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Stable Isotope Peptide Mass Spectrometry To Decipher Amino Acid Metabolism in *Dehalococcoides* Strain CBDB1

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Dehalococcoides **species are key players in the anaerobic transformation of halogenated solvents at contaminated sites. Here, we analyze isotopologue distributions in amino acid pools from peptides of *Dehalococcoides* strain CBDB1 after incubation with ^{13}C-labeled acetate or bicarbonate as a carbon source. The resulting data were interpreted with regard to genome annotations to identify amino acid biosynthesis pathways. In addition to using gas chromatography-mass spectrometry (GC-MS) for analyzing derivatized amino acids after protein hydrolysis, we introduce a second, much milder method, in which we directly analyze peptide masses after tryptic digest and peptide fragments by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC-ESI-MS/MS). With this method, we identify isotope incorporation patterns for 17 proteinaceous amino acids, including proline, cysteine, lysine, and arginine, which escaped previous analyses in *Dehalococcoides*. Our results confirmed lysine biosynthesis via the α-aminoadipate pathway, precluding lysine formation from aspartate. Similarly, the isotopologue pattern obtained for arginine provided biochemical evidence of its synthesis from glutamate. Direct peptide MS/MS analysis of the labeling patterns of glutamine and asparagine, which were converted to glutamate and aspartate during protein hydrolysis, gave biochemical evidence of their precursors and confirmed glutamate biosynthesis via a *Re*-specific citrate synthase. By addition of unlabeled free amino acids to labeled cells, we show that in strain CBDB1 none of the 17 tested amino acids was incorporated into cell mass, indicating that they are all synthesized *de novo*. Our approach is widely applicable and provides a means to analyze amino acid metabolism by studying specific proteins even in mixed consortia.**

Dehalococcoides species are strictly anaerobic bacteria known for the ability to use a variety of halogenated aliphatic and aromatic compounds as respiratory electron acceptors. Many of these organohalides are persistent and toxic groundwater pollutants. *Dehalococcoides* isolates use hydrogen as the sole electron donor and acetate plus bicarbonate as carbon sources. While some biochemical details of the respiratory electron chain have been studied, knowledge of the carbon metabolism of *Dehalococcoides* spp. is scarce. Sequenced and annotated genomes of several *Dehalococcoides* strains provide a basis for the generation of hypotheses for carbon metabolism but also highlight gaps in our understanding (12, 15, 21). For instance, the genome annotations lack key genes for the biosynthesis of methionine, alanine, serine, glycine, and threonine. In addition, genes may be annotated incorrectly as evidenced by the recent identification of a gene encoding a *Re*-citrate synthase previously annotated as homocitrate synthase (14). A modeling approach has used a pan-genome of all available *Dehalococcoides* sequences to develop a model for the central metabolism and growth of *Dehalococcoides* species (2). This study emphasized the need for biochemical evidence of amino acid biosynthetic pathways. Metabolic flux analysis using ^{13}C tracers has proven to be a key methodology to aid in the identification of unannotated pathways (28). To analyze amino acid isotopologue distributions after feeding with ^{13}C-labeled carbon sources (amino acid stable isotope probing [amino acid SIP]), the direct isolation of free amino acids from cell lysates and the total hydrolysis of bulk protein preparations have been described. In both approaches, amino acids are analyzed after derivatization by gas chromatography with mass spectrometric detection (GC-MS). By applying the protein hydrolysis method to cultures of *Dehalococcoides* strain 195, several amino acid biosynthesis pathways were postulated (24). However, methodological limitations such as degradation of amino acids during the hydrolysis step or weak mass spectrum signal intensities resulted in the lack of evidence for several amino acids, including proline, cysteine, lysine, histidine, arginine, and tryptophan, several of which have pivotal importance for structural or catalytic properties of proteins. In addition, the amino acid pairs glutamate-glutamine and aspartate-asparagine could not be differentiated.

Here, we investigate central metabolic pathways in our model organism *Dehalococcoides* sp. strain CBDB1 by using ^{13}C-labeled carbon sources as precursors to trace amino acid anabolism via amino acid SIP. We first use GC-MS for isotopologue analysis of labeled amino acids after complete protein hydrolysis. To overcome the limitations stated above and to extend the number of amino acids susceptible for analysis, we introduce a novel approach for isotopologue analysis based on the mass spectrometric data from peptide precursors and peptide fragment ions analyzed by nano-liquid chromatography-electrospray ionization-tandem mass spectrometry (nano-LC-ESI-MS/MS) (LTQ-Orbitrap). Although this technique has a number of performance advantages over standard mass spectrometry, including high mass accuracy (<2 ppm) and high resolution (up to 100,000) (17, 26), its direct application for amino acid isotopologue analysis remains largely unexplored. Our approach, here called the peptide MS/MS ap-

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proach for amino acid SIP, combines high sensitivity with mild treatment that prevents undesired modification of amino acids.

MATERIALS AND METHODS

Chemicals. Sodium acetate labeled with 99% atom ^{13}C at the first ([1-^{13}C]acetate) or second ([2-^{13}C]acetate) position and labeled sodium bicarbonate ([^{13}C]bicarbonate, 98% atom ^{13}C) were acquired from Sigma-Aldrich Isotec (Munich, Germany). Amino acid standard solution was obtained from Sigma-Aldrich (Munich, Germany).

Cultivation of strain CBDB1 with ^{13}C-labeled substrates. *Dehalococcoides* strain CBDB1 was cultured under strictly anaerobic conditions in 60-ml glass serum bottles containing 30 ml gas phase and 30 ml synthetic bicarbonate-buffered mineral medium, as previously described (1). Perchloroethylene was used as an electron acceptor in doses of 50 μM, and all cultures were amended with H_2 (+30 kPa) as an electron donor (13). Cells were harvested at the mid-exponential growth phase and contained 5×10^7 to 8×10^7 cells ml^{-1} as determined by direct epifluorescence microscopy of SYBR green-stained cells (13). Four experimental conditions were set up, each in triplicate, to study amino acid formation: (i) cultures containing 5 mM [1-^{13}C]acetate plus 30 mM unlabeled bicarbonate, (ii) cultures with [2-^{13}C]acetate plus 30 mM unlabeled bicarbonate, (iii) cultures with 5 mM unlabeled acetate plus 30 mM [^{13}C]bicarbonate, and (iv) cultures with 5 mM unlabeled acetate plus 30 mM unlabeled bicarbonate as controls. [^{13}C]bicarbonate-containing cultures were gassed with nitrogen (+20 kPa), and all others were gassed with N_2/CO_2 (4:1, vol/vol, +20 kPa). pH was between 7.0 and 7.1. To remove unlabeled carbon traces from the inoculum, strain CBDB1 was transferred four times with a 5% inoculum into fresh medium containing the corresponding labeled substrate before being collected for amino acid analysis.

To analyze the uptake of externally added amino acids, cultures growing for several transfers with [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate were amended with a mix of 17 unlabeled amino acids not including tryptophan, asparagine, and glutamine at a final concentration of 0.67 μM each, while cysteine was added at 0.34 μM. Here, two replicates were done for each treatment, and in each case, one control with unlabeled carbon sources and unlabeled amino acids was included.

GC-MS analysis. Amino acids were analyzed according to a previously described method (22). We collected cells by filtering 210 ml of strain CBDB1 cultures through a 0.2-μm filter. Then, 2 ml of 6 M HCl was used to backflush the filters and to incubate cells at 110°C for 22 h, resulting in lysis of the cells and concurrent hydrolysis of proteins. Hydrolysates were dried under nitrogen. The resulting amino acids were derivatized to isopropyl esters and subsequently acetylated as described elsewhere (14). An external standard containing 500 μl of a commercial amino acid standard solution was used for identification of amino acids.

The derivatized samples were analyzed by gas chromatography (Agilent 7890A) with mass spectrometric detection (Agilent 5975C; Agilent, Palo Alto, CA) using a DB5 column (30 m by 0.25 mm by 0.25 μm; Agilent) as previously described (14).

Nano-ultrahigh-pressure liquid chromatography (UPLC)–ESI-MS/MS analysis. For mass spectrometric analysis, at least three biological replicates of labeled and nonlabeled samples were measured by mass spectrometry. Thirty milliliters of CBDB1 culture was harvested for protein extraction by filtration through a 0.2-μm filter. Cells were suspended in 50 mM ammonium bicarbonate, and cell lysis was performed by a freeze-thaw step and 30 s of an ultrasonic bath treatment. Samples were incubated in 100 mM iodoacetamide-50 mM dithiothreitol at 30°C for 1 h in the dark to prevent methionine oxidation and to carbamidomethylate cysteine residues. Then, 0.6 μg trypsin was added and incubated at 37°C for 16 h. The reaction was stopped by adding 0.1% formic acid. Peptides were purified with C_{18} Zip Tip columns (Millipore, Schwalbach, Germany).

Peptides were reconstituted in 0.1% formic acid. Six microliters was used for injection with an autosampler and concentrated on a trapping column (nanoAcquity UPLC column, C_{18}, 180 μm by 2 cm by 5 μm; Waters, Eschborn, Germany) with water containing 0.1% formic acid at a flow rate of 15 μl min^{-1}. After 6 min, the peptides were eluted onto the separation column (nanoAcquity UPLC column, C_{18}, 75 μm by 100 mm by 1.7 μm; Waters, Eschborn, Germany). Chromatography was performed by using 0.1% formic acid in solvents A (100% water) and B (100% acetonitrile), with peptides eluted over 90 min with a 6 to 40% solvent B gradient using a nano-high-pressure liquid chromatography (nano-HPLC) system (nanoAcquity; Waters, Eschborn, Germany) coupled to an LTQ-Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Continuous scanning of eluted peptide ions was carried out between 300 and 1,400 *m/z*. MS/MS collision-induced dissociation (CID) mode was automatically performed with normalized collision energy of 35.0 on the top 6 ions exceeding a minimum signal threshold of 3,000 counts.

Bioinformatics applied to the amino acid SIP peptide MS/MS approach. Raw data from unlabeled samples were analyzed using Mascot (Matrix-Science, Boston, MA) as described previously (14) to identify expressed proteins in strain CBDB1. Peptides were considered to be identified by Mascot when a probability of <0.05 (probability-based ion score threshold of >40) was achieved. The precursor ion tolerance was set to 10 ppm, and the MS^2 fragment ion tolerance was 0.8 Da. For the analysis of labeled samples, data sets were compared with data sets from unlabeled samples (Fig. 1). For that, raw data sets were reformatted to mzXML format, and scan overviews and single MS or MS^2 scans were extracted with the tools ReAdW and readmzXML within the Trans Proteomic Pipeline suite, respectively (11). Then, a specific peptide from the unlabeled data set was picked for which solid evidence from MS^2 patterns was available in our data. To identify the corresponding peptide in the labeled samples, the MS^2 scans of the labeled data sets were filtered (i) for the retention time (±5 min) and (ii) for *m/z* values of the precursor ions (limited to *m/z* values between the *m/z* value of the doubly charged unlabeled precursor ion $[m/z + 2]^{2+}$ and the *m/z* value of the doubly charged peptide in its fully ^{13}C-labeled form). A mass deviation of 0.5 Da was tolerated. This resulted in a list of possible candidate MS^2 scans for the sought peptide. The set of data was further reduced using a model for the biosynthesis of amino acids, which predicted the additional masses included by the different amino acids in a peptide. The model was based on the genome annotations of strain CBDB1 (12) and physiological and biochemical evidence from previous studies. With this model, we predicted the *m/z* values of labeled precursor ions and compared the results with the filtered list of precursor ions, selected for MS^2 analysis. Using the model, also the *m/z* values of fragment y-ions were calculated and compared with a list of the 100 most intensive peaks in each MS^2 scan of the filtered list. With application of a mass tolerance of 1 Da, the number of hits between a predicted y-ion series and the different measured MS^2 spectra was calculated. Then, the filtered list of MS^2 scans was ranked according to the number of hits. If no hits or a low number of hits were obtained, the model was iteratively adjusted. The described steps were all automated by Microsoft Excel VBA routines, and the calculation was quickly done for each peptide or as a batch overnight. Finally, the best-matching MS^2 scans were manually examined with mMass version 3.8 (23) to quantify ^{13}C incorporation into single amino acids. Because the chosen mass and time tolerances were relatively wide, manual examination also was used to confirm the results from the filtering process.

RESULTS AND DISCUSSION

Cultivation. Bacterial cultures with *Dehalococcoides* strain CBDB1 were grown on acetate and bicarbonate as carbon sources using only nonlabeled compounds, or substituting the respective carbon source with labeled [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate. Cell extracts were then used to determine amino acid masses.

Isotopologue analysis of amino acids by GC-MS after total hydrolysis of proteins. To allow direct comparison with previously published data, we analyzed isotopologue distribution in

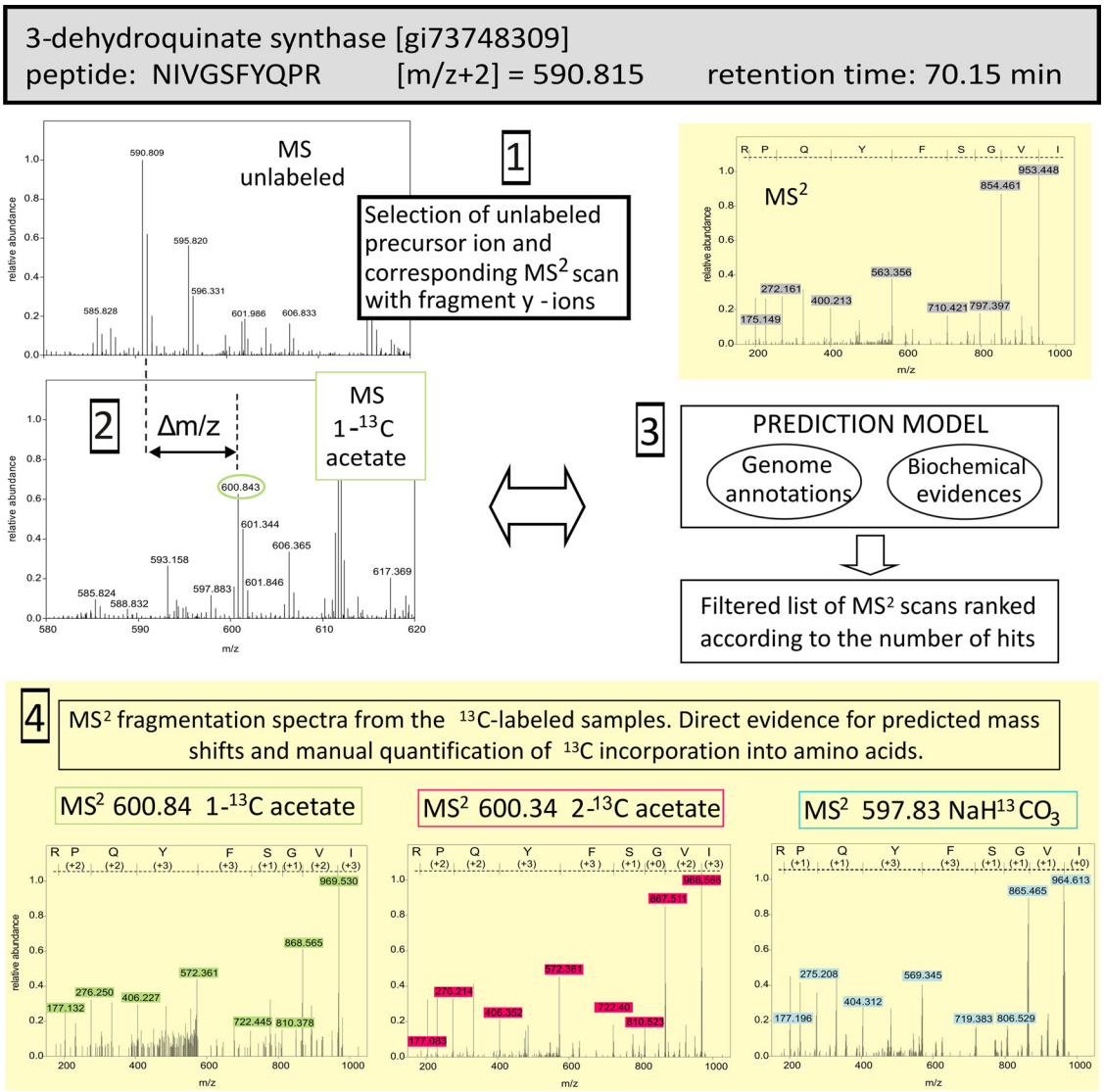
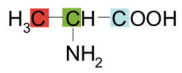
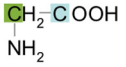
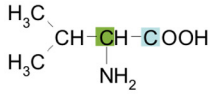
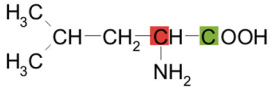
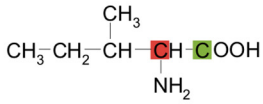
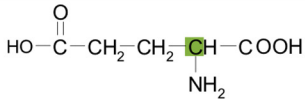
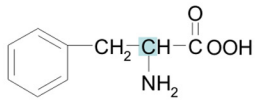


FIG 1 Determination of ^{13}C incorporation from differently labeled carbon sources into amino acids of *Dehalococcoides* strain CBDB1 by analysis of peptide (MS) and peptide fragment (MS^2) mass spectra from nano-LC-MS/MS shotgun proteome data. Boxes with yellow backgrounds show MS^2 scans. Gray, green, red, and blue boxes indicate no labels, labels from [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate, respectively. Step 1: a specific peptide with high identification score from cells grown on unlabeled carbon sources was selected. Step 2: the data set from labeled carbon sources contained the corresponding peak at a higher *m/z* value. Here, this is shown only for the peptide labeled from [1-^{13}C]acetate. MS^2 scans with their precursor ion information were screened to identify the correct precursor ion. First, we filtered for the expected retention time window and possible *m/z* values of precursor ions. Step 3: the 100 most intense peaks of each MS^2 scan in the filtered list were then compared with calculated y-ions using a model for the biosynthesis of amino acids based on genome annotations of strain CBDB1 and biochemical evidence from previous studies. This iteratively developed model predicted the additional masses included by the different amino acids in a peptide (Table 2). The MS^2 scan list was then ranked according to the number of matches with expected y-ions. For steps 2 and 3, automated scripts were used. Step 4: the best matching MS^2 scans were manually examined to quantify ^{13}C incorporation into single amino acids.

amino acids released from proteins of *Dehalococcoides* strain CBDB1 by total hydrolysis of protein preparations using GC-MS. Four types of positively charged fragments of derivatized amino acids were identified (see Fig. S1 in the supplemental material): (i) fragment $[M-59]^+$, which contained the intact carbon skeleton of the amino acid but had released the 59-Da isopropyl group of the derivatizing agent; (ii) fragment $[M-87]^+$, which had released the C-1 carboxyl group of the amino acid together with the isopropyl group; (iii) fragment $[f153]^+$, which was obtained after the isopropyl group and the side chain of the amino acid were removed; and (iv) fragment $[f126]^+$, which was freed after the isopropylated C-1 group and the side chain of the amino acid were removed. In the case of glutamine/glutamate, an additional positively charged fragment, $[f139]^+$, was identified. Fragments for nine different amino acids were found (Table 1). The data revealed the exact distribution of ^{13}C labels in alanine and glycine. For seven other amino acids, the number of incorporated ^{13}C atoms and information on the labeling of the first two carbons were obtained. The data corresponded well with results obtained from GC-MS analysis of protein hydrolysates of *Dehalococcoides* strain 195 (24). A major disadvantage of GC-MS analysis after total protein hydrolysis, however, was the exclusion of several amino acids due to

TABLE 1 Isotopologue analysis of amino acids by GC-MS measurement of hydrolyzed proteins from *Dehalococcoides* strain CBDB1[b]

Amino acid	Fragment ion	Non labeled experiment	[1-^{13}C] acetate		[2-^{13}C] acetate		[^{13}C bicarbonate]		Inferable ^{13}C positions
		m/z	*m/z*	Number of ^{13}C	*m/z*	Number of ^{13}C	*m/z*	Number of ^{13}C	
Ala	$[M-59]^+$	168.0	169.0	**1**	169.0	**1**	169.0	**1**	
	$[M-87]^+$	140.0	141.0	**1**	141.0	**1**	140.0	**0**	
	$[f126]^+$	126.0	127.0	**1**	126.0	**0**	126.0	**0**	
Gly	$[M-59]^+$	154.0	155.0	**1**	154.0	**0**	155.0	**1**	
	$[M-87]^+$	126.0	127.0	**1**	126.0	**0**	126.0	**0**	
Val	$[M-59]^+$	196.0	198.0	**2**	198.0	**2**	197.0	**1**	
	$[M-87]^+$	168.1	170.1	**2**	170.1	**2**	168.0	**0**	
	$[f153]^+$	153.0	154.0	**1**	153.0	**0**	154.0	**1**	
Leu	[M-59]+	210.1	213.0	**3**	213.0	**3**	WS	**0**[a]	
	[M-87]+	182.1	184.0	**2**	185.1	**3**	182.1	**0**	
	[f153]+	153.0	154.0	**1**	154.0	**1**	153.0	**0**	
Ile	[M-59]+	WS	WS	-	WS	-	WS	-	
	[M-87]+	182.0	184.1	**2**	185.1	**3**	182.0	**0**	
	[f153]+	153.0	153.9	**1**	154.0	**1**	153.0	**0**	
	[f126]+	126.0	126.0	**0**	127.0	**1**	126.0	**0**	
Glu (Gln)	[M-59]+	226.0	228.0	**2**	228.0	**2**	227.0	**1**	
	[M-87]+	198.0	199.0	**1**	200.0	**2**	199.0	**1**	
	[f139]+	139.0	140.0	**1**	139.9	**1**	139.0	**0**	
Phe	[M-59]+	244.0	247.0	**3**	247.0	**3**	WS	**3**[a]	
	[M-87]+	216.1	219.1	**3**	219.1	**3**	WS	**2**[a]	
Thr	[M-59]+	198.0	199.0	**1**	199.0	**1**	200.0	**2**	
Tyr	[M-59]+	260.1	263.0	**3**	263.0	**3**	WS	**3**[a]	

[a] The number of ^{13}C atoms was indirectly calculated by subtracting the number of carbons deriving from experiments with [1-^{13}C]acetate and [2-^{13}C]acetate from the total number of carbons containing the amino acid.

[b] Shown are mass-to-charge ratio (*m/z*) values of fragments, inferred numbers of incorporated ^{13}C atoms, and ^{13}C positions in those amino acids that could be assessed by this method after growth on unlabeled carbon sources, [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate. To calculate the number of ^{13}C atoms in each fragment ion, *m/z* values from the nonlabeled culture were subtracted from those obtained with labeled carbon sources. On the basis of these data and the fragmentation pattern given in Fig. S1 in the supplemental material, the positions of the ^{13}C labels in the amino acids were deduced. The color background indicates the origin of the carbon: C-1 of acetate (green), C-2 of acetate (red), or carbon of bicarbonate (blue). WS, weak signals due to high background on the GC-MS.

their degradation during total hydrolysis of proteins with chloridic acid. Deamination of glutamine and asparagine to glutamate and aspartate, respectively, and degradation of cysteine and tryptophan are typical undesired reactions and were also observed in our study. Several amino acids were not identified due to very weak or overlapping signals (24).

Isotopologue analysis by the peptide MS/MS approach for amino acid SIP. To overcome the limitations of protein hydrolysis approaches, nano-LC-ESI-MS/MS was employed to track carbon fluxes in amino acid biosyntheses of strain CBDB1. This approach targets intact peptides, prepared from proteins via well-defined mild tryptic digestion procedures, to analyze their masses and their collision-induced fragment ions to identify amino acid masses. Because we focused on y-type ions, we did not obtain information about the position of a label in an amino acid. The fragment ion mass detection was based on an incorporation model elaborated according to the annotated genome of strain CBDB1 (Fig. 1). The model was validated by analyzing three independent measurements from each of the four cultivation conditions. While in the three unlabeled samples many proteins were identified (275, 144, and 175 proteins with 14,27, 434, and 495 peptides, respectively), only a very few peptides were identified by the Mascot search in the samples from cultures with labeled carbon sources, indicating incorporation of ^{13}C. For the three sample

TABLE 2 Comparison of isotopologue analysis results obtained from *Dehalococcoides* strain CBDB1 by nano-LC-MS/MS of digestion peptides with isotopologue analysis results obtained by Tang et al. (24) from *Dehalococcoides* strain 195 by GC-MS of completely hydrolyzed proteins[a]

Amino acid	No. of carbons	Strain CBDB1 peptide MS/MS approach			Strain 195 (24) GC-MS of hydrolyzed proteins		
		[1-^{13}C]acetate	[2-^{13}C]acetate	[^{13}C]bicarbonate	[1-^{13}C]acetate	[2-^{13}C]acetate	[^{13}C]bicarbonate
Glycine	2	1	0	1	1	0	1
Alanine	3	1	1	1	1	1	1
Serine	3	1	1	1	1	1	1
Cysteine	3	1	1	1	Unknown	Unknown	Unknown
Aspartate	4	1	1	2	1	1	2
Asparagine	4	1	1	2	1	1	2
Threonine	4	1	1	2	1	1	2
Glutamate	5	2	2	1	2	2	1
Glutamine	5	2	2	1	2	2	1
Methionine	5	Unknown	Unknown	Unknown	1	2	2
Proline	5	2	2	1	Unknown	Unknown	Unknown
Valine	5	2	2	1	2	2	1
Arginine	6	2	2	2	Unknown	Unknown	Unknown
Lysine	6	2	2	2	Unknown	Unknown	Unknown
Leucine	6	3	3	0	3	3	0
Isoleucine	6	3	3	0	3	3	0
Histidine	6	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown
Phenylalanine	9	3	3	3	3	3	3
Tyrosine	9	3	3	3	3	3	3
Tryptophan	11	Unknown	Unknown	Unknown	Unknown	Unknown	Unknown

[a] Both approaches used [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate to label amino acids. Values show the number of ^{13}C carbon atoms incorporated into the respective amino acid. By nano-LC-MS/MS, isotopologues could be assigned for all amino acids except methionine, histidine, and tryptophan. The labeling pattern of glutamine and asparagine in strain 195 was indirectly deduced from glutamate and aspartate, respectively.

sets with ^{13}C labels, 103, 78, and 59 peptides from 46, 27, and 17 proteins, respectively, were analyzed to evaluate the model (see Table S1 in the supplemental material). With this approach, the number of integrated ^{13}C atoms was determined for each of the amino acids in a peptide. The experiments gave solid data for 17 different amino acids. In contrast to the GC-MS approach, isotopologues of asparagine, glutamine, lysine, cysteine, proline, and arginine were detected (Table 2; see also Table S2 in the supplemental material). Our data demonstrate that isotope labels are stably incorporated into the amino acids of *Dehalococcoides* species and that amino acid pools are not mixtures of different isotopologues (Fig. 1 and Table 1). Therefore, in strain CBDB1 amino acids do not underlie a continuous turnover to central intermediates which would disturb labeling patterns.

Analysis of single amino acid biosynthesis pathways. The peptide MS/MS approach allowed direct determination of ^{13}C incorporation into glutamate, glutamine, aspartate, and asparagine (Table 2). The labeling patterns of glutamate and glutamine as well as the labeling patterns of aspartate and asparagine were identical, which indicates that they were converted into each other by glutamine synthetase (cbdbA1050) and an unidentified aspartate aminase or transaminase, respectively (Fig. 2). GC-MS after protein hydrolysis confirmed that the C-1 of glutamate originates from acetate rather than from bicarbonate (Table 1), indicating that a *Re*-type citrate synthase is active and that strain CBDB1 does not catalyze a reductive tricarboxylic acid cycle in which 2-oxoglutarate is synthesized via oxaloacetate (14). The labeling pattern for aspartate is consistent with its biosynthesis from acetyl coenzyme A (acetyl-CoA) via two carboxylations to pyruvate and oxaloacetate, also highlighting the pivotal role of bicarbonate as a carbon source.

Solid labeling data were obtained for arginine and proline by the peptide MS/MS approach but not by GC-MS of protein hydrolysates in this study and a previous study (24). Arginine was labeled with two carbons each from the C-1 of acetate, the C-2 of acetate, and bicarbonate, whereas proline contained 2 carbons from the C-1 of acetate, 2 carbons from the C-2 of acetate, and 1 carbon from bicarbonate. The genomes of strain CBDB1 and other *Dehalococcoides* strains encode a complete pathway for arginine biosynthesis from glutamate via *N*-acetyl-glutamate, *N*-acetyl-ornithine, ornithine, and citruline, and our isotopologue data are consistent with this biosynthetic pathway. All sequenced *Dehalococcoides* strains with the exception of strain 195 contain a gene, annotated in strain CBDB1 as ornithine cyclodeaminase (*arcB*, cbdbA155), by which proline can directly be synthesized from ornithine. Ornithine cyclodeaminase has been described in several anaerobic bacteria and is mostly inhibited by oxygen (4). A second proline biosynthesis pathway via glutamylphosphate (*proB*, *proA*, *proC*) is encoded in all *Dehalococcoides* strains. The two different proline biosynthesis pathways lead to identical carbon labeling patterns, and their contributions to proline biosynthesis cannot be differentiated by our methods.

The peptide MS/MS data show that lysine contained 2 carbon atoms each from the C-1 of acetate, from the C-2 of acetate, and from bicarbonate. Two lysine biosynthesis pathways are described, originating from aspartate via diaminopimelate and from 2-oxoglutarate via α-aminoadipate, respectively. Whereas the labeling pattern of lysine formed via the diaminopimelate pathway is in accordance with the one obtained from peptide MS/MS analysis (Fig. 2), lysine derived via the α-aminoadipate pathway would contain two carbon atoms from the C-1 of acetate, three carbon atoms from the C-2 of acetate, and one carbon atom from bicarbonate. The genome of strain CBDB1 encodes all enzymes of the diaminopimelate pathway but none of the enzymes of the α-ami-

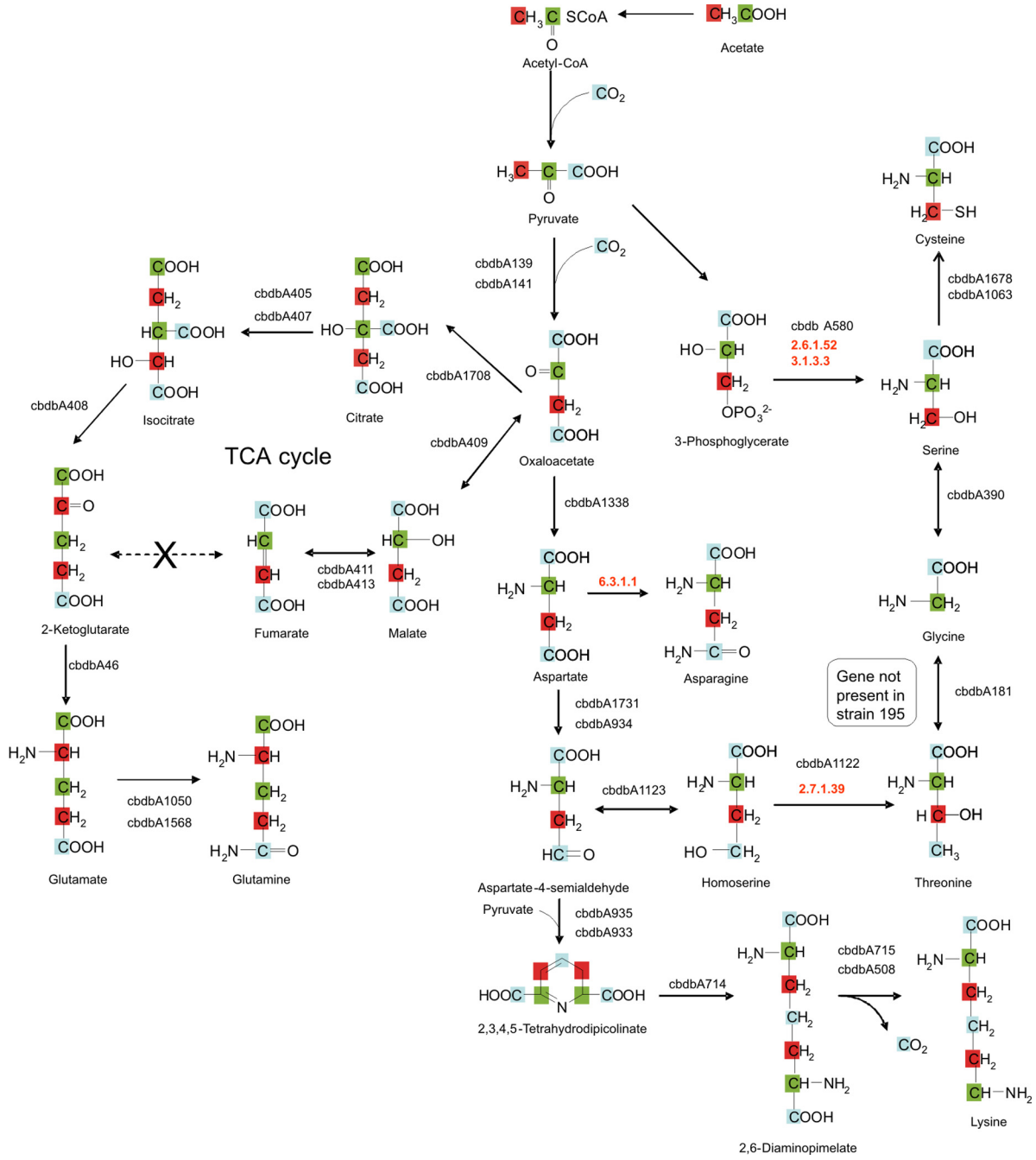


FIG 2 Pathway for serine, glycine, threonine, cysteine, and lysine biosynthesis and tricarboxylic acid (TCA) cycle in *Dehalococcoides* strain CBDB1 inferred from annotation of the genome and isotope labeling patterns. Colors indicate that the carbon was synthesized from the C-1 of acetate (green), from the C-2 of acetate (red), or from bicarbonate (blue). EC numbers in red mark genes that have not been annotated in CBDB1, so far.

noadipate pathway. However, strain CBDB1 seems to catalyze a variant of the diaminopimelate pathway which operates via LL-diaminopimelate aminotransferase (*dapL*, EC 2.6.1.83, cbdbA714) (Fig. 2). It has been speculated that this pathway is an ancient pathway and is mainly conserved in slow-growing organisms because it relies on the spontaneous formation of the acyclic structure of the substrate (7). This is in accordance with the ecophysiology of strain CBDB1. Formation of lysine via the α-aminoadipate pathway is common in eukaryotes, although the thermophilic prokaryotes *Thermus thermophilus*, *Ignicoccus hospitalis*, and *Thermoproteus neutrophilus* were also shown to use this unconventional pathway (8, 16, 20).

Cysteine is a rare amino acid, amounting to 1.4% of the amino acids in proteins of strain CBDB1 (http://www.ebi.ac.uk/integr8/), possibly explaining why cysteine was missed in the analyses of protein hydrolysates by GC-MS in the present study (Table 1) and in other studies (24). In contrast, peptide MS/MS analysis allowed determination that the three carbon atoms in

cysteine stem from the C-1 of acetate, the C-2 of acetate, and bicarbonate, respectively. Strain CBDB1 encodes the enzymes required to synthesize cysteine from serine via *O*-acetylserine in a two-step reaction (locus tags cbdbA1678 and cbdbA1063, Fig. 2). This is in agreement with the results obtained by peptide MS/MS experiments showing that cysteine and serine labeling were identical. Methionine is also a rare amino acid in proteins of strain CBDB1, amounting to 2.3% of the amino acids when AUG codons at the N-terminal ends of proteins are disregarded, and we were not able to detect methionine in either of our two approaches. Although growth of strain CBDB1 in purely synthetic medium clearly demonstrates that methionine is synthesized *de novo*, genome annotations do not allow the reliable prediction of its biosynthetic pathway. Tang et al. reported the detection of labeled methionine in strain 195 by GC-MS (24). One label originated from the C-1 of acetate, two labels originated from the C-2 of acetate, and two originated from bicarbonate (Table 2).

For alanine, isoleucine, leucine, valine, phenylalanine, tyrosine, glycine, threonine, and serine, the peptide MS/MS approach with strain CBDB1 proteins resulted in the same incorporation numbers as those previously shown for amino acids of strain 195 (24). Also, the results obtained from strain CBDB1 with the peptide MS/MS approach and the GC-MS approach after protein hydrolysis were mutually supporting. Briefly summarized, these results suggest the following amino acid biosynthesis pathways in strain CBDB1: (i) alanine synthesis via pyruvate or aspartate; (ii) isoleucine biosynthesis from pyruvate and acetyl-CoA via the citramalate pathway instead of the canonical threonine pathway as shown for several other anaerobic microorganisms such as *Leptospira interrogans* (4), *Methanococcus jannaschii* (6), *Geobacter sulfurreducens* (19), *Thermoanaerobacter* strain X514 (5), or *Clostridium acetobutylicum* (3); this is also supported by the lack of threonine ammonia-lyase (EC 4.3.1.19) in the genome annotation of strain CBDB1 but the presence of a gene, cbdbA803, that has a high similarity (53% sequence identity, blastp E value of 10^{-152}) to a recently biochemically identified citramalate synthase (*cimA*, EC 2.3.1.182) in *Geobacter sulfurreducens* (19); (iii) leucine biosynthesis via isopropylmalate synthase (*leuA*), possibly encoded by cbdbA808; (iv) valine biosynthesis via the canonical acetolactate pathway; and (v) biosynthesis of phenylalanine and tyrosine via the shikimate pathway from erythrose-4-phosphate and phosphoenolpyruvate, for which all genes are included in the genome. In most *Dehalococcoides* strains, including strain CBDB1, serine, glycine, and threonine are predicted to be converted into each other by serine hydroxymethyltransferase (EC 2.1.2.1, cbdbA390) and threonine aldolase (EC 4.1.2.5, cbdbA181). Only threonine aldolase is deleted without substitution in the genome of strain 195 (12). On the basis of genome annotations in strain CBDB1, the three amino acids could be synthesized either from 3-phosphoglycerate via serine or from aspartate via homoserine and threonine (Fig. 2), and the obtained labeling pattern is consistent with the use of both pathways. A similar situation was found for *Clostridium acetobutylicum*, where both pathways were shown to be active (3).

Tryptophan and histidine are described to be unstable under typical protein hydrolysis conditions and could therefore not be detected by GC-MS in hydrolyzed samples by us or others (24). In addition, the abundance of tryptophan and histidine in the total predicted proteome is low, at 1.1% and 1.9%, respectively. Although both of these issues should not be problematic with the peptide MS/MS approach, we could also not find labeling evidence from intact peptides. The canonical pathways of the two amino acids have in common the incorporation of ribose-5-phosphate (Rib5P). In *Dehalococcoides*, all enzymes required for the biosynthesis of Rib5P through the nonoxidative part of the pentose phosphate cycle from glyceraldehyde-3-phosphate and fructose-6-phosphate are encoded in the genome but not the enzymes of the oxidative part. Taking this into account, Rib5P will be synthesized to two different isotopologues (see Fig. S2 in the supplemental material). Thus, the measured tryptophan and histidine pools are expected to be a mixture of two different isotopologues. This mixing of different isotopologues lowers the sensitivity of the analysis and complicates the analysis of detected peaks. However, this hypothesis assumes that the transketolase and transaldolase reactions in the cell occur almost unidirectionally because Rib5P under both label patterns would react back to glyceraldehyde-3-phosphate and fructose-6-phosphate. Indeed, the finding that the labels in phenylalanine and tyrosine are stably distributed also shows that erythrose-4-phosphate, which is the precursor of the aromatic amino acids, must be stably labeled, indicating that in fact the transaldolase and transketolase reactions are essentially unidirectional in strain CBDB1. A similar conclusion has recently been reported for pentose phosphate intermediates in *Clostridium acetobutylicum* on the basis of intermediate analysis (3).

Effect of unlabeled amino acid addition to strain CBDB1 growing with ^{13}C-labeled substrates. One challenging aspect of the biotechnical application of *Dehalococcoides* species for the treatment of contaminated groundwater and soil is the optimization of growth conditions to prepare large inocula. In the previous sections, we demonstrate that strain CBDB1 is able to synthesize all 20 amino acids *de novo* from acetate and bicarbonate. However, it can be hypothesized that growth yields might be higher if free amino acids were directly assimilated. To assess whether strain CBDB1 takes up amino acids from the medium, we grew strain CBDB1 on [1-^{13}C]acetate, [2-^{13}C]acetate, or [^{13}C]bicarbonate over several transfers to label almost all of its proteins. Then, we prepared medium with acetate and carbonate as carbon sources in which one of the carbons always was labeled plus a mixture of 17 unlabeled amino acids (all 20 with the exception of tryptophan, asparagine, and glutamine). After incubation for several weeks, all cultures were analyzed using the peptide MS/MS approach to determine the dilution of labeled amino acids in the peptides of strain CBDB1 with unlabeled amino acids. This experiment was performed in duplicate, and the peptides selected for each replicate were the same peptides as shown in Table S1 in the supplemental material for replicates 1 and 2, respectively. However, the masses of the precursor ions and the obtained amino acid masses fitted perfectly with the predictions made for cultures without any incorporated unlabeled amino acids. Dilution of the labeled amino acid pool with unlabeled amino acids did not occur, and therefore, no incorporation from the medium was evident (see Table S3 in the supplemental material).

Our experimentally obtained results are in contrast to the conclusion drawn from a metabolic model, based on the published genomes of *Dehalococcoides* strains, that predicted that *Dehalococcoides* species are able to unselectively take up all amino acids, leading to an increase of the growth yield by a maximum of 55% (2). A recent isotopomer-based dilution study, in which *Dehalococcoides* strain 195 was incubated with ^{13}C-labeled acetate and unlabeled amino acids and harvested proteins were completely

hydrolyzed and analyzed by GC-MS, reported incorporation of many amino acids from the medium, including phenylalanine, isoleucine, leucine, and methionine at percentages of more than 30% (29). In the light of this discrepancy, we reassessed our data; however, the incorporation of a single unlabeled amino acid into an accurately labeled peptide would change the mass of the precursor ion and also the masses of y-type ions, and therefore, we do not see an alternative to our conclusions. On the other hand, we recognized a correlation of the nonlabeled mass fraction in protein hydrolysates from strain 195 with the hydrophobicity of the respective amino acids (29). In fact, the four amino acids phenylalanine, isoleucine, leucine, and methionine reported to be taken up most by strain 195 are among the most hydrophobic amino acids. It would therefore be important to confirm that such unlabeled hydrophobic amino acids do indeed stem from protein-integrated amino acids and not from unlabeled free amino acids that were dissolved in or attached to the lipid membrane. A contamination of protein hydrolysates with free unlabeled amino acids would explain (i) the finding that phenylalanine but not tyrosine was highly diluted, although both share a largely common biosynthesis pathway, and (ii) the reported insignificant transcriptional feedback from phenylalanine onto *pheA* (29). Our approach avoids such effects as all amino acids are measured as a part of intact peptides. In summary, our results indicate that strain CBDB1 synthesizes all 20 amino acids *de novo* from acetate and bicarbonate and does not import exogenous amino acids under the tested conditions. This may be the result of an adaptation of the metabolism to nutrient-depleted environments such as sediments and groundwater.

Evaluation of the peptide MS/MS approach for amino acid SIP. With the peptide MS/MS approach, we introduce a new approach to track isotope incorporation from labeled carbon sources into individual amino acids in proteins. In contrast, existing protein or proteomic stable isotope probing (SIP) techniques track and quantify label incorporation into proteins (9, 18) but do not track labels into individual amino acids with which amino acid biosynthesis pathways can be determined. The standard technique to analyze such isotope incorporation into amino acids is the analysis of protein hydrolysates by GC-MS, which is currently cheaper and quicker than the peptide MS/MS approach and, in contrast to the peptide MS/MS approach, provides position-specific isotope incorporation information for the first and second carbon of an amino acid. We therefore do not propose the peptide MS/MS approach as a substitute but as a complement to the analysis of protein hydrolysates, as peptide MS/MS also has many advantages over GC-MS analysis of protein hydrolysates. The first and probably most important advantage is that the amino acids are measured for each protein separately and that amino acid-specific incorporation can be traced for each single peptide. The incorporation might be similar for all peptides of a given organism, as shown in our case with strain CBDB1; however, in many cases this will not be true. For example, when labeled substrates are added at the same time as changes in a physiological state, it will be possible to analyze the expression pattern by analyzing protein-specific incorporation. A similar use of stable isotope labels in a protein SIP experiment has been proposed (10), but the peptide MS/MS approach for amino acid SIP described here can provide incorporation data without the need for massive amounts of protein data as shown for determination via a decimal place method (9), or for extensive computer processing hours when calculated via a stepwise comparison with hypothetical incorporation percentages (18). Similarly to protein SIP (25), turnover rates of proteins can be analyzed. Also, because orthologous proteins, i.e., homologous proteins with the same function in different organisms, have different sequences, phylogenetic information of microbial consortia can be retrieved, e.g., by comparing incorporation of labels into strain-specific marker proteins such as GroEL, PolA, or citrate synthase. This could even be exploited for the description of food webs in a microbial community. The second main advantage of our method is that more amino acids are amenable to analysis than by GC-MS of protein hydrolysates, and in fact, we do not see a principal obstacle to assessing all proteinaceous amino acids, although the existence of isotopically mixed pools of tryptophan and histidine will significantly complicate the analysis of these amino acids. We were not yet able to analyze methionine, possibly due to its high susceptibility to oxidation even after addition of dithiothreitol to protect the C—S bond in combination with its low abundance in proteins. The third advantage of the peptide MS/MS approach is the extraordinarily high sensitivity. In our standard protocol, 5×10^8 to 15×10^8 cells of strain CBDB1 were sufficient to generate an overwhelming amount of raw data. The protein amount roughly correlates with that from 10^7 cells of *Escherichia coli* (2). In contrast, standard numbers of *Dehalococcoides* cells used for GC-MS analysis after protein hydrolysis were 1.5×10^{11} to 2.3×10^{11} cells (24, 29). The high sensitivity of our approach is of utmost importance when analyzing very slowly growing organisms or organisms in mixed cultures. A fourth advantage is the direct detection of intact amino acids in their peptide environment, which avoids mixing of amino acids that are incorporated into the protein pool with free amino acids. This also allows the detection of labels after posttranslational modifications. A fifth advantage of the method is that a complete set of shotgun proteomics data is underlying the analysis so that apart from isotope incorporation analysis, expression data for hundreds of proteins can be identified simultaneously (27).

For future application and extension of the approach, advanced bioinformatic routines to extract spectral information will be important. With such advancements, the inclusion of more ion types can be envisaged, such as a-type or immonium ions that would allow gathering information on the C-1 carbon of an amino acid or on c- and z-type ions which can give information on ^{15}N labels incorporated at the α-amino group of an amino acid in a protein.

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