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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†

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A novel type of denitrifying bacterium (strain HxN1) with the capacity to oxidize *n*-alkanes anaerobically with nitrate as the electron acceptor to CO_2 formed (1-methylpentyl)succinate (MPS) during growth on *n*-hexane as the only organic substrate under strict exclusion of air. Identification of MPS by gas chromatography-mass spectrometry was based on comparison with a synthetic standard. MPS was not formed during anaerobic growth on *n*-hexanoate. Anaerobic growth with [1-^{13}C]*n*-hexane or d_{14}-*n*-hexane led to a 1-methylpentyl side chain in MPS with one ^{13}C atom or 13 deuterium atoms, respectively. This indicates that the 1-methylpentyl side chain originates directly from *n*-hexane. Electron paramagnetic resonance spectroscopy revealed the presence of an organic radical in *n*-hexane-grown cells but not in *n*-hexanoate-grown cells. Results point at a mechanistic similarity between the anaerobic initial reaction of *n*-hexane and that of toluene, even though *n*-hexane is much less reactive; the described initial reaction of toluene in anaerobic bacteria is an addition to fumarate via a radical mechanism yielding benzylsuccinate. We conclude that *n*-hexane is activated at its second carbon atom by a radical reaction and presumably added to fumarate as a cosubstrate, yielding MPS as the first stable product. When 2,3-d_2-fumarate was added to cultures growing on unlabeled *n*-hexane, 3-d_1-MPS rather than 2,3-d_2-MPS was detected, indicating loss of one deuterium atom by an as yet unknown mechanism.

Alkanes as metabolites from organisms (10) and constituents of petroleum (49) and its refined products are widespread compounds in our environment. Containing exclusively apolar σ-bonds, alkanes are among the chemically least reactive compounds. Bacteria and fungi that utilize alkanes as growth substrates in the presence of molecular oxygen have been known for about 100 years (14, 15). Aerobic microorganisms all initiate the metabolism of alkanes by monooxygenase reactions. These enzymes generate a highly reactive oxygen species by partial reduction of O_2, resulting in the introduction of a hydroxyl group into the alkane molecule by a radical mechanism (22, 51). The alkanol formed is further oxidized and metabolized via the β-oxidation pathway.

It was shown only relatively recently that special types of bacteria, which are physiologically and phylogenetically distinct from previously described aerobic hydrocarbon-degrading microorganisms, degrade *n*-alkanes under strict exclusion of oxygen. *n*-Alkanes were anaerobically oxidized in pure cultures using sulfate (2, 3, 41, 43) or nitrate (18) as the electron acceptor or in enrichment cultures with sulfate (16) or nitrate (13, 39). Also, anaerobic conversion of long-chain *n*-alkanes to methane and CO_2 in associations of enriched bacteria and archaea was demonstrated (4, 55). Oxidation of the isoprenoid-like alkane 2,5,9,13-tetramethylpentadecane (pristane) was demonstrated in a nitrate-reducing bacterial community (13). None of the hitherto isolated alkane-degrading anaerobic bacteria utilize aromatic hydrocarbons such as toluene, whereas anaerobic bacteria that degrade aromatic hydrocarbons do not utilize alkanes (24).

First investigations into the anaerobic metabolism of *n*-alkanes were conducted with two phylogenetically related sulfate-reducing bacteria, strains Hxd3 and Pnd3 (3). Adaptation studies and analysis of cellular fatty acids with strain Hxd3 suggested that anaerobic degradation of alkanes does not occur via desaturation to 1-alkenes, a questionable mechanism that has been speculated about in some early studies on the possibility of anaerobic *n*-alkane degradation (for an overview, see reference 3). Strain Hxd3 formed mainly C-odd cellular fatty acids during growth with *n*-hexadecane and C-even cellular fatty acids during growth with *n*-heptadecane. One explanation for these fatty acid patterns was the assumption of an alteration in the carbon chain length during the initial anaerobic reactions by a C-odd carbon unit, e.g., by terminal addition of a one-carbon moiety. In strain Pnd3, however, cellular fatty acids were mainly C-even upon growth with C-even alkanes and mainly C-odd upon growth with C-odd alkanes; in addition, unidentified fatty acids were formed. The findings

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† This article is dedicated to Wolfgang Buckel on the occasion of his 60th birthday.

suggested different modes of initial reactions in the two *n*-alkane-degrading sulfate-reducing strains (3). Still, a common principle in the mechanism of initial reactions of *n*-alkanes in both strains was considered by assuming that the site of carbon addition may also be the subterminal position in the chain (3). In a third isolate of an *n*-alkane-degrading sulfate-reducing bacterium (strain AK-01), the *n*-alkanes serving as growth substrates influenced the pattern of cellular fatty acids in a similar manner as in strain Pnd3; in addition, 2-, 4-, and 6-methyl-branched fatty acids were identified (44). By labeling studies, the methyl branch of the fatty acids was shown to be the original terminal carbon of the *n*-alkane, suggesting addition of a carbon compound to the subterminal position (carbon-2) of the *n*-alkanes; the carboxyl group was not derived from bicarbonate (44).

So far, no metabolites have been detected that could represent the direct product of the initial anaerobic reaction of an *n*-alkane. The present study was undertaken to identify such a metabolite and to gain insights into the mechanism of its formation. For the experiments, the recently isolated denitrifying strain HxN1 (18) was chosen. In contrast to other *n*-alkane-degrading anaerobic bacteria, strain HxN1 grows relatively rapidly (doubling time of 11 h under optimal conditions) and does not adhere to the insoluble alkane phase, so that cells can be harvested easily. Strain HxN1 utilizes *n*-alkanes with relatively short chains (C_6 through C_8) that are oxidized to CO_2; other anaerobic *n*-alkane-degrading strains grow preferentially with chain lengths between C_8 and C_{18}. Recently, two-dimensional gel electrophoresis of cell extracts of strain HxN1 revealed specific formation of proteins during growth on *n*-hexane that were not formed on *n*-hexanoate. These proteins were therefore supposed to be specifically involved in initial reaction steps of *n*-alkanes. In the N terminus, one of these proteins exhibited a similarity to the small subunit (BssC) of benzylsuccinate synthase in denitrifying bacteria (A. Behrends, P. Ehrenreich, J. Heider, T. Hurek, S. Ratering, and F. Widdel, unpublished data). This enzyme activates toluene anaerobically by addition of the methyl group to fumarate, yielding benzylsuccinate (24), and there is evidence for the involvement of a glycyl radical in this reaction (17, 31; C. Leutwein, A. J. Pierik and J. Heider, personal communication). Hence, we expected a parallel between the mechanism of the initial reaction of *n*-alkanes and that of toluene in anaerobic bacteria. Still, a reaction of an alkane in the absence of oxygen would present a novel type of biochemical mechanism, because a saturated hydrocarbon is much less reactive than toluene. In this paper, we report the identification of (1-methylpentyl)succinate (MPS) formed during strictly anaerobic growth of strain HxN1 with nitrate and with *n*-hexane as the only organic substrate. Furthermore, an electron paramagnetic resonance (EPR) signal characteristic of a glycyl radical was detected in *n*-hexane-grown cells of strain HxN1. We therefore propose the formation of MPS from *n*-hexane and fumarate via a radical mechanism as the initial reaction in strain HxN1.

MATERIALS AND METHODS

Organism and cultivation. The denitrifying strain HxN1 has been subcultured since its isolation from an enrichment culture (18). It was cultivated under anoxic conditions in chemically defined, bicarbonate-buffered medium with nitrate (9 mM) as the electron acceptor, as described previously (18, 38). Freshly prepared sodium ascorbate (4 mM) was added as the reductant and allowed to scavenge traces of oxygen by incubation for >1 day of the medium before anaerobic inoculation (18). Cultures were grown in stopper-sealed flat glass bottles (500 ml) containing 400 ml of medium under a head space of N_2-CO_2 (90:10, vol/vol). Since pure *n*-hexane is inhibitory, it was diluted (5% [vol/vol]) in sterile, deaerated 2,2,4,4,6,8,8-heptamethylnonane as an inert, nontoxic carrier phase (18) before addition to the culture (10 ml of mixture per bottle) by means of an N_2-flushed plastic syringe; [1-^{13}C]*n*-hexane was applied in the same manner. d_{14}-*n*-Hexane was mixed with an equal volume of unlabeled *n*-hexane, and the isotopomer mixture was applied in the carrier phase like unlabeled *n*-hexane alone. The hydrocarbons were filter sterilized (regenerated cellulose, 0.2-μm pore size; Schleicher & Schuell, Dassel, Germany) before addition. Sodium *n*-hexanoate (caproate) in control experiments was added from an autoclaved stock solution (final concentration, 3 mM). Cells were passaged (inoculum size, 5% [vol/vol]) with *n*-hexane or *n*-hexanoate as organic substrates at least 10 times before the cultures for the experiments described were inoculated. Cultures with *n*-hexane were incubated on a slowly rotating shaker (50 rpm) nearly horizontally, with the orifices below the medium surface, so that contact of the overlying, hydrophobic *n*-hexane/carrier phase mixture with the stoppers was avoided. The incubation temperature was 28°C. In labeling experiments with potential cosubstrates, cultures were grown on *n*-hexane to an optical density at 660 nm (OD_{660}) of 0.1. Then, disodium 2,3-d_2-fumarate or 2,2,3,3-d_4-succinate was added (final concentration, 10 mM), and the culture was allowed to grow to a higher OD. All cultures were used for the described investigations (see following sections) immediately after depletion of nitrate and formed nitrite; the final OD_{660} was usually 0.3 to 0.4. The presence of nitrate and nitrite was examined by using disposable test strips (Merck, Darmstadt, Germany).

Analysis of metabolites. Cultures for metabolite analysis were heated in closed bottles in a water bath to 85°C for 15 min to inactivate eventually present enzymes that may catalyze reactions with oxygen during further handling in the air. Also, release (or additional release) of metabolites into the medium was expected from this treatment. In the case of *n*-hexane-grown cultures, the aqueous phase was separated from the overlying carrier phase via a separatory funnel. The heated cells were removed by centrifugation (7,000 × *g*, 20 min) to avoid possible interference with phase separation during subsequent ether extraction. The supernatant from each culture (400 ml) was extracted with diethyl ether (80 ml), first at pH 7.5 and subsequently at pH 1.5 (adjusted with phosphoric acid); extraction at each pH was performed three times. The three corresponding ether extracts were pooled, dried over anhydrous Na_2SO_4, and stored at 4°C in glass bottles sealed with Teflon-coated screw caps until further analysis. Directly before analysis, each ether extract was evaporated to dryness, taken up in 1 ml of dichloromethane, and methylated using an etheral solution of diazomethane that was freshly prepared from Diazald (Sigma-Aldrich, Deisenhofen, Germany) according to standard procedures (Technical Bulletin AL113; Aldrich Chemical Co., Milwaukee, Wis.).

Gas chromatographic-mass spectrometric (GC-MS) analysis of methylated culture extracts was performed on a type 5890 gas chromatograph (Hewlett Packard, Waldbronn, Germany) connected to a type 95SQ mass spectrometer (Finnigan MAT/ThermoQuest, Egelsbach, Germany). The gas chromatograph was equipped with a KAS 3 injection system (Gerstel, Mülheim, Germany) and a BPX5 fused silica capillary column 50 m long, 0.22-mm inner diameter, and 0.25-μm film thickness. Helium was used as the carrier gas. The oven temperature was programmed from 60 to 340°C (8-min isotherm) at a heating rate of 3°C/min. The mass spectrometer was operated in electron impact mode at an electron energy of 70 eV and a source temperature of 260°C. Full-scan mass spectra were recorded over a mass range from 50 to 600 Da at a scan rate of 0.74 s per decade, with an interscan delay of 0.2 s and a scan cycle rate of 1 s.

Identity of metabolites with synthetic MPS or (1-ethylbutyl)succinic acid (EBS) was verified by coinjection of the methyl esters. Each coinjection was performed twice using two different gas chromatography columns. In addition to the column used for GC-MS analysis (see above), an Ultra 1 fused silica capillary column (50 m long, 0.22-mm inner diameter, and 0.33-μm film thickness) was used, with hydrogen as the carrier gas. All coinjection analyses were performed on the same model of gas chromatograph and with the same temperature program as described for GC-MS; however, peaks were detected via flame ionization. 5α-Androstane served as an internal standard for quantification of extracted metabolites.

EPR spectroscopy. Cells for EPR analysis were harvested from six cultures (400 ml each) freshly grown with *n*-hexane or *n*-hexanoate. All steps were performed under strictly anoxic conditions either inside an anoxic chamber (Coy, Ann Arbor, Mich.) with a fresh oxygen-scavenging catalyst, or under gassing of tubes with pure N_2 (52). Cells from 2.4 liters of culture volume were centrifuged

(7,000 × *g*, 20 min) and resuspended in anoxic water (10 ml) containing sodium dithionite (0.1 mM) to a final volume of approximately 5 ml. One part of this concentrated suspension was disrupted by passage through a French pressure cell (9.5-mm piston, 120 MPa). Removal of cell debris by centrifugation (20,000 × *g*, 20 min) yielded the crude extract. From this, another aliquot was used to sediment membranes (100,000 × *g*, 60 min); this supernatant is referred to as the soluble extract. Protein in lysed cells and extracts was quantified by the method of Bradford (12) with bovine serum albumin as the standard. Aliquots (each approximately 0.25 ml) from the cell suspension, the crude extract, and the soluble extract were transferred to anoxic EPR tubes, sealed under N_2, and immediately frozen and stored in liquid N_2.

EPR spectroscopy was carried out using an EMX-6/1 X-band EPR spectrometer (Bruker, Karlsruhe, Germany) with a standard TE102 rectangular cavity and an ESR-900 helium flow cryostat with variable temperature (Oxford Instruments, Oxford, U.K.). EPR spectra were recorded under nonsaturating conditions and present averages of 5 to 20 scans. Data acquisition was done with the software supplied by Bruker (WINEPR acquisition program, version 2.3.1); data manipulation (determination of *g* values, subtraction, baselining, and integration) was done with the WINEPR program version 2.11. Spin integration with correction for *g* anisotropy in field-swept spectra (1) was performed using a solution of 10 mM $CuSO_4$, 2 M $NaClO_4$, and 10 mM HCl as the standard.

Chemical synthesis of MPS and EBS. MPS and EBS as standard compounds were synthesized from succinic acid dimethyl ester and 2-hexanone or 3-hexanone, respectively. The reaction sequences involved Stobbe condensation of the carbonyl compounds with succinic acid dimethyl ester, dehydration yielding the unsaturated intermediate, and catalytic hydrogenation with Raney nickel, analogous to the synthesis of 3-hydroxybenzylsuccinate described elsewhere (35). This procedure of chemical synthesis yields racemic diastereomers of MPS and EBS. Methylation of the substituted succinic acids using a solution of diazomethane in diethyl ether yielded the respective dimethyl esters. The diastereomer mixtures of the dimethyl esters of MPS and EBS were isolated as colorless oily liquids from the reaction mixture by column chromatography on silica gel with a dichloromethane-methanol mixture (99:1). Structures were confirmed by ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra recorded on a Varian Gemini300 instrument (Varian, Darmstadt, Germany). Signals from (1-methylpentyl) succinic acid dimethyl ester (mixture of diastereomers) were as follows: ^1H NMR (300 MHz, $CDCl_3$, trimethylsilane [TMS]): δ = 3.697 (s, 3H), 3.693 (s, 3H), 3.676 (s, 3H), 3.673 (s, 3H), 2.92-2.67 (m, 4H), 2.43-2.31 (m, 2H), 1.86-1.95 (m, 1H), 1.72-1.84 (m, 1H), 1.15-1.35 (m, 12H), 0.84-0.92 (m, 12H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 175.10, 174.68, 173.12, 172.96, 51.72, 51.68, 51.53, 46.03, 45.61, 35.03, 34.50, 34.01, 33.55, 33.27, 31.32, 29.39, 29.28, 22.67, 16.85, 16.19, and 13.98 ppm. Signals from (1-ethylbutyl)succinic acid dimethyl ester (mixture of diastereomers) were as follows: ^1H NMR (300 MHz, $CDCl_3$, TMS): δ = 3.690 (s, 6H), 3.684 (s, 6H), 2.87-3.05 (m, 2H), 2.69-2.79 (m, 2H), 2.29-2.36 (m, 2H), 1.62-1.72 (m, 2H), 1.15-1.38 (m, 12H), 0.85-0.94 (m, 12H) ppm; ^{13}C NMR (75 MHz, $CDCl_3$, TMS): δ = 175.34, 173.18, 51.79, 51.70, 43.07, 41.21, 33.17, 32.97, 31.99, 31.78, 24.17, 23.70, 20.41, 20.26, 14.24, 14.19, 11.81, and 11.65 ppm.

Chemicals. Chemicals were of analytical grade and mostly obtained from Fluka (Deisenhofen, Germany) or Merck (Darmstadt, Germany). Purity of *n*-hexane was ≥99.5%. d_{14}-*n*-Hexane, 2,2,3,3-d_4-succinic acid, 2-hexanone, 3-hexanone, and succinic acid dimethyl ester were purchased from Sigma-Aldrich (Deisenhofen, Germany). [1-^{13}C]*n*-Hexane and 2,3-d_2-fumaric acid were purchased from Campro Scientific (Berlin, Germany). 2,2,4,4,6,8,8-Heptamethylnonane was from Alfa/Johnson Matthey (Karlsruhe, Germany). Acids used as substrates (*n*-hexanoic acid, labeled fumaric acid, and labeled succinic acid) were titrated to yield aqueous solutions (1 M, pH 8) of sodium salts prior to addition to the medium. Diethyl ether of analytical grade was obtained from Riedel-de Haën (Seelze, Germany).

Calculation of free energies. ΔG° values of reactions were calculated from G_f° values. The G_f° values (in kilojoules per mole) of the following species are given in the literature: fumarate^{2-} (aq), −604.2 (48); methane (g), −50.8 (48); *n*-hexane (lq), −4.3 (47); and toluene (lq), +114.2 (48). G_f° values of substituted succinates, which are not given in the literature, were calculated from increments using two different algorithms (34, 47). The G_f° values obtained (in kilojoules per mole) were as follows: methylsuccinate^{2-} (aq), −681.6 and −685.5; MPS^{2-} (aq), −647.3 and −644.0; and benzylsuccinate^{2-} (aq), −525.4 and −521.1. Respective G_f° values calculated via increments for succinate^{2-} (aq) as a test case were −687.5 and −690.0, compared to the literature value of −690.2 kJ/mol (48). The equilibrium constant was calculated as $K = \exp(-\Delta G^\circ/RT)$, where R is the gas constant and T is the absolute temperature.

RESULTS

Identification of MPS as a metabolite specifically formed from *n*-hexane. GC-MS analysis of ether extracts from cultures of strain HxN1 grown with *n*-hexane and subsequently pasteurized without access to air revealed 12 pronounced peaks of metabolites in the supernatant. These were not detectable in cultures grown with *n*-hexanoate. Peaks were only detectable if the culture (pH of about 7.5) was acidified before ether extraction, indicating that metabolites were carboxylates. No significant portions of metabolites were left in the cell pellet of the heat-treated culture.

Because of the hypothesized initial reaction of *n*-alkanes with fumarate yielding an alkyl-substituted succinate (see the introduction), we focused in the present study on the identification of dicarboxylic acids. Methyl esters of these eluted in the gas chromatogram after 33 min (Fig. 1) behind peaks of various monocarboxylic acids (not shown). The mass spectra corresponding to the three depicted peaks (Fig. 1) were very similar and thus provided evidence for a close structural relationship between them (Table 1). The relatively simple fragmentation patterns were essentially in agreement with the structure of C_6 alkyl-substituted succinic acid dimethyl esters. Weak but definite and specific signals at *m/z* 173 ($M^+ - C_4H_9$; from compounds 2 and 3 in Fig. 1) and *m/z* 187 ($M^+ - C_3H_7$; from compound 1 in Fig. 1) pointed at methyl esters of MPS and EBS, respectively. Details of the fragmentation pattern are shown for MPS dimethyl ester in Fig. 2A. To confirm the interpretation of the GC-MS results, authentic standards of MPS and EBS dimethyl esters were synthesized. Based on coinjection on two different GC columns and identity of the mass spectra, MPS and EBS dimethyl esters were identified unambiguously. No evidence for the presence of *n*-hexylsuccinate (as dimethyl ester), another hypothetical transformation product of *n*-hexane (see Discussion), was obtained. On the basis of structural considerations it is very unlikely that *n*-hexylsuccinate dimethyl ester, which is expected to have a mass spectrum similar to those of MPS and EBS dimethyl esters, would coelute with either of these two compounds. However, this assumption has to be verified by synthesis and analysis of an authentic standard of *n*-hexylsuccinate dimethyl ester. The concentration of MPS in the supernatant of the heat-treated culture was approximately 5 μM.

Both MPS and EBS possess two chiral carbon atoms and can in principle exist as two racemic diastereomers. The two adjacent peaks that were observed with the methylated culture extract (compounds 2 and 3, Fig. 1) as well as with the synthetic standard had essentially the same mass spectra and were therefore assigned to the two diastereomeres of MPS dimethyl ester. In contrast, synthetic EBS dimethyl ester yielded only one peak, suggesting that diastereomers were not separable on the columns used; it is therefore presently unknown whether EBS from strain HxN1 consists of diastereomers like MPS. Assignment of the configuration to the two diastereomers of MPS was not possible due to the lack of standards of the pure diastereomers. At present it is also unknown whether the diastereomers of MPS from strain HxN1 are racemic.

Labeling studies. To examine the formation of MPS in more detail, cultures were provided with labeled *n*-hexane or with labeled fumarate or succinate as possible precursors of the

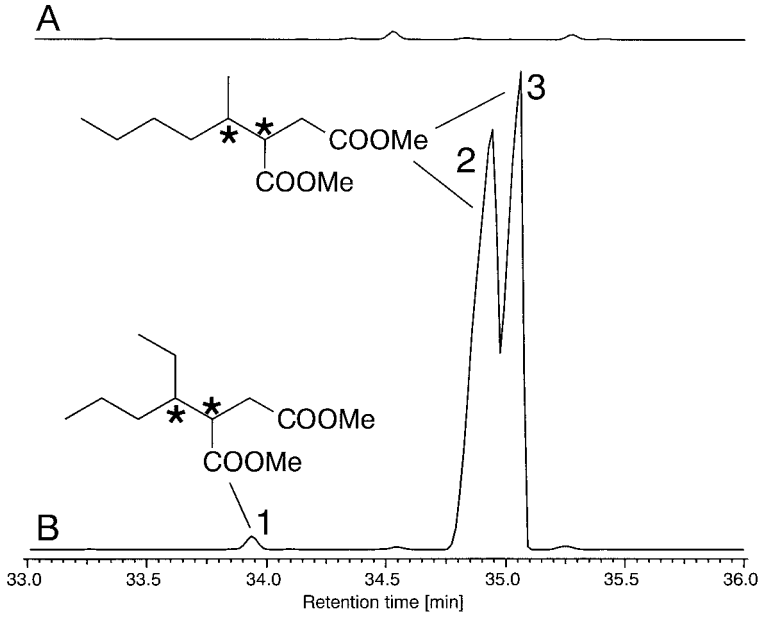

FIG. 1. Partial gas chromatograms of methylated extracts from heat-treated, acidified cultures of strain HxN1 after anaerobic growth with nitrate as the electron acceptor and with *n*-hexanoate (A) or *n*-hexane (B) as the only organic substrate. Both chromatograms were recorded at the same detection sensitivity. Mass spectrometric data from peaks are shown in Table 1; the mass spectrum of compound 3, which was essentially the same as of compound 2, is shown in Fig. 2A. Compounds 2 and 3 are interpreted as separable diastereomers of MPS dimethyl ester; possible diastereomers of compound 1, viz., of EBS dimethyl ester, were not separable on the gas chromatography column. Asterisks (∗) indicate chiral carbon atoms; assignment of the absolute stereochemistry of diastereomers was not achieved in the present study.

dicarboxylate moiety in MPS. Growth of cultures was not affected when unlabeled *n*-hexane was completely replaced by [1-^{13}C]*n*-hexane. In contrast, when d_{14}-*n*-hexane was provided as the only organic substrate, no growth occurred within 2 weeks. Growth tests with mixtures of unlabeled *n*-hexane and d_{14}-*n*-hexane at various ratios revealed a 1:1 mixture (equal volumes) as the highest portion of deuterated *n*-hexane that still allowed growth; this ratio was therefore applied for labeling studies. Addition of 2,3-d_2-fumarate or 2,2,3,3-d_4-succinate to cultures growing with *n*-hexane did not impede growth; in contrast, these dicarboxylic acids even stimulated growth, because strain HxN1 uses them readily as carbon sources and electron donors (Behrends et al., unpublished).

Assignment of the structures obtained from the labeling experiments was always straightforward due to the observation of relevant fragments in the mass spectra. In all these experiments, the diastereomers of MPS dimethyl ester exhibited the same labeling patterns.

Utilization by strain HxN1 of [1-^{13}C]*n*-hexane as the only organic substrate yielded isotope peaks in the fragmentation patterns of extracted MPS methyl ester (not shown) that indicated full preservation of the label in the 1-methylpentyl side chain.

Growth with the mixture of *n*-hexane and d_{14}-*n*-hexane yielded two polydeuterated MPS isotopomers in addition to monodeuterated and unlabeled MPS isotopomers. The former two displayed a significantly shorter retention time and could be clearly separated by gas chromatography from the isotopically lighter compounds. In contrast, complete separation of the two polydeuterated isotopomers or the two lighter isotopomers from each other could not be achieved with the gas chromatographic method used. Nevertheless, the labeling patterns of the polydeuterated and the lighter isotopomers of MPS dimethyl ester could be resolved by careful selection of mass spectra from the ascending and descending parts of the gas chromatographic peaks. One of the polydeuterated prod-

TABLE 1. Mass spectral data and structural assignment of metabolites (after methylation) formed during growth of strain HxN1 on *n*-hexane and nitrate under anoxic conditions

Peak[a]	Key ions, *m/z* (%)	Molecular mass (M_r)	Formula	Assigned compound
1	M^+ absent; 199 (11.0), 187 (0.1), 157 (21.7), 146 (47.8), 114 (100), 87 (11.4), 69 (7.7), 59 (7.4), 55 (15.8)	230	$C_{12}H_{22}O_4$	EBS dimethyl ester, mixture of diastereomers
2	M^+ absent; 199 (11.0), 173 (0.5), 157 (27.5), 146 (40.6), 114 (100), 87 (8.1), 69 (5.6), 59 (5.9), 55 (12.3)	230	$C_{12}H_{22}O_4$	MPS dimethyl ester
3	M^+ absent; 199 (10.5), 173 (0.6), 157 (21.7), 146 (42.6), 114 (100), 87 (8.4), 69 (6.1), 59 (6.7), 55 (13.8)	230	$C_{12}H_{22}O_4$	MPS dimethyl ester

[a] Numbers correspond to peaks in Fig. 1.

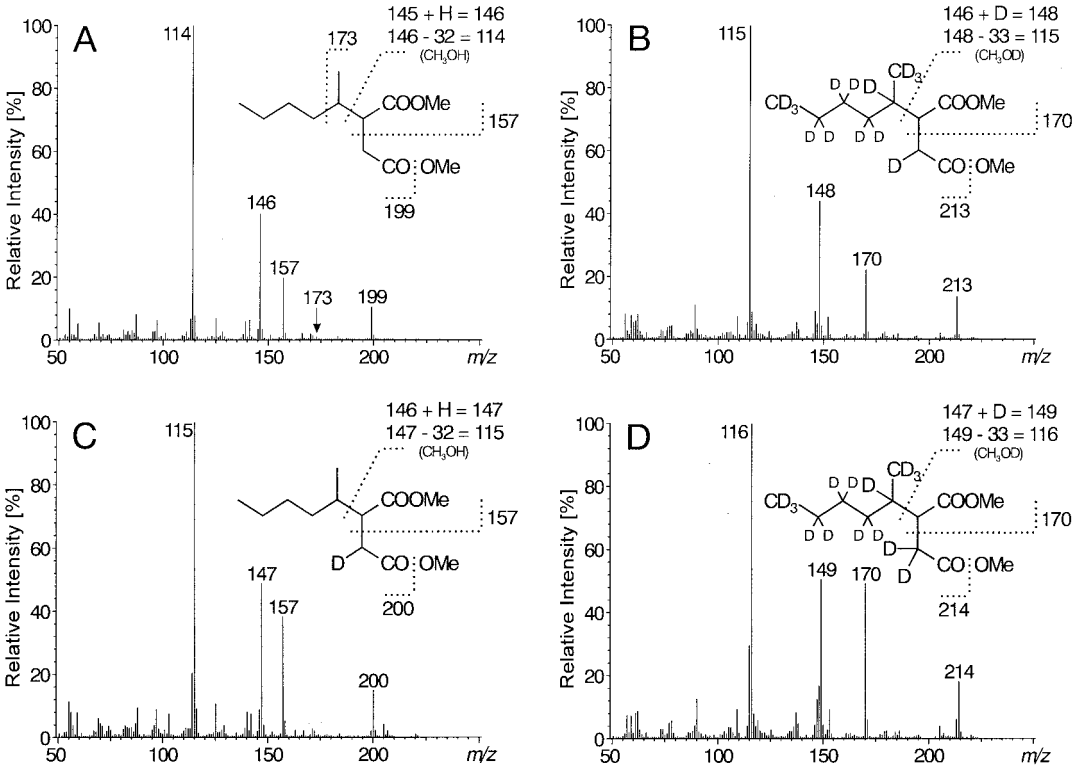

FIG. 2. Mass spectra of methylated MPS (diastereomer corresponding to peak 3 in Fig. 1B) from anaerobic cultures of strain HxN1 grown with nitrate as the electron acceptor and unlabeled and/or deuterium-labeled organic substrates. The following experiments were conducted (panel of recorded mass spectra indicated in parentheses): unlabeled *n*-hexane (A); mixture of unlabeled *n*-hexane and d_{14}-*n*-hexane (A, B, C, and D); and unlabeled *n*-hexane and 2,3-d_2-fumarate or 2,2,3,3-d_4-succinate (A and C).

ucts (Fig. 2B compared to Fig. 2A) showed the presence of 14 deuterium atoms and preservation of 13 deuterium atoms in the fragment (*m/z* 170) carrying the 1-methylpentyl side chain of MPS. Fragments at *m/z* 148 and 115 (Fig. 2B) localized the remaining deuterium atom at carbon-2 or carbon-3, whereas the crucial fragment at *m/z* 170 allowed a definite localization at carbon-3 of the succinate moiety. Hence, there was always a hydrogen atom and never a deuterium atom at carbon-2 of the succinate moiety. The mass spectrum of the other polydeuterated isotopomer of MPS showed the presence of 15 deuterium atoms (Fig. 2D). Again, 13 deuterium atoms were present in the fragment carrying the 1-methylpentyl side chain. The two remaining deuterium atoms were located at carbon-3 of the succinate moiety.

Upon addition of 2,3-d_2-fumarate to cultures utilizing unlabeled *n*-hexane, an unlabeled and a monodeuterated isotopomer of MPS dimethyl ester (Fig. 2A and C) were detected and identified as described in the experiment with the mixture of *n*-hexane and d_{14}-*n*-hexane (see above). Also in this experiment, the deuterium in the labeled MPS could be definitely localized at carbon-3 of the succinate moiety, due to the crucial fragment at *m/z* 157 (Fig. 2C). Location of the deuterium atom at any position other than carbon-3 of the succinate moiety would not be in agreement with the observed fragmentation pattern. When 2,2,3,3-d_4-succinate was added instead of 2,3-d_2-fumarate, the same products (MPS and 3-d_1-MPS) but no MPS isotopomers containing more than one deuterium atom were detected.

Evidence for the presence of a glycyl radical. Dense cell suspensions, crude extract, and soluble extract from strain HxN1 grown on either *n*-hexane or *n*-hexanoate were studied by EPR spectroscopy. A characteristic intense EPR signal was observed in cells of *n*-hexane-grown cultures. (Fig. 3B). In crude and soluble extracts of *n*-hexane-grown cells, a signal with identical EPR parameters could also be observed, with intensity losses of 7 and 27%, respectively (data not shown). The *g* value (2.0032), line width, relaxational behavior ($P_{1/2}$ at 77 K is 0.05 mW), and strong hyperfine coupling to a single proton (1.5 ± 0.1 mT) are indicative of an organic radical centered at the α-carbon of an amino acid. With *n*-hexanoate-grown cells, the signal indicative of such an organic radical was less than 4% of the signal from *n*-hexane-grown cells (Fig. 3A), and only a very weak nonhyperfine resolved isotropic signal at $g = 2.004$ with a line width of 1.7 ± 0.2 mT was present. The signal persisted in the crude and soluble extract, with similar amplitude and EPR parameters (not shown), and is tentatively assigned to a soluble flavoprotein in the semiquinone form. Quantification of the intense EPR signal by double integration and correction for the assumed presence of a flavin semiquinone background signal as in *n*-hexanoate-grown cells (only 10 to 20% of the double integral) indicated a concentration of the radical of 5 μM in the cell suspension. The protein content in

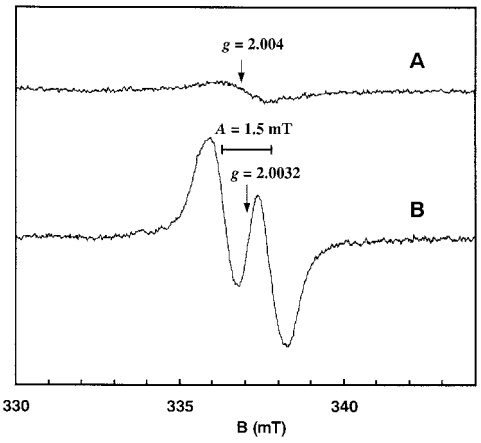

FIG. 3. EPR spectra of suspensions of cells of strain HxN1 grown anaerobically with *n*-hexanoate (A) and *n*-hexane (B) as the only organic substrates and nitrate as the electron acceptor. The relative amplitudes were normalized for equal concentrations of soluble protein. Conditions for recorded EPR spectra: 60 K; microwave power, 13 μW; modulation amplitude, 0.45 mT; microwave frequency, 9.46 GHz; modulation frequency, 100 kHz.

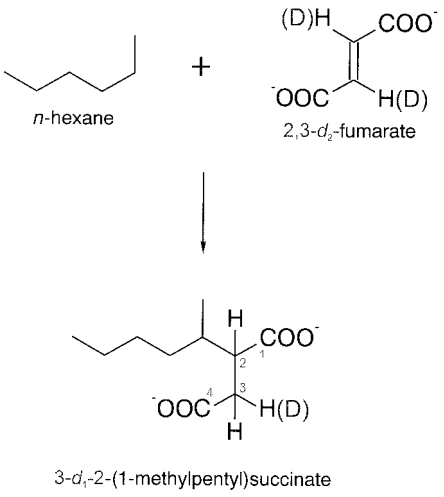

FIG. 4. Proposed initial reaction of *n*-hexane with fumarate yielding MPS during anaerobic growth of denitrifying strain HxN1. Deuterium-labeled positions in a parallel experiment with 2,3-d_2-fumarate added to an *n*-hexane-utilizing culture are indicated in grey.

the undiluted soluble extracts was 12.4 and 9.4 mg/ml for cells grown on *n*-hexanoate and *n*-hexane, respectively.

DISCUSSION

Supposed initial reaction of *n*-hexane. MPS was identified as a prominent metabolite in cultures of strain HxN1 grown anaerobically with *n*-hexane and nitrate. In parallel cultures grown on *n*-hexanoate, MPS was not detectable, indicating that this compound is specifically formed during *n*-hexane metabolism. The finding of the ^{13}C label from [1-^{13}C]*n*-hexane in the 1-methylpentyl side chain proves that this moiety originates from the *n*-alkane. This assumption is further confirmed by the observed preservation of 13 deuterium atoms from d_{14}-*n*-hexane in the 1-methylpentyl side chain of MPS. If *n*-hexane is the only organic substrate, the dicarboxylate moiety in MPS also has to be derived, at least in part, from the carbon of *n*-hexane; this transformation has to involve various metabolic steps that may include oxidation/reduction, cleavage, and carboxylation reactions. The origin of the dicarboxylate moiety of MPS is best explained if a C_4 dicarboxylic acid is assumed to be the direct precursor. Such an assumption agrees well with the hypothesized mechanistic relationship between the initial reactions of *n*-alkanes and of toluene in denitrifying bacteria (see the introduction). Toluene is activated by addition of the methyl group to fumarate as a cosubstrate, yielding benzylsuccinate (7, 9, 24, 31). In analogy to this, the formation of MPS can be explained by an addition of *n*-hexane with its subterminal carbon atom to fumarate (Fig. 4). Another metabolite, EBS, was identified in cultures of strain HxN1, and the amount of EBS extracted was 100-fold lower than that of MPS. It is true that a low pool size of a metabolite under nonequilibrium conditions in active cells does not necessarily indicate that the compound is of minor relevance. Nevertheless, there are hints that EBS is a by-product (formed by "accidental" reaction) rather than a true intermediate and that MPS is the principal product of the initial reaction of *n*-hexane degradation. Monocarboxylic organic acids (with fewer carbon atoms than MPS and EBS) detected in extracts from *n*-hexane-grown cultures and tentatively identified as possible subsequent metabolites contain methyl branches rather than ethyl branches (H. Wilkes, R. Rabus, T. Fischer, A. Armstroff, A. Behrends, and F. Widdel, unpublished data).

Experiments with the mixture of unlabeled *n*-hexane and d_{14}-*n*-hexane demonstrated the formation of isotopomers of MPS with one and two deuterium atoms at carbon-3 of the succinate moiety. These results suggest that the deuterium at carbon-3 of MPS originates via two different routes. First, the deuterium atom that has to be removed from d_{14}-*n*-hexane to enable formation of the new C-C bond may be attached at carbon-3 of the succinate moiety in MPS rather than being diluted in a general pool of hydrogen ions. Such a deuterium transfer would be in accordance with a radical mechanism (see next section) and the assumed mechanistic similarity with the initial reaction of toluene in anaerobes. The proposed reaction mechanism of toluene includes abstraction of a hydrogen (or deuterium) atom from toluene (or methyl-d_3-toluene), addition of the benzyl radical formed to carbon-2 of fumarate, and final recombination of the hydrogen (or deuterium) atom with the radical at carbon-3, yielding benzylsuccinate (6, 24). Second, formation of d_{15}-MPS with two deuterium atoms at carbon-3 indicates conservation of the label from d_{14}-*n*-hexane in fumarate as the assumed cosubstrate via presently uncertain metabolic reactions (without or with partial proton/deuteron exchange with H_2O).

The formation of two diastereomers of MPS in strain HxN1 is remarkable, since most enzymatic reactions are highly stereoselective. MPS has two chiral carbon atoms (Fig. 1), so that two racemic diastereomers (viz., four stereoisomers) do in principle exist. Formation of all four stereoisomers in an enzymatic reaction is highly unlikely. However, each of the two observed diastereomers of MPS from strain HxN1 may represent only one enantiomer. Formation of two pure, nonracemic diastereomers would occur in an initial reaction with stereose-

lectivity with respect to only one of the two newly formed chiral centers. In the initial reaction of toluene, the formation of benzylsuccinate (which has only one chiral center) was shown to be stereospecific, with addition of the benzyl moiety at the *re* face of fumarate yielding *R*-(+)-benzylsuccinate (6, 32). In view of this and other highly stereoselective biochemical reactions of fumarate, such as hydration to malate (50), it is likely that MPS formation is also stereospecific at the fumarate carbon (viz., at either the *si* or *re* face). One may therefore envision that the MPS-forming enzyme exhibits relaxed stereospecificity with respect to carbon-2 of the *n*-hexane moiety. Confirmation of this hypothesis requires unequivocal assignment of the absolute configurations of MPS stereoisomers.

The formation of 3-d_1-MPS upon addition of 2,3-d_2-fumarate to *n*-hexane-utilizing cultures of strain HxN1 indicates a loss of the deuterium atom/ion at the carbon atom that is linked to the alkane chain. Such a loss is not in agreement with the formal addition of the 1-methylpentyl group to the double bond of fumarate in analogy with the presently assumed mechanism of toluene activation. Such a formal addition of unlabeled *n*-hexane to 2,3-d_2-fumarate would yield 2,3-d_2-MPS, which was, however, never detected. Two explanations for the loss of deuterium at carbon-2 of MPS may be envisioned. (i) Fumarate is not the actual cosubstrate but is converted to another, presently unknown compound that is the direct reactant and that has not kept the hydrogen or deuterium at the reacting carbon atom. (ii) Fumarate is the actual cosubstrate, but the deuterium or hydrogen atom at the reacting carbon-2 exchanges with external hydrogen species in an unknown, eventually ionic reaction step immediately before, during, or after the formation of the novel C-C bond. It is unlikely that the observed loss of deuterium at carbon-2 of MPS occurs via a D/H exchange at deuterated fumarate in a reversible (back and forth) reaction at succinate dehydrogenase. Such a reaction would yield a mixture of 2,3-d_2-, 2-d_1-, and unlabeled fumarate and, upon formal addition of *n*-hexane to the double bond, a mixture of 2,3-d_2-, 2-d_1-, and 3-d_1-labeled as well as unlabeled MPS; however, such a mixture of MPS isotopomers has never been detected. In conclusion, our experiments indicate that the hydrogen atom of not only the hydrocarbon substrate but also of the assumed cosubstrate fumarate is involved in the mechanism of MPS formation. In the presently proposed mechanistic model of the initial reaction of toluene, the hydrogen atoms of fumarate are assumed to be maintained (24), even though this has not been proven. Experiments with labeled fumarate and unlabeled toluene are needed to prove whether and to what extent the mechanisms of *n*-alkane and toluene activation differ in some details. An activated succinate derivative is unlikely as the direct cosubstrate in the initial reaction of *n*-hexane; the loss of three of the four deuterium atoms from 2,2,3,3-d_4-succinate suggests succinate oxidation to fumarate prior to a reaction with *n*-hexane.

In view of the observed benzylsuccinate formation in a variety of toluene-degrading anaerobic microorganisms, such as denitrifying (7, 9, 37), sulfate-reducing (8, 37), and anoxygenic phototrophic bacteria (54), one may assume that the initial reaction of *n*-alkanes in various groups of anaerobes also follows the same principle, with fumarate as the direct reactant. Different responses of the fatty acid patterns in strains of sulfate-reducing bacteria to growth with long-chain *n*-alkanes (3) may be due to different sites of initial attack and fumarate addition at the carbon chain. The formation of methyl-branched fatty acids in sulfate-reducing strain AK-01 grown on long-chain *n*-alkanes and indication of subterminal carbon addition to the chain (44) may also be considered a result of an addition to fumarate analogous to that in strain HxN1. A more detailed and comparative study of metabolic pathways is needed to elucidate the further metabolism of MPS and initial reaction products of other *n*-alkanes.

Significance of the identified radical. The distinct EPR signal in *n*-hexane-grown cells and its virtual absence in *n*-hexanoate-grown cells of strain HxN1 provide evidence for the specific involvement of a radical in the anaerobic initial reaction of *n*-hexane. The observed signal is from a radical species which has a strong coupling to a single hydrogen atom. The average *g* value, line shape, and magnitude of the hyperfine coupling is atypical for radicals at carbon side chains of amino acids (viz., at amino acids other than glycine), flavin radicals, or sulfur-centered radicals. To our knowledge, there are presently no biochemically purified cell components other than glycyl radical enzymes which exhibit such an EPR signal. However, it cannot be excluded that a 1-methylpentyl radical rather than a glycyl radical is present in a protein that is specifically formed during growth of strain HxN1 on *n*-hexane. It is true that α-carbon-centered radicals may occur at amino acids other than glycine (40); however, these would not have the observed strong hyperfine coupling to a single hydrogen atom. In anaerobic bacteria, signals like the present one have thus far only been observed in the case of glycyl radical enzymes (42). Such enzymes are pyruvate formate-lyase (27–29), anaerobic ribonucleotide reductase (36, 45, 46, 53), and the toluene-activating benzylsuccinate synthase (C. Leutwein, A. J. Pierik, and H. Heider, personal communication). Before the EPR measurement with benzylsuccinate synthase, evidence for the presence of a glycyl radical in benzylsuccinate synthase was provided by sequence similarity with other glycyl radical enzymes (17, 31), and polypeptide fragmentation upon exposure to oxygen (31). Hence, the EPR spectrum obtained from *n*-hexane-grown cells of strain HxN1 is in favor of the presence of a glycyl radical in this bacterium and further substantiates the assumed parallel between the initial reactions of *n*-alkanes and toluene in anaerobes. A small *n*-hexane-induced protein in strain HxN1 with N-terminal sequence similarity to the small subunit (BssC) of benzylsuccinate synthase (Behrends et al., unpublished) may be part of the assumed *n*-alkane-activating enzyme. Still, a definite assignment of the EPR signal to a glycyl radical would be premature at present and has to be substantiated by further experiments, such as EPR measurements upon growth with labeled glycine and purification of the radical-bearing species.

The determined concentration of the radical in cells of strain HxN1 allows an estimation of the cellular content of the radical-harboring protein. Using the presently determined protein content in the soluble cell extract and a molecular mass of 60 kDa as an average value for cytoplasmic proteins, it can be estimated that the radical enzyme in *n*-hexane-metabolizing cells of strain HxN1 amounts to 3.6% (by mass) of the soluble protein; the amount would be 6% if 100 kDa was used as the average molecular mass of glycyl radical enzymes as the basis for the estimation. Two other catabolic glycyl radical enzymes, pyruvate-formate lyase in fermentatively growing *Escherichia*

coli and benzylsuccinate synthase in toluene-degrading *Thauera aromatica*, were also proportionately significant proteins, with a cellular content of 2 to 3% (30, 31). The rather high content of all these proteins may compensate for the relatively low specific activity of radical enzymes that catalyze and control intricate anaerobic reactions.

If a 1-methylpentyl radical is generated in strain HxN1, this reaction does not necessarily have to occur by direct reaction of the glycyl radical with *n*-hexane. The glycyl radical in pyruvate-formate lyase of *E. coli* is regarded as a storage radical. A thiyl radical subsequently generated from a cysteyl residue is supposed to perform the actual attack on the substrate (5). Purification of the MPS-forming enzyme from strain HxN1 and structural investigations are needed to prove the assumed involvement of a glycyl radical.

Energetic aspects of anaerobic initial reactions of hydrocarbons. Even if the mechanisms of the initial reactions of *n*-alkanes and toluene in anaerobes follow the same principle, the activation steps are not energetically equivalent. The energetic expenditure for homolytic (and also heterolytic) cleavage of a C-H bond is higher with an alkane than with the methyl group of toluene. The resulting alkyl radical cannot be stabilized like the benzyl radical with its π-electron system. The C-H bond energies at the secondary carbon atom of an *n*-alkane and the methyl group of toluene are 401 and 368 kJ/molmol, respectively (33). However, these absolute values are not direct indicators of the actual activation energies of the hydrocarbons, because the transition state and its bond energies (such as that of transiently bonded $H^{\cdot}$ radical) at the enzyme are unknown. These values only indicate that the bond energy to be overcome in the case of *n*-hexane is 33 (= 401 − 368) kJ/mol higher than in the case of toluene. The bond energy to be overcome for the activation of an *n*-alkane at the primary (terminal) carbon (C-H bond energy, 419 kJ/mol) would be higher by as much as 51 kJ/mol in comparison to activation of toluene. This may explain why enzyme evolution has not favored *n*-hexane activation at the terminal carbon. A similar principle has been observed in the anaerobic synthesis of diabolic acid (15,16-dimethyltriacontanedioic acid), the only known reaction (19, 21) that resembles the anaerobic initial reaction of an alkane. Diabolic acid, a major lipid component in *Butyrivibrio fibriosolvens*, is formed by radical condensation of two *n*-hexadecanoic (palmitic) acid molecules at the subterminal carbon atom of their apolar residues. The reaction is assumed to proceed via simultaneous formation of a radical at each chain and hence differs in some detail from the suggested anaerobic alkane activation. Furthermore, radical formation in the synthesis of diabolic acid has been suggested to involve coenzyme B_{12} (21), whereas the glycyl radicals investigated so far are generated with *S*-adenosylmethionine (20, 23). Among chemical reactions, the addition of alkanes (e.g., of propane at carbon-2) to alkenes via a radical mechanism (33) has some similarity to the suggested route of MPS formation. However, the more rapid chemical reaction is performed at 500°C and 10 MPa and yields an uncontrolled variety of products. In addition, an ionic mechanism for alkane-alkene addition catalyzed by very strong acids is known (33).

Irrespective of the high energy barrier during alkane activation, the net reaction of *n*-hexane and fumarate yielding MPS is energetically favorable, with a standard free energy change ($\Delta G°$) of −35 to −39 kJ/mol (depending on the algorithm used for calculation of the $G_f°$ value for MPS); this corresponds to an equilibrium constant of $10^{6.1}$ to $10^{6.8}$. The $\Delta G°$ value for the reaction of toluene with fumarate yielding benzylsuccinate is −31 to −35 kJ/mol. This is in agreement with the observation that benzylsuccinate formation needs no coupling to an energy-yielding reaction such as ATP hydrolysis (9, 31), and the same may be true for MPS formation. Hence, fumarate with its π-bond is not only an acceptor to bind the unstable intermediate of hydrocarbon activation (viz., the postulated radical), but also a compound that "forces" the reaction to the side of the product. A reaction of fumarate with any hydrocarbon, even with methane as the least reactive one, would be energetically feasible (exergonic). A hypothetical reaction with methane yielding methylsuccinate would have a $\Delta G°$ value of −26.6 to −30.5 kJ/mol. However, there are no hints of such a reaction from studies of the anaerobic oxidation of methane in sediments. Anaerobic utilization of methane could not be demonstrated in any pure culture, including strain HxN1. Presently, the oxidation of methane in anoxic habitats is assumed to include a reversal of the steps of methanogenesis in archaea and scavenge of the product by associated sulfate-reducing bacteria (11, 25, 26).

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R. Rabus, H. Wilkes, and A. Behrends contributed equally to this study.

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ADDENDUM IN PROOF

A recent examination of metabolites in an anaerobic sulfate-reducing enrichment culture growing on *n*-dodecane also provided evidence for the formation of alkyl-substituted succinates (K. G. Kropp, I. A. Davidova, and J. M. Suflita, Appl. Environ. Microbiol. **66:**5393–5398, 2000).

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