proposed that hydration across the double bond to produce an alcohol was the most likely reaction. However, Schink (1989) has noted that this may be either a biological or an abiotic chemical reaction and that there is no evidence for or against either of these possibilities. Thus, it can be concluded that attack upon alkenes appears feasible but substrate range has so far been found to be limited. For alkanes it would still appear that conclusive evidence is lacking. Certainly the potential metabolic reactions are energy-yielding but no enzymes have yet been described which are capable of initiating attack. It is possible that the proposed reduction of alkanes to alkenes could be a feasible route but this has not yet been conclusively demonstrated (Singer & Finnerty 1984a) and has certainly not been seen anaerobically.

One special case of anaerobic aliphatic hydrocarbon degradation is that of ethyne. This compound is commonly degraded anaerobically (Hartmans et al. 1989) and may also be converted by a variety of non-specific mechanisms, including that of nitrogenase. Degradation of ethyne has been obtained in pure culture. Schink (1985b) reported a new spe-

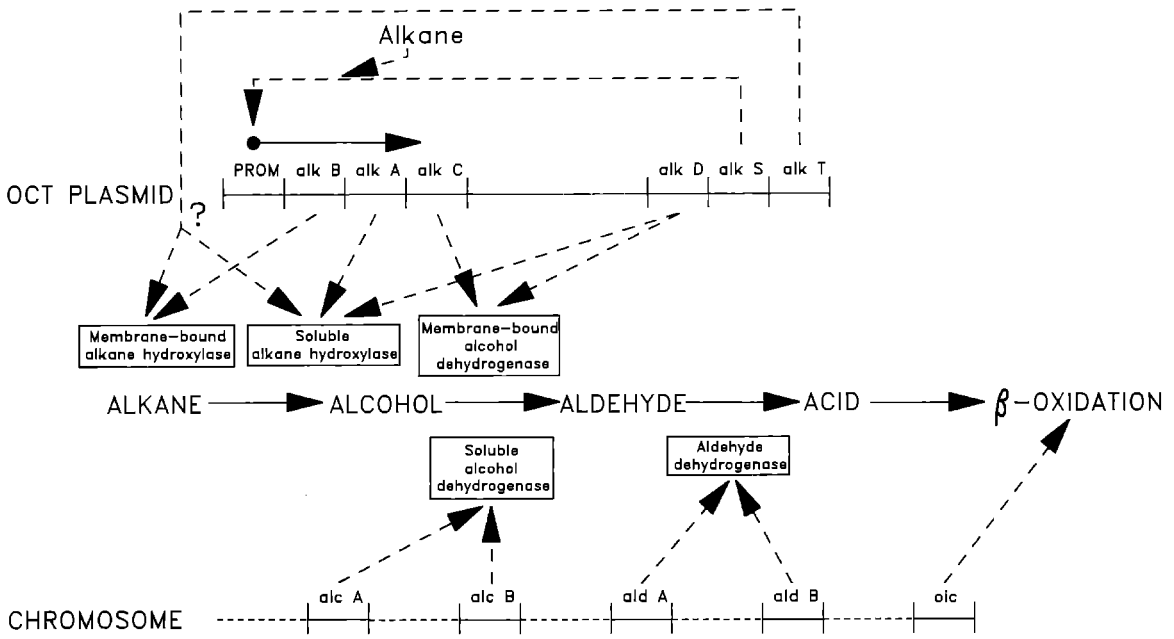

Fig. 4. Structure and function of the *OCT* plasmid and associated chromosomal genes in *Pseudomonas putida* PpG6. The *alk S* and *alk R* loci correspond to the area originally identified as the *alk R* regulatory locus. The *alk S* gene product is a regulatory protein that controls transcription of the *alk BAC* operon which is induced by n-alkanes. The role of the *alk T* gene product is unclear but it appears to be part of the alkane hydroxylase enzyme complexes. Chromosomal genes code for the soluble alcohol dehydrogenase, aldehyde dehydrogenase and β-oxidation enzymes. Information from Singer & Finnerty (1984a) and Eggink et al. (1987a, 1988).

cies (*Pelobacter acetylinicus*) that could grow on ethyne as a sole carbon and energy source. The degradation probably involved hydration to acetaldehyde followed by disproportionation to acetate and ethanol.

Genetics of aliphatic hydrocarbon degradation

The genetics of aliphatic hydrocarbon degrading organisms has only been studied in detail in a small number of microorganisms. The best studied system in bacteria is the *OCT* plasmid which codes for a number of proteins involved in growth on C_6 to C_{10} n-alkanes. This plasmid was originally intensively studied in *Pseudomonas putida* PpG6 (ATCC 17633) and the induction of the system, basic metabolic pathways and genetics were reported in a number of papers, most notably Chakrabarty et al. (1973), Nieder & Shapiro (1975), Grund et al. (1975) and Benson et al. (1977). The metabolic pathway for n-alkanes was found to involve an initial monooxygenation reaction by an alkane hydroxylase to produce the corresponding 1-ol. This was further oxidised to the corresponding aldehyde by an aldehyde dehydrogenase and then to the fatty acid by means of an aldehyde dehydrogenase. The fatty acids were then metabolized *via* the β-oxidation pathway. Fennewald and co-workers (Fennewald & Shapiro 1977, 1979; Fennewald et al. 1979) identified a number of loci on the plasmid and the chromosome of *Pseudomonas aeruginosa* which were involved in coding for the pathway. The designation of these was as follows: *alk* for alkane hydroxylase; *ald* for aldehyde dehydrogenase; *alc* for alcohol dehydrogenase; *oic* for a locus involved in the β-oxidation pathway. Genes identified on the chromosome were: *alc A* coding for soluble alcohol dehydrogenase with a substrate range of C_7 and above; *alc B* coding for soluble alcohol dehydrogenase with a substrate range of C_3 to C_6; *ald A* and *ald B* coding for the corresponding aldehyde dehydrogenases; *oic*. Among the apparent loci located on the plasmid were a promoter

sequence, regulatory genes and genes coding for subunits of alkane hydroxylase and alcohol dehydrogenase.

Extensive further research has enabled the construction of a detailed picture of the operation of the *OCT* system as illustrated in Fig. 4. Eggink et al. (1987a) cloned a region of *OCT* DNA containing the promoter, the *alk BAC* operon and the regulatory locus (then known as *alk R*) into *Escherichia coli* and non-degradative strains of *Pseudomonas putida*. Expression of the genes occurred demonstrating that this fragment contained all of the necessary information for production of the alkane hydroxylase and alcohol dehydrogenase activity. Using a 16.9 kilobasepair *Eco* RI fragment, Eggink et al. (1987b) were able to translate the genes of the *alk BAC* operon in an *E. coli* minicell preparation. They detected six proteins, of which four could be assigned a definite function: 41 kDa, membrane-bound alkane dehydrogenase; 15 kDa and 49 kDa, subunits of soluble alkane hydroxylase; 58 kDa, probably involved in membrane-bound alcohol dehydrogenase activity; 59 kDa; 20 kDa. The 41 kDa protein was associated with the *alk B* locus, the soluble alkane hydroxylase subunits with the *alk A* locus and the *alk C* locus appeared to code for the 58 kDa polypeptide. Kok et al. (1989) further studied the *alk B* region by sequencing. They defined a 401 amino-acid polypeptide with 8 hydrophobic regions of suitable size for spanning cell membranes. Extensive investigation of the *alk R* region suggested that there may be several operons present at this location (Owen 1986). Eggink et al. (1988) translated a 4.9 kilobasepair fragment from this region in an *E. coli* minicell preparation and identified two cistrons designated *alk S* and *alk T*. The former coded for a 99 kDa polypeptide which directly promoted transcription of the *alk BAC* operon. The *alk T* gene product was a 48 kDa protein of uncertain function although it was necessary for activity of alkane hydroxylase and did not act as a regulator of gene expression. It was concluded that this protein was part of the alkane hydroxylase complex.

Genetics of other alkane-oxidising bacteria has been far less extensively studied. Singer & Finnerty (1984b) reviewed observations on the genetics of alkane degradation in *Acinetobacter* HO1-N which can only utilise alkanes with a carbon chain length of C_{10} or above. Despite an extensive search no plasmids coding for any regions of the degradative pathway have been detected and it has been concluded that the pathway is encoded on the chromosome. Unlike the *OCT* system, only one set of enzymes is involved in oxidation of compounds of all chain lengths. Analysis of non-degradative mutants has demonstrated that the region of the chromosome coding for the alcohol and aldehyde dehydrogenase enzymes is totally independent of that coding for the initial alkane-oxidising enzyme.

The genetics of hydrocarbon degradation in yeasts has been little studied. Bassel & Mortimer (1985) illustrated the complexity of the genetic system in yeasts by identifying at least 16 loci that control functions affecting n-alkane uptake in *Yarrowia lipolytica*. One particular area that is receiving increasing interest has been the alkane-inducible cytochrome P-450 detected in certain *Candida* species. In *C. tropicalis* sequence analysis has shown that this inducible cytochrome is distinctly different from other P-450 cytochromes previously reported and it has been concluded that this represents a member of a new family of cytochrome P-450 (Sanglard et al. 1987; Sanglard & Loper 1989). A second cytochrome of this family has now been detected in alkane-induced *C. tropicalis*. The family has been termed P-450 LII and the cytochromes designated P-450 *alk 1* and *alk 2* (Sanglard & Fiechter 1989). A very similar inducible cytochrome P-450 has also been observed in *C. maltosa* (Suniari et al. 1988). Sequencing of this has shown that it is very closely related to the LII gene family of *C. tropicalis* and has shown that there are two highly hydrophobic regions of the molecule towards the N-terminus which apparently serve to anchor the molecule in the endoplasmic reticulum (Takagi et al. 1989; Schunk et al. 1989).

Biotechnological applications of aliphatic hydrocarbon metabolism

Aliphatic hydrocarbon-degrading microorganisms may have a number of potential biotechnological

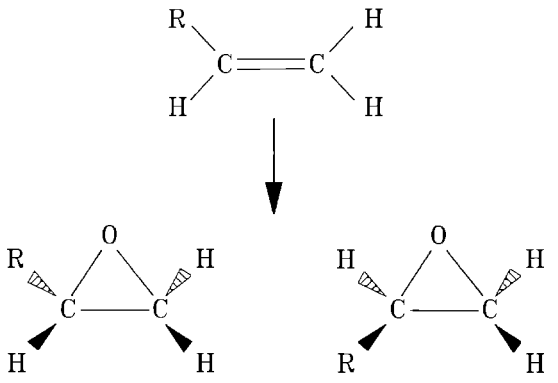

Fig. 5. Illustration of production of stereoisomers during epoxidation across the double bond of alkenes with a chain length of three carbon atoms or more.

applications. Much of the early research on biotechnology based upon these organisms focused upon the use of hydrocarbon substrates as feedstocks for single cell protein production but this proved to be an uneconomical process following the oil price rises of the early 1970s. More recent research has highlighted three areas where the physiology of aliphatic hydrocarbon degradation may have possible biotechnological application and these will be considered in turn.

Epoxide production

Epoxides are widely used as feedstocks in the synthetic chemical industry. The production of epoxides from alkenes has been extensively documented. When produced by co-oxidation reactions (e.g. by methane monooxygenase; MMO), by some alkane oxidation enzymes or by mutant strains blocked in the subsequent stage of alkene degradation, they may accumulate in the culture medium (Hartmans et al. 1989). Although epoxides are relatively toxic to microorganisms, developments in strain selection and bioengineering mean that it is possible to utilise bioreactor systems for the synthesis of epoxides. In particular, biocatalytic conversion of hydrocarbon substrates in two-phase culture systems have been shown to be widely applicable (de Smet et al. 1983a; Witholt et al. 1990). For the extensively used compounds epoxyethane and epoxypropane it is unlikely that biotechnologies will be able to compete on cost with chemical syntheses. However, alkenes of three carbon atoms or more are asymmetric when converted to epoxides and this permits the production of stereoisomers (Fig. 5). Chemical syntheses and some microbiological enzymes produce racemic mixtures but some microorganisms produce epoxides which are virtually optically pure (Weijers et al. 1988a). There is extensive interest in the use of specific stereoisomers as substrates for chemical syntheses in the pharmaceutical and fine chemicals industries and the potential application of biotechnology for stereospecific syntheses has been widely considered (Hartmans et al. 1989; Weijers et al. 1988b,c).

Production of aliphatic hydrocarbon-based metabolites

It has long been known that growth on specific hydrocarbon substrates results in the enrichment of specific fatty acids in the cell envelope (Ratledge 1978; Finnerty 1984). This enrichment could conceivably have a biotechnological role if the enriched compounds included a high-value fatty acid that was difficult to obtain from other sources, but is otherwise unlikely to be economically viable as a technology. The same would appear to be true of other compounds that can be obtained readily from other sources. For example, Shennan (1984) and Miall (1980) review the efforts devoted to the biotechnological production of citric acid and amino-acids by alkane-degrading microorganisms which were subsequently rendered uneconomical. Other metabolites may be of greater interest. Biosurfactants produced during growth on hydrocarbons have received a good deal of attention and their potential is reviewed elsewhere in this volume by Hommel. Ratledge (1984) reviewed work on the composition of wax esters produced by microorganisms during growth on alkanes. The composition and degree of unsaturation of these esters varies according to the substrate provided and certain waxes may represent viable alternatives to materials currently derived from plant or animal

sources. Extensive further work is necessary to develop this technology towards actual deployment.

De Smet et al. (1983b) reported the production of a poly-3-hydroxyalkanoate intracellular storage heteropolymer during the growth of *Pseudomonas oleovorans* on n-octane. The composition and degree of unsaturation of this polymer can be varied by cultivation of this organisms on C_6 to C_{12} n-alkanes and C_8 to C_{10} n-alkenes (Lageveen et al. 1988). By selection of the substrate and growth conditions, yields of up to approximately 25 g polymer per 100 g cell dry weight can be obtained in a fermentation process lasting 20 to 30 hours. These compounds have potential application in the production of biologically-derived plastics.

Environmental biotechnology

The use of biodegradative microorganisms to clean oil-contaminated soil and water is receiving increasing attention (Morgan & Watkinson 1989a). Exploitation of the physiology of aliphatic hydrocarbon-degrading organisms will of necessity be part of this process, but at the majority of locations it is probable that the indigenous microbial population will be adapted for degradation of the aliphatic hydrocarbons present. However, in special circumstances, when the indigenous population is prevented from developing by local environmental conditions or when particular substrates are to be degraded, it may be necessary to inoculate contaminated locations with specific degradative phenotypes. The co-oxidation of aliphatic hydrocarbons by methylotrophic bacteria has potential applications in environmental cleanup (Morgan & Watkinson 1989b) and may also be useful in biofilters for the cleaning of hydrocarbon-containing waste gas streams (Hartmans et al. 1989). Further research into these areas is necessary.

Conclusions

The microbial degradation of aliphatic hydrocarbons has long been known and many of the basic features of the physiology of degradative organisms had been considered to be well documented. Emphasis in recent years has tended to move away from studying aliphatic hydrocarbon degradation but recent developments in a number of key areas have shown that there is much still to be studied, for example pathways for the degradation of branched alkanes. The genetics and regulation of the degradation pathways for aliphatic hydrocarbons has recently made major advances, particularly in our understandings of the structure and function of the genes in the *OCT* plasmid. However, our knowledge of the pathways in other organisms has been little studied. Also requiring further elucidation are the detailed molecular processes involved in hydrocarbon transport into microbial cells. The potential for anaerobic degradation of most aliphatic hydrocarbons remains unclear and raises a number of fundamental scientific questions about the physiology of hydrocarbon degradation. Finally, attempts are now being made to exploit the physiological activity of alkane and alkene-metabolizing microorganisms in a number of areas of biotechnology. It is evident that much research is still necessary in these and other fields. It is hoped that future results will continue to be as interesting and novel as those discussed in this article.

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