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# Metabolic diversity of aromatic hydrocarbon-degrading bacteria from a petroleum-contaminated aquifer

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## Abstract

We characterized bacteria from contaminated aquifers for their ability to utilize aromatic hydrocarbons under hypoxic (oxygen-limiting) conditions (initial dissolved oxygen concentration about 2 mg/l) with nitrate as an alternate electron acceptor. This is relevant to current intense efforts to establish favorable conditions for *in situ* bioremediation. Using samples of granular activated carbon slurries from an operating groundwater treatment system, we isolated bacteria that are able to use benzene, toluene, ethylbenzene, or *p*-xylene as their sole source of carbon under aerobic or hypoxic-denitrifying conditions. Direct isolation on solid medium incubated aerobically or hypoxically with the substrate supplied as vapor yielded $10^3$ to $10^5$ bacteria $ml^{-1}$ of slurry supernatant, with numbers varying little with respect to isolation substrate or conditions. More than sixty bacterial isolates that varied in colony morphology were purified and characterized according to substrate utilization profiles and growth condition (i.e., aerobic vs. hypoxic) specificity. Strains with distinct characteristics were obtained using benzene compared with those isolated on toluene or ethylbenzene. In general, isolates obtained from direct selection on benzene minimal medium grew well under aerobic conditions but poorly under hypoxic conditions, whereas many ethylbenzene isolates grew well under both incubation conditions. We conclude that the conditions of isolation, rather than the substrate used, will influence the apparent characteristic substrate utilization range of the isolates obtained. Also, using an enrichment culture technique, we isolated a strain of *Pseudomonas fluorescens,* designated CFS215, which exhibited nitrate dependent degradation of aromatic hydrocarbons under hypoxic conditions.

*Abbreviations:* BTEX – benzene, toluene, ethylbenzene, and *p*-xylene, HPLC – high performance liquid chromatography, GAC – granular activated carbon

## Introduction

Petroleum contamination of groundwater is widely recognized as a serious environmental problem. Surface spills, leaking pipelines, and leaking underground fuel storage tanks can lead to subsurface contaminant plumes containing significant amounts of the hazardous aromatic hydrocarbons benzene, toluene, ethylbenzene, and xylenes. This group of compounds, referred to collectively as BTEX, ranks second only to trichloroethylene in occurrence in groundwater in the U.S.A. In recent years it has been recognized that biodegradation of environmental contaminants such as BTEX is the

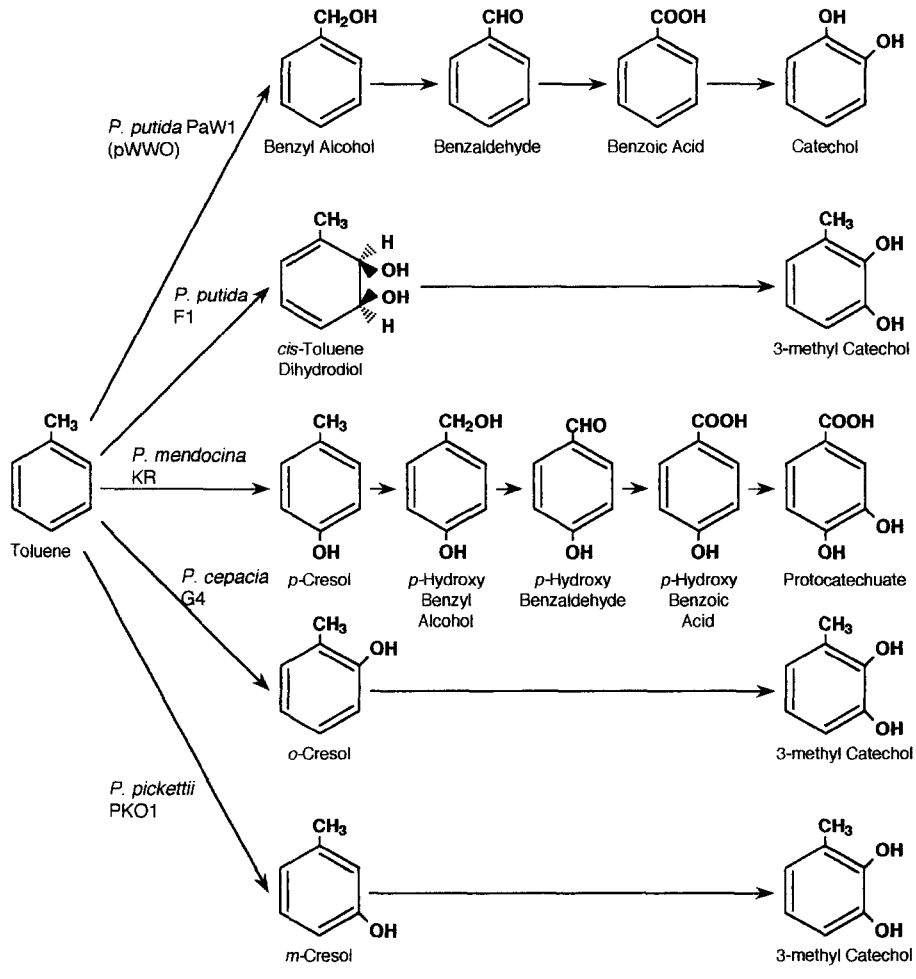


*Fig. 1.* Five pathways for the aerobic metabolism of toluene by bacteria.

most significant determinant of their eventual fate and impact.

Biodegradation of BTEX by bacteria under aerobic conditions has been studied for many years (Gibson & Subramanian 1984) and several pathways have been described. Five pathways have been demonstrated for the aerobic metabolism of toluene, for example, with initial hydroxylation at the *ortho* (Shields et al. 1989), *meta* (Kaphammer et al. 1991), or *para* (Whited & Gibson 1991) positions, or on the methyl group (Worsey & Williams 1975), or with dioxygenation at the 2,3 positions (Gibson et al. 1970). These five pathways are illustrated in Fig. 1. It is important to note that not all of the BTEX compounds are metabolized by each of the mechanisms described here. The TOL plasmid pathway metabolizes *m*- and *p*-xylene, but not *o*-xylene; the dioxygenase-catalyzed reaction is broad in specificity, but only partially metabolizes xylenes; and the three monooxygenase reactions have unique specificities that limit their range of activity (Shields et al. 1989; Kaphammer et al. 1991; Whited & Gibson 1991; Worsey & Williams 1975; Gibson et al. 1970). It is also important to note that each of these pathways requires molecular oxygen at two points, the initial oxidation and the ring cleavage steps.

In contrast to the oxygen-saturated environments discussed above, biodegradation of BTEX under anaerobic (reduced) or anoxic conditions (reviewed by Evans & Fuchs 1988) has largely been limited to studies with mixed bacterial populations,

such as soil or aquifer microcosms (Major et al. 1988; Hutchins 1991a, b; Edwards et al. 1992; Edwards & Grbic-Galic, 1992), flow-through systems (Zeyer et al. 1986; Kuhn et al. 1985), and sewage sludge enrichments (Grbic-Galic & Vogel 1987). In recent years, reports have appeared describing pure cultures of bacteria able to utilize BTEX compounds under iron-reducing (Lovley et al. 1989, 1990) and denitrifying (Dolfing et al. 1990; Evans et al. 1991; Altenschmidt & Fuchs 1991; Chee-Sanford et al. 1992) conditions. The pathways utilized have been described in some of these cases, and appear to involve oxidation of the methyl group to the carboxylic acid followed by conversion to the coenzyme A derivative (Altenschmidt & Fuchs 1991), or direct attack on the methyl group by acetyl-coenzyme A (Evans et al. 1992).

Our recent work has focused on BTEX degradation in hypoxic environments in which $O_2$ is present at approximately 2 mg/l or less, about 25% of the concentration present in air-saturated water. This carries significant implications for aquifer bioremediation strategies. It is possible that the addition of nitrate as an alternate electron acceptor could result in more $O_2$ being available as a substrate for oxygenase reactions. That is, nitrate may have an 'oxygen sparing' effect on hydrocarbon metabolism by denitrifying bacteria. This concept of 'oxygen sparing' is thus relevant to potential treatment strategies in which mixed electron acceptor regimes, namely $O_2$ plus nitrate, could be employed. The enhancement of aerobic biodegradation by nitrate addition has been observed in the metabolism of phenol in a pure bacterial culture (Britton 1987) and for the degradation of alkylbenzenes in aquifer microcosms (Hutchins 1991a, b). The practical advantages offered by such a scheme are manifest, not only because delivery of nitrate to the subsurface is more convenient than that of $O_2$, but also because traces of $O_2$ are difficult to exclude completely from the commonly used groundwater recirculation systems.

Strategies for the bioremediation of hydrocarbon-contaminated aquifers rely mainly on providing oxygen to the contaminant plume, easing the most commonly encountered limitation on biodegradation, namely, the inadequate supply of an electron acceptor. This may be accomplished by adding air or pure oxygen or by adding hydrogen peroxide as a source of oxygen. The utility of adding gaseous oxygen is limited by its expense and its low solubility, and by the fact that oxygen consumption in contaminant plumes can be rapid (Wilson et al. 1986). Whether the source of oxygen is the added gas or the decomposition of hydrogen peroxide, a major problem is the reaction with metal oxides, leading to reduced permeability of the aquifer and plugging of delivery systems (Lee et al. 1988). Since nitrate can serve as an electron acceptor at reduced oxygen concentrations, resulting in denitrification, the possibility of exploiting denitrifying bacteria for the bioremediation of groundwater contaminated with aromatic hydrocarbons is attractive (Britton 1987). The high water solubility of nitrate and the comparative ease with which it can be delivered to contaminated zones, in contrast to oxygen, enhance its potential feasibility as a candidate for addition to a contaminated aquifer for the purpose of stimulating *in situ* biodegradation.

To evaluate factors affecting the effectiveness of using denitrification as a component in a groundwater bioremediation strategy, our objective in this work was to investigate the metabolic diversity of bacteria from a hydrocarbon-contaminated aquifer. As part of a commonly-used type of remediation strategy, hydrocarbon-contaminated groundwater is pumped through beds containing granular activated carbon (GAC) particles (Voice 1989). It is well known that in this sort of 'pump and treat' operation GAC systems adsorb aquifer bacteria as well as contaminants such as BTEX, and that biodegradation by adsorbed bacteria can be credited with extending the duration of usefulness of such systems (Bouwer & McCarty 1982; DeLaat & Bouanga 1985). We were given access to samples of such a GAC adsorption system, and since it was likely that, due to the presence of organic contaminants (BTEX), oxygen levels were depleted in the aquifer as well as in the GAC beds, we were interested in examining the GAC bacteria with regard to the metabolic diversity present under hypoxic, denitrifying conditions as well as under aerobic conditions.

## Materials and methods

*Samples*

Granular activated carbon (GAC) samples were obtained from an aquifer treatment system composed of two trains operated in parallel, each with three stages arranged in series. This system had been receiving petroleum-contaminated groundwater containing BTEX for approximately three years. Samples of about 200 ml of GAC slurry were collected aseptically from each of the six GAC beds.

*Isolation procedures*

Bacteria able to utilize benzene, toluene, ethylbenzene, and *p*-xylene were isolated directly from the GAC samples under aerobic and hypoxic (denitrifying) conditions. The isolaton protocol is presented schematically in Fig. 2. GAC was mixed with sterile water (1 : 1 wt.: vol.) on a vortex mixer for 1 min.; after settling, 0.1 ml of the resulting supernatant was spread on the surface of solid minimal medium containing (per liter) 20 g purified agar, 0.1 g $(NH_4)_2SO_4$, 0.02 g $MgSO_4 \cdot 7H_2O$, 0.01 g $CaCl_2$, 5 mg $FeSO_4 \cdot 7H_2O$, and 25 mg $Na_2MoO_4 \cdot 2H_2O$, and buffered at pH 6.8 with 40 mM potassium phosphate buffer. For hypoxic incubations, in which the initial dissolved oxygen concentration was approximately 2 mg/l, the medium contained nitrate at 10 mM (140 mg/l nitrate-N). Aerobic incubations with volatile substrates (BTEX) took place inside glass dessicators with the hydrocarbon supplied as vapor by saturating a piece of filter paper (2 × 2 cm) with 100 µl of pure compound. For the comparatively more soluble, non-volatile substituted aromatic compounds such as the cresols and catechols the substrate was incorporated into the medium at a concentration of 0.05%. Hypoxic incubations were performed in GasPak jars (BBL Microbiology Systems, Cockeysville, MD) with the substrate provided in the same manner as in the aerobic incubations. *Pseudomonas putida* mt-2, a nondenitrifier containing the TOL plasmid pWWO, was included as a biological control for hypoxic conditions. The hydrocarbons (BTEX) were provided individually as the sole source of carbon. Aerobic and hypoxic plates were incubated at 30° C for 48 and 170 hours, respectively. Of the colonies appearing on each plate, eight were selected, picked and streaked for purification under the same conditions as in the initial incubation. The colonies were selected on the basis of colony morphology as well as the extent of growth so that a variety of organisms could be obtained. After a third streaking for purity each isolate (eight on each substrate under each of the two incubation conditions, hypoxic and aerobic) was incubated with each of the other substrates (benzene isolates incubated with toluene, ethylbenzene, and *p*-xylene, etc.). Finally, the aerobic isolates were incubated with all substrates under denitrifying conditions, and vice versa. The extent of growth for the isolates was judged by visual inspection of standard plate inoculations; growth was rated on a scale of one to ten, a score of one corresponding to negligible growth, a ten denoting lush growth. Strain characterization was facilitated by use of Rapid

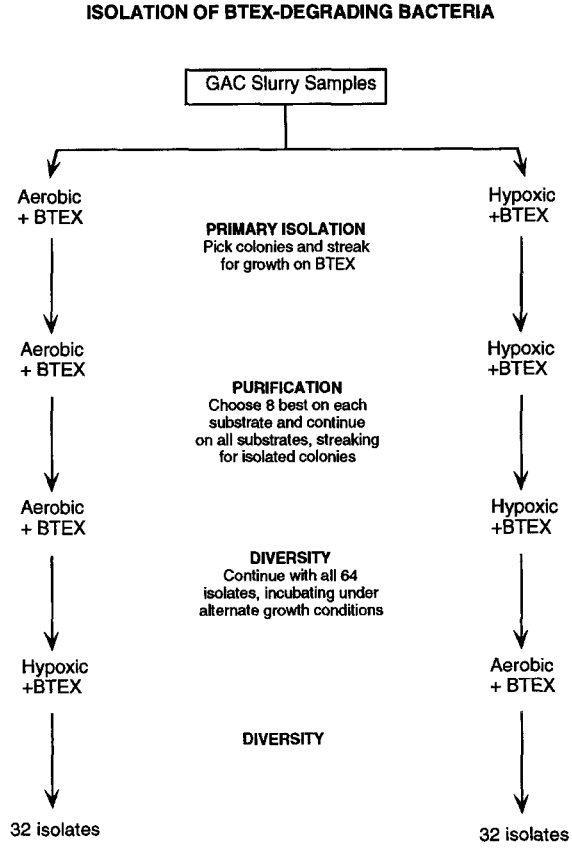


*Fig. 2.* Protocol for the isolation and characterization of BTEX-utilizing bacteria from granular activated carbon (GAC).

NFT strips (Analytab Products, Inc., Plainview, NY).

*Chemicals*

All aromatic hydrocarbons and other growth substrates were obtained from Aldrich Chemical Co. (Milwaukee WI) and were used without further purification.

*Enrichment culture and batch growth*

For incubation in liquid culture, serum bottles (160 ml capacity) with Teflon-lined rubber stoppers and aluminum crimp seals (all from Wheaton, Millville, NJ) were used. The composition of the medium was the same as that used for the direct plate isolations (above) but without agar. After the addition of 0.5 g GAC particles to 100 ml medium the bottles were flushed for 10 minutes at approximately 1.5 l/min with $O_2$-free gas mixture (85% $N_2$, 10% $H_2$, 5% $CO_2$). Before sealing, toluene was added to a concentration of 1 mM (92 mg/l). The toluene was monitored by high performance liquid chromatography and was replenished when the concentration fell below 200 µM. When the toluene had been replenished five times the enrichments were transferred (10% v/v) to freshly prepared medium and the process continued. Isolates were obtained by inoculating solid medium and incubating hypoxically as described above.

Batch incubation of pure cultures was performed in serum bottles as described above, but the bottles contained 150 ml of minimal medium and were flushed with $O_2$-free gas for 15 minutes after addition of an inoculum to an optical density ($O.D._{425}$) of 0.1. The inoculum was obtained by growing the strain on solid minimal medium containing nitrate (10 mM) and incubated with toluene vapors for 48 h. The batch cultures were incubated with 250 µM each of benzene, toluene, ethylbenzene, and *p*-xylene and sampled with a syringe at 1d, 3d, and 6d.

*Analytical methods*

Aromatic hydrocarbons and potential degradation products were analyzed by reverse-phase high performance liquid chromatography. Separation was performed on a 25 cm × 4.6 mm Spherisorb $C_{18}$ column (Phase Separations, Norwalk CT) using a mobile phase consisting of methanol and 1% aqueous acetic acid in varying proportions depending on the separation requirements. For benzene, toluene, ethylbenzene, and *p*-xylene, for example, good resolution was achieved using 70% methanol at 1.5 ml/min. Compounds were detected by UV absorbance and identified by co-chromatography with analytical standards.

The initial dissolved oxygen concentration in hypoxic batch cultures of approximately 2 mg/l was determined by polarographic $O_2$ measurement using a Clark $O_2$ electrode (Yellow Springs Instrument Co., Yellow Springs, OH).

## Results and discussion

Inoculation of minimal medium with GAC slurry supernatant resulted in the formation of numerous colonies utilizing each of the four hydrocarbons under both aerobic and hypoxic conditions. Numbers of isolated colonies were similar on all four substrates, ranging from $2 \times 10^3$ to $> 1 \times 10^5$ organisms/ml of GAC slurry supernatant. A more precise determination of total numbers of BTEX-utilizing bacteria is not possible, since our procedure for detaching cells was not intended to be exhaustive. Some samples yielded as many as four colony types while others appeared to contain a practically pure culture, based on the colony morphology of the primary isolates. At this stage, no clear pattern emerged to distinguish the isolates obtained on the four substrates or the two incubation conditions. Also, the two GAC trains and the three stages in each train appeared to be similar in the numbers and types of organisms obtained.

The isolation protocol (Fig. 2) resulted in the selection of eight isolates on each substrate for each set of growth conditions (e.g., toluene/aerobic, toluene/hypoxic, etc.). We tested each of the isolates for growth under both conditions and on all four substrates, yielding results in the form of a set of tables. Examples are shown in Tables 1 and 2, which describe the strains isolated on ethylbenzene under aerobic and hypoxic conditions, respectively. Many differences are apparent in the substrate utilization

profiles for these isolates. For overall cell growth, a clear contrast is provided by strain EA13, a poor grower, and EA17, which grew well on all substrates under both incubation conditions; EA21 and EN5 grew well aerobically but not hypoxically, while the opposite is true for strain EN37. Strain EN23 exhibited strong growth aerobically and fair growth under hypoxic conditions except on *p*-xylene, on which aerobic growth was poor and hypoxic growth was strong.

These examples of substrate range diversity are not unique to the ethylbenzene isolates for which the results are shown; similar results were obtained on the other substrates, although some qualitative differences are revealing. For example, growth of strains originally isolated by aerobic incubations with benzene was relatively poor under hypoxic conditions, and hypoxic benzene isolates grew poorly under aerobic conditions. Toluene isolates gave results similar to those isolated on ethylbenzene (Tables 1 and 2), while the *p*-xylene isolates showed distinct characteristics, having some similarities to the benzene group but with better growth in general, and better hypoxic growth in particular. Isolation of many diverse catabolic groups of gasoline-degrading bacteria was reported by Ridgeway et al. (1990), who found that a large majority of the identifiable isolates were members of the genus *Pseudomonas*. Although the authors did not attempt to determine if activity was present at reduced oxygen concentrations or if denitrification was occurring, it is likely that their isolates include many denitrifying strains.

The substrate used for the initial isolation and purification of strains did not always support the strongest growth. For example, benzene isolates generally grew better with toluene or ethylbenzene. This may be attributable to varying strength of the compounds as effectors for the degradative pathway(s) involved (R.H. Olsen et al. unpublished results). When the results for all the isolates, aerobic and hypoxic, are taken in aggregate ethylbenzene

*Table 1.* Growth on benzene, toluene, ethylbenzene, or *p*-xylene (B, T, E, or X) for bacterial strains isolated on ethylbenzene under aerobic conditions.

| Isolate | (sample)[b] | Incubation conditions | Growth substrate[a] | | | |
|---|---|---|---|---|---|---|
| | | | B | T | E | X |
| EA3 | (CFN 1) | Aerobic | 10 | 10 | 7 | 7 |
| | | Hypoxic | 5 | 10 | 8 | 5 |
| EA7 | (CFN 1) | Aerobic | 7 | 10 | 7 | 10 |
| | | Hypoxic | 5 | 7 | 9 | 5 |
| EA13 | (CFN 2) | Aerobic | 5 | 7 | 7 | 5 |
| | | Hypoxic | 1 | 6 | 9 | 5 |
| EA17 | (CFN 3) | Aerobic | 10 | 10 | 10 | 7 |
| | | Hypoxic | 10 | 10 | 10 | 10 |
| EA21 | (CFN 3) | Aerobic | 10 | 10 | 10 | 7 |
| | | Hypoxic | 5 | 6 | 8 | 3 |
| EA29 | (CFS 1) | Aerobic | 10 | 8 | 8 | 10 |
| | | Hypoxic | 7 | 7 | 10 | 5 |
| EA35 | (CFS 2) | Aerobic | 7 | 10 | 8 | 10 |
| | | Hypoxic | 10 | 10 | 8 | 9 |
| EA39 | (CFS 2) | Aerobic | 10 | 10 | 10 | 10 |
| | | Hypoxic | 10 | 8 | 8 | 8 |

[a] Growth is scored on a scale of 1 to 10, with a score of 1 for negligible growth, and 10 for lush growth. Incubations were 48 h for aerobic and 170 h for hypoxic conditions.

[b] Sample refers to the GAC bed from which the isolate originates; CFN, CFS – carbon filter north and south, respectively.

*Table 2.* Growth on benzene, toluene, ethylbenzene, or *p*-xylene (B, T, E, or X) for bacterial strains isolated on ethylbenzene under hypoxic conditions.

| Isolate | (sample)[b] | Incubation conditions | Growth substrate[a] | | | |
|---|---|---|---|---|---|---|
| | | | B | T | E | X |
| EN5 | (CFN 1) | Aerobic | 10 | 10 | 10 | 3 |
| | | Hypoxic | 3 | 8 | 7 | 4 |
| EN7 | (CFN 1) | Aerobic | 6 | 10 | 7 | 7 |
| | | Hypoxic | 1 | 5 | 10 | 4 |
| EN15 | (CFN 2) | Aerobic | 7 | 8 | 8 | 5 |
| | | Hypoxic | 5 | 7 | 9 | 8 |
| EN23 | (CFN 3) | Aerobic | 10 | 10 | 10 | 2 |
| | | Hypoxic | 7 | 8 | 9 | 10 |
| EN31 | (CFS 1) | Aerobic | 3 | 8 | 5 | 10 |
| | | Hypoxic | 8 | 9 | 8 | 8 |
| EN37 | (CFS 2) | Aerobic | 3 | 5 | 5 | 4 |
| | | Hypoxic | 8 | 9 | 7 | 7 |
| EN45 | (CFS 3) | Aerobic | 7 | 8 | 2 | 4 |
| | | Hypoxic | 8 | 8 | 7 | 8 |
| EN47 | (CFS 3) | Aerobic | 3 | 6 | 4 | 3 |
| | | Hypoxic | 7 | 9 | 7 | 7 |

[a] Growth is scored on a scale of 1 to 10, with a score of 1 for negligible growth, and 10 for lush growth. Incubations were 48 h for aerobic and 170 h for hypoxic conditions.

[b] Sample refers to the GAC bed from which the isolate originates; CFN, CFS – carbon filter north and south, respectively.

stands out clearly as the best growth substrate, followed by toluene, with benzene and *p*-xylene the least effective. In the work described by Ridgeway et al. (1990) initial isolation was performed using gasoline, a mixture containing hundreds of aromatic and aliphatic hydrocarbons. Almost 75% of their isolates were able to grow on toluene, which was followed by *p*-xylene and ethylbenzene in frequency utilized. These gasoline-degrading isolates were generally limited to two or three structurally related compounds.

To select the best growing out of these 64 isolates we arbitrarily chose as a limit an average score of seven for growth on each of the four substrates. The results of this manipulation are summarized in Table 3. All eight of the aerobic benzene isolates grew well aerobically but none did so under hypoxic conditions. This is in contrast to the toluene and particularly the ethylbenzene isolates, of which several were able to grow well on all four substrates under both sets of incubation conditions. From the data summarized in Table 3 it is clear that the conditions of isolation, i.e., aerobic or hypoxic, will determine the most probable optimal growth conditions for the resulting isolates: aerobic *p*-xylene isolates are more likely to grow well under aerobic conditions and hypoxic *p*-xylene degraders are more likely to grow well under hypoxic conditions. This trend, however, does not hold for the ethylbenzene isolates for which the numbers in the two categories are more nearly equal. In a study conducted by Britton (1987), soil enrichments performed with phenol, dodecane, toluene and naphthalene, with and without nitrate, and at 0.5 and 21% oxygen, resulted in no qualitative microbial population differences. This led to the conclusion that many of the organisms associated with hydrocarbon degradation are also capable of nitrate respiration. These results are in consonance with ours, insofar as many of the isolates we characterized, regardless of the substrate and conditions used for isolation, were active under aerobic as well as hypoxic, denitrifying, conditions.

*Table 3.* Number of isolates in each category scoring an average of seven or better for growth on all four aromatic hydrocarbons[a].

| Isolation | | Number of fast-growing strains | | |
|---|---|---|---|---|
| | | Incubation Conditions | | |
| Substrate | Conditions | Aerobic | Hypoxic | Both |
| Benzene | Aerobic | 8 | 0 | 0 |
| | Hypoxic | 0 | 0 | 0 |
| Toluene | Aerobic | 6 | 2 | 2 |
| | Hypoxic | 2 | 6 | 2 |
| Ethylbenzene | Aerobic | 7 | 5 | 5 |
| | Hypoxic | 4 | 6 | 2 |
| *p*-Xylene | Aerobic | 5 | 0 | 0 |
| | Hypoxic | 1 | 6 | 1 |

[a] Growth is scored on a scale of 1 to 10, with a score of 1 for negligible growth and 10 for lush growth. Incubations were 48 h for aerobic and 170 h for hypoxic conditions.

The twelve strains that grew well (average score of seven or better for growth on all four substrates) under both aerobic and hypoxic conditions, i.e. those indicated in the righthand column in Table 3, were tested for their ability to grow on a variety of substituted aromatic compounds. The test compounds were selected because of their occurrence as intermediates in known toluene metabolic pathways. In two cases (EA3 and EA39) intermediates of the pathway encoded by the TOL plasmid pWWO (Worsey & Williams 1975), benzyl alcohol, benzoic acid, and catechol, supported lush growth, but not under hypoxic conditions. Also, some isolates grew well aerobically on *p*-hydroxybenzyl alcohol and *p*-hydroxybenzoic acid, intermediates in the pathway found in *P. mendocina* KR for toluene metabolism (Whited & Gibson 1991). In most cases, however, the pattern of substrate utilization provided no insight into which pathway(s) might be operating. In general, hypoxic growth on the substituted aromatics was poor, possibly due to a failure to induce metabolic pathways.

Attempts to grow these twelve strains in hypoxic liquid culture were unsuccessful. After incubation of the strains for 14 days in liquid minimal medium containing toluene at a concentration of 250 μM, we observed neither an appreciable increase in optical density nor significant substrate depletion as measured by HPLC.

Faced with this difficulty, we began an enrichment strategy to obtain a strain from the GAC samples that was able to grow at reduced $O_2$ concentrations using toluene as a carbon source and nitrate as an electron acceptor. This procedure resulted in the

isolaton of a bacterial strain able to metabolize BTEX under oxygen-limited denitrifying conditions. This strain has been identified as a *Pseudomonas fluorescens* and is designated CFS215 (Table 4). Although several substitued aromatic compounds have been screened as growth substrates (Table 4), we have not yet determined the pathway (s) used for BTEX metabolism in this strain.

The nitrate dependence for metabolism of BTEX by strain CFS215 under hypoxic conditions is illustrated in Fig. 3. Under aerobic conditions, BTEX compounds are completely degraded (unpublished results; Alvarez & Vogel 1991). Clearly each of the BTEX compounds examined is metabolized under hypoxic conditions when nitrate is present and not with ammonia only. The rate of degradation for all four compounds taken together, normalized for cell density, is 83 μmoles per day per $O.D._{425}$ 1.0 in the presence of nitrate, compared to 12 μmoles per day per $O.D._{425}$ 1.0 in the absence of nitrate. There was no change in the cell density over the course of the incubation. Based on the amount of dissolved oxygen present initially, 2 mg/l, and assuming that the headspace of the bottles contained oxygen in the same proportion (25% air saturation), a total of about 0.15 mmoles of $O_2$ was present. At the six day sampling point, about 0.05 mmoles of BTEX had been degraded, and the dissolved oxygen concentration was about 0.2–0.3 mg/l. Therefore, the ratio of dissolved oxygen available to hydrocarbon degraded was about 3 to 1, a quantity that is more than adequate for transformation as far as the ring cleavage step or farther. The presence of nitrite in the batch cultures confirms that the reduction of nitrate is occurring (data not shown). Further studies will be directed at more complete measurements including monitoring oxygen, nitrate, nitrite, and the hydrocarbon over

*Table 4.* Characteristics of *Pseudomonas fluorescens* strain CFS215.

- Isolated from hypoxic denitrifying enrichment culture using granular activated carbon as the inoculum and toluene as the sole carbon source
- No growth at 4° or 42° C
- Produces diffusible fluorescent pigment
- Denitrifier
- Biochemical Characteristics:

| | |
|---|---|
| Tryptophanase | − |
| Glucose fermentation | − |
| Arginine dihydrolase | + |
| Urease | + |
| Esculinase | − |
| Gelatinase | − |
| β-galactosidase | − |
| Oxidase | + |
| Substrate utilization | |
| D-Glucose | + |
| L-Arabinose | + |
| D-Mannose | + |
| D-Mannitol | + |
| N-Acetyl-D-glucosamine | + |
| Maltose | + |
| D-Gluconate | + |
| Caprate | + |
| Adipate | − |
| L-Malate | + |
| Citrate | + |
| Phenylacetate | − |

Aromatic growth substrates

| Aerobic | | Hypoxic |
|---|---|---|
| + | Benzene | + |
| + | Toluene | + |
| + | Ethylbenzene | + |
| + | *p*-Xylene | + |
| − | *o*-Cresol | − |
| + | *m*-Cresol | + |
| + | *p*-Cresol | + |
| + | Benzoate | − |
| + | *p*-Hydroxybenzoate | + |
| − | Phenol | − |
| − | 3-Methylcatechol | − |
| − | 4-Methylcatechol | − |
| − | Catechol | − |

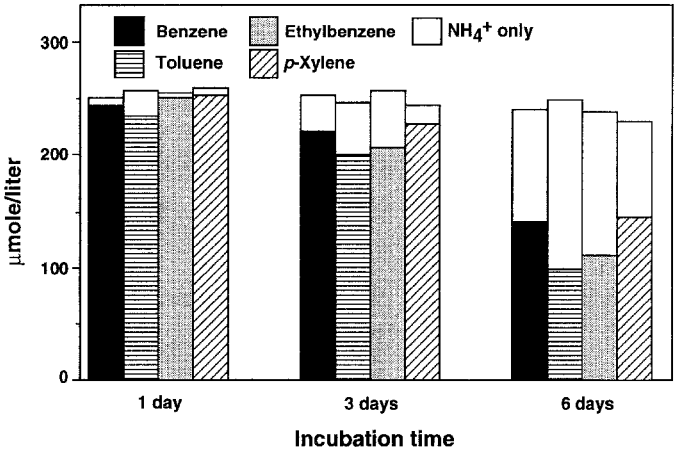


*Fig. 3.* Nitrate-dependence of BTEX utilization under hypoxic conditions by *P. fluorescens* strain CFS215. The cells were added to an $OD_{425}$ of 0.1 to minimal medium containing nitrate and ammonium or containing ammonium only. The initial dissolved $O_2$ level in the bottles was approximately 2 mg $l^{-1}$.

time, allowing more complete understanding of the process.

It is likely that organisms such as strain CFS215 and the other isolates described above which were obtained from a GAC-based groundwater remediation operation, would be present in $O_2$-depleted ground water containing BTEX. Since we have shown that such strains are capable of BTEX degradation under hypoxic denitrifying conditions, it is reasonable to speculate that *in situ* biodegradation of BTEX could be enhanced by the addition of nitrate to contaminated aquifers. Recent work by Hutchins (1991a, b) demonstrated that the extent of alkyl benzene degradation under conditions of limited oxygen was enhanced by the presence of nitrate. This study showed that degradation of aromatic hydrocarbons ceased as oxygen was depleted, unless an alternate electron acceptor (nitrate) was available. Although the rate of degradation of aromatic hydrocarbons was slower under denitrifying conditions than under aerobic conditions, it is clear that mixed electron acceptor regimes may be useful in remediation schemes.

## Conclusions

A diverse population of BTEX-degrading bacteria developed on the surface of GAC particles utilized in a pump-and-treat restoration scheme for a petroleum-contaminated aquifer. We isolated and partially characterized many bacteria able to utilize benzene, toluene, ethylbenzene, and *p*-xylene under either aerobic or hypoxic (2 mg $l^{-1}$ $O_2$) conditions. Many of the isolates were characterized by a broad substrate range, and activity under both aerobic and hypoxic conditions was dependent on the substrate used for isolation, with ethylbenzene and toluene most likely to yield the strains with the broadest range of activity. Using an enrichment culture technique we isolated a strain of *P. fluorescens* with which we demonstrated nitrate-dependent BTEX metabolism under hypoxic conditions.

These results, together with results published elsewhere, indicate that strategies for *in situ* bioremediation may require less oxygen than has been assumed to be necessary, providing nitrate is present to serve as an alternate electron acceptor when conditions become hypoxic.

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