

Anaerobic Degradation of Hexadecan-2-one by a Microbial Enrichment Culture under Sulfate-Reducing Conditions

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A microbial enrichment culture from marine sediment was able to grow on hexadecan-2-one as the sole source of carbon and energy under sulfate-reducing conditions. Oxidation of the ketone involved carboxylation reactions and was coupled to sulfide production. This enrichment culture also grew on 6,10,14-trimethylpentadecan-2-one.

Degradation of hydrocarbons under anoxic conditions has been demonstrated in microbial communities and pure cultures of sulfate-reducing (1, 2, 7, 15, 18, 26, 31), denitrifying (5, 17, 27), and iron(III)-reducing bacteria (19). Most of these studies involved aromatic hydrocarbons. Indeed, there are only a few reports on the anaerobic degradation of aliphatic hydrocarbons, particularly alkanes (1, 5, 20, 31). Due to the lack of unsaturations in alkanes, their degradation is difficult in the absence of molecular oxygen.

While alkanes cannot be directly photooxidized by solar light, there are several compounds in seawater (10) or petroleum (29) which are capable of introducing oxygen atoms into these hydrocarbon molecules by photochemical mechanisms. The photosensitized oxidation of *n*-alkanes generates mainly ketones and secondary alcohols with the same number of carbon atoms as the alkanes (11, 14), while oxidation of branched alkanes leads mainly to the formation of tertiary alcohols and ketones (30).

When photooxidation products are not fully degraded under aerobic conditions, they will reach anoxic environments where they might be degraded. Further, in the literature, there are relatively few reports dealing with degradation under anaerobic conditions of ketones and secondary alcohols resulting from hydrocarbon photooxidation. Acetone degradation has been shown to occur in an enrichment culture under methanogenic conditions (23) and also in pure cultures using nitrate (3, 24) or sulfate (25) as the electron acceptor. Higher ketones, such as butanone, pentan-2-one, hexan-2-one, and hexan-3-one, were degraded anaerobically by pure cultures of denitrifying bacteria (24). A pure culture of sulfate-reducing bacteria was also able to degrade butanone (13). A recent study showed the degradation of a branched isoprenoid ketone, 6,10,14-trimethylpentadecan-2-one, by a denitrifying marine bacterium (28). However, no evidence for microbial degradation of ketones higher than butanone or secondary alcohols under sulfate-reducing conditions has been presented. Owing to the abundance of sulfate in anoxic marine environments, experiments were conducted under sulfate-reducing conditions with

hexadecan-2-one, nonadecan-10-one, and two pristane photooxidation products (6,10,14-trimethylpentadecan-2-one and 2,6,10,14-tetramethylpentadecan-2-ol).

Two of the compounds tested, hexadecan-2-one and 6,10,14-trimethylpentadecan-2-one, were degraded under sulfate-reducing conditions in microcosms and enrichment cultures.

Chemicals. Hexadecan-2-one and nonadecan-10-one were purchased from Aldrich (Milwaukee, Wis.). 6,10,14-Trimethylpentadecan-2-one was produced by oxidation of phytol (Riedel de Haën) with KMnO_4 in acetone (6). 2,6,10,14-Tetramethylpentadecan-2-ol was obtained by condensation of 6,10,14-trimethylpentadecan-2-one and methylmagnesium iodide in anhydrous diethyl ether. Reduction of 6,10,14-trimethylpentadecan-2-one with LiAlH_4 in diethyl ether gave the corresponding secondary alcohol. The synthesis of 2-(1-hydroxyethyl)pentadecanoic acid required three steps: (i) condensation of ethyl acetoacetate with 1-bromotridecane in the presence of potassium *tert*-butoxide (12), (ii) reduction of the resulting β -keto-ester with NaBH_4 (which did not reduce the carboxyl group of esters) for 15 min in methanol, and (iii) alkaline hydrolysis of the produced β -hydroxyester (5% KOH in 50% CH_3OH , reflux for 1 h).

Sediment incubation protocol. Microcosms were prepared in 60-ml serum bottles from a volume of sediment and a volume of sterilized site water enriched as described below. Coastal sediment showing little oil contamination (0.06 kg/kg of sediment) was collected at Fos Bay (France). Site water was enriched with 3.7 mM ammonium chloride, 2 mM sodium thiosulfate, 14 mM morpholinepropanesulfonic acid (pH 7.2), and 1 mg of resazurin per liter. The fresh sediment and water were homogenized under an $\text{N}_2\text{-H}_2\text{-CO}_2$ (85:10:5) atmosphere in an anaerobic chamber (La Calhène). A mixture of four compounds, hexadecan-2-one, nonadecan-10-one, 6,10,14-trimethylpentadecan-2-one, and 2,6,10,14-tetramethylpentadecan-2-ol, had previously been added to the empty bottles; the compounds were dissolved in dichloromethane and distributed so as to provide a final concentration of 200 mg/liter in each microcosm. Dichloromethane was then allowed to evaporate from the serum bottles. Killed controls were prepared with autoclaved sediments. The bottles were sealed with Teflon-coated butyl rubber stoppers (Serflam, Marseille, France) and incubated at 30°C in the dark.

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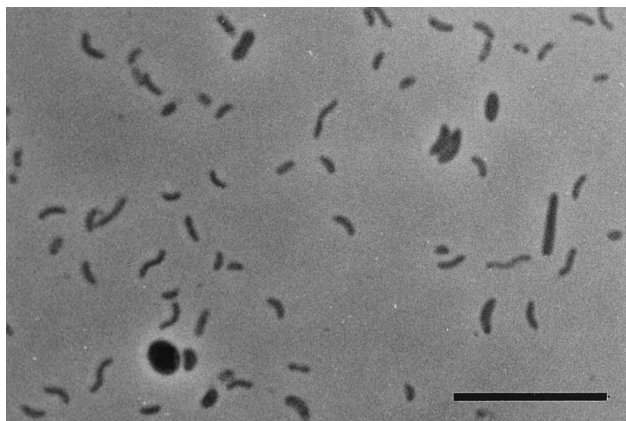


FIG. 1. Phase-contrast photomicrograph of an enrichment culture grown on hexadecan-2-one and sulfate. Bar, 10 μ m.

Subculture procedure. A homogeneous fraction (3 ml) of the microcosm was transferred to serum bottles containing the substrate hexadecan-2-one at a final concentration of 200 mg/liter in artificial seawater medium (27 ml) with 0.4 mM $\text{Na}_2\text{S} \cdot 9\text{H}_2\text{O}$. Hexadecan-2-one was added as described above and autoclaved under an $\text{N}_2\text{-CO}_2$ (90:10) atmosphere. The seawater medium contained (per liter, unless otherwise noted) 23.5 g of NaCl, 10.6 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 3.9 g of Na_2SO_4 , 0.19 g of NaHCO_3 , 0.66 g of KCl, 0.1 g of KBr, 0.024 g of H_3BO_3 , 0.040 g of $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 0.2 g of NH_4Cl , 50 mg of yeast extract, 1 mg of resazurin, and 3 g of morpholinepropanesulfonic acid (pH 7.2). After autoclaving, 10 ml of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (140 g/liter), 1 ml of trace element solution SL12 (21), 1 ml of selenite-tungstate solution (33), 1 ml of 7-vitamin solution (22), and 4 ml of phosphate buffer (4.4 g of $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$ and 25.8 g of $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ per liter) were added from sterile stock solutions. Prior to inoculation, the medium was incubated overnight in an anaerobic chamber. The bottles were incubated at 30°C in the dark on a reciprocal shaker (100 strokes/min).

Analytical procedures. Sulfide was quantified with Cu^{2+} ions yielding CuS (8).

For analyses of the ketones and alcohol, the sediment and the aqueous phase from the microcosm were separated by decantation. The wet sediment was extracted under sonication with isopropanol-hexane (4:1, vol/vol) (9), and the aqueous phase was extracted three times with chloroform. For analysis of the substrate of the subculture without sediment, the entire contents of the vial were extracted with chloroform. The combined hexane and chloroform extracts were dried with anhydrous Na_2SO_4 , filtered, and concentrated by means of rotary evaporation to give extract E1 (containing residual substrates and eventual nonacidic metabolites). The aqueous phase was then acidified with hydrochloric acid (pH 1) and extracted three times with chloroform to give extract E2 (containing acidic compounds). After evaporation of the solvents, extracts E1 and E2 were taken up separately in 250 μ l of a mixture of pyridine and *N,O*-bistrimethylsilyl-trifluoroacetamide (3:1, vol/vol) and allowed to silylate at 50°C for 1 h. Following evaporation to dryness under nitrogen, the residues were dissolved in hexane and analyzed by gas chromatography-mass spectrometry (GC/MS). Compounds were identified (by comparison of their retention times and mass spectra with those of standards) and quantified (after calibration with external standards) by GC/MS. The GC/MS analyses were carried out with an HP 5890 series II plus gas chromatograph connected to an HP 5972 mass spectrometer. The operating conditions in-

cluded a capillary measuring 15 m by 0.25 mm (inside diameter), a column coated with BPX 35 (SGE), an oven temperature programmed to go from 60 to 150°C at 30°C/min and then from 150 to 320°C at 3°C/min, a carrier gas (He) pressure of 0.48 bar, an injector temperature of 320°C, an electron energy of 70 eV, and a source temperature of 170°C.

The microbial biomass was estimated indirectly by quantifying the cellular protein (4) assuming a 50% protein content.

Depletion experiments in microcosms. In microcosms fed with the mixture of four compounds, hexadecan-2-one was the first compound to be depleted. Within 2 months, more than 80% was depleted while only 15% of the 6,10,14-trimethylpentadecan-2-one was depleted. After 10 more months, 42% of the 6,10,14-trimethylpentadecan-2-one was depleted with respect to a control incubated just as long. Neither nonadecan-10-one nor 2,6,10,14-tetramethylpentadecan-2-ol was depleted after 1 year of incubation. In the controls, none of the compounds disappeared within 6 months; however, after 1 year of incubation, only 80% of the four substrates remained. Successive subcultures confirmed those depletions. We conclude that of the compounds studied, hexadecan-2-one is the most rapidly depleted.

Evidence that hexadecan-2-one metabolism is linked to sulfate reduction. Successive transfers to defined medium with hexadecan-2-one were made to establish enrichment cultures without sediment. To link sulfate reduction and hexadecan-2-one oxidation, transfers were made in complete artificial seawater medium and in sulfate-depleted artificial seawater medium. After 1 month of incubation, 80% of the hexadecan-2-one was degraded in the complete artificial seawater medium while only 10% was degraded in the sulfate-depleted artificial seawater medium. Transfer of the enrichment culture to medium with hexadecan-2-one and/or yeast extract showed that yeast extract alone in artificial seawater medium did not support growth, while growth was the same on hexadecan-2-one with or without yeast extract. On the other hand, the weak

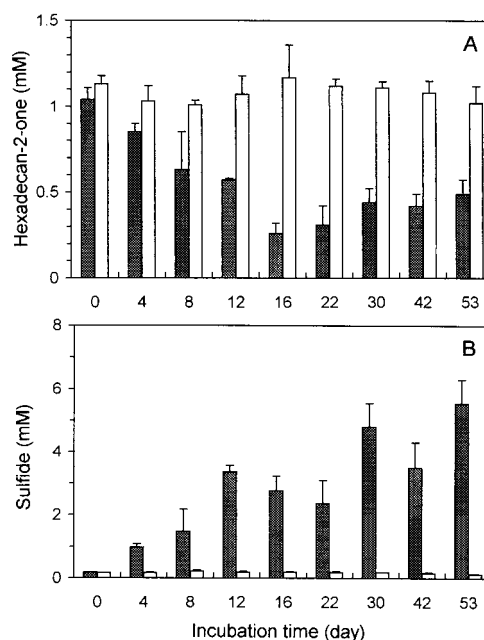


FIG. 2. Concentrations of hexadecan-2-one (A) and sulfide (B) in an enrichment culture growing in the presence of hexadecan-2-one. Open bars correspond to sterile controls. The data are mean values from three cultures. At each time, the entire contents of three vials were analyzed.

- 1- C_{12,0} fatty acid
- 2- C_{14,0} fatty acid
- 3- Hexadecan-2-one
- 4- C_{15,0} fatty acid
- 5- C_{16,1} fatty acid
- 6- C_{16,0} fatty acid
- 7- C_{17,0} fatty acid
- 8- 2-(1-Hydroxyethyl)pentadecanoic acid
- 9- C_{18,1} fatty acid
- 10- C_{18,0} fatty acid

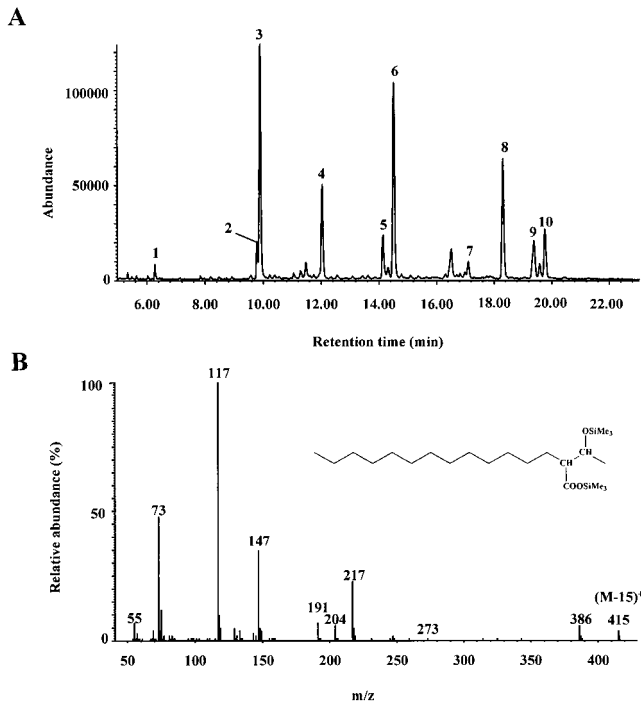


FIG. 3. (A) Total ion chromatogram of (silylated) extract E2 obtained after the growth of an enrichment culture on hexadecan-2-one. (B) Electron impact mass spectrum of metabolite 8, identified as (silylated) 2-(1-hydroxyethyl)pentadecanoic acid.

growth in subcultures without sulfate was detectable only in the presence of both hexadecan-2-one and yeast extract. In conclusion, the experiments show that efficient anaerobic degradation of hexadecan-2-one requires sulfate and the activity of sulfate-reducing bacteria. Nevertheless, some degradation occurred in the absence of sulfate, and hence, other microorganisms are probably involved.

The community growing in yeast extract-free artificial seawater medium with hexadecan-2-one as the sole source of carbon was composed of curved rods and of some oval and rod-shaped cells (Fig. 1). The culture grown on hexadecan-2-one as the sole source of carbon and energy reached its optimum growth within about 20 days with an optical density at 450 nm of about 0.20. The curve is not shown, as the culture often formed aggregates or biofilm on a glass surface. Nevertheless, increased optical density was linked to an increase in microbial biomass, as about 16 mg (dry weight) per liter was produced in a month of growth while 0.6 mM hexadecan-2-one was consumed. At optimum growth, 60% of the hexadecan-2-one was degraded. Although sulfate was not limiting, about 35% of the hexadecan-2-one remained at the end of the experiment (Fig. 2A). The degradation of hexadecan-2-one was linked to sulfide production (Fig. 2A and B). In sterile controls, no depletion occurred and no sulfide was produced. Furthermore,

in inoculated controls without substrate, the sulfide produced never exceeded 0.2 mM. At the end of the experiment, 5.42 mM sulfide was produced during the degradation of 0.53 mM hexadecan-2-one. The stoichiometry of complete hexadecan-2-one oxidation through sulfate reduction is as follows: $\text{CH}_3(\text{CH}_2)_{13}\text{COCH}_3 + 11.75 \text{SO}_4^{2-} \rightarrow 11.75 \text{S}^{2-} + 16 \text{CO}_2 + 16 \text{H}_2\text{O}$. Therefore, the amount of sulfide produced will allow the oxidation of 0.46 mM hexadecan-2-one. The remaining 0.07 mM will be converted to cell carbon, and possibly to energy, by the nonsulfidogenic part of the community. Since it was consistent with the cell mass produced (0.04 mM hexadecanone turned into cell carbon), we concluded that oxidation was complete.

After growth of the enrichment culture on hexadecan-2-one, we detected 2-(1-hydroxyethyl)pentadecanoic acid in extract E2 (Fig. 3A). This metabolite, which was undetectable in controls with a sterilized inoculum, was identified unambiguously by comparison of its retention time and mass spectrum (Fig. 3B) with those of a synthesized standard. The presence of this compound clearly shows that the metabolism of hexadecan-2-one by the enrichment culture involves carboxylation reactions. Such a mechanism has already been described for the anaerobic oxidation of acetone (3, 23, 24). Partial reduction of the carbonyl group to the corresponding secondary alcohol was also observed. The involvement of this "blind-alley" pathway probably results from nonspecific enzyme activity not related

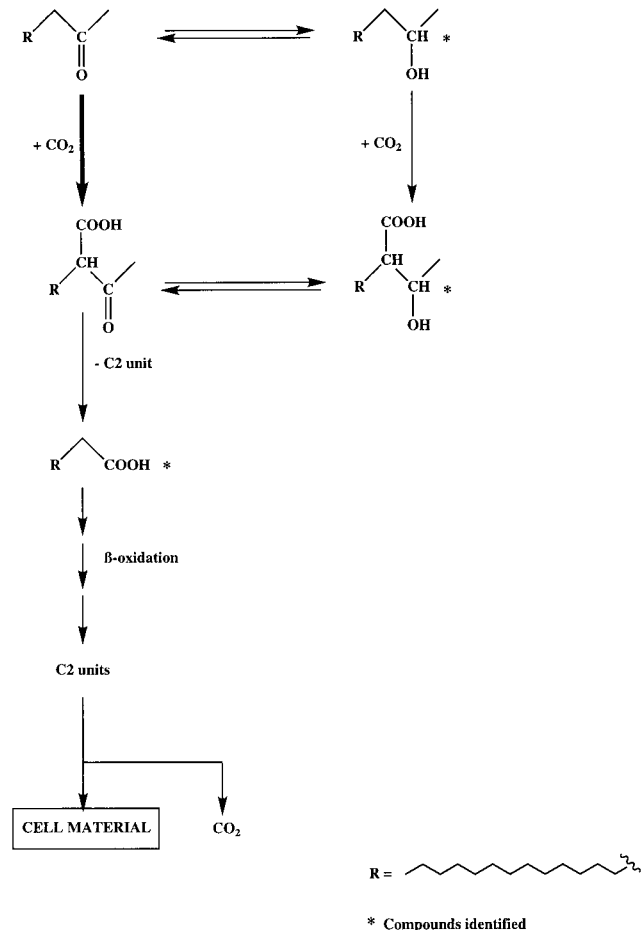


FIG. 4. Proposed pathway for the metabolism of hexadecan-2-one by an enrichment culture.

to hexadecan-2-one degradation (16). On the basis of these results, we propose the pathway described in the Fig. 4 for the metabolism of hexadecan-2-one by the enrichment culture. The detection of a relatively strong proportion of pentadecanoic acid in extract E2 (Fig. 3A) is in good agreement with such a pathway. If carboxylation of ketones is easier than that of the corresponding alcohols, due to activation of the carbon α relative to the carbonyl group by keto-enol tautomerism, the possibility of hexadecan-2-ol carboxylation cannot be completely excluded (3, 32).

Although the carboxylation takes place on the secondary carbon in the α position relative to the carbonyl group of hexadecan-2-one, the enrichment culture was unable to degrade nonadecan-10-one. An attempt at hexadecan-3-one biodegradation confirmed that only ketones with the keto group at position 2 could be degraded by the enrichment culture.

These results confirm once more the importance of the community of sulfate-reducing bacteria in anoxic environments and, in particular, in the degradation of hydrocarbons. Furthermore, it is the first demonstration of the degradation of a typical hydrocarbon photooxidation product under sulfate-reducing conditions.

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