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Stable Hydrogen and Carbon Isotope Fractionation during Microbial Toluene Degradation: Mechanistic and Environmental Aspects†

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Primary features of hydrogen and carbon isotope fractionation during toluene degradation were studied to evaluate if analysis of isotope signatures can be used as a tool to monitor biodegradation in contaminated aquifers. D/H hydrogen isotope fractionation during microbial degradation of toluene was measured by gas chromatography. Per-deuterated toluene-d_8 and nonlabeled toluene were supplied in equal amounts as growth substrates, and kinetic isotope fractionation was calculated from the shift of the molar ratios of toluene-d_8 and nondeuterated toluene. The D/H isotope fractionation varied slightly for sulfate-reducing strain TRM1 (slope of curve [b] = −1.219), *Desulfobacterium cetonicum* (b = −1.196), *Thauera aromatica* (b = −0.816), and *Geobacter metallireducens* (b = −1.004) and was greater for the aerobic bacterium *Pseudomonas putida* mt-2 (b = −2.667). The D/H isotope fractionation was 3 orders of magnitude greater than the $^{13}C/^{12}C$ carbon isotope fractionation reported previously. Hydrogen isotope fractionation with nonlabeled toluene was 1.7 and 6 times less than isotope fractionation with per-deuterated toluene-d_8 and nonlabeled toluene for sulfate-reducing strain TRM1 (b = −0.728) and *D. cetonicum* (b = −0.198), respectively. Carbon and hydrogen isotope fractionation during toluene degradation by *D. cetonicum* remained constant over a growth temperature range of 15 to 37°C but varied slightly during degradation by *P. putida* mt-2, which showed maximum hydrogen isotope fractionation at 20°C (b = −4.086) and minimum fractionation at 35°C (b = −2.138). D/H isotope fractionation was observed only if the deuterium label was located at the methyl group of the toluene molecule which is the site of the initial enzymatic attack on the substrate by the bacterial strains investigated in this study. Use of ring-labeled toluene-d_5 in combination with nondeuterated toluene did not lead to significant D/H isotope fractionation. The activity of the first enzyme in the anaerobic toluene degradation pathway, benzylsuccinate synthase, was measured in cell extracts of *D. cetonicum* with an initial activity of 3.63 mU (mg of protein)$^{-1}$. The D/H isotope fractionation (b = −1.580) was 30% greater than that in growth experiments with *D. cetonicum*. Mass spectroscopic analysis of the product benzylsuccinate showed that H atoms abstracted from the toluene molecules by the enzyme were retained in the same molecules after the product was released. Our findings revealed that the use of deuterium-labeled toluene was appropriate for studying basic features of D/H isotope fractionation. Similar D/H fractionation factors for toluene degradation by anaerobic bacteria, the lack of significant temperature dependence, and the strong fractionation suggest that analysis of D/H fractionation can be used as a sensitive tool to assess degradation activities. Identification of the first enzyme reaction in the pathway as the major fractionating step provides a basis for linking observed isotope fractionation to biochemical reactions.

Biological isotope fractionation leads to unequal distribution of heavier and lighter isotopes between the educts and products of a reaction. In most cases, the lighter isotopes are preferentially used and the heavier isotopes are enriched in the residual substrate fraction. This is a well-known phenomenon for CO_2 fixation during photosynthesis (25) and methanogenesis (11, 17) and for methane oxidation (5, 8, 36). In recent years, isotope fractionation has been described for bacterial degradation of environmental contaminants, such as chlorinated hydrocarbons (6, 10, 16) and aromatic compounds (1, 18, 21, 22, 32, 38). In addition, $^{13}C/^{12}C$ isotope fractionation of chlorinated and aromatic hydrocarbons was demonstrated in contaminated aquifers (9, 16, 29), and this finding was taken as an indication of microbial degradation. Assessment of isotope fractionation was therefore discussed as a method for monitoring biological degradation directly in the aquifer. However, in natural environments factors like redox conditions and temperature can vary and may influence isotope fractionation. To assess the contributions of bacterial degradation activities in situ to natural attenuation, further understanding of the possible effects of physical and chemical parameters on isotope fractionation has to be attained.

In addition to carbon isotope fractionation of organic contaminants, some studies have reported on chlorine (34) or deuterium/hydrogen isotope fractionation (8, 35). Kinetic D/H isotope fractionation has been shown to be 2 orders of mag-

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nitude greater than carbon isotope fractionation (8). However, single-compound isotope analysis by gas chromatography-combustion-isotope ratio monitoring mass spectrometry (GC-C-IRMS), which is the standard method used for carbon isotope analysis (12, 23), became available for hydrogen fractionation only recently (14), and hydrogen isotope analysis is still more expensive and less precise than carbon isotope analysis.

Here, we describe a method for investigating D/H isotope fractionation during bacterial toluene degradation by gas chromatography (GC). The depletion of lighter toluene species and the enrichment of labeled toluene in the residual fraction were determined to assess hydrogen isotope fractionation. The effects of different electron acceptors or temperatures on isotope fractionation were checked, and the major fractionating step in anaerobic bacterial toluene degradation was identified. Furthermore, the proposed reaction mechanism of benzylsuccinate synthase was confirmed by D/H isotope analysis with GC and mass spectroscopy.

MATERIALS AND METHODS

Strains and cultivation. Sulfate-reducing strain TRM1 was isolated in our laboratory (20), and *Desulfobacterium cetonicum* DSM 7267, *Thauera aromatica* K172 (= DSM 6984), and *Geobacter metallireducens* GS-15 (= DSM 7210) were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). *Pseudomonas putida* mt-2 was a kind gift from J. R. van der Meer, Dübendorf, Switzerland.

Anaerobic strains were cultivated in carbonate-buffered freshwater mineral medium, pH 7.2 to 7.4 (37). This medium was prepared in the absence of oxygen under an N_2-CO_2 atmosphere (80:20) and was reduced with Na_2S (1 mM). Either Na_2SO_4 (10 mM) or $NaNO_3$ (10 mM) was added as an electron acceptor. $FeCl_2$ (3 mM) was added to the medium for strain TRM1 in order to scavenge the sulfide produced. The same freshwater medium was used to cultivate *Geobacter metallireducens*, but $FeCl_2$ (1 mM) was added as a reducing agent instead of sulfide and Fe(III) citrate (50 mM) was used as an electron acceptor. Aerobic bacteria were cultivated in mineral medium M9 (30). Serum bottles (120 and 500 ml) were half filled with mineral medium and tightly sealed with Viton rubber stoppers (Maag Technik, Dübendorf, Switzerland). Toluene was injected into the culture bottles with syringes through the stoppers and was allowed to equilibrate overnight before inoculation. All substrates, including nonlabeled toluene, perdeuterated toluene-d_8 (Fluka, Buchs, Switzerland), ring-deuterated toluene-d_5, and methyl-deuterated toluene-d_3 (Campro Scientific, Berlin, Germany), were analytical grade. Growth experiments were performed in triplicate at 30°C unless indicated otherwise. Aerobic cultures were shaken at 100 strokes min^{-1}.

Analysis and sampling. Growth of sulfate-reducing bacteria and *G. metallireducens* was monitored by measuring the formation of sulfide or Fe(II) (7, 33). Growth of all other strains was determined by measuring the increase in optical density at 578 nm. Toluene concentrations were analyzed by high-performance liquid chromatography (System Gold; Beckman, Fullerton, Calif.) with a C_{18} reversed-phase column (GROM-SIL; ODS-5 ST; length, 250 mm; particle size, 5 μm; Grom, Herrenberg, Germany) and UV detection (206 nm), using acetonitrile–100 mM ammonium phosphate buffer (pH 3.5) (70:30, vol/vol) as the eluent. Culture samples (250 μl) were diluted 1:5 with ethanol (99.9%, p. a.) and centrifuged (14,000 × *g*, 5 min) to remove precipitates.

Isotope analysis. D/H isotope fractionation in experiments with mixtures of deuterium-labeled toluene and nonlabeled toluene could be assessed by separation of the different toluene species in a GC capillary column. GC-C-IRMS is sensitive enough to detect changes in the deuterium ratio in the per mille range and was used to analyze D/H isotope fractionation in experiments with nonlabeled toluene. $^{13}C/^{12}C$ isotope fractionation was also measured by GC-C-IRMS.

Liquid samples (2 to 7 ml) for GC analysis were taken with a syringe through the stoppers and were extracted with 0.3 ml of pentane (Suprasolve; Merck, Darmstadt, Germany). Aliquots (4 μl) of the pentane phase were analyzed in three replicates with a GC equipped with a flame ionization detector (Carlo Erba Instruments, Milan, Italy). The GC was equipped with a fused silica capillary column (optima δ-3; length, 60 m; inside diameter, 0.32 mm; film thickness, 0.35 μm; Macherey-Nagel, Düren, Germany). Baseline separation of the different toluene species was achieved at 60°C and 80 kPa with a N_2 flow rate of 1.5 ml min^{-1}. Samples were injected into the heated injector (200°C) with a split of 1:15.

$^{13}C/^{12}C$ isotope ratios were determined by GC-C-IRMS. The system consisted of a GC (HP-5890; Hewlett-Packard Co., San Diego, Calif.) which was connected via a combustion unit (GC-combustion interface; Finnigan, Bremen, Germany) to an isotope mass spectrometer (Finnigan MAT 252; Finnigan). The GC was equipped with a fused silica capillary column (BPX-5; length, 50 m; inside diameter, 0.32 mm; film thickness, 0.5 μm; SGE, Darmstadt, Germany). The temperature program consisted of 2 min at 40°C , followed by a linear increase to 180°C at a rate of 6°C min^{-1}. Samples were injected splitless at 250°C.

D/H isotope ratios were determined with an Isochrome GC-C-IRMS system (Micromass, Manchester, United Kingdom) consisting of a GC unit which was connected via a chromium furnace to a mass spectrometer (Isoprime, Manchester, United Kingdom). The temperature of the furnace was adjusted to 1,800°C. Samples were injected splitless at 20°C in a KAS4 cooled on column injector (Gerstel, Germany), and subsequently the injector was heated to 300°C at a rate of 12°C s^{-1}; the temperature was kept at 300°C for 5 min. The GC conditions were the same as those described above for determination of carbon isotope ratios.

Calculations. Calculations were based on the Rayleigh equation for closed systems, which was developed to describe distillation processes (28) and was adapted for isotope fractionation (equation 1) (15). D/H isotope fractionation with labeled toluene was determined by calculating the GC peak areas of pentane-extracted toluene species. The hydrogen isotope ratio (R_t) was the quotient of the concentrations of toluene ([toluene-d_8]/[toluene]) at time *t*. If the logarithms of the isotope ratios (R_t/R_0) were plotted over the respective logarithms of the concentrations (c_t/c_0), according to equation 2 (15, 22, 29), the kinetic isotope fractionation factor (α) could be determined from the slopes of the curves (*b*), with $b = 1/\alpha - 1$ (equation 3). c_t and c_0 were the total toluene concentrations at time *t* and time zero, respectively. When only nondeuterated toluene was supplied in isotope fractionation experiments, isotope ratios (R_t) were determined from the common δ values with equations 4 and 5 (22). The international PDB and SMOW standards were used to calculate δ values from GC-C-IRMS analysis data. δ_0 was the initial isotope signature of the substrate.

$$R_t/R_0 = (c_t/c_0)^{(1/\alpha - 1)} \quad (1)$$

$$\ln (R_t/R_0) = (1/\alpha - 1) \times \ln (c_t/c_0) \quad (2)$$

$$b = 1/\alpha - 1 \quad (3)$$

$$\delta_t = \frac{(R_{\text{sample}} - R_{\text{standard}})}{R_{\text{standard}}} \times 1{,}000 \quad (4)$$

$$(\delta_t + 1{,}000)/(\delta_0 + 1{,}000) = R_t/R_0 \quad (5)$$

The fractionation factor (α) is a nonlinear function of *b* given in $\alpha = 1/(b + 1)$ and not defined if $b = -1$. For carbon isotope fractionation during toluene degradation, *b* usually was between −0.001 and −0.01, a range where α is almost linearly dependent on *b*. The slopes of D/H isotope fractionation were within a range of −5 to 0. For *b* approaching −1, α diverged against $\pm\infty$ and became an abstract term for quantitative descriptions. It is therefore more illustrative to describe D/H fractionation by the slope, which is used throughout this paper. The slope decreases with increasing isotope fractionation. The results of $^{13}C/^{12}C$ isotope fractionation experiments are given in the common α notation.

Benzylsuccinate synthase assay. A culture of *D. cetonicum* (0.8 liter; optical density at 578 nm, 0.3) was harvested in the late exponential growth phase. The cells were washed in anoxic potassium phosphate buffer (50 mM; pH 7.5) which had been supplemented with NaCl (175 mM) and $MgCl_2$ (25 mM), reduced with H_2 (10^5 Pa)–palladium-charcoal (1 mg $liter^{-1}$) (Merck, Darmstadt, Germany) and titanium(III) citrate (1 mM), and filtered. Cells were suspended in 3 ml of buffer and broken with a French pressure cell (9 MPa) in the presence of 2 mM fumarate. Cell debris was removed by centrifugation (14,000 × *g*, 5 min). The cell extract was diluted to a volume of 6 ml with buffer. One milliliter of a solution containing 0.5 mM toluene-d_8 and 0.5 mM toluene (each in reduced potassium phosphate buffer) was added to the diluted extract, and the preparation was mixed.

The benzylsuccinate synthase test was performed at 30°C in 8-ml glass vials sealed with Viton rubber stoppers. At each sampling time, 500 μl of buffer was injected into the reaction vial and mixed. Subsequently, a 500-μl sample was removed with a syringe through the stopper, and aliquots (200 μl) of the sample were transferred into two 1.7-ml glass vials. The reaction was stopped with ice-cold 1 M NaOH (20 μl), and the mixture was overlaid with 100 μl of pentane (Suprasolve) containing 2.5 mM ethylbenzene (Fluka, Buchs, Switzerland) as an

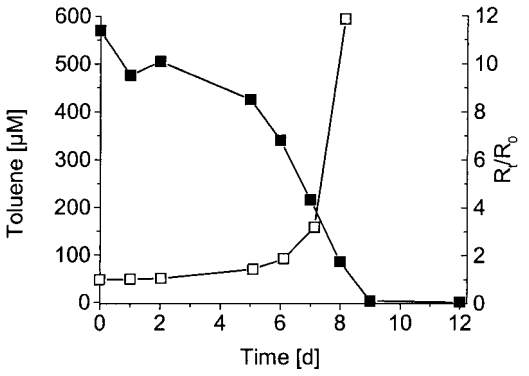

FIG. 1. Substrate conversion during growth of *D. cetonicum* with a mixture of 50% toluene-d_8 and 50% nonlabeled toluene as the sole carbon and energy source. Toluene concentrations (■) and related toluene-d_8/toluene ratios in the residual nondegraded substrate fraction (□) were monitored over time. R_t is the isotope ratio calculated as follows: [toluene-d_8]/[toluene].

internal standard. A control assay was run without cell extract. Toluene-d_8/toluene ratios and overall toluene concentrations were determined by GC as described above. The aqueous phase remaining from the extraction was diluted fivefold with ethanol, and benzylsuccinate was analyzed by high-performance liquid chromatography as described above with acetonitrile-ammonium phosphate buffer (30:70) as the eluent. Protein concentrations were determined with a protein assay kit (Bio-Rad, Munich, Germany). The identity of benzylsuccinate in the extracts was confirmed by coelution with a benzylsuccinate reference (Sigma, Deisenhofen, Germany).

Mass spectroscopic analysis. Benzylsuccinate was converted to its methyl ester with trimethylchlorosilane (Supelco, Bellefonte, Pa.) in methanol (8:1, vol/vol) at 60°C for 1 h. The reaction mixture was extracted with dichloromethane for a mass spectrometric analysis performed with a quadrupole system (MSD; Hewlett-Packard Co.). The GC was equipped with a fused silica capillary column (DB-1; length, 30 m; inside diameter, 0.32 mm; film thickness, 0.25 μm; J&W Scientific, Folsom, Calif.). The injection mode was splitless. The temperature program was as follows: 2 min at 60°C, followed by an increase to 250°C at a rate of 4°C min^{-1}.

RESULTS

Separation of isotopic toluene species. A method was developed to determine D/H isotope fractionation upon toluene degradation by GC analysis. Batch cultures were grown with mixtures of deuterated toluene-d_8 and nonlabeled toluene (50:50, vol/vol). The nondegraded residual toluene fraction in the cultures was extracted with pentane and analyzed by GC. The different toluene species were separated by GC; the elution time for toluene-d_8 was 13.8 min, and this compound was followed by methyl-labeled toluene-d_3 (13.95 min), ring-labeled toluene-d_5 (14.0 min), and nonlabeled toluene (14.2 min). During growth with a mixture of per-deuterated toluene-d_8 and nonlabeled toluene, the different bacterial strains degraded nonlabeled toluene faster, and consequently the hydrogen isotope ratios (R_t) of [toluene-d_8] to [toluene] increased substantially, as illustrated by a degradation experiment performed with *D. cetonicum* (Fig. 1).

D/H isotope fractionation factors for various bacterial strains cultivated with toluene. The D/H isotope fractionation factors obtained in growth experiments with different bacterial strains were all of the same order of magnitude. The greatest fractionation for toluene degradation was observed in growth experiments with the aerobic bacterium *P. putida* mt-2, with fractionation of $b = -2.667 \pm 0.163$. The D/H isotope fractionation by anaerobic bacteria was slightly less, with $b = -1.219 \pm 0.254$ for sulfate-reducing strain TRM1, $b = -1.196 \pm 0.075$ for *D. cetonicum*, $b = -1.004 \pm 0.077$ for *G. metallireducens*, and $b = -0.816 \pm 0.133$ for *T. aromatica* (Fig. 2). All of the anaerobic strains showed similar degrees of isotope fractionation, although they used different electron acceptors.

D/H isotope fractionation of toluene with the natural deuterium abundance. *D. cetonicum* and strain TRM1 were grown with nonlabeled toluene, and the D/H isotope compositions were determined by GC-C-IRMS. D/H isotope fractionation of $b = -0.728$ was obtained for strain TRM1 with nonlabeled toluene; this fractionation was 1.7 times less than the fractionation observed when toluene-d_8 and nonlabeled toluene were supplied. In experiments with *D. cetonicum* grown with nonlabeled toluene, the D/H isotope fractionation was $b = -0.198$, or six times less than the value obtained in growth experiments with per-deuterated toluene-d_8 and nonlabeled toluene (50:50) ($b = -1.196$) (Fig. 2), indicating that isotope fractionation with deuterated toluene was not identical to fractionation with nonlabeled toluene but was of the same order of magnitude.

Temperature dependence of isotope fractionation. To assess the effects of temperature on isotope fractionation, bacterial D/H isotope fractionation upon toluene degradation was investigated in growth experiments performed with the aerobic bacterium *P. putida* mt-2 and per-deuterated toluene-d_8 and nonlabeled toluene in equal amounts at five different temperatures between 15 and 35°C. The highest isotope fractionation value was obtained at 20°C, with $b = -4.086 \pm 0.127$; the value decreased to $b = -2.138 \pm 0.667$ at 35°C (Fig. 3A). With the anaerobic bacterium *D. cetonicum,* the D/H isotope fractionation value differed between $b = -1.092 \pm 0.239$ at 18°C and $b = -1.260 \pm 0.009$ at 37°C, a difference which was not significant within the range of the standard deviation (Fig. 3A).

Temperature effects on carbon isotope fractionation were analyzed in growth experiments performed with *P. putida* mt-2, *D. cetonicum*, and strain TRM1 cultivated with nonlabeled toluene. Similar to D/H isotope fractionation, there was a

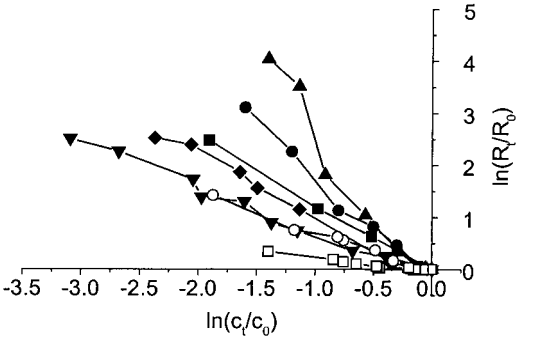

FIG. 2. D/H isotope fractionation during degradation of a mixture of toluene-d_8 and nonlabeled toluene in batch cultures. Representative curves from three replicates are shown for *P. putida* mt-2 (▲), strain TRM1 (●), *D. cetonicum* (■), *G. metallireducens* (◆), and *T. aromatica* (▼). For *D. cetonicum* (□) and strain TRM1 (○) D/H isotope fractionation was determined in batch cultures grown with nonlabeled toluene, and the natural abundance of deuterium was measured by GC-C-IRMS.

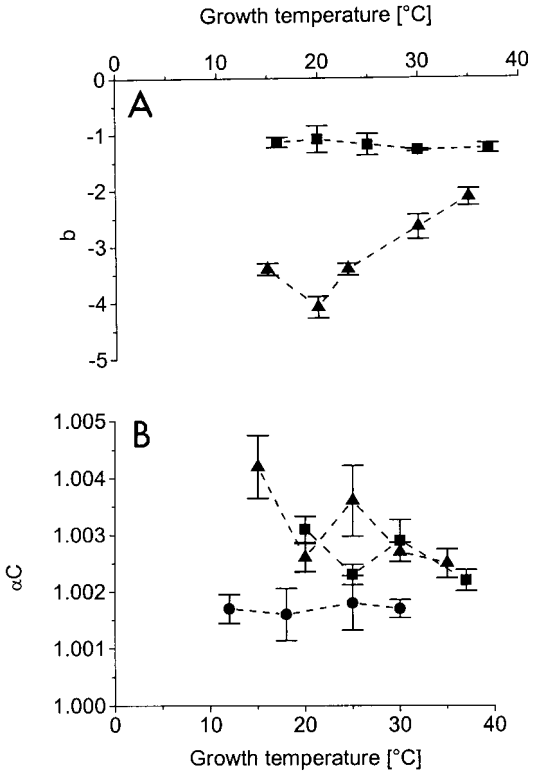

FIG. 3. Influence of temperature on D/H isotope fractionation (A) and $^{13}C/^{12}C$ isotope fractionation (B) during toluene degradation by *D. cetonicum* (■), sulfate-reducing strain TRM1 (●), and *P. putida* mt-2 (▲). (A) D/H isotope fractionation factors were determined by determining the slope ($b = 1/\alpha - 1$) of the double-logarithmic plot of equation 2. For D/H isotope fractionation, each data point represents the average fractionation factor calculated from three independent growth experiments; the error bars indicate the standard deviations. (B) Carbon isotope fractionation factors were calculated from the regression curves in a double-logarithmic plot of equation 2. Each data point represents a $^{13}C/^{12}C$ isotope fractionation factor (αC). The error bars indicate the reliability of αC calculated from the slopes ($b = 1/\alpha - 1$) of the regression curves.

slight decrease in the $^{13}C/^{12}C$ isotope fractionation factors with decreasing temperatures for *P. putida* mt-2 from $\alpha C = 1.0042 \pm 0.0006$ at 15°C to $\alpha C = 1.0025 \pm 0.0003$ at 35°C. The $^{13}C/^{12}C$ isotope fractionation factors for the anaerobic organisms *D. cetonicum* and TRM1, however, did not vary with temperature within the observed standard deviations (Fig. 3B).

Identification of the fractionating step. We examined whether the observed strong D/H isotope fractionation during bacterial toluene degradation could be attributed to the first enzyme reaction of the toluene degradation pathway or to other parameters, like substrate diffusion to the cells or substrate uptake. Therefore, different toluene species with deuterium labels either at the aromatic ring (toluene-d_5) or at the methyl group (toluene-d_3) were used as growth substrates in equal amounts with per-deuterated toluene-d_8 or nondeuterated toluene. The three bacteria used initiate degradation with an attack on the methyl group; this has been proven for *P. putida* mt-2 (31) and *D. cetonicum* (24) and is assumed for strain TRM1. *D. cetonicum* (Fig. 4), sulfate-reducing strain TRM1, and *P. putida* mt-2 showed strong D/H isotope effects ($b = -1.251$, $b = -1.280$, and $b = -4.218$, respectively) (Table 1) if methyl-deuterated toluene-d_3 was used in combination with nonlabeled toluene. When ring-deuterated toluene-d_5 was used in combination with nonlabeled toluene, the D/H isotope fractionation factors for *D. cetonicum*, strain TRM1, and *P. putida* mt-2 were negligible within the analytical errors. In addition, no D/H isotope fractionation within the standard deviations could be demonstrated for the three strains if methyl-deuterated toluene-d_3 was provided in equal amounts with per-deuterated toluene-d_8. When ring-deuterated toluene-d_5 was used in combination with per-deuterated toluene-d_8, the D/H isotope fractionation factors were $b = -0.679$ for *D. cetonicum*, $b = -0.917$ for strain TRM1, and $b = -2.696$ for *P. putida* mt-2 and thus slightly lower than the values obtained in experiments with toluene-d_3 and nonlabeled toluene.

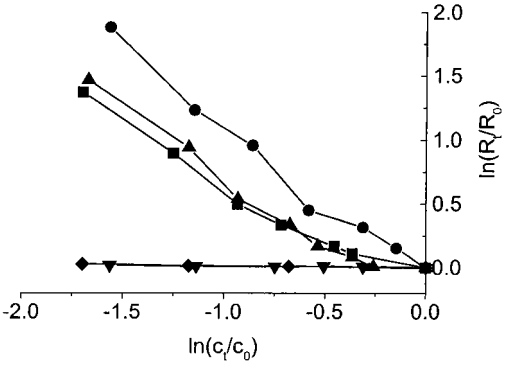

FIG. 4. D/H isotope fractionation during degradation of various combinations of selectively deuterated toluene species. Representative curves from three replicates are shown for *D. cetonicum* grown with equal amounts of toluene-d_8 and toluene-d_5 (■), with toluene-d_3 and nonlabeled toluene (●), with toluene-d_8 and nonlabeled toluene (▲), with toluene-d_5 and nonlabeled toluene (◆), and with toluene-d_8 and toluene-d_3 (▼). The curves were plotted by using equation 2.

TABLE 1. D/H isotope fractionation during growth of *D. cetonicum*, sulfate-reducing strain TRM1, and *P. putida* mt-2 with mixtures (50:50) of two selectively deuterated toluene species

Substrate mixture	*D. cetonicum*		Strain TRM1		*P. putida* mt-2	
	b	α*D*	*b*	α*D*	*b*	α*D*
Toluene-d_8 and toluene-d_3	−0.002 ± 0.003[a]	1.002 ± 0.003	0.167 ± 0.219	0.857 ± 0.148	−0.016 ± 0.003	1.016 ± 0.007
Toluene-d_3 and nonlabeled toluene	−1.251 ± 0.034	−3.991 ± 0.331	−1.280 ± 0.080	−3.572 ± 0.922	−4.218 ± 0.125	−0.311 ± 0.015
Toluene-d_8 and toluene-d_5	−0.679 ± 0.115	3,117 ± 2,947	−0.917 ± 0.336	12.005 ± 3.379	−2.696 ± 0.163	−0.590 ± 0.057
Toluene-d_5 and nonlabeled toluene	−0.005 ± 0.004	1.005 ± 0.004	−0.012 ± 0.005	1.042 ± 0.042	−0.079 ± 0.041	1.088 ± 0.048
Toluene-d_8 and nonlabeled toluene	−1,196 ± 0.075	−5.090 ± 1.990	−1.219 ± 0.254	−4.566 ± 4.145	−2.667 ± 0.163	−0.600 ± 0.652

[a] Mean ± standard deviation based on three independent growth experiments.

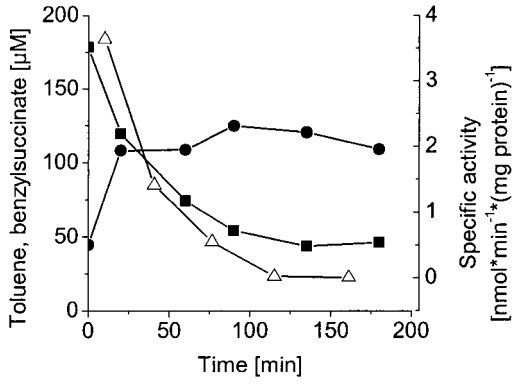

FIG. 5. Discontinuous benzylsuccinate synthase activity assay with *D. cetonicum* cell extract. The total concentrations of toluene-d_8 and nonlabeled toluene (■) and of benzylsuccinate (●) shown are means based on two samples per time point. The specific benzylsuccinate synthase activity (△) was calculated by determining the toluene degradation rate between two adjacent sampling points.

Benzylsuccinate synthase assay with *D. cetonicum*. The activity and D/H isotope fractionation of benzylsuccinate synthase were determined in discontinuous enzyme assays at 30°C. About 80 μM toluene was consumed, and 70 μM benzylsuccinate was produced (Fig. 5). At the beginning of the experiment the enzyme activity in the cell extracts was 3.63 mU (mg of protein)$^{-1}$, but the activity decreased to zero within 180 min. The maximum toluene turnover rate determined was 12% of the in vivo degradation activity (29.3 nmol min^{-1} mg of protein^{-1}). The D/H isotope fractionation obtained in the benzylsuccinate synthase assay was $b = -1.580$ with 50% toluene-d_8 and 50% nonlabeled toluene as the substrates, or 30% greater than the fractionation by growing *D. cetonicum* cells (Fig. 6). Mass spectroscopic analysis revealed that benzylsuccinate-d_8 and nondeuterated benzylsuccinate were produced. The maximum rate of nondeuterated benzylsuccinate production was 4.4 nmol min^{-1} mg of protein $^{-1}$ and was 11 times higher than the rate of production of benzylsuccinate-d_8 (0.4 nmol min^{-1} mg of protein^{-1}). The mass peaks *m/z* 243 (benzylsuccinate-d_7) and *m/z* 237 (benzylsuccinate-d_1) could not be detected.

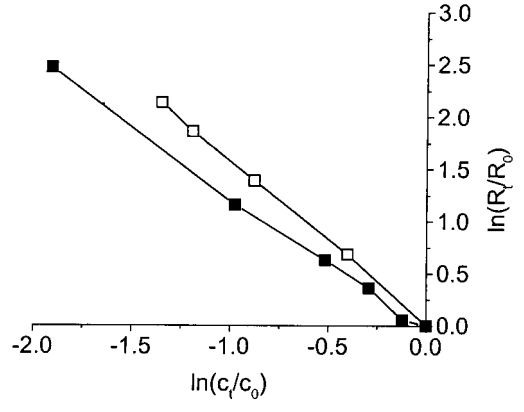

FIG. 6. Toluene D/H isotope fractionation in the cell-free benzylsuccinate synthase reaction (□) and in a growth experiment (■) with *D. cetonicum*. Equal amounts of toluene-d_8 and nonlabeled toluene were used in both experiments.

DISCUSSION

Mechanistic aspects of hydrogen and carbon isotope fractionation were studied to assess whether isotope fractionation could serve as a tool to estimate biological degradation in contaminated aquifers. Therefore, isotope fractionation was investigated with several bacterial strains using different electron acceptors during toluene degradation. The influence of growth temperature was determined, and the fractionating step in anaerobic toluene degradation was identified.

Hydrogen isotope fractionation was studied by a method in which the strains were grown on mixtures of different deuterated toluene species under defined laboratory conditions. Thus, basic questions of bacterial isotope fractionation could be determined by GC analysis without using the more expensive and time-consuming technique of single-compound isotope analysis by GC-C-IRMS.

Many biochemical reactions are known to cause significant partitioning of isotopes between substrates and products. Isotope effects not only are due to sheer mass differences but also are due to the impact of additional neutrons on the mechanism and velocity of a biochemical reaction. Direct involvement of a bond which is substituted with a heavy isotope is known as a primary isotope effect. The dissociation energy needed to cleave a heavy-isotope-substituted bond is higher and is reflected in a decrease in the reaction rate (26). Weaker, secondary isotope effects occur when the heavy-atom isotope is located close to the bond but is not directly involved in the reaction mechanism.

D/H isotope fractionation factors for various bacterial strains cultivated with toluene. In contrast to carbon isotope fractionation, which was almost the same for all of the anaerobic strains tested (22), the 100- to 1,000-times-greater hydrogen isotope fractionation factors varied among the different species within the same order of magnitude. As the mass of deuterium is 100% greater than the mass of a hydrogen atom, deuterium isotope effects are more pronounced. Small differences in the toluene-degrading enzymes are multiplied by these effects and are reflected in the variations in the D/H isotope fractionation factors for the bacterial strains studied. These variations in D/H isotope fractionation are unfavorable for quantitative evaluation of biological degradation at natural sites by analysis of hydrogen isotope ratios. Nevertheless, as the hydrogen isotope fractionation was at least 100-fold greater than the corresponding carbon isotope fractionation, D/H isotope ratios might be promising indicators for detection of low bacterial activities in natural environments.

D/H isotope fractionation of toluene for natural deuterium abundance. D/H isotope fractionation by the sulfate-reducing organisms strain TRM1 and *D. cetonicum* grown with toluene-d_8 and nonlabeled toluene was compared to fractionation when nonlabeled toluene was used as the sole carbon source, in order to determine the reliability of using labeled toluene species in D/H isotope fractionation studies. The isotope fractionation values determined for growth with nonlabeled toluene were 1.7 and 6 times lower than the values determined for growth with toluene-d_8 and nonlabeled toluene for strain TRM1 and *D. cetonicum*, respectively. The reason for this might be different reactivity of a methyl group labeled with three deuterium atoms compared to the reactivity of nonla-

beled toluene molecules which have only one deuterium atom at the methyl group. In the case of per-deuterated toluene-d_8 the primary isotope effect resulting from cleavage of a C—D bond is enhanced by secondary isotope effects caused by two additional deuterium atoms in the methyl group. For statistical reasons the natural occurrence of toluene with two or three deuterium atoms at the methyl group is negligible.

Another factor contributing to the differences in the results of isotope fractionation between nonlabeled toluene and toluene-d_8–nonlabeled toluene is the two types of isotope analysis used. Determination of isotope fractionation by GC is based on separation of methyl-labeled toluene and non-methyl-labeled toluene. Therefore, every deuterium atom of the labeled molecule is directly or indirectly subject to fractionation. GC-C-IRMS analysis of toluene for natural deuterium abundance detects every deuterated toluene molecule without taking into account the localization of the deuterium atoms. Statistically, 0.8% of all toluene molecules carry a deuterium atom. The probability that the deuterium label is located at the methyl group is 3/8, which means that five-eighths of the molecules detected are not subject to fractionation. Thus, the isotope fractionation factors determined with labeled compounds should be two to three times greater than the factors obtained with nonlabeled toluene because of the different methods used, which indeed was the case.

The relative concentrations of deuterium-labeled molecules did not influence isotope fractionation; this conclusion was reached because the natural abundance of deuterium in nonlabeled toluene was 4 orders of magnitude lower than the abundance in experiments performed with deuterated toluene. The results indicate that the use of deuterium-labeled toluene to study D/H isotope fractionation is a valid technique, although the fractionation obtained is not identical to the fractionation obtained with nonlabeled toluene.

Temperature dependence of isotope fractionation. In geosciences, temperature is a well-known parameter that affects the degree of isotope fractionation (15, 26). We examined whether temperature alters the extent of isotope fractionation during toluene degradation. No significant dependence on temperature for hydrogen or carbon isotope fractionation was observed for the anaerobic bacteria *D. cetonicum* and strain TRM1. In contrast, hydrogen isotope fractionation by *P. putida* mt-2 increased with decreasing temperature and showed the greatest fractionation at 20°C. No clear effect of temperature on $^{13}C/^{12}C$ carbon isotope fractionation by *P. putida* mt-2 could be found. The reason why the temperature effect on isotope fractionation was so small might be the temperature range of mesophilic bacterial activity (10 to 40°C), a more limited temperature range than that of geochemical processes. It has been shown that undefined cultures of methane-oxidizing bacteria exhibit greater $^{13}C/^{12}C$ isotope fractionation at 30°C ($\alpha C = 1.025$) than at 11.5°C ($\alpha C = 1.013$) (8), although thermodynamics suggests that fractionation decreases with increasing temperature. However, since in this study an undefined mixture of methanotrophic bacteria was used, the observed effect might have been due to different subpopulations with alternate enzyme systems enriched at the two different temperatures. Our results showed that variations in temperature should not significantly affect isotope fractionation in contaminated anoxic aquifers. There was also no correlation between toluene degradation rates and isotope fractionation because growth of our strains was closely linked with the growth temperature; e.g., the generation time of the sulfate-reducing strain TRM1 decreased from 25.5 days at 12°C to 5 days at 30°C (data not shown), but the $^{13}C/^{12}C$ isotope fractionation factors were identical ($\alpha C = 1.0017$).

Identification of the fractionating step. Isotope fractionation may be caused by transport of the substrate to the cell, by uptake into the cell, or by the first enzyme reaction or a subsequent enzyme reaction in the degradation pathway. Growth experiments with selectively deuterated toluene species as carbon sources were performed to identify which of the processes mentioned above is relevant for fractionation. *P. putida* mt-2, *D. cetonicum*, and strain TRM1 all initiate toluene degradation with an enzymatic attack on the methyl group (3, 4, 24, 31, 39). The greatest hydrogen isotope fractionation effects were observed if the bacteria grew with a mixture of methyl-labeled toluene-d_3 or per-deuterated toluene-d_8 and nonlabeled toluene. If the deuterium label was located on the aromatic ring or if toluene-d_3 was used in combination with per-deuterated toluene-d_8, isotope fractionation was not detectable. These findings indicate that higher molecular masses did not influence processes such as transport to the cells and substrate uptake and that the D/H isotope fractionation determined was not due to the differences in the overall molecular masses. Significant fractionation occurred only when the methyl group was labeled, which shows that the initial enzymatic attack at the methyl group was the major step which led to hydrogen isotope fractionation.

Benzylsuccinate synthase assay with *D. cetonicum*. Benzylsuccinate synthase in a cell extract of *D. cetonicum* exhibited 30% greater D/H isotope fractionation with per-deuterated toluene-d_8 and nonlabeled toluene than benzylsuccinate synthase in growing cells exhibited, confirming that the benzylsuccinate synthase reaction is the major isotope-fractionating step. This is consistent with previous reports which showed that isotope fractionation decreases when the supply of the enzyme with educts is less than saturation (11, 26, 27).

It has been proposed that benzylsuccinate synthase is a glycyl radical enzyme which abstracts one hydrogen radical from toluene in a primary step and returns the identical hydrogen to the benzylsuccinate molecule produced (Fig. 7) (2, 13, 19). To study the benzylsuccinate synthase mechanism, the products of the enzyme assay performed with per-deuterated toluene-d_8 and nondeuterated toluene in equal amounts were analyzed by mass spectroscopy. Benzylsuccinate-d_8 and nonlabeled benzylsuccinate were detected, and nondeuterated benzylsuccinate was produced 11 times faster than benzylsuccinate-d_8. Not even traces of benzylsuccinate-d_7 and benzylsuccinate-d_1 masses were detectable, which had to be expected if a hydrogen was attached to the glycyl radical enzyme before substrate binding and was exchanged with a hydrogen atom from benzylsuccinate before product release. Absence of these masses derived from transfer of hydrogen radicals to other substrate molecules was predicted from the proposed reaction mechanism (Fig. 7) (13). A similar experiment was performed earlier with the toluene-degrading, denitrifying strain T and with toluene-d_3 and nondeuterated toluene as the substrates (2). However, the hydrogen isotope fractionation effects described above were not considered. Unless the different deuterated toluene species are turned over completely, the distribution of

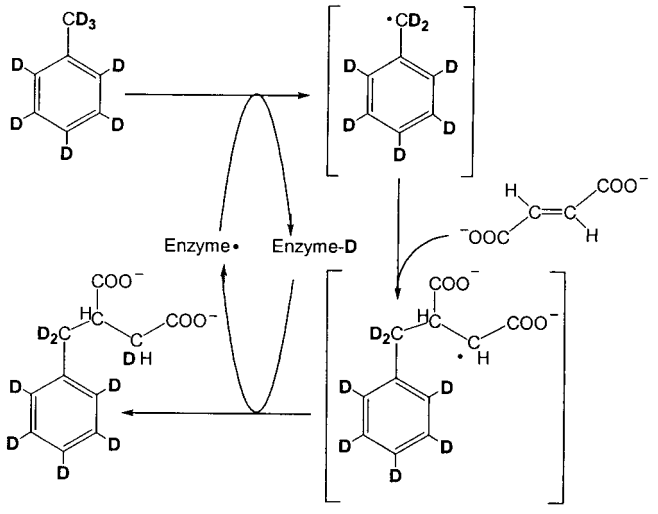

FIG. 7. Reaction mechanism of benzylsuccinate synthase as proposed by Heider et al. (13). The reaction shown is the reaction in which per-deuterated toluene-d_8 is the substrate. The deuterium radical subtracted from one molecule of toluene-d_8 by the glycyl radical enzyme is transferred from the enzyme to the same molecule to generate benzylsuccinate-d_8.

benzylsuccinate-d_3 and benzylsuccinate should be about 90:10 because of the different turnover rates. Consequently, the masses of benzylsuccinate-d_1 and benzylsuccinate-d_2 that are produced by a possible alternative enzyme mechanism must be extremely low. The distributions of benzylsuccinate species are evident only if the different reaction rates of benzylsuccinate synthase with deuterated and nondeuterated substrates are known and taken into account.

In this study, we showed that the first enzymatic reaction in anaerobic toluene degradation is the major process leading to hydrogen isotope fractionation. No significant effects of temperature or changes in the composition of the bacterial community are expected as long as anoxic conditions prevail in the aquifer. Isotope fractionation during aerobic degradation of toluene could be influenced by growth temperatures. The D/H isotope fractionation during toluene degradation was 3 orders of magnitude greater than the $^{13}C/^{12}C$ isotope fractionation for the same bacterial strains described previously, and analysis of hydrogen isotope fractionation in natural environments might be considered an appropriate tool for detecting low bacterial benzene-toluene-ethylbenzene-xylene degradation activities at contaminated sites.

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