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DEGRADATION OF 2-METHYLNAPHTHALENE BY A SULFATE-REDUCING ENRICHMENT CULTURE OF MESOPHILIC FRESHWATER BACTERIA

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Granules of expanded clay ("Lecaton") serving as a mineral filter bed in a groundwater purification plant (Lübeck, Metallhüttengelände) were investigated for the presence of mesophilic freshwater microorganisms able to degrade monomethylated naphthalenes under oxygen- and sulfate-reducing conditions. Microorganisms inhabiting the granules readily degraded 1-methylnaphthalene and 2-methylnaphthalene in the presence of oxygen. An enrichment culture of sulfate-reducing bacteria was obtained which grew on 2-methylnaphthalene but not on 1-methylnaphthalene. The enriched bacteria were sensitive toward high concentrations of sulfide and

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2-methylnaphthalene in the medium. Naphthyl-2-methyl-succinate and 2-naphthoate were major metabolites that accumulated in cells during growth of the enrichment culture. This finding proposed that the initial reactions of 2-methylnaphthalene metabolism included addition of fumarate to the methyl group of the compound and further degradation to 2-naphthoate, analogous to toluene metabolism under anoxic conditions. However, the addition reaction was specific for 2-methylnaphthalene because none of the bacteria present in the enrichment culture was able to grow on toluene. 2-Methylnaphthalene might be degraded through cooperation of aerobic and sulfate-reducing bacteria on the basis of 2-naphthoate production and its utilization, respectively.

Keywords aerobic, anaerobic degradation, 1-methylnaphthalene, 2-methylnaphthalene, 2-naphthoate, 1-naphthoate, naphthyl-2-methyl-succinate, sulfate-reducing bacteria

Monomethylated naphthalenes (MNs) are ubiquitous bicyclic aromatic hydrocarbons that reach natural environments from numerous natural and anthropogenic sources (1). They occur at high concentration in crude oil, coal tar, and in a number of commercial products including the wood preservative creosote (2, 3). Exposure of macroorganisms to elevated concentrations of MNs causes symptoms of toxicity (4–6).

Degradation of MNs in contaminated environments is mainly achieved by microorganisms. A variety of aerobic microorganisms (fungi, cyanobacteria, and eubacteria) can metabolize 1-MN and 2-MN via action of oxygenases (7–12). The presence of a methyl substituent at the aromatic ring affects the site of the initial oxygenation reaction and leads to either methyl group hydroxylation or dioxygenation of the non-substituted aryl ring (9–12).

In the past decade it has been shown that degradation of MNs is also possible under anoxic conditions (13–17). During bioremediation of groundwater in the area of a former gas plant at Düsseldorf, Germany, the production of naphthylmethylsuccinate was detected (18). That suggested that MNs were metabolized through fumarate addition to the methyl group (18). So far, enrichment cultures of freshwater and marine sulfate-reducing bacteria were obtained that mineralized 2-MN but not 1-MN (16, 17, 20). Analysis of metabolites and conversion of

the substrate in dense cell suspension of the enrichment cultures by freshwater bacteria confirmed that degradation of 2-MN proceeded via fumarate addition to the methyl substituent (15). It was reported that marine sulfate-reducing bacteria could also carboxylate carbons of aromatic rings (20).

Observation of sulfate reduction in the groundwater purification plant of a tar oil-contaminated site (Lübeck, Metallhüttengelände, Germany) (21) caused us to investigate degradation of MNs by bacteria present in the granules of expanded clay taken from a reactor that had been operating 6 months. Here we report on the enrichment of anaerobic mesophilic freshwater bacteria degrading 2-MN with concomitant reduction of sulfate to sulfide and on the determination of organic acids extracted from bacterial cells in order to understand the degradation pathway.

MATERIALS AND METHODS

Granules of expanded clay "Lecaton" (Fibo ExClay Deutschland GmbH, Frankfurt am Main, Germany) taken from a mineral filter bed in a tar oil-contaminated groundwater purification plant (Lübeck, Metallhüttengelände, Germany) were tested for the presence of aerobic and anaerobic sulfate-reducing bacteria capable of 1-MN and 2-MN degradation.

Techniques for preparation of the medium and for cultivation of sulfate-reducing bacteria under anoxic conditions were as described previously (22). Cultures were grown in defined bicarbonate-buffered freshwater mineral medium reduced with sodium dithionite. Several grains of $Na_2S_2O_3$ were added by a sterile spatula to the medium before inoculation. 1-MN and 2-MN were dissolved in sterile deaerated 2,2,4,4,6,8,8-heptamethylnonane (HMN) (1.0 to 4.0 ml in 100 ml of the solvent). Because 2-MN is solid at room temperature, it was melted at 37°C shortly before preparation of the solution. Viton rubber-sealed tubes (20 ml) with a headspace (50% of total tube volume) of N_2-CO_2 (90:10 [v/v]) were used for routine cultivation. Each tube contained 9 ml of medium and 0.5 ml of HMN with the corresponding substrate. Inoculated tubes were incubated in vertical position in the dark at 28°C. Cultures were shaken by hand for a few seconds each day.

The presence of aerobic bacteria utilizing 1-MN and 2-MN in the granules was tested by incubation of the granules in Viton rubber-sealed 50 ml bottles containing 15 ml of nonreduced bicarbonate-buffered freshwater mineral medium and 1.5 ml of 1-MN or 2-MN solution in HMN

(6 ml of the hydrocarbon in 100 ml of the solvent). The headspace of the bottles was filled with air.

Growth of bacteria was followed as change in optical density in the aqueous phase of the medium at 578 nm wavelength measured in a Bausch & Lomb GmbH Spectronic 70 photometer (Rochester, New York, USA). Sulfide in the medium was determined photometrically (23). Protein content was measured with bicinchoninic acid according to an enhanced protocol (BCA protein assay kit; Pierce, Rockford, Illinois, USA).

The cultures of aerobic and anaerobic bacteria were tested for the presence of metabolites. Samples of the aqueous phase of the medium were taken through the stoppers by syringes. Samples of cultures of aerobic bacteria (100 μl) were diluted in 900 μl of ethanol (99.8%) and centrifuged for 15 min at 20,000 $\times$ g to remove precipitates. Samples of sulfate-reducing enrichment cultures were subjected to extraction of free aromatic acids as described (24) and concentration by evaporation. Extracts were analyzed by high-performance liquid chromatography (HPLC) (Beckman Instruments Inc., München, Germany). Samples were injected on a Grom-Sil 120 ODS 5 column (250 by 4 mm) with 5 μm packing (Grom Analytik + HPLC GmbH, Herrenberg, Germany). A gradient elution at a constant flow rate (1 ml/min) was performed. The initial mobile phase was a mixture of 20% acetonitrile and 80% water with 0.01 vol % trifluoroacetic acid. This was kept for 1.5 min, then the mobile phase was changed linearly to 95% acetonitrile and 5% aqueous solution of trifluoroacetic acid in 20 min. This eluent was kept for 2.5 min, whereafter the mobile phase was linearly switched back to the initial ratio of components (80/20 v/v) for a 4.5 min interval to prepare the column for the next injection. Elution of compounds was monitored by measuring the absorption at 220 nm and spectra were analyzed by an UV/VIS photodiode array detector (SRD-M10Avp, Shimadzu Deutschland GmbH, Duisburg, Germany). In addition, the extract was analyzed by high-performance mass spectrometry with a MAT-95XL spectrometer (Thermo Finnigan MAT GmbH, Bremen, Germany). This analysis was performed by Ruey-an Doong and Hsiao-Ling Wu at National Tsing Hua University (Taiwan). Synthesis of naphthyl-2-methyl-succinic acid was performed as described earlier (15). All chemicals were of analytical grade. 1-MN and 2-MN used in this study were 97.0% pure and obtained from Fluka Chemie AG (Buchs, Switzerland) and Sigma-Aldrich Chemie GmbH (Steinheim, Germany), respectively. HMN was 99% pure and purchased from Alfa Johnson Matthey (Karlsruhe, Germany). Green Viton rubber stoppers were purchased from Glasgerätebau Ochs GmbH (Bovenden-Lenglern, Germany).

RESULTS

A steel production plant with integrated coking and tar-distilling facilities as well as cement and zinc sulfate production plants were situated for more than 60 years in the northern Lübeck area. The complex ("Metallhüttengelände") was finally shut down in 1989 leaving a large contaminated site on the bank of River Trave right before entering the Baltic Sea. The area of the plant was deeply ($\leq$15 m in sandy ground) and heavily ($\leq$2,000 mg/kg soil) contaminated by policyclic aromatic hydrocarbons from tar oil besides spots of monocyclic aromatic hydrocarbons (benzene and its alkylated derivatives) and cyanides from deposition, spillage, and war damage.

A purification procedure for the oily groundwater was developed that included oil skimming and tar oil sedimentation in a mechanical phase separator and further filtration through soft coal coke (21). To show the coke's function as a fixed bed reactor besides its adsorptive capacity, the carbonaceous filter bed was replaced by a mineral bed. The reactor's fixed bed consisted of granules of expanded clay "Lecaton." Removal of naphthalene (NAPH), 1-MN, 2-MN, and of sulfate from water passing the experimental reactor is shown in Figure 1. The oxygen content of the water decreased from 62.5 to 3.1 μM.

Incubation of "Lecaton" granules taken from the reactor after operating for 6 months in bottles with nonreduced medium, a headspace of air and 1-MN and 2-MN supplied in HMN revealed the presence of aerobic bacteria capable to degrade both substrates. Growth of bacteria on MNs started after 2 days of incubation with simultaneous accumulation of metabolites in the aqueous phase of the medium. Two major metabolites of 1-MN and 2-MN degradation were identified as 1-naphthoate (53 μM) and 2-naphthoate (40 μM), respectively. Identification was performed by comparison of HPLC retention

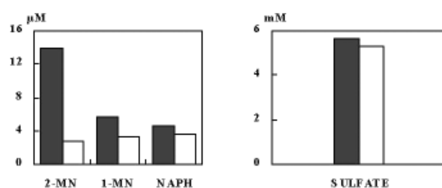

FIGURE 1. Removal of bicyclic aromatic hydrocarbons and sulfate from wastewater by a fixed bed reactor. Black columns show concentration of compounds in incoming water, white columns in outcoming water. Data are from 11 December 1997.

time values and ultraviolet (UV) spectra with those of reference compounds.

Enrichments of 1-MN- and 2-MN-utilizing sulfate-reducing bacteria were attempted with granules from the reactor as the inoculum and substrates being provided either in an inert carrier phase or as pure substances. Three granules per tube were placed in 10 ml of anoxic freshwater medium containing 20 mM of sulfate. After 6 months of incubation, there was no growth of bacteria at all to be observed in control tubes with granules but without hydrocarbons and as well as in tubes with granules and MNs added as pure substances. However in tubes with 2-MN in a carrier phase, a slight turbidity appeared (up to 0.1 of optical density at 578 nm) in the aqueous phase of the medium and up to 3.5 mM of sulfide was detected at the end of incubation. Examination of the medium under the phase-contrast microscope showed the presence of numerous rod-shaped bacterial cells. In tubes supplied with 1-MN in the carrier phase, neither turbidity nor sulfide production were detectable.

The tube with bacteria grown on 2-MN was kept at 4°C for 1 week. When bacterial cells settled down at the bottom of the tube, around 7 ml of the medium as well as organic carrier phase were carefully removed with a sterile pipette. Cells suspended in the rest of the medium (around 3 ml) were transferred into a tube containing 7 ml of the medium overlaid by 0.5 ml of 2-MN dissolved in HMN. Further cultivation of bacteria was routinely initiated by transfer of 1 ml of the culture grown on 2-MN and resulted in a stable enrichment of a sulfate-reducing bacterial culture (named culture RS2MN).

Two major morphological types of bacteria dominated after six successive transfers in the enrichment culture: thick and thin rods of 0.6–1.0 or 0.3–0.4 μm width and 3.5–3.7 μm length, respectively. Sulfide concentrations measured in the aqueous phase of the medium never exceeded 6–7 mM at the end of growth. Several attempts to isolate a pure culture of a sulfate-reducing bacterium growing on 2-MN from this enrichment by using a technique similar to the one described earlier for the isolation of a naphthalene-degrading sulfate-reducing bacterium (25) were not successful.

Bacteria grew homogeneously suspended in the aqueous phase: attachment of cells to the organic carrier phase was never observed. Therefore, it was possible to measure growth by following the optical density of the medium. Growth of the enrichment culture in the presence of different concentrations of 2-MN in the organic carrier phase is shown in Figure 2. No growth was observed in the absence of substrate. As seen in Figure 2, the growth rate of the enrichment culture was proportional

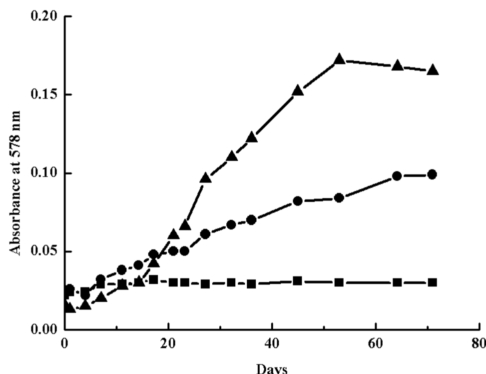

FIGURE 2. Growth of the enrichment culture RS2MN (shown as an increase in optical density) with different initial concentrations of 2-MN in HMN (■, control without substrate; ●, 1 ml; and ▲, 4 ml of substrate in 100 ml of HMN. The substrate was supplied as described in the Materials and Methods section.

to the amount of 2-MN added, whereas final optical density measured in the tubes were not.

Free aromatic acids were extracted from cells of the sulfate-reducing enrichment culture at different growth phases and analyzed by HPLC separation with detection of compounds with a UV photodiode array detector. Several compounds were found to be present in the extracts (Figure 3). Two aromatic compounds (marked on Figure 3 as A and B) were identified to be either one isomer of naphthyl-2-methyl-succinate and 2-naphthoate, respectively, on the basis of their retention time and

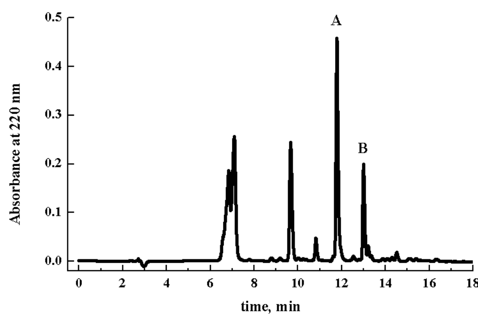

FIGURE 3. Chromatogram of high-performance liquid chromatography (HPLC) analysis of organic acids extracted from cells of the enrichment culture RS2MN. Peaks were identified as (A) naphthyl-2-methyl-succinate and (B) 2-naphthoate.

UV spectra which were identical to those of the reference compounds. The identity of these compounds was confirmed by high-performance mass spectrometry. The concentration of organic acids in the cultures during growth was estimated as 6.7 μmol of naphthyl-2-methyl-succinate and 2.6 μmol of 2-naphthoate per mg of cell protein. At the late stationary phase, the content of these metabolites decreased: Naphthyl-2-methyl-succinate was still present at rather high concentration (3.9 μmol per mg of cell protein) whereas the amount of 2-naphthoate substantially dropped (from 0.025 μmol per mg of cell protein to nondetectable level). Neither 1-naphthoate nor 1,2,3,4-tetrahydro-2-naphthoate was detected in the extracts. Other compounds present in the extracts were not identified.

Sulfate-reducing bacteria present in the enrichment culture grew on 2-naphthoate and benzoate but not on 1-naphthoate or on toluene, the structural analogue of the MNs supplied in the test tubes dissolved in HMN (4 ml of toluene per 100 ml of solvent). A pure culture of a sulfate-reducing bacterium was isolated that grew on benzoate. Unfortunately, the bacterium did not grow on either 2-MN or 2-naphthoate.

DISCUSSION

In this study, we attempted to enrich freshwater sulfate-reducing bacteria capable of using 1-MN and 2-MN as growth substrates. For this purpose, we used granules of expanded clay taken from a fixed bed reactor operating for 6 months. The large volume of pores (~71% of total volume) favored a deep penetration of bacteria into the granules. This property of the granules allowed us to maintain a population of microorganisms within the granules adapted to metabolize nutrients from the passing water and promoted various types of symbiotic cooperation between metabolically different bacteria. In particular, aerobic bacteria respiring oxygen created anoxic zones in the granules where sulfate-reducing bacteria could survive. The granules indeed lowered the oxygen content in the passing water thus, sheltering viable sulfate-reducing bacteria as we have shown in this study. To mineralize organic compounds, sulfate-reducing bacteria used sulfate present in wastewater (Figure 1) and also in granules matter (200 mg in 100 g of granules).

The reactor removed both 1-MN and 2-MN from the passing water with concomitant reduction of oxygen due to the action of aerobic bacteria present in the granules. We also found that the granules contained bacteria capable of mineralization of 2-MN but not of 1-MN under

sulfate-reducing conditions. Earlier studies have reported that 1-MN is persistent to degradation by sulfate-reducing bacteria (15, 19, 27) and only limited mineralization was observed under methanogenic conditions (14). The reason for that is yet unknown.

It is a common practice to cultivate MN-utilizing aerobic bacteria in a medium completely saturated with the substrates (9, 10, 12). We succeeded to develop the sulfate-reducing enrichment of 2-MN-utilizing bacteria only if the substrate was supplied in the organic carrier phase which helped to avoid toxic effects of the aromatic hydrocarbon on the bacteria. Growth of bacteria during cultivation of the granules in such a medium under batch conditions resulted in appearance of cells in the aqueous phase of the medium. Our data suggest that the enriched bacteria were sensitive toward high sulfide concentrations in the medium; therefore, the culture never showed significant growth.

Detection of naphthyl-2-methyl-succinate and 2-naphthoate in the enrichment culture indicated that the initial reactions of 2-MN metabolism probably include addition of fumarate to the methyl group of the compound and further degradation to 2-naphthoate as it was suggested earlier (15, 18). Such a pathway was elucidated in detail for the degradation of toluene by nitrate-reducing bacteria, and also occurred in sulfate-reducing and anoxygenic phototrophic bacteria (19, 26–30). It turned out that the initial reactions of the 2-MN metabolism were very substrate-specific because the enrichment culture did not grow on toluene and 1-MN. No evidence was obtained for carboxylation of aromatic ring carbons by high-performance mass spectrometry. However, our data do not allow us to exclude 2-naphthoate formation from 2-MN by means of methyl group oxidation.

Some bacteria in the enrichment grew on benzoate which is a structural analogue of 2-naphthoate. Assuming that degradation of benzoate and 2-naphthoate could be performed in similar enzymatic reactions, we tested the utilization of 2-naphthoate and 2-MN by a pure culture of benzoate-degrading sulfate-reducing bacterium isolated from the enrichment. Unfortunately, the bacterium did not grow on these substrates.

It is not certain whether a single species of a sulfate-reducing bacterium is responsible for the 2-MN degradation in the enrichment culture or if it is performed as a syntrophic cooperation between several anaerobic bacteria.

Mineralization of 2-MN in granules of the groundwater purification plant might be performed by interaction of aerobic and anaerobic bacteria. Microorganisms found in the granules utilized 1-MN and 2-MN in the presence of oxygen with the production of 1-naphthoate

and 2-naphthoate, respectively. These metabolites often accumulate in pure cultures of aerobic bacteria grown on MNs (9–12) or in fungal cultures exposed to these compounds (8, 31). Both aromatic acids could be degraded by oxygen-respiring bacteria (32, 33). To our knowledge only 2-naphthoate was known to be degraded by a pure culture of a marine sulfate-reducing bacterium (25) and we found such bacteria in the enrichment culture of freshwater bacteria described in this study. Removal of 1-naphthoate produced during 1-MN oxidation therefore might be due to mineralization by aerobic bacteria alone (32).

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