APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 2004, p. 1222–1225 Vol. 70, No. 2
0099-2240/04/$08.00+0 DOI: 10.1128/AEM.70.2.1222–1225.2004


# Biodegradation of Benzene by Halophilic and Halotolerant Bacteria under Aerobic Conditions

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Received 9 September 2003/Accepted 12 November 2003

**A highly enriched halophilic culture was established with benzene as the sole carbon source by using a brine soil obtained from an oil production facility in Oklahoma. The enrichment completely degraded benzene, toluene, ethylbenzene, and xylenes within 1 to 2 weeks. Also, [$^{14}$C]benzene was converted to $^{14}CO_2$, suggesting the culture's ability to mineralize benzene. Community structure analysis revealed that *Marinobacter* spp. were the dominant members of the enrichment.**

Large volumes of oily wastewater are generated during oil exploration and production activities. Produced waters display a wide range of salinities, ranging from low to high. Spilled brine inhibits plant growth, leading to increased erosion and loss of topsoil and contamination of groundwater by both salt and hydrocarbons. Because conventional microbiological treatment processes do not function at high salt concentrations, bioremediation of oilfield brine can only be accomplished by using indigenous bacteria capable of degrading petroleum compounds or through bioaugmentation of halophiles or halotolerants. Unfortunately, information on the ability of such organisms to degrade petroleum compounds is limited. Only recently have there been indications that the halophilic bacteria may have a greater potential in the degradation of pollutants than was previously assumed. A halophilic archaea (strain EH4) was found to be capable of degrading a wide range of *n*-alkanes and aromatic hydrocarbons in the presence of high salt (1, 12, 15). *Marinobacter hydrocarbonoclasticus* degraded a variety of aliphatic and aromatic hydrocarbons (7). A halotolerant *Streptomyces* sp., isolated from an oil field in Russia, degraded crude petroleum (9). In addition, bacteria isolated from salt-impacted material degraded polycyclic aromatic hydrocarbons (PAHs) (13). While these observations provide evidence for the degradation of *n*-alkanes, PAHs, and other simple aromatic compounds, little is known about the degradation of benzene, toluene, ethylbenzene, and xylene (BTEX) compounds. The primary objective of this work was to evaluate the ability of halophilic and halotolerant bacteria present in oilfield brine to degrade BTEX compounds. BTEX are most problematic, because they are highly water soluble and are toxic. Among BTEX, benzene is of major concern, because it is one of the most stable aromatic compounds and is a known carcinogen.

**Enrichment culture.** A stable and highly enriched aerobic microbial consortium that degraded benzene as the sole carbon and energy source was developed from a soil sample obtained from Seminole County in Oklahoma. Hereafter this culture is referred to as the Sem-2 enrichment culture. The enrichment was initiated by adding 10 g of soil (wet weight) to duplicate 1-liter-capacity bottles containing 500 ml of mineral salts medium (MSM). MSM contained (in grams/liter): NaCl, 145; $MgCl_2$, 0.5; $KH_2PO_4$, 0.45; $K_2HPO_4$, 0.9; $NH_4Cl$, 0.3; KCl, 0.3. Air in the headspace served as the source of oxygen. The bottles were closed with a black rubber stopper with a hole in the middle that fit a cut 3-in. Hungate tube. The tubes were closed with Teflon-coated septa and aluminum caps. A 100-μl gas-tight glass syringe was used to introduce 22 μl of undiluted benzene (~245 μmol) to each bottle. The bottles were incubated static in the dark at room temperature. After 7 to 8 months of continuous enrichment, the culture consistently degraded benzene within 18 days at a rate of approximately 12 μmol/day.

**Biodegradation assay.** Unless otherwise mentioned, all experiments involving the Sem-2 enrichment culture were performed with 160-ml-capacity serum bottles filled with 45 ml of MSM containing 2.5 M NaCl. Bottles were inoculated with 5 ml of Sem-2 enrichment culture and were spiked with 2 μl (17 to 22 μmol) of undiluted benzene, toluene, ethylbenzene, or xylenes. The bottles were then closed with Teflon-coated septa and aluminum caps and were incubated under static conditions in the dark at 30°C. The headspace gas was withdrawn periodically, and degradation of BTEX was monitored by gas chromatography (GC).

Biodegradation of BTEX compounds were assayed by using a Hewlett Packard 6890 GC equipped with a flame ionization detector and a DB-1 capillary column (30 m by 0.320 mm by 1 μm; J&W Scientific, Inc.). Helium served as both carrier and makeup gas at flow rates of 10 and 40 ml/min, respectively. The flow rates of hydrogen and air were set at 40 and 450 ml/min, respectively. The operating GC conditions were the following: oven temperature, 70°C for 7 min; inlet temperature, 150°C; and detector temperature, 220°C. Approximately 200 μl of headspace gas from microcosms was injected into the GC for quantification. The GC response for each compound tested was calibrated to give the total mass in that bottle. Standards were prepared in 160-ml bottles filled with 50 ml of NaCl solution (0 to 4 M) and were closed with Teflon-faced septa and aluminum caps. After equilibration at room temperature the GC response for a range of mass (in micromoles/bottle) of

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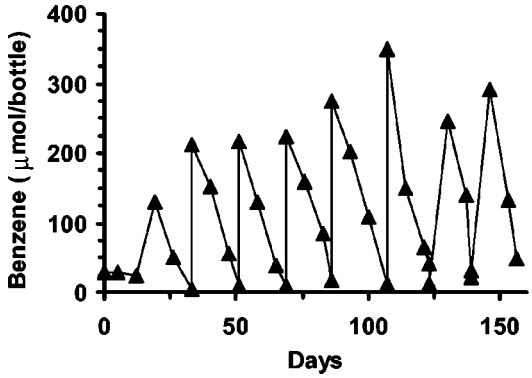


FIG. 1. Repeated use of benzene (▲) as the sole carbon and energy source in the presence of 2.5 M NaCl by the Sem-2 enrichment. The enrichments were maintained in 1-liter-capacity bottles containing 500 ml of MSM at room temperature. After an initial lag period, the enrichment degraded 200 to 300 μmol of added benzene/bottle consistently in 2.5 weeks. Results for only one bottle are shown; duplicate enrichments behaved similarly.

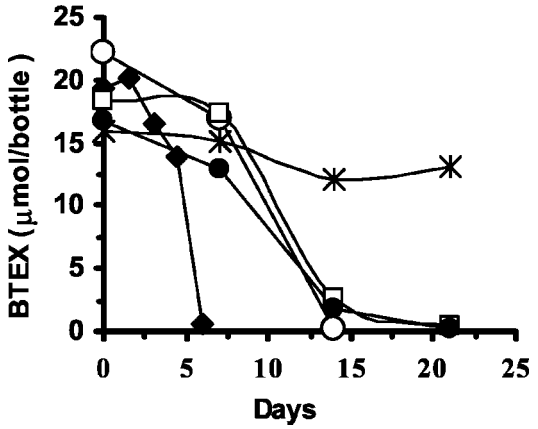


FIG. 2. Biodegradation of benzene (○), toluene (◆), ethylbenzene (□), and xylenes (●) in 160-ml-capacity bottles containing 45 ml of MSM containing 2.5 M NaCl and inoculated with 5 ml of Sem-2 enrichment. The microcosms were incubated at 30°C. The data are means ± standard deviations for triplicate active microcosms and average of duplicate autoclaved control bottles. Since all controls behaved similarly, only the control for xylenes (asterisks) is shown.

each compound tested was plotted, and the slopes were used to quantify the unknown. The quantification of benzene in enrichment bottles was accomplished as described above by using a calibration curve prepared with 1-liter bottles containing 500 ml of 2.5 M NaCl solution. The GC detection limit for benzene using our method was <1.0 μmol/bottle.

**Microbial community structure.** Community structure of the Sem-2 culture grown on benzene in the presence or absence of salt was analyzed by using denaturing gradient gel electrophoresis (DGGE) by Microbial Insights, Inc. (Rockford, Tenn.). PCR-amplified eubacterial 16S ribosomal DNA (rDNA) fragments were obtained by nested PCR using primer sets corresponding to *Escherichia coli* base pair positions 27 to 1492 and 341 to 534 (the forward primer contained a 40-bp GC clamp). PCR products were separated on a DGGE gel, and each prominent band was excised, purified, and sequenced.

**Degradation BTEX by halophilic and halotolerant bacteria.** The Sem-2 enrichment consistently degraded all added benzene (250 μmol/bottle) in 2.5 weeks at room temperature (Fig. 1). Experiments also evaluated the ability of the Sem-2 enrichment to mineralize [$^{14}$C]benzene to $^{14}CO_2$. Roughly 46% of the initially added radiolabeled benzene was converted to $^{14}CO_2$ in 4 weeks (data not shown). In addition, the enrichment also degraded toluene, ethylbenzene, or *o-m-p*-xylenes as the sole carbon source when grown in MSM with 2.5 M NaCl at 30°C (Fig. 2). Among the tested compounds, toluene was degraded fastest. Approximately 20 μmol of toluene was completely degraded in less than 1 week, while benzene, ethylbenzene, or xylenes required roughly 2 to 3 weeks. Autoclaved control bottles showed no evidence of degradation. Although a few reports have documented the ability of halophiles or halotolerants to degrade hydrocarbons, including phenol (16), nitrophenols (12), benzoate (5), pesticides (2), herbicides (11), *n*-alkanes (1), and PAHs (1, 13), the authors are unaware of any report demonstrating the aerobic degradation of BTEX by halophiles or halotolerant bacteria. The ability of the enrichment to degrade BTEX is significant, because these compounds are highly soluble and can contaminate large volumes of soil or aquifer material at exploration and production sites. Also, these compounds are toxic and carcinogenic and are among the U.S. Environmental Protection Agency's priority pollutants.

Our study also evaluated the effect of low concentrations of yeast extract (YE), vitamins, or trace elements on benzene degradation rate. Benzene was completely removed within 8 days in microcosms amended with 0.02% YE, 1 μl of vitamin solution/ml, or 1 μl of trace elements/ml (10) compared to 15 days in the absence of the stimulants (Fig. 3). It was suggested that halophiles have more demanding nutritional requirements at high salt concentrations, and complex media may help stimulate growth of halophilic bacteria at high salt concentrations

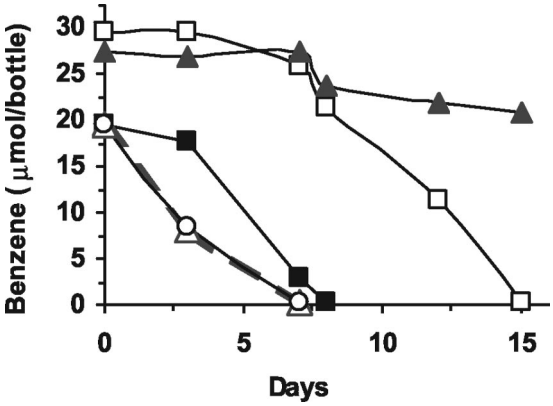


FIG. 3. Biostimulation of benzene degradation by YE, vitamin, or trace elements. Microcosms (160 ml) were established with 45 ml of MSM containing 2.5 M NaCl and were inoculated with 5 ml of Sem-2 enrichment. Biodegradation of benzene (22 μmol/bottle) was evaluated in the presence or absence of growth stimulants. Symbols: ■, YE; △, vitamins; ○, trace elements; □, no amendments. The autoclaved microcosms were also amended with filter-sterilized 0.02% YE, 1 μl of vitamin solution/ml, or 1 μl of trace elements/ml. The results are means ± standard deviations for triplicate active microcosms and the average of two autoclaved bottles. Because all controls behaved similarly, only control data for YE (▲) are shown.

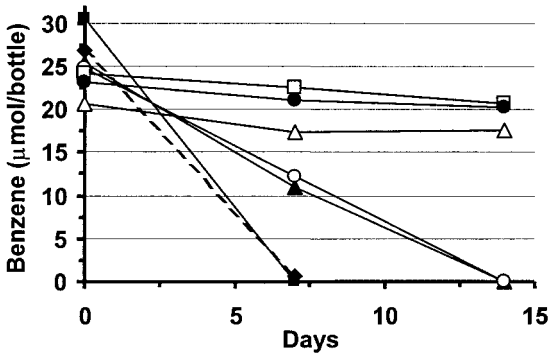


FIG. 4. Biodegradation of benzene by the Sem-2 enrichment at different salt concentrations (NaCl). Microcosms were established with 160-ml-capacity serum bottles filled with 45 ml of MSM and were inoculated with 5 ml of Sem-2 culture. All microcosms were spiked with benzene (22 μmol/bottle) and various concentrations of NaCl, including 0 (△), 0.5 (◆), 1 (■), 2 (▲), 2.5 (○), 3 (□), or 4 M (●). The results are means ± standard deviations for triplicate active microcosms. Although results for 0, 3.0, and 4.0 M NaCl bottles are shown for only 2 weeks of incubation, these bottles were incubated for more than 4 weeks. No benzene degradation occurred even after 4 weeks of incubation (data not shown).

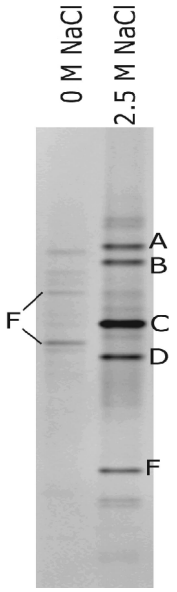


FIG. 5. DGGE gel image of amplimers of bacterial 16S rDNA extracted from microcosms amended with 0 and 2.5 M NaCl. Each microcosm was filled with 45 ml of MSM and 22 μmol of benzene and was inoculated with 5 ml of Sem-2 enrichment. When all the added benzene was degraded (in bottles with 2.5 M NaCl), the culture was analyzed for community composition by DGGE. Bottles without salt did not degrade benzene even after 4 weeks of incubation, and these bottles were analyzed after 4 weeks of incubation. The bands were excised and sequenced. The sequences were compared with closely related sequences in the database. Results show that the dominant members of the Sem-2 culture grown in the presence of 2.5 M NaCl had 97 to 100% sequence similarity to the members of the genus *Marinobacter* (bands A, B, C, and D). Similar bands were missing in DGGE gel from the Sem-2 culture that did not degrade added benzene (0 M NaCl). Bands labeled F failed to yield usable sequences.

(14). An archaea (strain EH4) isolated from a salt marsh was able to degrade a higher percentage of eicosane ($C_{20}H_{42}$) in the presence of YE, peptone, and Casamino Acids (1).

Biodegradation of benzene was evaluated at various salt concentrations, ranging from 0 to 4 M (Fig. 4). The Sem-2 degraded 25 to 30 μmol of benzene in 7 to 14 days with no apparent lag time in bottles containing 0.5, 1.0, 2.0, or 2.5 M NaCl. No degradation occurred in bottles containing 0, 3.0, or 4.0 M NaCl, even after 4 weeks of incubation (data not shown). Degradation of benzene required the addition of at least 0.5 M NaCl in MSM while no degradation occurred in its absence, indicating that the enrichment harbored true halophiles. Also, the ability of the culture to degrade benzene over a wide range of NaCl (0.5 M to 2.5 M) suggests that this culture is well suited for field bioremediation applications, because many produced waters or oilfield brines display a wide range of temporal and spatial salinity flux. The reason for the lack of benzene degradation at 3.0 and 4.0 M NaCl is not known. Few studies have dealt with the effect of salinity on microbial degradation of hydrocarbons. These studies (3, 4, 15) have found that biodegradation of hydrocarbons declined with increasing NaCl concentrations. In contrast, Fernandez-Linares et al. (6) showed that increasing salt level had no effect on eicosane degradation by a *Marinobacter* sp.

The community structure of the Sem-2 grown in the presence or absence of added NaCl was characterized by profiling the 16S rDNA gene by using DGGE (Fig. 5). Multiple bands were amplified, and sequences from each of these bands aligned well (>99%) with the *Marinobacter* spp. sequences (GenBank accession nos. AY136121, AF513448, and AF237685). From analysis, it appears that bands A and B were heteroduplexes of bands C and D (accession nos. AJ294359, AF212213, AY136121, AY129889, and AF546961). It may be that bands C and D represent two different yet closely related bacteria or that there are two ribosomal sequences for *Marinobacter*. Also, similar bands were missing in the DGGE obtained from the enrichment devoid of NaCl. These results are consistent with the degradation activity; benzene was not degraded in bottles with no added salt, thus suggesting that perhaps the *Marinobacter* sp. was responsible for the observed degradation of benzene. *Marinobacter* spp. have been isolated from geographically different locations, including the French Mediterranean coast, at the mouth of a petroleum refinery outlet, from deep-sea sediments in the western Pacific, and from oil wells off the coasts of Vietnam and California (7, 8).

Overall our research has demonstrated the ability of halophilic and halotolerant bacteria to rapidly degrade BTEX compounds under aerobic conditions, and such activity could be enhanced markedly by the addition of growth promoting nutrients such as YE. Thus, these observations provide hope for the establishment of cost-effective methods to remediate brine-impacted soil and aquifers.

This research was funded through grants from the Integrated Petroleum Environmental Consortium and the Environmental Institute's Center for Water Research at Oklahoma State University.We gratefully acknowledge the assistance of Steve Sower, Oklahoma Environmental Resources Board, for providing contaminated oilfield samples. We also thank Alok Bhandari, Greg Wilber, Vijai Elango, and Kanchan Joshi for their assistance with radioisotopic and GC analyses.

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