NOVEL METABOLITES OF ANAEROBIC NAPHTHALENE BIODEGRADATION BY PURE CULTURES

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ABSTRACT: We have previously reported on anaerobic pure cultures that degrade naphthalene in the strict absence of oxygen. One of these pure cultures NAP-3 was phylogenetically most closely related to Pseudomonas stutzeri based on 16S rDNA sequences. Further studies in our laboratory have shown that the degradation of naphthalene was slow or nearly absent when volatile fatty acids (VFAs) were not present. To investigate this further, we analyzed NAP-3 during incubations with acetate and found that it stimulates naphthalene transformation. Naphthalene significantly increased denitrification in nearly exact stoichiometric balances of nitrate and nitrite as would be expected based on the metabolism. NAP-3 was sensitive to the amount of naphthalene in the culture; with naphthalene removal rates higher when present at 20mg/l compared to 40mg/l. Investigation of the culture supernatant by GC/MS showed the transient production and consumption of the nitrogen-containing bicyclic indole. Production of indole was repeatable and found to be affected by naphthalene concentration; with the 20mg/l culture producing the most indole. The production of indole was strictly biotic (it was not formed in either killed or blank controls). It is not immediately apparent which metabolic pathway could form indole from naphthalene, but it possibly could involve the biotic incorporation of either nitrate or a reduced metabolite of nitrate into the nitrogen-containing ring of indole. To date, the only published metabolic pathways for anaerobic PAH biodegradation is ring hydroxylation or carboxylation by sulfate-reducers. These results show that other metabolic pathways exist for denitrifiers, and may require stimulation of the organisms by more-readily utilizable VFA substrates.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are of concern in the environment because of their persistence and their potential carcinogenic effects. PAHs are introduced into the environment through natural and anthropogenic processes causing them to be ubiquitous in the environment. Over the past 20 years, numerous studies have reported the capacity of various bacteria, fungi and algae to degrade PAHs (See Makkar and Rockne, 2003 for a review). However, few studies have focused on the contribution of individual microorganisms in reducing the toxicity of PAH compounds. With recent results clearly demonstrating some bicyclics and PAHs can be degraded without oxygen, further progress in understanding this process will be achieved by the identification of pure cultures of anaerobic PAH degrading bacteria. Many pure cultures of denitrifying and sulfate reducing bacteria able to degrade mono-aromatics like toluene have been determined (Karthikeyan, 2001).

Anaerobic degradation of monoaromatic hydrocarbons proceeds in every case investigated through the benzoyl-coenzyme A (benzoyl-CoA) pathway and differs only with respect to the peripheral degradation pathways (Johann et al, 1999). In contrast to
the degradation of monoaromatic compounds, much less information exists on the anaerobic degradation of polycyclic aromatic hydrocarbons.

In one study, naphthalene was microbially transformed in sulfate-reducing laboratory microcosms established under strictly anaerobic conditions with the formation of naphthol as a potential metabolic intermediate, suggesting that hydroxylation may be a first step in the sulfate-mediated transformation of naphthalene (Bedessem and Colberg, 1997). Earlier studies were done on naphthalene degradation by sulfate-reducing bacterial cultures with a marine enrichment culture from harbor sediment (Zhang and Young, 1997, Zhang and Young, 2000) and a freshwater culture enriched from a tar-oil contaminated aquifer (Annweiler and Meckenstock, 2000, Meckenstock, 2000, Morasch and Meckenstock, 2001). These studies showed that 2-naphthoic acid was an intermediate formed by the incorporation of bicarbonate into the carboxy group, suggesting that carboxylation is the initial step for degradation of naphthalene by sulfate-reducers (Zhang and young, 2000, Makkar and Rockne, 2003). It has been also proposed that degradation of PAHs (mainly bicyclic) compounds goes through carboxylation and then through ring saturation with 2-naphthoic acid is the central intermediate (Annweiler and Meckenstock, 2002).

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\text{CO}_2 + \begin{array}{c} \text{Naphthalene} \\ \text{2-naphthoic acid} \end{array} \rightarrow \begin{array}{c} \text{2-naphthoic acid} \\ \text{CO}_2 \end{array}
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**FIGURE 1. Proposed pathway for naphthalene transformation by sulfate-reducing bacteria.**

We previously reported on the isolation of pure cultures of anaerobic PAH-degrading bacteria derived from a denitrifying enrichment culture (Rockne and Strand, 1999). The pure culture was phylogenetically most closely related to *Pseudomonas stutzeri* based on 16S rDNA sequences. We subsequently found that the degradation of naphthalene by NAP-3 (the pseudomonad) was slow or nearly absent when volatile fatty acids (VFAs) were not present.

To investigate this further, studies were performed to demonstrate the biotransformation of naphthalene by NAP-3 during incubations with acetate present. In this report we also studied in detail the nitrate transformation kinetics by Nap 3 culture with and without naphthalene.

**MATERIALS AND METHODS**

**Bacterial strain.** The microorganism used (NAP-3) was a pure culture previously shown to degrade naphthalene with stochiometric amounts of nitrate reduction under strictly anaerobic conditions (Rockne et al, 2000). NAP-3 was phylogenetically most closely related to *Pseudomonas stutzeri* based on 16S rDNA sequences.
Medium. A nitrate reducing mineral salts medium was prepared incorporating the following compounds in DI water: 22.79 g/l NaCl, 3.97 g/l Na₂SO₄, 0.25g/l NH₄Cl, 0.30 g/l NaNO₃, 0.72 g/l KCl, 0.19 g/l NaHCO₃, 0.08 g/l KBr, 0.026 g/l H₃BO₃ 0.002 g/l NaF, 0.10 g/l of yeast extract. 10ml each of 100mM sodium acetate and 100mM sodium pyruvate were added to the above salt solution. The stock solution was boiled and cooled to 50°C under de-oxygenated N₂. 0.1mL of resazurin solution, 0.5mL of anaerobic metal solution (containing 0.2g/l FeCl₂·4 H₂O, 1g/l MnCl₂·4H₂O, 1g/l CoCl₂·5H₂O, 0.1 g/l ZnCl₂, 0.06 g/l CuCl₂, 0.1 g/l of H₃BO₃, 0.2 g/l of Na₂MoO₄·2H₂O, 2 g/l NiCl₂·H₂O, 0.1 g/l Na₂SeO₃·5H₂O, 0.1 g/l Na₂WO₄·2H₂O), 10ml/l of anaerobic phosphate solution (8.89g/l Na₂HPO₄·7H₂O) and 20ml/L of divalent cation solution (55.91g/100ml MgCl₂·6H₂O, 7.23g/100ml CaCl₂·2H₂O and 0.12 g/100ml SrCl₂·6H₂O) was added to the medium. The medium was reduced with the addition of 2.5ml/l of reductant solution (4g/l Na₂S·9H₂O and 4g/l cysteine-HCl). 125mL of medium was dispensed in 160ml serum bottles anaerobically and sealed and autoclaved.

Analytical procedures. Nitrate and nitrite quantification was done using ion chromatography with conductivity detection (Dionex IC-25/AS-50). The serum bottles with 125mL of medium were inoculated with 3ml of Nap-3 culture grown overnight on the anaerobic marine salt medium with 100mg/l of yeast extract with the addition of 1.25ml of vitamin solution. Samples were taken at regular time intervals and were filtered and centrifuged at 8228 rcf for 20 min (Eppendorf Centrifuge 5810R). The samples were diluted in the ratio of 1:10 with water. 2mL of the diluted samples were dispensed in IC vials and measured for the ion concentration.

For the quantification of naphthalene and screening of metabolic intermediates, 0.8ml of the samples were removed from the enrichments with a disposable syringe and extracted in the same volume of hexane. Gas chromatographic/mass spectrometric (GC/MS) analyses were performed on an Agilent 6890 GC coupled to an Agilent 5937 mass selective detector. Separation was achieved using an HP-5 MS fused silica capillary column (30m x 0.25mmID; 0.25μm film thickness) and a 1-μl injection volume. The injector temperature was 250°C. The column temperature was kept at 50°C for the initial 5 min, followed by a temperature increase of 10°C/min to a temperature of 150°C. Helium was used as a carrier gas at a flow rate of 1.2 ml/min. Indole was identified by comparison of its spectrum with that in the NIST library and by comparison with the authentic standard of indole. Indole was quantified by preparing a calibration curve using indole 99+% pure (Aldrich Chemical Company Inc., St. Louis, MO). All the chemicals used were purchased either from Aldrich (St. Louis, MO) or Sigma (St. Louis, MO) and were of purity of at least 99.9%.
RESULTS AND DISCUSSION

The pure culture NAP-3 was grown on mineral salts medium with or without naphthalene with sodium acetate as an additional electron donors under strict anaerobic conditions. Naphthalene significantly increased nitrate reduction (denitrification) in near stiochiometric balances of nitrate and nitrite as would be expected based on the metabolism. The cultures were sensitive to the concentration of naphthalene added to them. It was observed that nitrate utilization by NAP-3 was much faster when naphthalene was present at a concentration of 20mg/l rather than at a concentration of 40mg/l (Figure 2). The nitrate concentration decreased rapidly when naphthalene was present at a concentration of 20mg/l in contrast to when the naphthalene concentration was 40mg/l. Thus, a higher concentration of naphthalene inhibited the activity of NAP-3 cultures. No utilization of nitrate was observed in the blank and killed controls as expected. The removal of nitrate when naphthalene was not present was due to the utilization of acetate. The nitrate was completely utilized by NAP-3 within

FIGURE 2. Nitrate utilization by NAP-3 cultures with and without naphthalene.

FIGURE 3. Production of Nitrite by NAP-3 with time.
50 hrs when grown on 20 mg/l of naphthalene. In contrast, nitrate was still present after 150 hrs when only acetate was present.

An increase in the nitrite concentration was quantified under various growth conditions. The pattern of production of nitrite was in concurrence with the utilization of nitrate (Figure 3). Because NAP-3 is a denitrifier, nitrite production was only seen transiently. This was seen particularly with 20mg/l naphthalene, where nitrite peaked and was completely gone by 50 hrs.

Initial rates of naphthalene biotransformation varied for the first 50 hrs to 200 hrs for almost complete degradation (Figure 4).

A large peak was observed in the total ion chromatogram (TIC) in incubations with naphthalene (Figure 5a). This peak had a retention time of 14.9 min and increased with time. The peak was present at higher mass in 20mg/l naphthalene experiment. This peak was never observed in the killed and blank controls. All other peaks in the TIC shown in Figure 5a are silanes. The spectrum of the unknown peak was compared with the standard mass spectra of indole using the NIST library (Figure 5b, c). The upper plot shows the mass spectrum of indole from the NIST library and the lower plot shows the difference between the standard mass spectra of indole and the unknown peak. Based on the high degree of similarity (nearly 100%) we have identified the peak as indole. The production of indole was repeatable and found to be affected by the concentration of naphthalene concentration, with the 20mg/l culture producing the most indole (Figure 6).

To date, the only published metabolic pathway for anaerobic naphthalene degradation of PAHs initiates either through ring hydroxylation or carboxylation by sulfate reducers. Thus, it is not apparent which metabolic pathway could form indole from naphthalene, but it could be possible that there may be some biotic incorporation of either nitrate or a reduced metabolite of nitrate in the formation of indole-containing a ring with nitrogen. Indole is known to be an intermediate in the production of the amino acid tryptophan. Neither naphthol nor naphthoic acid were found at detectable levels in this culture.
FIGURE 5. a) Total ion chromatogram showing both naphthalene and indole. b) Mass spectrum of the unknown peak. c) The upper plot shows the standard mass spectrum of indole and the lower shows the difference between the standard and scanned peak of indole.
SUMMARY

Production of indole during the biotransformation of naphthalene anaerobically under denitrifying conditions was strictly biotic and was in concurrence with nitrate utilization. Coupled with the lack of naphthol and naphthoic acid production, these results suggest that there are metabolic pathways for the biotransformation of naphthalene other than the hydroxylation and carboxylation carried out by sulfate reducers that may require the stimulation of the organisms by more readily utilizable volatile fatty acids substrates.

REFERENCES


