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Identification and Characterization of a Succinyl-Coenzyme A (CoA): Benzoate CoA Transferase in *Geobacter metallireducens*

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Geobacter metallireducens **is a Fe(III)-respiring deltaproteobacterium and serves as a model organism for aromatic compound-degrading, obligately anaerobic bacteria. In this study, a genetic system was established for *G. metallireducens* using nitrate as an alternative electron acceptor. Surprisingly, disruption of the benzoate-induced *bamY* gene, encoding a benzoate coenzyme A (CoA) ligase, reproducibly showed an increased biomass yield in comparison to the wild type during growth with benzoate but not during growth with acetate. Complementation of *bamY* in *trans* converted the biomass yield back to the wild-type level. Growth of the *bamY* mutant with benzoate can be rationalized by the identification of a previously unknown succinyl-CoA:benzoate CoA transferase activity; it represents an additional, energetically less demanding mode of benzoate activation. The activity was highly enriched from extracts of cells grown on benzoate, yielding a 50-kDa protein band; mass spectrometric analysis identified the corresponding benzoate-induced gene annotated as a CoA transferase. It was heterologously expressed in *Escherichia coli* and characterized as a specific succinyl-CoA:benzoate CoA transferase. The newly identified enzyme in conjunction with a benzoate-induced succinyl-CoA synthetase links the tricarboxylic acid cycle to the upper benzoyl-CoA degradation pathway during growth on aromatic growth substrates.**

Aromatic compounds are widely distributed in nature and comprise many components that are harmful to human health and/or the environment. They can only be fully degraded to CO_2 by aerobic and anaerobic microorganisms. The strategies for the catabolism of aromatic growth substrates differ fundamentally in aerobic and anaerobic bacteria. Aerobic bacteria make use of oxygenases that are involved in aromatic ring hydroxylation/cleavage. In contrast, a completely different set of degradation pathways and enzymes is used in aromatic compound-degrading anaerobes. The anaerobic degradation pathways of monocyclic aromatic compounds have initially been studied in facultative anaerobes such as *Thauera* sp., *Azoarcus* sp., *Magnetospirillum* sp., *Rhodopseudomonas palustris*, and "*Aromatoleum aromaticum*" (for recent reviews, see references 11, 19, 20, and 37). In contrast, most information about the anaerobic aromatic degradation pathways in obligate anaerobes derive from studies with the Fe(III)-respiring model organism *Geobacter metallireducens*. This organism was isolated and described by Lovley et al. and uses, e.g., phenol, toluene, *p*-cresol, or benzoate as carbon sources and metal oxides or nitrate as electron acceptors in anoxic respiratory chains (33, 34).

In anaerobic bacteria the majority of monocyclic aromatic growth substrates are converted to the central intermediate benzoyl-coenzyme A (BCoA) by enzymes of the peripheral aromatic catabolism. The anaerobic degradation of benzenes, phenols, cresols, benzoates, aromatic amino acids, and many other monocyclic aromatic compounds involves the activation of aryl carboxylate substrates/intermediates to the corresponding CoA esters. In all cases investigated thus far this activation is catalyzed by AMP-forming aryl carboxylate CoA ligases. Benzoate CoA ligases have been isolated and characterized in both facultatively and obligately anaerobic bacteria (4, 6, 17, 21, 23, 29, 31, 40, 46, 50). For example, *G. metallireducens* contains a single gene, *bamY*, encoding a benzoate CoA ligase. The expression of *bamY*, as well as the synthesis and activity of its product, was induced during growth on benzoate and other aromatic compounds (39, 50).

The next step in the so-called benzoyl-CoA degradation pathway involves the energetically and mechanistically difficult reduction of benzoyl-CoA to cyclohexa-1,5-diene-1-carboxyl-CoA (1,5-dienoyl-CoA) (7, 8). The benzoyl-CoA/1,5-dienoyl-CoA redox pair is the most negative one of a substrate/product couple in biology, and the highly endergonic electron transfer from a physiological donor to the aromatic ring has to be coupled to an exergonic reaction (27). There are two completely different classes of benzoyl-CoA reductases (BCRs), which both yield 1,5-dienoyl-CoA. Facultative anaerobes use an ATP-dependent class I BCR (9, 49), whereas obligate anaerobes are supposed to contain an ATP-independent, high-molecular-weight subunit complex harboring W, Zn, FAD, FeS clusters, and selenocysteine as cofactors (28, 32). The subsequent steps involved in 1,5-dienoyl-CoA degradation comprise modified β-oxidation reactions that are catalyzed by similar enzymes in facultatively and obligately anaerobic bacteria (30, 41). In bacteria with an anaerobic respiratory chain, the product acetyl-CoA is finally oxidized to CO_2 in the tricarboxylic acid (TCA) cycle.

To study the unprecedented enzymes involved in the anaerobic degradation of aromatic compounds, genetic systems have successfully been developed in a number of facultatively anaerobic bacteria (1, 14, 18, 22, 42, 51). Very recently, a first genetic system was described for the obligately anaerobic *G. metallireducens* that included growth on solid medium with Fe(III)-citrate as an electron acceptor, markerless gene mutation, and gene complementation (48). The limitations of this system included poor growth

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on solid medium with Fe(III)-citrate as an electron acceptor, the requirement for harvesting cells in early-log phase, and the dependency on supplements such as yeast extract.

In the present study, we establish an alternative genetic system for *G. metallireducens* using a pure mineral medium with nitrate as a highly soluble electron acceptor allowing high growth yields. Using this system, the gene coding for a benzoate CoA ligase, previously considered essential for benzoate utilization, was found to be dispensable. Instead, a previously unknown succinyl-CoA:benzoate CoA transferase activity was identified and characterized as an additional enzyme involved in benzoate activation.

MATERIALS AND METHODS

Bacterial strains and plasmids. *G. metallireducens* was obtained from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ 7210). Chemically competent *Escherichia coli* JM109 was purchased from Promega (Madison, WI). The Champion pET101 directional TOPO expression kit and chemically competent *E. coli* OneShotTOP10 and BL21Star(DE3)OneShot were purchased from Life Technologies (Grand Island, NY).

Growth media, culturing conditions, and determination of antibiotic resistance. *E. coli* strains were cultivated as described previously (43). *G. metallireducens* was cultivated at 30°C under strictly anoxic conditions in a mineral salt medium (35), in which nitrate (15 mM) was used as the electron acceptor and either acetate (30 mM) or benzoate (5 mM) was used as the electron donor. Plating on solid medium containing 1.5% agar was performed in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI) under an H_2/N_2 atmosphere (5:95 [vol/vol]). Cultivation on solid medium was performed in anaerobic containers that were flushed with N_2/CO_2 (80:20 [vol/vol]) at a 0.5-bar overpressure; the containers were transferred to the anaerobic glove box during cultivation. The sensitivity of *G. metallireducens* to antibiotics was tested in liquid mineral medium. All antibiotic stock solutions were sterile and anoxic.

In order to estimate the plating efficiency of *G. metallireducens* the cell number of a pre-stationary-phase liquid culture was determined by using the cell counting chamber. The number of CFU in these cultures was determined by plating 10-fold serial dilutions. For the assessment of plasmid stability *G. metallireducens* was electrotransformed with broad-host-range IncQ plasmids RSF1010 (45) and pCD342 (16), respectively. The resulting streptomycin- and kanamycin-resistant liquid cultures were transferred over at least 10 passages (>100 generations) into nonselective liquid medium. Tenfold serial dilutions of each passage were plated onto selective and nonselective solid medium, respectively. The resulting CFU ml^{-1} on selective and nonselective media were compared.

Preparation of electrocompetent *G. metallireducens* and electroporation procedure. Preparation of electrocompetent *G. metallireducens* cells was performed in a glove box in an H_2/N_2 atmosphere (5:95 [vol/vol]), and this was followed a modified protocol described previously (13). All steps were performed anaerobically. Portions (100 ml) of mid-log-phase cultures in mineral medium with acetate (optical density at 600 nm [OD_{600}] of 0.4 to 0.5 corresponding to 4×10^8 to 5×10^8 cells ml^{-1}) were harvested by centrifugation at 4°C for 10 min at 7,000 × *g*. The cells were washed twice in 100 ml of electroporation buffer (13) and suspended in 1 ml of the same buffer containing 10% dimethyl sulfoxide (DMSO). The resulting electrocompetent cells were stored in liquid nitrogen. Electroporation was performed in 1-mm-gap electroporation cuvettes using an Eporator (Eppendorf, Germany). Then, 100-μl portions electrocompetent *G. metallireducens* were pulsed at 1.7 kV cm^{-1} for 4 ms. After electroporation, the cells were immediately transferred into 1 ml of room temperature mineral medium with acetate as a carbon source and allowed to recover for around 5 h, prior to plating on selective solid medium or cultivation in liquid growth medium.

General DNA techniques. The genomic DNA of *G. metallireducens* was isolated as described previously by Cheng and Jiang (12). PCR products were purified from agarose gels using the Wizard SV Gel and PCR Clean-Up system (Promega). Plasmid DNA was purified by using the Wizard Plus Minipreps DNA purification system (Promega). Primers were provided by Metabion (Martinsried, Germany). For cloning reactions using the Champion pET101 directional TOPO expression kit, PCR amplification was performed using *Pfu* DNA polymerase (Thermo Scientific). All other PCR amplifications were performed using the GoTaq Green Master Mix (Promega) according to the manufacturer's instructions. The sequences of all of the primers used are listed in Table S1 in the supplemental material.

Homologous expression and purification of *bamY*. For homologous expression of *bamY* (gi78194605), plasmid pCD342 was used (16). The *bamY* gene was amplified from the *G. metallireducens* genome using the primers bamY_Bam+SDGm_for and bamY_Hind+cHis_rev (for oligonucleotide primer sequences see Table S1 in the supplemental material). The forward primer included restriction sites for BamHI and the Shine-Dalgarno sequence of *bamY* from *G. metallireducens* (26) in addition to the annealing nucleotides. The reverse primer included the restriction site for HindIII and a C-terminal 6-fold His tag sequence in addition to the annealing nucleotides. The resulting PCR product was digested with BamHI and HindIII and inserted into the corresponding sites of pCD342 to form the expression plasmid pCD*bamY*. After transformation, the resulting kanamycin-resistant cultures were grown at 30°C in 2 liters of mineral medium containing 30 mM acetate as electron donor. Stationary-phase cultures were harvested by centrifugation at 7,000 × *g* for 10 min at 4°C. The cells were suspended in 50 mM Tris-HCl (pH 6.8), $MgCl_2$, 10% glycerol (vol/vol), DNase I (10 μg/g of cells), and lysozyme (50 μg/g of cells) and were subsequently disrupted by sonication (7 W, 5 to 7 pulses of 30 s). After centrifugation at 100,000 × *g*, His-tagged BamY was purified from the supernatant at 4°C by Ni-Sepharose high-performance affinity column (HisTrap HP; GE Healthcare). After equilibration of the 1-ml column with 20 mM Tris-HCl (pH 7.4), 500 mM NaCl, 20 mM imidazole, and 10% (vol/vol) glycerol, the extract was applied to the column at a flow rate of 0.75 ml min^{-1} and washed with 10 column volumes, followed by a linear gradient over 30 column volumes from 20 to 500 mM imidazole in buffer.

Enrichment of succinyl-CoA:benzoate CoA transferase and heterologous expression of its gene. The succinyl-CoA:benzoate CoA transferase activity was partially purified from benzoate-grown cells of wild-type *G. metallireducens* in four chromatographic steps. Since the enzyme was not sensitive to oxygen, all purification steps were carried out under oxic conditions. Cells (10 g [wet mass]) were suspended in 15 ml of 50 mM Tris-HCl (pH 6.8) containing 4 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM dithioerythritol (DTE), and 10% (vol/vol) glycerol. After the addition of DNase I and lysozyme, the cells were disrupted by sonication as described above. The extract was centrifuged at 100,000 × *g*, and the supernatant was dialyzed overnight in suspension buffer.

The dialyzed protein solution was applied to a DEAE-Sepharose column at a flow rate of 0.75 ml min^{-1} (Fast Flow [GE Healthcare]; diameter, 16 mm; volume, 10 ml), which had been equilibrated with 10 mM Tris-HCl (pH 7.8) containing 2 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM dithioerythritol, and 10% (vol/vol) glycerol (buffer A). The column was washed with 30 ml of buffer A at a flow rate of 1 ml min^{-1} and afterward with 70 ml of 50 mM KCl in buffer A. The succinyl-CoA:benzoate CoA transferase activity eluted in a linear gradient of 50 to 120 mM KCl in buffer A (100 ml). Pooled fractions with succinyl-CoA:benzoate CoA transferase activity were applied to a hydroxyapatite column (ceramic hydroxyapatite type I, 40 μm [MacroPrep; Bio-Rad]; diameter, 16 mm; volume, 7 ml) that had been equilibrated with buffer A. The transferase activity eluted at 50 mM KH_2PO_4 in buffer A at a flow rate of 1 ml min^{-1}. Next, 15 ml of the active hydroxyapatite fraction was concentrated (Vivaspin 6 [Sartorius], 10,000 molecular weight cutoff) to a volume of 5 ml and subsequently applied to a MonoQ column (GE Healthcare; volume, 1 ml), which had been equilibrated with buffer A. The column was washed with 10 ml of buffer A, followed by 100 mM KCl in buffer A at a flow rate of 0.5 ml min^{-1}. The

transferase activity eluted in a linear gradient of 100 to 200 mM KCl in buffer A. The active fractions were concentrated, and 0.5 ml were applied to a Reactive Green Agarose affinity chromatography column (GE Healthcare; diameter, 5 mm; volume, 1 ml) which had been equilibrated with buffer A. The column was washed with 10 ml of buffer A and 10 ml of 50 mM KCl in buffer A at a flow rate of 0.5 ml min^{-1}. Activity eluted in a step gradient of 100 mM KCl in buffer A. Purity control was carried out by SDS-PAGE analysis.

For the heterologous expression of the gene putatively coding for succinyl-CoA:benzoate CoA transferase (gi78194516), the Champion pET101 Directional TOPO Expression Kit was used (Life Technologies). The primers Gm_act-pET/D_for and Gm_act-pET/D_rev were designed according to the manufacturer's instructions. Ligation, cloning, and expression were performed according to the manufacturer's protocol. Cells were suspended and disrupted, after which the His-tagged succinyl-CoA:benzoate transferase was purified by Ni-affinity chromatography as described above for BamY.

Construction of a *bamY*::*kan* disruption mutant and complementation in *trans*. For the construction of a *bamY* disruption mutant, the plasmid pK18*mob* was selected (44). The plasmid contains a kanamycin resistance cassette and a pMB1 origin of replication which prevents the replication in *G. metallireducens*.

A central 672-bp-comprising fragment of *bamY* (referred to as *bamY*′) was amplified from the *G. metallireducens* chromosome using primers bamYDelHind_for and bamYDelXba_rev (see Fig. S3 in the supplemental material). In addition to annealing nucleotides, the primer sequences contained restriction sites for subsequent cloning of the PCR product. The resulting PCR product was digested with HindIII and XbaI and inserted into the corresponding sites of pK18*mob* to form plasmid pK*bamY*′. After transformation with 125 ng of pK*bamY*′, positive clones that had incorporated the plasmid into the chromosome were identified by growth with kanamycin-containing medium. For the verification of the *bamY* disruption, several PCRs using the primers bamY_Bam+SDGm_for (P1), R24 (P2), and F24 (P3) and bamY_Hind+cHis_rev (P4) were performed using GoTaq Green Master Mix (Promega) (see Table S1 and Fig. S3 in the supplemental material). In addition, PCR control reactions were performed using Phusion High-Fidelity DNA polymerase (Finnzymes, Finland) for large PCR products according to the manufacturer's instructions.

In order to complement the *bamY*::*kan* mutation, *bamY* was expressed in *trans* using the plasmid pCD*bamY*Sm. This plasmid was obtained from pCD*bamY* by replacing the kanamycin resistance cassette by a streptomycin resistance cassette. It was amplified from plasmid RSF1010 (45) using the primers Sm_for_BglII and Sm_rev_BglII, which included the restriction site for BglII. The resulting PCR product was digested with BglII and inserted into the corresponding sites of pCD*bamY*. For the complementation of the *bamY*::*kan* mutation, 100 ng of plasmid pCD*bamY*Sm was introduced into the mutant strain by electroporation.

Enzyme assays. Benzoate CoA ligase activity was assayed in a coupled spectrophometric assay as described previously (46). Succinyl-CoA:benzoate CoA transferase activity was determined in a discontinuous high-pressure liquid chromatography (HPLC) assay (C_{18} reversed-phase HPLC; Eurosphere-100 10 μm, Waters Alliance 2695 [Waters, Eschborn, Germany]) by monitoring the substrate consumption and product formation at 30°C by diode array detection. The assay solution contained 100 mM Tris-HCl (pH 7.8), 5 mM $MgCl_2$, and 0.2 mM succinyl-CoA or benzoyl-CoA. After the addition of 1 to 3 mg of cell extract or 10 to 50 μg of enriched protein to 200 μl of assay solution, the reaction was started by the addition of 0.15 mM carboxylic acid. Samples (50 μl) were taken at different time points, and the reaction was stopped by the addition of 5 μl of 10% (vol/vol) formic acid.

Determination of kinetic parameters and molecular mass. K_m values for benzoate and succinate were estimated by varying the concentration of the respective carboxylic acid from 0.025 to 0.5 mM. The data obtained were analyzed by fitting them to the Michaelis-Menten equation using the Prism software package (GraphPad, CA). The conversion of succinate, acetate, malate, fumarate, crotonate, glutarate, cyclohexanecarboxylate, cylohex-1-ene-1-carboxylate, and *p*-hydroxybenzoate as CoA acceptors was tested at 0.125 mM concentrations. The native molecular mass of the purified His-tagged succinyl-CoA:benzoate CoA transferase was determined by using an FPLC Superdex 200 gel filtration column (GE Healthcare; diameter, 10 mm; volume, 25 ml). After equilibration with 50 mM Tris-HCl (pH 6.8) containing 4 mM $MgCl_2$, 5 mM KH_2PO_4, 1 mM DTE 10% (vol/vol) glycerol, and 150 mM KCl, 200-μl portions of the protein solution (~2.5 mg) were applied at a flow rate of 0.3 ml min^{-1}.

Mass spectrometry analysis. Protein identification was performed by in-gel tryptic digestion, followed by UPLC-LTQ Orbitrap MS/MS analysis as described previously (29). Mascot searches of the raw data were done against the genome entry of *Geobacter metallireducens* GS-15.

RESULTS

Gene expression system in *G. metallireducens* with nitrate as an electron acceptor. *G. metallireducens* is known to grow with nitrate as an alternative electron acceptor in liquid cultures, which is reduced to ammonia (35). Under these conditions, an increased requirement for Fe(III) in the culture medium was described previously (47). However, no growth on solid medium with nitrate as electron acceptor has yet been described, which could be explained by an increased sensitivity to oxidative stress caused by reactive nitrogen oxide intermediates. In particular during growth on aromatic compounds, enzymes involved in oxidative stress defense are known to be induced in *G. metallireducens* (25).

In order to establish a genetic system, including growth on solid medium with nitrate (15 mM) as electron acceptor, the preparation of agar plates and the inoculation of *G. metallireducens* was carried out in an anaerobic glove box under an N_2/H_2 atmosphere (95:5 [vol/vol]). Incubation of plates was performed in custom-made gas-tight containers in an N_2/CO_2 atmosphere with 0.5-bar overpressure using acetate (30 mM) or benzoate (5 mM) as electron donors. Under these conditions, the formation of red colonies was observed after 4 to 6 days at 25 to 30°C that were 1 to 2 mm in diameter (see Fig. S1 in the supplemental material). The plating efficiency was ca. 60%. No growth of *G. metallireducens* was observed in the presence of the following antibiotics at the concentrations as indicated: kanamycin (100 μg ml^{-1}), streptomycin (100 μg ml^{-1}), ampicillin (100 μg ml^{-1}), tetracycline (15 μg ml^{-1}), chloramphenicol (30 μg ml^{-1}), and gentamicin (50 μg ml^{-1}).

For establishing a protocol for homologous expression of a gene in *G. metallireducens* during growth on nitrate, *bamY*, encoding benzoate-CoA ligase, was introduced into vector pCD342 (16) with a 6-fold His tag. This gene was previously heterologously expressed in *E. coli* (50) and served as a proof of principle. The resulting pCD*bamY* was introduced into *G. metallireducens* by electroporation with transformation efficiencies of ~10^4 CFU μg of DNA^{-1}. To assess the plasmid stability, the colonies formed on kanamycin were transferred over at least 10 passages. Comparison of the CFU ml^{-1} on selective and nonselective media indicated that >70% of the cells still contained the plasmid displaying a high stability of pCD342 in *G. metallireducens*.

The homologously expressed, His-tagged BamY was purified using a Ni-chelating affinity chromatography column. A protein band of ~60 kDa was highly enriched that contained a benzoate CoA ligase activity of 13 μmol min^{-1} mg^{-1}, which is comparable to the previously described BamY heterologously produced in *E. coli* (50) (see Fig. S2 in the supplemental material).

Characterization of a *bamY* disruption mutant. A central

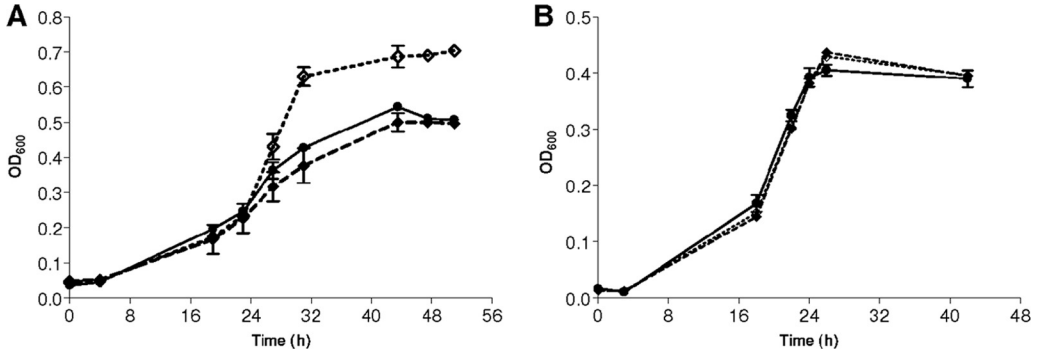

FIG 1 Growth of *G. metallireducens* strains on A. benzoate and B. acetate. Wild type (●), *bamY*::*kan* mutant (◇), and complemented mutant (◆). Error bars indicate the standard deviations of biological triplicates.

672-bp fragment of *bamY* was inserted into plasmid pK18*mob* carrying a kanamycin resistance cassette; it is not replicated in *G. metallireducens* due to the pMB1 origin of replication (see Fig. S3 in the supplemental material). The resulting plasmid was introduced into *G. metallireducens* by electroporation. *G. metallireducens bamY*::*kan* strains that had incorporated the knockout plasmid into the chromosome by homologous recombination were identified by growth on mineral medium containing kanamycin. The insertion of the plasmid into the *bamY* gene was verified by sequencing PCR amplicons obtained using appropriate oligonucleotide primer pairs (see Fig. S3 in the supplemental material).

Extracts from cells of the *G. metallireducens bamY*::*kan* strain fully lost benzoate CoA ligase activity (<0.01 nmol min^{-1} mg^{-1}; activity in the wild type, 0.5 to 1 nmol min^{-1} mg^{-1}). However, the *G. metallireducens bamY*::*kan* strain was still able to grow with benzoate and nitrate as the sole sources of cell carbon and energy. Surprisingly, the maximal OD_{600} reached (5 mM benzoate) was reproducibly 1.35- to 1.5-fold higher than in the wild-type strain (Fig. 1A). This unexpected finding suggested that the expression of *bamY* was dispensable or even disadvantageous for growth on benzoate under the conditions used. In contrast, growth on acetate was highly similar in the wild type and in the *bamY*::*kan* strain (Fig. 1B). In summary, these findings suggest that, next to BamY, an additional, thus-far-unknown enzyme had to be involved in the initial step of benzoate degradation.

The *bamY* gene was complemented in *trans* in the *G. metallireducens bamY*::*kan* mutant using the plasmid pCD*bamY*Sm. This plasmid was constructed from the expression plasmid pCD*bamY* by replacing the kanamycin resistance with a streptomycin resistance cassette for selection of the complemented mutant from the kanamycin-resistant *bamY*::*kan* mutant. In the corresponding complemented *bamY*::*kan* mutant strain that expressed an intact *bamY* gene in *trans*, the observed phenotype of an increased biomass yield was reverted back to the wild-type level (Fig. 1A). This result confirms that disruption of *bamY* had a positive effect for growth on benzoate. Again, no effect was observed during growth on acetate (Fig. 1B).

***In vitro* carboxylic acid activation activities.** The potential alternative formation of benzoyl-CoA by a CoA transferase was tested by *in vitro* HPLC assays using extracts of the wild type and the *bamY*::*kan* mutant; acetyl-CoA, crotonyl-CoA, and succinyl-CoA served as potential CoA donors, and benzoate served as the CoA acceptor. Only with succinyl-CoA was time- and protein-dependent formation of benzoyl-CoA observed during HPLC analysis of CoA ester substrates consumed and products formed. The specific activity was 13 nmol min^{-1} mg^{-1} (for the results of representative HPLC analyses with enriched enzyme, see Fig. 2A). The activity was nearly identical in the wild type and in the *bamY*::*kan* mutant; virtually no activity was determined in extracts from wild-type cells grown on acetate (<0.01 nmol min^{-1} mg^{-1}). The reaction could also be monitored in the reverse reaction at a similar rate (Fig. 2B). It leveled off when succinyl-CoA and benzoyl-CoA were present in an almost 1:1 ratio, indicating that the reaction proceeded close to thermodynamic equilibrium ($\Delta G = 0$, Fig. 2C). In conclusion, the presence of the benzoate-induced succinyl-CoA:benzoate CoA transferase activity enabled growth on benzoate, even when the benzoate CoA ligase activity was knocked out.

In addition to this succinyl-CoA-dependent transferase, a benzoate induced, ATP-dependent succinyl-CoA synthetase activity was determined (16.5 nmol min^{-1} mg^{-1}), which was missing in extracts from cells grown on acetate (<0.1 nmol min^{-1} mg^{-1}). The induction of the putative genes coding for this TCA enzyme during growth on benzoate were previously described (10, 50). The combined induction of genes coding for a succinyl-CoA synthetase/CoA transferase during growth on aromatic compounds directly links benzoate activation to the TCA cycle.

G. metallireducens was predicted to contain three different enzymes involved in acetyl-CoA formation during growth on acetate, including the essentially irreversible AMP-forming acetyl-CoA synthetase and the fully reversible succinyl-CoA:acetate CoA transferase and acetate kinase/phosphotransacetylase (2, 10). Determination of *in vitro* activities of the putative acetate activating enzymes in extracts from cells grown on acetate confirmed the presence of a highly active succinyl-CoA:acetate CoA transferase (550 nmol min^{-1} mg^{-1}) and an acetate CoA ligase (60 nmol min^{-1} mg^{-1}), whereas no acetate kinase activity was determined (<0.1 nmol min^{-1} mg^{-1}). In summary, *G. metallireducens* appears to use two different modes for acetate and benzoate activation via specific carboxylic acid CoA synthetases and CoA transferases.

Enrichment of succinyl-CoA:benzoate CoA transferase from the wild type and identification of its gene. The succinyl-CoA:benzoate CoA transferase activity was 53-fold enriched from extracts of wild-type cells grown on benzoate using four chromatographic steps (Table 1 and Fig. 3A). After affinity chromatography on Reactive Green agarose, the pooled protein fractions containing succinyl-CoA:benzoate CoA transferase activity showed a

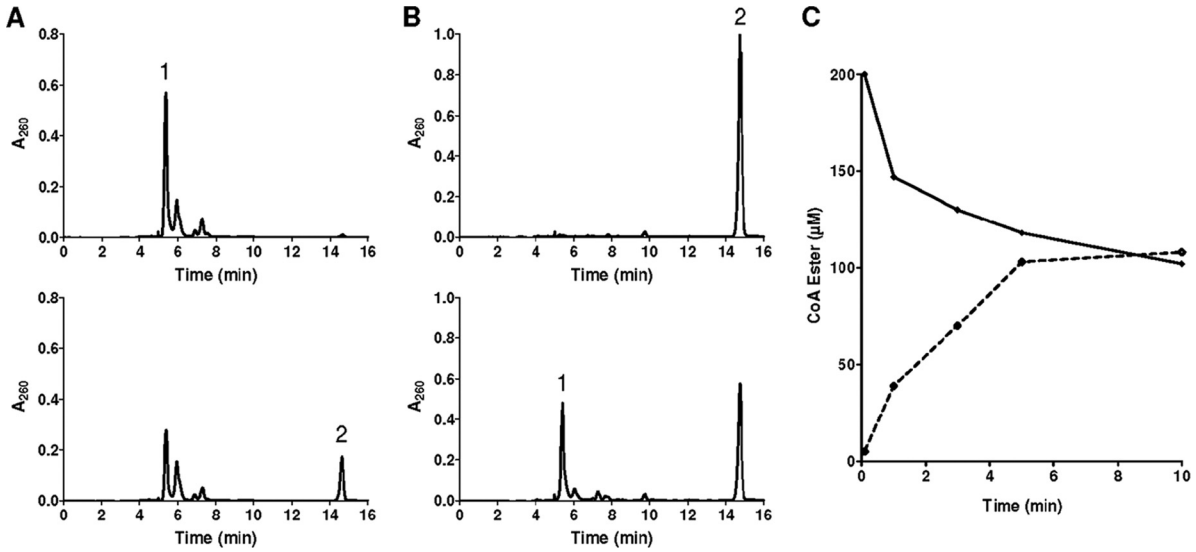

FIG 2 HPLC analysis of succinyl-CoA:benzoate CoA transferase reaction. (A) Time-dependent consumption of 0.2 mM succinyl-CoA (peak 1) and formation of benzoyl-CoA (peak 2) in the presence of 0.5 mM benzoate. Upper panel, after 0.1 min of incubation; lower panel, after 10 min of incubation. (B) Time-dependent consumption of 0.2 mM benzoyl-CoA (peak 2) and formation of succinyl-CoA (peak 1) in the presence of 0.5 mM succinate. Upper panel, after 0.1 min of incubation; lower panel, after 10 min of incubation. (C) Time course of succinyl-CoA:benzoate CoA transferase reaction starting with succinyl-CoA. Solid line, succinyl-CoA; dashed line, benzoyl-CoA.

highly enriched protein band on SDS polyacrylamide gels at ~50 kDa. The protein was excised from the gel and digested with trypsin, and the peptides obtained were subsequently analyzed by mass spectrometry. Using the MASCOT platform, the best matches were obtained with peptides from a deduced gene product in the genome of *G. metallireducens*, which was annotated as a CoA transferase (gi78194516; theoretical mass, 48.1 kDa). The protein identification was based on four peptides (sequence coverage, 13%; protein summary score, 208.5). The gene coding for succinyl-CoA:benzoate CoA transferase was previously identified as a gene at the downstream edge of the benzoate-induced cluster IB that includes genes encoding enzymes involved in β-oxidation reactions (50). The genomic context is shown in Fig. 4.

Using BLAST, highly homologous genes with deduced amino acid sequence identities greater than 75% were only found in aromatic compound-degrading members of the *Geobacteraceae*, including *G. bemidjensis*, *G. daltonii*, and *Geobacter* species M21. Notably, in each of the genomes two copies of such highly similar transferase genes are present. Similarities to experimentally characterized CoA transferases from other organisms clearly indicate that the enzyme from *G. metallireducens* belongs to the Ia family of CoA transferases. Members of this family contain a conserved active-site glutamate (E248 in the transferase of *G. metallireducens*), which forms a covalent mixed anhydride intermediate during catalysis (24, 36).

Heterologous production and characterization of succinyl-CoA:benzoate CoA transferase. The putative gene coding for succinyl-CoA:benzoate CoA transferase identified by mass spectrometry was heterologously expressed in *E. coli* with a 6-fold C-terminal His tag (Fig. 3B). After purification by Ni-chelating affinity chromatography, a highly enriched protein band that migrated at a slightly higher mass than wild-type succinyl-CoA:benzoate CoA transferase was obtained which can be explained by the additional 6-fold His tag. The specific activity of the heterologously expressed CoA transferase was 265 nmol min^{-1} mg^{-1} and ca. 40% of the highly enriched wild-type enzyme.

The native molecular mass as determined by gel filtration was ~90 kDa, suggesting an α_2-composition. The K_m value for ben-

TABLE 1 Enrichment of succinyl-CoA:benzoate CoA transferase[a]

Purification step	Protein (mg)	Sp act (mU mg^{-1})	Total activity (mU)	Recovery (%)	Purification (fold)
Cell extract	630	13	7,900	100	1
DEAE	118	74	8,750	110	6
Hydroxyapatite	44	98	4,330	55	8
MonoQ	1.2	261	315	4	21
Reactive Green	0.06	673	37	0.5	53

[a] The starting material was 10 g (wet mass) of wild-type *G. metallireducens* grown on benzoate. "1 mU" corresponds to the formation of 1 nmol of benzoyl-CoA min^{-1}.

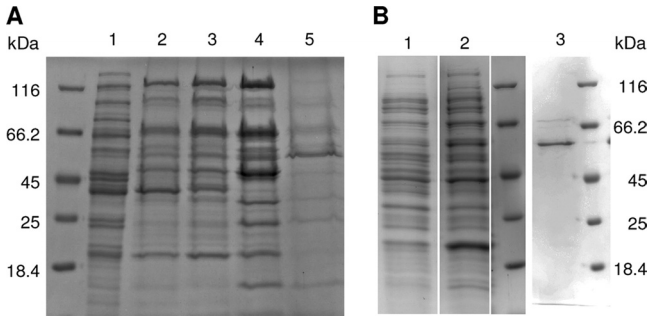

FIG 3 SDS-PAGE analysis of succinyl-CoA:benzoate CoA transferase enrichment from the wild type (A) and after heterologous expression of the gene in *E. coli* (B). (A) Lanes: 1, cell extracts of *G. metallireducens*; 2, after DEAE chromatography; 3, ceramic hydroxyapatite; 4, MonoQ; 5, Reactive Green agarose. (B) Lanes: 1 and 2, cell extract of *E. coli* before (lane 1) and after (lane 2) IPTG induction; 3, purified His-tagged protein after Ni-chelating chromatography.

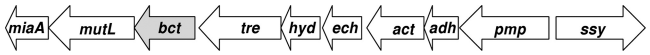

FIG 4 Genomic context of the gene coding for succinyl-CoA:benzoate CoA transferase. The gene referred to as *bct* is indicated by the gray-shaded arrow (gi78194516). Abbreviations for annotated predicted gene products: *miaA*, DNA mismatch repair protein; *mutL*, tRNS isopentenyl transferase; *tre*, transcriptional regulator with PAS sensor; *hyd*, hydrolase; *ech*, enoyl-CoA hydratase/isomerase; *act*, acetyl-CoA transferase; *adh*, short-chain dehydrogenase; *pmp*, fusaric acid resistance protein; *ssy*, sodium symporter.

zoate was 55 ± 5 μM (mean value ± the standard deviation). Next to benzoate, only cyclohex-1-ene-1-carboxylate was used as a CoA acceptor at a comparable rate, whereas other nonaromatic carboxylic acids tested were virtually not converted (Table 2). Together, these findings suggest a high preference for benzoate or closely related analogues as CoA acceptor. In the reverse reaction with benzoyl-CoA as CoA donor no nonaromatic carboxylic acid other than succinate was converted (Table 2).

DISCUSSION

Genetic system for *G. metallireducens* grown with nitrate as an electron acceptor. Very recently, two different genetic systems became available for an obligately anaerobic, aromatic compound-degrading bacterium. The systems described by Tremblay et al. (48) and in the present study have different strengths and weaknesses and may be suitable for different individual purposes. The system described by Tremblay et al. enables the introduction of markerless mutations, which is recommended for the insertion of multiple markerless mutations and the corresponding multiple complementations. For example, the formation of a *bamY bct* double mutant cannot be achieved in the system established here due to its dependency on the introduction of antibiotic resistance cassettes. On the other side, the advantage of the system established here enabled a much higher growth yield due to the use of nitrate as an electron acceptor and, in contrast to the system by Tremblay et al., the cells can be harvested in the mid-exponential-growth phase. Moreover, it does not depend on supplements such as yeast extract, which may have a negative effect on the expression of catabolic enzymes.

The system established here will in particular be useful for studying the physiology of deltaproteobacteria during growth on aromatic growth substrates, including Fe(III)- and sulfate-reducing as well as fermenting bacteria. For example, the study of the highly complex class II BCRs, which are composed of eight subunits with W, FeS, and selenocysteine cofactors requiring complex cofactor assembly machineries will benefit from the system established (28, 50). Although *G. metallireducens* does not use polycyclic aromatic hydrocarbons (PAHs), the system established may be helpful for expressing enzymes from related deltaproteobacteria that are involved in the degradation of PAHs, e.g., established anaerobic pure/enrichment cultures that are capable of using naphthalene or phenanthrene as the sole carbon source belong to the sulfate-reducing deltaproteobacteria (5, 15, 38).

TABLE 2 Substrate preference of heterologously expressed succinyl-CoA:benzoate CoA transferase from *G. metallireducens*

Substrate	Activity (%)[a]	
	CoA donor: benzoyl-CoA	CoA donor: succinyl-CoA
Succinate	100	ND
Acetate	<0.1	<0.1
Malate	<0.1	ND
Fumarate	<0.1	ND
Crotonate	ND	<0.1
Glutarate	ND	<0.1
Benzoate	ND	100
Cyclohex-1-ene-carboxylate	ND	126
Cylohexanecarboxylate	ND	<0.1
p-Hydroxybenzoate	ND	11

[a] Carboxylic acids (0.125 mM) were tested as CoA acceptors in the presence of benzoyl-CoA or succinyl-CoA as CoA donors. ND, not determined.

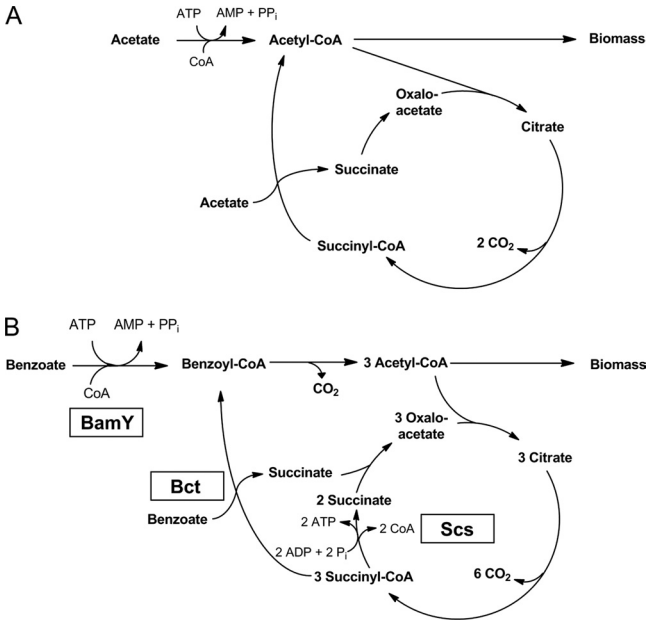

FIG 5 Activation of carboxylic acid growth substrates and TCA cycle in *G. metallireducens*. (A) Acetate as carbon source; (B) benzoate as carbon source. During growth on benzoate a succinyl-CoA synthetase is required as only maximally one of the three succinyl-CoA formed per benzoyl-CoA oxidized is regenerated by succinyl-CoA:benzoate CoA transferase. BamY, benzoate-CoA ligase; Bct, succinyl-CoA:benzoate CoA transferase (benzoate CoA transferase); Scs, succinyl-CoA synthetase.

Benzoyl-CoA formation in aromatic compound-degrading anaerobes. Thus far, the activation of benzoate or hydroxylated, aminated, methylated, or halogenated analogues was always found to be catalyzed by AMP-forming aryl carboxylate CoA ligases. This finding was in particular surprising as obligate anaerobes such as sulfate-reducing or fermenting bacteria have a very poor energy yield during growth on aromatic compounds. With the succinyl-CoA:benzoate CoA transferase, an alternative, energetically less demanding enzyme has been identified to be involved in aryl carboxyl-CoA formation. It appears to be present in all aromatic compound degrading members of the Fe(III)-respiring *Geobacteraceae* but might also be present in sulfate-reducing or fermenting bacteria.

Since three acetyl-CoAs are formed per benzoate oxidized, the succinyl-CoA:benzoate CoA transferase as the only catabolic enzyme involved in succinate formation from succinyl-CoA would not be sufficient for maintaining the TCA cycle (Fig. 5B). For this reason, the additional induction of a succinate regenerating suc-

cinyl-CoA synthetase is essential for growth on benzoate. In contrast, during growth on acetate no succinyl-CoA synthetase is required as per acetyl-CoA oxidized one succinate is formed by succinyl-CoA:acetate CoA transferase (Fig. 5A). In conclusion, no succinyl-CoA synthetase activity was determined during growth on acetate.

Disruption of the *bamY* gene in *G. metallireducens* resulted in the unexpected phenotype of the observed increased biomass yield (i.e., the OD_{600} reached per mol of benzoate consumed). This, on first view, curious finding suggests that the loss of BamY activity was apparently rather advantageous to the cell, since benzoate activation by a CoA transferase enables a higher ATP yield during growth on benzoate. The *bamY* gene is integrated in the benzoate induced *bam* gene cluster II, which is supposed to be acquired by *G. metallireducens* as a whole by lateral transfer. This assumption is substantiated by the finding that in *G. metallireducens* the genes coding for the degradation of aromatic compounds are organized in a genomic island (2, 10). The gene coding for succinyl-CoA:benzoate CoA transferase in *G. metallireducens* is part of the benzoate induced cluster IB, and its acquisition could have made *bamY* dispensable under certain conditions. This hypothesis is supported by the finding that the closely related, benzoate-degrading *G. bemidjiensis* contains only a nonfunctional *bamY*, in which a mutation caused an internal TGA stop codon (3). Functional *bamY* genes contain a codon for glutamate at this position. Indeed, cells of *G. bemidjiensis* grown on benzoate do not contain a benzoate CoA ligase activity but a succinyl-CoA:benzoate CoA transferase activity (our unpublished results). In conclusion, the artificial inactivation of the *bamY* gene carried out here during the establishment of a genetic system obviously occurred in nature in a related *Geobacter* species.

The availability of genes coding for both a benzoate CoA ligase and a succinyl-CoA:benzoate CoA transferase could be advantageous under certain conditions in nature. The essentially irreversible benzoate CoA ligase should facilitate growth on aromatic compounds at very low concentrations compared to the fully reversible CoA transferase. Low concentrations of aromatic compounds probably prevail more often at natural habitats than the high concentrations that are generally applied in pure/enrichment laboratory cultures. In conclusion, the apparent advantageous inactivation of *bamY* observed under experimental conditions might be rather disadvantageous at low substrate concentrations at environmental sites.

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