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MINI-REVIEW

Bacterial metabolism of long-chain *n*-alkanes

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Abstract Degradation of alkanes is a widespread phenomenon in nature, and numerous microorganisms, both prokaryotic and eukaryotic, capable of utilizing these substrates as a carbon and energy source have been isolated and characterized. In this review, we summarize recent advances in the understanding of bacterial metabolism of long-chain *n*-alkanes. Bacterial strategies for accessing these highly hydrophobic substrates are presented, along with systems for their enzymatic degradation and conversion into products of potential industrial value. We further summarize the current knowledge on the regulation of bacterial long-chain *n*-alkane metabolism and survey progress in understanding bacterial pathways for utilization of *n*-alkanes under anaerobic conditions.

Keywords Bacterial alkane metabolism · Alkane degradation · Long-chain *n*-alkanes · Anaerobic · Aerobic · Wax ester

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Introduction

Alkanes are saturated hydrocarbons representing the main constituents of mineral oil. They can be linear (*n*-alkanes), circular (*cyclo*-alkanes), and branched (*iso*-alkanes), and are virtually insoluble in water. Besides their high inflammability, alkanes are probably the least reactive class of organic compounds. As main components in fuels and oils, they are of outstanding value for modern life, but the relative inertness of alkanes poses ecological problems upon their release to the environment. However, microorganisms have established effective strategies involving specialized enzyme systems and metabolic pathways to access *n*-alkanes as a carbon and energy source. Thus, such microorganisms are capable of degrading alkanes and converting them to easily metabolizable substrates. This review focuses on the metabolism of long-chain *n*-alkanes by bacteria, summarizes the knowledge about metabolic pathways involved in long-chain *n*-alkane degradation and conversion, and discusses perspectives for the development of industrial biotechnological processes based on genetically engineered long-chain *n*-alkane degrading bacteria.

In scientific literature on microbial alkane degradation, *n*-alkanes have frequently been referred to as short-chain, medium-chain, long-chain, and very long-chain *n*-alkanes, according to the length of their linear carbon chain. However, definitions of these classes vary dramatically and are often used in a rather imprecise and relative manner. This article will follow the broad consensus definition of long-chain *n*-alkanes starting with decane (C_{10}) as the member with the shortest chain length. Throughout this review, *n*-alkanes of defined chain lengths will be referred to as C_{10} (decane), C_{12} (dodecane), C_{16} (hexadecane), etc, in accordance with the number of carbon atoms they contain.

The microbiology of long-chain *n*-alkane utilization

Extensive knowledge has been accumulated on the microbiology of long-chain *n*-alkane degradation, and numerous novel strains capable of degrading these hydrocarbons have been described. In addition, in-depth characterization of genes and enzyme systems involved in the utilization of long-chain *n*-alkanes has led to an improved understanding of microbial long-chain *n*-alkane metabolism. A large number of microorganisms belonging to the phyla of eubacteria, yeast, and fungi and also some algae capable of using long-chain *n*-alkanes as carbon and energy source have been described (reviewed in van Beilen et al. 2003). Many of these microorganisms show an extended long-chain *n*-alkane substrate range, including also those *n*-alkanes, which are solid at ambient temperature (paraffin waxes, C_{18} and longer). A listing of isolated bacterial strains characterized for the degradation of those long-chain *n*-alkanes is presented in Table 1.

Some long-chain *n*-alkane-degrading species have been described to play an important role in bioremediation of oil-polluted marine environments. *Alcanivorax borkumensis*, capable of growing on long-chain *n*-alkanes up to C_{32}, is the most abundant species being found under such conditions (Kasai et al. 2001, 2002; Syutsubo et al. 2001). The genome sequence of *A. borkumensis* strain SK2 has been recently published (Schneiker et al. 2006), and a comparative proteome analysis of the strain grown on pyruvate and C_{16}, respectively, has been performed, providing interesting insights into the genetics of *n*-alkane degradation by this ubiquitous bacterium specialized in the degradation of hydrocarbons (Sabirova et al. 2006). In addition to *Alcanivorax*, an important role of the genus *Thalassolituus* in *n*-alkane degrading microcosms of temperate marine environments has lately been suggested (Coulon et al. 2007; McKew et al. 2007). *Thalassolituus oleivorans* was found to obligately utilize long-chain *n*-alkanes with a substrate range up to C_{20} (Yakimov et al. 2004). Not listed in Table 1 is the increasing number of not closer characterized microbial strains, shown to degrade long-chain *n*-alkanes of C_{18} and longer in complex mixtures like crude oil (Chaillan et al. 2004; Meintanis et al. 2006), fuel oil (Chaineau et al. 1999), diesel oil (Zhuang et al. 2003; Wongsa et al. 2004; Lee et al. 2006), light oil (da Cunha et al. 2006), lubricating oil (Wongsa et al. 2004), and paraffinic petroleum (Antic et al. 2006).

Thermophilic long-chain *n*-alkane-degrading strains are of special interest for future biotechnological applications (see below). Since the first description of a thermophilic *n*-alkane degrading bacterium by Mateles et al. (1967), alkane-degrading bacterial strains have been isolated from several high-temperature habitats like oil reservoirs and volcanic environments. Thermophilic *n*-alkane-utilizing strains have been identified as *Thermooleophilum* (Zarilla and Perry 1984; Perry 1985), *Thermomicrobium* (Phillips and Perry 1976), *Bacillus* (Zarilla and Perry 1987; Sorkhoh et al. 1993; Kato et al. 2001; Nazina et al. 2001; Feitkenhauer et al. 2003; Meintanis et al. 2006), *Geobacillus* (Nazina et al. 2001; Marchant et al. 2006; Meintanis et al. 2006; Wang et al. 2006), and *Thermus* species (Feitkenhauer et al. 2003; Hao et al. 2004). Several of these are also capable of using long-chain *n*-alkanes of C_{18} and longer (Table 1).

Microbial strategies for accessing long-chain *n*-alkanes as a carbon and energy source

Most substrates promoting microbial growth need to undergo cellular uptake or attachment to become accessible by the cell's catabolic machinery. This need, however, can be surpassed if hydrolytic enzymes (e.g., chitinases) are being secreted by bacteria and fungi to perform extracellular breakdown of substrates before uptake of the reaction products. In the case of water-insoluble substrates like *n*-alkanes, the hydrophobic nature of the bacterial cell surface has been reported to play an important role (Fletcher 1996). Here, the cell contact with hydrophobic substrates is crucial because the initial step in aliphatic and aromatic hydrocarbon degradation is often mediated by oxidation reactions catalyzed by cell-surface-associated oxygenases (Foster 1962; Rosenberg 1993). In the case of long-chain *n*-alkanes, two mechanisms for accessing these substrates are generally considered for bacteria: (1) interfacial accession by direct contact of the cell with the hydrocarbon and (2) biosurfactant-mediated accession by cell contact with emulsified hydrocarbons (Boulton and Ratledge 1984; Singer and Finnerty 1984; Haferburg et al. 1986; Hommel 1994; Bouchez-Naitali et al. 1999). For *Acinetobacter* sp. RAG-1 (Reisfeld et al. 1972), fimbriae on the cell surface were found to be crucial for growth of this strain on C_{16} (Rosenberg et al. 1982). These fimbriae were postulated to enable this strain to adhere to hydrophobic surfaces like long-chain *n*-alkane droplets, rendering these accessible for cellular uptake and metabolism. Concerning surfactant-mediated accession, most of the *n*-alkane-degrading bacteria studied produce and secrete biosurfactants of diverse chemical nature that allow emulsification of hydrophobic compounds (Desai and Banat 1997; Rosenberg and Ron 1999). The mode of action of biosurfactants has been extensively studied and reviewed earlier (Neu 1996; Ron and Rosenberg 2002; Maier 2003). For comprehensive recent reviews on the variety of biosurfactants, their production and biotechnological applications, we refer to Mukherjee et al. (2006), van Hamme et al. (2006), and Singh et al. (2007).

Table 1 Bacterial strains capable of utilizing long-chain *n*-alkanes of C_{18} and longer as a sole carbon source

Species, strain	Documented *n*-alkane substrate range	Reference
Acinetobacter baylyi ADP1	$-C_{36}$	(Throne-Holst et al. 2007)
Acinetobacter calcoaceticus EB104	C_9–C_{18}	(Kleber et al. 1983)
Acinetobacter calcoaceticus RR8	C_{10}–C_{34}	(Yuste et al. 2000)
Acinetobacter lwoffi	C_{12}–C_{28}	(Amund and Higgins 1985)
Acinetobacter sp. M-1	C_{13}–C_{44}	(Sakai et al. 1994)
Acinetobacter sp. ODDK71	C_{12}–C_{30}	(Koma et al. 2001)
Acinetobacter sp. S30	$-C_{33}$	(Lal and Khanna 1996)
Acinetobacter sp. DSM17874	C_{10}–C_{40}	(Throne-Holst et al. 2006)
Alcaligenes odorans P20	$-C_{33}$	(Lal and Khanna 1996)
Alcanivorax borkumensis AP1	$C_{10-}C_{20}$	(van Beilen et al. 2004)
Alcanivorax borkumensis SK2	C_8–C_{32}	(Schneiker et al. 2006)
Arthrobacter nicotianae KCC B35	C_{10}–C_{40}	(Radwan et al. 1996)
Bacillus thermoleovorans B23 and H41	C_9–C_{30}	(Kato et al. 2001)
Bacillus thuringiensis/cereus A2	C_6–C_{28}	(Chaerun et al. 2004)
Brachybacterium sp.	C_{10}–C_{20}	(Yan 2006)
Burkholderia cepacia RR10	C_{12}–C_{34}	(Yuste et al. 2000)
Desulfatibacillum aliphaticivorans CV2803	C_{13}–C_{18}	(Cravo-Laureau et al. 2004)
Dietzia cinnamea P4	C_{11}–C_{24}	(von der Weid et al. 2006)
Dietzia psychralcaliphila	C_{13}–C_{24}	(Yumoto et al. 2002)
Geobacillus thermodenitrificans NG80-2	C_{15}–C_{36}	(Wang et al. 2006)
Gordonia sp. TY-5	C_3, C_{13}–C_{22}	(Kotani et al. 2003)
Marinobacter hydrocarbonoclasticus 617	C_{16}–C_{30}	(Doumenq et al. 2001)
Marinobacter sp. BC36, BC38, and BC42	C_{18}	(Bonin et al. 2004)
Mycobacterium sp. CH1	C_{12}–C_{28}	(Churchill et al. 1999)
Mycobacterium sp. HXN 600	C_6–C_{24}	(van Beilen et al. 2002)
Mycobacterium sp. OFS	C_{11}–C_{28}	(Dunlap and Perry 1968)
Paracoccus sereniphilus/marcusii A7	C_6–C_{28}	(Chaerun et al. 2004)
Paracoccus sp. strains Ophe1 and Sphe1	C_{10}–C_{28}	(Zhang et al. 2004)
Planococcus alkanoclasticus MAE2	C_{11}–C_{33}	(Engelhardt et al. 2001)
Pseudomonas aeruginosa PAO1	C_{12}–C_{24}	(Smits et al. 2002)
Pseudomonas aeruginosa RR1	C_{12}–C_{34}	(Yuste et al. 2000)
Pseudomonas aeruginosa strains A1, A3, A4, A5, A6	C_6–C_{28}	(Chaerun et al. 2004)
Pseudomonas fluorescens CHA0	C_{12}–C_{32}	(Smits et al. 2002)
Pseudomonas sp. PUP6	C_{12}–C_{28}	(Naik and Sakthivel 2006)
Rhodococcus erythropolis	C_{10}–C_{30}	(Milekhina et al. 1998)
Rhodococcus erythropolis 23-D	C_6–C_{36}	(van Beilen et al. 2002)
Rhodococcus erythropolis NRRL B-16531	C_6–C_{36}	(van Beilen et al. 2002)
Rhodococcus erythropolis strains 42-O and 50-V	C_6–C_{32}	(van Beilen et al. 2002)
Rhodococcus fascians 115-H	C_6–C_{32}	(van Beilen et al. 2002)
Rhodococcus fascians 154-S	C_6–C_{24}	(van Beilen et al. 2002)
Rhodococcus rhodochrous	C_{12}–C_{20}	(Sorkhoh et al. 1990)
Rhodococcus sp. 1BN	C_6–C_{28}	(Andreoni et al. 2000)
Rhodococcus sp. NCIM5126	C_{13}–C_{20}	(Sharma and Pant 2000)
Rhodococcus sp. RR12 and RR14	C_{14}–C_{34}	(Yuste et al. 2000)
Rhodococcus sp. strain Q15	C_{10}–C_{32}	(Whyte et al. 1998)
Rhodococcus sp. strains T12 and TMP2	C_9–C_{22}	(Kunihiro et al. 2005)
strain AK01	C_{13}–C_{18}	(So and Young 1999b)
strain HdN1	C_{14}–C_{20}	(Ehrenreich et al. 2000)
strain Hxd3	C_{12}–C_{20}	(Aeckersberg et al. 1991)
Thalassolituus oleivorans	C_7–C_{20}	(Yakimov et al. 2004)
Thermooleophilum album	C_{13}–C_{20}	(Zarilla and Perry 1984)
Thermus sp. C2	C_9–C_{39}	(Hao et al. 2004)
Weeksella sp. RR7	C_{12}–C_{34}	(Yuste et al. 2000)
Xylella fastidiosa RR15	C_{14}–C_{34}	(Yuste et al. 2000)

Aerobic bacterial long-chain *n*-alkane degradation pathways

During the past decades, research related to alkane degradation has focused on the identification and characterization of enzymes involved in the initial step of aerobic bacterial catabolic pathways. In most described cases, the *n*-alkane is oxidized to the corresponding primary alcohol by substrate-specific terminal monooxygenases/hydroxylases. However, subterminal oxidation has also been described both for long-chain *n*-alkane substrates up to C_{16} (Britton 1984; Whyte et al. 1998) and for *n*-alkanes of shorter chain-lengths (Ashraf et al. 1994; Sullivan et al. 1998). The class of alkane hydroxylases involved in bacterial aerobic *n*-alkane metabolism has recently been reviewed in detail by van Beilen and Funhoff (2007). In that review, two unrelated classes of enzymes for long-chain *n*-alkane oxidation were proposed: (1) the class of cytochrome-P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (2) the class of bacterial particulate alkane hydroxylases (pAHs). The latter class of integral membrane non-heme diiron monooxygenases of the AlkB-type allows a wide range of *Proteobacteria* and *Actinomycetales* to grow on *n*-alkanes with carbon chain lengths from C_5 to C_{16}. AlkB-type enzymes function in complex with two electron transfer proteins, a dinuclear iron rubredoxin, and a mononuclear iron rubredoxin reductase channeling electrons from NADH to the active site of the alkane hydroxylase (van Beilen et al. 2003). After initial oxidation of the *n*-alkane, the corresponding alcohol is subsequently oxidized further by alcohol dehydrogenase and aldehyde dehydrogenase to the corresponding aldehyde and carboxylic acid, respectively. The carboxylic acid then serves as a substrate for acyl-CoA synthetase, and the resulting acyl-CoA enters the β-oxidation pathway (Fig. 1). The most extensively studied bacterial alkane degradation pathway of this sequence of enzymatic conversions is that of *Pseudomonas putida* GPo1 (formerly *Pseudomonas oleovorans*) encoded on the OCT plasmid (Baptist et al. 1963; Chakrabarty et al. 1973; van Beilen et al. 1994a, 2001). For details and a comprehensive overview on the *alk* pathway, we refer to the review by van Hamme et al. (2003).

For many *n*-alkane degraders, multiple alkane hydroxylases have been reported exhibiting overlapping substrate ranges, including both pAHs and cytochrome P450 enzymes. For example, *Rhodococcus erythropolis* contains up to five pAHs and two CYP153s (van Beilen et al. 2006), whereas *A. borkumensis* SK2 contains two pAHs with overlapping substrate specificity ($alkB_1$: C_5–C_{12}, $alkB_2$: C_8–C_{16}) and three CYP153 enzymes with yet undetermined substrate range (Sabirova et al. 2006; Schneiker et al. 2006). Further enzyme candidates involved in long-chain *n*-alkane oxidation up to C_{32} have been deduced from the *A. borkumensis* genome sequence, including putative monooxygenases and oxidoreductases. Also, for other bacterial isolates shown to metabolize long-chain *n*-alkanes with C-chains of C_{18} and longer (Table 1), additional enzyme systems have been proposed. However, only a few yet uncharacterized enzymes have been suggested to catalyze oxidation of these long-chain *n*-alkanes. For example, for *P. fluorescence*, a yet uncharacterized long-chain *n*-alkane oxygenase was postulated, enabling this strain to grow on *n*-alkanes ranging from C_{18} to C_{28} (Smits et al. 2002). For *Acinetobacter* sp. M-1, a flavin-containing *n*-alkane dioxygenase with a proposed substrate range from C_{10} to C_{30} has been reported (Maeng et al. 1996). This strain was later

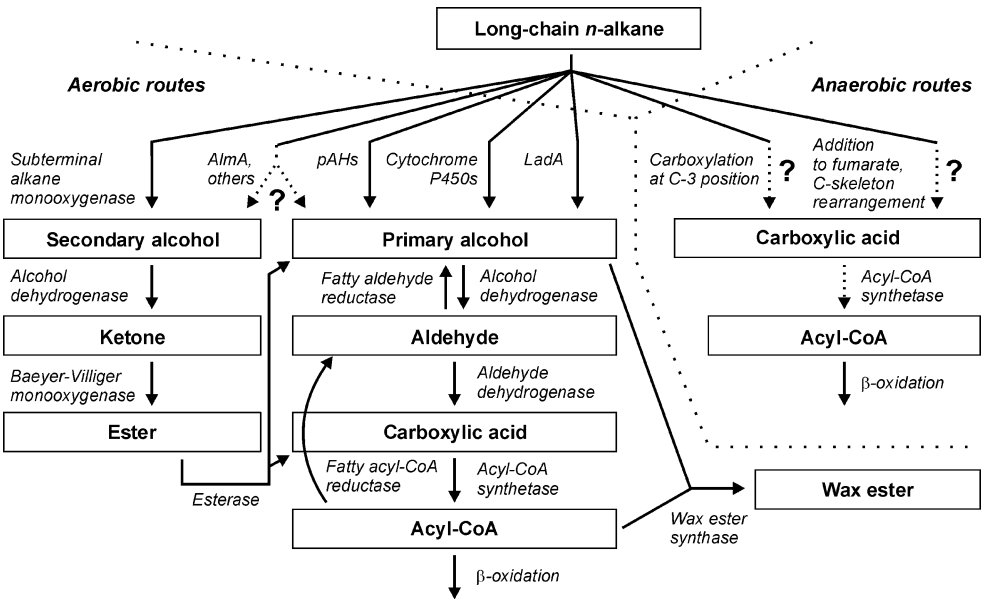

Fig. 1 Pathways for aerobic and anaerobic bacterial degradation of long-chain *n*-alkanes and for synthesis of wax esters. *Dotted arrows* represent suggested metabolic routes

shown to also harbor two integral membrane *n*-alkane hydroxylases covering the same substrate range (Tani et al. 2001). Using a novel high-throughput screening system for analyzing growth of transposon mutants of another *Acinetobacter* strain, *Acinetobacter* sp. DSM 17874, on solid *n*-alkanes, several genes with potential functions specifically in long-chain *n*-alkane metabolism have lately been identified (Throne-Holst et al. 2007). This strain, growing on *n*-alkanes from C_{10} to C_{40}, had earlier been described to also harbor two AlkB-type pAHs with overlapping substrate preferences ranging from C_{10} to C_{18} (Throne-Holst et al. 2006). One of the novel genes identified, designated *almA*, encodes a putative monooxygenase of the flavin-binding family. This gene was analyzed in more detail and found to be involved in the utilization of *n*-alkanes with a chain length of C_{32} or longer. *almA* represents the first cloned gene encoding an enzyme specifically involved in the degradation of *n*-alkanes with carbon chains longer than C_{30}. Genes homologous to *almA* have been identified in and cloned from *Acinetobacter* sp. RAG-1 (Reisfeld et al. 1972), *Acinetobacter* sp. M-1 (Sakai et al. 1994), and *Acinetobacter baylyi* ADP1 (Juni and Janik 1969; Patel et al. 1975; Vaneechoutte et al. 2006). In addition, sequence homology analysis has suggested the presence of similar enzymes also in *Marinobacter aquaeolei* VT8, *Oceanobacter* sp. RED65, *Ralstonia* spp., *Mycobacterium* spp., *Photorhabdus* sp., *Psychrobacter* spp., and *Nocardia farcinica* IFM10152. Further investigations will be necessary to characterize AlmA with respect to its possible function in the oxidation of long-chain *n*-alkanes. Lately, genome and proteome analysis of *Geobacillus thermodenitrificans* strain NG80-2 isolated from a deep-subsurface oil reservoir (Wang et al. 2006) revealed a plasmid-encoded novel thermophilic enzyme for terminal oxidation of *n*-alkanes, with no detectable similarity to other alkane oxidizing enzymes known to date (Feng et al. 2007). This enzyme, designated LadA, was found to be expressed in NG80-2 when grown on crude oil and hexadecane. By complementation of the AlkB functionality in an *alkB* knockout mutant strain of *Pseudomonas fluorescence* CHA0 and in vitro analysis of the LadA protein purified after heterologous expression in *Escherichia coli*, this enzyme was characterized as a thermophilic soluble long-chain alkane monooxygenase for terminal oxidation of long-chain *n*-alkane substrates ranging from C_{15} to C_{36} (Feng et al. 2007).

Regulation of genes involved in aerobic *n*-alkane utilization in bacteria

Early studies by Thijsse and Linden (1958) have shown that *n*-alkane utilization by *Pseudomonas aeruginosa* is inducible, and later the same trait has been demonstrated for other *n*-alkane-oxidizing bacterial species (Perry and Scheld 1968). However, some uncertainty remains regarding the nature of the inducers, which seem to be species-specific. Utilization of *n*-alkanes has been shown to be induced by both alkanes and alkanols in *P. putida* and *Burkholderia cepacia* (Grund et al. 1975; Marin et al. 2001), while in *Pseudomonas butanovora* only alkanols and aldehydes have been identified as inducers (Sayavedra-Soto et al. 2005).

The initial studies on the genetics of regulation of the *n*-alkane utilization have been performed on *P. putida* GPo1, described to utilize *n*-alkanes from C_5 to C_{14} (van Beilen et al. 1994b). *P. putida* GPo1 was shown to harbor an OCT plasmid carrying the *alkBAC* operon containing the genes for *n*-alkane utilization later identified as *alkBFGHJKL* (Kok et al. 1989a, b; van Beilen et al. 1992), and a regulatory locus *alkR* (Fennewald et al. 1979). The latter locus was shown to contain the *alkS* gene responsible for activation of the *alkBFGHJKL* operon expression (Eggink et al. 1988). Notably, the expression of the *alkBFGHJKL* genes involved in *n*-alkane utilization upon induction by *n*-alkanes have been shown to significantly affect the physiology of *P. putida* GPo1, leading to lower growth rate and filamentation on glucose as a carbon source (Chen et al. 1996). Authors have suggested that overproduction of the AlkB hydroxylase, which is a membrane protein, may be responsible for the observed phenotype. Furthermore, it has been shown that prolonged incubation of *P. putida* GPo1 on *n*-alkane-containing medium resulted in the loss of *n*-alkane-oxidizing activity, which was suggested to be due to the downregulation of the *alkBFGHJKL* operon (Chen et al. 1996). This phenomenon can potentially cause a problem for a long-term biotechnological process based on *n*-alkane utilization/degradation because it would rely on an enzyme system required for good productivity while at the same time being deleterious to the host if it is being overexpressed.

To better understand the regulation of the *alkBFGHJKL* genes expression, Yuste et al. (1998) constructed a reporter *P. putida* strain carrying the *lacZ* reporter gene under control of the P*alkB* promoter, which initiates the expression of the *alk* operon. It has been demonstrated that the expression from the P*alkB* promoter depends strongly on the carbon source available to *P. putida*, implying the involvement of a catabolite repression in regulation of the *n*-alkane degradation.

Further work on the molecular mechanism of *alk* operon regulation in *P. putida* has demonstrated that the positive regulator AlkS is expressed poorly during the exponential phase of growth, while its expression increases considerably when cells enter the stationary phase (Canosa et al. 1999). The subsequent study has revealed that AlkS expression is controlled by two promoters, and that this protein regulates its own expression both positively and

negatively (Canosa et al. 2000). In the absence of *n*-alkanes, expression of the *alkS* gene occurred mostly from the σ^S-dependent P*alkS1* promoter, which provides for relatively high level of expression only during stationary phase. When the *n*-alkanes have been present in the growth medium, AlkS was shown to strongly repress the P*alkS1* promoter, while activating its own expression from a second promoter, P*alkS2*. Transcription from P*alkS2* has been shown to be a subject for catabolite repression, and it has been suggested that the expression of *alkS* is regulated by a positive feedback mechanism. Such mechanism should allow both rapid induction of the *n*-alkane utilization pathway, and a fast downregulation thereof when the *n*-alkanes are consumed.

Regulation of *n*-alkane utilization has been studied in some detail for *A. baylyi* ADP1, which is capable of degrading long-chain *n*-alkanes with carbon chain length of C_{12} and more. Studies by Ratajczak et al. (1998) revealed that expression of alkane hydroxylase *alkM* in ADP1 is induced by C_7 to C_{11}, which are not utilized by this strain, and long-chain *n*-alkanes from C_{12} to C_{18}, which are metabolized. Transcription of *alkM* depended strictly on the AlkR, positive regulator of the AraC-XylS family normally expressed at a low level. The mechanism of regulation of *n*-alkane degradation by AlkR in *A. baylyi* ADP1 must therefore be different from the one involving AlkS in *P. putida* GPo1. While AlkS induces the expression of the *P. putida* GPo1 *alkBFGHJKL* operon in response to *n*-alkanes and the respective primary alcohols (Grund et al. 1975), the ability of AlkR to activate *alkM* expression in *Acinetobacter* depends on the presence of *n*-alkanes with chain lengths above C_6 (Ratajczak et al. 1998). Moreover, an inhibitory effect of oxidized *n*-alkane derivatives on the expression of *alkM* in ADP1 has been noticed. It seems interesting that the genes for rubredoxin, and rubredoxin reductase, which are components of the AlkM hydroxylase complex, are constitutively expressed in ADP1 and do not seem to be subject to regulation in response to the presence of *n*-alkanes (Geissdorfer et al. 1999). It seems plausible that both these proteins, in addition to alkane utilization, are involved in other redox reactions important for normal cell functioning. Constitutive expression of rubredoxin and rubredoxin reductase genes have also been demonstrated in *P. aeruginosa* (Marin et al. 2003) and *Acinetobacter* sp. M-1 (Tani et al. 2001). In the latter strain, capable of degrading *n*-alkanes with chain length up to C_{44}, the expression of two alkane hydroxylases, AlkMa and AlkMb, has been shown to be modulated by two independent regulatory proteins, AlkRa and AlkRb, respectively. It has been demonstrated that, while *alkMa* expression was induced by long-chain *n*-alkanes $>C_{22}$, *alkMb* expression was preferentially induced by the *n*-alkanes with chain lengths of C_{16} to C_{22}. A regulation strategy different to that reported for the *P. putida* GPo1 *alk* genes was also found in *A. borkumensis*. In strain AP1, expression of the two genes encoding pAHs (*alkB1*, *alkB2*) was induced by *n*-alkanes, but transcription levels were found to be significantly different, possibly due to binding of transcriptional activator AlkS only to promoter P*alkB1* of the *alkB1* gene. Unlike in GPo1, the expression of the *alkS* gene was not induced by *n*-alkanes, and an AlkS binding site was not detected upstream of *alkS* (van Beilen et al. 2004).

Anaerobic bacterial long-chain *n*-alkane metabolism

Aerobic bacteria use molecular oxygen as terminal electron acceptor and convert *n*-alkanes by oxidation from nonpolar substrates into compounds acceptable by the central oxidative catabolic pathways. In anoxic environments, however, such transformation must be ensured without oxygen as a reactant. Knowledge on the processes of biological breakdown of hydrocarbons under anoxic conditions still remains limited. Energetics of anaerobic long-chain *n*-alkane degradation have been repeatedly calculated for denitrification, iron(III) reduction, sulfate reduction, and methanogenesis, rendering these processes theoretically suitable for bacterial energy conservation (reviewed in Spormann and Widdel 2000). Indeed, during the past two decades, anaerobic oxidation of *n*-alkanes has repeatedly been reported under strictly anoxic conditions with pure cultures of sulfate-reducing (Aeckersberg et al. 1991; Rueter et al. 1994; Aeckersberg et al. 1998; So and Young 1999a, b; So et al. 2003; Cravo-Laureau et al. 2005) and denitrifying bacteria (Ehrenreich et al. 2000). Many of these strains are capable of using long-chain *n*-alkanes as substrates (Table 2). In these organisms, sulfate and nitrate serve as terminal electron acceptors, analogous to molecular oxygen in aerobic bacterial *n*-alkane oxidation. Utilization of only a distinct, narrow range of *n*-alkanes by individual strains under anaerobic conditions seems to be a common feature. In addition to sulfate and nitrate reduction, long-chain *n*-alkane degradation has also been shown by methanogenic bacterial communities (Zengler et al. 1999; Alain et al. 2006). In general, anaerobic bacterial alkane degradation seems to be a slow process resulting in low growth rates, compared to aerobic long-chain *n*-alkane-degrading strains. Strains Hxd3 and Pnd3, for example, were reported to have estimated doubling times of 9 days on hexadecane (Aeckersberg et al. 1998), and strain AK-01 showed doubling within 3 days when grown on hexadecane even under optimized cultivation conditions (So and Young 1999b).

Mechanisms of anaerobic *n*-alkane degradation have been studied in some detail for a number of anaerobic consortia and isolates. In mixed sulfate-reducing cultures,

Table 2 Anaerobic bacterial strains capable of *n*-alkanes utilization

Phylum	Species, strain	Documented *n*-alkane substrate range	Type of metabolism	Reference
β-Prot.	Strain HxN1	C_6–C_8	Denitr.	(Ehrenreich et al. 2000)
β-Prot.	Strain HdN1	C_{14}–C_{20}	Denitr.	(Ehrenreich et al. 2000)
β-Prot.	Strain OcN1	C_8–C_{12}	Denitr.	(Ehrenreich et al. 2000)
γ-Prot.	*Marinobacter* sp. BC36	C_{18}	Denitr.	(Bonin et al. 2004)
γ-Prot.	*Marinobacter* sp. BC38	C_{18}	Denitr.	(Bonin et al. 2004)
γ-Prot.	*Marinobacter* sp. BC42	C_{18}	Denitr.	(Bonin et al. 2004)
δ-Prot.	Strain TD3	C_6–C_{15}	Sulfate-red.	(Rueter et al. 1994)
δ-Prot.	Strain Hxd3	C_{12}–C_{20}	Sulfate-red.	(Aeckersberg et al. 1991)
δ-Prot.	Strain Pnd3	C_{14}–C_{17}	Sulfate-red.	(Aeckersberg et al. 1998)
δ-Prot.	Strain AK01	C_{13}–C_{18}	Sulfate-red.	(So and Young 1999b)
δ-Prot.	*Desulfatibacillum aliphaticivorans* CV2803	C_{13}–C_{18}	Sulfate-red.	(Cravo-Laureau et al. 2004)
δ-Prot.	Clone B1–B3	C_{16}	Met.gen.	(Zengler et al. 1999)

Prot. Proteobacteria, *Denitr*. denitrification, *Sulfate-red*. sulfate reduction, *Met.gen*. methanogenesis

simultaneous occurrence of multiple anaerobic *n*-alkane degradation pathways was detected (Callaghan et al. 2006). In general, two different reaction mechanisms for the activation of *n*-alkanes have been proposed, *n*-alkane carboxylation and the addition of *n*-alkane to fumarate (Fig. 1). The former pathway for anaerobic *n*-alkane activation was proposed for sulfate-reducing strain Hxd3 (Aeckersberg et al. 1991, 1998). This strain utilizes *n*-alkanes from C_{12} to C_{20} and has been shown to carboxylate C_{16} at the C-3 position, subsequently eliminating the two subterminal carbon atoms and leading to C-even and C-odd fatty acids from C-odd and C-even *n*-alkane substrates, respectively (So et al. 2003). In contrast to this, C-even and C-odd fatty acids resulted from C-even and C-odd *n*-alkanes, respectively, in other sulfate-reducing strains like AK-01 (So and Young 1999a), Pnd3 (Aeckersberg et al. 1998), and *Desulfatibacillum aliphaticivorans* strain CV2803 (Cravo-Laureau et al. 2005). Strains AK-01 and CV2803 also produce 2-, 4-, and 6-methyl branched fatty acids (So and Young 1999a; Cravo-Laureau et al. 2005), and therefore anaerobic activation of C_{16} via addition of carbon to a subterminal substrate carbon by strain AK-01 had been proposed (So and Young 1999a). Metabolite analysis after ^{13}C-labeled bicarbonate addition demonstrated that in these cases the carbon addition was not the result of a carboxylation reaction (So and Young 1999a; Cravo-Laureau et al. 2005). As in sulfate-reducing consortia (Kropp et al. 2000; Davidova et al. 2005) and a denitrifying isolate (Rabus et al. 2001), evidence for a reaction with fumarate was lately described for strain AK-01 (Callaghan et al. 2006). By this mechanism, the *n*-alkane is added to the double bond of fumarate via its subterminal carbon atom giving succinates substituted with *n*-alkane-derived alkyl chains. A similar addition of cyclic alkanes to fumarate has been reported (Rios-Hernandez et al. 2003; Wilkes et al. 2003). A mechanism for the reaction of fumarate with long-chain *n*-alkane has been proposed, involving a radical formation, wherein the abstracted hydrogen from the parent compound is retained (Kropp et al. 2000; Rabus et al. 2001; Cravo-Laureau et al. 2005). A carbon skeleton rearrangement after the addition of the *n*-alkane to fumarate has been proposed (Wilkes et al. 2002; Cravo-Laureau et al. 2005), and a recent study involving deuterated methylpentadecylsuccinic acid from hexadecane addition to fumarate supported this proposed mechanism (Callaghan et al. 2006). However, further studies are necessary to confirm the proposed reaction.

Biotechnological applications of long-chain *n*-alkane utilizing microorganisms

Since the 1960s, potentials have been discussed and attempts have been undertaken to channel capabilities of microorganisms to convert aliphatic compounds like *n*-alkanes into commercial biotechnological applications. Especially the well-studied *alk* system of *P. putida* (formerly known as *P. oleovorans*) has been subject to extensive efforts to produce intermediate value compounds from alkane oxidation products (reviewed in Witholt et al. 1990). Recombinant strains of *E. coli* carrying *alk* genes of *P. putida* have been employed to study and optimize the production of alkanoates in two-liquid systems (Favre-Bulle and Witholt 1992; Wubbolts et al. 1996). Since then, the spectrum of potential applications of microbial alkane degradation capabilities has broadened widely especially due to a rapidly expanding knowledge on the genetics and biochemistry of long-chain *n*-alkane-utilizing strains throughout the past two decades.

In this section, two recent directions of utilizing long-chain alkane metabolic capabilities of bacteria for biotechnological applications will be discussed in detail: (1) the bacterial production of high-value wax esters and (2) the biodegradation of paraffin waxes from complex mixtures like crude oils.

Bioconversion of *n*-alkanes into commercially valuable wax esters

Storage of neutral lipids like triacylglycerols, wax esters, and steryl esters is common in plants, animals, fungi, and bacteria (Murphy and Vance 1999; Alvarez and Steinbüchel 2002). Wax esters, oxoesters of long-chain fatty acids esterified with long-chain alcohols, are of particular commercial interest because they have a wide variety of applications in medicine, cosmetics, and food industry, and as lubricants. For a long time, the jojoba plant (*Simmondsia chinensis*) has been the main source of wax esters for commercial applications. Though the development of biotechnological processes for wax ester production based on immobilized lipases has increased the availability of jojoba-like wax esters (Hills 2003), a strong demand for inexpensive production still remains.

Predominantly, strains of the genus *Acinetobacter* have been reported to accumulate wax esters (Gallagher 1971; Fixter et al. 1986; Ishige et al. 2002). These show chain-lengths from C_{30} to C_{36} of unsaturated and monounsaturated fatty acid and fatty alcohol moieties (Gallagher 1971; Fixter et al. 1986). Because the composition with respect to the hydrocarbon chains appears quite flexible, dependent on the cultivation conditions, a similar chemical composition as in jojoba oil could be achieved (DeWitt et al. 1982; Ervin et al. 1984). The pathway for wax ester synthesis in *A. baylyi* ADP1 has been studied in detail involving three different enzymatic steps (Fig. 1). The first step comprises reduction of long-chain acyl-CoAs by an NADPH-dependent acyl-CoA reductase resulting in the corresponding fatty aldehyde (Reiser and Somerville 1997). This latter substrate is further reduced to the fatty alcohol by an NADPH-dependent fatty aldehyde reductase (Singer and Finnerty 1985a; Fox et al. 1992). Finally, the fatty alcohol is esterified with acyl-CoA by means of a rather unspecific wax ester synthase, resulting in synthesis of wax esters (Kalscheuer et al. 2003). The wax ester synthase/acyl-CoA: diacylglycerol acetyltransferase of *A. baylyi* ADP1 mediates synthesis of both wax esters and triacylglycerols (Kalscheuer and Steinbüchel 2003). Both educts of wax ester synthesis are also intermediates in the bacterial *n*-alkane degradation pathway. The supply of fatty alcohols is also achieved by terminal oxidation of long-chain *n*-alkanes by means of alkane hydroxylases and formation of acyl-CoAs from fatty acids by acyl-CoA synthetase.

Because the wax ester production by the natural *Acinetobacter* isolates seems to be limited to up to 14% of the cellular dry weight (Fixter et al. 1986), construction of recombinant strains for more efficient production may be envisaged. The recombinantly expressed wax ester synthase from *A. baylyi* ADP1 was found to be active in both *E. coli* and *Saccharomyces cerevisiae* (Kalscheuer et al. 2003, 2006). Moreover, the enzyme was also shown to be capable of using hexadecanethiol as substrate for the formation of hexadecyl thioesters (Uthoff et al. 2005), underlining a broad biocatalytic potential of this enzyme in biotechnological production of a variety of lipids. By simultaneous recombinant expression of the bifunctional acyl-CoA reductase from the jojoba plant producing fatty alcohol and the wax ester synthase from *A. baylyi* ADP1 in *E. coli*, substantial heterologous production of jojoba-like wax esters could be achieved (Kalscheuer et al. 2006). For further information on bacterial wax ester synthesis, we refer to a review article by Ishige et al. (2003).

Microbial paraffin wax degradation in crude oil

Crude oils represent complex mixtures predominantly composed of linear and branched alkanes, polyaromatic hydrocarbons, and asphaltenes of varying shapes. Though *n*-alkanes represent the major part of all conventional crude oils, the particular distribution of *n*-alkanes of different chain lengths can vary greatly between oils from different reservoirs, leading to drastically different physical properties of the respective oils. Paraffinic oils with high proportions of long-chain *n*-alkanes of C_{18} and longer (paraffin waxes) cause problems during extraction, transportation, and handling due to the formation of wax deposits, which lead to clogging of, e.g., transportation pipes (Burger et al. 1981; Singh et al. 2001; Azevedo and Teixeira 2003; Correra et al. 2007). Although chemical and mechanical/physical means to tackle these problems exist, these are often relatively costly and may represent environmental hazards. Biotechnological processes may provide new and more environmentally friendly alternatives for value enhancement of heavy oils, highly paraffinic crudes, and partially distilled petroleum products (Leon and Kumar 2005). The use of microorganisms, which can specifically reduce the fraction of paraffin waxes of long-chain *n*-alkanes in biotechnological processes for upgrading of waxy crude oil has been suggested (Lazar et al. 1999). However, to date, no biological processes for upgrading of challenging crude oils have been described or implemented (Leon and Kumar 2005).

Within the oil industry, interest increases to develop biotechnological processes along the crude oil value chain that will give the lowest cost/benefit ratio, competing with or being superior to existing conventional methods. In

particular, a process for controlled biodegradation of waxes in crude oils is highly desirable, as this process could lead to a substantial economical gain. Such a process may take place anywhere from down-hole to the refinery, in the reservoir, at the wellhead, during tanking, transport, and storage. The prerefining opportunity is to utilize the time slot from the start of drainage in the reservoir to the point when the crude reaches the refinery stage. At any of these stages, a specially designed biocatalyst could be introduced. In addition, besides processing during the prerefining stage, periorefining or postrefining technologies might also be of interest. This would be of great importance for extraction/transportation of highly paraffinic crudes and for improving wintering properties of traditional diesel and possibly other fuel types. In future refinery processes, biocatalytic approaches might to some extent substitute for energy-expensive distillation processes. Results from a successful implementation of microbial cracking of aromatic rings by a genetically modified mutant strain under "near ambient conditions" (Foght et al. 1997) indicate that bioreactor systems have the potential for upgrading of hydrocarbon refinery fractions, heavier distillates, and crude oils.

Further comprehensive studies on in particular thermophilic and anaerobic bacterial long-chain *n*-alkane metabolism may provide the basis for genetic engineering of strains for biocatalytic processes under extreme conditions. Especially strains like the lately identified thermophilic strain NG80-2 of *G. thermodenitrificans* (Wang et al. 2006), with its capabilities to specifically degrade long-chain *n*-alkanes from C_{15} to C_{36} in crude oil involving its monooxygenase LadA (see above), may provide an excellent basis for further strain improvement leading to, e.g., facilitated oil recovery (Feng et al. 2007). Recombinant and functionally improved strains have the potential to be used for improving crude oil properties already in the oil reservoir or near the production wells. Novel in situ approaches employing organisms with engineered microbial *n*-alkane metabolism may circumvent problems caused by wax precipitation in transportation pipes or vessels and simplify the subsequent refinery processes (Kotlar et al. 2007).

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