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Derek Lovley, *University of Massachusetts - Amherst*
Carla Risso
Stephen J. Van Dien
Amber Orloff
Maddalena V Coppi

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Elucidation of an Alternate Isoleucine Biosynthesis Pathway in
Geobacter sulfurreducens

Carla Risso,1* Stephen J. Van Dien,2 Amber Orloff,1 Derek R. Lovley,1 and Maddalena V. Coppi1

Department of Microbiology, 203N Morrill Science Center IVN, University of Massachusetts Amherst, Amherst, Massachusetts 01003, and Genomatica, Inc., 5405 Morehouse Drive, Suite 210, San Diego, California 921212

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The central metabolic model for Geobacter sulfurreducens included a single pathway for the biosynthesis of isoleucine that was analogous to that of Escherichia coli, in which the isoleucine precursor 2-oxobutan-2-olate is generated from threonine. 13C labeling studies performed in G. sulfurreducens indicated that this pathway accounted for a minor fraction of isoleucine biosynthesis and that the majority of isoleucine was instead derived from acetyl-coenzyme A and pyruvate, possibly via the citramalate pathway. Genes encoding citramalate synthase (GSU1798), which catalyzes the first dedicated step in the citramalate pathway, and threonine ammonia-lyase (GSU0486), which catalyzes the conversion of threonine to 2-oxobutan-2-olate, were identified and knocked out. Mutants lacking both of these enzymes were auxotrophs for isoleucine, whereas single mutants were capable of growth in the absence of isoleucine. Biochemical characterization of the single mutants revealed deficiencies in citramalate synthase and threonine ammonia-lyase activity. Thus, in G. sulfurreducens, 2-oxobutan-2-olate can be synthesized either from citramalate or threonine, with the former being the main pathway for isoleucine biosynthesis. The citramalate synthase of G. sulfurreducens constitutes the first characterized member of a phylogenetically distinct clade of citramalate synthases, which contains representatives from a wide variety of microorganisms.

The Geobacteraceae are a family of dissimilatory Fe(III)- reducing Deltaproteobacteria that are predominant members of microbial communities in a diversity of environments where dissimilatory iron reduction is the primary terminal electron accepting process (3, 10, 23, 26, 33, 34, 44). They have been found to play an important role in the natural cycling of Fe(III) and organic compounds, the bioremediation of both organic and metal contamination, and the generation of electricity from organic matter in microbial fuel cells (3, 4, 16, 18, 25, 26, 32). A model of central metabolism was constructed for the genetically tractable Geobacter species, Geobacter sulfurreducens, based on the results of comparative genomic analyses coupled with physiological and genetic studies (29). This network contained 522 biochemical reactions and 541 unique metabolites, including all 20 amino acids, and was used to create a constraint-based model of G. sulfurreducens metabolism which accurately simulated growth via acetate oxidation and the reduction of either Fe(III) citrate or fumarate (29). Many of the amino acid biosynthetic pathways included in the network were analogous to those of Escherichia coli, including that for isoleucine (Fig. 1A). In E. coli, the first dedicated step in isoleucine biosynthesis is the conversion of threonine to 2-oxobutan-2-olate by the enzyme threonine ammonia-lyase (37). However, alternate precursors for the synthesis of isoleucine have been identified in other organisms, including 2-methylbutyrate, propionate, and citramalate (17, 19, 31, 34, 47). In this study, evidence of two pathways for the biosynthesis of 2-oxobutan-2-olate in G. sulfurreducens is presented. Our results indicate that 2-oxobutan-2-olate can be synthesized either from citramalate or threonine, with the former being the main pathway for isoleucine biosynthesis in G. sulfurreducens. Homologs of the citramalate synthase of G. sulfurreducens (cimA) have been found in a wide diversity of microorganisms, particularly, in the Deltaproteobacteria.

MATERIALS AND METHODS

Bacterial strains and growth conditions. G. sulfurreducens (ATCC 51753) (9) strain DL1 was obtained from our laboratory culture collection and used to construct strains DLCR5 (tdcB::Kn′), DLCR6 (cimA::Gmr′) and the double mutant DLCR7 (tdcB::Kn′ cimA::Gmr′) as described below. Strains were cultured under strict anaerobic conditions at 30°C in an atmosphere of N2 and CO2 (80%/20%), as previously described (6), in either fresh-water medium (27) or NBAF medium (electron donor, 20 mM acetate; electron acceptor, 80 mM fumarate) (9). Isoleucine was added to a final concentration of 0.02% when required. Antibiotics were added at the following final concentrations: 50 μg/ml for kanamycin and 20 μg/ml for gentamicin.

Nucleic acid manipulations. Genomic DNA preparations and gel extractions were carried out using the Qiagen Genomic-tip 100G and Qiaquick Gel Extrac-

kits, respectively (Qiagen Inc, Valencia, CA).

Construction of mutants via single-step gene replacement. Single-step gene replacement was performed essentially as previously described (9, 24). The sequences of all primers used for the construction and screening of strains DLCR5, DLCR6, and DLCR7 are listed in Table 1. To create a linear DNA fragment for the construction of mutant DLCR5, three primary fragments were generated independently by PCR. The first fragment was amplified from DL1 chromosomal DNA using primers 486-1b and 486-2b (Table 1). The middle segment containing a kanamycin resistance cassette was amplified from plasmid pBBR1MCS-2 (21) with hybrid primers 486-3bKn and 486-4bKn (Table 1). The third fragment was amplified from DL1 chromosomal DNA using primers 486-5b and 486-6b (Table 1). PCR conditions were as follows: 35 cycles of 95°C for 30 s, 58°C for 90 s, and 72°C for 45 s. The reactions were preceded by a 5-min incubation at 95°C during which Taq polymerase was added (“hot start”) and followed by a 10-min extension period at 72°C. The amplified fragments were gel purified and joined by recombinant PCR. The resulting linear fragment was amplified with distal primers 486-1b and 486-6b (Table 1). PCR conditions during these two steps were as described above except that an extension time of 3 min at 72°C was employed. A similar strategy was used to create a linear DNA

* Corresponding author. Mailing address: Department of Microbi-
ology, 203N Morrill Science Center IVN, University of Massachusetts
Amherst, Amherst, MA 01003. Phone: (413) 577-2439. Fax: (413) 577-4660. E-mail: crisso@microbio.umass.edu.

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fragment for the construction of mutant DLCR6 (cimA::Gm$^r$). For this mutant, the first fragment was amplified from DL1 chromosomal DNA using primers 1798-1b and 1798-2b (Table 1). The middle fragment containing a gentamicin resistance cassette was amplified from plasmid pBSL141 (1) with hybrid primers 1798-3Gm2/1798-4Gm2. The third fragment was amplified from DL1 chromosomal DNA with primers 1798-5b and 1798-6b. The individual pieces were joined by recombinant PCR and amplified using the distal primer pairs 1798-1b and 1798-6b. The double mutant DLCR7 was constructed by knocking out the cimA gene in DLCR5 using the linear fragment described above.

Electroporation and mutant isolation were carried out as previously described (9, 24) except that the plating medium was supplemented with 0.02% isoleucine. In the case of double mutant DLCR7, the recovered colonies were replica plated onto plates with and without isoleucine, and colonies that failed to grow without 0.02% isoleucine were selected for further analysis. One of each of the mutants was selected as a representative.

In order to confirm the genotypes of the strains, PCRs were carried out with distal primers using chromosomal DNA from DL1 and each of the mutants as template. Distal primers 486-1b and 486-6b span a 1.2-kb fragment in DL1 and a 2.8-kb fragment in DLCR5 (tdcB::Knr). Similarly, 1798-1b and 1798-6b span a 1-kb fragment in DL1 and a 2.1-kb fragment in DLCR6 (cimA::Gm$^r$) and DLCR7 (cimA::Gm$^r$tdcB::Knr). In all cases bands of the expected sizes were obtained. The mutant and wild-type strains were also screened with combinations of primers that annealed outside and inside the two mutagenic constructs and thus were expected to yield amplicons only in specific mutants. Primers 486-1b/486-4bKn and 486-3bKn/486-6b were used to confirm the presence of the tdcB::Knr mutation in strains DLCR5 and DLCR7, while primers 1798-1b/1798-4Gm2 and 1798-3Gm2/1798-6b were used to confirm the presence of the cimA::Gm$^r$ mutation in strains DLCR6 and DLCR7. As expected, bands of the correct sizes were obtained from all the mutants but not from the wild type (data not shown).

![FIG. 1. Pathways for isoleucine biosynthesis in *G. sulfurreducens*. (A) Threonine-dependent pathway (29, 39). (B) Citramalate-dependent pathway, based on that of *L. interrogans* (47).](http://jb.asm.org/)

## TABLE 1. Primers used for mutant construction by recombinant PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Coordinate$^a$</th>
<th>Length (no. of residues)</th>
<th>Sequence (5' → 3')$^b$</th>
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<tr>
<td>486-1b</td>
<td>517645</td>
<td>21</td>
<td>CATCACCGGGCATTCACACTAG</td>
</tr>
<tr>
<td>486-2b</td>
<td>516976</td>
<td>20</td>
<td>CTTTTTGACGGGATATGTGG</td>
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<td>CCACATTTGCGTGGAAAAAGGAGCAGTACTGGGGTTATGGAACAA</td>
</tr>
<tr>
<td>486-4bKn</td>
<td>Hybrid primer</td>
<td>41</td>
<td>GATTGAGGAAAAAGGTGGGCTATCGTGTCGTCATTTC</td>
</tr>
<tr>
<td>486-5b</td>
<td>515892</td>
<td>21</td>
<td>GTGAAACCTTCTTCCATCTAC</td>
</tr>
<tr>
<td>486-6b</td>
<td>515413</td>
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<td>1964589</td>
<td>19</td>
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</tr>
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<td>GGTCAGGGGAAGGATGGCACTTCAGTCTGTCGTCGTCATCACCC</td>
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<tr>
<td>1798-4Gm2</td>
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<tr>
<td>1798-6b</td>
<td>1963728</td>
<td>20</td>
<td>TCCACACTGTCCGGAAGAAG</td>
</tr>
</tbody>
</table>

$^a$ Negative strand nucleotide position.

$^b$ Hybrid primers are composed of *G. sulfurreducens* sequences and antibiotic resistance cassette sequences. The underlined sequences in primers 486-3bKn, 486-4Kn, 1798-3Gm2, and 1798-4Gm2 correspond to the reverse complements of primers 486-2b, 486-5b, 1798-2b, and 1798-5b, respectively.
TABLE 2. Comparison of experimental and predicted mass isotopomer distributions during growth of wild-type
G. sulfurreducens on acetate-fumarate medium containing 30% [13C]acetate and unlabeled fumarate.

<table>
<thead>
<tr>
<th>Amino acid and mass isotopomer&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Experimental mass isotopomer distribution&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Predicted MDV&lt;sup&gt;c&lt;/sup&gt;</th>
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<tbody>
<tr>
<td></td>
<td>Threonate pathway</td>
<td>Citramalate pathway</td>
</tr>
<tr>
<td>Aspartate</td>
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<td></td>
</tr>
<tr>
<td>M&lt;sub&gt;0&lt;/sub&gt;</td>
<td>0.9350</td>
<td>0.9572</td>
</tr>
<tr>
<td>M&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0</td>
<td>0.0339</td>
</tr>
<tr>
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<td>0.0080</td>
</tr>
<tr>
<td>M&lt;sub&gt;3&lt;/sub&gt;</td>
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<td>0.0009</td>
</tr>
<tr>
<td>Isoleucine</td>
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<td></td>
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<td>0.0115</td>
</tr>
<tr>
<td>M&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>0.0023</td>
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<tr>
<td>M&lt;sub&gt;5&lt;/sub&gt;</td>
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<td>0.0003</td>
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<tr>
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<tr>
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<td>0.2813</td>
</tr>
<tr>
<td>M&lt;sub&gt;3&lt;/sub&gt;</td>
<td>0.1213</td>
<td>0.1312</td>
</tr>
<tr>
<td>M&lt;sub&gt;4&lt;/sub&gt;</td>
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<td>0.0594</td>
</tr>
<tr>
<td>M&lt;sub&gt;5&lt;/sub&gt;</td>
<td>0</td>
<td>0.0250</td>
</tr>
<tr>
<td>Total error&lt;sup&gt;d&lt;/sup&gt;</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.3608</td>
<td>0.1495</td>
</tr>
</tbody>
</table>

<sup>a</sup> The unlabeled molecule is shown as M<sub>0</sub>, and numbers above zero correspond to the numbers of additional mass units ([13C] atoms).

<sup>b</sup> Experimental values are averages of two fragments for each amino acid, both missing the carboxy-terminal carbon atom. The mass isotopomer distributions for these two fragments were the averages of single derivatization reactions performed on extracts prepared from three replicate cultures.

<sup>c</sup> MDVs were calculated by running the optimization routine assuming each pathway separately, and then allowing the optimization routine to select the best-fitting ratio between them.

<sup>d</sup> Errors are the sum of squared differences between predicted and experimental values for aspartate, isoleucine, and leucine fragments.

**Analytical techniques.** Growth of fumarate cultures was assessed by measuring optical density at 600 nm with a Genesys 2 spectrophotometer (Spectronic Instruments, Rochester, NY). The organic acid content of the culture medium was determined by high-pressure liquid chromatography using an LC-10AT high-pressure liquid chromatograph (Shimadzu, Kyoto, Japan) equipped with an Aminex HPX-87H column (300 by 7.8 mm; Bio-Rad, Hercules, CA). Organic acids were eluted in 8 mM H<sub>2</sub>SO<sub>4</sub> and quantitated with an SPD-10VP UV detector (Shimadzu, Kyoto, Japan) set at 215 nm. Fe(II) concentrations were determined with a ferrozine assay as previously described (26). Protein concentrations were determined by the bicinchoninic acid method with bovine serum albumin as a standard (40).

**13C labeling.** For the 13C labeling experiments described in Tables 2 and 3, cells were grown in fresh-water medium containing either 10 mM acetate (30% [13C]acetate–70% unlabeled acetate [mol/mol]) and 27.5 mM unlabeled fumarate or 10 mM unlabeled acetate and 27.5 mM fumarate (30% [13C]fumarate–70% unlabeled fumarate [mol/mol]). The labeled compounds are abbreviated hereafter as [13C]<sub>acetate</sub> and [13C]<sub>fumarate</sub>, respectively. Note that “unlabeled” in this context means containing the natural abundance of 1.07% [13C] in each carbon atom. For the 13C labeling experiments performed to compare the wild-type and the mutant strains, cells were grown in NBAF medium containing 20 mM acetate (30% [13C]acetate–70% unlabeled acetate [mol/mol]) and 40 mM unlabeled fumarate. 13C compounds were purchased from Sigma-Aldrich Corporation (St. Louis, MO).

**Processing and GC-mass spectrometry analysis of 13C-labeled samples.** Cells were cultured for a minimum of two 5% transfers in 13C-labeled medium prior to harvesting at mid-log phase by centrifugation at 3,000 × g. Cell pellets were resuspended in isotonic buffer, pH 7 (4.19 g/liter morpholinepropanesulfonic acid, 0.6 g/liter NaH<sub>2</sub>PO<sub>4</sub> · H<sub>2</sub>O, 0.1 g/liter KCl, 5 g/liter NaCl, 10 ml of Mg-Ca mix [pH 7]). Mg-Ca mix contains 3 g/liter MgSO<sub>4</sub> · 7H<sub>2</sub>O and 0.1 g/liter CaCl<sub>2</sub> (Mg-Ca mix containing unlabeled acetate and unlabeled fumarate. 13C compounds were purchased from Sigma-Aldrich) (2, 12) and incubated for 60 min at 75°C. The unlabeled molecule is shown as M<sub>0</sub>, and numbers above zero correspond to the number of additional mass units ([13C] atoms).

**Errors** are the sum of squared differences between predicted and experimental values for aspartate, isoleucine, and leucine fragments.

**Development of the isotopomer balance model.** A reduced metabolic model of G. sulfurreducens, containing 209 reactions and 143 metabolites, was derived from the genome-scale model (29) by retaining only those reactions involved in central carbon and amino acid metabolism. The reduced network contained 79 reversible reactions, which added an additional level of complexity, since both...
forward and reverse reaction rates affect the observed isotopomer distribution. This is typically addressed by using net fluxes and exchange coefficients (11, 45). The 79 exchange coefficients for the reversible reactions, therefore, constituted additional adjustable parameters which had to be determined using the isotopomer model. Finally, redox and ATP balances were incorporated into the flux analysis model as constraints in order to make the calculated optimum feasible. For simplicity, NADH and NADPH were considered equivalent, and the electron transport chain was represented by a single reaction. Isotopomer mapping matrices (IMMs) describe the transfer of carbon atoms from the reactants to products and are a property of a given reaction independent of the particular model (38). IMMs for the majority of the reactions were obtained from an E. coli isotopomer model of similar size (41). The IMMs for the remaining Geobacter-specific reactions were created by hand, based on known biochemistry.

Calculation of bounds on exchange fluxes. In order to establish bounds on the exchange fluxes, the rates of acetate and fumarate uptake as well as succinate and biomass production were determined by periodically sampling the cultures. Effective fumarate uptake was determined by two measurements: (i) fumarate depletion minus malate accumulation or (ii) succinate accumulation. In order to maintain consistency among experiments, all rates were then normalized to a flux of 10 mmol g (dry weight)\(^{-1}\) h\(^{-1}\) acetate uptake. Because of variability in the measurements, constraints were set as ranges rather than fixed values, with the ranges determined by error propagation of the standard deviations of the measurements. The values calculated for fumarate uptake were very consistent, and for each case the wider range of the two calculated was used.

Flux analysis calculations. The isotopomer balance algorithm calculated the predicted set of isotopomer distribution vectors (IDVs) for all metabolites in the network for a given flux distribution. The input was a random flux distribution and set of exchange coefficients that were within specified bounds and satisfied the overall metabolite balance, \( S \cdot v = 0 \), where \( S \) is the stoichiometric matrix for the reaction network and \( v \) is the vector of net reaction fluxes. Through each iteration, the IDV of compound \( i \) was calculated as follows:

\[
\text{IDV}_i = \frac{1}{M} \sum_{k=1}^{M} \frac{v_{i,k}}{v_{\text{final}}^i} \times (\text{IMM}_{i,k} \times \text{IDV}_j)
\]

where \( M \) is the total number of reactions in the network, \( n_k \) is the number of substrates in reaction \( k \), \( v_{i,k} \) is the flux of metabolite \( i \) in reaction \( k \) if it is consumed, and \( v_{\text{final}}^i \) is the flux if it is produced. The resulting program was model independent. It generated the isotopomer balance once the stoichiometric matrix and list of IMMs were supplied and calculated all IDVs given the input flux distribution and isotopomer distribution of the feed molecules acetate and fumarate (41). Subsequently, these IDVs were converted to mass distribution vectors (MDVs), column vectors containing mole fractions for groups of isotopomers with the same mass for all observable products (46). The genetic strategy that creates diversity in a “population” of flux distributions through small changes in the parameter values (mutation) or combination of parameters from two different “parent” flux distributions (7, 15) was used as the optimization routine for flux analysis. An initial population size of 1,000 was used, and optimizations were run until the routine converged on a minimum, usually within 100 generations. The objective value was the sum-of-squares difference between the measured and calculated MDV values, weighted by the standard deviations of the measurements, constraints were set as ranges rather than fixed values, with the ranges determined by error propagation of the standard deviations of the measurements. The values calculated for fumarate uptake were very consistent, and for each case the wider range of the two calculated was used.

Enzymatic assays. For the initial biochemical characterization of the wild-type strain, cells were cultured in fresh-water medium containing 10 mM acetate and 27.5 mM fumarate and harvested at mid-log phase. For biochemical assays comparing the wild-type and mutant strains, cells were grown in NBAF medium and harvested at early stationary phase. Soluble extracts were prepared as described above, aliquoted, and stored at \(-80°C\). Threonine and serine ammonia-lyase activities were assayed essentially as previously described (22) by measuring the production of ketone (either 2-oxobutanoate or pyruvate) colorimetrically with 2,4-dinitrophenyl hydrazine, except that NH\(_4\)Cl was omitted, and the final concentration of threonine was 50 mM. The citramalate synthase activity was assayed by monitoring the pyruvate-dependent release of coenzyme A (CoA) from acetyl-CoA as previously described (17) with the following modifications: the samples were incubated at 37°C, the concentration of acetyl-CoA was 0.5 mM, and 0.1% sodium dodecyl sulfate was added to the stop solution. The assay for isopropylmalate synthase was identical to the citramalate synthase assay, except that pyruvate was replaced with \(\alpha\)-ketoglutarate.

RESULTS AND DISCUSSION

Evidence for an alternate isoleucine biosynthesis pathway in \(G.\) sulfurreducens. \(^{13}\)C metabolic flux analysis can be used as a tool for verifying genome annotation, optimizing metabolic models, and elucidating the physiological state of microorganisms (11). In order to test the accuracy of the reconstructed central metabolic network of \(G.\) sulfurreducens, \(^{13}\)C labeling studies were initiated. An isotopomer balance model for \(G.\) sulfurreducens, with the IMMs taken largely from an \(E.\) coli model (41), was developed.

In these studies, \(G.\) sulfurreducens was cultured in freshwater acetate-fumarate medium containing either 30% (mol/mol) \([13\text{C}]\)acetate (labeled at both carbons) or \([13\text{C}]\)fumarate (labeled at carbons 2 and 3). During growth on this medium, the tricarboxylic acid cycle (TCA) cycle functions as an open loop in which the succinate dehydrogenase reaction is bypassed (13). Continual flux through the remaining reactions of the TCA cycle is maintained by coupling the secretion of succinate to the uptake of fumarate via the dicarboxylate exchanger, DcuB (5, 13, 29, 42). As a result, the TCA cycle intermediate, oxaloacetate, is derived primarily from exogenous fumarate. In fact, during growth on acetate-[\(^{13}\text{C}]\)fumarate medium, the mass isotopomer distribution of aspartate, which derives from oxaloacetate, matched that of the feed (30% doubly labeled/70% unlabeled). In contrast, during growth on \([13\text{C}]\)acetate-fumarate medium, aspartate was primarily unlabeled (Tables 2 and 3), confirming the presence of the open loop. Pyruvate is another common amino acid precursor. In \(G.\) sulfurreducens, pyruvate biosynthesis occurs primarily via the condensation of acetyl-CoA and CO\(_2\) by the pyruvate-ferredoxin oxidoreductase (39). In fact, leucine, which was predicted to be derived from acetyl-CoA and pyruvate, was labeled during growth on \([13\text{C}]\)acetate-fumarate, and essentially unlabeled during growth on acetate-[\(^{13}\text{C}]\)fumarate, confirming the central role of the pyruvate-ferredoxin oxidoreductase in pyruvate biosynthesis.

According to the annotated pathway (Fig. 1A), both oxaloacetate and pyruvate serve as precursors for isoleucine biosynthesis, and thus this amino acid should be labeled in the presence of both [\(^{13}\text{C}]\)acetate and \([13\text{C}]\)fumarate (Tables 2 and 3, threonine-dependent pathway). However, the isotopomer mass distribution of isoleucine did not match the expected pattern: isoleucine was extensively labeled in the presence of [\(^{13}\text{C}]\)acetate but poorly labeled in the presence of \([13\text{C}]\)fumarate (Tables 2 and 3). This suggested that the annotated threonine-dependent pathway did not play a major role in isoleucine biosynthesis and that acetyl-CoA and/or pyruvate was the predominant precursor for this amino acid.

In the spirochete \(Leptospira\) interrogans and in methanogenic \(Archaeae\), the key isoleucine precursor, 2-oxobutanate, is synthesized from acetyl-CoA and pyruvate via the citramalate pathway (17, 34, 47) (Fig. 1B). The first dedicated step in this pathway is the condensation of pyruvate and acetyl-CoA by the enzyme citramalate synthase (CimA; EC 4.1.3.22). The introduction of this pathway into the \(G.\) sulfurreducens isotopomer
balance model significantly improved the agreement of experimental and predicted isotopomer mass distributions (Tables 2 and 3). The best fit was generated by allowing flux through both pathways, with the citramalate pathway serving as the primary route of isoleucine biosynthesis (Fig. 2), accounting for 68 to 77% of the total flux to isoleucine.

In order to determine whether the citramalate pathway was active in *G. sulfurreducens*, crude soluble extracts were prepared from mid-log, freshwater acetate-fumarate cultures grown under the same conditions as those used for 13C flux analysis studies and tested for the presence of citramalate synthase activity. These extracts contained 5.94 ± 0.49 nmol min⁻¹ of citramalate synthase activity, measured as the pyruvate-dependent release of CoA from acetyl-CoA (47). The citramalate synthase appeared to have a high affinity for pyruvate, with 52.5% ± 4.9% of the activity remaining when the pyruvate concentration was reduced from 1 mM to 0.1 mM. Although these results were consistent with the presence of citramalate synthase, they were not conclusive due to the fact that isopropylmalate synthase, which catalyzes the first step in leucine biosynthesis, has residual citramalate synthase activity (20, 34, 47). In addition, high levels of pyruvate-independent CoA release interfered with detection of the enzyme activity and accurate determination of the *K_m* for pyruvate.

Because both 13C labeling studies and preliminary biochemical studies were consistent with the presence of citramalate synthase in *G. sulfurreducens*, we examined the genome for candidate genes. Only two citramalate synthases had been characterized, those of *L. interrogans* and *Methanocaldococcus jannaschii* (17, 47). Both citramalate synthases were homologous to isopropylmalate synthase (LeuA), which catalyzes the first step in the biosynthesis of leucine (47). Examination of the genome failed to reveal any obvious candidates. In order to address this issue, we performed a phylogenetic analysis of threonine ammonia-lyases (Fig. 3). The result was consistent with the presence of citramalate synthase in *G. sulfurreducens*, since candidate genes were found in the genome of that organism.
G. sulfurreducens genome revealed three LeuA family members: GSU1906, GSU1798, and GSU0937. Comparison to characterized enzymes suggested that GSU1906, which has 65% sequence similarity to LeuA of Salmonella enterica serovar Typhimurium, encoded an isopropylmalate synthase. The remaining candidate, GSU1798, which was annotated as a homolog (30), is equally similar to characterized isopropylmalate synthases. Each determination represents the average and standard deviation of triplicate cultures.

Because flux analysis indicated that the threonine-dependent pathway was a relatively minor contributor to isoleucine biosynthesis, we reexamined the genomic evidence for this pathway. Threonine ammonia-lyase (GSU0486) was the only biosynthetic threonine ammonia-lyase (TdcB; EC 4.3.1.19), which encoded a homocitrate synthase. The G. sulfurreducens genome revealed three LeuA family members: GSU1906, GSU1798, and GSU0937. Comparison to characterized enzymes suggested that GSU1906, which has 65% sequence similarity to LeuA of Salmonella enterica serovar Typhimurium, encoded an isopropylmalate synthase. The remaining candidate, GSU1798, which was annotated as a homolog (30), is equally similar to characterized isopropylmalate synthases; it is 46% similar to LeuA homolog (30), is equally similar to characterized isopropylmalate synthases. Each determination represents the average and standard deviation of triplicate cultures.

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Genetic evidence for two isoleucine biosynthetic pathways.

In order to corroborate the results of the preliminary biochemical analysis and evaluate the functions of the putative threonine ammonia-lyase (GSU0486; \textit{tdcB}) and citramalate synthase (GSU1798; \textit{cimA}) genes, three mutant strains were constructed: a threonine ammonia-lyase knockout mutant (DLCR5; \textit{tdcB::Knr}), a citramalate synthase knockout mutant (DLCR6; \textit{cimA::Gmr}), and a double knockout mutant (DLCR7; \textit{tdcB::Knr cimA::Gmr}) (Fig. 4A). The single mutants grew on the standard plating medium, whereas the double mutant grew only on plates supplemented with 0.02% isoleucine. This indicated that there were no other pathways generating the key precursor 2-oxobutanoate and that both pathways contributed to the biosynthesis of isoleucine. Moreover, these genes could compensate for each other. During growth on acetate-fumarate medium (Fig. 4B), the growth rate and biomass yields of both single mutants were very similar to wild type, albeit there was a small increase in the doubling time of the citramalate synthase-deficient mutant relative to wild type (6 ± 0.13 h versus 5.25 ± 0.18 h). During growth on acetate-Fe(III) citrate medium, the rate of Fe(III) citrate reduction (Fig. 4C) and the final biomass yields of the two single mutants (data not shown) were essentially identical to the wild type.

In order to confirm that GSU0486 and GSU1798 coded for threonine ammonia-lyase and citramalate synthase, respectively, soluble extracts of the wild-type and the three mutant strains were prepared from early-stationary-phase NBAF cultures, and enzymatic assays were performed (Table 4). In the wild-type strain, the two activities were comparable to those obtained from extracts prepared from mid-log fresh-water medium cultures. As expected, threonine ammonia-lyase and
serine ammonia-lyase activities were undetectable in DLCR5. Likewise, citramalate synthase activity was greatly reduced in DLCR6. Neither activity could be detected in the isoleucine auxotroph DLCR7. Isopropylmalate synthase activity was assayed as an internal control and was found to be identical or higher than wild type in the three mutant strains. These results indicate that the current annotation of GSU0486 as ilvA and GSU1798 as a leuA homolog does not reflect their actual enzymatic activities. We propose that they be reannotated as threonine/serine ammonia-lyase (tdcB) and citramalate synthase (cimA), respectively.

Unlike the closely related catabolic ammonia lyases of E. coli and S. enterica serovar Typhimurium, which have a strictly biodegradative role in these organisms (37), the threonine ammonia-lyase of G. sulfurreducens clearly participates in isoleucine biosynthesis. Despite the fact that the contribution of the threonine-dependent pathway to isoleucine biosynthesis in the wild-type strain was relatively minor (18 to 30%) (Fig. 2), the amount of threonine ammonia-lyase activity in soluble extracts was about 25-fold higher than that of citramalate synthase activity. This discrepancy was due to low intracellular concentrations of threonine and/or to inhibition of the enzyme by pyruvate, which occurs in E. coli (37). A detailed biochemical characterization of these enzymes coupled with measurements of intracellular concentrations of amino acids and metabolites is therefore warranted.

In order to corroborate the roles of the citramalate synthase and the threonine ammonia-lyase in isoleucine biosynthesis, 13C labeling studies were conducted in all three mutants and the wild-type strain. Isoleucine was 90 to 95% unlabelled in the isoleucine auxotroph DLCR7 (data not shown). A clear shift in the isoleucine flux ratio to primary use of the citramalate-dependent pathway in DLCR5 and the threonine-dependent pathway in DLCR6 was observed (Table 5 and Fig. 5). The residual fluxes in the deleted pathway in each case were likely due to imperfect model fit to the experimental data. Error in flux ratios can result from random variations in mass spectrometry data, loss of information in converting positional isotopomer distributions to mass isotopomer distributions of measurable fragments, and the nonlinearity of the optimization problem (45).

**Distribution of the citramalate synthase.** The CimA protein from G. sulfurreducens constitutes the first characterized member of a phylogenetically distinct clade of citramalate synthases (Fig. 6, clade III). This clade contains representatives from a wide range of bacteria including Deltaaproteobacteria, Alphaproteobacteria, Cyanobacteria, Deinococci, and Clostridia as well as members of the Archaea, such as Thermococcales. Clade III representatives were also found in the Actinobacteria, Sphingobacteria, Chlorobia, and the Chloroflexi. Inclusion of the LeuA and CimA sequences from these organisms did not affect the structure of the phylogenetic tree (data not shown). Clade III CimA homologs appear to be absent from the Beta-, Epsilon-, and Gammaproteobacteria. In some organisms that lack homologs of threonine ammonia-lyase, this class of citramalate synthases may constitute the only route for isoleucine biosynthesis; examples include Pelobacter propionicus, Pyrococcus furiosus, and all sequenced members of the Desulfobivirionaceae.

**Implications.** There are two pathways involved in isoleucine biosynthesis in G. sulfurreducens: the threonine-dependent pathway and the citramalate-dependent pathway (Fig. 1). Our results indicate that the citramalate-dependent pathway is the major route for isoleucine biosynthesis in G. sulfurreducens. This conclusion is supported by genetic, biochemical, and 13C-labeling data. The citramalate synthase of G. sulfurreducens represents a novel phylogenetic variant of the enzyme. Furthermore, the wide distribution of this novel class of citramalate synthases throughout the microbial world indicates that the citramalate-dependent pathway of isoleucine biosynthesis is fairly common.

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