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Characterization of the Central Metabolic Pathways in *Thermoanaerobacter* sp. Strain X514 via Isotopomer-Assisted Metabolite Analysis▽†

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***Thermoanaerobacter* sp. strain X514 has great potential in biotechnology due to its capacity to ferment a range of C_5 and C_6 sugars to ethanol and other metabolites under thermophilic conditions. This study investigated the central metabolism of strain X514 via ^{13}C-labeled tracer experiments using either glucose or pyruvate as both carbon and energy sources. X514 grew on minimal medium and thus contains complete biosynthesis pathways for all macromolecule building blocks. Based on genome annotation and isotopic analysis of amino acids, three observations can be obtained about the central metabolic pathways in X514. First, the oxidative pentose phosphate pathway in X514 is not functional, and the tricarboxylic acid cycle is incomplete under fermentative growth conditions. Second, X514 contains (*Re*)-type citrate synthase activity, although no gene homologous to the recently characterized (*Re*)-type citrate synthase of *Clostridium kluyveri* was found. Third, the isoleucine in X514 is derived from acetyl coenzyme A and pyruvate via the citramalate pathway rather than being synthesized from threonine via threonine ammonia-lyase. The functionality of the citramalate synthase gene (*cimA* [Teth514_1204]) has been confirmed by enzymatic activity assays, while the presence of intracellular citramalate has been detected by mass spectrometry. This study demonstrates the merits of combining ^{13}C-assisted metabolite analysis, enzyme assays, and metabolite detection not only to examine genome sequence annotations but also to discover novel enzyme activities.**

Rising global energy demand and the depletion of fossil energy resources have resulted in significant environmental, economic, and social impacts. The production of renewable, biomass-derived energy sources has been suggested to be a partial solution to this problem. Among renewable energy sources, ethanol is an attractive short-term solution owing to its strong research foundation and its ready integration with the current petroleum-based infrastructure (7, 21). Plant-based cellulose is the most attractive raw material for bioethanol production (29). However, the use of anaerobic cellulosic bacteria in consolidated bioreactors has been proposed to be an efficient means of the rapid conversion of cellulosic biomass to ethanol (14). Thermophilic bacteria of the genus *Thermoanaerobacter* have the ability to naturally ferment a wide variety of monomeric and polymeric carbohydrates, including D-xylose, into ethanol (13, 16, 22). While not cellulose-utilizing bacteria themselves, *Thermoanaerobacter* species in coculture with thermophilic cellulose-utilizing *Clostridium* species have significantly higher yields of ethanol from both cellulose and hemicellulose than from monoculture alone (5, 15). Therefore, the investigation of carbon metabolism in *Thermoanaerobacter* sp. strain X514 has implications for an understanding of the potential of X514 for bioenergy production.

Despite the potential importance of X514 for biofuel production, a rigorous investigation of the central metabolic pathways of X514 has yet to be conducted. Although an array of functional genomic tools has been applied to predict the metabolism of this species (1, 28, 31), a precise description of cellular metabolism is complicated by misannotation and by the posttranscriptional regulation of protein synthesis (6, 9). The complete genome sequence of X514 from the KEGG database (http://www.genome.jp/kegg/) suggests a few gaps in several essential pathways involved in the biosynthesis of amino acids (e.g., isoleucine) and in the tricarboxylic acid (TCA) cycle (e.g., citrate synthase). Therefore, X514 would not survive without supplements of isoleucine or other essential nutrients. However, X514 can actually grow in a completely minimal medium. Hence, the metabolism of X514 cannot be precisely revealed by genome sequence annotation alone. At this time, one of the most physiologically reliable methods for determining cell metabolism remains ^{13}C-based isotopic analysis (6, 19, 24, 26). Based on ^{13}C-labeling patterns in key amino acids, the active pathways can be traced back, and new enzymes can be revealed. In this study, ^{13}C-based isotopic analysis was applied to accurately examine the annotated pathways in X514 and to investigate gaps in key biosynthetic pathways (25–27). Specifically, glucose (the first or sixth carbon labeled) and pyruvate (the first carbon labeled) were used, respectively, as the sole source of carbon to grow X514. By analyzing the mass spectra of different fragmentations in proteogenic amino acids derived from various pathways, we have determined the

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active fluxes of intracellular pathways (e.g., the pentose phosphate pathway and citric acid cycle). Meanwhile, misannotations in the genome sequence were checked, and unknown enzymes involved in the pathway were identified. The isotopomer analysis linked the genome annotation to the final enzyme-functional output and thus significantly improved our understanding of the regulation of the central metabolism of X514.

MATERIALS AND METHODS

Medium and cultivation conditions. *Thermoanaerobacter* sp. strain X514 was grown anaerobically at 60°C without shaking (18). The minimal medium contained (per liter) 1 g of NaCl, 0.5 g of $MgCl_2$, 0.2 g of KH_2PO_4, 0.3 g of NH_4Cl, 0.3 g of KCl, 0.015 g of $CaCl_2$, 0.25 mg of resazurin, 0.031 g of L-cysteine-HCl, 0.048 g of Na_2S, 2.52 g of $NaHCO_3$, and 1 ml trace-element solution. One liter of trace-element solution included 10 ml 25% (wt/wt) HCl solution, 1.5 g of $FeCl_2$, 0.19 g of $CoCl_2$, 0.1 g of $MnCl_2$, 70 mg of $ZnCl_2$, 6 mg of H_3BO_3, 36 mg of Na_2MoO_4, 24 mg of $NiCl_2$, 2 mg of $CuCl_2$, 6 mg of Na_2SeO_3, 8 mg of Na_2WO_4, and 0.5 g of NaOH. The pH of the medium was adjusted with NaOH to pH 7.2 to 7.3. The vitamin solution was prepared according to a method developed previously by Wolin et al. (32). Rich medium was prepared by adding 0.1% yeast extract to the minimal medium. Three types of ^{13}C-labeled carbon substrates were obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA), and used for cell culture: pyruvate (1-^{13}C; 98%), glucose (1-^{13}C; 98%), or glucose (6-^{13}C; 98%). The medium was flushed with N_2 and was filter sterilized. All gases, including nitrogen and a nitrogen-CO_2 mixture, were obtained from Airgas, Inc. (Radnor, PA). The strain was initially grown in a 50-ml culture medium with an unlabeled carbon source (glucose or pyruvate). At the mid-log phase of growth, a 3% inoculum was added to a 50-ml culture containing one of the following carbon sources: 2.1 g/liter of [1-^{13}C]glucose, 2.1 g/liter of [6-^{13}C]glucose, or 2.2 g/liter first-position-labeled pyruvate. At the mid-log phase of growth in this culture, 3% inoculum from the first ^{13}C-labeled culture medium was used to inoculate a 50-ml subculture (with the same labeled carbon source), which reduced the effect of unlabeled carbon from the initial stock.

Analytical methods. Biomass was harvested at the late-log phase of growth by centrifugation at 8,000 × *g* for 15 min at 10°C. The concentrations of glucose, acetate, ethanol, and lactate were analyzed with a high-performance liquid chromatograph (Agilent Technologies, CA) equipped with a variable-wavelength (190- to 600-nm) detector (the UV absorption at 245 nm) and an ion exclusion column (Aminex HPX-87H, 300 mm by 7.8 mm; Bio-Rad Laboratories, CA) operating at 55°C. The mobile phase consisted of 0.025% sulfuric acid at a flow rate of 0.6 ml/min.

Isotopic analysis. The preparation and isotopic analysis of proteogenic amino acids were performed as previously described (23, 27). In brief, biomass was hydrolyzed in a solution containing 6 M HCl at 100°C for 24 h. The amino acid solution was dried under air flush overnight, and amino acid samples were derivatized in tetrahydrofuran and *N*-(*tert*-butyl dimethylsilyl)-*N*-methyl-trifluoroacetamide (Sigma-Aldrich, St. Louis, MO) at 70°C for 1 h. A gas chromatograph (Hewlett-Packard model 7890A; Agilent Technologies, CA) equipped with a DB5-MS column (J&W Scientific, Folsom, CA) and a mass spectrometer (model 5975C; Agilent Technologies, CA) was used for analyzing amino acid labeling profiles. Three types of charged fragments were detected by gas chromatography-mass spectrometry (MS) for most amino acids: the $[M-57]^+$ group, which contained unfragmented amino acids; the $[M-159]^+$ group, which contained amino acids losing an α-carboxyl group; and the $[f302]^+$ group, which contained only the first (α-carboxyl group) and second carbons in an amino acid backbone. (However, $[f302]^+$ cannot be detected in some amino acids.) Previously reported algorithms were used to correct the effects of natural isotopes on the mass distributions of amino acids (30), and the final isotopomer distribution is shown in Tables S1 and S2 in the supplemental material. Ion mass fractions shown in Tables S1 and S2 in the supplemental material were given for the amino acid fragments. M0, M1, and M2, etc., are fractions of unlabeled, singly labeled, and doubly labeled amino acids, respectively.

Confirmation of citramalate synthase. The citramalate synthase activity was assayed by monitoring the pyruvate-dependent production of coenzyme A (CoA) over time (8, 17). In brief, X514 cells from a 10-ml culture in the mid-log phase were centrifuged (19,000 × *g* for 10 min at 4°C). The total protein content in the biomass was estimated using a Bradford protein assay (Bio-Rad Laboratories, Inc., CA). X514 cell extracts were then prepared by sonication of X514 pellets for 3 min (30 s on and 20 s off) in 2 ml of a 0.1 M TES [*N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.5)] buffer. Samples to be measured were brought to a final volume of 1,000 μl by mixing with cell extracts (100 μl), TES buffer (0.2 M [pH 7.5]; 500 μl), pyruvate (10 mM; 100 μl), acetyl-CoA (50 mM; 20 μl), and distilled water. The resulting solutions were then incubated in an oven at 60°C for 2 h. At intervals of 20 min, 100 μl of either test samples or blank samples was taken from the oven and mixed with a 900-μl stop solution. The stop solution was prepared in distilled water with 50 μl of 10 mM DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] in 0.1 M Tris-HCl and with 70 μl of 1 M Tris-HCl. The absorbance at 412 nm was recorded immediately and blanked against an identical incubation sample without pyruvate. The micromoles of CoA produced was calculated from a standard curve generated with known concentrations (0 to 1 mM) of 2-mercaptoethanol and based on the linear function between product formation and the amount of enzyme added over the 2-h time period of the assay. All the chemicals employed in this measurement were obtained from Sigma-Aldrich.

Liquid chromatography (LC)-tandem MS (MS/MS) was further applied to confirm the presence of citramalate in X514. In this study, the free intracellular metabolites were extracted with a cold methanol-water (60%, vol/vol)–chloroform mixture (1:1) held at −20°C overnight. Subsequently, cold water was added to separate free metabolites from those components that might interfere with the results. The extracts were then lyophilized. After being dissolved with 1% formic acid in water, 50-μl samples were injected into the LC-MS/MS system for separation and detection. The LC-MS/MS system was composed of a Shimadzu LC system, a Leap CTC PAL autosampler, and an Applied Biosystems 4000 QTRAP mass spectrometer equipped with a TurbolonSpray electrospray ion source. A total of 5 μM of citrate, malate, and citramalate standards (Sigma) in water was separately infused into the mass spectrometer to optimize compound-dependent parameters for multiple-reaction monitoring (MRM) and to obtain corresponding MS/MS spectra. LC separation was achieved by coupling three 4.6- by 300-mm Onyx Monolith C_{18} columns (Phenomenex, CA) in tandem. The LC gradient was delivered at 1 ml/min with solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol). The gradient started from 5% solvent B and was kept isocratic for 4 min, ramped to 20% within 7 min, and then increased to 95% solvent B within 1 min. Finally, after being held at 95% solvent B for 8 min, the gradient was ramped down to 5% solvent B, where it remained for 4 min to reequilibrate the column.

RESULTS

Growth and metabolite curves. When X514 was cultivated in minimal medium, the lag phase for X514 in ^{13}C-labeled glucose was 12 h, which was followed by an exponential growth phase with a doubling time of ~15 to 19 h (Fig. 1a). This rate was much slower than the growth rate of X514 in rich medium (with 0.1% yeast extract), which measured a doubling time of 6 h. Figure 1b shows both the glucose consumption and the production of ethanol, acetate, and lactate when X514 was grown in minimal glucose medium. No formate was detected during the culturing. The ability to grow in minimal medium using glucose (or pyruvate [data not shown]) indicates that X514 contains the necessary biosynthetic pathways for all macromolecule building blocks (i.e., for synthesizing amino acids).

Confirmation of amino acid biosynthetic pathways. According to the genome annotation from the KEGG database (http://www.genome.jp/kegg/), two amino acid biosynthetic pathways (isoleucine and proline) were incomplete. To examine biosynthetic pathways in X514, the labeling profiles of 14 proteogenic amino acids were analyzed (see Tables S1 and S2 in the supplemental material). Since pyruvate is the key metabolite in the central pathway (i.e., glycolysis, pentose phosphate pathway, and TCA cycle), the labeling profiles of amino acids from tracer experiments that use first-position-labeled pyruvate as the carbon source can easily identify the precursors of several key amino acids (see Table S2 in the supplemental material). For example, alanine, valine, and serine demonstrated the same carbon molecule-labeling pattern as that of pyruvate (see

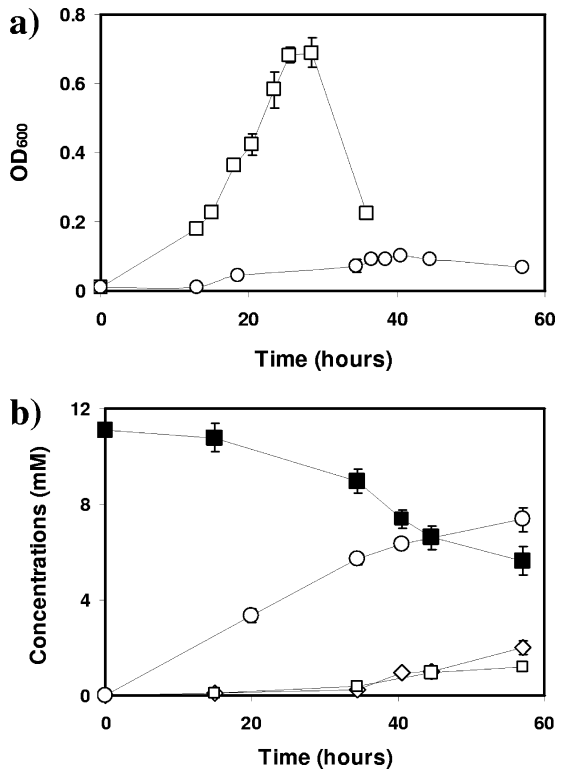

FIG. 1. *Thermoanaerobacter* sp. strain X514 growth and metabolite secretion. (a) Growth curves of *Thermoanaerobacter* sp. strain X514 in minimal glucose medium (○) and rich medium (with 1 g/liter yeast extract) (□). OD_{600}, optical density at 600 nm. (b) Glucose consumption and metabolite production by *Thermoanaerobacter* sp. strain X514 in minimal glucose medium. ■, glucose; ◇, acetate; □, lactate; ○, ethanol.

Table S2 in the supplemental material), which confirms that pyruvate is the common precursor of these three amino acids (Fig. 2). The aromatic amino acids phenylalanine and tyrosine were derived from phosphoenolpyruvate (also synthesized from pyruvate). The labeling patterns of the $[f302]^+$ group (containing the first and second carbons of fragmented amino acids) for alanine, phenylalanine, and tyrosine were all identical. This fact indicates that the first and second carbons of all three amino acids are originally derived from the first and second carbons of pyruvate.

In tracer experiments using the first-position-labeled pyruvate, the similar labeling patterns of aspartate, methionine, and threonine shown in Table S2 in the supplemental material suggest the same precursor (oxaloacetate) for the three amino acids. Based on the KEGG-generated pathway map, oxaloacetate is expected to be synthesized from pyruvate (labeled at its first position) and CO_2 (labeled because it is cleaved from the first carbon of pyruvate), leading to the labeling of two carbons in oxaloacetate and aspartate (both α- and β-carboxyl groups) (Fig. 2). In tracer experiments using [1-^{13}C]glucose or [6-^{13}C] glucose as the carbon source, the labeling percentage of methionine was higher than that of aspartate from the same experiment as a result of the addition of a ^{13}C-enriched C-1 pool (5,10-methyl-tetrahydrofuran) into the carbon backbone of methionine (synthesis route of aspartate + C-1 pool→ methionine). The labeled carbon entered the C-1 pool via glucose→serine→glycine + C-1 pool (Fig. 2), which caused more methionine to be labeled.

Alternate isoleucine pathway. Anaerobic bacteria such as *Methanococcus jannaschii* and *Leptospira* can biosynthesize isoleucine from citramalate by the direct condensation of acetyl-CoA and pyruvate (8, 33). Recently, Risso et al. (17) first used ^{13}C-assisted metabolic flux analysis and biochemistry assays to discover an alternate isoleucine pathway in *Geobacter sulfurreducens*. Interestingly, X514 may also contain a similar alternate isoleucine pathway. The labeling patterns of leucine and isoleucine in both glucose and pyruvate tracer experiments were similar. For example, the pyruvate experiment demonstrated that the M0 ($[M-159]^+$, without the carboxyl group) of both leucine and isoleucine was >91%, indicating that the C_2~C_6 carbons in both leucine and isoleucine were mostly unlabeled. Such labeling patterns in isoleucine are unexpected unless isoleucine shares the same precursors (pyruvate and acetyl-CoA) as leucine. According to the genome annotation, X514 lacks threonine ammonia-lyase (EC 4.3.1.19), which is necessary for the biosynthesis of isoleucine from threonine; this result is supported by our labeling data: when [1-^{13}C]pyruvate was used as the carbon source, threonine was labeled with two carbons (M2 $[M-57]^+$, >93%), while isoleucine (C_2~C_6) was not labeled. (Note that gas chromatography-MS could not detect the labeling information for the first carbon of isoleucine due to the peak overlap [3].) We propose an alternate isoleucine synthesis pathway via the citramalate pathway that uses pyruvate and acetyl-CoA as precursors (Fig. 3). The key enzyme regulating the citramalate pathway (citramalate synthase [*cimA*]) is annotated in X514 (Teth514_1204) (17, 26).

In order to determine whether the citramalate pathway was active in X514, crude soluble extracts from mid-log-phase cells were tested for the presence of citramalate synthase activity (about 27 ± 9 nmol/mg protein/min). Furthermore, we applied high-sensitivity MS to detect whether there was any intracellular citramalate in X514. Figure S1 in the supplemental material indicates that malate, citrate, and citramalate were clearly detected in the X514 soluble extracts by LC-MS/MS in the MRM mode. The retention time and the ratio of multiple MRM transitions selected for each of the three targeted compounds are in agreement with those of the respective authentic standards. Although collision-induced dissociation spectrum signals for the three targeted compounds in X514 soluble extracts were lower than the spectrum signals of authentic standards, the major fragments and their relative abundances agreed with their corresponding standards (see Fig. S2 in the supplemental material). This result provides additional evidence to prove the presence of citramalate synthase in X514.

(*Re*)-type citrate synthase. In spite of the production of citrate by X514 (see Fig. S1 in the supplemental material), the genome annotation indicates that the enzyme in the first step of the TCA cycle, i.e., the (*Si*)-type citrate synthase, is missing. Interestingly, the isotopomer data from the first-position-labeled pyruvate experiment suggests that the α-carboxyl group of glutamate is not labeled (see Table S2 in the supplemental material). Such an observation of the labeling pattern of glutamate is consistent with the main characteristics of (*Re*)-type citrate synthase, which has been discussed in detail in our previous study of (*Re*)-type citrate synthase in *Desulfovibrio*

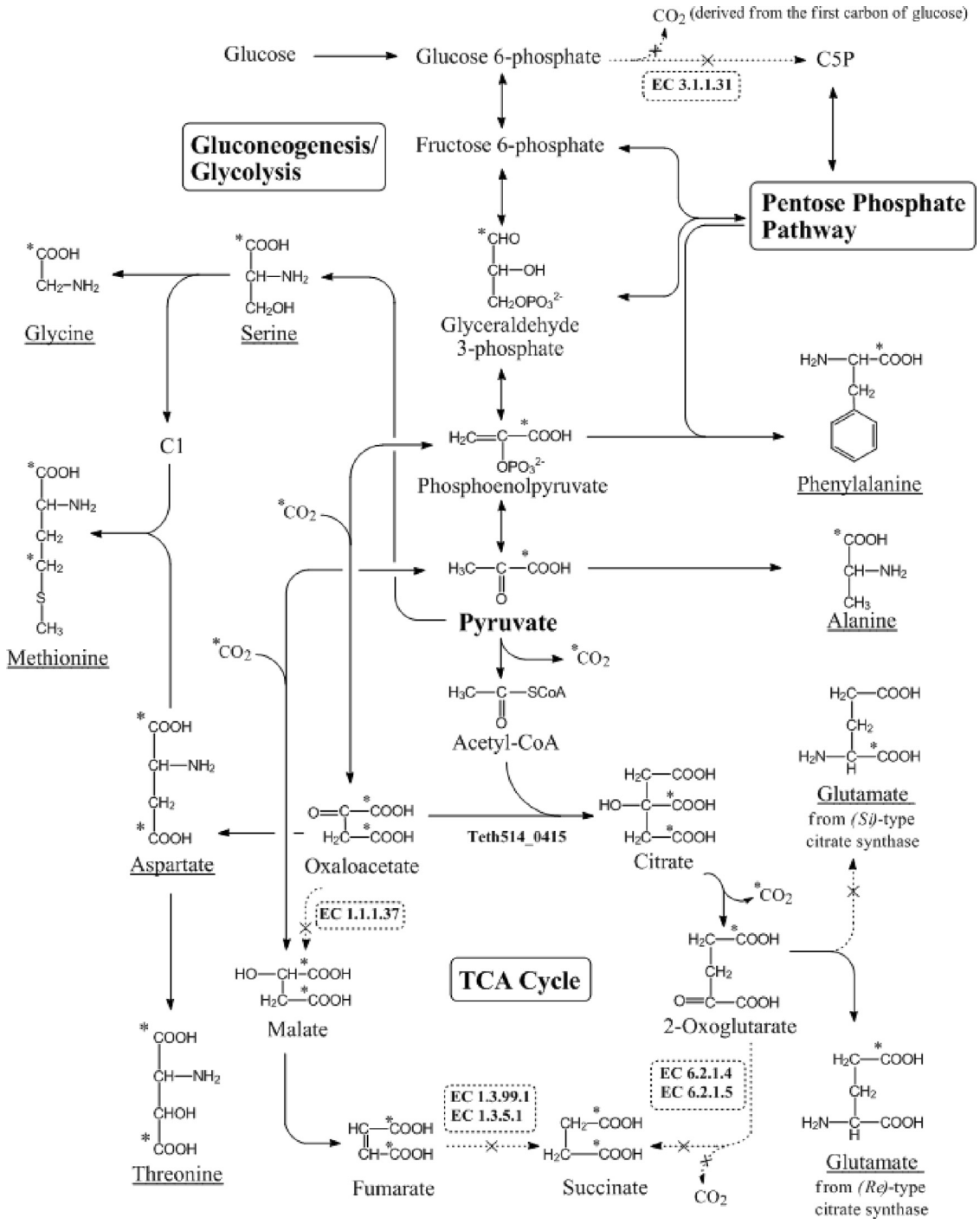

FIG. 2. Proposed scheme of central metabolic pathways in *Thermoanaerobacter* sp. strain X514. ^{13}C-labeled positions in metabolites are marked with an asterisk for the first-position-labeled pyruvate experiment. The inactive pathways are marked with dashed lines. Based on the genome annotation in the KEGG database, some genes that are missed in the pathways are marked by dashed boxes. C5P includes ribulose 5-phosphate, ribose 5-phosphate, and xylulose 5-phosphate.

vulgaris Hildenborough (26). Due to the lack of a regular (*Si*)-type citrate synthase, X514 may employ the (*Re*)-type citrate synthase. Figure 2 shows the proposed carbon transition routes from labeled pyruvate (α-carboxyl group) to doubly labeled oxaloacetate (both α- and β-carboxyl groups). The β-carboxyl group of 2-oxoglutarate and the β-carboxyl group of glutamate were ultimately derived from the β-carboxyl group of oxaloacetate.

Pentose phosphate pathway. About 50% of alanine was not labeled ($[M-57]^+$; M0 = 0.51) when [1-^{13}C]glucose was used as the carbon source (see Table S1 in the supplemental material). The glucose carbon backbone loses the first carbon as CO_2

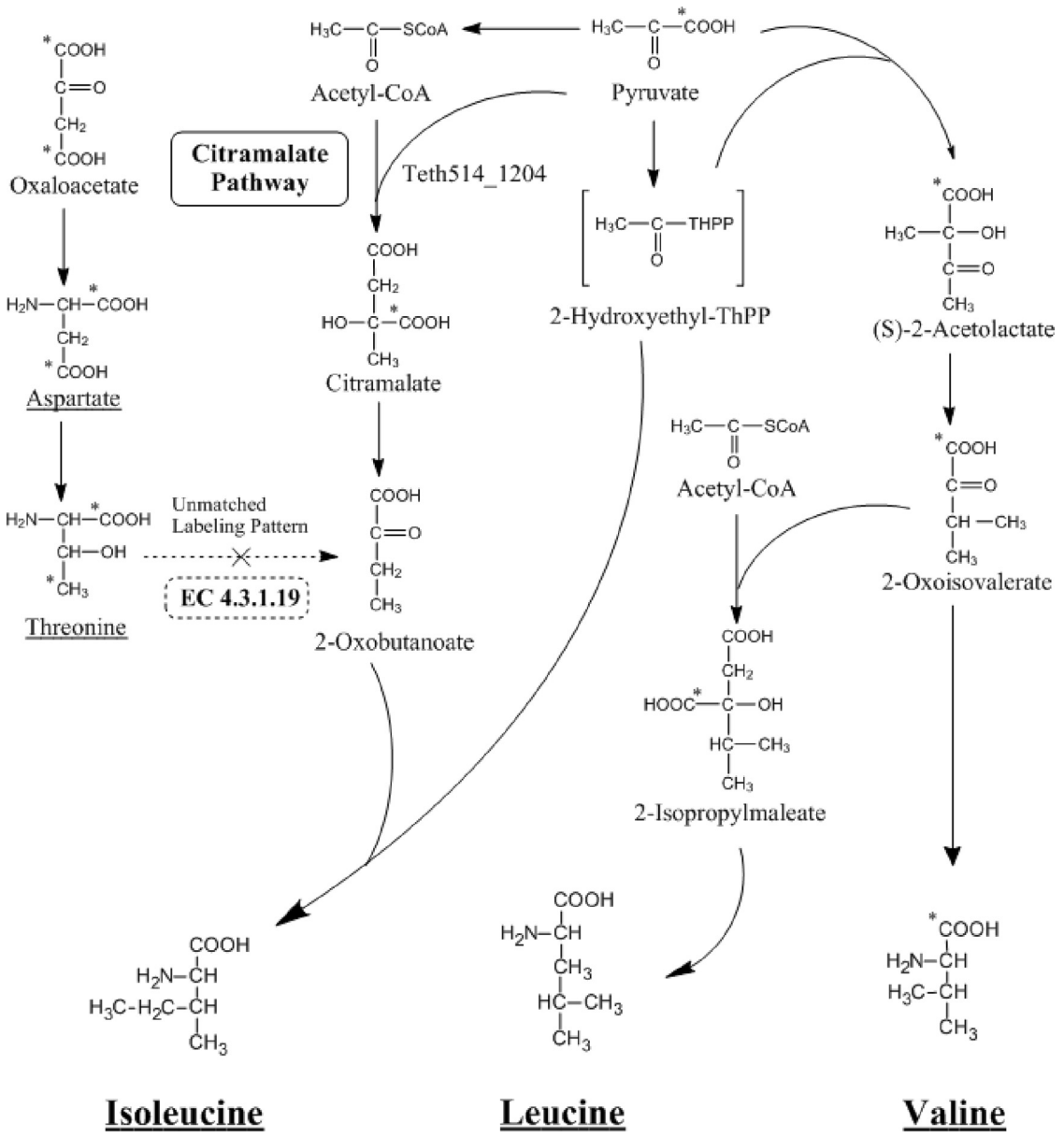

FIG. 3. Proposed scheme of isoleucine biosynthesis in *Thermoanaerobacter* sp. strain X514 (using first-position-labeled pyruvate as the carbon source). ^{13}C-labeled positions are marked with asterisks. The inactive pathway is marked by a dashed line.

when it is metabolized via the oxidative pentose phosphate pathway to synthesize five-carbon sugars (C5P) (Fig. 2). If the oxidative pentose phosphate pathway is not active, one glucose molecule (with either the first or sixth carbon labeled) converts to two pyruvate molecules, and thus, ~50% of pyruvate (inferred from alanine) is expected to be unlabeled. The fact that the fraction of unlabeled alanine was 51% ± 2% indicates the very low activity of the oxidative pentose phosphate pathway; i.e., the carbon flux split ratio between G6P→C5P (oxidative pentose phosphate pathway) and G6P→G3P (glycolysis) was <3% (the algorithm for calculating the split ratio is provided in the supplemental material). Since the oxidative pentose phosphate pathway was not active, the isotopomer labeling patterns of most amino acids in the [1-^{13}C]glucose experiment were identical to those in the 6-^{13}C experiments (see Table S1 in the supplemental material).

DISCUSSION

This study has examined the pentose phosphate pathway, amino acid biosynthesis, and the TCA cycle in *Thermoanaerobacter* sp. strain X514 by ^{13}C-labeling experiments. X514 shows very low levels of activity in the oxidative phase of the pentose phosphate pathway under glucose fermentation conditions. Such an observation is consistent with a missing 6-phosphogluconolactonase (EC 3.1.1.31) gene, which catalyzes 6-phospho-D-glucono-1,5-lactone to 6-phospho-D-gluconate. Considering the important role of the oxidative

TABLE 1. BLAST searches for key genes in an alternate isoleucine synthesis pathway (citramalate synthase; GSU 1798), for (*Si*)-type citrate synthase (EC 2.3.3.1 from *E. coli* K-12) and for (*Re*)-type citrate synthase (CKL 0973 from *Clostridium kluyveri*) in the Joint Genome Institute database[a]

Species (domain, genome completion)[b]	% Identity		
	EC 2.3.3.1 (b0720) [(*Si*)-type citrate synthase]	EC 2.3.3.3 (CKL 0973) [(*Re*)-type citrate synthase]	EC 2.3.1.182 (GSU1798)
Thermoanaerobacter sp. strain X514 (*Bacteria*, F)*	0	27	49
"*Anaerocellum thermophilum*" DSM 6725 (*Bacteria*, D)	0	68	51
Caldicellulosiruptor saccharolyticus DSM 8903 (*Bacteria*, F)	0	68	51
Caldivirga maquilingensis IC-167 (*Archaea*, F)	39	41	47
"*Candidatus* Kuenenia stuttgartiensis" (*Bacteria*, F)	0	55	54
Clostridium acetobutylicum ATCC 824 (*Bacteria*, F)	0	65	50
Clostridium botulinum A ATCC 3502 (*Bacteria*, F)	0	73	22
Clostridium kluyveri DSM 555 (*Bacteria*, F)	28	100	29
Clostridium cellulolyticum H10 (*Bacteria*, F)	30	60	50
Clostridium thermocellum ATCC 27405 (*Bacteria*, F)	30	60	51
Clostridium phytofermentans ISDg (*Bacteria*, F)	30	62	25
Desulfovibrio desulfuricans G20 (*Bacteria*, F)	0	48	55
Desulfovibrio vulgaris Hildenborough (*Bacteria*, F)	0	48	55
Desulfovibrio vulgaris DP4 (*Bacteria*, F)	0	48	55
Desulfovibrio vulgaris Miyazaki F (*Bacteria*, F)	0	49	55
"*Dehalococcoides ethenogenes*" 195 (*Bacteria*, F)*	0	28	53
Syntrophus aciditrophicus SB (*Bacteria*, F)	0	49	55
Syntrophobacter fumaroxidans MPOB (*Bacteria*, F)	32	50	59
Pelotomaculum thermopropionicum SI (*Bacteria*, F)*	0	29	56
Ignicoccus hospitalis KIN4/I (*Archaea*, F)	0	50	45

[a] BLAST search done in May 2009.

[b] F, finished genome; D, draft genome. An asterisk indicates that both (*Si*)- and (*Re*)-type citrate synthases in the species may be missing due to the low level of identity (<30%) of polypeptide amino acid sequences to the documented (*Si*)- and (*Re*)-type citrate synthases.

pentose phosphate pathway in providing NADPH for biosynthesis, alternate NADPH pathways should be present in X514. Some bacteria utilize transhydrogenase PntAB or UdhA for NADPH generation (20), but a BLASTP search (2) indicates that neither of the two transhydrogenases is encoded in the X514 genome (see Fig. S3 in the supplemental material). Although NADPH-dependent isocitrate dehydrogenase is annotated in X514 (Teth514_0327), it may provide only limited NADPH since the TCA cycle is branched and is used mainly for biosynthesis. On the other hand, ferredoxin-$NADP^+$ reductase (e.g., Teth514_0652) and pyruvate:ferredoxin oxidoreductase (e.g., Teth514_0781, the enzyme that catalyzes the production of ferredoxin from pyruvate) are annotated in the X514 genome and may be key sources of NADPH in X514. Ferredoxin-$NADP^+$ reductase activity for NADPH production has been well documented for certain thermophilic anaerobes (11).

The discovery of an alternative pathway for isoleucine synthesis and (*Re*)-type citrate synthase activity demonstrates the unique metabolism of X514. Isoleucine is synthesized from the citramalate pathway so that both leucine and isoleucine share the same precursors (pyruvate and acetyl-CoA). Multiple lines of evidence support an alternate isoleucine biosynthesis pathway via citramalate in X514: (i) labeling patterns in key amino acids, (ii) genomic evidence (i.e., the presence of the citramalate synthase gene but the absence of the threonine deaminase gene), (iii) detection of citramalate synthase activity, and (iv) detection of citramalate via LC-MS/MS. In some organisms, citramalate is used for reactions other than isoleucine biosynthesis. For example, during the phototrophic growth of *Rhodospirillum rubrum* on acetate and CO_2, citramalate (formed via the condensation of acetate and pyruvate) can be degraded to glyoxylate and propionate (10). This pathway allows *R. rubrum* to assimilate acetate and synthesize intermediates in the TCA cycle (i.e., succinate). On the other hand, Atsumi and Liao (4) previously introduced citramalate synthase (*cimA*) into *Escherichia coli* and successfully evolved a new 2-oxobutanoate synthetic pathway for both 1-propanol and 1-butanol production (9- and 22-fold-higher productions, respectively). Therefore, the citramalate pathway in X514 could be potentially utilized for biobutanol production.

An (*Re*)-type citrate synthase was recently reported for both *Clostridium kluyveri* (CKL 0973) and *Desulfovibrio* spp. (12). (*Re*)-type citrate synthase and (*Si*)-type citrate synthase are phylogenetically unrelated. (*Re*)-type citrate synthase is O_2 sensitive and thus is restricted to anaerobic microorganisms. Using a Joint Genome Institute database search in May 2009 (http://img.jgi.doe.gov) (with a BLAST search score of >400 and with identity of amino acid sequences of >40%), we found that ~200 microbial species may be annotated with citramalate synthase (a key step for an alternate isoleucine synthesis pathway), while ~40 strains may have (*Re*)-type citrate synthase (with a BLAST search score of >290 and with an identity of >40%). A few species may contain both citramalate synthase and the (*Re*)-type citrate synthase (Table 1), including *Desulfovibrio desulfuricans* and *Desulfovibrio vulgaris* Hildenborough. *Thermoanaerobacter* sp. strain X514, "*Dehalococcoides ethenogenes*," and *Pelotomaculum thermopropionicum* contain neither (*Si*)-type citrate synthase nor the documented (*Re*)-type citrate synthase, but all encode citramalate synthase. Table 1 indicates that the citramalate pathway and (*Re*)-type

citrate synthase may be more widespread in microorganisms than originally thought.

(*Re*)-type citrate synthase-like activity was observed for X514. A candidate (*Re*)-type citrate synthase gene in X514 should be identifiable through a BLAST search of the polypeptide amino acid sequences (see Fig. S4 in the supplemental material). However, no gene candidate is identical to the reported (*Re*)-type citrate synthase in *Clostridium kluyveri* (CKL 0973), and thus, X514 may contain an undocumented (*Re*)-type citrate synthase. However, X514 contains homocitrate synthase (Teth514_0415) (2-oxoglutarate + acetyl-CoA→homocitrate) and isopropylmalate synthase (Teth514_0014) (3-methyl-2-oxobutanoate + acetyl-CoA→2-isopropylmalate), which are phylogenetically related to the reported (*Re*)-type citrate synthase (oxaloacetate + acetyl-CoA→citrate). More importantly, Teth514_0416 is annotated as aconitate hydratase (citrate→isocitrate), and this gene is in the same operon with homocitrate synthase (Teth514_0415). Therefore, homocitrate synthase (Teth514_0415) can be a potential (*Re*)-type citrate synthase candidate, and further experimentation is required to test this hypothesis. Interestingly, citramalate synthase (Teth514_1204) (pyruvate + acetyl-CoA→citramalate) condenses acetyl-CoA and organic acids to form metabolites that are structurally similar to citrate. This enzyme also belongs to the isopropylmalate synthase/homocitrate synthase family.

In summary, ^{13}C isotopic analysis is a powerful tool to examine the metabolic networks of sequenced species and to predict novel enzymes. Our results suggest an inactive pentose phosphate pathway and an alternate isoleucine biosynthesis pathway via citramalate in X514. Furthermore, X514 also demonstrates (*Re*)-type citrate synthase activity. A comprehensive understanding of metabolism of *Thermoanaerobacter* sp. strain X514 could have dual significance for both rational genetic engineering of microorganisms for biofuel production (4) and investigations of the evolution of phylogenetically related pathways.

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