Physiological Attributes of Microbial BTEX Degradation in Oxygen-limited Environments

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Our work has focused on the determination of physiological traits that may facilitate in situ degradation of xenobiotic compounds by indigenous microorganisms. For this our interests center on the following questions: What are the ambient conditions in a benzene, toluene, ethylbenzene, and xylene (BTEX)-contaminated aquifer? What is the behavior of indigenous bacteria under these conditions? What are the attributes of bacterial strains that are functional under hypoxic conditions? How do these strains compare with other BTEX-degrading strains? — Environ Health Perspect 103(Suppl 5):49-51 (1995)

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Aquifer Characteristics

For several years we sampled three sites in which groundwater was contaminated by petroleum products, including benzene, toluene, ethylbenzene, and xylene (BTEX) components (1,2). All three of these contaminated aquifers were characterized by their low dissolved oxygen levels in the contaminated regions (~ 1–2 mg/l), in contrast to uncontaminated adjacent regions in the aquifer (~ 3–4 mg/l). The occurrence of dissolved nitrate also showed a corresponding relationship when the contaminated and uncontaminated regions were compared. The nitrate concentrations were low or undetectable in the contaminated regions, but were higher in the uncontaminated regions. These relationships were observed for all three aquifers, leading to the conclusion that oxygen and nitrate depletion characteristic of the contaminant plume regions was indicative of an active, ongoing biological process.

Activity of Indigenous Microbial Communities

Interesting correlations between the presence of denitrifying bacteria competent for growth on BTEX were observed when cores of aquifer material that had been sampled vertically were tested for microbial activity as a function of depth and location within the plume regions. Bacterial strains growing on BTEX aerobically were observed for all samples independent of depth or BTEX concentration. However, when such samples were incubated under hypoxic conditions (i.e., <2 mg/l dissolved oxygen), bacterial growth and denitrification matched BTEX degradation observed with microcosm cultures inoculated with bacteria eluted from the soil core samples. Therefore, differences were observed between bacteria that would degrade BTEX under hypoxic-denitrifying conditions and those that grow on BTEX under aerobic conditions.

Diversity of Bacteria from Hypoxic Environments

Samples from the BTEX-contaminated aquifers were incubated in mineral salts buffer overnight to elute bacteria from particles. These eluates were then plated on solid mineral salts medium followed by incubation aerobically or under hypoxic conditions in the presence of BTEX vapors. Aerobic cultures were incubated 48 hr and hypoxic cultures were incubated 1 week. Colonies were replated onto the same medium and incubated as before. These cultures then became the source for subsequent tests for the mineralization of individual BTEX components under both aerobic and hypoxic conditions. For this work, p-xylene was used as a representative xylene. The colonies selected for such tests were selected on the basis of their distinctive morphologies and colony size to facilitate estimates of the diversity of the microflora that might be isolated from BTEX-contaminated environments.

A subset of these bacterial isolates that showed good growth on all four of the BTEX substrates was analyzed, as shown in Table 1. These isolates were compared with respect to their growth on BTEX under either aerobic or hypoxic conditions as a further indication of the diversity of microorganisms resident in the samples.

The data in Table 1 show that not all bacteria isolated under one condition (e.g., aerobiosis, carbon source) grow under dissimilar conditions. For example, of the eight isolates selected for growth on benzene under aerobic conditions, none grew on BTEX under hypoxic conditions. In contrast, however, of the seven bacteria...
selected for growth on ethylbenzene under aerobic conditions, five strains also grew on BTEX under hypoxic conditions. These results show how the substrate used to select such strains and also the gas atmosphere may provide an estimate of the diversity that occurs in BTEX-contaminated aquifers. Moreover, bacteria that perform well under hypoxic conditions can be isolated.

**Pure Culture Studies**

Studies with single bacterial strains were also performed as for the microcosm cultures described above. For this work we used three strains designated W31, CFS215, and PKO1 that we isolated from BTEX-contaminated soil; we compared their behavior with other BTEX-degrading strains described previously by Olsen et al. (3). These studies showed that the ability to degrade BTEX was not simply a matter of denitrifying ability since not all denitrifiers degraded BTEX under hypoxic conditions. Moreover, these studies suggested fundamental enzymological differences occurring between the two groups of microorganisms, as shown in Figure 1. In this regard, all of the strains used had in common the production of catechols from toluene or benzene.

Moreover, such catechols were metabolized to ring fission products with the obligatory requirement for one molecule of oxygen. The ring fission enzyme, catechol dioxygenase, accordingly offered a good subject for comparing the two groups of organisms depicted above with regard to an oxygen-requiring enzyme's kinetic characteristics both for oxygen and substrate. When Michaelis constants (K_m) were determined for oxygen, they were approximately one order of magnitude lower for the bacterial strains active under hypoxic-denitrifying conditions (W31, CFS215, PKO1) than the other *Pseudomonas* strains depicted above. Thus such organisms that are functional under oxygen-limiting conditions may be comprised to more efficiently use low ambient concentrations of dissolved or atmospheric oxygen necessary for critical oxygen-requiring steps.

**Physiology of Pseudomonas pickettii PKO1**

The toluene pathway from a strain typical of those growing under hypoxic conditions on toluene has been cloned into vector pRO1727 and designated pRO1957. The 27.5 kbp DNA fragment cloned from *Pseudomonas pickettii* PKO1 encodes for the enzymes of the pathway reported previously (4–6) and is organized as depicted in Figure 2.

Using deleted versions of pRO1957, its gene order has been determined, as illustrated in Figure 3. Inducers (effectors) and substrates for the three regulons comprising the toluene-3-monoxygenase pathway are listed in Figure 3. This path-

![Figure 1](image1.png)

**Figure 1.** The effect of nitrate on toluene degradation under hypoxic conditions (< 2 mg O_2/l).

![Figure 2](image2.png)

**Figure 2.** Mapping and regulation of the *tbu* pathway. Abbreviations: R, activator encoded by *tbuR*; S, activator encoded by *tbuS*; T, activator encoded by *tbuT*; E, effectors (inducers). Gene designations are as described for Figure 3.

![Figure 3](image3.png)

**Figure 3.** Metabolism of alkyl-substituted benzenes by *P. pickettii* PKO1. Abbreviations: TTM, toluene-3-monoxygenase; PH, phenol hydroxylase; C230, catechol 2,3 dioxygenase; HMSD, 2-hydroxyoxymonocate semialdehyde dehydrogenase; HMSC, 2-hydroxyoxymonocate semialdehyde hydratase; 40I, 4-oxalocrotonate isomerase; 40D, 4-oxalocrotonate decarboxylase; DEH, 2-hydroxypent-2,4-dienoate hydratase; HOA, 4-hydroxy-2-oxovalerate aldolase. Gene designations corresponding to each enzyme are shown in parentheses.
way is unlike those previously described in view of the initial carbon 3 hydroxylation reaction with the production of the corresponding cresols from toluene and xylene(s). The *tbu* pathway is arranged in three regulons: one for toluene-3-monoxygenase (*tbu*A–C), one for phenol hydroxylase (*tbu*D), and another for the catechol pathway (*tbu*E–K). Regulatory loci and the juxtaposition of these regulons is shown in Figure 2. Putative starting points for the initiation of transcription of the regulons are depicted as circles with arrows for each of the three regulons.

We have sequenced the genes associated with loci designated *tbu*A, B, C, and D and compared them with other oxygenase enzyme sequences (7). In this regard, *tbu*A, B, C, and D encode for six reading frames, some of which show remarkable homology with other procaryote oxygenase genes that have been sequenced. The gene for phenol/cresol hydroxylase, *tbu*D, has little overall homology with other aromatic hydroxylases, although three domains show homology with other flavoprotein monoxygenases.

**Summary**

Our work to date suggests that a group of microorganisms has evolved which is, for the most part, indistinguishable from closely related species, but which has adapted to growth and metabolism in low-oxygen environments. Such microorganisms, then, may facilitate the natural bioremediation of toxic xenobiotic molecules *in situ*, thus reducing the impact of such substances on human health and the environment.

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