Evidence for Benzylsuccinate Synthase Subtypes Obtained by Using Stable Isotope Tools

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We studied the benzylsuccinate synthase (Bss) reaction mechanism with respect to the hydrogen–carbon bond cleavage at the methyl group of toluene by using different stable isotope tools. A values (slopes of linear regression curves for carbon and hydrogen discrimination) for two-dimensional compound-specific stable isotope analysis (2D-CSIA) of toluene activation by Bss-containing cell extracts (in vitro studies) were found to be similar to previously reported data from analogous experiments with whole cells (in vivo studies), proving that A values generated by whole cells are caused by Bss catalysis. The Bss enzymes of facultative anaerobic bacteria produced smaller A values than those of obligate anaerobes. In addition, a partial exchange of a single deuterium atom in benzylsuccinate with hydrogen was observed in experiments with deuterium-labeled toluene. In this study, the Bss enzymes of the tested facultative anaerobes showed 3- to 8-fold higher exchange probabilities than those for the enzymes of the tested obligate anaerobic bacteria. The phylogeny of the Bss variants, determined by sequence analyses of Bssa, the gene product corresponding to the α subunit of Bss, correlated with the observed differences in A values and hydrogen exchange probabilities. In conclusion, our results suggest subtle differences in the reaction mechanisms of Bss isoenzymes of facultative and obligate anaerobes and show that the putative isoenzymes can be differentiated by 2D-CSIA.

Aromatic compounds such as alkylbenzenes are an important class of hydrocarbons occurring in crude oil-, coal-, and mineral oil-related products, or in residues of incomplete combustion events. They are widespread in the environment, and their relatively high water solubility makes them amenable for transport with the water flow. Therefore, they are commonly found in subsurface systems such as groundwater, sediments, oil, and coal deposits. The small amounts of oxygen penetrating these habitats are rapidly consumed by degradation reactions, leading to anoxic environmental conditions. Therefore, alkylbenzenes are predominantly metabolized anaerobically in subsurface environments, which is an important practical aspect of bioremediation of fuel-contaminated aquifers. One of the most critical steps in the degradation of alkylbenzenes is their initial activation in the absence of molecular oxygen, which excludes the involvement of mono- or dioxygenases as described for aerobic degradation. Toluene has been used widely as a model compound for studying anaerobic alkylbenzene metabolism. About 20 years ago, the biodegradation of toluene in the absence of oxygen was reported for the first time (1–4). Several isolates capable of anaerobic toluene degradation have been described since then, including both facultative and obligate anaerobic bacterial strains. Toluene degradation was shown to be coupled to anaerobic respiration, with nitrate, sulfate, iron(III), manganese(IV), or carbonate serving as an electron acceptor (5–10). Most of the currently known facultative anaerobic toluene degraders belong to the betaproteobacterial genera Thauera and Azoarcus, whereas most obligate anaerobic toluene degraders represent sulfate- or ferric iron-reducing Deltaproteobacteria, e.g., belonging to the genera Desulfosarcina and Geobacter. In all currently investigated anaerobic toluene degraders, the first step of the toluene degradation pathway is the formation of benzylsuccinate, resulting from addition of the methyl group of toluene to the double bond of the fumarate cosubstrate (11, 12) (see Fig. S1 in the supplemental material). Several other aromatic and aliphatic compounds can be activated anaerobically by an analogous reaction (12), and it was recently shown by sequence analysis that numerous clades of fumarate-adding isoenzymes exist that activate different substrates (13–15). Anaerobic toluene activation is catalyzed by benzylsuccinate synthase (Bss), a member of the glycol radical enzyme family, which also includes pyruvate formate lyase and anaerobic ribonucleotide reductase (16). Bss is a heterohexamer with an α2β2γ composition. The large α subunit carries the catalytically active site, which is characterized by a conserved glycine motif located near the C-terminal end of the subunit and a conserved cysteine residue in the middle of the protein sequence (17, 18).

Consequently, the gene sequence of the α subunit (Bssa) has been used to elucidate the diversity and distribution of Bss enzymes in nature (5, 13). The proposed mechanism for this reaction is given in Fig. S1 in the supplemental material. The detailed structure of Bss is hitherto unknown, and insights into the reaction mechanism are based on kinetic or theoretical studies (12, 19, 20).

Biochemical activation mechanisms for hydrocarbons can be characterized by compound-specific stable isotope analysis (CSIA) (21), making use of kinetic isotope effects (KIEs) occurring during C-H bond breakage. During such isotope fractionation, heavier isotopes are usually enriched in the residual fraction of the reactant and depleted in the product fraction. The extent of stable isotope fractionation depends largely on the isotope-sensi-
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In vitro assay with cell extract. A discontinuous enzymatic assay as described by Verfürth and colleagues (18) was performed to monitor the formation of benzylsuccinate directly by high-pressure liquid chromatography (HPLC). Toluene (final concentration, 1 mM) was dissolved in a buffer containing 5 mM fumarate and 20 mM triethanolamine-NaOH (pH 7.5) by shaking at 150 rpm for 12 h. This buffer was distributed in portions of 480 μl in anoxic 800-μl glass vials which were sealed gastight. The reaction was started by the addition of 120 μl cell extract (final concentration, 5 to 15 mg protein ml⁻¹). Samples were subsequently incubated at 30°C within an anaerobic glove box. The enzymatic reaction was stopped by adding 1% H₂SO₄ (1 M). Precipitated protein was removed by centrifugation twice at 4°C and 16,000 g for 10 min (Sorvall RC5B Plus centrifuge; Eppendorf, Hamburg, Germany). Supernatants were analyzed by HPLC using an Agilent 1200 system (Agilent, Waldbronn, Germany). To this end, 35 μl of supernatant was mixed with 75 μl distilled and filtered water (0.22-μm pore size). HPLC analyses were conducted using a Eurospher 100-5 C₁₈ column (Wissenschaftlicher Gerätebau Dr. Ing. Herbert Knauer GmbH, Berlin, Germany). The separation of the reaction mixture was carried out under isocratic conditions with 30% (vol/vol) acetonitrile in 30 mM sodium formate (pH 3.0) at a flow rate of 1 ml min⁻¹. The elution of formed benzylsuccinate was monitored spectrophotometrically by determining the UV absorbance at 210 nm. Benzylsuccinate eluted after 6.1 min, as verified by coelution of an authentic standard. The peak areas were used to determine the concentrations of benzylsuccinate, using an external calibration curve with ml benzylsuccinate.

For each organism tested in this study, in vitro assays were performed in at least 10 biological replicates for compound-specific stable isotope analysis.

Determination of toluene concentrations. Toluene concentrations were determined by automated headspace gas chromatography (GC) with a gas chromatograph (Varian 3800; Varian, Germany) coupled with a flame ionization detector. Samples were separated on a CP Sil 5 CB column (length, 25 m; inner diameter, 0.12 mm; film thickness, 0.12 μm).

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Evidence was investigated in facultative and obligate anaerobes. Usually, isotope fractionation in complex biogeochemical systems is influenced by nonfractionating processes prior to the isotope-sensitive bond cleavage (e.g., transport or binding of the substrate to the enzyme), so the resulting apparent kinetic isotope effects (AKIEs) are often smaller than their corresponding KIEs (22). Accordingly, these processes may lead to a masking of the KIEs, frequently resulting in observed variabilities of AKIEs for specific enzymatic reactions (23, 24). Masking effects can be canceled out by two-dimensional isotope fractionation analysis (2D-CSIA); in this case, the enrichments of the isotopic signatures of two elements in a residual substrate fraction during the reaction are compared and expressed by the lambda (λ) value, which is defined as the slope of the linear regression for carbon and hydrogen discrimination (25). Assuming that both isotopes are affected in similar ways (22), λ values can be regarded as fingerprints of distinct biochemical reactions, providing useful parameters for elucidating specific degradation pathways in laboratory cultures and contaminated sites (26).

In previous studies with whole cells, data from CSIA indicated that the Bss-mediated reaction is always related to a significant change in the isotopic signature of toluene (27, 28), and the cleavage of a C–H bond of the methyl group of toluene was postulated to be the isotopically sensitive step (29). 2D-CSIA has been used to compare degradation pathways of various facultative and obligate anaerobic toluene degraders. Although toluene in the investigated cultures was always activated by benzylsuccinate synthases, the determined λ values were significantly different, indicating that Bss subtypes in the investigated cultures may show slightly different reaction mechanisms. For facultative anaerobic toluene degraders, the observed λ values were generally smaller than those for obligate anaerobic toluene degraders (23). This result motivated us to investigate the isotope-sensitive steps in the overall reaction in more detail.

In this study, we analyzed whether the isotope fractionation effects observed in vivo are identical to those caused by Bss in vitro. First, isotope fractionation experiments were carried out with cell extracts of different facultative and obligate anaerobic toluene degraders, and the carbon and hydrogen isotope fraction factors (εC and εH) for toluene conversion to benzylsuccinate, as well as their respective in vitro λ values, were determined. Second, the extent of an enzymatically mediated hydrogen exchange in benzylsuccinate was investigated in facultative and obligate anaerobes.

MATERIALS AND METHODS

Chemicals. The chemicals used in this study were of the highest available purity (generally ≥99%). If not otherwise specified, the chemicals were purchased from AppliChem (Darmstadt, Germany), Fluka (Steinheim, Germany), Merck (Darmstadt, Germany), Roth (Karlsruhe, Germany), and Sigma-Aldrich (Taufkirchen, Germany). Stable isotope-labeled α,α,α-D₃-toluene was obtained from Isotec (Miamisburg, OH). Deuterium oxide was received from Armar GmbH (Leipzig, Germany). Both deuterium-labeled compounds were purchased with an isotopic purity of 99 atom% (D isotope) and a chemical purity of 99%, respectively.

Growth of bacteria and preparation of cell extracts. Thauera aromatica strain K172 (DSM 6984) (30), Azotobac sp. strain T (DSM 9506) (31, 32), Desulfoarsonia cetonica (DSM 7267) (33), and Geobacter metallireducens strain GS-15 (DSM 7210) (10) were obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). For preparing cell extracts, the strains were cultivated in 4 to 6 liters of anoxic mineral salt medium spiked with tolune as the sole source of carbon and energy. Due to its poor water solubility, toluene was supplied by a paraffin carrier phase (4 ml paraffin/liter medium). The final toluene concentration within the paraffin was 0.5 M. All cultures were incubated at 30°C. G. metallireducens was cultivated in a mineral salt medium (9) in which iron(III)-citrate-H₂O₂ (50 mM) was used as an electron acceptor. T. aromatica and Azotobac sp. were grown under denitrifying conditions with 10 mM sodium nitrate in a freshwater mineral medium as described previously by Tschech and Fuchs (34). D. cetonica was cultivated in a sulfide-reduced carbonate-buffered mineral saltwater medium (35), with 20 mM sodium sulfate as an electron acceptor. The growth of T. aromatica, Azotobac sp., and D. cetonica was monitored by measuring the optical density at 578 nm. Due to the high intrinsic absorption of iron(III)-citrate, the growth of G. metallireducens was monitored by cell counting with a Neubauer counting chamber (Karl Hecht KG, Sondheim, Germany).

All following steps (except the centrifugation steps in airtight beakers) were carried out at 25°C under strictly anoxic conditions in a glove box with an N₂-H₂ (95:5 vol/vol) atmosphere (Coy Laboratory Products Inc.). Cultures were harvested in the exponential growth phase by centrifugation at 4°C and 10,000 × g for 10 min (Sorvall RC5B Plus centrifuge with an SLA 3000 rotor; Du Pont Instruments and Sorvall, Bad Homburg, Germany) and then were suspended in 2 to 3 volumes (wt/vol) of 10 mM triethanolamine-NaOH buffer (pH 7.5; 10% [vol/vol] glycerol) containing 0.05 mg ml⁻¹ D1ase I. The suspension was transferred to a French pressure cell (American Instruments Company, MD), and the cells were lysed by one passage at 137 MPa. The lysate was centrifuged at 4°C and 100,000 × g for 60 min (Ultracentrífuge L-60 with a 90 Ti fixed-angle rotor; Beckmann, Munich, Germany). Protein concentrations of supernatants (cell extracts) were determined by the method of Bradford (36), using bovine serum albumin as a standard. The cell extracts prepared in this way were used immediately for the in vitro assay.
(Varian, Germany) with the following temperature program: 70°C for 2 min, 10°C min⁻¹ to 90°C, and 60°C min⁻¹ to 220°C. One-hundred-microliter liquid test samples were mixed with 10 ml H₂SO₄ (1.6 mM) in 20-ml glass vials; each vial was incubated for 30 min at 70°C in an agitator (rotation regimen of 250 rpm for 5 s and no rotation for 2 s) prior to analysis. One milliliter of each sample’s headspace was injected.

**Determination of stable isotope ratios of toluene.** For analysis of the stable isotope ratios of toluene, 400-µl liquid test samples were mixed with 1.6 ml distilled water in 5-ml glass vials after termination of the reaction. The remaining toluene was extracted with 1 ml n-pentane with continuous shaking at 12°C for at least 72 h. Aliquots (1 to 5 µl) of these n-pentane extracts of samples were used for isotope analyses.

The carbon and hydrogen isotope ratios of toluene were analyzed by use of an Agilent Technologies 7891A GC system (Agilent, Waldbronn, Germany) coupled with a MAT 253 stable isotope ratio mass spectrometer (Thermo Fisher Scientific Germany Ltd. & Co. KG, Bonn, Germany). Samples were separated on a Zebron ZB-1 column (length, 60 m; inner diameter, 0.23 mm; film thickness, 1 µm) (Phenomenex, Torrance, CA) with the following temperature program: 40°C for 5 min, 3°C min⁻¹ to 60°C, and 20°C min⁻¹ to 250°C, with a hold for 5 min.

The isotope ratios were expressed in delta notation (δ¹³C and δ²H) as per mille (%o) units according to equation 1.

\[ \delta^{13}C_{\text{sample}} \text{ or } \delta^2H_{\text{sample}}[‰] = \left( \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \right) \times 1000 \quad (1) \]

In equation 1, \( R_{\text{sample},i} \) and \( R_{\text{standard},i} \) are the \( \delta^{13}C \) or \( \delta^2H \) ratios of the sample and an international standard, respectively. Vienna Pee Dee belemnite (VPDB) was used as a reference for analysis of carbon isotope signatures, and Vienna standard mean ocean water (VSMOW) was used as a reference for the hydrogen isotope ratios.

The isotopic composition of each sample was measured in at least 3 technical replicates. The total analytical uncertainties for compound-specific hydrogen and carbon isotope analyses with respect to both accuracy and reproducibility were ±0.5‰ and ±0.5‰ respectively.

**Quantification of isotope fractionation.** Detailed information on determination of the enrichment factor (ε) is given in the supplemental material. For determination of the lambda value (Λ), the hydrogen versus carbon isotope discrimination (\( \Delta \delta^2H/\Delta \delta^{13}C \)), given by the equation \( \Delta \delta = \delta^2H - \delta^{13}C \), was plotted according to equation 2, and the slope of linear regression was derived (25).

\[ \Lambda = \frac{\Delta \delta^2H}{\Delta \delta^{13}C} = \frac{\delta^2H - \delta^2H_i}{\delta^{13}C - \delta^{13}C_i} \quad (2) \]

The uncertainty of the Λ value, given as the 95% confidence interval (CI), was derived from regression analysis (37).

**Determination of hydrogen atom exchange during Bss reaction.** To investigate a Bss-mediated exchange of hydrogen atoms between the educt, \( \text{H}_2\text{O} \), and the formed product, the \textit{in vitro} assay was performed with a final concentration of 1 mM \( \text{H}_2\text{O} \), α-D-\textit{d}-toluene instead of unlabeled toluene.

For analyses of the isotopic composition of benzylsuccinate, the samples were acidified with \( \text{H}_2\text{SO}_4 \) to pH 1 and extracted three times with diethyl ether. The organic phases were combined and evaporated under a continuous nitrogen stream. The residual fraction was dissolved in 450 µl methanol, and 50 µl trimethylsilylsilane (Sigma-Aldrich, Steinheim, Germany) was added. This mixture was incubated for 1.5 h at 65°C to obtain carboxylic acid methyl esters. Excesses of solvent and reagent were removed under a continuous nitrogen stream, and the remaining carboxylic acid methyl esters were dissolved in 100 µl n-hexane. Hydrogen-related isotopologues of benzylsuccinate were detected mass spectrometrically using an HP 6890 gas chromatograph coupled with an HP 5973 quadrupole mass spectrometer (Agilent Technologies, Palo Alto, CA). For separation, a Zebron BPX-5 column (length, 30 m; inner diameter, 0.32 mm; film thickness, 0.25 mm) (Phenomenex, Torrance, CA) with the following temperature program was used: 40°C for 5 min, 20°C min⁻¹ to 150°C, 2°C min⁻¹ to 250°C, and 20°C min⁻¹ to 300°C, with a hold for 2 min. Identification of benzylsuccinate was done by coelution of an authentic \( \text{L}-\text{benzylsuccinic acid} \) standard. The concentrations of molecular ions of benzylsuccinate were recorded, and the relative abundance of each deuterated species was calculated using the peak areas of mass traces and compared to the relative abundance mass traces for the corresponding mass peaks of the standard.

For analysis of the enrichment of deuterium in the medium, the water phase was treated with 1 mg activated carbon and shaken for 10 min to remove dissolved organic material; the activated carbon was subsequently removed by sedimentation. The abundance of deuterium in the water phase was analyzed by use of an elemental analyzer (EA1108; CE Instruments [formerly Carlo Erba], Rodano, Italy) with a specially designed furnace (Hekatech, Wegberg, Germany) allowing a maximum temperature of 1,550°C for pyrolysis. The EA unit was coupled to a Finnigan MAT 253 stable isotope ratio mass spectrometer (Thermo Fisher Scientific Germany Ltd. & Co. KG, Bonn, Germany). Samples were pyrolyzed in the presence of nickelized graphite (ca. 10% Ni, carbon powder [Merck, Darmstadt, Germany], mixed with nickel powder, 100 mesh [Aldrich, Steinheim, Germany]). A detailed description of the method can be found elsewhere (38-40). The hydrogen and oxygen isotope ratios of the water were reported in delta notation (δ²H, δ¹⁸O) in per mille (%) SMOW according to equation 1. The precision for the δ²H and δ¹⁸O signatures of water was better, in general, than 2‰ and 0.1‰, respectively.

To investigate a Bss-mediated exchange of hydrogen atoms between the solvent and the formed product, the \textit{in vitro} assay was performed in buffer containing 10% deuterium oxide or water with a natural abundance of hydrogen isotopes. For analyses of the isotopic composition of the metabolites, methyl esters of carboxylic acids were synthesized as mentioned above. The magnitude of deuterium incorporation into the metabolites was measured by GC-isotope ratio mass spectrometry (IRMS) analysis and expressed as atom%. Samples were separated on a Zebron ZB-1 column with the following temperature program: 40°C for 5 min, 20°C min⁻¹ to 220°C, 5°C min⁻¹ to 270°C, and 20°C min⁻¹ to 320°C, with a hold for 5 min. Identification of metabolites was done by coelution of authentic \( \text{L}-\text{benzylsuccinic acid}, \) fumaric acid, and \( \text{L}-\text{malic acid} \) standards.

**Phylogenetic analysis using BssA sequences.** BssA amino acid sequences from the investigated organisms and closely related reference strains (available in the GenBank database under the following accession numbers: AF441130, EF123664, EF123666, EU780921, EF123667, EF123662, AJ001848, CR555306, AB066263, AB285034, andAY032676; additional information regarding the lengths of the sequences is given in the supplemental material) were aligned using the ClustalW algorithm. Based on this alignment, a neighbor-joining tree was constructed in MEGA 5, using the Jones-Taylor-Thornton model and performing 1,000 bootstrap replicates (41).

**RESULTS**

Specific activities of benzylsuccinate synthases from different organisms. The specific activity of Bss in cell extracts of \textit{T. aromatica} cells grown in the presence of toluene was 19 nmol min⁻¹ mg protein⁻¹ (see Table S1 in the supplemental material). The Bss activity of cell extracts of \textit{Azoarcus} sp. strain T was marginally lower (17 nmol min⁻¹ mg protein⁻¹). Both results are in the range of previously reported Bss activities for these strains (18). Analogously, the specific activity of the Bss of \textit{D. acetovorans} cell extracts (4 nmol min⁻¹ mg protein⁻¹) showed a good agreement with the hitherto described Bss activity for this strain (29). In contrast, extracts of toluene-grown \textit{G. metallireducens} cells showed a significantly higher Bss activity (15 nmol min⁻¹ mg protein⁻¹) than that previously reported (42) (see Table S1).

Carbon and hydrogen stable isotope fractionation during toluene conversion by cell extracts. The Rayleigh equation was
used to calculate the respective enrichment factors (εC and εH). All examined cell extracts showed a significant stable isotope fractionation for carbon and hydrogen in the course of toluene conversion to benzylsuccinate (Table 1; see Fig. S2 and S3 in the supplemental material). In general, toluene concentrations and isotope signatures were fairly well correlated (with R² values between 0.72 and 0.97). In controls containing a similar concentration of proteins but deactivated Bss, a time-dependent decrease of toluene was observed, from 1.2 mM initially to 0.7 mM. However, such controls never produced benzylsuccinate and, furthermore, showed no significant time-dependent change of toluene isotopic signatures (data not shown).

The enrichment factors for carbon obtained in the assays were almost identical for T. aromatica, Azoarcus sp. strain T, and D. cetonica, falling between −2.8 and −2.9‰; the C value of −1.8‰ obtained for G. metallireducens was slightly lower. The observed hydrogen fractionation factors appeared to differ more profoundly between cell extracts of facultative and obligate anaerobic strains. The two examined facultative anaerobes showed εH values of −38‰ ± 5‰ (Azoarcus sp. strain T) and −35‰ ± 11‰ (T. aromatica), whereas the obligate anaerobes yielded εH values of −59‰ ± 19‰ (D. cetonica) and −55‰ ± 8‰ (G. metallireducens) (Table 1; see Fig. S2 and S3 in the supplemental material).

Comparing enrichment factors obtained in vitro with those determined in vivo, a complete agreement of εC and εH was observed only in the case of T. aromatica. In vitro hydrogen enrichment factors for Azoarcus sp. strain T, D. cetonica, and G. metallireducens were significantly lower than the in vivo values. The corresponding carbon enrichment factors matched for T. aromatica and D. cetonica, whereas the in vitro εC values for Azoarcus sp. strain T and G. metallireducens were considerably lower than the previously determined in vivo values (Table 1).

Two-dimensional isotope fractionation analysis. The individual hydrogen and carbon isotope enrichments were correlated by two-dimensional isotope fractionation analysis. The Δ values express the slope of the linear regression for Δδ²H versus Δδ¹³C. For all enzyme assays, a linear regression was observed for carbon and hydrogen discrimination (Δδ²H versus Δδ¹³C) (Table 1; Fig. 1). The Δ values for cell extracts of facultative toluene-degrading organisms were 15 ± 2 (Azoarcus sp. strain T) and 11 ± 3 (T. aromatica). Extracts of the obligate anaerobic toluene degraders produced significantly higher Δ values (23 ± 4 for D. cetonica and 26 ± 3 for G. metallireducens). Notably, all these Δ values were similar to the corresponding in vivo Δ values of growing cultures reported previously by Vogt et al. (23) and Tobler et al. (43) (Table 1). It therefore appears that Δ values are more robust in the face of experimental fluctuations than the individual isotope fractionation values.

Exchange of hydrogen atoms during the Bss reaction. To investigate the extent of hydrogen atom exchange during Bss reactions, two complementary experiments were done. First, the in vitro assay was performed using α,α,α-D₃-toluene, and the formed benzylsuccinate as well as the solvent (water) was subsequently analyzed with regard to deuterium content. Second, to check whether hydrogen isotopes of the solvent (water) were incorporated into benzylsuccinate, the in vitro assay was performed with unlabeled toluene in the presence of deuterium oxide.

Experiments using α,α,α-D₃-toluene. The molecular ion peak pattern of a derivatized unlabeled benzylsuccinate standard
is shown in Fig. 2A. It is composed of a main signal at m/z 236 and three minor signals, at m/z 237, m/z 238, and m/z 239, with relative abundances of 86%, 12%, 1.5%, and 0.1%, which represent the $^{13}$C and $^{18}$O stable isotope signatures expected from the natural isotope abundance. This characteristic pattern with comparable relative abundances was also observed in benzylsuccinate formed by cell extracts and unlabeled toluene (data not shown). As expected from previous reports (11), the benzylsuccinate produced from $\alpha$,$\alpha$,$\alpha$-D$_3$-toluene revealed an overall shift of m/z 3 (D$_3$-benzylsuccinate) compared to unlabeled benzylsuccinate (see Fig. S4 in the supplemental material). Therefore, the pattern of the benzylsuccinate molecular ion peaks was shifted to m/z 239, m/z 240, m/z 241, and m/z 242 (Fig. 2B). In addition to these expected mass peaks, three further peaks, at m/z 236, m/z 237, and m/z 238, were recorded. The peaks at m/z 236 and m/z 237 showed the same ratio as the equivalent mass peaks in samples with unlabeled benzylsuccinate, which can be explained by the presence of remaining unlabeled toluene in the extracts carried over from cell cultivation.

However, the peaks at m/z 238 were consistently larger than the equivalent mass peaks of unlabeled benzylsuccinate, suggesting that they represent the production of significant amounts of D$_3$-benzylsuccinate from $\alpha$,$\alpha$,$\alpha$-D$_3$-toluene (Table 2). This suggested that one hydrogen atom of the product was partially exchanged during the course of the Bss reaction. Notably, the peak at m/z 238 was 3- to 8-fold more abundant in experiments with the facultative anaerobes T. aromatica and Azurarcus sp. strain T than in those with the obligate anaerobes D. cetonica and G. metallireducens (Table 2). Thus, the Bss of the tested facultative anaerobes showed a higher tendency for hydrogen exchange than the Bss of the tested obligate anaerobes. The observed partial loss of deuterium from $\alpha$,$\alpha$,$\alpha$-D$_3$-toluene was not accompanied by a significant parallel deuterium enrichment in the solvent, probably because the detection threshold was not reached (data not shown).

**Experiments using deuterium oxide.** The overall exchange of hydrogen between water and benzylsuccinate was additionally analyzed by performing the *in vitro* assay in the presence of 10% deuterium oxide with cell extracts of T. aromatica, which had generated the largest amount of D$_2$-benzylsuccinate in the previous experiments. The produced benzylsuccinate showed an $^2$H fraction of 0.013% in control samples without added deuterium oxide, whereas the fraction shifted to 0.318% when deuterium oxide was present, suggesting that deuterium atoms of the solvent were indeed incorporated into benzylsuccinate. Notably, two further deuterium-enriched compounds were detected in these experiments. Based on comparison with authentic standards, these compounds were identified as fumarate and malate; fumarate shifted from an $^2$H fraction of 0.024 to 0.595%, and malate shifted from 0.025 to 1.080%. Because of this extensive isotope exchange in the cosubstrate, the exchange values in benzylsuccinate have to be treated with caution.

**BssA sequence diversity.** The phylogenetic affiliation of the BssA amino acid sequences revealed a clustering into two major branches. One branch represents the Bss of facultative anaerobic toluene degraders, including different *Thauera* and *Azurarcus* species, whereas the other branch contains the enzymes of obligate anaerobic toluene degraders, such as *Geobacter* species and different sulfate-reducing bacteria (Fig. 3). Notably, the phylogenetic clustering of the BssA sequences correlated with biochemical

![FIG 2 Distributions of the molecule ion peaks of an unlabeled benzylsuccinate standard (A) and benzylsuccinate formed using a cell extract of *T. aromatica* and $\alpha$,$\alpha$,$\alpha$-D$_3$-toluene (B).](http://jb.asm.org/)

<table>
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<th>Mass peak (m/z)</th>
<th>Benzylsuccinate standard</th>
<th>T. aromatica (nitrate reducer)</th>
<th>Azurarcus sp. strain T (nitrate reducer)</th>
<th>D. cetonica (sulfate reducer)</th>
<th>G. metallireducens (ferric iron reducer)</th>
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*For comparison, the distribution of molecule ion peaks of an unlabeled benzylsuccinate standard is listed.*
characteristics expressed by the observed differences in $\Lambda$ values and hydrogen exchange probabilities of the reaction.

**DISCUSSION**

Stable isotope fractionation indicates the existence of two functionally distinct Bss subgroups. It was observed in previous *in vivo* studies that facultative and obligate anaerobes produced different two-dimensional isotope fractionation factors ($\Lambda$ values) during toluene conversion (23, 43, 44). Here we show that the former results likely display actual differences in the Bss reaction mechanisms, since cell extracts produced similar $\Lambda$ values under *in vivo* conditions (Table 1). Moreover, the differences in $\Lambda$ values between obligate and facultative microorganisms also correlate with differences in the phylogenetic clustering of BssA sequences (Fig. 3). Since the observed isotope fractionation was caused solely by the C-H bond cleavage reaction of toluene (see Fig. S1C in the supplemental material), and the reaction is believed to become irreversible after the addition of fumarate to the benzyl radical (19) (see Fig. S1D), the isotope fractionation patterns (characterized by the $\Lambda$ values) are probably influenced by partial reactions preceding the C-H bond cleavage reaction, thus generating various AKIE values. Therefore, we propose that toluene transformation by Bss isoenzymes of obligate and facultative anaerobes may occur via slightly different reaction paths leading to the rate-limiting transition state preceding the C-H cleavage (23).

The current mechanistic model for the Bss reaction mechanism predicts that the cleavage of the C-H bond is preceded by the formation of a thiol radical intermediate (see Fig. S1B in the supplemental material). Hence, effects regarding this partial reaction would have a major impact on the overall isotope fractionation. As the reaction mechanism characteristics of the investigated Bss enzymes match their phylogenetic affiliations (Fig. 3), we suggest that small sequence variations between the investigated facultative and obligate anaerobes affect either the rates of C-H bond cleavage or those of the preceding thiol radical generation. To our best knowledge, this is the first report correlating phylogenetic information to enzymatic reactivity by use of cell-free assays and two-dimensional isotope fractionation analysis. Our observations are supported by the results of Callaghan and colleagues (14) and von Netzer and colleagues (45), which indicate that the diversity of Bss-A-like sequences in environmental samples may reflect the presence of several Bss isoenzymes in nature. However, only protein exchanges modifying the active center are expected to affect stable isotope fractionation patterns. In the absence of structural information on Bss, the effects on hydrogen exchange and isotope fractionation observed in this study cannot be linked with the sequence variations and rationalized yet. In any case, our results indicate that $\Lambda$ values are useful tools for fingerprinting specific mechanistic subtypes of Bss. In a similar approach, Nikolausz and colleagues (49) determined different *in vivo* carbon isotope fractionation factors of dichloromethane (DCM) degradation for different strains and conditions; they showed that the variability of isotope fractionation was related to different isoenzymes of the DCM-transforming enzyme DCM dehalogenase.

Notably, the individual measured carbon and hydrogen enrichment factors ($\varepsilon$) expressing the extent of bulk isotope fractionation were slightly lower in the *in vitro* analyses than the *in vivo* data for three of the four investigated species; only *T. aromatica* Bss showed similar *in vivo* and *in vitro* enrichment factors. These observed differences between *in vivo* and *in vitro* carbon and hydrogen enrichment factors might be explained by rate-limiting factors proceeding during the *in vivo* assay, as previously reported, e.g., for methane oxidation by whole cells (46). In contrast, the observed $\Lambda$ values agreed very well between *in vivo* and *in vitro* experiments. These values are not expected to be affected by rate limitations, since the isotopic composition of different elements of one compound is biased similarly (22). Accordingly, the investigation of conserved $\Lambda$ values corroborates the strength of the two-dimensional isotope fractionation analysis approach compared to one-dimensional approaches.

**Exchange of hydrogen atoms during Bss reaction using deuterium-labeled toluene.** Mass spectroscopic analysis of benzylsuccinate formation with $\alpha,\alpha,\alpha$-D$_3$-toluene indicated that none of the tested Bss enzymes fully retained the deuterium atoms in the product as previously reported (47), but they always replaced one deuterium partially with hydrogen (Table 2). The loss of deuterium atoms was calculated to amount to 0.3 to 2.9% of total benzylsuccinate by the relative abundances of the peaks at $m/z$ 238. The correlated hydrogen exchange rate turned out to be too low to be traced as a gain of deuterons in the solvent. The deuteron ex-
change rates appeared to be a second physiological property discriminating the Bss enzymes from facultative and obligate anaerobes: the enzymes of *T. aromaticum* and *Azourcus* sp. strain T showed a 3- to 8-fold higher deuterium exchange rate than those of *D. cetonica* and *G. metallireducens* (Table 2). The most prominent opportunity for hydrogen exchange during the postulated enzymatic mechanism of Bss (11) might occur at the thiol group of the active-site cysteine. The observed exchange would then occur after abstraction of the deuterium atom from toluene to form the benzyl radical (see Fig. S1C in the supplemental material) and before the deuterium atom is transferred back to the benzylsuccinyl radical to form benzylsuccinate (see Fig. S1E). The hydrogen abstraction and its subsequent readdition are the only isotopically sensitive bond changes in the Bss reaction, and previously determined isotope effects of the overall reaction suggest that the hydrogen abstraction from toluene is a kinetically significant step (20).

**Hydrogen exchange during the Bss reaction using deuterium oxide.** The hydrogen isotope signature of the benzylsuccinate formed using deuterium oxide showed an incorporation of deuterium atoms into the product of the Bss reaction. However, the deuterium-labeled metabolites fumarate and malate were also detected, indicating that deuterium-labeled benzylsuccinate would also have been produced by an indirect process via the cosubstrate fumarate. In our cell-free *in vitro* assays, all expressed soluble enzymes are expected to be active. Thus, we suppose that the observed deuterium enrichment of benzylsuccinate is caused primarily by enzymes of the citric acid cycle. In this respect, fumarate would be converted to malate by adding one molecule of water catalyzed by fumarase. Subsequently, malate dehydrogenase would oxidize malate to oxaloacetate in an NAD-dependent reaction. Since both reactions are reversible and NAD should be present in the extract, fumarate can then be regenerated from oxaloacetate, leading to deuterium exchange into fumarate. Consequently, the deuterium-enriched fumarate would be converted by the Bss, leading to deuterium-enriched benzylsuccinate. Therefore, the observed hydrogen exchange in benzylsuccinate seems to originate mainly from proton exchange in the fumarate cosubstrate, which is large enough to overcome any subtle effect caused by partial proton exchange during the Bss reaction. Metabolic recycling of fumarate was also recently observed in isotope exchange experiments using cell extracts of *T. aromaticum* strain K172 (48).

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