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Biochemical and genetic evidence of benzylsuccinate synthase in toluene-degrading, ferric iron-reducing *Geobacter metallireducens*

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Abstract

In vitro assays demonstrated that toluene-grown cells of *Geobacter metallireducens* catalyzed the addition of toluene to fumarate to form benzylsuccinate under anaerobic conditions. The specific *in vitro* rate of benzylsuccinate formation was ca. 45% of the specific *in vivo* rate of toluene consumption. In addition, *bssA* and *bssB*, which code for the α and β subunits of benzylsuccinate synthase (BSS), respectively, were found to have sequences in *G. metallireducens* similar to the only sequences heretofore available (for three denitrifying strains). This is the first report of the presence of BSS in a ferric iron-reducing bacterium; BSS activity has previously been reported in denitrifying, sulfate-reducing, and anoxygenic phototrophic toluene degraders, as well as in a highly enriched methanogenic, toluene-degrading culture.

Introduction

The recent discovery of benzylsuccinate synthase (BSS), which catalyzes the addition of the methyl carbon of toluene to the double bond of fumarate, was an important development in the understanding of anaerobic toluene degradation (Heider et al. 1998). Furthermore, recent studies have shown that analogous fumarate addition reactions are involved in the anaerobic activation of a variety of other compounds, including *m*-xylene, *m*- and *p*-cresol, certain *n*-alkanes, and 2-methylnaphthalene (Krieger et al. 1999; Müller et al. 1999, 2001; Rabus et al. 2001; Kropp et al. 2000; Annweiler et al. 2000). To date, BSS has been purified from two toluene-degrading, denitrifying bacteria (Leuthner et al. 1998; Beller & Spormann 1999) and the genes encoding for the three subunits of BSS have been sequenced from three denitrifying bacteria (*Thauera aromatica* strains K172 and T1; *Azoarcus* sp. strain T) (Leuthner et al. 1998; Coschigano et al. 1998; Achong et al. 2001). BSS activity is distributed across a wide range of phylogenetically and physiologically diverse bacteria, including denitrifying (Biegert et al. 1996; Beller & Spormann 1997a), sulfate-reducing (Beller & Spormann 1997b; Rabus & Heider 1998), and anoxygenic phototrophic (Zengler et al. 1999) strains, as well as a highly enriched methanogenic culture (Beller & Edwards 2000). However, the presence of BSS has never been reported (or, to our knowledge, investigated) in toluene-degrading, ferric iron-reducing bacteria. In this article, we report *in vitro* BSS activity and the presence of *bssA* and *bssB* [also called *tutD* and *tutG*, respectively] in *Geobacter metallireducens*, which is the most extensively studied of the two known iron-reducing, toluene-degrading bacteria (Lovley et al. 1993; Coates et al. 2001).

Materials and methods

Cultivation of G. metallireducens for biochemical studies

G. metallireducens (Lovley et al. 1993) was cultured under strictly anaerobic conditions with a modified medium containing 2.5 g/L $NaHCO_3$, 0.25 g/L

NH_4Cl, 0.6 g/L $NaH_2PO_4 \cdot H_2O$, 0.1 g/L KCl, as well as vitamin and trace element mixtures (Lovley et al. 1984, 1988). Toluene (~1 mM) was provided as the sole electron donor. Electron acceptor in the form of solubilized Fe(III) (10–14 mM) was added to the medium from a sterile, anaerobic stock solution of Fe(III)-nitrilotriacetic acid. Ferric iron reduction was confirmed by monitoring Fe(II) production, as previously described (Lovley & Phillips 1986). Total protein concentration was determined from pelleted cells using the bicinchoninic acid method (Smith et al. 1985) with bovine serum albumin as the standard.

Determination of in vivo toluene degradation rate

In vivo and *in vitro* kinetic experiments were performed at 30 °C in an anaerobic glove box (Coy Laboratory Products, Inc., Grass Lake, MI) with a gas composition of 80% N_2, 10% CO_2, and 10% H_2. The *in vivo* rate of toluene consumption was determined immediately prior to the harvesting of cells for *in vitro* assays of BSS activity. *In vivo* toluene consumption was measured by a static headspace method involving gas chromatography-flame ionization detection (Beller & Edwards 2000).

In vitro assay for BSS activity

After the *in vivo* rate was determined, the culture was harvested and permeabilized cell assays were performed in a manner similar to that used previously for denitrifying, sulfate-reducing, and methanogenic cultures (Beller & Spormann 1997a, b; Beller & Edwards 2000). Briefly, 300 mL of cells were harvested anaerobically by centrifugation (7900 × *g*, 4 °C, 20 min) in sealed polycarbonate bottles, suspended in 2 mL of degassed morpholinopropanesulfonic acid (MOPS) buffer [20 mM MOPS, 10 mM $MgSO_4$ (pH 7.2); Beller & Spormann 1997a], and then permeabilized with Triton X-100 (2% [vol/vol] final concentration). To facilitate formation of a pellet in this low-biomass culture, 1.3 mM ferrous sulfate was added immediately prior to centrifugation to form a ferrous mineral precipitate (possibly siderite). The assay mixtures contained toluene-d_8 (0.33 μmol; 100 atom% D, Sigma Chemical Co., St. Louis, MO), sodium fumarate (1.4 μmol), dithiothreitol (as a reductant; 1.7 μmol), and ca. 0.4 mg protein in 1.35 ml of MOPS buffer. The reaction was halted at selected time intervals by rapid cooling on ice and injection with air (the enzyme is inactivated by molecular oxygen). After incubation, assay mixtures were treated with DNase I, acidified with concentrated HCl, and extracted four times with high-purity diethyl ether. The ether extracts were dried with anhydrous sodium sulfate, derivatized with ethereal diazomethane to convert carboxylic acids into methyl esters, exchanged into high-purity CH_2Cl_2, and analyzed by capillary gas chromatography-mass spectrometry in electron ionization mode (Beller & Spormann 1997a).

Southern hybridization

Ten-μg portions of *G. metallireducens* genomic DNA were subjected to digestion using three different restriction endonucleases: *Eco*RI, *Kpn*I and *Hin*dIII. One-half of each reaction (5 μg) was run on a 1% agarose gel along with genomic DNA from *T. aromatica* strain K172 digested with *Eco*RI as a positive control. The other half was stored at 4 °C for subsequent cloning. After electrophoresis, the gel was stained with ethidium bromide and visualized. The DNA was then transferred to a Hybond-N+ membrane (Amersham Pharmacia, Piscataway, NJ) in 20X SSC buffer (3 M sodium chloride, 0.3 M sodium citrate) by overnight capillary transfer and fixed by UV cross-linking. The membrane was pre-hybridized for 1 hr at 65 °C in a solution containing 7% sodium dodecyl sulfate (SDS), 0.5 M Na_2HPO_4 (pH 7.2), and 1 mM EDTA (Church & Gilbert 1984). The probe was generated by random-prime labeling a 1060-bp PCR product amplified from the *bssA* gene of *T. aromatica* strain K172 (GenBank Acc. No. AJ001848, position 8007-9066). The labeling was performed using a RediprimeII kit (Amersham Pharmacia) with 50 μCi [α-^{32}P]-dCTP (62.5 pmol) according to manufacturer's protocols. The probe was purified from unincorporated label and primers using a Microspin S-400HR column (Amersham Pharmacia), added to the prehybridization solution, and the membrane was hybridized overnight at 65 °C. High stringency washes were performed as follows (65 °C): 2X SSC, 0.1% SDS two times for 5 min; 1X SSC, 0.1% SDS for 10 min; and 0.1X SSC, 0.1% SDS three times for 15 min. The membrane was imaged using a Phosphorimager SI (Molecular Dynamics, Sunnyvale, CA).

DNA cloning and sequencing

The remaining 5 μg of *G. metallireducens* genomic DNA digested with each restriction endonuclease was subjected to electrophoresis as described and fragments that corresponded to the same size as those

that hybridized with the *bssA* probe were excised from the gel. The DNA fragments were extracted using a QIAquick gel extraction kit (Qiagen, Valencia, CA) and cloned into pPCR-Script Amp vector (Stratagene, La Jolla, CA) after making the ends blunt with *Pfu* DNA Polymerase (with 3′ to 5′ exonuclease activity), as described by the manufacturer. Transformants were selected on Luria-Bertani (LB) agar plates with 100 μg/mL carbenicillin and 32 μg/mL X-gal. White colonies were picked, replica-plated, and transferred to nitrocellulose membranes (Life Technologies, Rockville, MD) for hybridization with the 1060-bp *bssA* probe, as described above. Positive and negative controls included the 1060-bp *bssA* PCR product from strain K172 and the cloning vector, respectively. Plasmid DNA was isolated from clones with positive probe results using a Plasmid mini kit (Qiagen). DNA was sequenced using the ABI Prism BigDye^{TM} terminator sequencing kit on a Model 377 automated sequencer (Applied Biosystems, Foster City, CA). M13 reverse and T7 primers were used to sequence the ends of the inserts and internal primers were designed based on the initial sequence data. Alignment of sequences was performed with MacVector 7.0 software (Accelrys, San Diego, CA).

Results and discussion

In vitro BSS activity

Permeabilized cells of *G. metallireducens* catalyzed the formation of deuterium-labeled benzylsuccinate from labeled toluene and fumarate. A kinetic study of this reaction is depicted in Figure 1. A linear regression of these data ($r^2 = 0.99997$) indicated a rate of 1.4 nmol min^{-1}, or a specific rate of 3.4 nmol min^{-1} mg protein^{-1}, which is similar to specific rates determined for other anaerobic, toluene-degrading bacteria (e.g., 2.5 to 4.9 nmol min^{-1} mg protein^{-1}; Beller & Spormann 1997a, b). The specific *in vitro* rate of benzylsuccinate formation in *G. metallireducens* accounted for ~45% of the specific *in vivo* rate of toluene consumption; this *in vitro*/*in vivo* ratio is among the higher values that have been observed for diverse toluene-degrading cultures. Previously reported ratios have ranged from 1 to 90%, with only two cultures having ratios ≥45% (Beller & Edwards 2000). A relatively high *in vitro*/*in vivo* ratio for a given culture suggests that the BSS pathway is the dominant pathway for anaerobic toluene degradation in that culture,

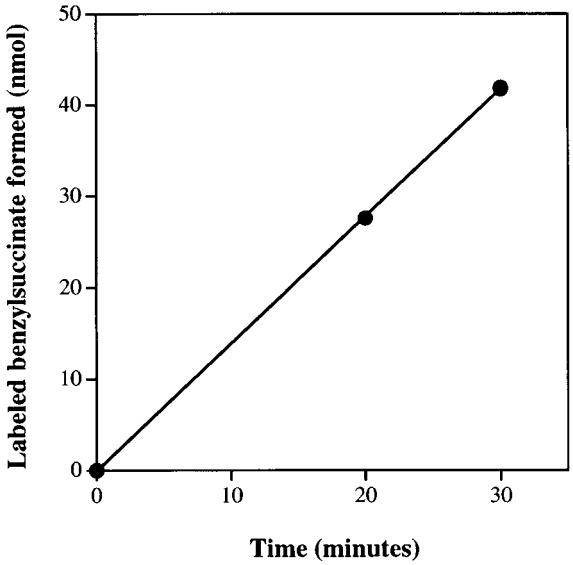

Figure 1. Kinetics of *in vitro* benzylsuccinate-d_8 formation from toluene-d_8 and fumarate by permeabilized cells of *G. metallireducens*. Note that two overlapping data points representing replicate assays are plotted at 30 min. The linear regression used to calculate the rate of benzylsuccinate formation is shown.

although a low ratio does not necessarily reflect the lack of importance of this pathway, since the enzyme may be unstable under *in vitro* assay conditions.

A distinctive characteristic of the BSS reaction observed during *in vitro* assays performed with a range of anaerobic, toluene-degrading cultures is that the H atom abstracted from the toluene methyl group during addition to fumarate is retained in the succinyl moiety of benzylsuccinate (Beller & Spormann 1997a, b; Beller & Edwards 2000; Krieger et al. 1999). This characteristic, which is consistent with the proposed reaction mechanism for BSS (Heider et al. 1998; Beller & Spormann 1998), was also observed in the present study. Specifically, mass spectral data showed conclusively that benzylsuccinate-d_8 (with a molecular ion at *m/z* 244 and a tropylium ion at *m/z* 98) was formed from toluene-d_8 and fumarate during *in vitro* assays (data not shown).

bssA hybridization and sequence analysis

In each of the three *G. metallireducens* restriction digests, a single band was observed to hybridize with the *bssA* probe from strain K172. The approximate sizes of the bands were 1.6, 8.5, and 7.5 kb for *Kpn*I, *Eco*RI, and *Hin*dIII digests, respectively. The appropriate size fragments were extracted from the second gel, and the ca. 1.6-kb *Kpn*I fragments were cloned as described.

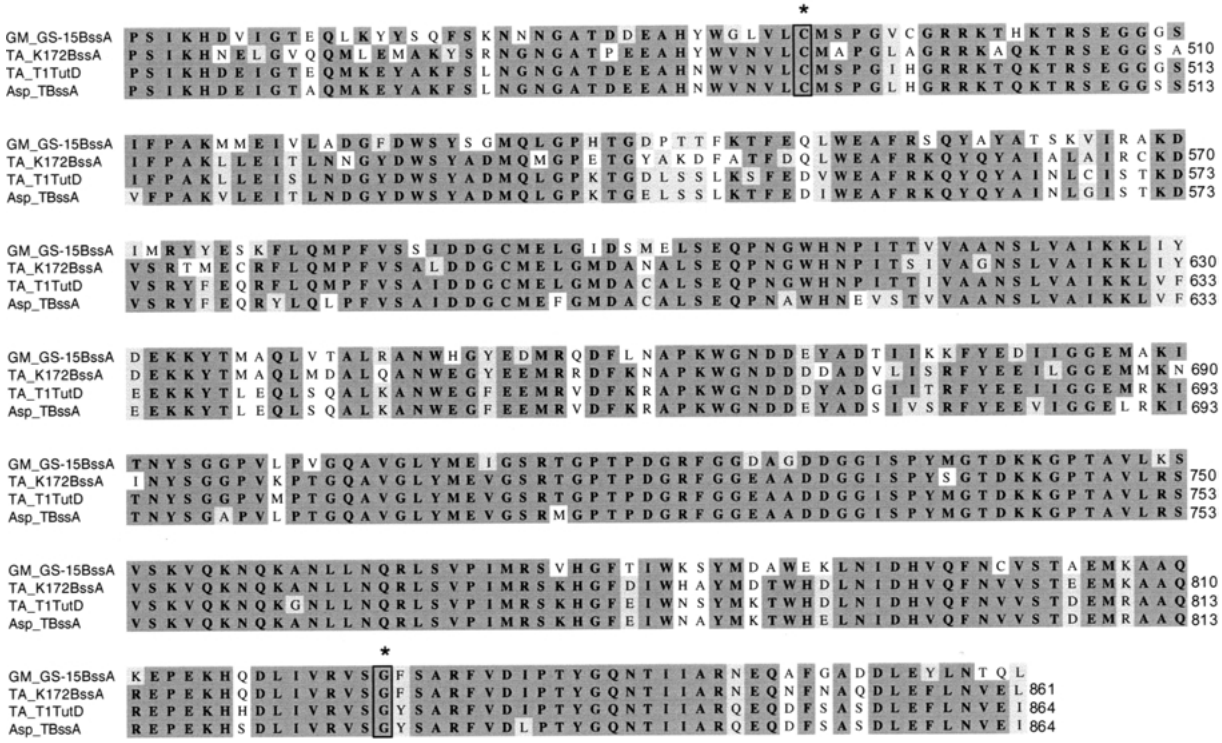

Figure 2. Alignment of the predicted amino acid sequence of the C-terminal region of BssA from *G. metallireducens* (GenBank Acc. No. AF441130) with the predicted sequences of BssA (or TutD) from *T. aromatica* strains K172 (GenBank Acc. No. AJ001848) and T1 (GenBank Acc. No. AF113168) and *A.* sp. strain T (GenBank Acc. No. AY032676). Identical residues are in boldface type and darkly-shaded; similar residues are lightly-shaded. A conserved cysteine residue (position 489, strain K172) and a conserved glycine residue (position 825, strain K172) are highlighted with asterisks; the relevance of these conserved residues to the catalytic activity of BSS is discussed in the text. Key: GM_GS-15 = *G. metallireducens* strain GS-15; TA_K172 = *T. aromatica* strain K172; TA_T1 = *T. aromatica* strain T1; Asp_T = *A.* sp. strain T.

Two hundred transformants were replica-plated and subjected to hybridization with the *bssA* probe. Two clones showed positive hybridization signals and their plasmid DNA was subsequently sequenced. Sequence analysis showed that both clones contained the same 1612-bp insert, which included 1236 bp from the C-terminal coding region of *bssA* and the entire coding region of *bssB*. Notably, *bssA* and *bssB* (or *tutD* and *tutG*) are similarly organized in the *bss* (or *tut*) operons of *A.* sp. strain T and *T. aromatica* strains K172 and T1 (Heider et al. 1998; Achong et al. 2001). The *bssA* and *bssB* sequences determined in this study have been assigned GenBank accession number AF441130.

The predicted amino acid sequence of BssA (TutD) from *G. metallireducens* is compared with those of *A.* sp. strain T and *T. aromatica* strains K172 and T1 in Figure 2. The predicted sequence from *G. metallireducens* contained 411 residues that corresponded to the C-terminal region of BssA (TutD) from strain K172 (residues 451–861) and strains T1 and T (residues 454–864). For this region of BssA, *G. metallireducens* had 74% identical and 12% similar amino acid residues with strain K172, 76% identical and 11% similar residues with strain T1, and 73% identical and 13% similar residues with strain T. The predicted C-terminal region of BssA in strains K172, T1, and T was reported to show strong sequence similarity with the glycyl radical enzymes pyruvate formate-lyase (PFL) and anaerobic ribonucleotide reductase (ARNR), including a conserved glycine at position 825 in strain K172 and position 828 in strains T1 and T, and a conserved cysteine at position 489 in strain K172 and position 492 in strains T1 and T (Heider et al. 1998; Leuthner et al. 1998; Coschigano et al. 1998; Achong et al. 2001). As expected, the predicted *G. metallireducens* BssA sequence also contained these two conserved residues (Figure 2). Evidence for the essential roles of the conserved cysteine and glycine residues in the radical catalytic activity of BSS and the analogy to their roles in PFL and ARNR have

been discussed elsewhere (Heider et al. 1998; Leuthner et al. 1998; Coschigano et al. 1998; Krieger et al. 2001). Briefly, the conserved glycine and cysteine residues are respectively the putative sites of a stable glycyl radical and a transient thiyl radical in the active site of BSS. Site-directed mutagenesis of these two conserved amino acids demonstrated that they were essential for the function of BSS (Coschigano et al. 1998), and electron paramagnetic resonance spectroscopy demonstrated the presence of a glycyl radical in active BSS (Krieger et al. 2001).

The predicted amino acid sequence of BssB (TutG) from *G. metallireducens* (GenBank Accession No. AF441130) was also compared with those of *A.* sp. strain T and *T. aromatica* strains K172 and T1. Although there were some differences in the N-terminal region, there was a high degree of similarity throughout most of the predicted amino acid sequence [of the 65 residues from the C-terminal end of BssB in *G. metallireducens*, 45 were identical with BssB (TutG) in both strains K172 and T1 and 44 were identical with BssB in strain T].

Thus, the predicted amino acid sequences of BssA and BssB (TutD and TutG) from *G. metallireducens* are similar to those from three denitrifying strains, despite the considerable phylogenetic distance that separates the ferric iron-reducing *δ-Proteobacterium* from the three denitrifying *β-Proteobacteria*. As BSS sequences from a wider range of anaerobic, toluene-degrading bacteria become available, it will become possible to evaluate the evolution of this intriguing enzyme.

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