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Investigation of Carbon Metabolism in "*Dehalococcoides ethenogenes*" Strain 195 by Use of Isotopomer and Transcriptomic Analyses▽‡

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Members of the genus "*Dehalococcoides*" are the only known microorganisms that can completely dechlorinate tetrachloroethene and trichloroethene to the innocuous end product, ethene. This study examines the central metabolism in "*Dehalococcoides ethenogenes*" strain 195 via ^{13}C-labeled tracer experiments. Supported by the genome annotation and the transcript profile, isotopomer analysis of key metabolites clarifies ambiguities in the genome annotation and identifies an unusual biosynthetic pathway in strain 195. First, the ^{13}C-labeling studies revealed that strain 195 contains complete amino acid biosynthesis pathways, even though current genome annotation suggests that several of these pathways are incomplete. Second, the tricarboxylic acid cycle of strain 195 is confirmed to be branched, and the Wood-Ljungdahl carbon fixation pathway is shown to not be functionally active under our experimental conditions; rather, CO_2 is assimilated via two reactions, conversion of acetyl-coenzyme A (acetyl coenzyme A [acetyl-CoA]) to pyruvate catalyzed by pyruvate synthase (DET0724-0727) and pyruvate conversion to oxaloacetate via pyruvate carboxylase (DET0119-0120). Third, the ^{13}C-labeling studies also suggested that isoleucine is synthesized from acetyl-CoA and pyruvate via citramalate synthase (CimA, EC 2.3.1.182), rather than from the common pathway via threonine ammonia-lyase (EC 4.3.1.19). Finally, evidence is presented that strain 195 may contain an undocumented citrate synthase (>95% *Re*-type stereospecific), i.e., a novel *Re*-citrate synthase that is apparently different from the one recently reported in *Clostridium kluyveri*.

Chlorinated organic compounds, such as tetrachloroethene (PCE) and trichloroethene (TCE), are among the most pervasive and persistent groundwater pollutants in the United States (40). Because of their toxicity and potential carcinogenicity, these chloroethenes pose significant risks to human health and the environment (5, 37). To date, only bacteria of the genus "*Dehalococcoides*" have been shown to completely reductively dechlorinate PCE, TCE, and their daughter compounds dichloroethene (DCE) and vinyl chloride (VC) to the nontoxic end product, ethene, in a process referred to as dehalorespiration (6, 9, 20, 21). Bioremediation using dehalorespiring bacteria has been shown to be a viable strategy to effectively treat chloroethene-contaminated groundwater (10, 16, 19, 28, 32). Although successful in situ bioremediation of chloroethenes in groundwater has been demonstrated at field sites containing *Dehalococcoides*, lack of knowledge about the physiology and metabolism of these microorganisms limits the optimization of these bioremediation strategies.

All *Dehalococcoides* strains identified thus far exhibit specific metabolic restrictions with respect to carbon source and respiratory activity. That is, all require acetate for biosynthesis, hydrogen as an electron donor, and halogenated compounds as electron acceptors (2, 6, 8, 9, 20, 33). To date, researchers have made substantial efforts to understand the metabolism of chlorinated compounds in *Dehalococcoides*, especially their functional reductive dehalogenases and hydrogenases (1, 13, 15, 18, 24, 26, 28, 29). In contrast, knowledge of carbon metabolism in *Dehalococcoides* is still limited. Sequences and annotations of the genomes of several *Dehalococcoides* strains have provided a beginning to understanding *Dehalococcoides* physiology (14, 31), but questions about carbon metabolism in this important genus remain unanswered. First, there are several key genes missing in the proposed amino acid biosynthetic pathways (e.g., methionine and glutamate) of the genome annotations (www.microbesonline.org), even though many *Dehalococcoides* isolates have been demonstrated to grow in minimal medium with only cysteine added as a reductant (1, 7, 8, 33). Second, the current genome annotations predict that *Dehalococcoides* strains possess an incomplete Wood-Ljungdahl acetyl coenzyme A (acetyl-CoA) carbon fixation pathway that is missing a functional formate dehydrogenase, since the gene annotated with this function is apparently associated with a different function (22, 23). Further, the subunit associated with carbon monoxide dehydrogenase activity in the carbon monoxide dehydrogenase/acetyl-CoA synthase/decarbonlyase (CODH/ACDS) enzyme complex is not annotated (31). Nevertheless, the function of this incomplete pathway has not been fully explored. Third, the *Dehalococcoides* annotations do not identify a gene

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coding citrate synthase, an enzyme required to functionalize the tricarboxylic acid (TCA) pathway for synthesizing glutamate family amino acids.

This study investigated the amino acid biosynthetic and central metabolic pathways of *Dehalococcoides* using "*Dehalococcoides ethenogenes*" strain 195 as a model strain. Strain 195 was cultivated either using ^{13}C-labeled acetate with unlabeled bicarbonate or with unlabeled acetate with ^{13}C-labeled bicarbonate and the resulting patterns of derivatized amino acids were determined by gas chromatography-mass spectrometry (GC-MS) to track the active pathways. The isotopomer data were then integrated with transcriptomic microarray analysis and reverse transcription-quantitative PCR data to link strain 195's physiology with functional genes and to provide significant insight into the central metabolism and biosynthetic pathways of *Dehalococcoides*.

MATERIALS AND METHODS

Chemicals. Ethenes, including TCE, *cis*-DCE (*c*DCE), VC, and ethene were purchased from Fisher scientific Co. (Pittsburgh, PA), Acros Chemical Co. (Pittsburgh, PA), Fluka Chemical Co. (Ronkonkoma, NY), and Alltech Associates, Inc. (Deerfield, IL), respectively. [1-^{13}C]sodium acetate and [2-^{13}C]sodium acetate (both of 99% purity) and [^{13}C]sodium bicarbonate (99% purity) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). All gases, including medical air, nitrogen, hydrogen, nitrogen-CO_2 mixture, hydrogen-CO_2 mixture, and nitrogen-hydrogen mixture were obtained from Praxair, Inc. (Oakland, CA).

Bacterial strain and culture conditions. *D. ethenogenes* strain 195 was grown aseptically in batch cultures at 34°C with a defined mineral salt medium and a H_2/CO_2 headspace (80/20 [vol/vol]) as described previously (7). The medium was amended with a modified Wolin vitamin solution containing 50 μg of B_{12}/liter in the final concentration (41). Sodium acetate (2 mM in final concentration) and liquid TCE (55 μmol per bottle) were added to 100 ml of culture medium as the carbon source and the electron acceptor, respectively. To unambiguously track the carbon transformations in the metabolic network, three tracer experiments with different carbon labeling were performed individually: [1-^{13}C]sodium acetate with unlabeled sodium bicarbonate, [2-^{13}C]sodium acetate with unlabeled sodium bicarbonate, and unlabeled sodium acetate with [^{13}C]sodium bicarbonate. In order to avoid unlabeled carbon introduced from inoculation, strain 195 biomass was subcultured with 5% inoculum in labeled medium three times before being harvested for isotopomer analysis.

Analytical methods. Approximately 3 liters of liquid culture (~7.7×10^7 cells/ml) was aseptically harvested by centrifugation at 22,000 × *g* for 15 min at 4°C. The cell pellet was washed three times and stored in a −80°C freezer before use. The preparation and isotopomeric analysis of proteogenic amino acids were performed as previously described (36). Biomass was hydrolyzed in 6 M HCl at 100°C for 24 h, and the resulting amino acid-HCl solution was dried under air flush overnight. Amino acid samples were derivatized in tetrahydrofuran and *N*-(*tert*-butyl dimethylsilyl)-*N*-methyl-trifluoroacetamide (MTBSTFA) (Sigma-Aldrich, St. Louis, MO) at 70°C for 1 h and analyzed by using a gas chromatograph (GC; Hewlett-Packard model 6890; Agilent Technologies, Palo Alto, CA) equipped with a DB5-MS column (30 m by 0.25 mm [inner diameter], 0.25-μm film thickness; J&W Scientific, Folsom, CA) and a mass spectrometer (MS; model 5973; Agilent Technologies). A 1-μl portion of the derivatized sample was injected into the GC-MS apparatus with a 1:10 split ratio. The GC column was initially held at 150°C for 2 min, followed by an increase of 3°C/min to 280°C, then by 20°C/min to 300°C, and finally held at 300°C for 5 min. Helium was used as carrier gas at a column flow rate of 1.2 ml/min. The fragmentation of targeted compounds was achieved via electron ionization. ChemStation software was used for data collection. The identification of individual amino acids was based on both the GC retention time of amino acid standards (unlabeled) and the NIST Library for Agilent ChemStations (34–36). Isotopomer analysis of amino acids was conducted as described by Wahl et al. (38).

Ethenes in culture headspace were measured by using a GC (Hewlett-Packard model 5890; Agilent Technologies) with a GC-GasPro capillary column (30 m by 0.32 mm [inner diameter], particle-free PLOT phase; J&W Scientific) and a flame ionization detector as described previously (7). Sodium acetate concentrations were determined by using an enzymatic bioanalysis and food analysis kit in accordance to the manufacturer's instructions (R-Biopharm, Germany). In brief, acetate was converted to citrate under sequential enzymatic reactions, while NADH was released as a by-product. The determination of acetate concentration was based on the formation of NADH measured by the increase of UV absorbance at 340 nm.

Quantification of genes and gene expression. To quantify the targeted genes, quantitative PCR was applied to the extracted genomic DNA (gDNA) (see the supplemental material). Briefly, each 20-μl reaction mixture contained 2.5 μl of sample or 10-fold serially diluted standard, 1× Fast SYBR green master mix (Applied Biosystems, Foster City, CA), and 0.1 μl of each forward and reverse primer (100 mM) (see Table S5 in the supplemental material). The standards for quantitative PCR analysis of genes were the gDNAs of strain 195 with known quantification. The theoretical copy number was calculated by using the length of the genome of strain 195 and an average molecular mass of 660 Da per DNA nucleotide. Gene concentrations were reported as copies of gene per milliliter of cultures. To quantify gene expression, transcripts from the total RNA (see the supplemental material) were reverse transcribed by using an RT Core Reagent kit (Applied Biosystems) as described previously (12). Each 10-μl reaction mixture contained 2.5 μl of sample or serially diluted RNA standard and 0.5 μM concentrations of random hexamer. The reaction mixture was incubated at 48°C for 30 min, followed by 5 min at 95°C. Contaminating gDNA in each sample was analyzed by using a minus reverse transcription reaction that did not contain reverse transcriptase. The reverse-transcribed samples and standards (2.5 μl) were then quantified using the quantitative PCR method described above. Standards for the quantification of gene expression were made using in vitro synthesized RNA of the corresponding genes with known copy numbers (see the supplemental material). Expression data were reported as copies of transcripts per copy of genes, which was calculated by dividing the quantity of transcripts per ml of cultures by the corresponding quantity of gene per ml of the cultures.

The Affymetrix GeneChip microarray that targets >99% of the 1,642 predicted genes within the genome of strain 195 had been applied for analysis of the strain 195 transcriptome as described previously (11). A complete description of the microarray was reported recently (39), and microarray data referred to in the present study was previously reported by Johnson et al. (11).

RESULTS

Strain 195 cultivation with ^{13}C-labeled acetate or bicarbonate. Strain 195 cells were cultivated in defined mineral medium as previously described (7) with 55 μmol of TCE (added to 100 ml of medium) and 2 mM ^{13}C-labeled sodium acetate or unlabeled acetate with 30 mM $NaH^{13}CO_3$. Strain 195 exhibited exponential growth with a mean doubling time of approximately 4 days (Fig. 1a) in both the ^{13}C-labeled acetate and the bicarbonate minimal medium. The cells of strain 195 were harvested in middle exponential phase (day 18), when more than 80% of the TCE had been reduced and 0.23 ± 0.12 mM concentration of the acetate had been consumed (Fig. 1b).

Analysis of amino acid biosynthetic pathways. Derivatized amino acids were cracked into three charged fragments during MS analysis. The three types of charged fragments for each amino acid were as follows (see Fig. S4 in the supplemental material): fragment $[M-57]^+$ that contains the entire amino acid but has released a 57-molecular-weight (MW) *tert*-butyl group, fragment $[M-159]^+$ that has released a 159-MW group that contains the first carbon (α carboxyl group) of the amino acid, and fragment $[f302]^+$ that consists of a 302-MW group containing the amino acid with its R group released (4, 38). The isotopic labeling data for these fragments from the three tracer experiments are summarized in Tables S1 to S3 in the supplemental material. The mass fractions for M0, M1, M2, M3, and M4, which represent fragments containing zero to four ^{13}C-labeled carbons, respectively, are reported. The three tracer experiments used [1-^{13}C]sodium acetate, [2-^{13}C]sodium acetate, or unlabeled sodium acetate as carbon source with [^{13}C]sodium bicarbonate added when the acetate was unla-

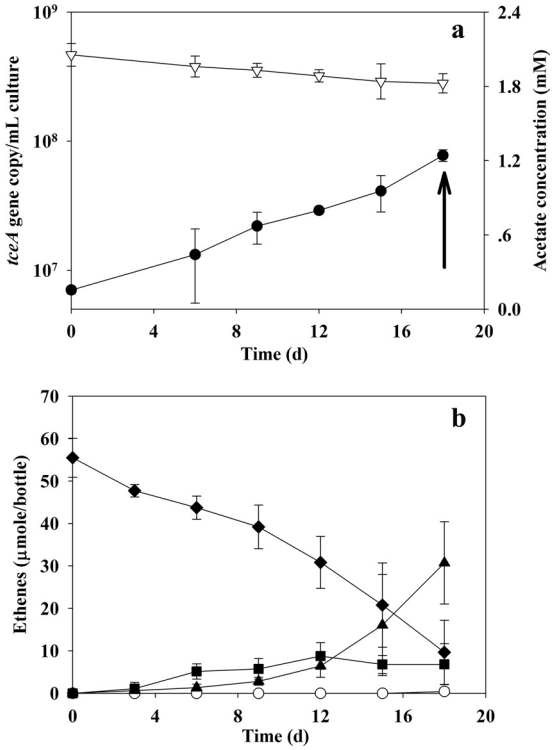

FIG. 1. Growth, acetate utilization, and TCE dechlorination by *D. ethenogenes* 195. (a) Growth and acetate utilization in mineral salt medium containing [1-^{13}C]acetate; (b) TCE dechlorination during growth on [1-^{13}C]acetate as the sole carbon source. An arrow indicates when the samples were collected for GC-MS analysis. Symbols: ▽, acetate; ●, *tceA* gene copies; ◆, TCE; ■, *cis*-DCE; ▲, VC; ○, ethene.

beled (see Tables S1 to S3 in the supplemental material). In each experiment, the fragmentation patterns of 14 of 20 proteogenic amino acids were analyzed in order to identify the labeling pattern of their precursors. Six amino acids were excluded from the analyses because cysteine and tryptophan were degraded during hydrolysis of biomass protein at 100°C, while histidine, arginine, lysine, and proline could not be measured precisely due to their very weak spectrum signals (35). Since the tracer experiments were not sufficient for the prediction of all ^{13}C-labeled positions, especially those within larger R groups, genome annotation was coupled with transcriptomic analysis (see Table S4 in the supplemental material) and known biochemistry to provide these labeling predictions. The predicted pathways and networks were then analyzed based on possible pathway links across all three labeling experiments.

The original genome annotation predicted that strain 195 might have incomplete pathways for the synthesis of methionine, glutamate (31), and probably for alanine and serine and that some key steps in gluconeogenesis (pathway important for nucleotide and sugars metabolism) were missing (such as fructose-bisphosphate aldolase and fructose-bisphosphatase) (http://www.microbesonline.org/). However, the fact that strain 195 can grow in minimal medium without the addition of amino acids (except for cysteine added as a reductant) and using acetate and CO_2 as carbon sources indicates that this organism must have evolved complete biosynthetic pathways for essential building blocks (Fig. 2).

When grown in [1-^{13}C]acetate, [2-^{13}C]acetate, or $NaH^{13}CO_3$, the labeling patterns of aspartate, methionine, and threonine (their second carbons were labeled) were similar, which suggested that these amino acids were derived from the same precursor (oxaloacetate). Identical labeling patterns for the aromatic amino acids phenylalanine and tyrosine across all three experiments confirmed that they were derived from the same precursors, whereas the side chain on the aromatic ring of the two amino acids was originally from pyruvate (same precursor as alanine) (see Tables S1 to S3 in the supplemental material) (25). Serine was predicted to be synthesized from 3-phosphoglycerate and, as such, was labeled on its second carbon (Fig. 2). *serA* (DET0599), the gene coding for the enzyme that catalyzes the first committed step in the phosphoserine pathway of serine biosynthesis, is highly expressed throughout the growth cycle of strain 195 (see Table S4 in the supplemental material). Labeling revealed that the first and the second carbons from the serine backbone were converted to glycine, whereas the third carbon (unlabeled) of serine was converted to 5,10-methylene-tetrahydrofuran (C1 pool), a finding consistent with high global expression of DET0436, the gene annotated for that reaction. The unlabeled C1 pool and aspartate were used to synthesize methionine, so methionine and aspartate were labeled identically in the [1-^{13}C]acetate experiment (Fig. 2). When [2-^{13}C]acetate was used as the carbon source, the C1 pool derived from the third carbon of serine became labeled so that methionine contained one more labeled carbon than aspartate (see Table S2 in the supplemental material), which is consistent with the serine results.

Although unlabeled cysteine was added to the minimal medium as a reductant to remove the trace oxygen during cell growth, unlabeled carbon from cysteine did not significantly dilute the labeling in other amino acids that could have been biosynthesized from cysteine metabolism. For example, unlabeled fractions (M0 ion) for alanine, serine, and phenylalanine from both [1-^{13}C]acetate and [2-^{13}C]acetate experiments (see Tables S1 and S2 in the supplemental material) were below 5%. This indicates that the unlabeled cysteine was not metabolized by strain 195 to synthesize other carbon metabolites.

Alternate isoleucine pathway in strain 195. Interestingly, the isotopomer labeling of isoleucine was identical to that of leucine in all tracer experiments (see Fig. 3 and Tables S1 to S3 in the supplemental material), indicating that isoleucine shares the same precursors (i.e., pyruvate and acetyl-CoA) as leucine. In most bacteria, isoleucine is synthesized from threonine via threonine ammonia-lyase (EC 4.3.1.19), but genome annotation predicts that strain 195 lacks this enzyme. In fact, isotopomer labeling indicates that strain 195 actually uses an alternate pathway for isoleucine synthesis from pyruvate (via the citramalate [(*R*)-2-methylmalate] route), which is analogous to findings recently reported for *Geobacter* spp. (30, 34). Furthermore, examination of strain 195's genome reveals that DET0825 has 53% amino acid sequence identity (see Fig. S1 in the supplemental material) to the recently characterized citramalate synthase (CimA) in *Geobacter sulfurreducens* (30). Reverse transcription-quantitative PCR demonstrates that this *cimA* candidate exhibits a strong and similar expression level to the 2-isopropylmalate synthase gene (*leuA*) throughout the growth cycle of

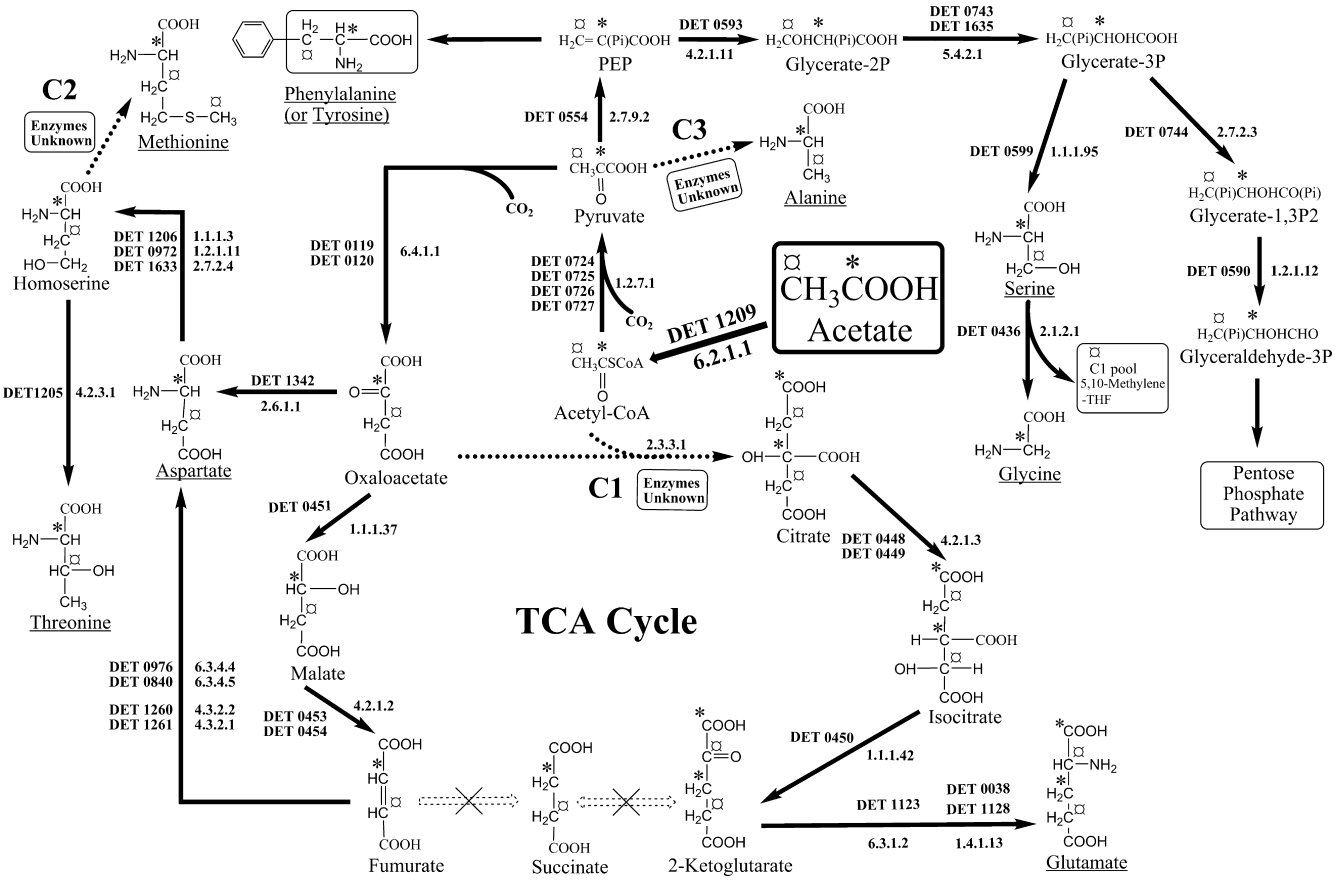

FIG. 2. Proposed central metabolic pathways in *D. ethenogenes* strain 195. ^{13}C-labeled isotopomers derived from [1-^{13}C]acetate are indicated with an asterisk (*), and those derived from [2-^{13}C]acetate are indicated with an "✳" symbol. The annotated pathways for amino acid synthesis were marked with solid arrows. Dotted arrows indicate incomplete amino acid pathways based on the genome annotation (www.genome.jp/kegg). C1 to C3 indicate the pathways that were confirmed to be functionalized based on the ^{13}C-labeling analysis in the present study.

strain 195 (see Fig. S2 in the supplemental material), suggesting that DET0825 may play an important role in the leucine-like pyruvate pathway for isoleucine biosynthesis.

Unusual (*Re*)-type citrate synthase in strain 195. Although genome annotation indicates the lack of a normal *Si*-type citrate synthase in strain 195 (31), *Re*-type citrate synthase activity was evident here based on the glutamate labeling pattern (>95% stereospecific). That is, in the [1-^{13}C]acetate experiment when acetyl-CoA would be labeled on its first carbon, glutamate was also mainly labeled on its first carbon (α-carboxyl group) (>95%) rather than the fifth carbon (β-carboxyl group) expected from an *Si*-type citrate synthase (Fig. 4). This observation was confirmed by both the [2-^{13}C]acetate and the [^{13}C]sodium bicarbonate experiments, since the first carbon of glutamate was not labeled if acetyl-CoA was not labeled on its first carbon (see Tables S2 and S3 in the supplemental material).

TCA pathway and CO_2 assimilation in strain 195. As shown in the labeled acetate experiments, the difference between the isotopic labeling in glutamate ($[M-57]^+$, M2 = 0.96) and aspartate ($[M-57]^+$, M1 = 0.93) precludes the possibility that strain 195 autotrophically assimilates CO_2 via a reductive citric acid cycle in a manner similar to phototrophic bacteria. A reversed TCA cycle would generate 2-ketoglutarate (precursor of glutamate) from oxaloacetate (precursor of aspartate) and unlabeled CO_2 and thus would result in similar labeling in aspartate and glutamate. This isotopomer information confirmed the genome annotation that the TCA cycle pathway was branched and broken at 2-ketoglutarate dehydrogenase (Fig. 2).

Although several genes (e.g., components of the CODH/ACDS complex [DET0665 to DET0667]) potentially involved in the Wood-Ljungdahl acetyl-CoA pathway of CO_2 fixation were predicted (31), the isotopic labeling results clearly indicate that this pathway was not active under the tested conditions. For example, the alanine and leucine labeling in the [1-^{13}C]acetate experiment indicates that acetyl-CoA (a precursor of alanine and leucine) was mostly labeled (Fig. 2 and 3) and thus was not derived from unlabeled CO_2 in the medium. In addition, when labeled sodium bicarbonate and unlabeled sodium acetate were used, the carboxyl group in leucine and isoleucine were not labeled (see Table S3 in the supplemental material). This evidence confirms that the enzymes in the Wood-Ljungdahl pathway were not playing a central role in generating acetyl-CoA by CO_2 fixation under the examined cultivation conditions, even though some of the genes related to the Wood-Ljungdahl pathway (i.e., DET0665 to DET0667) were highly expressed throughout the growth cycle of strain 195 (see Table S4 in the supplemental mate-

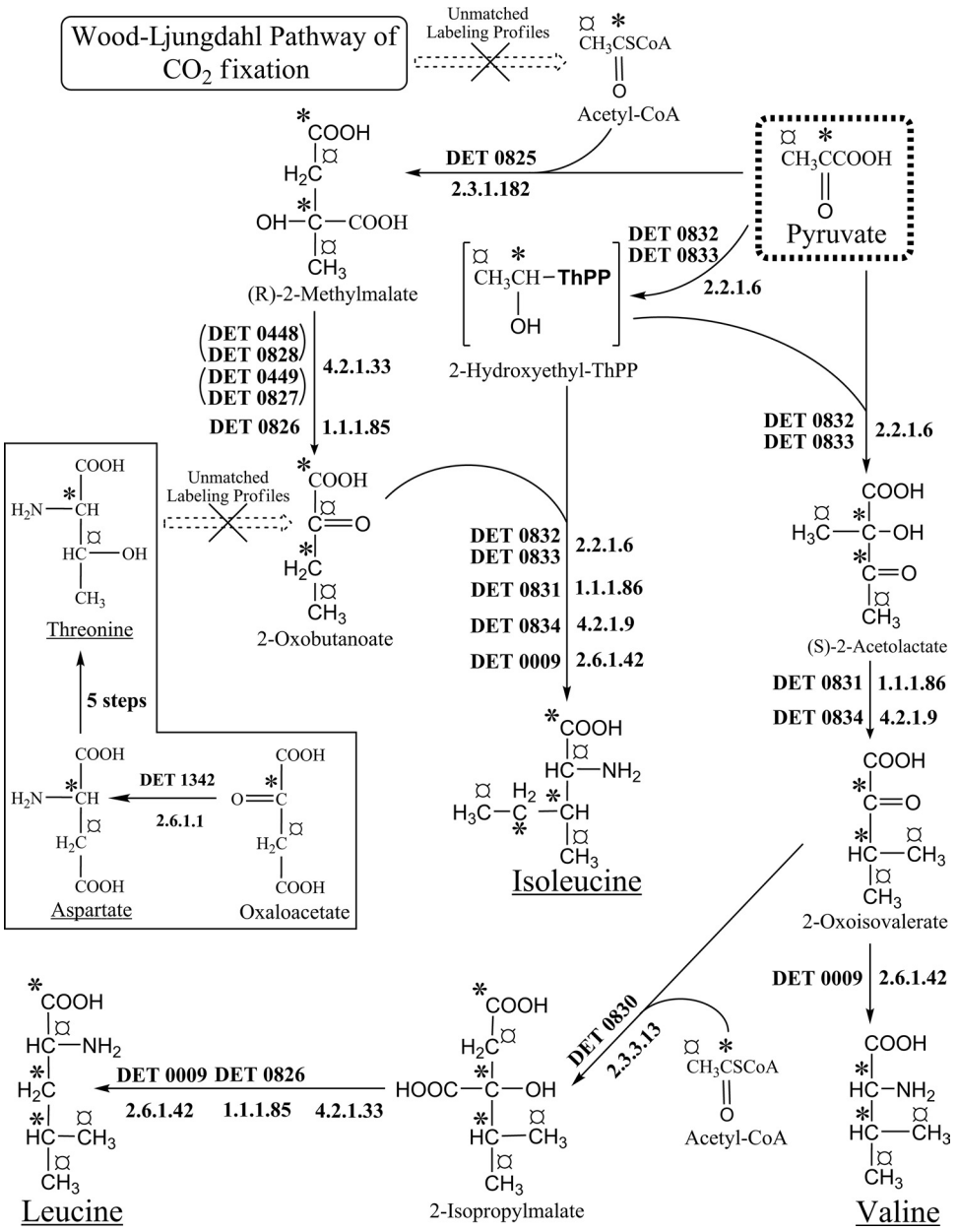

FIG. 3. Proposed mechanism for isoleucine biosynthesis in *D. ethenogenes* strain 195 via the citramalate [(*R*)-2-methylmalate] pathway. The positions of the ^{13}C label in the various amino acids from [1-^{13}C]acetate are indicated with an asterisk (∗), and those derived from [2-^{13}C]acetate are indicated by a "¤" symbol. Amino acids detected by GC-MS are underlined.

rial). On the other hand, several amino acids derived from the TCA cycle, such as alanine and aspartate, contained an unlabeled first carbon (α-carboxyl group) when the cells were grown on [1-^{13}C]acetate and [2-^{13}C]acetate (see Tables S1 and S2 in the supplemental material). This evidence indicates that the first carbons in these amino acids were derived primarily from unlabeled CO_2, possibly via pyruvic-ferredoxin oxidoreductase (DET0724 to DET0727) (Fig. 2). As indicated by the transcriptome, those genes were expressed at high levels throughout the growth cycle (see Table S4 in the supplemental material). In contrast, the experiment using labeled sodium bicarbonate and unlabeled acetate showed a very clear labeling of the first carbon in alanine, aspartate, and threonine. Furthermore, CO_2 assimilation can also occur via the route of pyruvate carboxylation based on the labeling information (Fig. 2). When unlabeled acetate and $^{13}CO_2$ was present in the medium, this reaction introduced a labeled carbon from $^{13}CO_2$ to the fourth carbon of aspartic acid, and consequently aspartic acid was labeled with two carbons ($[M-57]^+$, M2 = 0.48) as shown by Table S3 in the supplemental material. Thus, strain 195 assimilates CO_2 for the formation of cellular components possibly via pyruvic-ferredoxin oxidoreductase and pyruvate carboxylase, but autotrophic growth is clearly not evident.

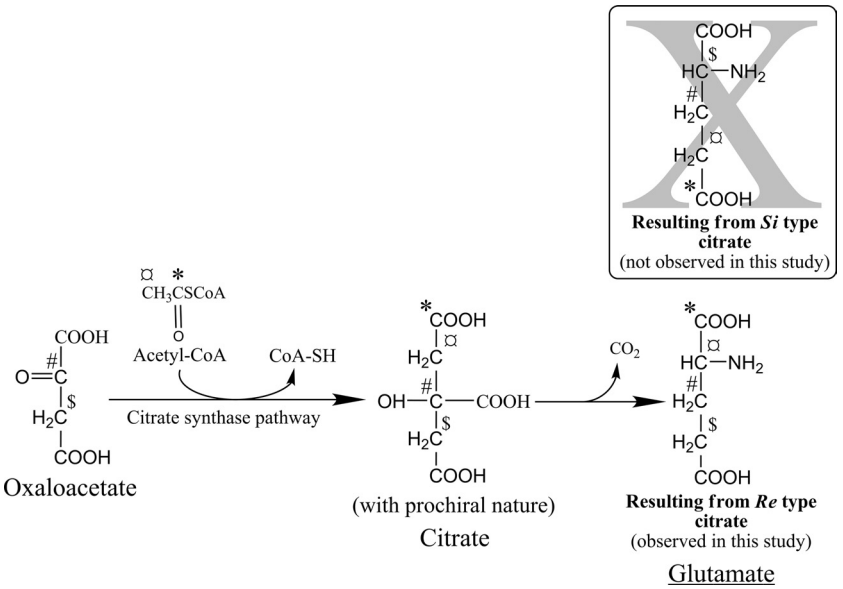

FIG. 4. Proposed mechanism for *Re*-citrate synthesis in *D. ethenogenes* strain 195. The positions of the ^{13}C label in acetyl-CoA and oxaloacetate from [1-^{13}C]acetate are indicated with "∗" and "#" symbols, respectively, and those derived from [2-^{13}C]acetate are indicated by "✶" and "$" symbols, respectively. The positions of the ^{13}C label in citrate and glutamate demonstrate the *Re* or *Si* types.

DISCUSSION

In this study, we used isotopomer analysis coupled with genomic and transcriptomic information to reveal the precursors of amino acids and to predict the active biosynthetic pathways in *D. ethenogenes* strain 195. For example, threonine ammonia-lyase (IlvA, EC 4.3.1.19), which catalyzes the conversion of threonine (synthesized from oxaloacetate) to 2-oxobutanoate in a common isoleucine pathway, was not identified in the strain 195 genome (31). We propose that strain 195 instead utilizes the citramalate pathway as an alternative isoleucine pathway, in which the key enzyme citramalate synthase (CimA) is probably encoded by DET0825. In fact, DET0825 is in a gene cluster with other key genes associated with leucine/isoleucine synthesis, including DET0826 (LeuB), DET0827 (LeuD), DET0828 (LeuC), and DET0830 (LeuA). Previous studies on a similar alternative isoleucine biosynthesis pathway in *Leptospira interrogans* showed that α-isopropylmalate isomerase (EC 4.2.1.33) (LeuC/D) and β-isopropylmalate dehydrogenase (EC 1.1.1.85) (LeuB) are functional in the biosynthesis of both leucine and isoleucine (42). That DET0825 to DET0828 are present in the same operon (www.microbesonline.org/operon) suggests that they may have coordinated regulation involved in isoleucine and leucine synthesis, since genes in a single pathway frequently reside in the same operon, exhibit similar expression patterns, and share transcriptional regulators (27). In addition, other *Dehalococcoides* spp., e.g., CBDB1 (CbdbA803) and BAV1 (DehaBAV1_0744), contain genes similar to the citramalate synthase gene that are also in the same operon with genes related to leucine and isoleucine synthesis (see Fig. S3 in the supplemental material).

The isotopomeric data in the present study suggests that strain 195 may contain a novel *Re*-citrate synthase, whose stereospecificity is opposite to that of the citrate synthase found in most organisms (*Si*-type, EC 2.3.3.1). An oxygen sensitive *Re*-citrate synthase (EC 2.3.3.3) was recently discovered in *Clostridium kluyveri* CKL0973, as well as in some *Desulfovibrio* spp. (17). When protein sequences of CKL0973 were compared to the strain 195 genome (www.jgi.doe.gov) using BLASTP (3), the resulting highest polypeptide amino acid sequence identity was very low: 28% for homocitrate synthase (DET1614) and 22% for 2-isopropylmalate synthase (LeuA, DET0830). Such low identity suggests that strain 195 might have a novel *Re*-citrate synthase. As suggested by a previous study, *Re*-citrate synthase is not only phylogenetically related to homocitrate synthase and isopropylmalate synthase families but also distantly related to citramalate synthase. These synthases all condense acetyl-CoA with other organic acids to form metabolites with molecular structure similar to that of citrate (Table 1). This suggests that the candidate gene encoding the *Re*-type

TABLE 1. Citrate, homocitrate, and isopropylmalate synthase

Enzyme	EC no.	Locus tag	Reaction
Si-citrate synthase	2.3.3.1	Missing	Oxaloacetate + acetyl-CoA → citrate
Re-citrate synthase	2.3.3.3	Missing	Oxaloacetate + acetyl-CoA → citrate
2-Isopropylmalate synthase	2.3.3.13	DET0825	3-Methyl-2-oxobutanoate + acetyl-CoA → 2-isopropylmalate
2-Isopropylmalate synthase	2.3.3.13	DET0830 (*leuA*)	3-Methyl-2-oxobutanoate + acetyl-CoA → 2-isopropylmalate
Homocitrate synthase	2.3.3.14	DET1614	2-Oxoglutarate + acetyl-CoA→ homocitrate

citrate synthase might be a homologue to the 2-isopropylmalate synthase/homocitrate synthase protein family. Further investigation of citrate synthase in strain 195 is under way.

Genome annotation and transcript profiling can provide a systematic description of metabolic pathways but may result in incomplete information due to undocumented functional genes and the functional gaps between genotype and phenotype. For example, some annotated genes in the Wood-Ljungdahl CO_2 fixation pathway are highly expressed throughout the growth cycle of strain 195, but ^{13}C-labeled isotopomer analysis indicates that this pathway does not generate significant acetyl-CoA, which was instead most likely generated from acetate by the highly expressed acetyl-CoA synthase/ligase (DET1209). That the Wood-Ljungdahl pathway genes are highly expressed when acetate is used as a carbon source is inconsistent with their serving a role in acetyl-CoA synthesis and, along with the pathway's being annotated as incomplete, highlights our poor understanding of their role.

The present study has demonstrated that in vivo ^{13}C-labeled metabolite analysis is a useful tool for studying fundamental metabolic pathways and provides complementary and direct information for genomic and transcriptomic analysis of cellular metabolism. Understanding *Dehalococcoides* metabolism is important for identifying potential bottleneck pathways for exploiting their capabilities in chloroethene bioremediation.

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