Anaerobic Degradation of 2-Methylnaphthalene by a Sulfate-Reducing Enrichment Culture†

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Aromatic hydrocarbons were considered to be recalcitrant in the environment under anoxic conditions until the first evidence of anaerobic BTEX degradation was reported in 1985 (17). Toluene turned out to be the most easily degradable aromatic hydrocarbon, and various pure bacterial strains have been isolated since then. Toluene can be degraded with nitrate, ferric iron, or sulfate as the electron acceptor, under fermentative conditions, and by phototrophic bacteria (7, 8, 20, 21, 25, 32). The pathway of toluene degradation has been investigated in detail for denitrifying bacteria of the genera Azotobacter and Thiobacillus, and it was shown that the first degradation step is the addition of fumarate to the methyl group (3, 4, 9, 13).

The reaction is catalyzed by benzylsuccinate synthase, which belongs to the glyoxyl radical enzyme family. The benzylsuccinate synthase reaction appears to be representative of the degradation pathways of a number of environmental pollutants, such as m-xylene, o-xylene, and m-cresol, which all exhibit the addition of fumarate to the methyl group as the first activation reaction (2, 16, 24). Further steps in anaerobic toluene degradation include a coenzyme A (CoA) transferase reaction of benzylsuccinate with succinyl-CoA, generating benzylsuccinyl-CoA and succinate (19). In the following reactions, benzylsuccinyl-CoA is probably oxidized through beta-oxidation to benzoyl-CoA, which enters the benzoyl-CoA degradation pathway.

Anaerobic degradation of polycyclic aromatic hydrocarbons has been demonstrated in a few microcosm studies (5, 6, 18, 23, 27), and recently, naphthalene-degrading denitrifying and sulfate-reducing cultures were reported (1, 10, 22, 26, 33). In experiments with one marine and one freshwater culture, 2-naphthoic acid was the major metabolite of anaerobic naphthalene degradation and was generated by incorporation of bicarbonate into the carboxyl group (22, 33). The further degradation pathway might proceed via reduction of the aromatic ring system in analogy to the benzoyl-CoA degradation pathway, as reduced 2-naphthoic acid derivatives have been identified in culture supernatants (22).

Here we report on the anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture from a freshwater sediment. Metabolites were extracted from culture supernatants and analyzed by gas chromatography-mass spectrometry (GC-MS). The first enzyme reaction in the anaerobic 2-methylnaphthalene degradation pathway, catalyzed by naphthalene-2-methylsuccinate synthase, was identified in dense cell suspensions.

MATERIALS AND METHODS

Cultivation of bacteria. A 2-methylnaphthalene-degrading, sulfate-reducing bacterial culture was enriched from a contaminated aquifer as described earlier (22). Subcultures were inoculated with 10% volume of the liquid phase in 100-ml serum bottles half filled with carbonate-buffered, sulfate-reduced freshwater medium, pH 7.4, with trace element solution SL10 (29, 30). Solid 2-methylnaphthalene crystals were added (2 to 4 mg/50 ml) together with 10 mM sulfate as the electron acceptor. The bottles were flushed with N2-CO2 (80/20), closed with Viton rubber stoppers (Maag Technik, Diibendorf, Switzerland), and incubated at 30°C in the dark.

Analysis of metabolites. Sample preparation, gas chromatographic analysis, and GC-MS measurements were performed as described previously (22). Culture growth was stopped with 100 mM NaOH, and samples were stored at −20°C until metabolite analysis. Naphthalene was extracted with hexane from the alkaline sample. After acidification to pH 2.0 with 6 M hydrochloric acid, the water phase was extracted three times with dichloromethane to isolate carboxylic acids and aromatic alcohols. The combined dichloromethane extracts were concent-

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trated by vacuum evaporation, dried over anhydrous sodium sulfate, and derivatized with ethereal diazomethane. The solvent was removed by a gentle stream of nitrogen, and products were transferred to hexane and analyzed by GC-MS.

GC-MS measurements were performed with a Hewlett Packard 6890 gas chromatograph coupled with a Quattro II mass spectrometer (Micromass, Amtrincham, United Kingdom). The chromatograph was equipped with a 30-m capillary column (0.32-mm inside diameter, 0.25-μm film thickness; DB-5; J & W Scientific), and helium was used as the carrier gas. The temperature program was 80°C (5 min, isothermal), 80 to 310°C (4°C/min), and 310°C (10 min, isothermal).

The following MS conditions were applied: ionization mode, EI⁺; ionization energy, 70 eV; emission current, 200 μA; source temperature, 180°C; mass range, m/z 50–400.

For identification of metabolites, instrumental library searches applying the National Institute of Standards and Technology/National Institutes of Health/U.S. Environmental Protection Agency mass spectral database, comparison with published mass spectra, and co-injection with commercially available authentic reference compounds were carried out. Reference compounds for GC-MS analyses were obtained from Fluka (Buchs, Switzerland). Decahydro-2-naphthoic acid was synthesized by reduction of 2-naphthoic acid with hydrogen as described earlier (22).

**Synthesis of naphthyl-2-methylene-succinic acid.** Synthesis of naphthyl-2-methylene-succinic acid (compound VI in Fig. 1 and 2) was performed according to references 11 and 28. Sodium metal (2.2 g of sodium, 96 mmol) was dissolved in absolute methanol (40 ml) and added dropwise within 40 min to the diethylsuccinate-methanol solution, which was heated under reflux cooling. After 2 h, NaOH (2 M, 160 ml) was added, and the mixture was heated for a further 6 h. The solution was concentrated by evaporation, HCl (37%, 40 ml) was added, and the aqueous phase was extracted three times with ethyl acetate. The combined organic layers were washed with saturation, HCl (37%, 40 ml) was added, and the aqueous phase was extracted three times with hexane. After the addition of hexane (30 ml) and benzene (30 ml), yellowish crystals precipitated. They were collected by filtration and recrystallized with 30 ml of ethanol (99.8%) and subjected to high-performance liquid chromatography (HPLC) analysis after removal of precipitates by centrifugation (5 min, 15,000 × g). Naphthyl-2-methylene-succinic acid concentrations were determined by HPLC analysis on a Beckman System Gold equipped with a C18 reversed-phase column and UV detection at 206 nm. Eluent was isocratic acetonitrile–100 mM ammonium phosphate buffer, pH 3.5 (40/60).

**RESULTS**

**Growth with 2-methylnaphthalene.** A sulfate-reducing culture that was enriched with naphthalene as the sole carbon and energy source was able to grow with crystalline 2-methylnaphthalene but not with 1-methylnaphthalene (22). The culture did not grow with 300 μM toluene as the sole carbon and energy source within 100 days of observation, either in the presence or in the absence of the solid adsorber resin Amberlite XAD7. XAD7 was used to provide the cells with toluene at a continuously low concentration, below toxic levels (30 to 50 μM). The identified metabolites naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid (see below) could also not be used as carbon sources at a concentration of 40 mg/liter.
Identification of metabolites. GC-MS analysis of metabolites from supernatants of 2-methylnaphthalene-grown cultures revealed two groups of metabolites. The first group consisted of the major metabolite naphthyl-2-methyl-succinic acid and of naphthyl-2-methylene-succinic acid (Fig. 1). Both compounds were identified by GC coinjection and comparison of the mass spectra with chemically synthesized reference substances (Fig. 2). The chromatogram of chemically synthesized naphthyl-2-methylene-succinic acid revealed two separate peaks with identical mass spectra probably representing the E and Z isomers. Both peaks were identified in culture supernatants (Fig. 1). The absolute configuration of the compounds was not determined. Naphthyl-2-methyl-succinic acid accumulated in culture supernatants up to 0.5 μM, as determined by HPLC.

The second group of metabolites was identified as 2-naphthoic acid and a series of reduced derivatives (Fig. 1) (for mass spectra see reference 22). 5,6,7,8-Tetrahydro-2-naphthoic acid and two isomers of octahydro-2-naphthoic acid were tentatively identified by their mass spectra. The most reduced metabolites were two decahydro-2-naphthoic acid isomers (decahydro-2-carboxylic acid). The compounds were identified by their mass spectra and by coelution with the chemically synthesized reference compounds (22).

Naphthyl-2-methyl-succinate synthase activity. The first reaction step in anaerobic 2-methylnaphthalene degradation was analyzed in dense cell suspensions. In the presence of 2-methylnaphthalene and fumarate, a continuous production of naphthyl-2-methyl-succinate was observed (Fig. 3A). Naphthyl-2-methyl-succinate was identified by HPLC with a diode array detector, by coelution with the chemically synthesized reference compound, and by its UV-visible-light absorption spectrum (Fig. 3B). Production of naphthyl-2-methyl-succinate was observed neither in the absence of cells nor in the absence of fumarate, indicating that fumarate is added to the methyl group of 2-methylnaphthalene as the first reaction step. The specific naphthyl-2-methyl-succinate synthase activity of three independent cell suspension experiments was 0.020 ± 0.003 mmol min⁻¹ mg of protein⁻¹. This represents 2.5% of the substrate turnover rate in the growing culture.

DISCUSSION

In this study we report on the anaerobic degradation of 2-methylnaphthalene by a sulfate-reducing enrichment culture and the identification of the first enzyme in the pathway, naphthyl-2-methyl-succinate synthase.

The 2-methylnaphthalene-degrading culture was enriched with naphthalene as sole carbon and electron source and sulfate as the electron acceptor (22). Subsequent substrate utilization tests revealed that the culture could also utilize 2-methylnaphthalene as a growth substrate.

In culture supernatants two groups of metabolites could be identified by GC-MS analysis. The first series consisted of naphthyl-2-methyl-succinic acid and naphthyl-2-methylene-succinic acid. Both compounds are structural analogs of the first metabolites in anaerobic toluene degradation, benzylsuccinic acid and phenylitaconic acid (13). Thus, it is very likely that naphthyl-2-methyl-succinate is generated by the addition of fumarate to the methyl group of 2-methylnaphthalene, a mechanism similar to the benzylsuccinate synthase reaction. Indeed, a fumarate addition to the methyl group of 2-methylnaphthalene to yield naphthyl-2-methyl-succinic acid could be confirmed in cell suspension experiments. The reaction with 2-methylnaphthalene depended on the presence of cells and fumarate. In the presence of all reactants, the specific activity was 0.020 ± 0.003 mmol min⁻¹ mg of protein⁻¹. The naphthyl-2-methyl-succinate synthase activity in the enzyme test represented 2.5% of the substrate turnover rate in the growing culture, which is comparable to data for the similar enzyme benzylsuccinate synthase obtained by other authors (4). As the culture was not able to grow with toluene as the sole carbon and energy source the measured enzyme reaction is not only a side reaction of a toluene-degrading capacity of the culture but an activity specifically related to 2-methylnaphthalene degradation. The second identified metabolite was naphthyl-2-methylene-succinic acid, which is probably generated by beta-oxidation of naphthyl-2-methyl-succinic acid. A first step therein may be a CoA-dependent activation by a succinyl-CoA transferase. This type of reaction has been shown for anaerobic toluene degradation by the denitrifying bacterium Thauera aromatica (19).
The second group of metabolites consisted of 2-naphthoic acid and reduced derivatives such as 5,6,7,8-tetrahydro-2-naphthoic acid, octahydro-2-naphthoic acid, and decahydro-2-naphthoic acid. These compounds were identified in an earlier study as metabolites of anaerobic naphthalene degradation by the same culture (22). In addition, some of these metabolites have been identified from a marine sulfate-reducing culture growing with naphthalene as the carbon source (33). As the two cultures were able to grow with 2-naphthoic acid by naphthalene and that of 2-methylnaphthalene share a common degradation pathway which is initiated by a reduction of the polycyclic aromatic ring system. At present, we do not know if ring cleavage is initiated from a monoaromatic ring system or if both rings are reduced before water addition.

Based on the present data, we propose an upper pathway for anaerobic degradation of 2-methylnaphthalene which is analogous to anaerobic degradation of toluene and other methylbenzenes (Fig. 4) (13, 19). In a first activation step, fumarate is added to the methyl group of 2-methylnaphthalene by naphthyl-2-methyl-succinate synthase, probably through a radical mechanism, in analogy to anaerobic toluene degradation. Naphthyl-2-methyl-succinic acid is likely to be activated by a succinyl-CoA-dependent CoA transferase and subsequent oxidation to yield naphthyl-2-methylenecsuccinyl-CoA. The following sequence of reactions proceeds via beta-oxidation and leads to the central intermediate 2-naphthoic acid CoA-ester.

Likewise, ring fission of the bicyclic system must not necessarily proceed via decahydro-2-naphthoic acid. The more reduced compounds may as well be dead-end metabolites. Nevertheless, the identification of common metabolites of both substrates suggests that anaerobic degradation of naphthalene and that of 2-methylnaphthalene share a common degradation pathway which is initiated by a reduction of the polycyclic aromatic ring system. At present, we do not know if ring cleavage is initiated from a monoaromatic ring system or if both rings are reduced before water addition.

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