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The abundance of *Geobacter* species in contaminated aquifers in which benzene is anaerobically degraded has led to the suggestion that some *Geobacter* species might be capable of anaerobic benzene degradation, but this has never been documented. A strain of *Geobacter*, designated strain Ben, was isolated from sediments from the Fe(III)-reducing zone of a petroleum-contaminated aquifer in which there was significant capacity for anaerobic benzene oxidation. Strain Ben grew in a medium with benzene as the sole electron donor and Fe(III) oxide (strain Ben) or Fe(III) citrate (strain Dechloromonas) as the electron acceptor, the cell yields of strain Ben were $3.2 \times 10^9$ and $8.4 \times 10^9$ cells/mmol of Fe(III) reduced, respectively. Strain Ben also oxidized benzene with anthraquinone-2,6-disulfonate (AQDS) as the sole electron acceptor with cell yields of $5.9 \times 10^9$ cells/mmol of AQDS reduced. Strain Ben serves as model organism for the study of anaerobic benzene metabolism in petroleum-contaminated aquifers, and *G. metallireducens* is the first anaerobic benzene-degrading organism that can be genetically manipulated.

Understanding the mechanisms for anaerobic benzene degradation may greatly aid the design and optimization of strategies for the bioremediation of subsurface environments contaminated with petroleum and is expected to reveal novel biochemistry for enzymatic attack on this highly stable molecule (9, 12, 20, 36, 38, 62). Monoaromatic hydrocarbons are often the petroleum contaminants of the greatest concern in groundwater because of their water solubility, and benzene is the most toxic of these contaminants (11). Aerobic benzene-degrading organisms are ubiquitous, and their degradation pathway and methods to detect them are well established (21, 30, 66). Many contaminated subsurface environments are, however, anoxic (29, 37, 38, 50, 54). The ability of microbial consortia to anaerobically degrade benzene has been demonstrated with a diversity of electron acceptors, and insights into potential metabolic pathways have been obtained (1, 2, 5, 8, 16, 17, 34, 45, 65). However, pure cultures are required for definitive genetic and biochemical analyses.

Strains of *Dechloromonas* and *Azoarcus* species metabolized benzene with nitrate as the electron acceptor (10, 11, 31, 32). However, analysis of the genome sequence of *Dechloromonas aromatica* strain RCB revealed a lack of genes for anaerobic degradation of expected intermediates in benzene degradation, such as benzoate or phenol, which in most organisms are metabolized via conserved pathways (55). Genes for aerobic benzene oxidation were present (55). These findings, and the possibility of microorganisms internally producing molecular oxygen during nitrate reduction (19, 68), have led to the suggestion that benzene degradation coupled to nitrate reduction might also involve oxygen (47, 55, 62, 64). This would be analogous to the degradation of benzene with chloride as the electron acceptor in *Aliicyciphilus denitrificans* in which molecular oxygen generated from (per)chlorate is used to activate benzene (63).

The one pure culture that clearly appears to anaerobically metabolize benzene is the hyperthermophile *Ferroglobus placidus* (27). Benzene was oxidized to carbon dioxide with Fe(III) serving as the sole electron acceptor. The use of strict anaerobic techniques and the abundance of Fe(III) in the medium ensured anoxic conditions. Furthermore, no monoxygenases were encoded in the genome. Analysis of metabolites, as well as whole-genome analysis of gene transcript abundances, suggested that benzene was first carboxylated to benzoate (27), which was then further metabolized via common pathways for anaerobic benzoate metabolism (28). However, the lack of tools for genetic manipulation has limited further study of benzene metabolism in this organism.

*Geobacter* species have been implicated in the degradation of aromatic hydrocarbons, including benzene, in contaminated subsurface environments (39, 53, 54) and *Geobacter* species capable of anaerobically oxidizing toluene and xylene with Fe(III) as the electron acceptor have been described (6, 39, 42). Stimulating Fe(III) reduction, presumably by *Geobacter* species, with chelators or electron shuttles is a potential mechanism for greatly accelerating anaerobic benzene degradation (40, 46). A benzene-degrading, Fe(III)-reducing *Geobacter* culture would be of interest not only because this environmental relevance, but also because it has been possible to develop genetic systems in *Geobacter* species (15, 44, 49, 59) that would be useful in further studying this unique metabolism. Here, we report on the anaerobic oxidation of benzene in two *Geobacter* species: a new isolate from a petroleum-contaminated aquifer and *Geobacter metallireducens*, which has already been shown to be genetically tractable (49, 59).
Sediment studies. Sediments were collected from the previously described (3, 39, 54) Fe(III) reduction zone of a petroleum-contaminated aquifer in Bemidji, MN. As previously described (5), sediment cores were obtained by drilling cores, which were transported immediately back to the laboratory, and sediment aliquots were stored anoxically in glass vessels overnight at 25°C for outlined experiments. For radionuclide tracer studies, 20-g sediment samples were transferred to 50-ml serum bottles under N2-CO2 (95:5) and incubated at 25°C. The sediments were amended with [Ring-UL-14C]benzene (1.48 \times 10^8 \text{ Bq} 14C) (radiochemical purity = 99.7%; Moravek Biochemicals, Brea, CA).

Enrichment and isolation of strain Ben. To further enrich benzene metabolic capacity, the benzene-degrading sediments were incubated at 30°C under anoxic conditions as previously described (43) and amended with 100 \mu M benzene (Sigma-Aldrich, St. Louis, MO; Chemical purity, \geq 99.9%) as the electron donor and 100 mM poorly crystalline Fe(III) oxide (43) as the electron acceptor. Fe(II) production was monitored and when sufficient Fe(II) to account for oxidation of the added benzene accumulated additional benzene was added. This was repeated three times. Sediments were then serially diluted in a bicarbonate-buffered freshwater medium (43) with benzene (100 \mu M) as the sole electron donor and poorly crystalline Fe(III) oxide (100 mM) as the sole electron acceptor. The liquid enrichments were transferred five times. Then enrichment cultures were inoculated (10% [v/vol]) into an anaerobic dilution series of the same medium amended with 2% Difco noble agar (BD, Franklin, NJ) to form roll tubes. A single isolated colony was picked and further purified by repeating serial dilution in roll tubes and restreaking of single colonies on solidified Fe(III) oxide medium as described previously (48) with the modification that benzene was the sole electron donor. A colony from this extended purification, designated strain Ben, was selected for further study. The purity of the culture was confirmed by analysis of 16S rRNA gene sequence. Overall, the enrichment process took nearly 1 year.

Acetate (10 mM) served as the electron donor to evaluate whether Fe(III) oxide, Fe(III) nitritriacetate, Fe(III) pyrophosphate, Fe(III) citrate, or anthraquinone-2,6-disulfonate (AQDS) could serve as an electron acceptor (41, 48). AQDS (5 mM) served as the electron acceptor for evaluating whether acetate, ethanol, fumarate, H2, lactate, pyruvate, benzene, or toluene could serve as electron donors (41, 48).

Geobacter metallireducens. Geobacter metallireducens strain GS-15 (ATCC 53774 and DSM 7210) (41) was obtained from our laboratory culture collection and was routinely cultured as previously described (43).

Analysis of growth and metabolism on benzene. Anaerobic pressure tubes (25 ml; 15-ml headspace) were used for benzene growth studies. Cells of strain Ben and G. metallireducens were grown for four successive transfers of a 20% inoculum in medium with 250 \mu M benzene as the sole electron donor, before conducting studies on the stoichiometry of growth on benzene. The electron acceptors in these studies were 5 mM AQDS or electron donor, before conducting studies on the stoichiometry of growth on benzene. The electron acceptors in these studies were 5 mM AQDS or

Additional studies of [U-14C]benzene metabolism were investigated with concentrated cells to provide for more rapid cell metabolism. Cells grown on acetate were concentrated under anaerobic conditions via centrifugation (4,400 \times g for 10 min at 15°C), the pellet was washed twice with medium and then resuspended to provide a 30-fold concentration of cells. A total of 3.7 \times 10^9 \text{ Bq} [U-14C]benzene was added to aliquots (3 ml) of the cell suspensions, which were then incubated anaerobically at 30°C. Controls for radiotracer experiments were heat-killed cultures with donor and acceptor.

Statistical analysis. Data are reported as the means and standard deviations of triplicate cultures. Statistical analysis was performed using Student t test. A P value of <0.05 was considered statistically significant.

Light and electron microscopy. Cells were routinely examined by phase-contrast microscopy with a Nikon E600 microscope as previously described (33). The Gram type was determined using Gram staining and bright-field microscopy as previously described (48). For transmission electron microscopy, strain Ben cultures were placed on 300-mesh carbon-coated copper grids, incubated for 15 min, and then stained with 2% aqueous uranyl acetate. The cells were observed using a JEOL 100S transmission electron microscope at an accelerating voltage of 80 kV. Images were taken digitally using the Maxlm-DL software and analyzed using ImageJ (http://rsbweb.nih.gov/j/index.html).

Phylogenetic analysis. For analysis of the 16S rDNA gene sequence of strain Ben, 10 ml of culture were collected by centrifugation, and genomic DNA was extracted with the FastDNA spin kit (Bio 101 Inc., Vista, CA) according to the manufacturer’s instructions. 16S rDNA gene sequences were amplified with primers 8 forward (18) and 1492 reverse (4) as previously described (25, 48). PCR products were agarose gel purified with a QIAquick gel extraction kit (Qiagen, Inc., Valencia, CA) and ligated into the TOPO TA cloning kit, version K2 (Invitrogen, Carlsbad, CA), according to the manufacturer’s instructions. Plasmid inserts were then amplified with M13 forward and reverse primers (Invitrogen) and the 519F internal primer (35) and sequenced at the University of Massachusetts sequencing facility.

The 16S rDNA gene from strain Ben was reconstructed from the sequenced fragments and compared to the GenBank nucleotide and protein databases with the BLASTN algorithm (3). Nucleotide sequences of strain Ben and the most similar isolates found by the BLAST search were aligned using CLC sequence viewer (CLC Bio, Cambridge, MA). Aligned sequences were imported into Seaview (22), where phylogenetic trees were inferred. Identical branching orders were observed with maximum parsimony, maximum-likelihood, and distance-based algorithms when 16S rDNA gene sequences were compared (not shown). Bootstrap values were calculated by all three analyses, and 1,309 nucleotide positions were considered for 16S rDNA gene comparisons.

Analytical techniques. Cell numbers were monitored with acridine orange staining and epifluorescence microscopy (42), and Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time as previously described (42). The reduction of AQDS was monitored by the absorbance of 2,6-anthrahydroquinone disulfonate produced at 525 nm (40).

Benzene concentrations in culture headspaces were quantified with a gas chromatograph equipped with a flame ionization detector. The samples were run through a Supelco VOCOL fused silica capillary column (60 m by 0.25 mm by 1.5 \mu m) held at 50°C for 0.5 min, followed by an increase to 200°C at 10°C/min. The concentrations of benzene in the aqueous phase were calculated with Henry’s law using the constant at 25°C of 0.25 for benzene (57).

[14C]Carbon dioxide was quantified with a gas proportional counter as previously described (14, 24).

RESULTS AND DISCUSSION

Isolation and characterization of Geobacter strain Ben. [U-14C]benzene was rapidly oxidized to 14CO2 in sediments from a zone in the petroleum-contaminated study site, which previous molecular studies (67) suggested were enriched in Geobacter species involved in the degradation of monoaromatic compounds (Fig. 1). An isolate was obtained from these sediments by (i) further enriching the benzene-degrading activity in the sediments with the addition of Fe(III) oxide and repeated benzene amendments, (ii) inoculation of enriched sediments into defined medium with benzene as the sole electron donor and Fe(III) oxide as the sole electron acceptor, (iii) repeated transfer of the enrichment

MATERIALS AND METHODS
culture, and (iv) isolation and purification with solidified benzene-Fe(III) oxide medium. The isolate was designated strain Ben.

Cells of strain Ben, were Gram-negative, slightly curved rods 1.5 to 2.5 μm by 0.5 μm with round ends (Fig. 2A). Analysis of the 16S rRNA gene of strain Ben revealed that it belongs to the genus Geobacter within the family Geobacteraceae (Fig. 2B). Strain Ben is most closely related to Geobacter daltonii (99% sequence similarity), an Fe(III)- and U(VI)-reducing bacterium isolated from the Oak Ridge Field Research Center (52). This phylogenetic placement of strain Ben was consistent in distance, parsimony algorithms, and maximum-likelihood analyses. The GenBank accession number for the 16S rRNA of strain Ben is JQ799138.

Strain Ben grew with Fe(III) oxide, Fe(III) nitrolitriacetic acid, Fe(III) pyrophosphate, or anthraquinone-2,6-disulfonate (AQDS), but not Fe(III) citrate, as electron acceptors. Various organic electron donors such as acetate (5 mM), ethanol (10 mM), fumarate (10 mM), H₂ (130 kPa with 100 μM acetate as the carbon source), lactate (10 mM), pyruvate (10 mM), benzoate (1 mM), or toluene (250 μM) served as sole electron donors.

**Benzene oxidation coupled to the reduction of Fe(III) oxide or AQDS by strain Ben.** Strain Ben grew when inoculated (20% inoculum) into a medium with benzene as the sole electron donor and poorly crystalline Fe(III) oxide, as the electron acceptor (Fig. 3). The results shown here for all studies are those for the fourth successive transfer (20% inoculum) on benzene medium. Benzene-dependent growth was slow, and between-tube differences in growth rates resulted in high replicate variability during growth on Fe(III) oxide, but there was a statistically significant increase in cell numbers and the accumulation of the reduced products of electron acceptor reduction (Fig. 3).

There was no growth in heat-killed controls (Fig. 3) or controls that contained benzene but no electron acceptor or contained an electron acceptor, but no benzene (see Fig. S1 in the supplemental material). There was some loss of benzene in heat-killed controls (Fig. 3), presumably due to adsorption into the butyl rubber stopper (11, 27). In live Fe(III) oxide cultures there was an increase in cell density over time that was accompanied by an accumulation of Fe(II) and a loss of benzene that was greater than the loss of benzene in the heat-killed controls (Fig. 3A). When this abiotic loss is considered, benzene depletion that could be attributed to strain Ben was 89 ± 15.9 μM benzene (mean ± standard deviation, n = 3), which could provide electrons sufficient to reduce 2.7 mM Fe(III) oxide if benzene was completely oxidized to carbon dioxide according to the reaction:

\[
\text{C}_6\text{H}_6 + 30 \text{Fe(III)} + 12 \text{H}_2\text{O} \rightarrow 30 \text{Fe(II)} + 6 \text{CO}_2 + 30 \text{H}^+ \quad (1)
\]

The observed accumulation of Fe(II) of 2.1 ± 0.2 mM was consistent with the fact that some of the substrate would be required for biomass formation. During the active growth phase (days 0 to 18) the cell yield per mol of Fe(II) produced [(3.23 ± 0.15) × 10⁹ cells/mmol Fe(II)] with benzene as the sole electron donor was comparable to the yield previously reported (42) for the growth of G. metallireducens with toluene [4.74 × 10⁹ cells/mmol Fe(II)] or phenol [5.11 × 10⁹ cells/mmol Fe(II)] as the sole electron donor and poorly crystalline Fe(III) oxide as the electron acceptor. In a similar manner, in medium with AQDS as the electron acceptor, cell growth was associated with an accumulation of reduced electron acceptor (anthrahydroquinone-2,6-disulfonate [AHQDS]) and loss of benzene (Fig. 3B). When abiotic benzene loss was considered, the loss of benzene that could be attributed to the activity of strain Ben (73 ± 6 μM, n = 3) could provide electrons sufficient to reduce 1.1 ± 0.1 mM AQDS if benzene was completely oxidized to carbon dioxide:

\[
\text{C}_6\text{H}_6 + 15 \text{AQDS} + 12 \text{H}_2\text{O} \rightarrow 15 \text{AHQDS} + 6 \text{CO}_2 \quad (2)
\]

The observed accumulation of AHQDS was 0.9 ± 0.1 mM, suggesting that AQDS was the sole electron acceptor for benzene oxidation. During the active growth phase (days 0 to 11) the cell yield per mol AHQDS produced (5.92 ± 0.27 10⁹ cells/mmol AHQDS) was comparable to that for growth on benzene with Fe(III) oxide as the electron acceptor when it is considered that two electrons are required to reduce AQDS versus one electron to reduce Fe(III).

In order to further evaluate the stoichiometry of benzene oxidation in a manner that alleviated concerns about adsorption of benzene into the rubber stoppers, a set of separate incubations were conducted in which [¹⁴C]benzene was added along with the standard deviation, n = 3) could provide electrons sufficient to reduce 1.1 ± 0.1 mM AQDS if benzene was completely oxidized to carbon dioxide:

\[
\text{C}_6\text{H}_6 + 15 \text{AQDS} + 12 \text{H}_2\text{O} \rightarrow 15 \text{AHQDS} + 6 \text{CO}_2 \quad (2)
\]
to a possible nonbenzene contaminant in the provided radiolabeled benzene, which had a radiochemical purity of 99.7%.

**Benzene oxidation coupled to the reduction of Fe(III) by *G. metallireducens***. The metabolism of aromatic compounds in *Geobacter* species has been studied most thoroughly in *G. metallireducens* (44). It was previously reported that *G. metallireducens* did not metabolize benzene when evaluated with an initial benzene concentration of 500 μM and Fe(III) oxide as the electron acceptor (41). However, when Fe(III) was supplied as Fe(III) citrate, which promotes faster growth of *G. metallireducens*, and the initial benzene concentration was lowered to 250 μM, *G. metallireducens* metabolized benzene with the reduction of Fe(III) (Fig. 4A). The increase in cell density over time was accompanied by an accumulation of Fe(II) and loss of benzene (Fig. 4A). There was no growth in the absence of added benzene or if no Fe(III) was provided (see Fig. S2 in the supplemental material). When the abiotic loss of benzene in heat-killed controls was considered, the benzene depletion that could be attributed to metabolism of *G. metallireducens* was 84.2 ± 2.4 μM, which could provide electrons sufficient to reduce 2.5 ± 0.1 mM Fe(III) if benzene was completely oxidized to carbon dioxide. The observed accumulation of Fe(II) was 2.2 ± 0.3 mM. The cell yield per mol of Fe(II) produced [(8.41 ± 0.19) × 10⁹ cells/mmol Fe(II)] was higher than for growth of strain Ben on benzene, which may be attributed to Fe(III) citrate rather than Fe(III) oxide serving as the electron acceptor. When [¹⁴C]benzene was added along with the unlabeled benzene (250 μM), [¹⁴C]CO₂ was produced when Fe(III) was supplied as the sole electron acceptor, but there was no [¹⁴C]CO₂ production if the Fe(III) was omitted (Fig. 4B). 28.0% ± 8.8% of the added [¹⁴C]benzene was recovered as [¹⁴C]CO₂. Oxidation of this
fraction of the added benzene would be expected to lead to the reduction of 2.1 \pm 0.7 \text{ mM Fe(II)} and 2.4 \pm 0.3 \text{ mM Fe(II)} was generated. Concentrated cell suspension of \textit{G. metallireducens} oxidized 80\% \pm 5\% of the added \textsuperscript{14}C-benzene to \textsuperscript{14}CO\textsubscript{2} with Fe(III) as the electron acceptor (Fig. 4B, inset).

**Implications.** The \textit{Geobacter} species described here provide additional model organisms for the study of anaerobic benzene degradation. The abundance of Fe(II) during growth on Fe(III) as the electron acceptor ensured anaerobic conditions, and there is no possibility of producing molecular oxygen intracellularly from Fe(III) as there is with nitrate. Further evidence for a lack of degradation of benzene via an aerobic pathway is the lack of monooxygenase genes in the genome of \textit{G. metallireducens} (49, 59) or in a draft genome of strain Ben (unpublished data).

The three primary mechanisms for anaerobic activation of benzene have been hypothesized to be hydroxylation to phenol (10, 23, 60, 61), carboxylation to benzoate (1, 2, 34, 51), or methylation to toluene (60). The \textit{G. metallireducens} genome encodes pathways for anaerobic benzoate, toluene, and phenol complete oxidation (7), and previous studies have shown that \textit{G. metallireducens} can anaerobically metabolize the hydrocarbon, toluene, and other monoaromatic compounds such as benzoate, phenol, and \textit{p}-cresol (39, 41, 42). Pathways for the anaerobic degradation of benzoate, phenol, toluene and \textit{p}-cresol are also encoded in the genome of \textit{Geobacter} strain Ben (unpublished data).

The finding that there are \textit{Geobacter} species that can anaerobically degrade benzene supports previous suggestions that \textit{Geobacter} species play an important role in the removal of benzene and other aro-
mantic hydrocarbon contaminants from polluted groundwater (6, 13, 26, 44, 53, 56, 58). Methods for genetically manipulating Geobacter \emph{metallireducens} have recently been developed (49, 59), making it the first genetically tractable organism known to be capable of anoxic benzene degradation under strict anaerobic conditions. This opens the possibility of definitively elucidating the pathway for anaerobic benzene degradation in this organism, identifying molecular signals that can be useful for monitoring rates of anaerobic benzene degradation in the subsurface, and developing new strategies for stimulating anaerobic benzene degradation from a better understanding of this metabolism.

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