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Formation of *n*-alkane- and cycloalkane-derived organic acids during anaerobic growth of a denitrifying bacterium with crude oil

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Abstract

The formation of metabolites during anaerobic biodegradation of saturated hydrocarbons directly from crude oil in the absence of oxygen was investigated using a denitrifying bacterium, the *Azoarcus*-like strain HxN1, which can utilise C_6–C_8 *n*-alkanes anaerobically as growth substrates. Various alkylsuccinates (apparently diastereomers) with alkyl chains (probably linked at C-2) ranging from C_4 to C_8 were detected by gas chromatography–mass spectrometry. These metabolites apparently result from the activation reaction of C_4–C_8 alkanes with cellular fumarate, analogous to the recently established reaction of pure *n*-hexane with fumarate in strain HxN1 to yield (1-methylpentyl)succinate. Other succinates carried substituents derived from cyclopentane and methylcyclopentane and hence indicated an activation of such cycloalkanes. Since *n*-butane, *n*-pentane or cycloalkanes as single compounds did not support growth of strain HxN1, their apparent products point to co-metabolic reactions during utilisation of the C_6–C_8 *n*-alkanes. Furthermore, methyl-branched and cyclopentyl-substituted fatty acids were detected. This finding is explained by a further metabolism of the substituted succinates via carbon skeleton rearrangement and decarboxylation. All metabolites detected in the oil-grown cultures were also identified in cultures grown with defined mixtures of saturated hydrocarbons. Results are of potential value for an understanding of metabolite formation in hydrocarbon-rich anoxic environments from the viewpoint of bacterial physiology.

1. Introduction

n-Alkanes represent the most abundant type of petroleum hydrocarbons. The disappearence of *n*-alkanes, like that of particular aromatic hydrocarbons, in oil reservoirs is generally regarded as an indication of in situ biodegradation (e.g. Peters and Moldowan, 1993). Since for many decades hydrocarbon-degrading microbial cultures could only be established in the presence of oxygen, the selective depletion of *n*-alkanes and other hydrocarbons in oil reservoirs has for a long time been attributed to aerobic bacteria (Palmer, 1993).

During the past decade, however, numerous novel isolates of anaerobic bacteria have been shown to degrade not only aromatic, but also the chemically less reactive saturated hydrocarbons under strict exclusion of O_2 (for overview see Heider et al., 1999; Spormann and Widdel,

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2000; Widdel and Rabus, 2001). Utilisation of *n*-alkanes was demonstrated with novel species of sulfate-reducing (Aeckersberg et al., 1991, 1998; Rueter et al., 1994; So and Young, 1999a) or nitrate-reducing (Ehrenreich et al., 2000) bacteria that oxidised *n*-alkanes. Anaerobic degradation of *n*-alkanes was in addition detected in enriched bacterial communities that reduce nitrate (Bregnard et al., 1997; Rabus et al., 1999) or sulfate (Caldwell et al., 1998), or form methane and CO_2 (Zengler et al., 1999; Anderson and Lovley, 2000). Even the branched alkane, pristane, is degraded anaerobically (Bregnard et al., 1997). The proven ability of anaerobic bacteria to degrade alkanes offers an explanation for the observed selective loss of these compounds from petroleum in reservoirs that are usually oxygen-depleted (Connan et al., 1996).

Reactive oxygen species derived from O_2, which are used in aerobic organisms to activate alkanes (Groh and Nelson, 1990; White and Coon, 1980), cannot be generated in an anaerobic metabolism. Therefore, anaerobic alkane activation must mechanistically differ completely from aerobic alkane activation. Indeed, recent investigations into anaerobic *n*-alkane metabolism point to an unprecedented reaction of hydrocarbons. Enriched sulfate-reducing bacteria utilising *n*-dodecane (Kropp et al., 2000) and a denitrifying isolate (strain HxN1) growing with *n*-hexane (Rabus et al., 2001) formed alkyl-substituted succinates. In case of strain HxN1, an organic radical was specifically detected in cells grown with *n*-hexane. These findings pointed at an addition of the *n*-alkane via a radical mechanism to fumarate, a common cell metabolite. Hence, there was an analogy with the anaerobic activation of toluene, the methyl group of which is added to fumarate by a radical mechanism, yielding benzylsuccinate (for overview see Heider et al., 1999; Spormann and Widdel, 2000). However, the *n*-alkanes were not activated at the methyl groups (Kropp et al., 2000; Rabus et al., 2001). With *n*-hexane, activation at C-2 was evident as the principal reaction that apparently yielded diastereomers of (1-methylpentyl)succinate. Further studies suggested that (1-methylpentyl)succinate is subsequently metabolised via activation to the coenzyme A (CoA) thioester and subsequent carbon skeleton rearrangement, yielding (2-methylhexyl)malonyl-CoA (Wilkes et al., 2002). The latter compound allows decarboxylation to 4-methyloctanoyl-CoA, an activated branched fatty acid that can undergo conventional β-oxidation and thus finally lead to CO_2 as end product (Wilkes et al., 2002). The simultaneously-derived reducing equivalents (''electrons'') are used to reduce nitrate to dinitrogen, which yields energy for growth. The specific formation of alkyl succinates (Kropp et al., 2000; Rabus et al., 2001; Gieg and Suflita, 2002) or methyl-branched fatty acids (Wilkes et al., 2002; So and Young, 1999b) during utilisation of different *n*-alkanes by various types of enriched or isolated anaerobic bacteria suggests that the pathway for *n*-hexane degradation can be generalised for the utilisation of *n*-alkanes by various anaerobic microorganisms as depicted in Fig. 1.

Succinates with hydrocarbon-derived substituents have been regarded as valuable indicators (biomarkers) of anaerobic biodegradation of aromatic and aliphatic hydrocarbons in subsurface environments contaminated with petroleum or other complex hydrocarbon mixtures (Beller, 2000; Elshahed et al., 2001; Gieg and Suflita, 2002; Reusser et al., 2002). Bacterial populations in or from such environments form metabolites that indicate the transformation of a wider range of alkylbenzenes (Rabus et al., 1999; Wilkes et al., 2000; Elshahed et al., 2001) or saturated hydrocarbons (Gieg and Suflita, 2002) than that utilised by cultures established with single compounds. This suggests the occurrence of so far uncultured anaerobic bacteria with degradative capacities that have not been observed in established cultures. On the other hand, certain hydrocarbons may also undergo by-reactions or co-metabolic reactions. It has been shown that, for instance, toluene-degrading anaerobic bacteria formed aromatic organic acids from xylene isomers that did not serve as growth substrates (Evans et al., 1992; Biegert and Fuchs, 1995; Rabus and Widdel, 1995a; Beller and Spormann, 1999). The latter probably reacted due to promiscuous activity of the toluene-activating enzyme (Beller and Spormann, 1999).

Here, we investigated, by chemical analysis of metabolites, whether anaerobic *n*-alkane-degrading bacteria can, in principle, also activate a wider range of saturated hydrocarbons than that used as direct growth substrates. We used crude oil as a natural hydrocarbon mixture and a denitrifying *Azoarcus*-like bacterium, strain HxN1, which is the most easily cultivated among the presently available anaerobic *n*-alkane-degrading isolates (Ehrenreich et al., 2000). Anaerobic bacterial growth with crude oil and simultaneous depletion of *n*-alkanes has been demonstrated (Rueter et al., 1994; Rabus et al., 1999), but formation of substituted succinates in such cultures has not been investigated. The present analyses were also intended as a further confirmation of the postulated generalised anaerobic degradation pathway for *n*-alkanes (Fig. 1).

2. Experimental

2.1. Cultivation

A pure culture of the denitrifying *Azoarcus*-like strain HxN1 has been maintained in our laboratory since the isolation of this organism with *n*-hexane and nitrate (Ehrenreich et al., 2000). The culture was grown at 28 °C in stoppered 500-ml bottles containing 400 ml of defined anoxic mineral medium with 9 mM $NaNO_3$

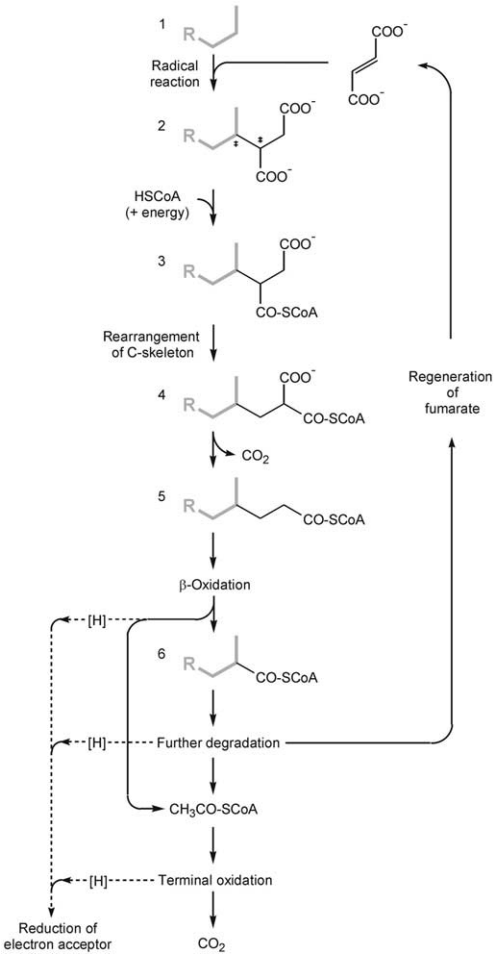

Fig. 1. Proposed generalised pathway for the anaerobic degradation of *n*-alkanes. The *n*-alkane (1) reacts at C-2 in a radical mechanism (not shown) with fumarate, yielding a (1-methylalkyl)succinate (2) which is then activated with HSCoA (coenzyme A, CoA) to (1-methylalkyl)succinyl-CoA (3). The latter undergoes carbon skeleton rearrangement (hydrogen-carboxyl-CoA exchange) yielding (2-methylalkyl)malonyl-CoA (4) that allows decarboxylation to 4-methylalkanoyl-CoA (5). This is further oxidised via conventional β-oxidation, yielding intermediates such as 2-methylalkanoyl-CoA (6), propionyl-CoA (not shown) and acetyl-CoA. Propionyl-CoA can be recycled to fumarate (details not shown; for further information see Wilkes et al., 2002) and acetyl-CoA is oxidised to CO_2 (details not depicted). Compounds 2, 5, and 6 have been identified in the present study (upon hydrolysis of the assumed thioesters and esterification with diazomethane during sample preparation). Formation of diastereomers of (1-methylalkyl)succinate (2) has been suggested to be due to reduced stereoselectivity at the *n*-alkane chain (Rabus et al., 2001); the two chiral carbon atoms of this compound are marked by double daggers. In the case of cyclopentane or methylcyclopentane as reacting hydrocarbons, the methyl esters of intermediates analogous to compounds 2 and 5 were observed (carbon chain marked in grey to be replaced by cyclic moieties); further processing is uncertain.

and 20 ml of crude oil (from the North Sea) as the only source of organic carbon under an anoxic headspace (9:1 mixture of N_2 and CO_2, v/v). Before addition, the crude oil was de-aerated and sterilised as described elsewhere (Rabus and Widdel, 1995b, 1996). Furthermore, strain HxN1 was grown with various defined mixtures of hydrocarbons. To avoid inhibitory effects of such hydrocarbons, they were diluted in 2,2,4,4,6,8,8-heptamethylnonane as inert carrier phase (5 ml per 400 ml medium) so as to achieve the indicated concentrations (percentages as v/v). The mixed hydrocarbons always included *n*-hexane (4%) and an additional hydrocarbon: methane, ethane, propane, *n*-butane (each 101 kPa in head space), *n*-pentane (1%), *n*-heptane (2%), *n*-octane (3%), *n*-nonane, *n*-decane (each 6%), cyclopentane, methylcyclopentane, or cyclohexane (each 1%). The inoculum size was 10% (v/v). The bottles were incubated nearly horizontally with orifices below the medium surface level so as to avoid contact between the hydrocarbon phase and the stopper (Rabus and Widdel, 1995b, 1996). Cultures were processed for chemical analysis towards the end of growth (with oil after 24 d, with defined alkanes after 6 d).

2.2. Sample preparation and gas chromatography-mass spectrometry

Extraction of acidified cultures with diethyl ether and derivatisation of extracts was carried out as described previously (Wilkes et al., 2000; Rabus et al., 2001). Methylated culture extracts were analyzed by gas chromatography–mass spectrometry (GC–MS) with a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan MAT/ThermoFinnigan, Egelsbach, Germany) as described by Wilkes et al. (2000) and Rabus et al. (2001). A standard of (1-methylpentyl)succinic acid dimethyl ester was synthesised as reported elsewhere (Rabus et al., 2001). Cyclopentylsuccinic acid dimethyl ester was prepared by analogy with the procedure described for cyclohexylsuccinic acid dimethyl ester (Giese and Meister, 1977; Giese and Kretzschmar, 1982, 1984) using dimethyl fumarate, cyclopentyl mercuric acetate, and sodium borohydride. ^{1}H- and ^{13}C-NMR data were measured on a Varian Inova 400 (Varian, Darmstadt, Germany): ^{1}H-NMR (400 MHz, $CDCl_3$) $\delta = 1.14$–1.34 (m, 2H), 1.50–1.82 (m, 6H), 1.92–2.02 (m, 1H), 2.50 (dd, $J = 3.0$, 15.7 Hz, 1H), 2.65–2.79 (m, 2H), 3.66 (s, 3H), 3.70 (s, 3H). ^{13}C-NMR (100 MHz, $CDCl_3$) = 24.85, 24.95, 30.41, 30.47, 35.37, 42.36, 46.16, 51.56, 51.68, 172.40, 175.13. Further standard compounds (used as methyl esters after derivatisation) were cyclohexylsuccinic, 3-cyclopentylpropionic, 2-methylpentanoic, 2-methylhexanoic, 4-methylheptanoic, and 4-methyloctanoic acids (Sigma-Aldrich, Deisenhofen, Germany). Coinjection

experiments were performed as described previously (Rabus et al., 2001).

3. Results

Strain HxN1 was able to grow in mineral medium with nitrate (being reduced to dinitrogen) and crude oil as the only source of organic compounds under conditions involving strict exclusion of air. No growth was observed in the absence of either crude oil or nitrate. Growth with crude oil was approximately 4 times slower (shortest doubling time, 40–50 h) than the previously documented growth with *n*-hexane as single substrate (Ehrenreich et al., 2000). If limiting amounts of crude oil were added, consumption of *n*-hexane, *n*-heptane and *n*-octane from crude oil was evident (Behrends and Widdel, unpublished data). To favour accumulation of metabolites for structural identification, a high (excess) amount of crude oil (see Section 2.1) was added such that *n*-alkanes were not significantly depleted during growth. With 9 mM nitrate added, optical cell densities (measured at 660 nm) of 0.15–0.2 were reached in these cultures.

GC–MS analysis of extracts from the grown culture revealed a complex suite of organic acids that were not

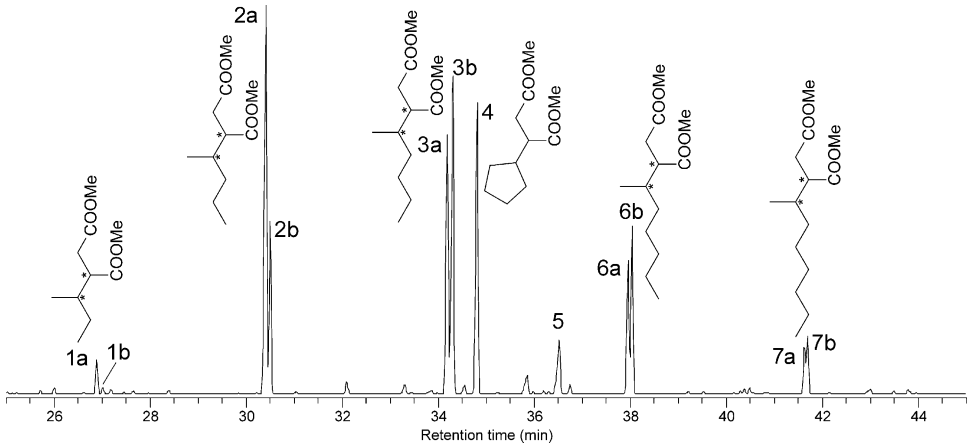

Fig. 2. Partial ion chromatogram (*m*/*z* 114 + 146) representing the alkyl succinic acid dimethyl esters in the methylated extract from the culture of denitrifying strain HxN1 upon anaerobic growth with crude oil. Peak numbers correspond to those used in Table 1.

Table 1
Selected characteristic mass spectral data of identified (1-methylalkyl)succinic acid dimethyl esters in the methylated extract from the culture of strain HxN1 after anaerobic growth with crude oil (a base peak at *m*/*z* 114 was observed for all listed compounds)

Peak no. [a]	Identified compound (as dimethyl ester)	Parent hydrocarbon	Formula	M_r	Relative intensity (%) of key ions			Kováts-Index
					m/*z* 146	$[M-31]^+$	$[M-73]^+$	
1a	(1-Methylpropyl)succinic acid	*n*-Butane	$C_{10}H_{18}O_4$	202	35	19	30	1306
1b					38	16	28	1309
2a	(1-Methylbutyl)succinic acid	*n*-Pentane	$C_{11}H_{20}O_4$	216	34	12	24	1387
2b					38	14	21	1389
3a	(1-Methylpentyl)succinic acid [b]	*n*-Hexane	$C_{12}H_{22}O_4$	230	42	12	28	1475
3b					42	11	20	1478
4	Cyclopentylsuccinic acid [b]	Cyclopentane	$C_{11}H_{18}O_4$	214	48	17	42	1490
5	(Methylcyclopentyl)succinic acid	Methylcyclopentane	$C_{12}H_{20}O_4$	228	66	15	42	1533
6a	(1-Methylhexyl)succinic acid	*n*-Heptane	$C_{13}H_{24}O_4$	244	49	11	32	1569
6b					48	11	24	1571
7a	(1-Methylheptyl)succinic acid	*n*-Octane	$C_{14}H_{26}O_4$	258	62	12	41	1664
7b					60	11	30	1666

[a] Peak numbers correspond to those in Fig. 2. Double peaks of identified compounds were interpreted as diastereomers (see text). Selected mass spectra are shown in Fig. 3.

[b] Identification was based on comparison of retention times and mass spectra with those of an authentic standard. Identification of all other peaks was based on relative retention times and similarity of fragmentation patterns.

detectable in controls lacking either nitrate or oil. Since the metabolism of *n*-hexane in strain HxN1 leads to a substituted succinate and branched fatty acids that carry the *n*-hexane-derived 1-methylpentyl moiety (Rabus et al., 2001; Wilkes et al., 2002; see also Section 1 and Fig. 1), chemical analysis of the organic acids was focused on analogous compounds.

To correlate unequivocally the metabolites formed with crude oil with the utilisation of particular hydrocarbons, strain HxN1 was grown in addition in separate cultures with *n*-hexane in combination with other hydrocarbons that were added individually. Since the metabolites of *n*-hexane are known (Wilkes et al., 2002), additional metabolites could be assigned to the utilisation of the second hydrocarbon in each experiment. The second hydrocarbon was methane, ethane, propane, *n*-butane, *n*-pentane, *n*-heptane, *n*-octane, *n*-nonane, *n*-decane, cyclopentane, methylcyclopentane, or cyclohexane.

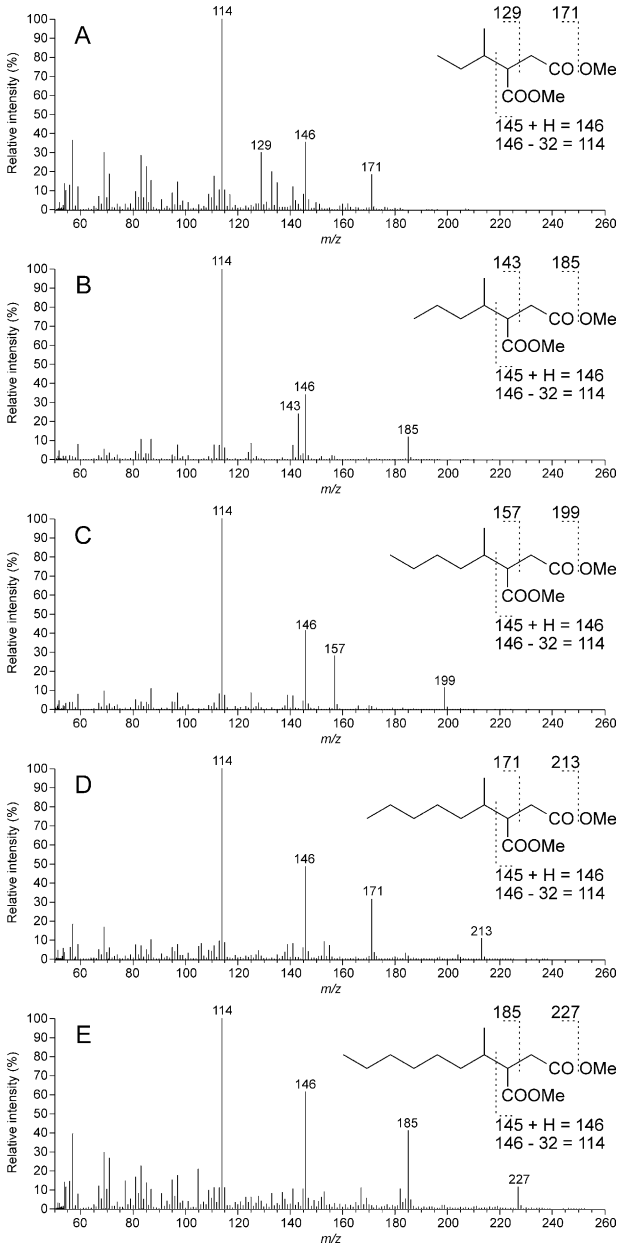

Fig. 3. Mass spectra of GC peaks (all from Fig. 2) representing dimethyl esters of (A) (1-methylpropyl)succinic acid (peak 1a), (B) (1-methylbutyl)succinic acid (peak 2a), (C) (1-methylpentyl)succinic acid (peak 3a), (D) (1-methylhexyl)succinic acid (peak 4a), and (E) (1-methylheptyl)succinic acid (peak 5a).

3.1. Alkyl- and cycloalkyl-substituted succinates

Since fragment ions at *m*/*z* 114 and *m*/*z* 146 from the *n*-hexane-derived (1-methylpentyl)succinic acid dimethyl ester (Rabus et al., 2001) do not include the alkyl moiety, they should be also detectable if anaerobic bacteria form alkyl succinates from saturated hydrocarbons other than *n*-hexane. Indeed, in the experiment with crude oil, the summed (partial) ion chromatogram for these two fragments revealed a series of seven GC peaks (Fig. 2, Table 1) that occurred singly or as doublets. The Kováts-indices provide evidence that these compounds represent a homologous series (Table 1). Characteristic mass spectral data of the components in these GC peaks are compiled in Table 1.

The two GC peaks from each double peak, i.e. from those numbered 1, 2, 3, 6 and 7 (Fig. 2), exhibited essentially the same fragmentation patterns (Fig. 3). By comparison with the mass spectrum of an authentic standard, peaks 3a and 3b were identified as the (postulated) diastereomers of (1-methylpentyl)succinic acid dimethyl esters (see also Rabus et al., 2001). Besides the common base peak at *m*/*z* 114 and the second most abundant fragment ion at *m*/*z* 146, the mass spectra of the doublet peaks exhibited specific fragment ions at *m*/*z* 129, 143, 157, 171, 185 and *m*/*z* 171, 185, 199, 213, 227 and thus revealed two series of homologues differing by 14 amu. This value is characteristic of CH_2-groups, and therefore the most probable interpretation of the double peaks 1a/1b, 2a/2b, 6a/6b and 7a/7b (Fig. 2; Table 1) is that they represent the diastereomers of (1-methylpropyl)succinic, (1-methylbutyl)succinic, (1-methylhexyl)-succinic and (1-methylheptyl)succinic acid dimethyl esters, respectively. We therefore conclude that *n*-butane, *n*-pentane, *n*-heptane and *n*-octane from crude oil were activated, in addition to *n*-hexane, by the postulated radical-catalyzed addition to fumarate (Rabus et al., 2001). It has been recently discussed that the formation of diastereomers of (1-methylpentyl)succinate may be due to reduced stereoselectivity at C-2 of *n*-hexane, whereas the fumarate carbon may react with high stereoselectivity (Rabus et al., 2001). The chromatogram of the various (1-methylalkyl)succinic acid dimethyl esters (Fig. 2) shows that in the case of the C_4 and C_5 alkyl chains the earlier eluting diastereomers clearly dominate; with longer alkyl chains, the diastereomers are formed in almost equal amounts. This suggests that in the case of short-chain *n*-alkanes the reaction at C-2 is more stereoselective.

GC peaks 4 and 5 (Fig. 2) yielded specific fragment ions at *m*/*z* 141, 183 and *m*/*z* 155, 197, respectively, in addition to the common ones at *m*/*z* 114 and 146 (Fig. 4A, B). The specific fragment ions are 2 amu lower than those of the dimethyl esters of (1-methylbutyl)succinate and (1-methylpentyl)succinate, respectively (Fig. 3B, C). This observation suggests that the substituents at the succinate moiety of GC peaks 4 and 5 also had five and six carbon atoms, respectively, but were either monocyclic or monounsaturated and hence derived from monocyclic or monounsaturated hydrocarbons. Since the latter are not common constituents of crude oil, these metabolites are interpreted as activation products of cycloalkanes. Indeed, the use of an authentic standard allowed a clear identification of GC peak 4 as cyclopentylsuccinic acid

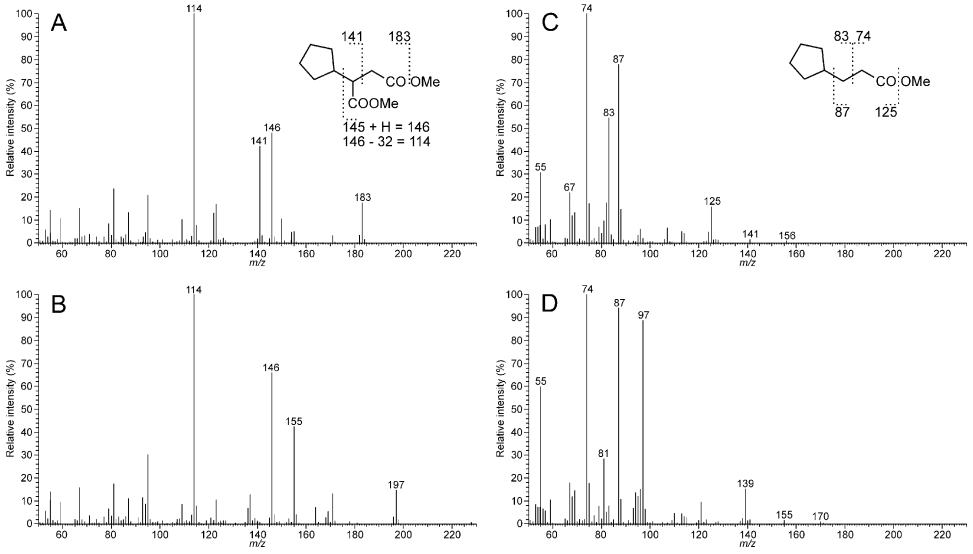

Fig. 4. Mass spectra of GC peaks representing dimethyl esters of (A) cyclopentylsuccinic acid (peak 4 from Fig. 2), (B) an assumed methylcyclopentylsuccinic acid (peak 5 from Fig. 2) and methyl esters of (C) 3-cyclopentylpropionic acid (peak 6 from Fig. 6), and an assumed 3-(methylcyclopentyl)propionic acid (peak 8 from Fig. 6). Identity of (A) and (C) was confirmed by analysis of authentic standards.

dimethyl ester. The fragmentation pattern (Fig. 4B) of GC peak 5 leaves open the possibility of a cyclohexane- or a methylcyclopentane-derived substituent. However, the retention time of an authentic standard of cyclohexylsuccinic acid dimethyl ester was different from that of GC peak 5, indicating that the substituent must have been derived from methylcyclopentane. Methylcyclopentane can theoretically be attacked at any of the four chemically distinct carbon atoms, leading to four structurally different succinate derivatives. However, it is speculated that the reaction preferentially takes place at carbon atoms 2 or 3. Carbon atom 1 is sterically hindered by the methyl group and an activation at the methyl group appears unlikely in view of the activation principle of *n*-alkanes (see Introduction). The succinate derivative of another alkyl-substituted alicyclic hydrocarbon, ethylcyclopentane, has been tentatively identified in a sulfate-reducing enrichment culture (Rios-Hernandez et al., 2003).

In the growth experiments with defined binary mixtures of hydrocarbons, substituted succinates other than (1-methylpentyl)succinate from the added *n*-hexane were identified in significant quantities if *n*-butane, *n*-pentane, *n*-heptane, *n*-octane, cyclopentane, or

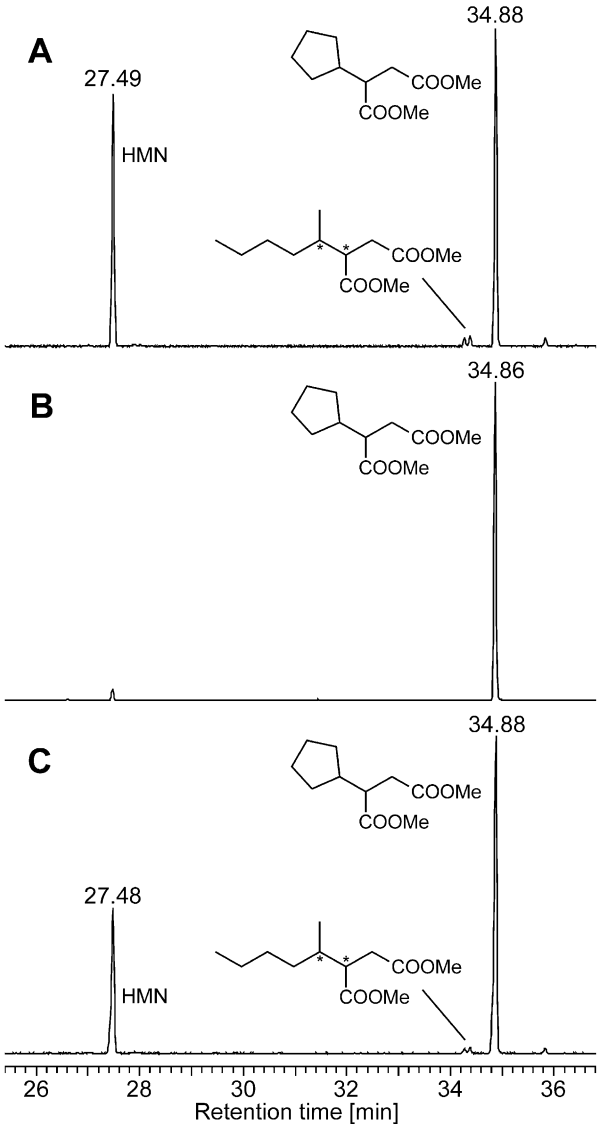

Fig. 5. Partial gas chromatograms of (A) methylated extract from a culture of strain HxN1 grown with a mixture of *n*-hexane and cyclopentane, (B) a synthetic standard of cyclopentylsuccinic acid dimethyl ester, and (C) a co-injection experiment of the samples shown under (A) and (B). HMN designates 2,2,4,4,6,8,8-heptamethylnonane that was used as an inert carrier phase and reservoir for the volatile hydrocarbons (see Experimental).

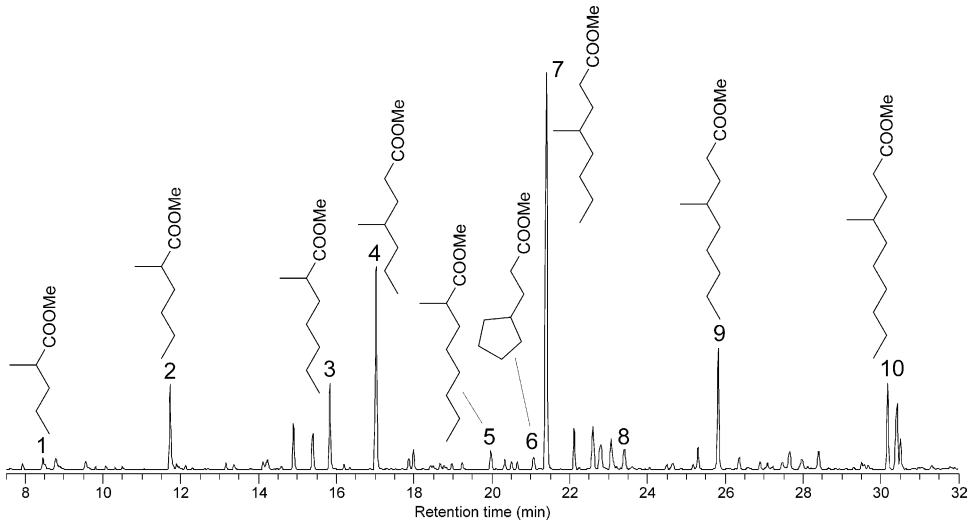

Fig. 6. Partial ion chromatogram (m/z 74 + 87 + 88 + 101) representing the 2- and 4-methyl-branched fatty acid methyl esters in the methylated extract from the culture of strain HxN1 upon anaerobic growth with crude oil. Peak numbers correspond to those in Table 2.

Table 2
Mass spectral data for methyl-branched and cyclic fatty acid methyl esters in methylated extract from the culture of strain HxN1 after anaerobic growth with crude oil

Peak no. [a]	Identified compound (as methyl ester)	Parent hydrocarbon	Formula	M_r	Key ions: m/z (% relative intensity)	Kováts-Index
1	2-Methylpentanoic acid[b]	*n*-Pentane	$C_7H_{14}O_2$	130	M^+ absent, 115 (3), 101 (16), 99 (18), 88 (100), 71 (33), 59 (10)	n.d.
2	2-Methylhexanoic acid[b]	*n*-Hexane	$C_8H_{16}O_2$	144	144 (M^+, <1), 113 (7), 101 (24), 88 (100), 87 (9), 85 (15), 59 (10)	969
3	2-Methylheptanoic acid[b]	*n*-Heptane	$C_9H_{18}O_2$	158	158 (M^+, <1), 127 (5), 115 (8), 101 (26), 99 (4), 88 (100), 71 (16), 59 (8)	1067
4	4-Methylheptanoic acid	*n*-Pentane	$C_9H_{18}O_2$	158	158 (M^+, <1), 129 (6), 127 (19), 115 (23), 87 (100), 85 (58), 74 (97), 59 (21)	1092
5	2-Methyloctanoic acid	*n*-Octane	$C_{10}H_{20}O_2$	172	M^+ absent, 141 (3), 115 (3), 113 (7), 101 (100), 88 (69), 85 (11), 71 (18), 59 (22)	1158
6	3-Cyclopentylpropionic acid[b]	Cyclopentane	$C_9H_{16}O_2$	156	156 (M^+, <1), 141 (2), 125 (16), 87 (78), 83 (54), 74 (100), 67 (22), 55 (31)	1180
7	4-Methyloctanoic acid[b]	*n*-Hexane	$C_{10}H_{20}O_2$	172	172 (M^+, <1), 143 (5), 141 (14), 115 (25), 99 (24), 87 (100), 74 (78), 59 (13)	1187
8	3-(Methylcyclopentyl) propionic acid	Methyl-cyclopentane	$C_{10}H_{18}O_2$	170	170 (M^+, <1), 155 (1), 139 (15), 97 (89), 87 (94), 81 (28), 74 (100), 55 (60)	1231
9	4-Methylnonanoic acid[b]	*n*-Heptane	$C_{11}H_{22}O_2$	186	186 (M^+, <1), 157 (4), 155 (10), 129 (11), 115 (16), 113 (19), 87 (100), 74 (73), 71 (29), 59 (14)	1283
10	4-Methyldecanoic acid	*n*-Octane	$C_{12}H_{24}O_2$	200	200 (M^+, <1), 171 (4), 169 (7), 143 (12), 127 (16), 115 (14), 87 (100), 85 (16), 74 (69), 59 (10)	1381

n.d., not determined.

[a] Peak numbers correspond to those used in Fig. 6.

[b] Identification was based on comparison of retention times and mass spectra with those of an authentic standard. Identification of all other peaks was based on relative retention times, similarity of fragmentation patterns and comparison with published mass spectra (McLafferty and Stauffer, 1989).

methylcyclopentane were also present. The dimethyl esters of the additional succinates formed with these hydrocarbons had the same retention times and fragmentation patterns as GC peaks 1a/1b, 2a/2b, 6a/b, 7a/7b, 4 or 5, respectively (Fig. 2) from the growth experiment with crude oil. Hence, this result confirmed that in addition to the C_6-C_8 *n*-alkanes, which are the only ones that definitely allow growth, *n*-butane, *n*-pentane, cyclopentane and methylcyclopentane were also activated. Cyclopentylsuccinate showed significant accumulation in comparison with the *n*-hexane-derived (1-methylpentyl)succinate (Fig. 5). In contrast, the experiment with *n*-hexane and cyclohexane yielded only traces of cyclohexylsuccinate, a compound which was not detectable in the experiment with crude oil.

3.2. Branched and cyclopentyl-substituted fatty acids

The further metabolism of *n*-hexane-derived (1-methylpentyl)succinate in strain HxN1 has been suggested to proceed through 4-methyloctanoate and 2-methylhexanoate (as thioesters; Wilkes et al., 2002; see also Section 1 and Fig. 1). Summed ion chromatograms for selected mass fragments of methyl esters of these fatty acids and postulated analogues in the oil-grown culture revealed a series of GC peaks; eight of these could be identified as methyl esters of different 2-methyl- and 4-methyl-branched fatty acids (Fig. 6, Table 2). These branched fatty acids are fully in agreement with an activation and further metabolism of different *n*-alkanes, analogous to that of *n*-hexane (see Section 3.1).

Two minor metabolites, visible as GC peaks 6 and 8 in the summed ion chromatogram (Fig. 6), revealed mass spectra with molecular ions at *m*/*z* 156 and *m*/*z* 170, respectively, specific fragment ions at *m*/*z* 83, 125 and *m*/*z* 97, 139, respectively, and common fragment ions at *m*/*z* 74, 87 (Fig. 4C, D). The ions from GC peak 6 suggest the component to be 3-cyclopentylpropionic acid methyl ester, which was confirmed by GC–MS of an authentic standard. The molecular ion and specific fragment ions of GC peak 8 were 14 amu higher than those of GC peak 6 and therefore indicate the formation of a fatty acid with a substituent derived from methylcyclopentane. There was no evidence for the formation of cyclohexylpropionate.

The experiments with the defined binary mixtures of hydrocarbons also confirmed the formation of methyl-branched fatty acids from *n*-alkanes other than *n*-hexane, and the formation of fatty acids containing the cyclic substituents from cyclopentane and methylcyclopentane.

4. Discussion

The present study not only confirmed the postulated generalised pathway for the anaerobic degradation of *n*-alkanes (Fig. 1), but also revealed the activation of short-chain and cyclic saturated hydrocarbons that do not serve as growth substrates for strain HxN1. It is unlikely that the organism possesses additional activating enzymes (alkyl succinate synthases) for saturated hydrocarbons that are not used for growth. Rather, the alkyl succinate synthase for the activation of the C_6–C_8 *n*-alkanes in strain HxN1 is assumed to exhibit promiscuous activity (relaxed substrate specificity) towards various saturated hydrocarbons. However, aromatic metabolites were not detectable in the present oil-grown cultures of strain HxN1, indicating that the relaxed substrate specificity does not extend to an activation of alkylbenzenes. It is also obvious from the detected fatty acids that the alkyl-substituted succinates are processed further also in case of the alkanes that do not serve as growth substrates.

It is presently unknown whether the biochemical processing of the hydrocarbons that alone do not support growth is an incomplete co-metabolism that ceases at some stage in an organic dead-end product, or whether this is a complete co-metabolism (leading to CO_2) that can contribute to a certain extent to the energy gain and growth of the bacterium. Either possibility may exist in microorganisms, depending on the type of hydrocarbon and bacterium. Even if such processes were to be very slow, they may contribute to the compositional alteration of crude oil in petroleum reservoirs on a geological time scale. Growth experiments in this study were carried out with a nitrate-reducing bacterium as a model organism grown at 28 °C. In oil reservoirs, the more relevant anaerobic organisms are most probably sulfate-reducing bacteria that usually thrive at the higher temperatures which prevail in situ. Nevertheless, the biochemical principles of anaerobic alkane degradation are expected to be the same for different bacterial groups (Kropp et al., 2000; Rabus et al., 2001). Hence, co-metabolic reactions resulting from relaxed specificity of involved enzymes may also occur in various hydrocarbon-degrading anaerobic bacteria. A more detailed investigation of the range and limits of co-metabolic reactions in anaerobic hydrocarbon-degrading bacteria would be a valuable contribution from the side of cultivation-based microbiology to a causal understanding and interpretation of hydrocarbon-derived biomarkers observed in situ.

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References

Aeckersberg, F., Bak, F., Widdel, F., 1991. Anaerobic oxidation of saturated hydrocarbons to CO_2 by a new type of sulfate-reducing bacterium. Archives of Microbiology 156, 5–14.

Aeckersberg, F., Rainey, F.A., Widdel, F., 1998. Growth, natural relationships, cellular fatty acids and metabolic adaptation of sulfate-reducing bacteria that utilize long-chain alkanes under anoxic conditions. Archives of Microbiology 170, 361–369.

Anderson, R., Lovley, D., 2000. Hexadecane decay by methanogenesis. Nature 404, 722–723.

Beller, H.R., 2000. Metabolic indicators for detecting in situ anaerobic alkylbenzene degradation. Biodegradation 11, 125–139.

Beller, H.R., Spormann, A., 1999. Substrate range of benzylsuccinate synthase from *Azoarcus* strain T. FEMS Microbiology Letters 178, 147–153.

Biegert, T., Fuchs, G., 1995. Anaerobic oxidation of toluene (analogues) to benzoate (analogues) by whole cells and by cell extracts of a denitrifying *Thauera* sp. Archives of Microbiology 163, 407–417.

Bregnard, T.A., Häner, A., Höhener, P., Zeyer, J., 1997. Degradation of pristane in nitrate-reducing microcosms and enrichment cultures. Applied and Environmental Microbiology 63, 2077–2081.

Caldwell, M., Garrett, R., Prince, R., Suflita, J., 1998. Anaerobic biodegradation of long-chain *n*-alkanes under sulfate-reducing conditions. Environmental Science and Technology 32, 2191–2195.

Connan, J., Lacrampe-Coulome, G., Magot, M., 1996. Origin of gases in reservoirs. In: Dolenc, D. (Ed.), Proceedings of the 1995 International Gas Research Conference. Government Institutes, Rockville, pp. 21–62.

Ehrenreich, P., Behrends, A., Harder, J., Widdel, F., 2000. Anaerobic oxidation of alkanes by newly isolated denitrifying bacteria. Archives of Microbiology 173, 58–64.

Elshahed, M.S., Gieg, L.M., McInerney, M.J., Suflita, J.M., 2001. Signature metabolites attesting to the in situ attenuation of alkylbenzenes in anaerobic environments. Environmental Science and Technology 35, 632–689.

Evans, P.J., Ling, W., Goldschmidt, B., Ritter, E.R., Young, L.Y., 1992. Metabolites formed during anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Applied and Environmental Microbiology 58, 496–501.

Gieg, L.M., Suflita, J.M., 2002. Detection of anaerobic metabolites of saturated and aromatic hydrocarbons in petroleum-contaminated aquifers. Environmental Science and Technology 36, 3755–3762.

Giese, B., Meister, J., 1977. Die Addition von Kohlenwasserstoffen an Olefine. Eine neue synthetische Methode. Chemische Berichte 110, 2588–2600.

Giese, B., Kretzschmar, G., 1982. Radikalische Addition an cyclische Derivate der Maleinsäure. Chemische Berichte 115, 2012–2014.

Giese, B., Kretzschmar, G., 1984. Radikalkettenreaktionen mit Maleinsäureanhydriden. Zur kontrathermodynamischen Stereoselektivität. Chemische Berichte 117, 3175–3182.

Groh, S., Nelson, M., 1990. Mechanisms of activation of carbon-hydrogen bonds by metalloenzymes. In: Davies, J., Watson, P., Liebman, J., Greenberg, A. (Eds.), Selective Hydrocarbon Activation. VCH, Weinheim, Germany, pp. 305–378.

Heider, J., Spormann, A.M., Beller, H.R., Widdel, F., 1999. Anaerobic bacterial metabolism of hydrocarbons. FEMS Microbiology Reviews 22, 459–473.

Kropp, K.G., Davidova, I.A., Suflita, J.M., 2000. Anaerobic oxidation of *n*-dodecane by an addition reaction in a sulfate-reducing bacterial enrichment culture. Applied and Environmental Microbiology 66, 5393–5398.

McLafferty, F.W., Stauffer, D.B., 1989. The Wiley/NBS registry of mass spectral data. Wiley, New York.

Palmer, S.E., 1993. Effect of biodegradation and water washing on crude oil composition. In: Engel, M.H., Macko, S.A. (Eds.), Organic Geochemistry. Plenum Press, New York, pp. 511–533.

Peters, K.E., Moldowan, J.M., 1993. The Biomarker Guide. Prentice Hall, Englewood Cliffs.

Rabus, R., Widdel, F., 1995a. Conversion studies with substrate analogues of toluene in a sulfate-reducing bacterium, strain Tol2. Archives of Microbiology 164, 448–451.

Rabus, R., Widdel, F., 1995b. Anaerobic degradation of ethylbenzene and other aromatic hydrocarbons by new denitrifying bacteria. Archives of Microbiology 163, 96–103.

Rabus, R., Widdel, F., 1996. Utilization of alkylbenzenes during anaerobic growth of pure cultures of denitrifying bacteria on crude oil. Applied and Environmental Microbiology 62, 1238–1241.

Rabus, R., Wilkes, H., Schramm, A., Harms, G., Behrends, A., Amann, R., Widdel, F., 1999. Anaerobic degradation of alkylbenzenes and *n*-alkanes from crude oil in an enrichment culture of denitrifying bacteria affiliating with the β-subclass of *Proteobacteria*. Environmental Microbiology 1, 145–157.

Rabus, R., Wilkes, H., Behrends, A., Armstroff, A., Fischer, T., Pierik, A.J., Widdel, F., 2001. Anaerobic initial reaction of *n*-alkanes in a denitrifying bacterium: evidence for (1-methylpentyl)succinate as initial product and for involvement of an organic radical in *n*-hexane metabolism. Journal of Bacteriology 183, 1707–1715.

Reusser, D.E., Istok, J.D., Beller, H.R., Field, J.A., 2002. In situ transformation of deuterated toluene and xylene to benzylsuccinic acid analogues in BTEX-contaminated aquifers. Environmental and Science Technology 36, 4127–4134.

Rios-Hernandez, L.A., Gieg, L.M., Suflita, J.M., 2003. Biodegradation of an alicyclic hydrocarbon by a sulfate-reducing enrichment from a gas condensate-contaminated aquifer. Applied and Environmental Microbiology 69, 434–443.

Rueter, P., Rabus, R., Wilkes, H., Aeckersberg, F., Rainey, F.A., Jannasch, H.W., Widdel, F., 1994. Anaerobic oxidation of hydrocarbons in crude oil by new types of sulphate-reducing bacteria. Nature 372, 455–458.

So, C.M., Young, L.Y., 1999a. Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Applied and Environmental Microbiology 65, 2969–2976.

So, C.M., Young, L.Y., 1999b. Initial reactions in anaerobic alkane degradation by a sulfate reducer, strain AK-01. Applied and Environmental Microbiology 65, 5532–5540.

Spormann, A.M., Widdel, F., 2000. Metabolism of alkylbenzenes, alkanes and other hydrocarbons in anaerobic bacteria. Biodegradation 11, 85–105.

White, R., Coon, M., 1980. Oxygen activation by cytochrome P-450. Annual Review of Biochemistry 49, 315–356.

Widdel, F., Rabus, R., 2001. Anaerobic biodegradation of saturated and aromatic hydrocarbons. Current Opinion in Biotechnology 12, 259–276.

Wilkes, H., Boreham, C., Harms, G., Zengler, K., Rabus, R., 2000. Anaerobic degradation and carbon isotopic fractionation of alkylbenzenes in crude oil by sulphate-reducing bacteria. Organic Geochemistry 31, 101–115.

Wilkes, H., Rabus, R., Fischer, Th., Armstroff, A., Behrends, A., Widdel, F., 2002. Anaerobic degradation of *n*-alkanes in a denitrifying bacterium: Further degradation of the initial intermediate (1-methylpentyl)succinate via C-skeleton rearrangement. Archives of Microbiology 177, 235–243.

Zengler, K., Richnow, H.H., Rosselló-Mora, R., Michaelis, W., Widdel, F., 1999. Methane formation from long-chain alkanes by anaerobic microorganisms. Nature 401, 266–269.