Metabolic Flux Analysis of the Mixotrophic Metabolisms in the Green Sulfur Bacterium *Chlorobaculum tepidum**1,9

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The photosynthetic green sulfur bacterium *Chlorobaculum tepidum* assimilates CO₂ and organic carbon sources (acetate or pyruvate) during mixotrophic growth conditions through a unique carbon and energy metabolism. Using a 13C-labeling approach, this study examined biosynthetic pathways and flux distributions in the central metabolism of *C. tepidum*. The isotope patterns of proteinogenic amino acids revealed an alternate pathway for isoleucine synthesis (via citramalate synthase, CimA, CT0612). A 13C-assisted flux analysis indicated that carbons in biomass were mostly derived from CO₂ fixation via three key routes: the reductive tricarboxylic acid (RTCA) cycle, the pyruvate synthesis pathway via pyruvate:ferredoxin oxidoreductase, and the CO₂-anaplerotic pathway via phosphoenolpyruvate carboxylase. During mixotrophic growth with acetate or pyruvate as carbon sources, acetyl-CoA was mainly produced from acetate (via acetyl-CoA synthetase) or citrate (via ATP citrate lyase). Pyruvate:ferredoxin oxidoreductase converted acetyl-CoA and CO₂ to pyruvate, and this growth-rate control reaction is driven by reduced ferredoxin generated during phototrophic growth. Most reactions in the RTCA cycle were reversible. The relative fluxes through the RTCA cycle were 80–100 units for mixotrophic cultures grown on acetate and 200–230 units for cultures grown on pyruvate. Under the same light conditions, the flux results suggested a trade-off between energy-demanding CO₂ fixation and biomass growth rate: *C. tepidum* fixed more CO₂ and had a higher biomass yield (Y/Xₚ, molar carbon in biomass/mole substrate) in pyruvate culture (Y/Xₚ = 9.2) than in acetate culture (Y/Xₚ = 6.4), but the biomass growth rate was slower in pyruvate culture than in acetate culture.

*Chlorobaculum tepidum* is a representative green sulfur bacterium that is ecologically significant in global cycling of carbon, nitrogen, and sulfur (1, 2). The *C. tepidum* genome has been sequenced, and the genetic tools for creating *C. tepidum* mutant strains have been developed to make transposon-based mutations or targeted gene disruptions, which offer great potential to engineer *C. tepidum* for future applications (3). The annotated genome reveals unique aspects in carbon and energy metabolism in *C. tepidum*. Instead of using the Calvin-Benson cycle for CO₂ assimilation as in most photosynthetic organisms, *C. tepidum* captures energy from light and uses it along with electrons, primarily derived from oxidation of sulfur compounds, to drive the reductive tricarboxylic acid cycle (RTCA)² for synthesis of building block molecules (3). *C. tepidum* can grow mixotrophically with acetate or pyruvate as the organic carbon source (2). Although recent research has been performed on the carbon and energy metabolism of *C. tepidum* (4, 5), rigorous quantification of the metabolic pathway activities has not yet been achieved. To provide quantitative readout of the metabolic functions and regulatory mechanisms, this study has performed 13C-assisted metabolic flux analysis of *C. tepidum* in the following steps: 1) growing cultures of *C. tepidum* with 13C-labeled acetate or pyruvate, 2) using gas chromatography (GC)-MS to measure the resulting labeling pattern in key metabolites, and 3) deciphering in vivo metabolisms via a flux model (6). Isotopic labeling and metabolic flux analysis have been developed to identify the active biosynthesis pathways (7–10) and measure the global enzymatic reaction rates (11, 12). The cell-wide quantification of intracellular metabolism can bridge the gap between genome annotations and final metabolic outputs and has been applied for characterizing numerous environmental microorganisms, including *Escherichia coli* (13, 14), *Saccharomyces cerevisiae* (15, 16), *Bacillus subtilis* (17), *Geobacter allireducens* (18), *Shewanella oneidensis* (19), and *Synechocystis* sp. (20), etc. This paper reports on the first studies of the fluxomics of mixotrophic metabolism in the green sulfur bacteria and provides complementary information to previous genomic and proteomic studies.

**EXPERIMENTAL PROCEDURES**

13C-labeled Experiments—*C. tepidum* cultures were grown anaerobically at temperatures ranging from 46–50 °C in low intensity light (100 ± 10 micromoles/m²/s). The medium composition (1 liter) was Na₂EDTA-2H₂O (0.015 g), MgSO₄·7H₂O (0.22 g), CaCl₂·2H₂O (0.08 g), NaCl (0.45 g), NH₄Cl (0.45 g), Na₂S₂O₃·5H₂O (2.6 g), KH₂PO₄ (0.57 g), MOPS buffer (2.4 g), trace element stock (1.2 ml), vitamin B₁₂ stock (0.08 mg), NaHCO₃ (2.3 g), and Na₂S·9H₂O (0.8 g). All chemicals were purchased from Sigma-Aldrich. The carbon source for the trace experiments was [1,13C]acetate (purity > 98%, 3).

1 The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1–S4 and Fig. S1.

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0.83 g/liter) or [2-13C] acetate (purity > 98%, 0.83 g/liter) for cultures growing on acetate, or [3-13C] pyruvate (purity > 98%, 1.1 g/liter) for cultures growing on pyruvate. The 13C-labeled acetate and pyruvate were purchased from Cambridge Isotope Laboratories. Cell growth was monitored at A605. 1% cultures (100-fold dilution) in the exponential growth phase were used to inoculate fresh media with 13C-labeled substrates. To reduce the effect of nonlabeled carbon from the initial stock, cells were subcultured twice in the same labeled medium. Biomass was sampled at two time points (4-hour interval) in the middle exponential growth phase, and the labeling patterns of proteinogenic amino acids in the biomass were measured. The invariance of amino acid labeling during the two time points confirmed the pseudo-steady-state metabolism in tracer experiments.

**Metabolites and Isotopomer Analysis**—The amount of pyruvate and acetate during the growth period was determined by enzymatic assays (21–23). For GC-MS measurement of amino acid labeling, the biomass was harvested by centrifugation and hydrolyzed using 6 M HCl (24 h at 100 °C) (19, 24). The amino acids were derivatized in 0.2 ml of tetrahydrofuran and 0.2 ml of N-(tert-butyl dimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich). A gas chromatograph (Hewlett-Packard model 7890A; Agilent Technologies) equipped with a DB5-MS column (J&W Scientific, Folsom, CA) and a mass spectrometer (model 5975C; Agilent Technologies) were used for analyzing metabolite labeling profiles. Four types of charged fragments were detected by GC-MS for all of the amino acids (see Table 1 and Fig. 1); the [M-57]⁺ or [M-15]⁺ group (containing unfragmented amino acids); and the [M-159]⁺ or [M-85]⁺ group (containing amino acids that had lost an α-carboxyl group). For each type of fragments, the labeling patterns were represented by 

\[ M_{\text{exch}} = \frac{v_i^{\text{exch}}}{1 - v_i^{\text{exch}}} \]  

(Eq. 3)

where \( v_i^{\text{exch}} \) is the exchange flux defined as the smaller of the forward and backward fluxes, and \( v_i^{\text{exch}} \) is the exchange coefficient with the range of 0–1. (If the reaction is irreversible, \( v_i^{\text{exch}} \) is 0; if the reaction is "freely" reversible, \( v_i^{\text{exch}} \) is close to 1.) The flux combinations were searched to minimize the objective function (19),

\[ \epsilon(v) = \sum_{i=1}^{n} \left( \frac{M_i - N_i(v)}{\delta_i} \right)^2 \]  

(Eq. 4)

where \( v_i \) are the unknown fluxes to be optimized in the program, \( M_i \) is the measured MS data, \( N_i \) is the corresponding model-simulated MS data, and \( \delta_i \) is the corresponding S.D. in GC-MS data (1–2%). The unknown metabolic fluxes were searched to minimize \( \epsilon \). The model was solved by IPOPT (Interior Point Optimizer), which is a software package for large-scale nonlinear optimization. To avoid getting trapped in a local optimal minimum, multiple initial guesses (100) were used for model calculation to obtain the global solution.

To estimate the confidence interval for the calculated fluxes, a Monte Carlo approach was employed (14). In brief, the isotopomer concentration data sets were generated by adding 2% of normally distributed measurement noise to actual measured isotopomer data. The same optimization routine was used to estimate flux distribution from these data sets. Confidence limits for each flux were obtained from the probability distribution of calculated fluxes resulting from the simulated data sets (\( n = 100 \)). To determine the requirements of cellular metabolism for energy...
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TABLE 1
Isotopic labeling patterns in amino acids from Chlorobaculum tepidum

The S.D. for GC-MS measurement were based on the duplicate experiments (n = 2), with an S.D. below 2%.

| Carbon source | Amino acids | Fragments | M₀ | M₁ | M₂ |
|---------------|-------------|-----------|-----|-----|-----|
| [1-13C]Acetate | Ala | [M-57]⁺ | 0.45 | 0.54 | 0.01 |
|                | Gly | [M-57]⁺ | 0.44 | 0.53 | 0.03 |
|                | Ser | [M-57]⁺ | 0.54 | 0.50 | 0.06 |
|                | Leu | [M-57]⁺ | 0.47 | 0.53 | 0.00 |
|                | Ile | [M-57]⁺ | 0.21 | 0.43 | 0.33 |
|                | Asp | [M-57]⁺ | 0.64 | 0.35 | 0.01 |
|                | Glu | [M-57]⁺ | 0.65 | 0.34 | 0.01 |
|                | Phe | [M-57]⁺ | 0.11 | 0.29 | 0.36 |
| [2-13C]Acetate | Ala | [M-57]⁺ | 0.43 | 0.56 | 0.01 |
|                | Gly | [M-57]⁺ | 0.42 | 0.54 | 0.04 |
|                | Ser | [M-57]⁺ | 0.45 | 0.55 | 0.00 |
|                | Leu | [M-57]⁺ | 0.13 | 0.25 | 0.36 |
|                | Ile | [M-57]⁺ | 0.14 | 0.25 | 0.36 |
|                | Asp | [M-57]⁺ | 0.61 | 0.38 | 0.00 |
|                | Glu | [M-57]⁺ | 0.62 | 0.37 | 0.00 |
|                | Phe | [M-57]⁺ | 0.63 | 0.34 | 0.03 |
|                | His | [M-57]⁺ | 0.16 | 0.35 | 0.36 |
| [3-13C]Pyruvate | Ala | [M-57]⁺ | 0.67 | 0.32 | 0.01 |
|                | Gly | [M-57]⁺ | 0.65 | 0.30 | 0.05 |
|                | Ser | [M-57]⁺ | 0.71 | 0.28 | 0.01 |
|                | Leu | [M-57]⁺ | 0.71 | 0.28 | 0.01 |
|                | Ile | [M-57]⁺ | 0.47 | 0.39 | 0.12 |
|                | Asp | [M-57]⁺ | 0.82 | 0.17 | 0.00 |
|                | Glu | [M-57]⁺ | 0.83 | 0.15 | 0.01 |
|                | Phe | [M-57]⁺ | 0.76 | 0.20 | 0.05 |
|                | His | [M-57]⁺ | 0.38 | 0.41 | 0.17 |

RESULTS AND DISCUSSION

Carbon Utilization during Mixotrophic Growth—During exponential growth with pyruvate and acetate, C. tepidum had the growth rates of 0.12 h⁻¹ and 0.17 h⁻¹, respectively. In the trace experiments, the labeled carbons were detected in all amino acids (Table 1), suggesting utilization of acetate or pyruvate to synthesize all building blocks. Table 2 shows the ratio of carbon utilization (organic carbon versus CO₂) for mixotrophic synthesis of proteinogenic amino acids. For acetate culture, the substrate utilization ratio for Ala (precursor, pyruvate) was 0.60, which indicated that carbons in pyruvate were mainly derived from labeled acetate. Pyruvate is the precursor for both the gluconeogenesis pathway and the pentose phosphate pathway, so the R ratios (Equation 1) of Ser and Phe are similar to that of alanine. The substrate utilization ratio of His was higher (0.96) for culture with [2-13C]acetate because the His precursor C1 pool (N⁵,N¹⁰-methylene-tetrahydrofolate) was highly labeled. This carbon was derived from acetate by following route [2-13C]acetate → [3-13C]pyruvate → [3-13C]Ser → [13C]C1 pool.

In the mixotrophic culture with acetate, the R ratios for aspartate and glutamate (~0.2) were significantly lower than the other amino acids. Aspartate and glutamate were synthesized from the RTCA cycle, so the small R values indicated that the unlabeled CO₂ was the dominant carbon source for synthesizing metabolites in the RTCA cycle. In pyruvate culture, the substrate utilization ratios of amino acids (Ala, Phe, Ser, and Asp) were all lower than in acetate culture, indicating a higher contribution of CO₂ to synthesize building blocks.

Analysis of Amino Acid Biosynthesis Pathways—Growing in a completely defined medium, C. tepidum was able to synthesize all amino acids from CO₂ and organic carbon sources using the annotated pathways. Interestingly, the labeling patterns of Leu and Ile from tracer experiments were found to be identical, which indicated that the two amino acids shared the same precursors. Ile is commonly synthesized via threonine ammonia-lyase, with Thr and pyruvate as the precursors, whereas Leu is synthesized from pyruvate and acetyl-CoA. In the [2-13C]acetate trace experiment, both Thr and pyruvate are singly labeled, which could lead only to doubly labeled Ile, instead of the triply labeled Ile detected by GC-MS. Such an observation is consistent with the fact that a gene encoding threonine ammonia-lyase has not been annotated in the genome of C. tepidum. On the other hand, an alternative pathway for Ile biosynthesis (i.e., threonine-independent pathway) has been recently identified in several bacteria (8, 9, 24, 30), where Ile is synthesized from acetyl-CoA and pyruvate (i.e.
using the same precursors as Leu) through the formation of citramalate as the intermediate (Fig. 1). A search in the genome of *C. tepidum* for the gene for citramalate synthase (*CimA*) as found in *Geobacter* (9) returned a high identity for CT0612 (52%), supporting the presence of the citramalate pathway for Ile synthesis.

Central Carbon Metabolic Pathways—The genome annotation from the KEGG database indicates that 1) pyruvate:ferredoxin oxidoreductase (*porA*, CT1628) converts pyruvate to acetyl-CoA (an end product from RTCA cycle); 2) phosphoenolpyruvate is derived from pyruvate via pyruvate phosphate dikinase (CT1682), and 3) phosphoenolpyruvate is fed into the TCA cycle via the CO2-anaplerotic pathway (Fig. 2). The fraction of nonlabeled Asp (M₀ = 0.64, Table 1) was much higher than that of Ala (M₀ = 0.45, Table 1) in trace experiments with [1-13C]acetate, which indicated that nonlabeled oxaloacetate was also generated through CO₂ fixation via the RTCA cycle. Furthermore, similar labeling patterns of Ala (precursor, pyruvate) and Ser (precursor, glycerate-3-P) confirmed that the carbon flux through the gluconeogenesis pathway was mainly from pyruvate.

The flux analysis results are shown in Fig. 2. In general, predicted labeling patterns of all amino acids were good matches with the measured isotopomer data (Fig. 3). Under acetate growth conditions, acetyl-CoA could be generated from acetate uptake or as the end product through the RTCA cycle. Fluxes through pyruvate:ferredoxin oxidoreductase, the RTCA cycle, and the CO₂-anaplerotic pathway were 152, 80–100, and 30 units, respectively (Fig. 2). Meanwhile, the flux ratio of the gluconeogenesis pathway to the RTCA cycle...
was close to 1:1. The oxidative pentose phosphate pathway (glucose-6-phosphate → ribulose-5-phosphate) was inactive under our experimental conditions. Compared with mixotrophic growth with acetate, the flux distribution during pyruvate growth demonstrated different patterns. In general, the fluxes into the RTCA cycle and CO₂-anaplerotic pathway were much higher (200–230 units and 50 units, respectively), and thus, more CO₂ was fixed. The flux ratio of the gluconeogenesis pathway to the RTCA cycle was reduced to 1:2, whereas acetyl-CoA was generated only from the RTCA cycle. The oxidative pentose phosphate pathway was not active (flux 1 unit) under pyruvate mixotrophic growth (or acetate mixotrophic growth).

In the mixotrophic cultures grown on acetate or pyruvate, most reactions in the RTCA cycle were freely reversible (with fluxes represented by arrows). The metabolic flux distribution in C. tepidum, A, net flux distribution in acetate growth conditions (based on [2-13C]acetate culture), B, net flux distribution in pyruvate growth conditions. The S.D. and exchange coefficients are marked by flux S.D. and exchange coefficient, respectively. The inactive pathways are marked with dashed lines. The calculated biomass yield (moles of carbon in biomass/ mol of substrate): pyruvate culture (YX/S = 9.2); acetate culture (YX/S = 6.4). S7P, sedoheptulose-7-phosphate; SUC, sucinate; SUCCoA, succinyl-CoA; Xu5P, xylulose-5-phosphate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; AC, intracellular acetate; ACCOA, acetyl-coenzyme A; AC.ext, extracellular acetate; AKG, α-keto-glutarate; CIT, citrate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; FNR, ferredoxin-NAD(P)⁺ reductase; FUM, fumarate; G6P, glucose-6-phosphate; GAP, glyceraldehyde 3-phosphate; ICT, isocitrate; MAL, malate; OAC, oxaloacetate; PEP, phosphoenolpyruvate; PYR, intracellular pyruvate; PYR.ext, extracellular pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; PP, pentose phosphate.

FIGURE 2. Metabolic flux distribution in C. tepidum. A, net flux distribution in acetate growth conditions (based on [2-13C]acetate culture). B, net flux distribution in pyruvate growth conditions. The S.D. and exchange coefficients are marked by flux S.D. and exchange coefficient, respectively. The inactive pathways are marked with dashed lines. The calculated biomass yield (moles of carbon in biomass/mol of substrate): pyruvate culture (YX/S = 9.2); acetate culture (YX/S = 6.4). S7P, sedoheptulose-7-phosphate; SUC, sucinate; SUCCoA, succinyl-CoA; Xu5P, xylulose-5-phosphate; 3PG, 3-phosphoglycerate; 6PG, 6-phosphogluconate; AC, intracellular acetate; ACCOA, acetyl-coenzyme A; AC.ext, extracellular acetate; AKG, α-keto-glutarate; CIT, citrate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; FNR, ferredoxin-NAD(P)⁺ reductase; FUM, fumarate; G6P, glucose-6-phosphate; GAP, glyceraldehyde 3-phosphate; ICT, isocitrate; MAL, malate; OAC, oxaloacetate; PEP, phosphoenolpyruvate; PYR, intracellular pyruvate; PYR.ext, extracellular pyruvate; R5P, ribose-5-phosphate; Ru5P, ribulose-5-phosphate; PP, pentose phosphate.

FIGURE 3. Model quality test for acetate metabolism (A) and pyruvate metabolism (B). ●, alanine; ○, serine; ▼, aspartate; ◯, glutamate; ■, leucine; △, histidine; ◇, phenylalanine; and ●, glycine.
an exchange coefficient close to 1). The reversibility is consistent with the physiological free energy changes in the RTCA cycle, where the standard Gibbs free energy for most reactions of the TCA cycle is positive so that the magnitude of the reaction equilibrium constants is small (31). Furthermore, pyruvate:ferredoxin oxidoreductase catalyzes a reversible reaction between pyruvate and acetyl-CoA (pyruvate + CoA + 2Fd\textsubscript{ox} \rightleftharpoons acetyl-CoA + CO\textsubscript{2} + 2Fd\textsubscript{red} + 2H\textsuperscript{+}). (Fd\textsubscript{red} indicates reduced ferredoxin, and Fd\textsubscript{ox} indicates oxidized ferredoxin.) However, metabolic flux analysis of the mixotrophic metabolism indicates that the net flux of this enzymatic reaction was from acetyl-CoA to pyruvate (32). The direction of the pyruvate:ferredoxin oxidoreductase pathway indicates that the reduced ferredoxin from phototrophic processes made the reaction for pyruvate synthesis thermodynamically favorable.

**Energy Metabolism of *C. tepidum***—The mixotrophic metabolism of *C. tepidum* consumes energy harvested from light. Because the oxidative pentose phosphate pathway and normal TCA cycle are not fully functional, the energy (NADPH and NADH) generation mainly depends on the light reactions. Based on the metabolic flux distribution and previous measurement of the absolute uptake rates of carbon substrates and biomass growth (26), the light energy harvested for central metabolism and biomass growth was approximately quantified (Fig. 4). In general, the photons absorbed by pigments (e.g. bacteriochlorophylls) initiate the electron flow and oxidize sulfide. The electrons are used to reduce ferredoxin and generate NAD(P)H (2, 3). Concomitant with the oxidation of the reduced ferredoxin, the essential cofactors for energy metabolism (i.e. NADPH and NADH) are mainly generated by ferredoxin-NAD(P)\textsuperscript{+} reductase (2, 33). Meanwhile, the oxidation of sulfide also creates a proton motive force for ATP production. The generated ATP, cofactors, and reduced ferredoxin then become the “driver” for the mixotrophic metabolism and the RTCA cycle for energy-demanding CO\textsubscript{2} fixation. Fig. 4 shows that the ATP, NADH, and NADPH fluxes into biomass and the RTCA cycle are stronger in acetate-grown cultures than in pyruvate-grown cultures, supporting a higher growth rate during mixotrophic growth with acetate. On the other hand, the calculated biomass yield (mol of carbon in biomass/mol of substrate) was higher for pyruvate culture (Y\textsubscript{X/S} = 9.2) than for acetate (Y\textsubscript{X/S} = 6.4), based on our previous experiments (26), which is consistent with the fact that the normalized fluxes through RTCA are higher in pyruvate culture than acetate culture. Accordingly, pyruvate metabolism has more CO\textsubscript{2} fixation (but slower growth rate).

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