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Authors

Tang, Yinjie J.
Chakraborty, Romy
Garcia-Martin, Hector
[et al.](#)

Publication Date

2007

Peer reviewed

Flux analysis of central metabolic pathways in *Geobacter metallireducens* during
reduction of soluble Fe(III)-NTA

†Yinjie J. Tang^{1,2,7}, †Romy Chakraborty^{3,7}, Héctor García Martín⁴, Jeannie Chu^{1,2}, Terry C.
Hazen^{3,7}, Jay D. Keasling^{1,2,5,6,7,*}

¹Synthetic Biology Department, Physical Biosciences Division, Lawrence Berkeley National
Laboratory, Berkeley, CA 94720

²Department of Chemical Engineering, University of California at Berkeley, Berkeley, CA
94720

³Center for Environmental Biotechnology, Lawrence Berkeley National Laboratory, Berkeley,
CA 94720

⁴DOE Joint Genome Institute, 2800 Mitchell Drive, Walnut Creek, CA 94598

⁵Department of Bioengineering, University of California at Berkeley, Berkeley, CA 94720

⁶California Institute for Quantitative Biomedical Research (QB3), University of California at
Berkeley, Berkeley, CA 94720

⁷ Virtual Institute for Microbial Stress and Survival, <http://vimss.lbl.gov>

*Corresponding author. Mailing address: Berkeley Center for Synthetic Biology, 717 Potter
.Street, Building 977, Mail code 3224, University of California, Berkeley, CA 94720-3224.
USA. Phone: (510) 495-2620. Fax: (510) 495-2630. E-mail: keasling@berkeley.edu.

† Equal contributions

1 **Abstract**

2 We analyzed the carbon fluxes in the central metabolism of *Geobacter metallireducens* strain
3 GS-15 using ^{13}C isotopomer modeling. Acetate labeled in the 1st or 2nd position was the sole
4 carbon source, and Fe-NTA was the sole terminal electron acceptor. The measured labeled
5 acetate uptake rate was 21 mmol/gdw/h in the exponential growth phase. The resulting isotope
6 labeling pattern of amino acids allowed an accurate determination of the *in vivo* global metabolic
7 reaction rates (fluxes) through the central metabolic pathways using a computational isotopomer
8 model. The tracer experiments showed that *G. metallireducens* contained complete biosynthesis
9 pathways for essential metabolism, and this strain might also have an unusual isoleucine
10 biosynthesis route (using acetyl-CoA and pyruvate as the precursors). The model indicated that
11 over 90% of the acetate was completely oxidized to CO_2 via a complete tricarboxylic acid (TCA)
12 cycle while reducing iron. Pyruvate carboxylase and phosphoenolpyruvate carboxykinase were
13 present under these conditions, but enzymes in the glyoxylate shunt and malic enzyme were
14 absent. Gluconeogenesis and the pentose phosphate pathway were mainly employed for
15 biosynthesis and accounted for less than 3% of total carbon consumption. The model also
16 indicated surprisingly high reversibility in the reaction between oxoglutarate and succinate. This
17 step operates close to the thermodynamic equilibrium possibly because succinate is synthesized
18 via a transferase reaction, and the conversion of oxoglutarate to succinate is a rate limiting step
19 for carbon metabolism. These findings enable a better understanding of the relationship between
20 genome annotation and extant metabolic pathways in *G. metallireducens*.

21

22 Key words: labeled acetate, minimal medium, isoleucine, TCA cycle, acetyl-CoA transferase

23

24

1 Introduction

2 Geobacter species have been known to be one of the dominant groups of microorganisms
3 mediating iron reduction in the environment (21). They have been found to be ubiquitous in a
4 myriad of subsurface environments. Detailed studies of their metabolism has revealed them to
5 be capable of bioremediation of several heavy metals including uranium, plutonium, technitium,
6 and vanadium as well as biodegradation of several organic contaminants including
7 monoaromatic hydrocarbons (16, 17, 26). More recently, *Geobacter* species have been used to
8 generate electricity from waste organic matter (2, 15, 19). These unique metabolisms make
9 *Geobacter* species important players in the contaminated subsurface environment (18).
10 *Geobacter metallireducens* was the first iron-reducing organism isolated that coupled complete
11 oxidation of organic acids to reduction of iron oxides (20, 21). It completely oxidizes organic
12 carbons such as fatty acids, alcohols, and monoaromatic compounds via the tricarboxylic acid
13 (TCA) cycle (3, 20) coupled with the reduction of iron. The genomes of several *Geobacter*
14 species have been sequenced, and proteome data are also available (5, 24). While the genome
15 sequence and proteome are important for understanding *Geobacter*, they are not necessarily
16 accurate representations of cell physiology and metabolism.

17 To quantitatively analyze central metabolism in *Geobacter sulfurreducens*, a constraint-
18 based model was developed using the annotated genome sequence and a series of
19 physicochemical constraints (thermodynamic directionality, enzymatic capacity and reaction
20 stoichiometry) (22). While the model provided important insight into energy conservation,
21 biosynthesis of building blocks (such as amino acids) and the relationship of the genotype to its
22 phenotype, underdetermined models require one to assume an objective function (i.e.,
23 maximizing the specific growth rate) that may or may not be accurate and have difficulty

1 predicting fluxes through reversible reactions or reactions that may form futile cycles (7, 32, 39).
2 Further, genes are often incorrectly annotated in sequenced genomes, and incorporation of these
3 reactions into the model can affect the flux calculation. Even when properly annotated, the
4 presence of a gene does not indicate if it is being expressed.

5 Here we report a different approach to analyze the fluxes in the central metabolic
6 pathways of *Geobacter metallireducens* GS-15. The cells were fed [¹³C]acetate, and the
7 distribution of the ¹³C was measured in amino acids. Interpreted in the light of the genome
8 annotation, a model based on the atom transitions between metabolites in biochemical reactions
9 calculated the fluxes through the central metabolic pathway (12, 32, 33, 35). The model did not
10 require energy balances for the calculation and resolved bidirectional or futile reactions. This
11 study provided complementary flux information to the recent *in silico* model predictions and
12 further extended our understanding of anaerobic carbon metabolism in *Geobacter* species.

13 **Materials and methods**

14 **Growth conditions.** All media and solutions were prepared using strict anaerobic
15 techniques. The standard *Geobacter metallireducens* bicarbonate-buffered freshwater medium
16 was used (21) with one exception: one-tenth of the vitamin mix solution was used to minimize
17 the isotopomer measurement “noise” introduced by unlabeled carbon from vitamins. Briefly, the
18 medium was boiled under a N₂-CO₂ (80-20, vol/vol) headspace in order to remove the dissolved
19 oxygen. It was then dispensed into anaerobic pressure tubes or serum bottles under a N₂-CO₂
20 (80-20, vol/vol) headspace. The anaerobic pressure tubes or serum bottles were capped with
21 thick butyl-rubber stoppers and sterilized. [1-¹³C] Sodium acetate and [2-¹³C] sodium acetate
22 (both of 99% purity) were obtained from Cambridge Isotope Laboratories Inc (Andover,
23 Massachusetts). Anoxic aqueous stock solutions were prepared of Ferric-NTA (1M), [1-¹³C] or

1 [2-¹³C] sodium acetate (1 M) under a headspace of N₂-CO₂. These stocks were delivered
2 anaerobically into culture tubes and serum bottles via a needle. *G. metallireducens* GS-15 was
3 routinely cultured on anaerobic basal medium (21) using 5 mM acetate and 15 mM Fe-NTA as
4 the electron donor and acceptor, respectively, under a N₂-CO₂ (80:20, vol:vol) headspace. A
5 10% inoculum from the unlabeled stock culture was made into the [1-¹³C] or [2-¹³C] acetate
6 medium containing equivalent amounts of electron donor and acceptor. After growth reached
7 the mid-log phase, cells were transferred again into the same labeled medium to minimize the
8 effect of unlabeled carbon from the initial inoculum. This sub-culture protocol was repeated
9 twice. All incubations were performed at 30°C.

10 **Determining metabolite concentrations and biomass composition.** The standard
11 ferrozine assay was used to measure Fe(II) concentration during growth on acetate and Fe-NTA
12 (21). Cell counts were performed using a microscope and acridine orange to stain cells. Briefly,
13 a 100- μ l sample was added to 900- μ l, 0.1% sodium polyphosphate solution and mixed well. Ten
14 μ l of this cell suspension was pipetted onto a 6 mm well of slide. The slide was dried and heat
15 fixed. Twenty-five (25) μ l of acridine orange stain was used to stain the wells containing several
16 dilutions of the cell samples. The slides were incubated in the dark for 2 minutes, washed, and
17 then dried; the cells were counted using fluorescent microscopy. The concentrations of acetate
18 in the culture supernatant (following centrifugation of the culture at 10,000 \times g for 20 minutes at
19 4°C) were measured using enzyme assays (r-Biopharm, Darmstadt, German). The amino acid
20 composition of the biomass protein was quantified using the Beckman 6300 amino acid analyzer
21 (Beckman Coulter, California), performed by the Molecular Structure Facility at the University
22 of California, Davis (<http://msf.ucdavis.edu>). Biomass constituents were taken from the
23 literature: protein (46%), RNA (10%), DNA (4%), lipids (15%), total carbohydrate (15%),

1 lipopolysaccharides (4%), and peptidoglycan (4%) (22), and those data were the initial estimates
2 for the isotopomer model to calculate fluxes into biomass.

3 **Isotopomer analysis of protein amino acids by GC-MS (33-35).** A 200-mL cell
4 culture (2×10^8 cells/ml) was harvested by centrifugation at $10,000 \times g$ for 20 minutes at 4°C and
5 sonicated subsequently for 3 minutes. The protein from the resulting lysate was precipitated
6 using trichloroacetic acid and then hydrolyzed in 6 M HCl at 100°C for 24 hours. The amino
7 acid/HCl solution was dried under nitrogen flow overnight. GC-MS samples were prepared in
8 100 μl tetrahydrofuran (THF) and 100 μl N-(tert-butyldimethylsilyl)-N-methyl-
9 trifluoroacetamide (Sigma-Aldrich, USA). These samples were derivatized at 70°C for 1 hour,
10 producing tert-butyldimethylsilyl (TBDMS) derivatives (33-35). One μL of the derivatized
11 sample was injected into a gas chromatograph (Agilent, model HP6890) equipped with a DB5-
12 MS column (J&W Scientific, Falsom CA) and analyzed using a mass spectrometer (Agilent,
13 model 5973). The GC column was held at 150°C for 2 minutes, heated at 3°C per minute to
14 280°C , heated at 20°C per minute to 300°C , and held for 5 minutes at that temperature.

15 **Annotated pathway map and algorithm for flux calculation.** The central biochemical
16 pathways in *Geobacter metallireducens* GS-15 include gluconeogenesis, the TCA cycle, and the
17 pentose phosphate (PP) pathway (1). Each reaction and its corresponding gene are listed in
18 Supplementary Table S-1. To reduce computational time, the fluxes through the pools of amino
19 acids, carbohydrate, and RNA/DNA were loosely constrained by the biomass production and the
20 measured average biomass composition (Supplementary Table S-2), and those fluxes were
21 optimized using the isotopomer model based on the amino acid label information. The reversible
22 reactions were characterized by their net flux, v_i , and their exchange flux, v_i^{exch} . The net flux was
23 defined as the difference between forward and backward fluxes, $(v_i^{\rightarrow} - v_i^{\leftarrow})$. The exchange flux,

1 v_i^{exh} , was the smaller of the forward and backward fluxes, $\min(v_i^{\rightarrow}, v_i^{\leftarrow})$, and was used to
2 calculate the exchange coefficient, exh_i , according to (33, 38):

3
$$v_i^{exh} = \frac{exh_i}{1 - exh_i} \quad (1)$$

4 Exchange coefficients for all reactions were searched in the range [0 1] (41). Since atom
5 transitions between metabolites in the biochemical network were known and the path that each
6 atom took through the network could be traced using the model, the steady-state isotopomer
7 distributions in the intracellular metabolite pools for a given flux distribution were obtained via
8 the isotopomer mapping matrices (29, 32) (using MATLAB 6.0, Mathworks, USA); these
9 isotopomer distributions were used to simulate MS data ($m/z=M0, M1, M2\dots$). Our isotopomer
10 model, like most non-linear systems, did not possess a simple analytic solution. The final
11 solution in our isotopomer model was searched based on an objective function defined as:

12
$$\varepsilon(v_n) = \sum_{i=1}^a \left(\frac{M_i - N_i(v_n)}{\delta_i} \right)^2 \quad (2)$$

13 where v_n were the unknown fluxes to be optimized in the model program, M_i were the measured
14 MS data when $[1-^{13}C]$ or $[2-^{13}C]$ acetate was used as the carbon source, respectively; δ_i were the
15 corresponding measurement errors; N_i were the corresponding model-simulated MS data when a
16 complete set of flux distribution v_n and exchange coefficients exh_i were given to the isotopomer
17 model. The optimal fluxes were calculated to be such that ε was minimized using a simulated
18 annealing approach with different initial conditions (27, 33). The initial annealing temperature
19 was set to 50 and the final one to 0.01, with the temperature being decreased 100 times by a set
20 fraction each time. In each run, 10,000~100,000 moves were used, and the algorithm was
21 restarted from the final position several times to check the reliability of the minimum. The
22 examples of MATLAB programs for calculation of flux and exchange coefficients are available

1 at http://vimss.lbl.gov/DvHFlux/AdvancedCodesWithAMM_IMM.rar. The solution produced
2 isotopomer predictions consistent with measured data from both [1-¹³C] and [2-¹³C] acetate
3 experiments.

4 **Result and discussion**

5 ***Geobacter metallireducens* GS-15 growth kinetics in minimal medium.** GS-15 grew
6 in minimal medium and completely oxidized acetate as the sole carbon and energy source by
7 reducing Fe³⁺ to Fe²⁺ (Figure 1). The doubling time was ~5 hours with a late mid-log phase
8 density of ~2.3×10⁸ cells/ml, and the corresponding biomass concentration was 4.8±0.3 mg/L
9 with a yield of 3.2 gdw/mol acetate. In the final sampling point, about 1.5 mM acetate was
10 consumed and 11 mM Fe²⁺ was generated (equivalent to dissimilating 1.4 mM acetate). This
11 result indicates that the *Geobacter*'s biomass yield from oxidization of acetate is three times
12 lower than the thermodynamic yield predictions (16.8 gdw/mol acetate) (36, 40). At standard
13 conditions (1 atm and 25°C indicated by the Θ superscript), Fe³⁺ ($\Delta G^\ominus=-24.38$ kcal/eq) has a
14 similar electron potential as oxygen ($\Delta G^\ominus=-25.28$ kcal/eq) (23). However, *Geobacter*'s Fe(III)
15 reduction site is extracellular (not in the cytoplasm); under Fe³⁺-NTA reduction, the electrons
16 have to be transported outside of the cell or into the periplasmic cytochrome pool, but the
17 protons remain in the cytoplasm (22). This could result in dissipating the membrane potential
18 and acidifying the cytoplasm, which in turn could reduce the biomass yield that results from
19 acetate via Fe³⁺ reduction compared to that obtained during oxygen or fumarate reduction (9,
20 22).

21 ***Isotopomer analysis of labeling pattern in protein amino acids by GC-MS.*** Labeled
22 acetate (1st position or 2nd position) was used in independent experiments. GS-15 was harvested
23 in the exponential growth phase from each batch culture (a quasi steady-state that the cells are

1 under a balanced growth condition) (10, 28, 31). Two types of positively charged amino acid
2 species from the biomass protein were clearly observed by GC-MS: unfragmented amino acids
3 [M-57] and fragmented species [M-159] that had lost the α -carboxyl group (4, 6, 14, 37). The
4 natural abundance of heavy isotopes common in organic molecules as well as the derivatization
5 agents was corrected for by using published algorithms (37). The corrected GC-MS data for
6 eight key amino acids useful for model calculation including [M-57]⁺ and [M-159]⁺ are provided
7 (Table 1). The isotopomer distributions in the amino acids from hydrolyzed protein were used to
8 examine the metabolic pathways. For example, the different labeling patterns of alanine and
9 serine indicate that their precursors were not same; i.e., alanine is derived from pyruvate, while
10 serine is derived from phosphoglyceric acid (PGA). In each type of experiment, isotopomer
11 patterns in some amino acids from the same precursor were similar and provided redundant
12 isotopomer information (11): i.e., threonine and aspartate from oxaloacetate, tyrosine and
13 phenylalanine from phosphoenolpyruvate and erythrose-4-phosphate. Therefore, only one amino
14 acid from each precursor listed in the table was used for model calculations. GC-MS cannot
15 accurately measure the ion fragment [M-57]⁺ (no loss, m/z=302) for leucine and isoleucine
16 because of the overlay of mass peaks (mass fragment with only the α and β carbons of
17 leucine/isoleucine also has an m/z of 302) (37).

18 ***Determination of the flux distribution using the isotopomer model.*** The published
19 annotated genome sequence of *Geobacter metallireducens* indicates that several amino acid
20 biosynthesis pathways (e.g., lysine, valine, leucine, isoleucine, and alanine) are incomplete (1).
21 However, *Geobacter metallireducens* is able to grow in minimal medium with acetate as its sole
22 carbon source, and therefore must contain complete energy and biosynthesis pathways for
23 essential metabolites, i.e., *G. metallireducens* may have undocumented genes encoding some

1 amino acid biosynthesis enzymes. The ^{13}C flux analysis can aid annotation of un-annotated
2 pathways. The isotopomer model calculation from two tracer experiments (with $[1-^{13}\text{C}]$ acetate
3 and $[2-^{13}\text{C}]$ acetate) gave similar flux distribution results (Figure 2). The predicted labeling
4 patterns of all metabolites (except isoleucine), based on calculated fluxes and exchange
5 coefficients, matched relatively well the measured data (deviations are within the noise from
6 triplicate tracer experiments), and this indicates that model calculations are of good quality
7 (Figure 3). The discrepancy between the measured isoleucine $[\text{M159}]^+$ data and the model
8 predictions indicates that isoleucine is not synthesized from oxaloacetate and pyruvate (Table 1).
9 This is consistent with the fact that a key step in the usual isoleucine biosynthesis pathway (EC
10 4.2.1.9, dihydroxy-acid dehydratase) in *G. metallireducens* is missing, and an alternative
11 isoleucine biosynthesis pathway may be present. The isotopomer labeling of isoleucine was
12 identical to that of leucine from both tracer experiments, and this indicates that isoleucine shares
13 the same precursors (i.e., pyruvate and acetyl-CoA) as leucine. This hypothesis is supported by
14 recent discovery of citramalate synthase in *Geobacter sulfurreducens*; thus, isoleucine is likely
15 derived from acetyl-CoA and pyruvate via citramalate as an intermediate in *Geobacter* species
16 (8) (personal communication with M. Coppi, University of Massachusetts, Amherst, MA and S.
17 Van Dien, Genomatica, San Diego, CA).

18 The conversion of acetate to acetyl-CoA (acetate uptake rate of 21 ± 1.6 mmol/gdw/h,
19 assumed to be 100 in the model calculation) may be catalyzed by two independent enzymes
20 (acetyl-CoA transferase or acetate kinase) (Figure 2). The acetyl-CoA produced branched into
21 three pathways: the major flow (19 mmol/gdw/h, relative flux $v = \sim 90$) was into a complete TCA
22 cycle; the second flow was (1.7 mmol/gdw/h, $v = \sim 8$) to pyruvate via pyruvate-ferredoxin
23 oxidoreductase; and the third flow was into biomass production (e.g., synthesis of leucine and

1 fatty acids). The genome annotation indicated that some key enzymes in gluconeogenesis were
2 missing (EC 4.1.2.13, fructose-bisphosphate aldolase; EC2.7.2.3, phosphoglycerate kinase;
3 EC5.4.2.4, bisphosphoglycerate synthase; i.e., no reactions for glycerate-3P \rightarrow glycerate-1,3-P₂
4 and glyceraldehyde-3P \rightarrow β -D-fructose-1,6-P₂). However, the tracer experiments indicated that
5 gluconeogenesis is actually complete, and the total flux was 0.5 mmol/gdw/h ($v = \sim 2.5$). The
6 pentose phosphate pathway (PPP) is mainly used for biosynthesis when acetate is used as the
7 sole carbon source. Although there are several alternative pathways to make C5P (ribose-5-
8 phosphate, i.e., precursors of histidine and nucleotides), the model indicates that the major
9 carbon flow to PPP is via the oxidative branch G6P \rightarrow 6PG \rightarrow C5P, which generates NADPH.
10 In general, the isotopomer model gave results consistent with the previous predictions from a
11 constraints-based model for a closely related species, *Geobacter sulfurreducens* (22). However,
12 the presence of phosphoenolpyruvate carboxykinase was not predicted by the constraints-based
13 model, but was found using the isotopomer model.

14 ***Characterization of GS-15 metabolism under Fe³⁺ reduction conditions.*** Previous
15 reports indicate that *Geobacter* possesses two acetyl-CoA production routes (via acetyl-CoA
16 transferase or acetate kinase/P-transacetylase) to secure sufficient flux for growth, whereas other
17 acetate-degrading anaerobic bacteria often use one pathway for acetyl-CoA formation. This
18 study also indicated the flexibility of central metabolism in other carbon utilization routes. For
19 example, pyruvate carboxylase activity was present (0.7 mmol/gdw/h, $v \sim 3.6$) — this is an
20 alternative pathway to feed carbon into the TCA cycle by consuming ATP. Second, two carbon
21 flows lead to phosphoenolpyruvate synthesis via pyruvate kinase / phosphoenolpyruvate
22 synthase (~ 0.4 mmol/gdw/h, $v = \sim 1.8$) or phosphoenolpyruvate carboxykinase (~ 0.1
23 mmol/gdw/h, $v = \sim 0.6$). The presence of redundant pathways may stabilize cellular metabolism

1 under conditions of environmental uncertainty (33). Meanwhile, the absence of the glyoxylate
2 shunt (this pathway has not been annotated) was confirmed by the isotopomer analysis. On the
3 other hand, the NADP⁺-dependent malic enzyme, which is inhibited by the presence of acetyl-
4 CoA (13) and whose corresponding gene was annotated in the genome, had no flux. These
5 results are consistent with the predictions from the genome-scale, constraints-based model (22).
6 With respect to energy production, zero flux through the glyoxylate shunt and malic enzyme
7 maximizes the total carbon flow through the oxidative TCA cycle and thus produces the most
8 reducing power (NADH).

9 In general, decarboxylation reactions, such as the oxidative reactions in the pentose
10 phosphate pathway and the TCA cycle, are frequently irreversible (30). However, the model
11 predicted extremely high reversibility (exch = 0.99) in the reaction that convert oxoglutarate to
12 succinate compared to those in other microorganisms (41). This reaction contains two steps and
13 is usually catalyzed by the enzymes oxoglutarate oxidoreductase (oxoglutarate → succinyl-CoA,
14 $\Delta G^\ominus = -33.5\text{kJ/mol}$) and succinyl-CoA synthetase (succinyl-CoA → succinate, $\Delta G^\ominus = -2.9$
15 kJ/mol) (25). The free energy of both steps indicates a positive driving force for converting
16 oxoglutarate to succinate. However, the succinyl-CoA synthetase activity is absent in *Geobacter*
17 *metallireducens*, and acetyl-CoA transferase instead is used to complete the reaction: succinyl-
18 CoA (+acetate) → succinate (+acetyl-CoA) (13). The reason for the very high reversibility
19 between oxoglutarate and succinate is likely that the accumulation of acetyl-CoA forces the
20 reaction in the reverse direction, and thus inhibits the rate of carbon metabolism through TCA
21 cycle. This may explain the slow growth of *Geobacter metallireducens* under iron-reducing
22 conditions, even though the organism can use the complete TCA cycle to oxidize carbon
23 substrates similar to other aerobic bacteria.

1 Growth of *G. metallireducens* while oxidizing acetate requires incorporation of CO₂ into
2 biomass (acetyl-CoA + CO₂ → pyruvate and pyruvate + CO₂ → oxaloacetate), and therefore our
3 model evaluated the fate of the labeled ¹³C of carbon dioxide. Both experiments with [1-
4 ¹³C]acetate and [2-¹³C]acetate indicated that the [¹³C]CO₂ in the medium was below 3% of total
5 CO₂ (Table 1). This is consistent with the fact that the labeled ¹³CO₂ produced from acetate
6 oxidization is negligible compared to the ¹²CO₂ from the headspace gases (N₂-CO₂). The
7 experiment performed with [1-¹³C]acetate introduced very little ¹³C (<3%) into the C1 pool
8 (5,10-Me-THF), while most of the C1 pool was labeled (82%) in the [2-¹³C]acetate experiments.
9 This result confirms that C1 metabolism is mainly via the serine pathway, i.e., serine is
10 converted to glycine and a C1-unit before being incorporated into protein. The carbon transition
11 routes are *CH₃COOH → *CH₃COCOOH → *CH₂(OH)CH(NH₂)COOH → CH₂NH₂COOH +
12 *C1 pool.

13 In conclusion, this study demonstrates ¹³C metabolic flux analysis as a tool for verifying
14 genome annotation, characterizing the physiological state of microorganisms, and mapping the
15 central metabolism in anaerobic bacteria. The results from our technique provide valuable
16 complementary information to genome-based modeling approaches resulting in a comprehensive
17 understanding of central carbon metabolism in microorganisms. Our study indicates that *G.*
18 *metallireducens* strain GS-15 utilizes the complete TCA cycle to oxidize acetate to CO₂ while
19 reducing soluble Fe(III)-NTA. A futile pathway (pyruvate → oxaloacetate → PEP) is also
20 evident by isotopomer data. Although the annotated genome indicates the absence of a few key
21 enzymes in *gluconeogenesis* and some amino acid synthesis pathways, our ¹³C-tracer
22 experiments demonstrate that those pathways are actually complete and *G. metallireducens* may
23 contain some undocumented metabolic routes, e.g., an unusual isoleucine biosynthesis pathway

1 possibly via citramalate as the intermediate is suggested by isotopic data. In combination with
2 physiological data on the environmentally relevant microbe *Geobacter metallireducens*, this
3 study helps our understanding of carbon assimilation in the survival of such organisms in the
4 environment.

5 **Acknowledgements**

6 We thank Dr. M. Coppi (Department of Microbiology, University of Massachusetts, Amherst)
7 and Dr. S. Van Dien (Genomatica, San Diego) for advice on *Geobacter* anaerobic pathways. We
8 also thank Michelle Chang (Department of Chemical Engineering, University of California,
9 Berkeley) for help with experiments. This work is part of the Virtual Institute for Microbial
10 Stress and Survival (<http://VIMSS.lbl.gov>) supported by the U.S. Department of Energy, Office
11 of Science, Office of Biological and Environmental Research, Genomics: GTL Program through
12 contract DE-AC02-05CH11231 between the Lawrence Berkeley National Laboratory and the US
13 Department of Energy.

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26

1 **Figure Captions**

2 **Figure 1.** *Geobacter metallireducens* GS-15 growth kinetics in minimal medium: □, total cell
3 number; ◆, Fe²⁺ concentration; ▲, acetate concentration.

4

5 **Figure 2.** Metabolic flux distribution in *Geobacter metallireducens* GS-15 under Fe³⁺ reduction
6 conditions. The upper number indicates flux based on [1-¹³C]acetate experiments, and the lower
7 number indicates flux based on [2-¹³C]acetate experiments. The acetate uptake rate was 21
8 mmol/gdw/h. The data in brackets are the exchange coefficients. The dotted arrows indicate the
9 absence of an annotated gene for the step. Abbreviations: 6PG, 6-phosphogluconate; ACoA,
10 acetyl-coenzyme A; C1, 5,10-Me-THF; C5P, ribose-5-phosphate (or ribulose-5-phosphate or
11 xylulose-5-phosphate); CIT, citrate; E4P, erythrose-4-phosphate; F6P, fructose-6-phosphate;
12 G6P, glucose-6-phosphate; ICT, isocitrate; MAL, malate; OAA, oxaloacetate; OXO, 2-
13 oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; PYR, pyruvate. S7P,
14 sedoheptulose-7-phosphate; SUC, succinate; and T3P, triose-3-phosphate.

15

16 **Figure 3.** Model quality test. ◆, glutamate data; □, aspartic acid data; ◇, alanine and leucine data;
17 Δ, serine and glycine data; ×, histidine data; ○, phenylalanine data; +, isoleucine (isoleucine data
18 were not used as constraints for the model calculation). The absolute GC-MS measurement
19 errors were based on the information in Table 1.

Figure 1.

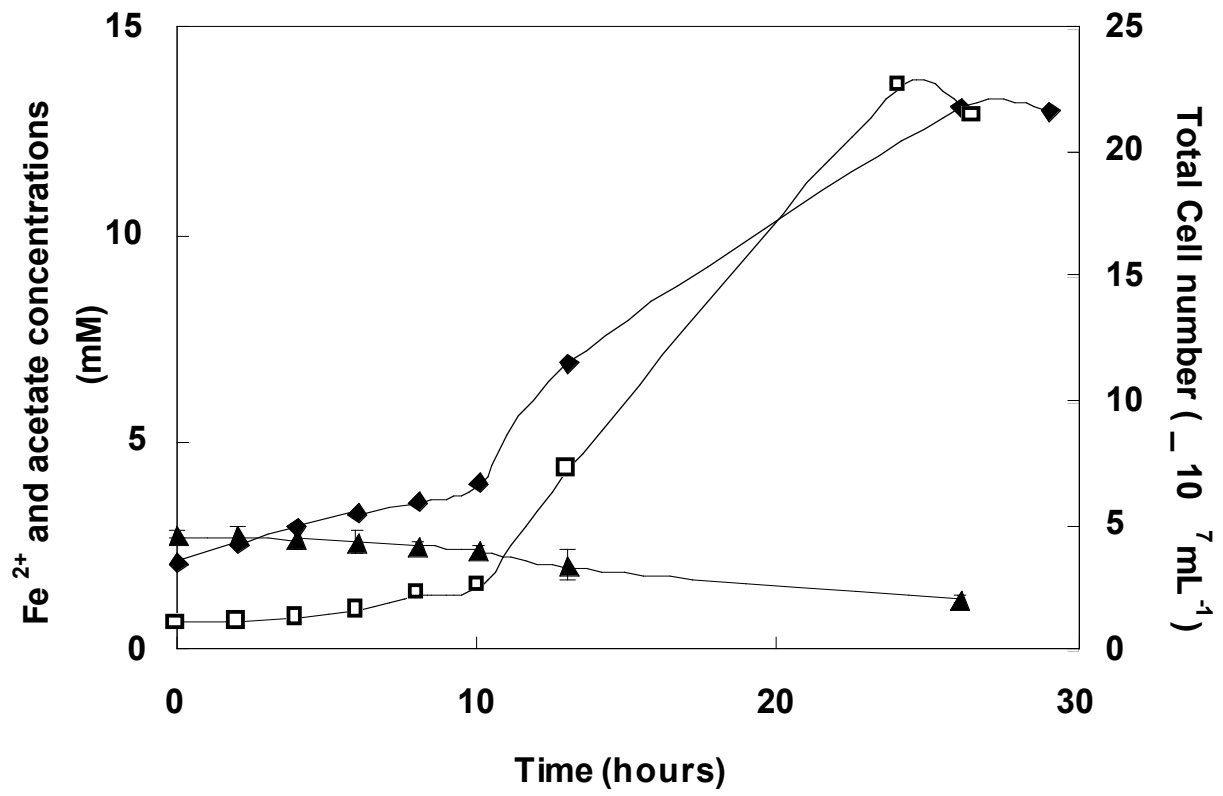


Figure 2.

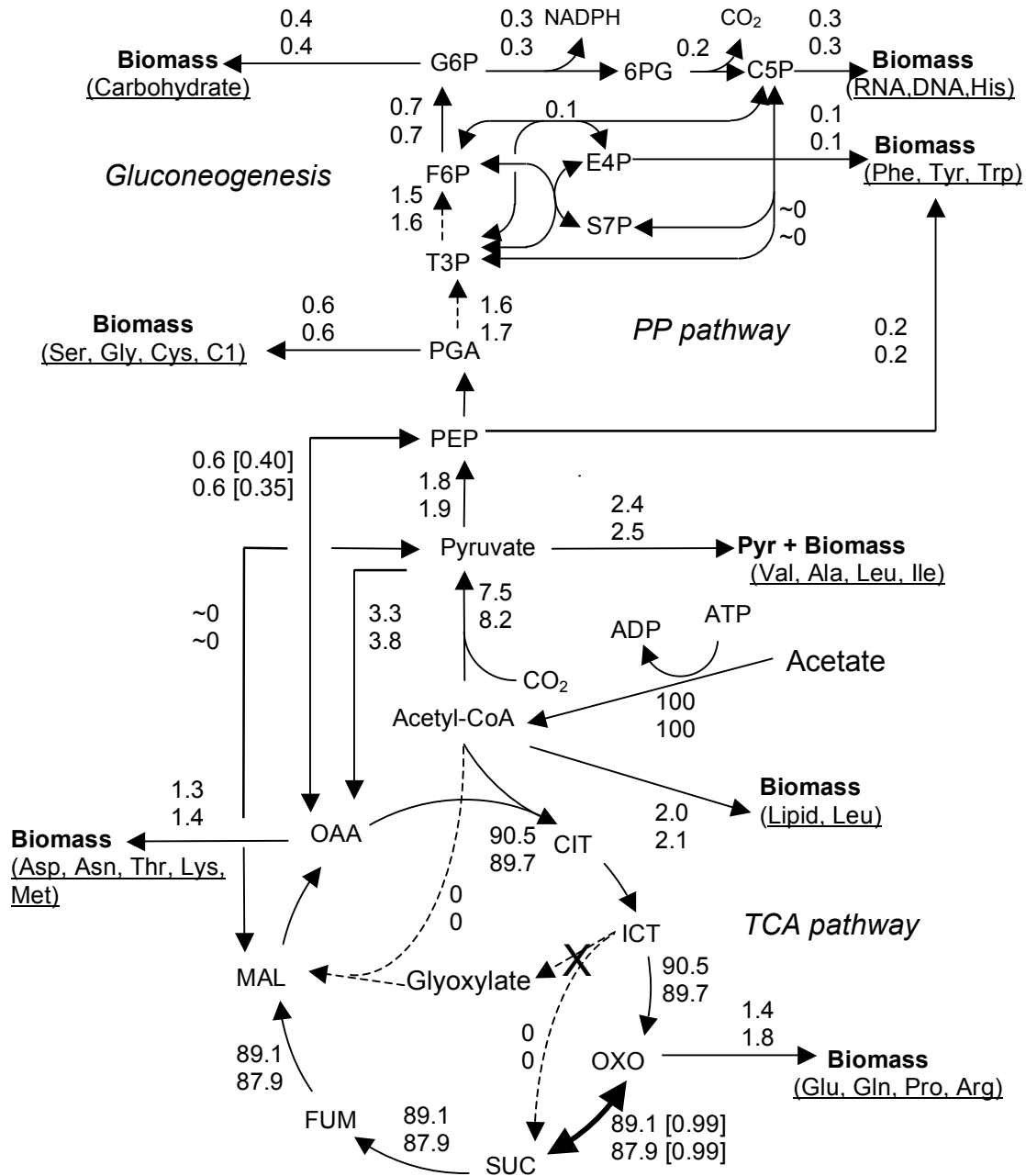


Figure 3.

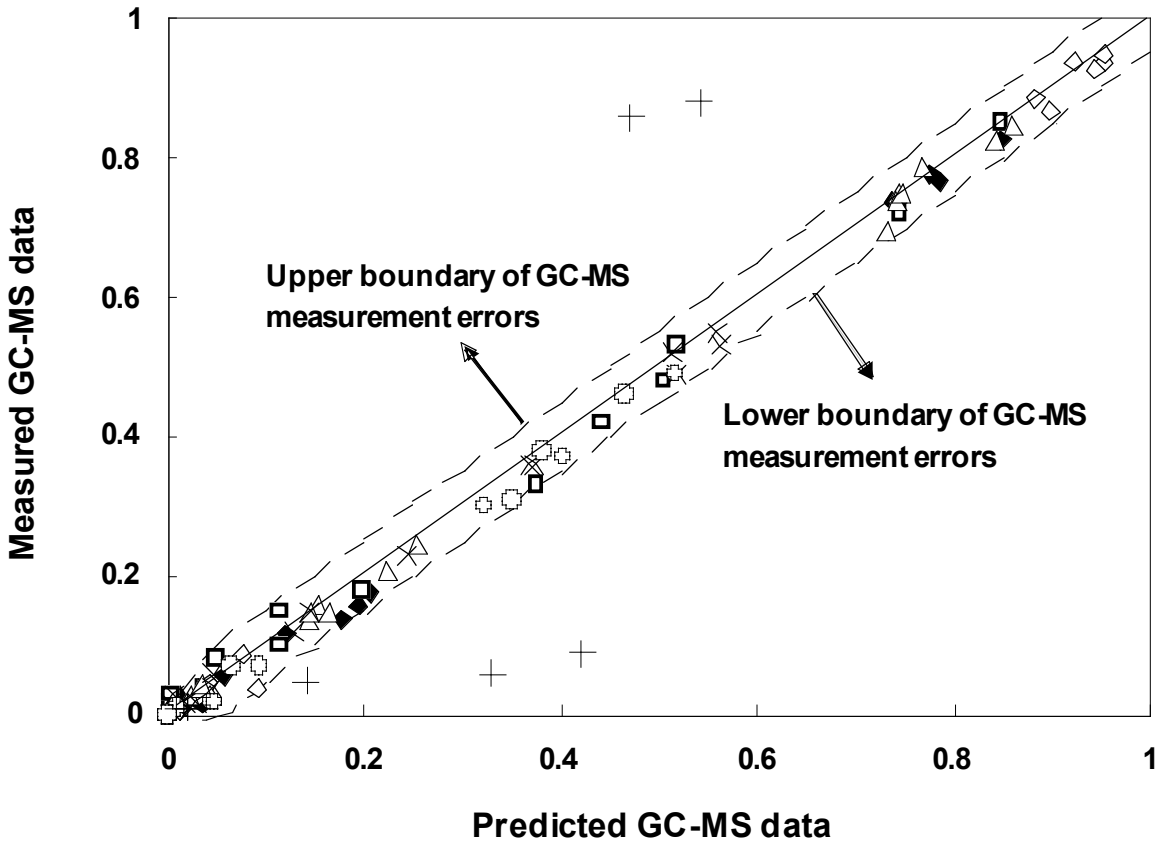


Table 1. Measured fragment mass distributions for ^{13}C -labeled amino acids from *Geobacter metallireducens* GS-15 hydrolysates¹.

Amino acids (Precursors)	Fragment	$[1-^{13}\text{C}]$ acetate culture			
		M ₀	M ₁	M ₂	M ₃
Glycine (PEP) ²	(M57) ⁺	0.15±0.02	0.85±0.03		
	(M85) ⁺	0.26±0.02	0.74±0.03		
Serine (PEP)	(M57) ⁺	0.14±0.02	0.83±0.03	0.03±0	
	(M159) ⁺	0.25±0.02	0.75±0.03	0.0±0	
Alanine (pyruvate)	(M57) ⁺	0.05±0.02	0.94±0.02	0.01±0	
	(M159) ⁺	0.05±0.02	0.95±0.01	0±0	
Leucine (pyruvate + acetyl CoA)	(M159) ⁺	0.01±0	0.09±0.02	0.87±0.02	0.02±0
Isoleucine (OAA + pyruvate)	(M159) ⁺	0.01±0	0.09±0.01	0.88±0.02	0.01±0.01
Glutamate (OXO)	(M57) ⁺	0.02±0	0.77±0.03	0.14±0.02	0.03±0.01
	(M159) ⁺	0.02±0.01	0.83±0.02	0.12±0.02	0.02±0.01
Asparate (OAA)	(M57) ⁺	0.04±0.02	0.85±0.03	0.10±0.02	0.01±0
	(M159) ⁺	0.42±0.03	0.53±0.03	0.03±0.01	0.02±0.01
Histidine (C5P)	(M57) ⁺	0.04±0.01	0.36±0.02	0.55±0.02	0.02±0
	(M159) ⁺	0.06±0.02	0.36±0.02	0.53±0.02	0.02±0
Phenylalanine (PEP+E4P)	(M57) ⁺	0.01±0	0.07±0.01	0.29±0.04	0.49±0.05
	(M159) ⁺	0.01±0	0.07±0.01	0.31±0.04	0.46±0.05
Predicted CO ₂			0.01		
Predicted C1pool			0.02		

Table 1 (Continued)

Amino acids (Precursors)	Fragment	[2- ¹³ C]acetate culture			
		M ₀	M ₁	M ₂	M ₃
Glycine (PEP) ²	(M57) ⁺	0.75±0.03	0.15±0.02	0.10±0.02	
	(M85) ⁺	0.79±0.03	0.21±0.02		
Serine (PEP)	(M57) ⁺	0.04±0.01	0.70±0.03	0.16±0.02	
	(M159) ⁺	0.05±0.02	0.79±0.03	0.16±0.02	
Alanine (pyruvate)	(M57) ⁺	0.03±0.01	0.93±0.01	0.02±0.01	
	(M159) ⁺	0.03±0	0.93±0.01	0.04±0.01	
Leucine (pyruvate + acetyl CoA)	(M159) ⁺	0.01±0	0.01±0	0.04±0.01	0.89±0.02
Isoleucine (OAA + pyruvate)	(M159) ⁺	0.01±0	0.01±0.01	0.05±0.01	0.87±0.01
Glutamate (OXO)	(M57) ⁺	0.02±0	0.01±0	0.16±0.01	0.74±0.02
	(M159) ⁺	0.02±0	0.02±0	0.18±0.02	0.78±0.03
Aspartate (OAA)	(M57) ⁺	0.02±0.01	0.08±0.01	0.18±0.02	0.72±0.03
	(M159) ⁺	0.03±0.02	0.15±0.02	0.48±0.02	0.33±0.02
Histidine (C5P)	(M57) ⁺	0.02±0	0.01±0	0.15±0.02	0.52±0.03
	(M159) ⁺	0.03±0.01	0.12±0.01	0.49±0.02	0.23±0.02
Phenylalanine (PEP+E4P)	(M57) ⁺	0.01±0	0±0	0.02±0	0.38±0.03
	(M159) ⁺	0.01±0	0±0	0.02±0	0.36±0.02
Predicted CO ₂			0.03		
Predicted C1pool			0.82		

- ¹³C-Labeled biomass was sampled in the middle log phase. The standard deviations for GC-MS measurement were based on the triplicate experiments (n=3).
- Glycine fragmentation (M159)⁺ was not observed. (M85)⁺ (Loss of carboxyl group) was used instead.