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Evidence for aromatic ring reduction in the biodegradation pathway of carboxylated naphthalene by a sulfate reducing consortium

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Abstract

Naphthalene was used as a model compound in order to study the anaerobic pathway of polycyclic aromatic hydrocarbon degradation. Previously we had determined that carboxylation is an initial step for anaerobic metabolism of naphthalene, but no other intermediate metabolites were identified (Zhang & Young 1997). In the present study we further elucidate the pathway with the identification of six novel naphthalene metabolites detected when cultures were fed naphthalene in the presence of its analog 1-fluoronaphthalene. Results from cultures supplemented with either deuterated naphthalene or non-deuterated naphthalene plus [^{13}C]bicarbonate confirm that the metabolites originated from naphthalene. Three of these metabolites were identified by comparison with the following standards: 2-naphthoic acid (2-NA), 5,6,7,8-tetrahydro-2-naphthoic acid, and decahydro-2-naphthoic acid. The presence of 5,6,7,8-tetrahydro-2-NA as a metabolite of naphthalene degradation indicates that the first reduction reaction occurs at the unsubstituted ring, rather than the carboxylated ring. The overall results suggest that after the initial carboxylation of naphthalene, 2-NA is sequentially reduced to decahydro-2-naphthoic acid through 5 hydrogenation reactions, each of which eliminated one double bond. Incorporation of deuterium atoms from D_2O into 5,6,7,8-tetrahydro-2-naphthoic acid suggests that water is the proton source for hydrogenation.

Abbreviations: gas-chromatography/mass spectroscopy, GC/MS; 2-napthoic acid, 2-NA; polycyclic aromatic hydrocarbons, PAH

Introduction

Polycyclic aromatic hydrocarbons (PAH) are a class of toxic and carcinogenic organic compounds listed by the EPA as priority pollutants (Keith & Telliard 1979). The majority of PAHs are introduced into the environment at industrial sites related to the combustion of fossil fuels, coke production, and petroleum refining. Other sources of contamination include chronic leakage of industrial effluents, urban run-off, and accidental release during transportation. The hydrophobic nature and low solubility of PAHs contributes to their long term persistence in the environment.

PAH removal in the environment has been shown to occur via bacterial degradation. The attempt to harness this activity for application in bioremediation has resulted in a solid understanding of both the key bacterial players and the biochemical pathways of *aerobic* PAH degradation (reviewed by Cerniglia 1992; Sutherland et al. 1995; Zylstra et al. 1997). In this process oxygen acts both as a terminal electron acceptor and as a reactant to break the aromatic ring. Much less is known about *anaerobic* PAH degradation, since just a decade ago the general consensus was that PAHs were recalcitrant once they entered an anoxic zone. Today this hypothesis has been disproven as an increasing body of literature on anaerobic microcosm

and column studies have confirmed mineralization of PAHs to CO_2 via ^{14}C-labeled substrate and/or the stoichiometric coupling of PAH degradation to alternative electron acceptors including nitrate (Bregnard et al. 1996; Rockne & Strand 1998) and sulfate (Langenhoff et al. 1996; Coates et al. 1996, 1997; Bedessem et al. 1997; Zhang & Young 1997; Rockne & Strand 1998). Numerous other studies support naphthalene degradation by nitrate-reducing consortia (Mihelicic & Luthy 1988a, 1988b; Al-Bashir et al. 1990; Durant et al. 1995) and expand the potential anaerobic environments to included methanogenic (Parker & Montheith 1995; Genthner et al. 1997), and manganese-reducing conditions (Langenhoff et al. 1996).

The microcosm experiments described above were all done with complex bacterial populations. Little is yet known about the bacteria within these communities that are responsible for the initial attack on the hydrocarbon ring, as only recently have the first descriptions of pure cultures of anaerobic PAH-degrading bacteria begun to appear in the literature. McNally et al. (1998) characterized the rates of both aerobic and anaerobic (nitrate-reducing) PAH degradation by three pseudomonads fed different PAHs (2–4 ring compounds). More recently, Galushko et al. (1999) published the first description of the isolation of a naphthalene-degrading, sulfate reducing bacterium. Interestingly, the authors found that the isolate was closely related to organisms enriched to degrade monoaromatic hydrocarbons (benzene and m-xylene) under sulfate-reducing conditions.

The lack of PAH-degrading pure cultures has hindered progress in the determination of anaerobic PAH metabolism, from a biochemical, and even more so, a genetic approach. The aerobic pathway offers little insight as oxygen plays a key role as a reactant in destabilizing the aromatic ring of PAHs before ring cleavage. One proposed reaction mechanism for the anaerobic naphthalene degradation under methanogenic conditions is naphthalene hydroxylation to 1-naphthol (Grbic-Galic 1989). Another study reported that hydroxy-naphthalene was detected in a sulfate-reducing enrichment (Bedessem et al. 1997). We previously established three sulfate-reducing enrichments capable of degrading naphthalene, 2-methylnaphthalene, and phenanthrene and demonstrated that naphthalene and phenanthrene were carboxylated as an initial reaction for metabolism of naphthalene (Zhang & Young 1997). Furthermore, inorganic bicarbonate was shown to be the source of the carboxyl group through the use of [^{13}C]bicarbonate in solution.

Biodegradation of the proposed carboxylated intermediate, 2-naphthoic acid (2-NA), has been observed in our naphthalene and 2-methyl naphthalene-degrading consortium (Zhang & Young 1997), but to the best of our knowledge anaerobic 2-NA biodegradation pathway is not documented. The closest model for anaerobic biodegradation of 2-NA is anaerobic benzoate degradation, which is the pathway by which most monoaromatic compounds are degraded (reviewed by Heider & Fuchs 1997; Harwood et al. 1999). Benzoate after combining with coenzyme A undergoes five reactions (2 reductions, followed by a hydration, a dehydrogenation, and a final hydration) prior to its ring breakage. Since 2-NA is structurally similar to benzoate, it was of interest to determine whether a similar reduction reaction occurs. In this paper we identify several intermediates of naphthalene degradation and present mass spectral evidence that 2-NA is reduced to decahydro-2-NA via 5,6,7,8-tetrahydro-2-NA, suggesting that the non-substituted ring of 2-NA is the first to be reduced. Based on our data, a pathway is presented for several of the initial steps of naphthalene degradation by a sulfate reducing consortium.

Materials and methods

Chemicals

[^{13}C]Bicarbonate was obtained from Cambridge Isotope Laboratories, Inc. (Andover, Massachusetts, USA). 2-NA, 1,2,3,4-tetrahydro-2-NA, deuterated naphthalene (D8), and 1-fluoronaphthalene were obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). 5,6,7,8-Tetrahydro-2-NA was synthesized by hydrogenation of 2-NA over platinum oxide (Sigma Chemical Co., St. Louis, MO, USA) in an alkaline solution (Levin & Pendergrass 1947; Dauben et al. 1951). The reagents, 200 mg 2-NA, 5 ml absolute ethyl alcohol, 48 mg platinum oxide, and 10 μl 10 N sodium hydroxide were transferred into a 60 ml serum bottle, which was then capped with a Teflon-coated rubber stopper and crimp-sealed. The bottle was flushed with pure hydrogen gas for 2 min and incubated overnight at 30 °C with shaking at 200 rpm. Based on gas chromatography/mass spectroscopy (GC/MS) analyses, the final solution contained 91% 5,6,7,8-tetrahydro-2-NA, 2% 1,2,3,4-tetrahydro-2-NA, and 6.5% 2-NA. Identification of 5,6,7,8-tetrahydro-2-NA was confirmed by compar-

ing its UV spectrum with that of a published standard (Dauben et al. 1951). Identification of 1,2,3,4-tetrahydro-2-NA was based on the GC retention time and mass spectrum of the standard. The procedure for synthesis of decahydro-2-NA was similar to the synthesis of 5,6,7,8-tetrahydro-2-NA except that acetic acid rather than sodium hydroxide was used. The yield of decahydro-2-NA generally reached 100%, and the four GC peaks detected were very similar in mass spectra indicating the presence of four isomers of decahydro-2-NA.

Microbial cultures and medium

The naphthalene-degrading consortium used for this study originated from PAH contaminated estuarine sediment collected from the Arthur Kill, NY/NJ harbor (Zhang & Young 1997). The active culture was maintained using the methods described in that paper. In brief, the culture remained active by routinely transferring a 30–50% inoculum every 2–4 weeks into minimal medium containing 10% autoclaved pristine sediment (from a local campus pond, Passion Puddle). All solutions, cultures, and media were prepared and maintained using strict anaerobic techniques with CO_2/N_2 gas (30%/70%) in the head space. The cultures were fed 200 μM naphthalene sorbed on dry Passion Puddle sediment and monitored for degradation by GC analysis with flame ionization detection. Subsequent transfers were conducted after one or two refeedings. The culture was maintained by this method for more than one year before the start of this research.

Metabolite detection

Metabolites of naphthalene were not commonly detected in transferred cultures unless growth conditions were altered by the addition of 1-fluoronaphthalene as shown previously (Zhang & Young 1997). Experiments were set-up by dividing active naphthalene-degrading cultures into four 30-ml aliquots using strict anaerobic techniques. Two bottles were autoclaved for 30 min at 121 °C to serve as abiotic sterile controls. 1-Fluoronaphthalene (50 μM) was added to all four bottles. Non-deuterated naphthalene (100 μM) was added to one active and one autoclaved bottle, while deuterated naphthalene (100 μM) was added to the other two bottles.

For experiments testing incorporation of [^{13}C]bicarbonate, 120-ml active culture grown in the presence of 1-fluoronaphthalene was washed and centrifuged anaerobically three times with 100, 100, and 120 ml phosphate buffered medium (20 mM) to remove the bicarbonate in the culture medium. The headspace was flushed with pure nitrogen gas rather than a mixture of CO_2/N_2 (30%/70%) used in normal maintenance of the cultures. Non-deuterated naphthalene (100 μM) was added before the washed culture was divided into four aliquots of 30 ml slurry each. Two of four subcultures were autoclaved. One active and one autoclaved subculture was injected with [^{12}C]bicarbonate (30 mM and 15 mM HCl) and the other two bottles were injected with [^{13}C]bicarbonate (30 mM and 15 mM HCl).

For experiments using deuterium oxide (D_2O), the medium was prepared by adding dry chemicals rather than stock solutions into D_2O or H_2O. Trace mineral and vitamin solutions were omitted, as were resazurin and reducing agent solution (Na_2S) to prevent introducing H_2O into D_2O medium. An active 120-ml culture grown in the presence of 1-fluoronaphthalene (50 μM) was divided into two and washed three times with either D_2O or H_2O medium. The dilution factor of the washing was at least 1,000X so that for example, the H_2O content was reduced to less than 0.1% in the culture washed with the D_2O medium. Each subculture was further divided into two, and either non-deuterated or deuterated naphthalene (100 μM) was added to each bottle. All the cultures were incubated at 30 °C in the dark.

HPLC analysis

High pressure liquid chromatography (HPLC; Beckman Instruments, Inc., Fullerton, CA) with an autosampler (Gibson Medical Electronics, Inc., Middleton, WI, USA) and a UV detector (280 nm) was used to monitor the loss of naphthalene and the accumulation of metabolites. A well-mixed 0.5 ml slurry sample was taken and added to 0.5 ml of 95% ethanol in a microcentrifuge tube. After vortexing and centrifugation, the supernatant was used for analysis. An eluent solution of methanol, water, and acetic acid in a ratio of either 80 : 19 : 1 or 60 : 38 : 2 flowed through a reverse-phase C18 column (Ultrasphere C-18 column; Beckman, USA).

GC-MS analysis

A slurry culture was centrifuged, and the supernatant was transferred to a 60-ml serum bottle and acidified with 1 ml of 6 N HCl. The sample was then extracted with 12 ml pentane, shaken, and centrifuged. The

pentane phase was transferred to a 13-ml serum bottle and evaporated under vacuum. This extraction process was repeated a second time. The dry residue was solubilized in 1 ml pentane, transferred to a 2-ml vial, and dried under vacuum. The extract was solubilized in 0.1 ml methylene chloride and derivatized with 0.1 ml bis(trimethylsilyl)trifluoroacetamide (BSTFA-1.0% trimethylchlorosilane; Sigma Chemical Co., USA) at 60 °C for 15 min. The derivatized sample was then subjected to GC-MS by injecting 1–2 μl into a Hewlett-Packard 5890 series II GC coupled with a mass-selective detector (Hewlett-Packard 5890 series) and autosampler. The column for the GC-MS analysis was a DB-5MS with a length of 30 m, a 0.25 mm inner diameter and a film thickness of 0.25 μm (J&W Scientific, Folsom, CA, USA). The injector temperature was 280 °C, and an isocratic program was used (120 °C).

Results

Chemical analysis of naphthalene metabolites

Addition of the naphthalene analog, 1-fluoronaphthalene, along with naphthalene caused partial inhibition of naphthalene degradation and accumulation of metabolites as revealed by HPLC chromatography. Concentrations of the intermediate 2-NA ranged from 0.5 to 6.0 μM as seen previously (Zhang & Young 1997). Culture incubation times lasted until the added naphthalene decreased to between 10–20% of the original concentration. GC/MS analysis of the metabolites derived from the culture supplemented with non-deuterated naphthalene revealed several GC peaks. Three of these peaks were not well resolved and their retention times were close to the derivatized standard 2-NA. By testing different temperature programs, an isocratic temperature program (120 °C) was found where all three peaks were completely separated.

The mass spectra of the compounds are shown in Figure 1A–C. It should be noted that all compounds analyzed by GC/MS were first derivatized with trimethylsilyl, which is reflected in the mass spectra. The mass spectrum shown in Figure 1A is the same as that of the standard 2-NA (Figure 2A). As reported previously, the metabolite 2-NA is produced by carboxylation of naphthalene (Zhang & Young 1997). The mass spectrum shown in Figure 1B is similar to that of Figure 1A and 2A, with the exception that its molecular ion is two mass units greater (246) than that of 2-NA (244). Since the mass spectrum shown in Figure 1C matched the mass spectrum of the standard 5,6,7,8-tetrahydro-2-NA (Figure 2B) and the GC retention time of both were also identical, we concluded that the metabolite shown in Figure 1C is 5,6,7,8-tetrahydro-2-NA. Since the difference between 2-NA and 5,6,7,8-tetrahydro-2-NA is the loss of two double bonds in the latter, it is reasonable to deduce that the metabolite shown in Figure 1B is dihydro-2-NA, which possesses one double bond less than 2-NA and one more than 5,6,7,8-tetrahydro-2-NA. The position where the reduction takes place can not be identified, since no corresponding standard was available. Nevertheless, these observations suggest that 2-NA is sequentially reduced first to dihydro-2-NA, then to 5,6,7,8-detrahydro-2-NA. The mass spectra of these three compounds is consistent with our hypothesis. In addition, the ring becomes less stable as the number of double bonds decreases, and as can be seen in Figure 1A, peak 127, which represents an intact ring structure of naphthalene, has disappeared in the mass spectrum of Figure 1C.

The mass spectra shown in Figure 1D to F represent three additional metabolites detected in cultures supplemented with naphthalene and 1-fluoronaphthalene. The mass spectrum shown in Figure 1F matches that of the decahydro-2-NA standard shown in Figure 2C, therefore identifying the metabolite as decahydro-2-NA. The latter is completely saturated and contains no double bonds. Four GC peaks (data not shown) exhibited the same mass spectra as shown in Figure 1F, indicating that there are four isomers for decahydro-2-NA. This is consistent with the synthesized standard decahydro-2-NA, which also displayed four GC peaks. Since the production of isomers in an enzymatic reaction is unexpected, their presence may be the result of abiotic reactions.

The mass spectra shown in Figure 1D and 1E are very similar to the decahydro-2-NA standard, with the exception that their molecular ion is shifted down 4 (250, Figure 1D) and 2 mass units (252, Figure 1E), respectively, relative to the decahydro-2-NA (254, Figure 2C). We conclude that Figure 1D–F represent the sequential and stepwise reduction of 5,6,7,8-tetrahydro-2-NA to hexahydro-2-NA, octahydro-2-NA, and decahydro-2-NA. Additional GC/MS data suggests that there are two and four isomers of hexahydro-2-NA and octahydro-2-NA, respectively (data not shown).

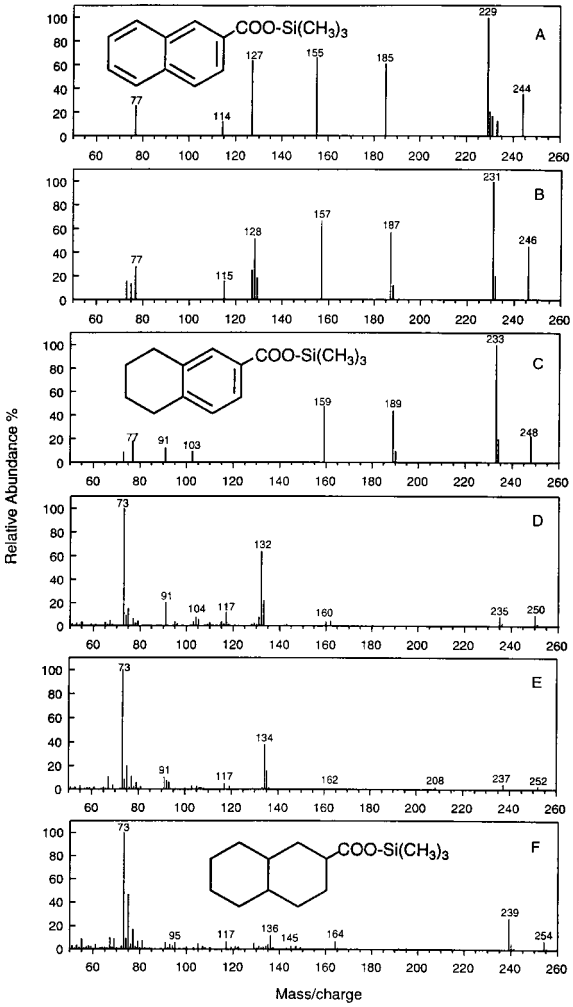

Figure 1. Mass spectra of derivatized metabolites detected in the consortium supplemented with non-deuterated naphthalene. The proposed chemical structures shown in A, C, and F are the results of comparison with the corresponding standards shown in Figure 2A, B, and C, respectively. The chemical structures for metabolites shown in B, D, and E were unknown due to unavailability of the corresponding standards.

Data supporting these observations were obtained from experiments using either deuterated naphthalene (D8) or non-deuterated naphthalene plus [^{13}C]bicarbonate. The same suite of deuterated metabolites were detected when deuterated naphthalene was used as the substrate and likewise, [^{13}C]carbon-labeled metabolites were detected with [^{13}C]bicarbonate. For example, in the latter case the molecular ions of each of the metabolites illustrated in Figure 3A–F were one mass unit larger. These results underscore the previous observation that naphthalene is first carboxylated (Zhang & Young 1997) during

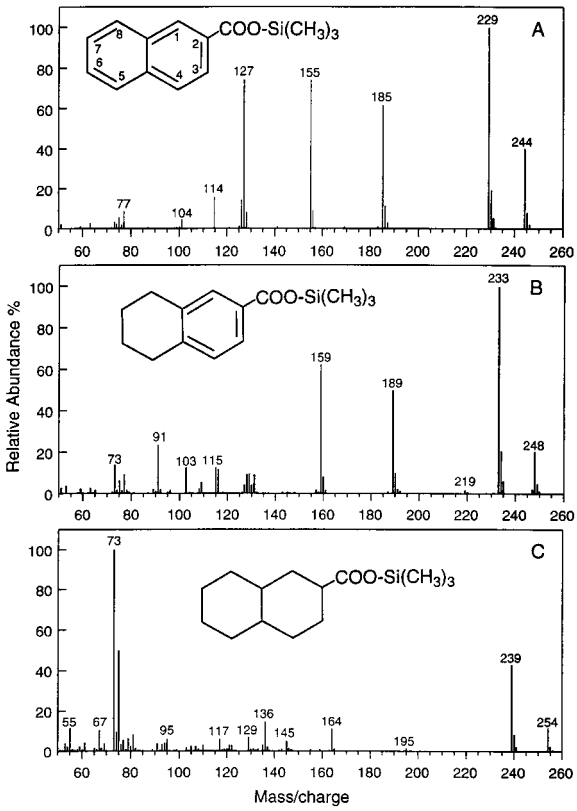

Figure 2. Mass spectra of derivatized standards 2-NA (A), 5,6,7,8-tetrahydro-2-NA (B), and decahydro-2-NA (C). The latter two compounds were chemically synthesized by methods described in the text.

anaerobic metabolism. None of the metabolites were found in the parallel autoclaved cultures.

Deuterium incorporation from D_2O into 5,6,7,8-tetrahydro-2-NA

To determine the proton source for hydrogenation, the mass spectrum of 5,6,7,8-tetrahydro-2-NA was compared between cultures fed either labeled (D8) or unlabeled naphthalene and incubated in the presence of either H_2O or D_2O. While the molecular ions of non-labeled 5,6,7,8-tetrahydro-2-NA is 248, the molecular ion for deuterated 5,6,7,8-tetrahydro-2-NA is 255 (248 + 7; 7 deuterons). By replacing H_2O with D_2O in the cultures when naphthalene was metabolized as the substrate, the molecular ion of the non-labeled 5,6,7,8-tetrahydro-2-NA was found to be 252 (248 + 4) and that for deuterated 5,6,7,8-tetrahydro-2-NA was 259 (255 + 4; data not shown). Thus, four deuterons from deuterated water were incorporated in 5,6,7,8-tetrahydro-2-NA indicating that

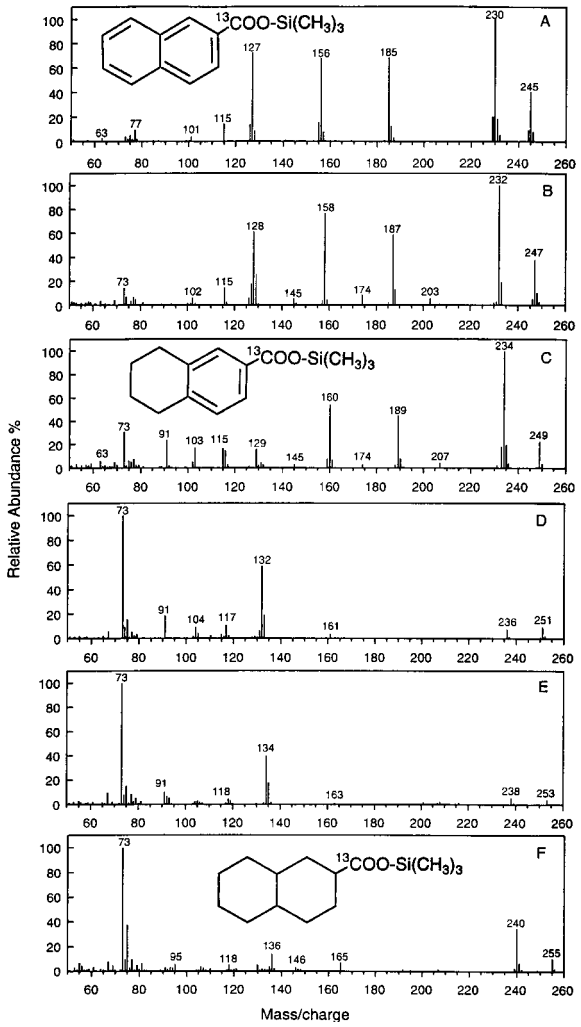

Figure 3. Mass spectra of the derivatized metabolites detected in the consortium supplemented with [^{13}C]bicarbonate. Note that the masses differ by one mass unit from the corresponding metabolite shown in Figure 1A-F because of labeled carbon.

water is the proton source for hydrogenation of 2-NA to 5,6,7,8-tetrahydro-2-NA.

Discussion

A sulfidogenic enrichment was previously shown to mineralize naphthalene to CO_2 via a carboxylation step (Zhang & Young 1997). In this paper we propose a more complete pathway for the degradation of naphthalene, based on the detection of additional intermediates. The accumulation of intermediates from naphthalene metabolism was enhanced by addition of the naphthalene analogue, 1-fluoronaphthalene. A total of six intermediates including 2-NA were detected by GC-MS. Using deuterated naphthalene (D8) and non-deuterated naphthalene plus [^{13}C]bicarbonate, we confirmed that all metabolites originated from naphthalene. Three of these intermediates were identified based on mass spectra identical to the known standards, 2-NA, 5,6,7,8,-tetrahydro-2-naphthoic acid, and decahydro-2-napththoic acid. Both 2-NA and 5,6,7,8,-tetrahydro-2-napthoic acid could be utilized as sole carbon sources; though this is not proof, it is consistent with their being intermediates. The intermediates for which no standards were available were identified through deduction based on their molecular ions and the similarity of their mass spectra to the known standard compounds.

The proposed pathway based on the detection of metabolites of naphthalene utilization is shown in Figure 4. Five reduction steps are involved in the complete reduction of 2-NA to decahydro-2-NA. The incorporation of D_2O into 5,6,7,8-tetrahydro-2-NA suggests that water is the proton source for the hydrogenation reactions. The presence of 5,6,7,8-tetrahydro-2-NA as an intermediate also indicates that the non-substituted ring of 2-NA is reduced first.

The pathway for anaerobic benzoate degradation, which is central to anaerobic degradation of monoaromatic hydrocarbons, offers an analogous pathway to compare with naphthalene metabolism (Heider & Fuchs 1997; Harwood et al. 1999). The presence of the intermediate, 2-NA, suggests that the carboxyl group may be thioesterified with coenzyme A (CoA), as it is with benzoate. CoA bonded intermediates were not the subject of this study; however the inability of the cells to grow on decahydro-2-naphthoic acid as a sole carbon source may be due to the absence of a mechanism to add CoA to this late intermediate in the pathway.

Despite the fact that these cultures have been subcultured and maintained in the laboratory for more than two years, the culture used for these studies remains complex with more than 55 different bacterial members as determined by molecular analysis (Palermo et al., manuscript in preparation). Indeed, degradation via the proposed pathway may be the result of one bacterium or the synergistic degradation by several. Interestingly, the first published description of a pure culture of a naphthalene-degrading, sulfate reducing bacterium reported utilization of 2-NA as the sole carbon source (Galushko et al. 1999). The ability of this strain to degrade naphthalene and 2-NA, but not 1-NA, suggests the pathway may be the same as the pathway proposed here.

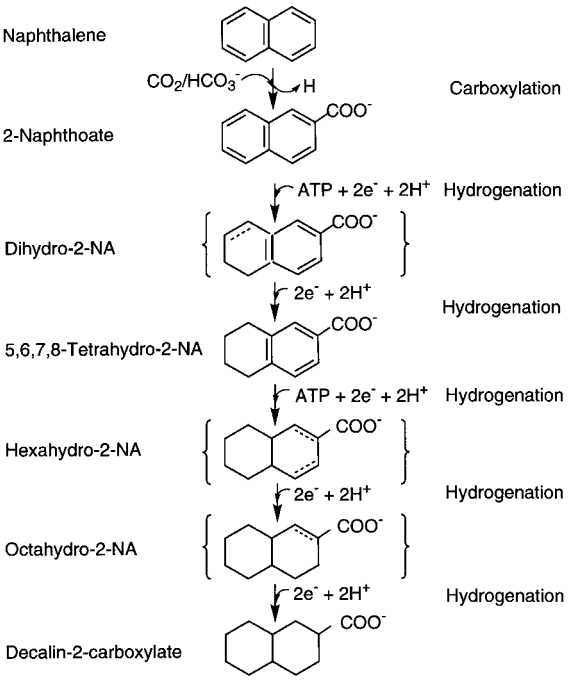

Figure 4. The proposed initial pathway for anaerobic naphthalene metabolism under sulfate-reducing conditions. Steps which are bracketed were deduced from comparison of mass spectra with the known standards. A dashed line is used to represent double bonds where the exact position of the bond within the aromatic ring is unknown.

In summary, this report describes the identification of several intermediates from naphthalene degradation by a sulfate reducing consortium. To the best of our knowledge, this is the first documentation presenting a detailed sequence of reactions for anaerobic naphthalene degradation and this work furthers our understanding of PAH degradation by anaerobic bacteria. Future research on the biochemistry and genetics of naphthalene metabolism using pure cultures will certainly advance the field even further.

Acknowledgments

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