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Anaerobic Benzene Oxidation via Phenol in *Geobacter metallireducens*

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Anaerobic activation of benzene is expected to represent a novel biochemistry of environmental significance. Therefore, benzene metabolism was investigated in *Geobacter metallireducens*, the only genetically tractable organism known to anaerobically degrade benzene. Trace amounts (<0.5 μM) of phenol accumulated in cultures of *Geobacter metallireducens* anaerobically oxidizing benzene to carbon dioxide with the reduction of Fe(III). Phenol was not detected in cell-free controls or in Fe(II)- and benzene-containing cultures of *Geobacter sulfurreducens*, a *Geobacter* species that cannot metabolize benzene. The phenol produced in *G. metallireducens* cultures was labeled with ^{18}O during growth in $H_2{}^{18}O$, as expected for anaerobic conversion of benzene to phenol. Analysis of whole-genome gene expression patterns indicated that genes for phenol metabolism were upregulated during growth on benzene but that genes for benzoate or toluene metabolism were not, further suggesting that phenol was an intermediate in benzene metabolism. Deletion of the genes for PpsA or PpcB, subunits of two enzymes specifically required for the metabolism of phenol, removed the capacity for benzene metabolism. These results demonstrate that benzene hydroxylation to phenol is an alternative to carboxylation for anaerobic benzene activation and suggest that this may be an important metabolic route for benzene removal in petroleum-contaminated groundwaters, in which *Geobacter* species are considered to play an important role in anaerobic benzene degradation.

Elucidating the pathways for anaerobic benzene degradation is important because of the environmental significance of this process and its potential biochemical novelty (1). For example, contamination of groundwater with hydrocarbons often leads to the development of anaerobic conditions, and benzene is one of the most mobile and toxic pollutants (1–4). Numerous studies with mixed microbial communities have documented that benzene can be degraded under anaerobic conditions (1, 5–12), but benzene is typically only slowly removed from anaerobic contaminated groundwater unless anaerobic microbial metabolism is artificially stimulated with the addition of Fe(III) chelators (13, 14), electron shuttles (15), sulfate (5), or an electrode (16).

It was previously found that the archaeon *Ferroglobus placidus* is capable of anaerobically oxidizing benzene to carbon dioxide with Fe(III) as the sole electron acceptor (17). Analysis of gene expression patterns indicated that benzene was first metabolized to benzoate (17), which was then metabolized via well-known pathways for anaerobic benzoate metabolism (18). The proposed carboxylation of benzene by *F. placidus* is consistent with evidence suggesting carboxylation in enrichment cultures (19–23). A gene encoding a putative benzene carboxylase in *F. placidus* was identified (17), but further analysis has been limited by a lack of a system for genetic manipulation of *F. placidus*.

A potential alternative for anaerobic benzene activation is conversion to phenol. This mechanism was previously proposed for *Dechloromonas aromatica*, which grows on benzene in anaerobic medium with nitrate as the electron acceptor (7, 24, 25). However, several lines of evidence suggest that the initial activation of benzene in *D. aromatica* involves molecular oxygen generated intracellularly from nitrate (1, 9, 26, 27). For example, the *D. aromatica* genome lacks genes for the anaerobic degradation of aromatic compounds that are highly conserved in all other organisms capable of anaerobic metabolism of monoaromatics but contains multiple genes for monooxygenases that could be involved in activation of benzene with oxygen (26). Furthermore, the oxygen incorporated into the benzene ring to produce phenol does not come from water, as would be expected for anaerobic metabolism of benzene to phenol (24). Another potential line of evidence for the possibility of anaerobic benzene activation to phenol is the presence of phenol in enrichment cultures anaerobically degrading benzene (12, 28, 29). However, it has been suggested that the phenol detected may have been an artifact of an abiotic reaction (19, 30).

Numerous studies have suggested that *Geobacter* species play an important role in the removal of aromatic hydrocarbons from contaminated aquifers, anaerobically oxidizing aromatic hydrocarbons to carbon dioxide with the reduction of Fe(III), which is abundant in many subsurface environments (6, 31–43). Recently two members of the *Geobacteraceae* family, *Geobacter metallireducens* and *Geobacter* sp. strain Ben, were shown to be capable of anaerobically metabolizing benzene (44). The development of methods for genetic manipulation of *G. metallireducens* (45) has made it possible to begin to evaluate the pathways for benzene metabolism with targeted gene deletions. Here we provide evidence from gene expression and gene deletion studies that indicate that *G. metallireducens* metabolizes benzene via a phenol intermediate.

MATERIALS AND METHODS

Organisms and culture conditions. The bacterial strains and plasmids used are listed in Table S1 in the supplemental material. *Geobacter metallireducens* (ATCC 53774 and DSM 7210) (46) was obtained from our

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laboratory culture collection and was routinely cultured under strict anaerobic conditions with 50 mM Fe(III) citrate as the electron acceptor, as previously described (47). Unless otherwise noted, the electron donor (concentration) for growing cultures was benzene (0.25 mM), phenol (0.5 mM), benzoate (1 mM), toluene (0.5 mM), or acetate (10 mM). The exception to this was that in the studies in which phenol concentrations during growth on benzene were monitored, the initial concentration of benzene was 0.1 mM.

For control studies, *G. sulfurreducens* cultures that were grown with acetate (10 mM) as the electron donor and Fe(III) citrate (50 mM) as the electron acceptor served as the inoculum, and a 10% inoculum was transferred into medium with benzene (100 μM) and Fe(III) citrate (50 mM). The acetate remaining in the inoculum (ca. 4 mM) served as an additional electron donor.

Cell suspensions. Studies of the metabolism of ^{14}C- or ^{18}O-labeled compounds were performed with cell suspensions to increase cell density and provide more rapid metabolic flux. Cells grown in acetate-Fe(III) citrate medium were concentrated under anaerobic conditions via centrifugation (4,400 × *g* for 10 min at 15°C). The cell pellets were washed with Fe(III) citrate medium devoid of an electron donor and then resuspended to provide a 30-fold concentration of cells. Aliquots (3 ml) of the cell suspension were incubated in 15-ml anaerobic pressure tubes. For studies on the origin of oxygen in phenol, $H_2{}^{18}O$ (95 atom% ^{18}O; Sigma-Aldrich, St. Louis, MO) was mixed with water to provide 9.5% $H_2{}^{18}O$. Additions for ^{14}C-labeled studies were 2.59×10^5 Bq [U-^{14}C]benzene (39 μM, 2.78×10^9 Bq mmol^{-1}; Moravek Biochemicals, Brea, CA), 2.22×10^5 Bq [U-^{14}C]benzoate (40 μM, 2.22×10^9 Bq mmol^{-1}; Moravek Biochemicals, Brea, CA), or 3.7×10^5 Bq [U-^{14}C]phenol (44 μM, 2.96×10^9 Bq mmol^{-1}; American Radiolabeled Chemicals Inc. [ARC], St. Louis, MO).

Analysis of gene expression. Cells were grown in 1-liter bottles and harvested during mid-exponential phase by centrifugation. The cell pellet was immediately frozen in liquid nitrogen and stored at −80°C.

RNA was isolated from triplicate cultures grown on each electron donor with a modification of a previously described method (17). Briefly, cell pellets were resuspended in HG extraction buffer (18) preheated to 65°C. The suspension was incubated for 10 min at 65°C to lyse the cells. Nucleic acids were isolated with a phenol-chloroform extraction followed by ethanol precipitation. The pellet was washed twice with 70% ethanol, dried, and resuspended in sterile diethylpyrocarbonate-treated water. RNA was then purified with an RNA cleanup kit (Qiagen, Valencia, CA) and treated with DNA-free DNase (Ambion, Woodward, TX). The RNA samples were tested for genomic DNA contamination by PCR amplification of the 16S rRNA gene. cDNA was generated with a TransPlex whole-transcriptome amplification kit (Sigma).

The sequences of all primers used for quantitative reverse transcription-PCR are listed in Table S2 in the supplemental material. Each reaction consisted of forward and reverse primers at a final concentration of 200 nM, 5 ng of cDNA, and 12.5 μl of Power SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Primer pairs with amplicon sizes of 100 to 200 bp were designed for the following: *bamY* (Gmet 2143), *ppsA* (Gmet 2100), and *bssA* (Gmet 1539). Expression of these genes was normalized to the expression of *proC*, a gene shown to be constitutively expressed in *Geobacter* species (48). Relative levels of expression of the studied genes were calculated with the $2^{-\Delta\Delta CT}$ threshold cycle (C_T) method (49).

Whole-genome microarray hybridizations were carried out by Roche NimbleGen, Inc. (Madison, WI). Triplicate biological and technical replicates were conducted for all microarray analyses. Cy3-labeled cDNA was hybridized to oligonucleotide microarrays based on the *G. metallireducens* genome and resident plasmid sequences (GenBank accession numbers NC_007515 and NC_007517). The microarray results were analyzed with Array 4 Star software (DNASTAR, Madison, WI). A gene was considered differentially expressed only if the *P* value determined by Student's *t* test analysis was less than or equal to 0.01.

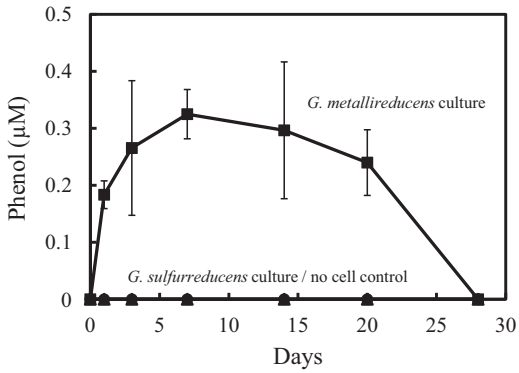

FIG 1 Transient phenol formation during anaerobic benzene degradation by *G. metallireducens* and a lack of phenol in cell-free and *G. sulfurreducens* controls. In the *G. metallireducens* culture, ca. 1.5 mM Fe(II) accumulated during the course of the incubation. The phenol results are the mean and standard deviation from triplicate cultures of each type.

Mutant construction and complementation studies. Sequences for all primers used for construction of the mutants are listed in Table S2 in the supplemental material. Genomic DNA was extracted with an Epicentre MasterPure DNA purification kit (Epicentre Biotechnologies, Madison, WI). Plasmids were extracted with a QIAprep Spin miniprep kit (Qiagen). PCR amplification was done with *Taq* polymerase (Qiagen). DNA gel purification was done with a QIAquick gel extraction kit (Qiagen). Mutant alleles were constructed by replacing the coding sequences with a spectinomycin resistance cassette as described previously (45). Briefly, upstream and downstream flanking regions of the genes to be deleted (ca. 500 bp) were amplified with *G. metallireducens* genomic DNA as the template. PCR products were mixed, digested with AvrII (NEB, Beverly, MA), and ligated with the T4 DNA ligase (NEB). The resulting construct (ca. 1kb) was cloned into pCR2.1-TOPO with a TOPO TA cloning kit (Invitrogen, Carlsbad, CA). The spectinomycin resistance cassette with AvrII sites at both ends was amplified with pRG5 (50) as the template. The spectinomycin resistance cassette was AvrII digested and ligated into the AvrII site located between the flanking regions of the genes to be deleted.

The sequences of the mutant alleles were verified by Sanger sequencing. Plasmids bearing the mutant alleles were linearized by restriction enzyme digestion. The linearized plasmids were concentrated by ethanol precipitation. The linearized plasmids were electroporated into *G. metallireducens* as described earlier (45). The genotypes of the mutant strains were confirmed by PCR using the genomic DNA of the mutant strains as the template.

For complementation studies, *ppsA* and *ppcB* with their respective native ribosome binding sites (RBSs) were cloned under the control of a constitutive *lac* promoter into pCM66 (51).

Analytical techniques. Fe(III) reduction was monitored by measuring the accumulation of Fe(II) over time with ferrozine, as previously described (52).

The benzene and toluene concentrations in the headspace 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 μm) held at 50°C for 0.5 min, followed by an increase to 200°C at 10°C/min, as previously described (16). 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 and 0.27 for toluene (53).

The acetate, benzoate, and phenol concentrations in the medium were measured with high-performance liquid chromatography as previously described (16). The [^{14}C]carbon dioxide in the headspace was quantified with a gas proportional counter, as previously described (54, 55).

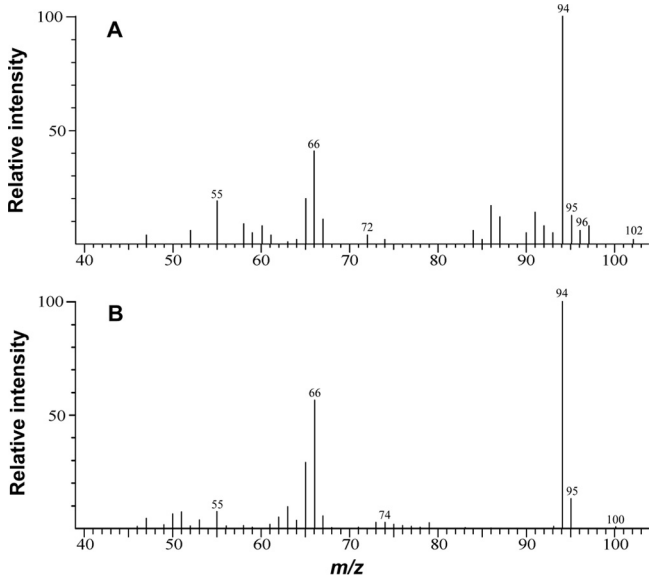

FIG 2 Mass spectra of phenol produced during metabolism of benzene (250 μM) in ^{18}O-labeled water (A) and unlabeled water (B).

Samples (5 ml) of culture for analysis of transient phenol were collected over time from benzene-degrading cultures and immediately extracted with hexane (2 ml) by vigorous shaking for 2 min. Hexane extracts from unlabeled water and ^{18}O experiments were immediately analyzed with a gas chromatograph-mass spectrometer (Shimadzu GCMS-QP2010Ultra). The injector port temperature was set at 250°C, and 1-μl sample extracts were injected through a SHRX1-5MS column (30 m by 0.25 mm by 0.25 μm) with the splitless mode. The column temperature was initially held at 60°C for 1 min, followed by an increase to 200°C at 4°C/min. Compounds were identified by comparison with chemical standards, and mass spectral data were characterized by comparison with data in the National Institute of Standards and Technology (NIST) library.

Microarray data accession number. Microarray data have been deposited with the NCBI GEO database under accession number GSE33794.

RESULTS AND DISCUSSION

Detection of phenol as an intermediate in benzene metabolism. Small quantities of phenol but not benzoate were detected in cultures of *G. metallireducens* growing on benzene (Fig. 1), suggesting that *G. metallireducens* metabolized benzene via a phenol intermediate. It was previously suggested that phenol may be abiotically produced from benzene in the presence of iron (30), but this was not observed in subsequent studies with *F. placidus* (17). In order to further evaluate this possibility, an inoculum of *G. sulfurreducens*, a *Geobacter* species which cannot metabolize aromatic compounds (56), was grown in acetate-Fe(III) citrate medium, and a 10% inoculum of this culture was added to Fe(III) citrate medium that contained 100 μM benzene. The Fe(II) from the inoculum, as well as the Fe(II) produced from the residual acetate in the inoculum, ensured the presence of substantial (>7 mM) Fe(II), yet no phenol was detected over 27 days (Fig. 1). These results further demonstrate that phenol is not abiotically produced from benzene reacting with iron under the culture conditions employed.

In order to investigate the source of oxygen for phenol production, cell suspensions were provided benzene and Fe(III) citrate in medium in which the water included 9.5% $H_2{}^{18}O$ (Fig. 2). The *m/z* 96 peak, representing ^{18}O-labeled phenol, had a relative intensity

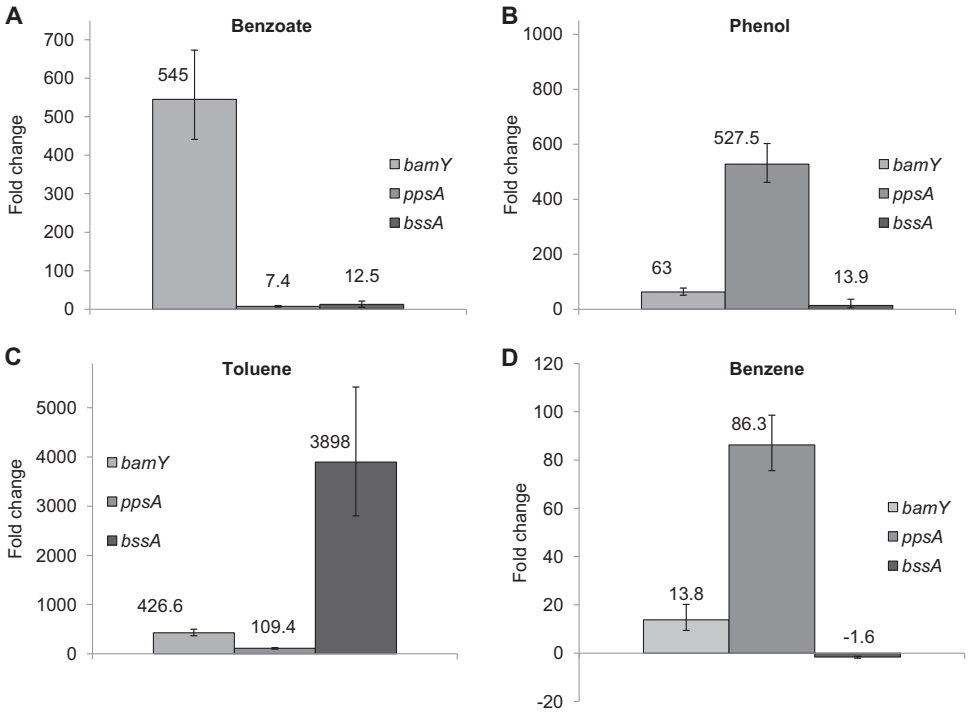

FIG 3 Relative transcript abundance of *ppsA*, *bamY*, and *bssA* during growth of *G. metallireducens* on benzoate (A), phenol (B), toluene (C), or benzene (D). The fold change shown is relative to the gene transcript abundance during growth on acetate, with gene expression normalized to that of the constitutively expressed gene *proC*. The results are the mean and standard deviation from triplicate cultures of each type.

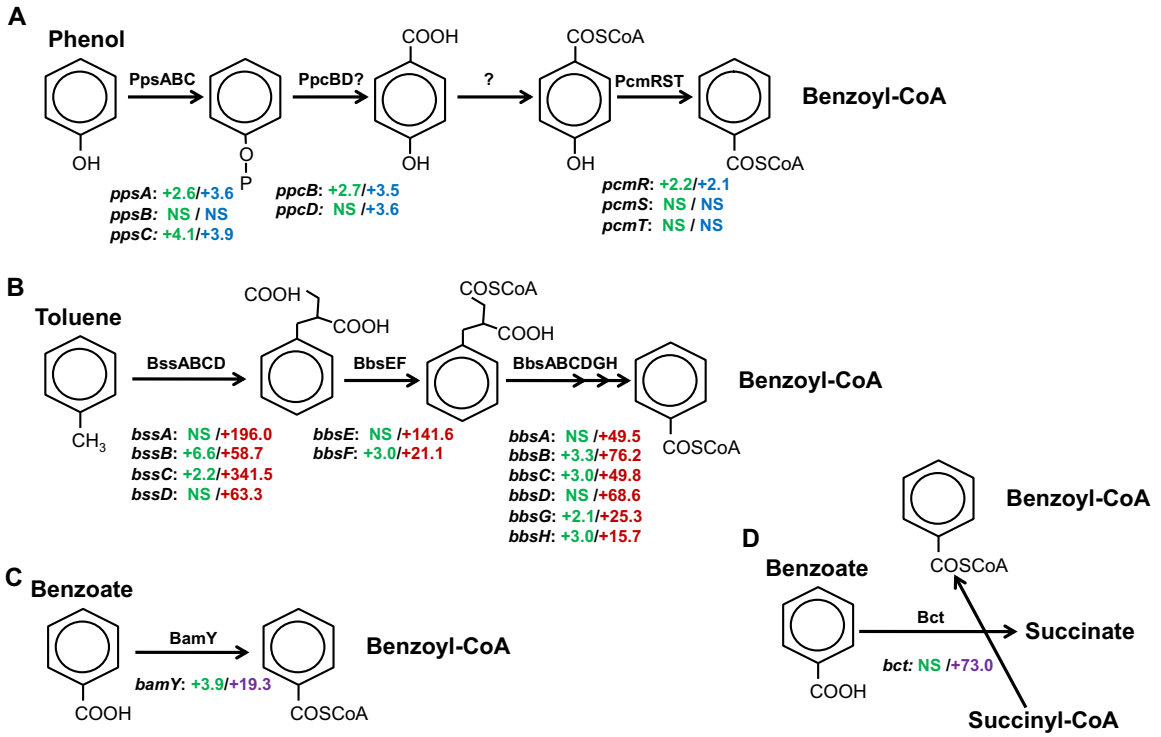

FIG 4 Transcriptomic analysis of benzene degradation in *G. metallireducens* evaluating expression for genes specifically involved in the phenol (A), toluene (B), or benzoate (C, D) degradation pathways. The numbers next to the gene designations represent the fold change in transcript abundance determined with microarray analyses in benzene- versus acetate-grown cells (green), phenol- versus acetate-grown cells (blue), toluene- versus acetate-grown cells (red), or benzoate- versus acetate-grown cells (violet). The results are the means of triplicate determinations from each of three replicate cultures. NS, no significant change in gene expression. COSCoA, carbon-oxygen-sulfur-coenzyme A.

that was 5% of the *m/z* 94 peak, representing unlabeled phenol (Fig. 2A). There was no *m/z* 96 peak when *G. metallireducens* was incubated in benzene medium without $H_2{}^{18}O$ (Fig. 2B). The production of ^{18}O-labeled phenol suggested that the hydroxyl group introduced into benzene during benzene metabolism was derived from water. This result is consistent with the fact that *G. metallireducens* does not possess monooxygenase genes, which would be required for activation of benzene with molecular oxygen. These results, coupled with the fact that strict anaerobic culturing techniques and the presence of abundant Fe(II) in the cultures ensured that molecular oxygen was not available, suggested that the formation of phenol in benzene-degrading cultures was likely to be an anaerobic enzymatic reaction.

Differential expression of genes associated with possible benzene degradation pathways. In order to gain additional insight into the pathway for benzene metabolism, the transcript abundance for genes coding for subunits of the enzymes catalyzing the first step of benzoate, phenol, or toluene metabolism was quantified with reverse transcription-quantitative PCR during growth with one of these aromatic compounds. Results were compared with expression during growth with acetate. As might be expected, each gene was the most highly expressed when its respective substrate was the electron donor that was added to support growth (Fig. 3). For example, *bamY* (Gmet 2143) encodes the benzoate coenzyme A (benzoate-CoA) ligase, an enzyme catalyzing the conversion of benzoate to benzoyl-CoA (57–59), and the transcript abundance of *bamY* was the highest during growth on benzoate (Fig. 3A). The transcript abundance for *ppsA* (Gmet 2100), which encodes the alpha subunit of phenylphosphate synthase, responsible for the conversion of phenol to phenylphosphate (60, 61), was the highest during growth on phenol (Fig. 3B). The transcript abundance of *bssA* (Gmet 1539), which encodes the alpha subunit of benzylsuccinate synthase, responsible for the activation of toluene with fumarate (60, 62), was the highest during growth on toluene (Fig. 3C).

As has previously been observed (63, 64), the genes for enzymes responsible for initiating the metabolism of benzoate, phenol, or toluene also had some increased expression during growth on the alternative aromatic compounds. This result demonstrates that the regulation of expression of aromatic degradation genes is not absolute but that relative gene expression levels might be diagnostic of the metabolic pathway employed.

During growth on benzene, the greatest increase in transcript abundance was for *ppsA* (Fig. 3D). There was a slight increase in transcript abundance for *bamY*, but this was much less than that in cells grown on any other aromatic compound. Transcript levels for *bssA* were comparable to those in acetate-grown cells. These results suggest that phenol, but not benzoate or toluene, is an intermediate in benzene degradation. The transcript abundance of *ppsA* was lower in the benzene-grown cells than in the phenol-grown cells, which may be linked to the higher phenol concentrations available to phenol-grown cells, as well as the greater phenol flux in phenol-grown cells, which grow much faster than benzene-grown cells.

Subsequent genome-scale analysis of gene expression with whole-genome microarrays (NCBI GEO accession number GSE33794) re-

vealed that genes for the putative phenol pathway in *G. metallireducens* that are upregulated in cells grown on phenol versus their regulation in cells gown on acetate are also upregulated during growth on benzene (Fig. 4A). The one exception was the gene for PpcD, which is thought to be a subunit in the enzyme phenylphosphate carboxylase (65). However, expression of the gene for the other subunit of this enzyme was upregulated in benzene-grown cells. Genes in the pathway for toluene degradation, which were highly upregulated in toluene-grown cells, had relatively little or no increase in transcript abundance in benzene-grown cells (Fig. 4B). In a similar manner, the microarray analysis confirmed the earlier findings from quantitative PCR analysis that *bamY* expression levels were much lower in benzene-grown cells than in cells metabolizing benzoate (Fig. 4C). Like *bamY*, *bct* (Gmet 2054), a gene coding for a succinyl-CoA:benzoate-CoA transferase involved in a recently discovered alternative mode of benzoate activation (66), was highly upregulated in benzoate-grown cells but not in cells grown on benzene (Fig. 4D). The results are consistent with a metabolism in which benzene is anaerobically hydroxylated to phenol, followed by a two-step carboxylation process to 4-hydroxybenzoate via phenylphosphate as an intermediate. 4-Hydroxybenzoate is further reductively converted via its coenzyme A ester to benzoyl-CoA. Free benzoate itself is not an expected intermediate.

Genetic evidence for benzene degradation via phenol. The possibility that benzene was metabolized via a phenol intermediate was further evaluated by deleting either *ppsA*, a gene predicted to encode a subunit for the enzyme necessary for the first step in phenol metabolism, or *ppcB* (65), a gene predicted to encode a subunit for the second step in phenol metabolism (Fig. 4). Cell suspensions of the wild-type strain readily utilized phenol, as expected (67), but cell suspensions of the *ppsA*- or *ppcB*-deficient strains could not (Fig. 5A), even though they metabolized benzoate as well as the wild type (Fig. 5B). Benzene oxidation was completely inhibited (Fig. 5C) in the absence of *ppsA* or *ppcB*. Complementation of either gene deletion by expression of the deleted gene on a plasmid restored the capacity for anaerobic phenol and benzene oxidation (Fig. 5). Rates of metabolism in the complemented strains were lower than those in the wild type, consistent with the general finding in previous studies with *Geobacter* species that the strategy for in *trans* expression does not yield wild-type activities when complementing deletions of genes for a wide variety of functions (45, 68–71). These results demonstrate that the phenol degradation pathway is essential for benzene degradation, further suggesting that phenol is an important intermediate in the metabolism of benzene.

Implications. The results demonstrate that phenol is a key intermediate in the anaerobic degradation of benzene by *Geobacter metallireducens*. The specific upregulation of genes for phenol metabolism during growth on benzene and the finding that the capacity for anaerobic benzene metabolism is lost when genes for phenol metabolism are deleted indicate that phenol is produced from benzene. The incorporation of oxygen from water in phenol demonstrates that molecular oxygen is not involved in the benzene activation. Potential mechanisms for conversion of benzene to phenol, an endergonic reaction, have previously been proposed (7, 24), but unlike the clustering of genes for the degradation of other monoaromatic compounds (60), no genomic regions specific for benzene degradation have been identified through genome annotation or gene expression patterns. Thus, further speculation on activation mechanisms are not warranted at this time.

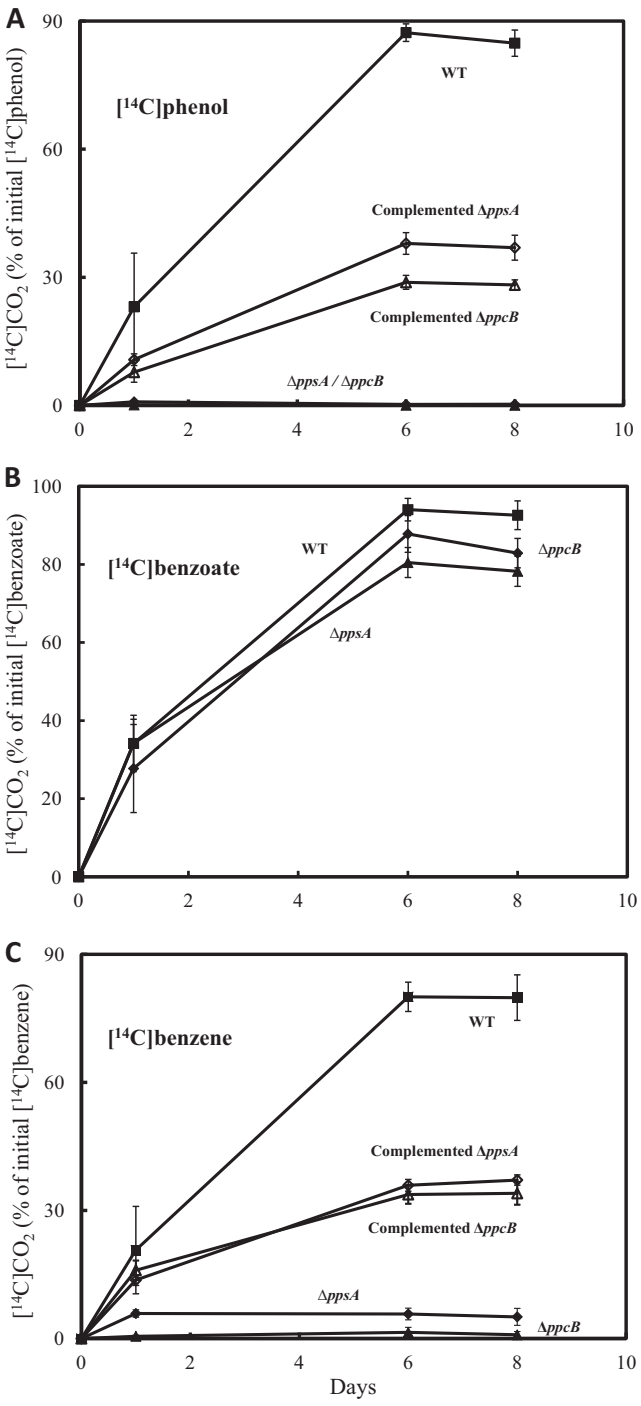

FIG 5 Production of $^{14}CO_2$ from [^{14}C]phenol (44 μM) (A), [^{14}C]benzoate (40 μM) (B), and [^{14}C]benzene (39 μM) (C) with in-cell suspensions of *G. metallireducens* wild-type (WT), *ppsA* mutant, *ppcB* mutant, complemented *ppsA* mutant, and complemented *ppcB* mutant strains. The results are the mean and standard deviation from triplicate cultures of each type.

The production of phenol as an intermediate in anaerobic benzene degradation in *G. metallireducens* contrasts with the production of benzoate as an intermediate in the hyperthermophile *Fer-*

roglobus placidus (17). A highly enriched benzene-oxidizing, Fe(III)-reducing enrichment culture also appears to activate benzene via carboxylation (19, 30).

It seems likely that benzene conversion to phenol will be the major route for anaerobic benzene degradation in petroleum-contaminated environments in which *Geobacter* species are specifically enriched (37). The ability to discern pathways for anaerobic benzene degradation through analysis of gene transcript abundance in pure cultures, as demonstrated previously in studies with *F. placidus* (17) and in this study with *G. metallireducens*, might be extended with metatranscriptomic analysis to elucidate how benzene is anaerobically degraded in other environments.

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