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The *Shewanella oneidensis* MR-1 fluxome under various oxygen conditions

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1 **Abstract**

2 The central metabolic fluxes of *Shewanella oneidensis* MR-1 were examined under
3 carbon-limited (aerobic) and oxygen-limited (micro-aerobic) chemostat conditions using ^{13}C
4 labeled lactate as the sole carbon source. The carbon labeling patterns of key amino acids in
5 biomass were probed using both GC-MS and ^{13}C -NMR. Based on the genome annotation, a
6 metabolic pathway model was constructed to quantify the central metabolic flux distributions.
7 The model showed that the tricarboxylic acid (TCA) cycle is the major carbon metabolism route
8 under both conditions. The Entner-Doudoroff and pentose phosphate pathways were utilized
9 primarily for biomass synthesis (flux below 5% of the lactate uptake rate). The anapleurotic
10 reactions (pyruvate to malate and oxaloacetate to phosphoenolpyruvate) and the glyoxylate shunt
11 were active. Under carbon-limited conditions, a substantial amount (9% of the lactate uptake
12 rate) of carbon entered the highly reversible serine metabolic pathway. Under micro-aerobic
13 conditions fluxes through the TCA cycle decreased and acetate production increased compared
14 to carbon-limited conditions, and flux from glyoxylate to glycine (serine-glyoxylate
15 aminotransferase) became measurable. Although flux distributions under aerobic, micro-aerobic,
16 and shake-flask culture conditions were different, the relative flux ratios of some central
17 metabolic reactions did not vary significantly (in particular, between shake flask and aerobic
18 chemostat). Hence, *S. oneidensis* central metabolism appears to be robust to environmental
19 changes. Our study also demonstrates the merit of coupling GC-MS with ^{13}C NMR for
20 metabolic flux analysis to reduce the use of ^{13}C labeled substrates and to obtain more accurate
21 flux values.

22 *Key words: micro-aerobic, isotopomer model, futile cycles, serine metabolism, flux ratio*

1 Introduction

2 *Shewanella oneidensis* MR-1 (ATCC70050) is a Gram-negative, facultative anaerobe that
3 was isolated from lake sediment (35). These bacteria are capable of utilizing many carbon
4 sources, including lactate, acetate, pyruvate, and some amino acids. Moreover, they are capable
5 of reducing a variety of electron acceptors besides oxygen, including Fe(III), Mn(IV), sulfur,
6 nitrate, and fumarate (26). There have been extensive studies of this strain, primarily focused on
7 its versatile respiration and its potential to engage in co-metabolic bioremediation of toxic metals
8 (17, 18, 34, 36, 37).

9 Recently, the complete genome was sequenced and annotated. Furthermore, key
10 phenotypic and molecular characteristics have been identified (13). *S. oneidensis* central carbon
11 metabolism under both aerobic and anaerobic conditions has been investigated using enzyme
12 assays and genome information (11, 26, 27), and there are several unusual features (26, 42).
13 First, a serine pathway is proposed to be active under anaerobiosis in *S. oneidensis* due to the
14 detection of high levels of hydroxypyruvate reductase, which is the key enzyme involved in
15 serine metabolism. Second, *S. oneidensis* shares some metabolic features with non-fermentative
16 Pseudomonads, such as utilizing the Entner-Doudoroff (ED) pathway instead of the Embden-
17 Meyerhof-Parnas (EMP) pathway for the oxidation of glucose. Third, because the TCA cycle
18 might be truncated under oxygen limited conditions, the glyoxylate shunt might be present to
19 synthesize TCA cycle intermediates. However, these features have not been rigorously verified
20 using ¹³C tracer experiments, and very little is known about the actual balances of intracellular
21 metabolic fluxes under different oxygen conditions. Metabolic flux analysis is necessary to
22 provide a detailed physiological characterization of *S. oneidensis* MR-1 and may be important

1 for improving its metal reduction ability through rational metabolic engineering or by stimulating
2 metal reduction in the environment through addition of growth supplements and electron donors.

3 ¹³C isotopomer analysis is a powerful approach to map intracellular fluxes. By feeding a
4 ¹³C-labeled carbon source to the cells, the labeling pattern of primary metabolites, often the
5 amino acids, can be measured. Based on these isotopomer data and the biochemical network of
6 *S. oneidensis* MR-1, a metabolic pathway model can quantify the rates of intracellular reactions
7 (16). In our study, labeling patterns of amino acids were analyzed by both nuclear magnetic
8 resonance (NMR) spectroscopy and gas chromatography-mass spectrometry (GC-MS). The
9 advantage of NMR is that it provides positional information about the labels in the isotopomers
10 even though detection sensitivity is low (2). GC-MS is a more sensitive detection method and
11 determines what fraction of a particular molecule or molecular fragment contains a specific
12 number of labels (38). By combining GC-MS and NMR data, a complete picture of the
13 isotopomer distribution in amino acids can be obtained. The main goal of this study was to
14 determine the fluxes through key metabolic pathways in *S. oneidensis* MR-1 under aerobic and
15 micro-aerobic conditions. The determination of fluxes was accomplished in three steps: 1) the
16 cells were grown in defined medium with ¹³C-labeled lactate as the sole carbon source; 2) the
17 labeling patterns in key amino acids of the total protein hydrolysate were characterized using
18 both GC-MS and NMR; and 3) a flux calculation algorithm was used to quantify the central
19 metabolic pathways. Two sets of conditions were probed to determine the flux distribution,
20 carbon limitation (DO>70%) and oxygen limitation (DO<10%). The results not only widen our
21 understanding of *Shewanella* metabolism but also demonstrate a powerful approach for
22 investigating metabolic flux analysis using both GC-MS and NMR techniques.

1 **Material and methods**

2 *Culture conditions*

3 *S. oneidensis* MR-1 was purchased from American Tissue Culture Center (ATCC 70050)
4 and was stored at -80°C prior to use. All cultures used the modified MR-1 defined medium (31)
5 (supplementary materials). The lactate used was either [3-¹³C] sodium L-lactate (98%,
6 Cambridge Isotope, USA), [1-¹³C] sodium L-lactate (99%, Cambridge Isotope, USA), or a
7 mixture of 10% [¹³C₃] sodium L-lactate (99%, Cambridge Isotope, USA) and 90% unlabeled
8 sodium lactate (Fisher, USA).

9 Fermentations were carried out in a 1-L New Brunswick Bioflo 110 fermentor. The off-
10 gas composition was analyzed using a mass spectrometer (Thermo Onix). The inoculum was
11 prepared in LB medium in shake flasks overnight (optical density at a wavelength of 600 nm,
12 OD₆₀₀>1.5). Fermentations were started with a 1% inoculated volume for optimal growth
13 kinetics. After three residence times in continuous mode, the amount of LB remaining would be
14 very small (<0.05%). The reactor temperature was maintained at 30°C. The working volume in
15 the bioreactor was kept at 500 mL, and agitation was set at 300 rpm. For the carbon-limited
16 condition, 30 mM [3-¹³C] L-lactate was used, and the dilution rate was set to 0.079 hr⁻¹ in order
17 to keep the DO level over 70% during continuous culture. For the oxygen-limited condition, 50
18 mM lactate composed of 10% [¹³C₃] L-lactate and 90% unlabeled lactate was used. The dilution
19 rate was set to 0.10 hr⁻¹, and the DO was controlled below 10% during continuous culture. In
20 both experiments, the continuous culture was started after 15 hours of batch culture and
21 continued for three generations. During both continuous cultures, the medium was controlled at
22 pH ~8 and final OD₆₀₀ is around one. ¹³C-Labeled biomass was sampled after three generation
23 times for biomass composition analysis and isotopomer measurements. For shake-flask

1 experiments, cells were grown in 10 ml of three differently labeled lactate media (shaking speed
2 = 200rpm): [3-¹³C] L-lactate, [1-¹³C] L-lactate, or [¹³C₃] lactate (10% [¹³C₃] L-lactate with 90%
3 unlabeled lactate). The final concentration of lactate was 30 mM. The MR-1 inoculum was
4 prepared in labeled modified MR-1 defined medium, and 2% of the final culture volume was
5 inoculated into the same medium in shake flasks. The biomass in shake flasks was harvested in
6 the mid-exponential growth phase (OD₆₀₀ ~0.5).

7 *Analytical methods for extracellular metabolites and biomass compositions*

8 Cell growth was monitored by measuring the OD₆₀₀. The harvested culture was
9 centrifuged at 4800 × g and 4°C for 20 min and lyophilized overnight. The dried biomass was
10 weighed and used for fatty acid quantification using fatty acid methyl ester (FAME) analysis
11 (33) (Microbial ID, Newark, Delaware). The total protein concentration was determined by the
12 Bradford Protein Assay (BioRad Cat#500-0006). The concentrations of lactate, acetate,
13 pyruvate, and succinate in the medium were measured using enzyme kits (r-Biopharm,
14 Darmstadt, German), and lactate, pyruvate, and acetate were also quantified using 1D ¹H
15 presaturation NMR spectra. The relaxation delay between scans was set to 20 s, and 100 μM
16 sodium 3-trimethylsilylpropionate (TSP) added to the sample was used as the reference
17 compound for quantification. The reported results are the average of both enzymatic and NMR
18 measurements.

19 All measurement methods for biomass constituents (protein, carbohydrates, RNA, and
20 DNA) were taken from previously reported protocols (4, 15, 16). Total protein content was
21 determined using the Bradford method, carbohydrate was determined by the phenol reaction,
22 RNA was assayed through a reaction involving orcinol, and DNA was obtained through the
23 colorimetric procedure that involves the reaction of DNA with diphenylamine in a mixture of

1 perchloric acid. Glucose, pure *E. coli* RNA (Ambion #7940), and deoxyribose were used as
2 standards for the carbohydrate, RNA, and DNA measurements, respectively. Quantification of
3 amino acids in protein was performed by the Molecular Structure Facility (University of
4 California, Davis).

5 *Gas chromatography-Mass spectrometry*

6 Before measuring amino acid labeling patterns in cellular protein, a 10 mL culture was
7 harvested and centrifuged down at 8000 g. The cell pellets were washed once with 0.9% NaCl;
8 then suspended in 1 ml of sterile nanopure water and sonicate using the microtip for 3 min with a
9 3 sec. on/1 sec. off cycle. The proteins from the resulting lysate were precipitated using
10 trichloroacetic acid and washed with cold acetone two times; then hydrolyzed in 6 M HCl at
11 100°C for 24 hours. GC-MS was carried out using a gas chromatograph (DB5 column, HP6890
12 series, Agilent Inc, USA) equipped with a mass spectrometer (5973 Network, Agilent Inc, USA).
13 GC-MS samples were prepared in 100 μ l of tetrahydrofuran (THF) and 100 μ l of N-(tert-
14 butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich, USA). All samples were
15 derivatized in a water bath at 65-80°C for 1 hour. Two types of positively charged species were
16 clearly observed by MS in this study: unfragmented molecules, $[M-57]^+$, and fragmented
17 molecules that had lost one carboxyl group, $[M-159]^+$. M is the total molecular mass of the
18 derivatized hydrolysate component, and 57 indicates the loss of 57 mass units, e.g. a tert-butyl
19 group. For amino acids that contain two carboxyl groups, the loss of the α -carboxyl group is
20 strongly favored because the amine group on the β -carbon allows the formation of an
21 entropically stable fragment (6, 12). The natural abundance of isotopes, including ^{13}C (1.13%),
22 ^{18}O (0.20%), ^{29}Si (4.70%) and ^{30}Si (3.09%) (Si occurs in amino acids derivatized for gas
23 chromatography separation), change the mass isotopomer spectrum. These changes were

1 corrected using a published algorithm before using the data for calculating the label distribution
2 (14).

3 *¹³C NMR sample preparation and analysis*

4 An aliquot (50 ml) of culture was harvested by centrifuging at 5000 × g for 20 min at
5 4°C. The cell pellet was washed twice with 20 mM NaH₂PO₄ (in D₂O) (pH 7) buffer. Washed
6 pellets were resuspended in the same buffer, and the cells were disrupted by sonication. Cells
7 were sonicated for 4-5 times with 15-20 seconds each time at sonication power 3 on Misonix
8 sonicator 300 (Misonix Inc., USA). Cell debris was removed by centrifugation at 11,250 × g for
9 30 minutes at 4°C. Cellular protein in the supernatant was then hydrolyzed in 6 M HCl by
10 incubation at 95-100°C for 24 hours. The hydrolysate was filtered through a 0.22-μm-pore-size
11 filter and lyophilized. The dried material was dissolved in 700 μl of 20 mM deuterium chloride
12 (DCl) in D₂O, filtered through a 0.22-μm-pore-size filter, and used for the NMR measurements.

13 Proton-detected 2D ¹³C-¹H heteronuclear single-quantum COSY (HSQC-COSY) spectra
14 were collected with the pulse sequence and parameters as described in (5). The spectra were
15 recorded at a ¹H frequency of 600 MHz on a Bruker DRX 600 spectrometer and analyzed with
16 the software NMRPipe and NMRDraw (5). For each sample, two spectra were taken: one for the
17 aliphatic resonances with the ¹³C carrier set to 43 ppm, and the other spectrum for the aromatic
18 resonances with the ¹³C carrier set to 125 ppm. The data sizes were 3,500 × 1,024 complex
19 points. The acquisition times were $t_{1\max} = 686$ msec and $t_{2\max} = 128$ msec. The relaxation delay
20 between scans was set to 2.2 - 2.3 s, and the spectra were collected at 25°C for all 2D NMR
21 experiments. The relative distribution of the isotopomers was determined from the intensities of
22 the individual multiplet components in ¹³C-¹³C scalar coupled multiplets (30).

1 *Algorithm for flux calculation and isotopomer modeling*

2 Central biochemical pathways for *S. oneidensis* MR-1 were selected based on the
3 internet-accessible genome data base MicrobesOnline (1). The complete list of key reactions in
4 the model is given in Supplementary Material, and the reaction network is shown in Figure 1.
5 The pathway map includes the tricarboxylic acid (TCA) cycle (including the glyoxylate shunt),
6 C₁ metabolism, the Entner-Doudoroff (ED) pathway, and the pentose phosphate (PP) pathway.
7 The shaded boxes represent the biomass pool containing key amino acids for which the
8 isotopomer distributions were measured by GC-MS and NMR. There are 36 free fluxes to be
9 determined in the pathway map. The extracellular fluxes, v₁ and v₆, were directly measured
10 using enzymatic methods. An isotopomer solution algorithm was developed using MATLAB
11 6.0 (The Mathworks, Massachusetts, U.S.A.). To search for a global solution, an iterative
12 procedure, which consisted of the following steps, was used. 1) A set of initial guesses for all
13 fluxes was input to the solution algorithm. The speed of converging to a global solution depends
14 on the guess of the initial value. The starting guess of independent fluxes was coarsely based on
15 MR-1 biomass composition or previous reported fluxes for the well-known microorganism,
16 *Escherichia coli*. After each round of iteration, a set of improved guessed fluxes was found and
17 then used as a new search point. The complete fluxes in the pathway map were solved using the
18 reaction stoichiometric matrix (29). 2) Concepts of atom mapping matrices (AMM) and
19 isotopomer mapping matrices (IMM) were used in an iterative scheme to calculate the steady-
20 state isotopomer distributions in the intracellular metabolite pools, and these were transformed to
21 MS and NMR data (24, 25). The simulated MS and NMR data for isotopomer distributions of
22 proteinogenic amino acids were compared to the experimental results. 3) For each search point,
23 the local optimal solution was found using Nelder-Mead method (via the fminsearch function in

1 MATLAB). 4) A simulated annealing strategy was used to obtain an optimal global solution.
 2 First, an optimal local solution was perturbed by taking a finite amplitude step away from it, and
 3 then steps 1-4 were repeated to see if a better solution could be obtained. The global search
 4 stopped when the objective function could not be further minimized (20). The objective function
 5 is defined as:

$$6 \quad \varepsilon(v_n) = \sum_{i=1}^a \left(\frac{M_{i,m} - M_{i,c}(v_n)}{\delta_i} \right)^2 + \sum_{j=1}^b \left(\frac{N_{j,m} - N_{j,c}(v_n)}{\delta'_j} \right)^2 \quad (1)$$

7 where v_n are the unknown fluxes to be optimized in the program, $M_{i,m}$ are the measured MS data,
 8 and $M_{i,c}$ are the corresponding model simulated values. $N_{i,m}$ are the measured NMR data, and
 9 $N_{i,c}$ are the corresponding model simulated values. δ and δ' are the experimental errors for
 10 measured MS and NMR data, respectively. To save computational time, biomass fluxes were
 11 constrained based on the uncertainty of the measurement. The confidence intervals of the
 12 calculated fluxes can be estimated by Monte Carlo methods (20): 1) the measured data were
 13 perturbed randomly within measurement noise; 2) the optimization routine described above was
 14 used to estimate the new flux distribution after each perturbation; 3) after testing over 100
 15 simulated measured data sets, the error bounds on the flux distributions resulting from errors in
 16 all measurements were obtained.

17 All reactions could be potentially reversible and make the system highly underdetermined
 18 (29). Several reactions (v_4 , v_5 , v_{11} , v_8 v_{12} , and v_{21}) were considered to be reversible because
 19 they have the most significant impact on the isotopomer distribution (2, 43). The reversible
 20 reactions are characterized by their net flux, v_i , and their exchange flux, v_i^{exch} . The net flux is
 21 defined as the difference between forward and backward fluxes. The exchange flux, v_i^{exch} , is the

1 smaller of the forward and backward fluxes. Equation 2 rescales v_i^{exch} (possible value range [0,
2 ∞]) to exchange coefficients, $exch_i$, which has a finite range [0, 1] (39):

$$3 \quad v_i^{\text{exch}} = \beta \frac{exch_i}{1 - exch_i} \quad (2)$$

4 where β is a constant of the order of magnitude of v_i . In our study, a value of one was assigned
5 to β . Exchange coefficients are limited to values between 0 and 0.95 in order to improve the
6 speed of convergence.

7 *Sensitivity test of isotopomer model*

8 The flux calculation is based on tracing the path of ^{13}C from labeled carbon substrate to
9 metabolites in the pathway network. Singly-labeled or fully-labeled ^{13}C substrate (often 10-20%)
10 can be used for tracer experiments in flux analysis (8). Although the labeling pattern of substrate
11 should not affect the actual flux distributions, it may affect the sensitivity of isotope data to
12 model calculations (2, 40). Some studies have shown that the ED and PP pathways are
13 particularly well resolved using singly-labeled carbon substrate, whereas the fully-labeled carbon
14 substrate is ideal for reactions in the TCA cycle because information from ^{13}C - ^{13}C connectivity
15 can be obtained (9). Other studies have shown that the use of a mixed labeling pattern
16 (containing certain percentages of unlabeled, fully-labeled, and doubly-labeled substrate) may be
17 the most useful to ascertain metabolic fluxes (2). However, most studies on sensitivity of
18 isotopomer distributions have used glucose as the carbon source and focused on GC-MS as the
19 only measurement technique. To avoid potential bias in calculated fluxes, our study utilized two
20 different labeling strategies: 10% fully-labeled lactate for the oxygen-limited chemostat and 98%
21 singly-labeled lactate for the carbon-limited chemostat. To minimize the cost of labeled lactate

1 for fermentations, these two types of labeled lactate medium were also used in shake-flask
2 cultures to provide an additional comparison for sensitivity analysis.

3 After obtaining global solutions for flux distributions of cells grown under different culture
4 conditions, a sensitivity test is necessary to check the reliability of the model results and to
5 estimate the confidence interval of the calculated fluxes. The sensitivity coefficient, which
6 reflects the sensitivity of mass distribution upon changes in fluxes and exchange coefficients, is
7 defined as:

$$8 \quad S_{i,j} = \frac{\partial I_i}{\partial v_j} \approx \frac{I_i' - I_i}{v_j' - v_j} \quad (3)$$

9 where I_i is the isotopomer data for amino acid (i) and v_j is the flux or exchange coefficient (j) (2).
10 Since the analytical expression is difficult to derive, an approximation has to be made. After the
11 global solution is found with the best fitted isotopomer data, I_i , the optimized flux distribution, v_j ,
12 is then perturbed with a small change in its independent fluxes and exchange coefficients. The
13 resulting new flux distribution (v_j') predicts a new set of isotopomer data (I_i'), and Equation 3 can
14 be applied to estimate the sensitivity of the model.

15 **Results**

16 *Aerobic chemostat and shake flask cultivations*

17 Continuous cultivation was performed under two conditions: carbon limitation (30 mM
18 lactate, DO>70%) and oxygen limitation (50 mM lactate, DO<10%). Cell growth, lactate
19 consumption, and acetate production were measured during the cultivation (Figure 2). During
20 the batch phases of both conditions, some of the lactate was converted to acetate. Under the
21 carbon-limited condition with a low growth rate ($D=0.079 \text{ hr}^{-1}$), neither lactate nor acetate was
22 detected in the effluent of the continuous culture; while under the oxygen-limited condition with

1 a higher growth rate ($D=0.10 \text{ hr}^{-1}$), 17.5 mM acetate was detected in the effluent of the
2 continuous culture. The CO_2 concentration in the off-gas remained constant after two
3 generations. A similar final OD_{600} and biomass concentration were the basis for our comparison
4 between these two chemostat cultures (Table 1). In parallel with the continuous culture, *S.*
5 *oneidensis* MR-1 was grown in shake flasks with labeled lactate. The profiles of growth, lactate
6 consumption, and acetate production were similar to those of the batch phase of chemostat
7 cultures (data not shown). The observed doubling time in defined medium was approximately 4
8 hrs, which was equivalent to a growth rate of 0.17 hr^{-1} . The results also reflect the growth
9 kinetics of *S. oneidensis* MR-1: a high growth rate and low dissolved oxygen level enhanced
10 acetate production under aerobic conditions (32).

11 *Biomass composition analysis*

12 Analysis of biomass composition not only helps understand the biosynthetic kinetics, but
13 also provides the initial guesses for some intracellular fluxes in our calculation algorithm. The
14 biomass compositions of *S. oneidensis* MR-1 grown under both shake flasks and chemostat
15 conditions are similar to those of *E. coli*, even though *E. coli* was grown on a different carbon
16 source under different growth conditions (Table 2). The RNA and DNA fractions of the biomass
17 from the shake-flask cultures were relatively similar to those observed in the chemostat cultures,
18 whereas the fatty acid fraction of the shake-flask cultures was much less than that of the
19 chemostat cultures. The undetermined weight fraction was probably soluble metabolites,
20 residual salts, and bound water that was not completely removed from the biomass by
21 lyophilization. FAME analysis showed that the fatty acid profile was dominated by even-
22 numbered fatty acids (Supplementary Table 1). The amino acid mole fractions from cellular
23 protein were very similar in the two chemostat cultures and the shake-flask cultures

1 (Supplementary Figure 1). This may indicate that even though specific proteins are different
2 under the two conditions, the amino acid fractions in total proteins do not change under those
3 growth conditions.

4 *Isotopomer distribution profiles*

5 Although NMR and MS analyses have both been used successfully in previous work, the
6 latter was thought to be high-throughput and more sensitive ($\leq 2\%$ errors) with comparatively
7 low cost (22). Application of GC-MS to separate the derivatized protein hydrolysate gave
8 chromatographic peaks of 15 proteinogenic amino acids (arginine, asparagine, cysteine,
9 glutamine, and tryptophan could not be determined). The possible alternative routes for leucine
10 and isoleucine synthesis suggested by the MR-1 genome information are complicated, and both
11 amino acids' MS peak $[M-57]^+$ were overlapped by other signals, so their isotopomer
12 distributions were not considered in the model calculation. Consistent with assumed amino acid
13 biosynthesis pathways, several amino acid pairs derived from the same precursor, such as proline
14 and glutamate (from precursor oxoglutarate), threonine and aspartate (from precursor
15 oxaloacetate), tyrosine and phenylalanine (from precursors phosphoenolpyruvate and erythrose-
16 4-phosphate), had similar isotopomer patterns from both MS and NMR measurements (29). This
17 redundant isotopomer information could be utilized to estimate the experimental errors (8).

18 Flux analysis requires the pools of intracellular metabolites to be in isotopomeric steady
19 state. Although flux analysis is best studied in physiological steady state by continuous
20 bioreactor culture, many studies have shown that a (quasi) steady state can also be achieved
21 during the exponential growth phase (or even at stationary phase) in batch culture (7, 23, 28). As
22 a convenient and less expensive approach to test the reproducibility of isotopomer distribution
23 determinations, biomass was also cultured in 10-mL shake flasks using the same medium as the

1 chemostat cultures. The isotopomer distributions of key amino acids from shake-flask and
2 chemostat cultures had relatively similar profiles with less than 10% difference (fragment [M-
3 57]⁺ in Table 3 and fragment [M-159]⁺ in Supplementary Table 2). There were larger
4 differences (10-15%) in the isotopomer ratios of the mass fragments of phenylalanine between
5 the carbon-limited chemostat and shake-flask cultures.

6 The ¹³C abundance for individual carbon positions is helpful to identify reactions in the
7 metabolic network and to provide additional constraints for the isotopomer model. In this study,
8 the labeling patterns of the key amino acids in the hydrolysate were analyzed by 2D [¹H-¹³C]
9 COSY NMR. In the indirectly detected ¹³C dimension, multiplets arise from scalar spin-spin
10 coupling between directly linked ¹³C carbon spins. For the α-carbon of a fractionally labeled
11 amino acid, up to four different patterns of multiplets (isotopomers) may be observed. Based on
12 the ratios of peak intensities, the relative populations of isotopomers can be determined (29).
13 However, because of some overlap in multiplet peaks and signals arising only from the natural
14 abundance ¹³C, estimations of the isotopomer distributions were not always unique. Therefore,
15 model calculations only considered the most reliable NMR data of nine amino acids for the
16 isotopomer model analysis, mainly from α and β carbons (Table 4).

17 *Flux calculation and model reliability test*

18 The isotopomer model provided flux distributions for both chemostat cultures and shake-
19 flask cultures. The lactate carbon flux (taken to be 100%) is split into two branches at pyruvate:
20 one flows towards the TCA cycle and the other flows towards the PP and ED pathways (Figure
21 3). For the carbon-limited condition, 61% of the substrate entered the TCA cycle via acetyl-CoA
22 condensing with oxaloacetate. For the micro-aerobic condition, the TCA cycle was weakened,
23 as the relative carbon flux through the TCA cycle dropped to 47%; approximately 35% of the

1 lactate was converted into acetate. Additionally, the calculations indicate that some fluxes might
2 be highly reversible, such as the serine metabolism route. The reversibility of the serine
3 metabolism route could alter the mass distribution of serine and glycine significantly. Although
4 all the reactions in the PP pathway were thought to be reversible, the total fluxes through the PP
5 reactions were small (< 4% of the lactate uptake); thus, the reversibility could be neglected in
6 the model in order to reduce computation time (43). Studies have shown that ¹³C tracer
7 experiments only give crude estimates of some exchange rates (often within one order of
8 magnitude); thus, there is no need to consider the reversibility of every reaction (25, 41).

9 The reliability of model results was checked using sensitivity coefficients, which are a
10 similar concept to the model objective function (Equation 1) (43). By making small changes to
11 specific optimized fluxes, the resulting new fluxes predicted the changes in isotopomer
12 distributions of specific amino acids. For example, by increasing or decreasing the pyruvate-to-
13 malate flux, the model predicted the change in isotopomer distributions of Asp and Glu. The
14 absolute value of the sensitivity coefficients was evaluated, and then the sum of their squares was
15 determined (Table 5). As for 98% 3rd position labeled lactate, the model was sensitive to both
16 GC-MS and NMR data based on the sum of the squared sensitivity coefficients (GC-MS:
17 0.00077 and NMR: 0.00082). On the other hand, the sum of the squared sensitivity coefficients
18 using 10% fully-labeled lactate (GC-MS: 0.00007) was one order-of-magnitude lower than that
19 using the singly-labeled lactate. This indicated that using GC-MS data from 10% fully-labeled
20 lactate experiments might produce biased results. However, the model was very sensitive to
21 NMR data when 10% labeled lactate was used. Coupling NMR analysis with GC-MS could
22 significantly improve the resolution of model calculations when a low fraction of fully-labeled
23 substrate is used.

1 In general, the predicted amino acid isotopomer distributions matched reasonably well
2 with both experimental GC-MS and NMR data (Tables 3 and 4 and Supplementary Table 2),
3 which indicated the accuracy of the model calculation. Besides the instrument errors (GC-MS
4 error: 1-2% and NMR error: 4-5%), differences between modeled and measured isotopomer data
5 could arise from other possible sources: 1) measurement errors of the extracellular fluxes, 2)
6 background noise from the 1.13% natural abundance ^{13}C , and 3) simplification of the model by
7 neglecting the reversibility of less influential pathways. All the above sources complicate the
8 error estimation. An in-depth analysis of the discrepancy between the model fitting and the
9 experimental data is beyond the scope of this paper.

10 **Discussion**

11 *The TCA cycle and serine oxidation pathway (energy production pathways)*

12 The TCA cycle was the main carbon metabolism route (fluxes >60% of lactate uptake)
13 and the flux toward a reversible serine oxidation pathway (PEP to serine to glycine to C1) was
14 almost 10% of lactate uptake under carbon-limited conditions. Serine metabolism has often been
15 shown to be reversible in other bacteria (24). But the flux through this pathway (PEP→serine) in
16 MR-1 was much higher compared to the same pathway in *E. coli*, where the flux is 0.9-3.5% of
17 total carbon utilization (43). High flux through serine metabolism suggested that MR-1 is able to
18 oxidize excess C1. In C1 metabolism, one ATP and one NADPH are produced when serine is
19 converted to formate via 5,10-Me-THF; an additional NADH is generated when the formate is
20 completely oxidized to CO_2 (10). There are two additional pieces of evidence to support the
21 serine oxidation route. First, high level of formate dehydrogenase (0.079 $\mu\text{mol}/\text{min}/\text{mg}$ protein)
22 has been reported for MR-1 under aerobic conditions (26). This enzyme is present in C1
23 oxidation route (Figure 4). Second, MR-1 can utilize glycine or serine as the sole carbon source

1 when grown in defined medium under aerobic condition (unpublished data). It would be
2 advantageous for the cell to utilize the serine oxidation pathway to obtain energy (ATP, NADPH,
3 and NADH). The same serine oxidation pathway has also been proposed in *Alteromonas*
4 *putrefaciens* NCMB 1735 (21).

5 Under oxygen-limited conditions, the flux through the TCA cycle was reduced (<50%).
6 Scott and Nealson proposed the existence of the serine pathway for MR-1 under anaerobic
7 conditions (26). This view was established based on high levels of hydroxypyruvate reductase
8 under anaerobic conditions. The fixation of carbon was postulated at the level of formaldehyde,
9 which combines with glycine to yield serine (Figure 4). Under carbon-limited conditions, the
10 labeling pattern of the α -carbon of serine is same as that of phenylalanine. This observation
11 suggested that the α -carbon of serine was derived from the precursor, phosphoenolpyruvate;
12 however, under micro-aerobic conditions, the labeling pattern of the α -carbon of serine is
13 different from that of phenylalanine and thus the calculated flux distribution showed another
14 reversible route to produce glycine and serine (glyoxylate \rightarrow glycine $\leftarrow \rightarrow$ serine). This proposed
15 pathway is consistent with the reported serine-glyoxylate aminotransferase activity when oxygen
16 is limited (26, 27). However, no significant net flux through the serine pathway (serine \rightarrow PEP
17 \rightarrow TCA cycle) was evident in oxygen-limited conditions based on the isotopomer model results.

18 *The Pentose Phosphate, Entner-Doudoroff, Embden-Meyerhoff-Parnas, and Gluconeogenesis*
19 *pathways*

20 For *E. coli* grown on glucose, the PP pathway flux was over 20% of total carbon uptake
21 and was utilized mainly for production of reducing equivalents (NADPH) and macromolecule
22 precursors (43). Grown on lactate, the PP pathway flux of MR-1 was very low and only for
23 biomass production. Comparing the two chemostat cultures, the average fluxes toward the ED

1 and PP pathways were higher under carbon-limited conditions than under oxygen-limited
2 conditions, because more lactate was used for biomass production under carbon-limited
3 conditions (no acetate production). The ED pathway flux was present in MR-1, consistent with
4 the presence of the active ED pathway enzyme, 2-keto-3-deoxygluconate aldolase, under aerobic
5 conditions (26). A few bacteria, including *Rhodobacter sphaeroides*, *Sinorhizobium meliloti*,
6 and *Agrobacterium tumefaciens*, have been shown to substitute the ED pathway for the common
7 EMP pathway (10, 19). These organisms usually lack two essential EMP enzymes, 6-
8 phosphofructokinase and 1,6-biphosphofructoaldolase, which preclude them from using the EMP
9 pathway. MR-1 does not contain phosphofructokinase but appears to contain 1,6-
10 bisphosphofructo-aldolase and fructose-1,6-bisphosphatase (13, 26), which would allow it to
11 synthesize glucose-6-phosphate using gluconeogenesis. As the Gibbs free energy of reaction
12 suggests that the reaction of glucose-6-phosphate to 6-phosphogluconate is unidirectional (39),
13 the reverse EMP pathway instead of the ED pathway is the only possible route to synthesize the
14 carbohydrate precursor, glucose-6-phosphate.

15 *Futile cycles*

16 Two anapleurotic reactions appeared to be present (pyruvate to malate catalyzed by
17 malate dehydrogenase and oxaloacetate to phosphoenolpyruvate catalyzed by
18 phosphoenolpyruvate carboxykinase) and formed a futile cycle. In a previous study, malate
19 dehydrogenase and phosphoenolpyruvate carboxylase of MR-1 were shown to be active under
20 aerobic conditions (26). In this study, the pyruvate-to-malate flux was around 13% of the lactate
21 uptake under the carbon-limited condition and less than half this value under the oxygen-limited
22 condition. A similar change in flux was also observed in the oxaloacetate to
23 phosphoenolpyruvate reaction under the two chemostat conditions.

1 Highly coupled to the anapleurotic reactions (via malate) is the glyoxylate shunt. The
2 flux through the glyoxylate shunt was below 4% of the lactate uptake rate under both chemostat
3 conditions. This finding correlated with the reported lower level (0.009 $\mu\text{mol}/\text{min}/\text{mg}$ protein) of
4 isocitrate lyase activity compared to other TCA cycle-related enzymes (26). The glyoxylate
5 shunt is necessary for synthesizing TCA cycle intermediates, such as succinate and malate, and is
6 also an important step for the serine pathway (isocitrate to glyoxylate to glycine) proposed for
7 MR-1 under oxygen-limited conditions.

8 There appears to be a futile cycle involving the reactions pyruvate \rightarrow malate, malate \rightarrow
9 oxaloacetate, and oxaloacetate \rightarrow phosphoenolpyruvate. It is not clear why the cell would
10 choose to route flux through this circuitous pathway than directly through the reaction pyruvate
11 \rightarrow phosphoenolpyruvate. These pathways might help to increase the flexibility in central carbon
12 metabolism, to allow MR-1 to utilize different electron acceptors, or to maintain stability in
13 central metabolism under environmental stresses (3, 34).

14 *Flux ratios analysis and verification of model results*

15 From GC-MS data (Table 3), the isotopomer distributions of key amino acids obtained
16 from shake-flask cultures were relatively similar to those from chemostat cultures. However,
17 based on the isotopomer data from the shake-flask cultures (using 3rd position labeled lactate),
18 the fluxes through the TCA cycle and the reactions that transform acetyl-CoA to acetate were
19 calculated to be 48% and 19% of lactate consumption, respectively. These values were very
20 different from those obtained from either the carbon-limited or oxygen-limited chemostat
21 cultures. As the shake-flask culture is non-steady state, the oxygen concentration changes from
22 fully aerobic to micro-aerobic (31). It is known that the relative flux ratios rather than the
23 absolute fluxes in key pathways determine the isotopomer distribution (23). The metabolic flux

1 ratios of key pathways were analyzed to reveal the similarity in the flux distribution of the
2 central metabolism under the two chemostat and shake-flask conditions (Figure 5). Although
3 acetate production, growth rate, and most intracellular fluxes were very different under these
4 three conditions, many flux ratios in the TCA cycle and futile cycles did not vary significantly
5 (the difference in the ratios between carbon-limited chemostat and shake-flask culture was below
6 5%). The same invariability in the flux ratios was also found in *Bacillus subtilis* and *E. coli* (7,
7 23). This observation suggests that central metabolism in some microorganisms is under specific
8 regulation and is robust to environmental changes (28). Even though the shake-flask culture
9 conditions were not identical to those of the chemostat cultures, the robust nature of bacterial
10 metabolism helps maintain its relative flux ratios. This supports the idea that shake-flask
11 cultures may sometimes substitute for continuous culture for metabolic flux analysis, at least to
12 obtain a reliable measurement of central metabolic flux ratios (23).

13 As our study showed that the isotopomer distributions in *S. oneidensis* MR-1 were not
14 sensitive to culture methods in the aerobic condition, the isotopomer distribution from shake
15 flasks with different labeled lactate substrates is an efficient and reliable approach to check the
16 accuracy of the flux estimations from chemostat cultures. First, using 99% [1-¹³C] L-lactate as
17 the carbon source, the model used the flux distribution from the carbon-limited conditions to
18 predict the isotopomer distribution of six key amino acids. The model prediction was then
19 compared with the experimental isotopomer distribution obtained from the culture grown
20 aerobically in shake flasks containing 30 mM 99% [1-¹³C] L-lactate. The accuracy of the flux
21 estimation was validated since the experimental isotopomer data was consistent with the model
22 predictions (Table 6).

1 GC-MS and NMR measurements of isotopomer distributions in proteinogenic amino
2 acids, the annotated genome, and mathematical algorithms enabled us to develop a metabolic
3 pathway model to quantify the intracellular fluxes of the central metabolic pathways. The results
4 revealed a general metabolic flux distribution under both carbon-limited and oxygen-limited
5 conditions. Our study also identified several active pathways, including a potential futile cycle,
6 the ED pathway, the serine oxidation pathway, and activity of serine-glyoxylate
7 aminotransferase (micro-aerobic condition). Furthermore, this research demonstrates successful
8 applications of NMR and GC-MS for metabolic flux analysis, particularly where low fractions of
9 fully labeled substrates were used.

10

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1 References

- 2 1. **Alm, E. J., K. H. Huang, M. N. Price, R. P. Koche, K. Keller, I. L. Dubchak, and A.**
3 **P. Arkin.** 2005. The MicrobesOnline Web site for comparative genomics. *Genome Res.*
4 **15:**1015-1022.
- 5 2. **Arauzo-Bravo, M. J., and K. Shimizu.** 2003. An improved method for statistical
6 analysis of metabolic flux analysis using isotopomer mapping matrices with analytical
7 expressions. *Journal of Biotechnology* **105:**117-133.
- 8 3. **Christiansen, T., B. Christensen, and J. Nielsen.** 2002. Metabolic network analysis of
9 *Bacillus clausii* on minimal and semirich medium using ¹³C-Labeled glucose. *Metabolic*
10 *Engineering:*159-169.
- 11 4. **Daniels, L., R. S. Hanson, and et al.** 1994. Chemical Analysis, p. 512-554. *In* P.
12 Gerhardt, R. Murray, W. Wood, and N. Krieff (ed.), *Method for General and Molecular*
13 *Bacteriology.* American Society of Microbiology, Washington DC.
- 14 5. **Delaglio, F., S. Grzesiek, G. Vuister, G. Zhu, J. Pfeifer, and A. Bax.** 1995. NMR pipe:
15 a multidimensional spectral processing system based on UNIX pipes. *Journal of*
16 *Biomolecular NMR* **6:**277-293.
- 17 6. **Dookeran, N. N., T. Yalcin, and A. G. Harrison.** 1996. Fragmentation reactions of
18 protonated α -amino acids. *Journal of Mass Spectrometry* **31:**500-508.
- 19 7. **Fischer, E., and U. Sauer.** 2005. Large-scale *in vivo* flux analysis shows rigidity and
20 suboptimal performance of *Bacillus subtilis* metabolism. *Nature Genetics* **37:**636-640.
- 21 8. **Fischer, E., and U. Sauer.** 2003. Metabolic flux profiling of *Escherichia coli* mutants in
22 central carbon metabolism using GC-MS. *Euro. J. Biochem* **270:**880-891.
- 23 9. **Fischer, E., N. Zamboni, and U. Sauer.** 2004. High-throughput metabolic flux analysis
24 based on gas chromatography-mass spectrometry derived ¹³C constraints. *Analytical*
25 *Biochemistry* **325:**308-316.
- 26 10. **Fuhrer, T., E. Fischer, and U. Sauer.** 2005. Experimental identification and
27 quantification of glucose metabolism in seven bacterial species. *Journal of Bacteriology*
28 **187:**1581-1590.
- 29 11. **Ghosal, D., M. Omelchenko, E. Gaidamakova, V. Matrosova, A. Vasilenko, A.**
30 **Venkateswaran, M. Zhai, H. Kostandarithes, H. Brim, K. Makarova, L. Wackett, J.**
31 **Fredrickson, and M. Daly.** 2005. How radiation kills cells: survival of *Deinococcus*
32 *radiodurans* and *Shewanella oneidensis* under oxidative stress. *FEMS Microbiology*
33 *Reviews* **29:**361-375.
- 34 12. **Harrison, A. G.** 2001. Ion chemistry of protonated glutamic acid derivatives.
35 *International Journal of Mass Spectrometry* **210/211:**361-370.
- 36 13. **Heidelberg, J. F., I. T. Paulsen, I. T. Nelson, E. J. Gaidos, W. C. Nelson, T. D. Read,**
37 **J. A. Eisen, R. Seshadri, N. Ward, B. Methe, R. A. Clayton, T. Meyer, A. Tsapin, J.**
38 **Scott, M. Beanan, L. Brinkac, S. Daugherty, R. T. DeBoy, R. J. Dodson, A. S.**
39 **Durkin, D. H. Haft, J. F. Kolonay, R. Madupu, J. D. Peterson, L. A. Umayam, O.**
40 **White, A. M. Wolf, J. Vamathevan, J. Weidman, M. Impraim, K. Lee, K. Berry, C.**
41 **Lee, J. Mueller, H. Khouri, J. Gill, T. R. Utterback, L. A. McDonald, T. V.**
42 **Feldblyum, H. O. Smith, J. C. Venter, K. H. Nealson, and C. M. Fraser.** 2002.
43 Genome sequence of the dissimilatory metal-ion reducing bacterium *Shewanella*
44 *oneidensis*. *Nature Biotechnology* **20:**1118-1123.

- 1 14. **Hellerstein, M. K., and R. A. Neese.** 1999. Mass isotopomer distribution analysis at
2 eight years: theoretical, analytic, and experimental considerations. *American Journal of*
3 *Physiology-Endocrinology and Metabolism* **276**:E1146-E1170.
- 4 15. **Herbert, D., P. J. Phipps, and e. al.** 1971. Chemical Analysis of Microbial Cells, p.
5 210-344. In J. R. Norris and D. W. Ribbons (ed.), *Methods in Microbiology*, vol. 5B.
6 Academic Press, Inc., New York.
- 7 16. **Marshal, J.** 2004. Production of secondary metabolites from acetyl Co-A precursors in
8 bacterial and fungal hosts. PhD thesis. University of California, Berkeley.
- 9 17. **Middleton, S. S., R. B. Latmani, M. R. Mackey, M. H. Ellisman, B. M. Tebo, and C.**
10 **S. Criddle.** 2003. Cometabolism of Cr(VI) by *Shewanella oneidensis* MR-1 produces
11 cell-associated reduced chromium and inhibits growth. *Biotechnology and*
12 *Bioengineering* **83**:627-637.
- 13 18. **Neal, A. L., K. Lowe, T. L. Daulton, J. Jones-Meehan, and B. J. Little.** 2002.
14 Oxidation state of chromium associated with cell surfaces of *shewanella oneidensis*
15 during chromate reduction. *Applied Surface Science* **202**:150-159.
- 16 19. **Nicklin, J., K. Graeme-Cook, T. Paget, and R. A. Killington.** 1999. *Instant Notes in*
17 *Microbiology*. Bios Scientific Publishers, London, UK.
- 18 20. **Press, W. H., S. A. Teukolsky, W. T. Vetterling, and B. P. Flannery.** 1992. *Numerical*
19 *Recipes in FORTRAN*, 2nd ed. Cambridge University Press, Cambridge.
- 20 21. **Ringo, E. E., E. Stenberg, and A. R. Strom.** 1984. Amino acid and lactate catabolism in
21 trimethylamine oxide respiration of *Alteromonas putrefaciens* NCMB 1735. *Applied and*
22 *Environmental Microbiology* **47**:1084-1089.
- 23 22. **Sauer, U.** 2004. High-throughput phenomics: experimental methods for mapping
24 fluxomes. *Current Opinion in Biotechnology* **15**:58-63.
- 25 23. **Sauer, U., D. R. Lasko, J. Fiaux, M. Hochuli, R. Glaser, T. Szyperski, K. Wuthrich,**
26 **and J. E. Bailey.** 1999. Metabolic flux ratio analysis of genetic and environmental
27 modulations of *Escherichia coli* central carbon metabolism. *Journal of Bacteriology*
28 **181**:6679-6688.
- 29 24. **Schmidt, K., J. Nielsen, and J. Villadsen.** 1999. Quantitative analysis of metabolic
30 fluxes in *Escherichia coli*, using two-dimensional NMR spectroscopy and complete
31 isotopomer models. *Journal of Biotechnology* **71**:175-190.
- 32 25. **Schmidt, K., L. C. Norregaard, B. Pedersen, A. Meissner, and J. Q. Nielsen.** 1999.
33 Quantification of intracellular metabolic fluxes from fractional enrichment and ¹³C-¹³C
34 coupling constraints on the isotopomer distribution in labeled biomass components.
35 *Metabolic Engineering* **1**:166-179.
- 36 26. **Scott, J. H., and K. H. Neelson.** 1994. A Biochemical Study of the Intermediary Carbon
37 Metabolism of *Shewanella putrefaciens*. *Journal of Bacteriology* **176**:3408-3411.
- 38 27. **Serres, M. H., and M. Riley.** 2006. Genomic Analysis of Carbon Source Metabolism of
39 *Shewanella oneidensis* MR-1: Predictions versus Experiments. *Journal of Bacteriology*
40 **188**:4601-4609.
- 41 28. **Stelling, J., U. Sauer, Z. Szallasi, F. Doyle, and J. Doyle.** 2004. Robustness of cellular
42 functions. *Cell* **118**:675-685.
- 43 29. **Stephanopoulos, G. N., A. A. Aristidou, and J. Nielsen.** 1998. *Metabolic Engineering*
44 *Principles and Methodologies*. Academic Press, San Diego.

- 1 30. **Szyperski, T.** 1995. Biosynthetically directed fractional ¹³C labeling of proteinogenic
2 amino acids: an efficient analytical tool to investigate intermediary metabolism. *Euro J*
3 *Biochem* **232**:433-448.
- 4 31. **Tang, Y. J., D. Laidlaw, K. Gani, and J. D. Keasling.** 2006. Evaluation of the effects of
5 various culture conditions on Cr(VI) reduction by *Shewanella oneidensis* MR-1 in a
6 novel high-throughput mini-bioreactor. *Biotechnology and Bioengineering* **95**:176-184.
- 7 32. **Tang, Y. J., A. L. Meadows, and J. D. Keasling.** 2006. A kinetic model describing
8 *Shewanella oneidensis* MR-1 growth, substrate consumption, and product secretion.
9 *Biotechnology and Bioengineering* **In press**.
- 10 33. **Teece, M. A., M. L. Fogel, M. E. Dollhopf, and K. H. Nealson.** 1999. Iostopic fraction
11 associated with biosynthesis of fatty acids by a marine bacterium under oxic and anoxic
12 conditions. *Organic Geochemistry* **30**:1571-1579.
- 13 34. **Tiedje, J.** 2002. *Shewanella*- the environmentally versatile genome. *Nature*
14 *Biotechnology* **20**:1093-1094.
- 15 35. **Venkateswaran, K., D. P. Moser, M. E. Dollhopf, D. P. Lies, D. A. Saffarini, B. J.**
16 **MacGregor, D. B. Ringelberg, D. C. White, M. Nishijima, H. Sano, J. Burghardt, E.**
17 **Stackebrandt, and K. H. Nealson.** 1999. Polyphasic taxonomy of the genus *Shewanella*
18 and description of *Shewanella oneidensis* sp. *International Journal of Systematic*
19 *Bacteriology* **49**:705-724.
- 20 36. **Viamajala, S., B. M. Peyton, W. A. Apel, and J. N. Petersen.** 2002. Chromate/nitrite
21 interactions in *Shewanella oneidensis* MR-1: Evidence for multiple hexavalent chromium
22 [Cr(VI)] reduction mechanisms dependent on physiological growth conditions.
23 *Biotechnology and bioengineering* **78**:770-778.
- 24 37. **Viamajala, S., B. M. Peyton, R. K. Sani, W. A. Apel, and J. N. Petersen.** 2004. Toxic
25 effect of chromium(VI) on anaerobic and aerobic growth of *Shewanella oneidensis* MR-
26 1. *Biotechnology Progress* **20**:87-95.
- 27 38. **Wiechert, W.** 2001. ¹³C Metabolic Flux Analysis. *Metabolic Engineering* **3**:195-206.
- 28 39. **Wiechert, W., and A. A. de Graaf.** 1997. Bidirectional reaction steps in metabolic
29 networks I. Modeling and simulation of carbon isotope labeling experiments.
30 *Biotechnology and Bioengineering* **55**:101-117.
- 31 40. **Wiechert, W., M. Mollney, S. Petersen, and A. A. de Graaf.** 2001. A universal
32 framework for ¹³C metabolic flux analysis. *Metabolic Engineering* **3**:265-283.
- 33 41. **Wiechert, W., C. Siefke, A. A. de Graaf, and A. Marx.** 1997. Bidirectional reaction
34 steps in metabolic networks: II. Flux estimation and statistical analysis. *Biotechnology*
35 *and Bioengineering* **55**:118-135.
- 36 42. **Zhang, C. L., Y. Li, Q. Ye, J. Fong, A. D. Peacock, E. Blunt, J. Fang, D. R. Lovley,**
37 **and D. C. White.** 2003. Carbon isotope signatures of fatty acids in *Geobacter*
38 *metallireducens* and *Shewanella algae*. *Chemical Geology* **195**:17-28.
- 39 43. **Zhao, J., and K. Shimizu.** 2003. Metabolic flux analysis of *Escherichia coli* K12 grown
40 on ¹³C-labeled acetate and glucose using GC-MS and powerful flux calculation method.
41 *Journal of Biotechnology* **101**:101-117.

1 **Figures Captions**

2 **Figure 1.** Pathways of lactate metabolism in *S. oneidensis* MR-1. The shaded boxes represent
3 biomass. The dashed arrow (reaction 21e) is not present in the annotated genome sequence. The
4 amino acids used for isotopomer models are in parenthesis. Numbers represent the reactions
5 included in the model (corresponding to the reactions listed in the Supplementary Material).
6 Abbreviations: 6PG, 6-phosphogluconate; ACoA, acetyl-coenzyme A; C1, 5,10-Me-THF; C5P,
7 ribose-5-phosphate (or ribulose-5-phosphate or xylulose-5-phosphate); CIT, citrate; E4P,
8 erythrose-4-phosphate; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; ICT, isocitrate;
9 MAL, malate; OAA, oxaloacetate; OXO, 2-oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-
10 phosphoglycerate; PYR, pyruvate. S7P, sedoheptulose-7-phosphate; SUC, succinate; T3P, triose-
11 3-phosphate;

12
13 **Figure 2.** Time courses of biomass growth (◆), lactate concentration (■), and acetate secretion
14 (▲) in chemostat experiments under carbon-limited conditions (A) and oxygen-limited
15 conditions (B). All data points have error bars representing the errors in the measurement; some
16 error bars are not visible due to the very small measurement errors.

17
18 **Figure 3.** (A) *In vivo* flux distribution in the central metabolism of *S. oneidensis* MR-1 under
19 carbon-limited (upper number), shake-flask (middle number, in parentheses), and oxygen-limited
20 (lower number) conditions. The abbreviations are the same as used in Figure 1. (B) The
21 exchange coefficients for significant reversible fluxes estimated in carbon-limited (upper
22 number) and oxygen-limited (lower number) chemostat cultures.

23

1 **Figure 4.** The proposed serine pathway under anaerobic conditions is shown by the solid
2 arrows. The direction of the serine oxidation pathway under aerobic conditions is shown by the
3 dashed arrows.

4

5 **Figure 5.** Relative flux ratios in the central metabolic pathways under carbon-limited chemostat,
6 oxygen-limited chemostat, and shake-flask cultures. The flux ratio represents the relative
7 relationships between key metabolic routes. Acetate production, v_6/v_1 ; serine-glyoxylate
8 aminotransferase, v_{21e}/v_{12} ; malate synthase/TCA, v_8/v_{14} ; serine metabolism/glycolysis,
9 $(v_{21}+v_{21e})/v_3$; glyoxylate shunt/TCA, v_{15}/v_{10} ; phosphoenolpyruvate synthase/TCA, v_4/v_{14} ;
10 ED pathway/glycolysis, v_{28}/v_{22} . Empty bars, oxygen-limited; stippled bars, carbon-limited;
11 filled bars, shake flasks.

12

13

14

Figure 2

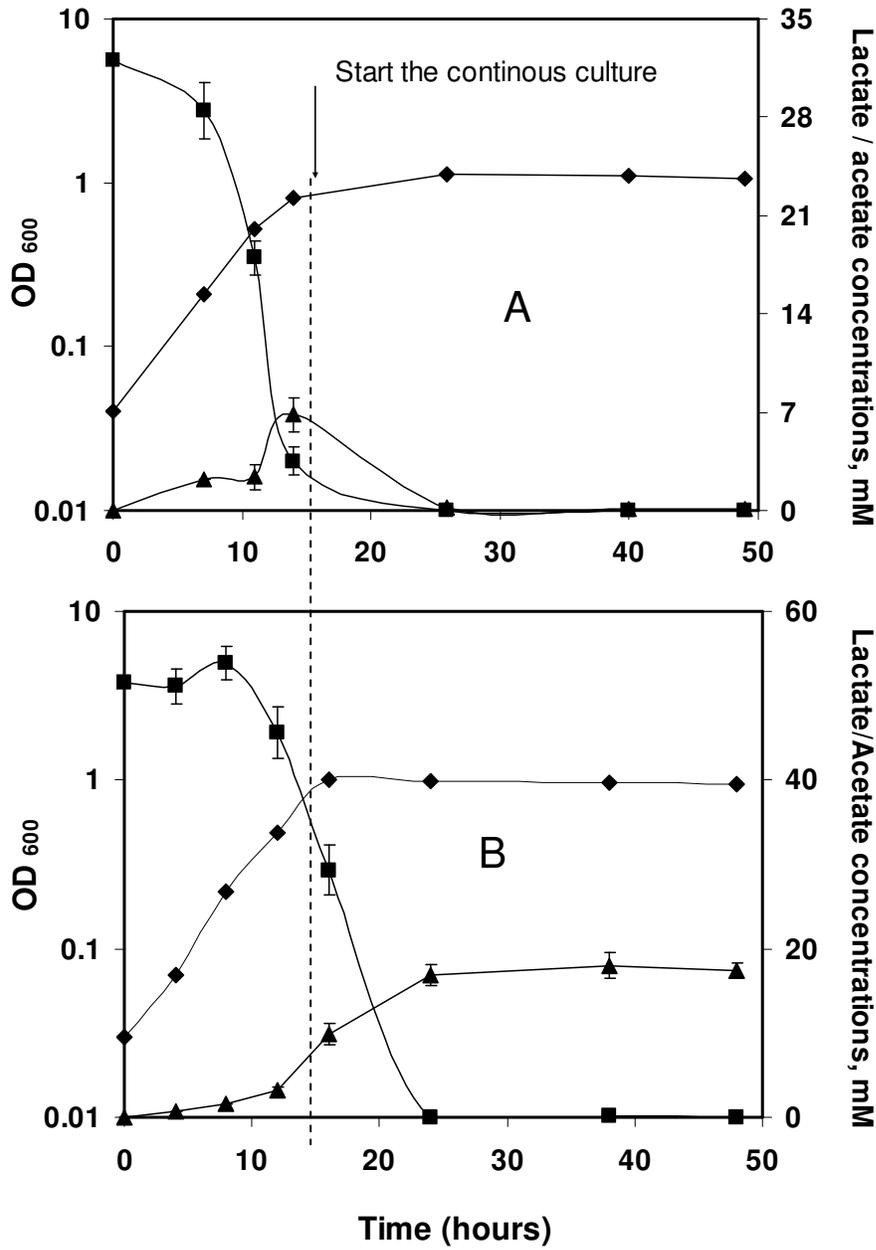
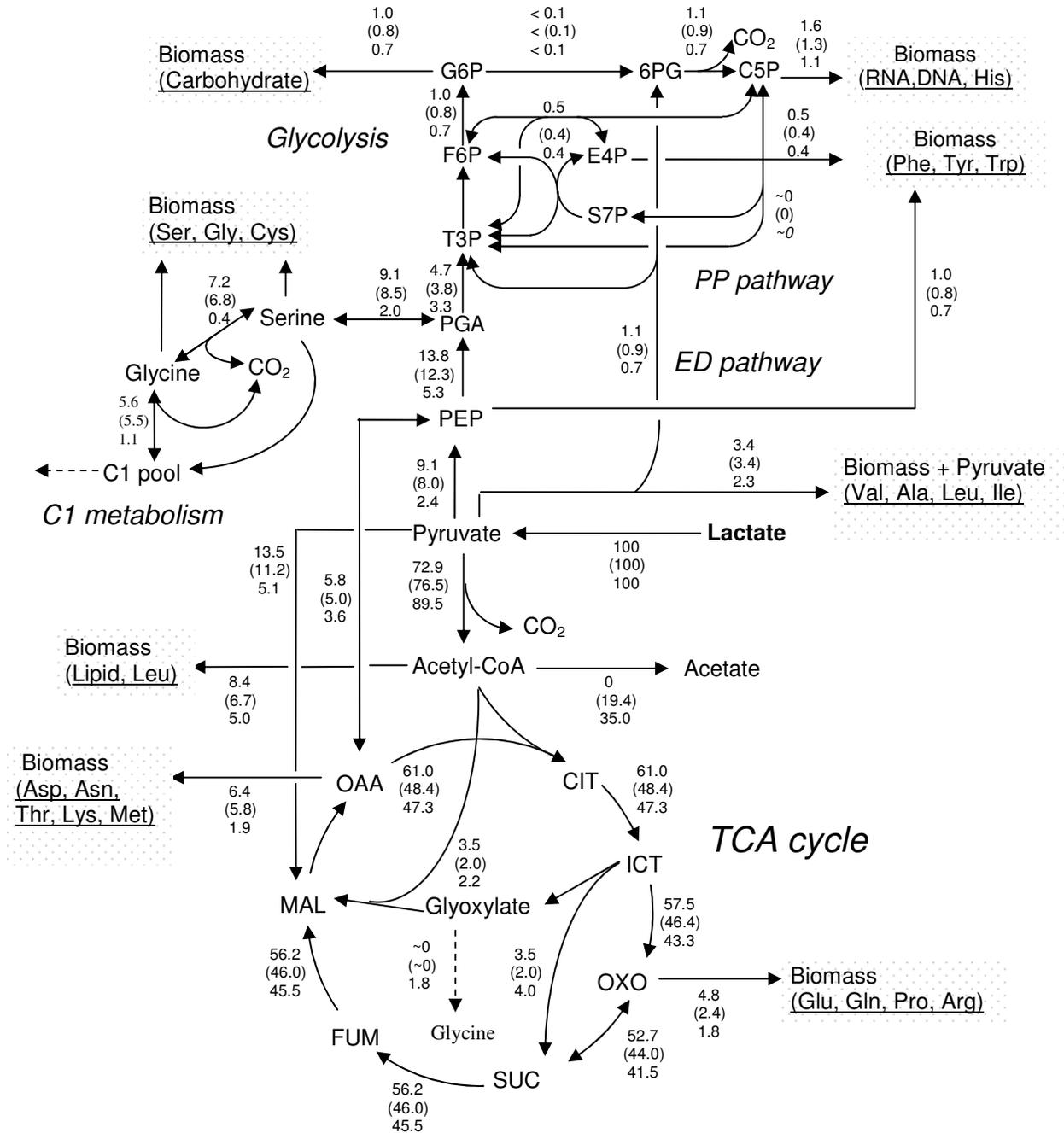


Figure 3

A



B



Figure 4

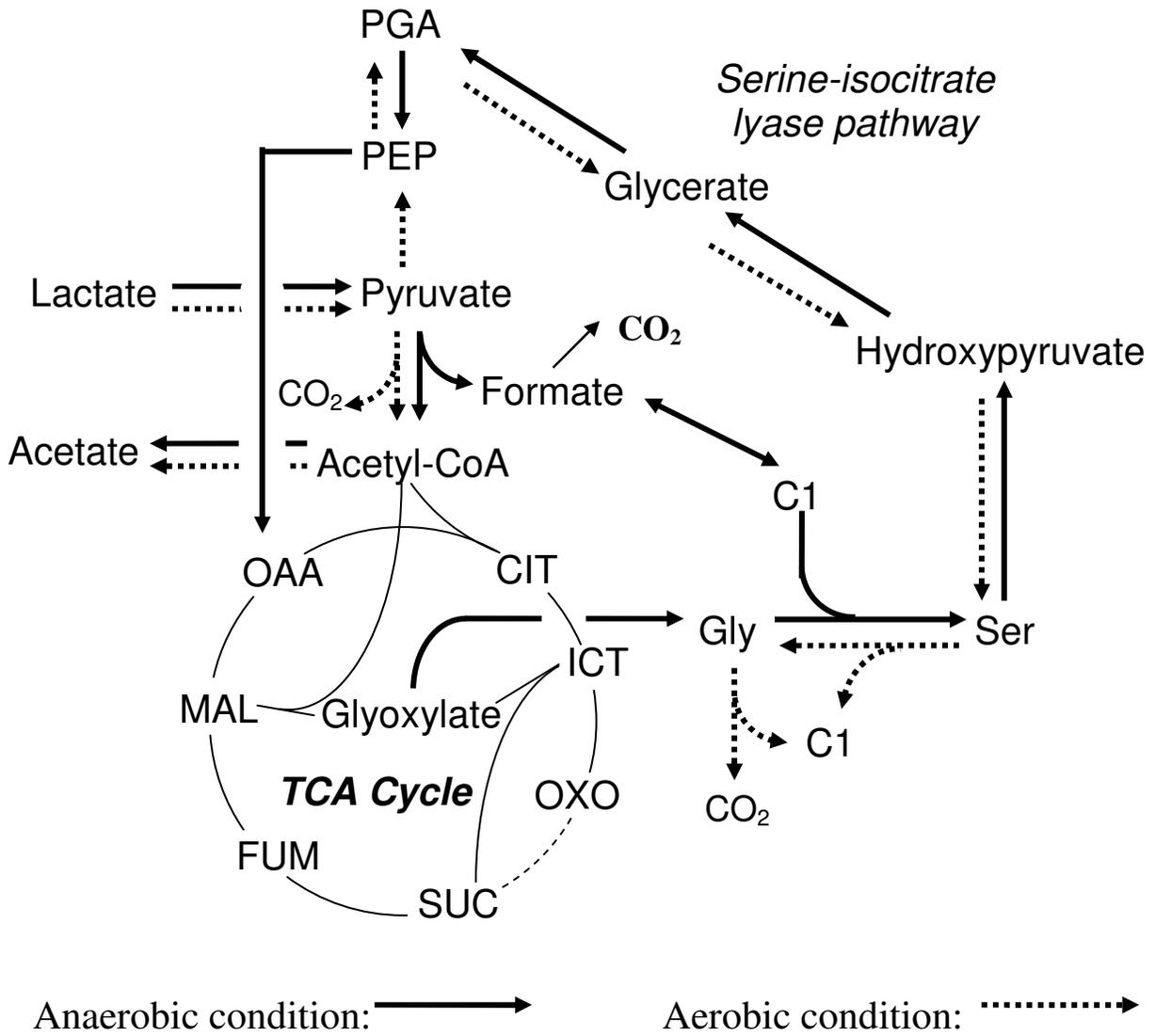
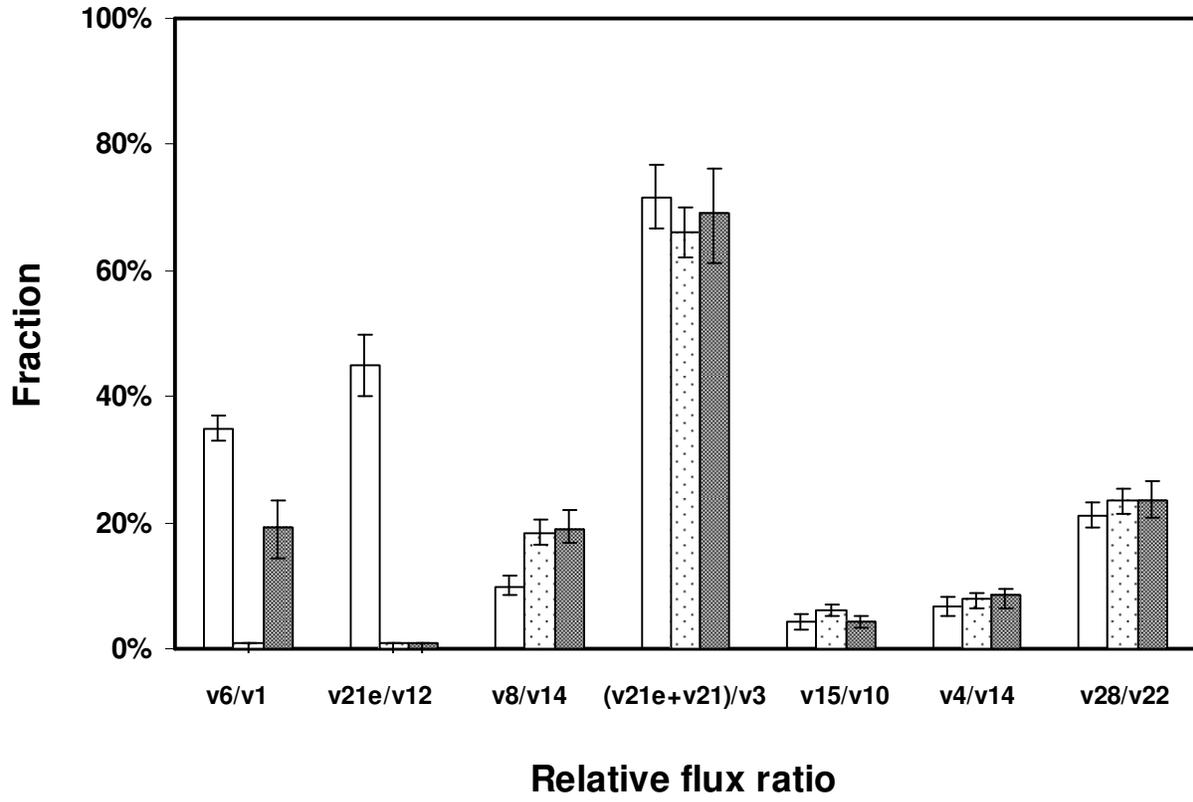


Figure 5



Tables

Table 1. Comparison of cultivation parameters of *S. oneidensis* MR-1 cultures.

Cultivation conditions	Shake flasks, n=5	Chemostat, oxygen-limited	Chemostat, carbon-limited
Growth or dilution rate (hr ⁻¹)	0.17	0.10	0.079
Sampling OD ₆₀₀	~0.5	~1.0	~1.0
Dissolved oxygen level	ND*	>70%	0~10%
Biomass concentration (g/L)	0.26±0.04	0.63 ± 0.05	0.58 ± 0.03
Lactate remained (mM)	13.5 ± 1.1	~0.3	~0
Acetate excreted (mM)	3.2 ± 0.6	17.5 ± 0.9	~0
Pyruvate excreted (mM)	~0.5	~0.1	~0
Lactate consumption rate (mM/g DCW h)	7.2 ± 0.7	7.9 ± 0.6	4.1 ± 0.3
CO ₂ formation rate (mM/g DCW h)	ND	13.7 ± 1.5	9.8 ± 1.2

*ND, not determined. Dissolved oxygen dropped during the exponential growth phase in shake flasks (31).

Table 2. Comparison in biomass composition between *S. oneidensis* MR-1 and *E. coli*.

Cultivation conditions	<i>S. oneidensis</i> MR-1			<i>E. coli</i>
	Shake flask ^a	Chemostat, oxygen-limited	Chemostat, carbon-limited	Reported value ^b
Protein (%)	51 ± 5	48 ± 3	48 ± 4	52
RNA (%)	16.2 ± 1.9	14.0 ± 2.5	13.6 ± 2.3	16
DNA (%)	2.7 ± 0.4	2.5 ± 0.4	2.1 ± 0.5	3
Fatty acids (%)	9.2 ± 1.2	15.1 ± 1.2	14.8 ± 0.8	9.1 (lipid fraction)
Carbohydrate (%)	12 ± 2	11 ± 3	10 ± 3	17
Others (%)	~8.9	~9.4	~9.5	3 (ash weight)

^a30mM lactate was used.

^bGlucose medium under aerobic culture, values adapted from Stephanopoulos, et al. (1998).

Table 3. Measured and predicted (in parenthesis) mass fragment [M-57]⁺ distribution of TBDMS-derivatized amino acids from *S. oneidensis* MR-1 hydrolysates*.

Amino Acid	Ions	Chemostat, carbon-limited	Shake flask, 3-¹³C¹	Chemostat, oxygen-limited	Shake flask, 10% ¹³C₃²
Ala	M0	0.01 (0.02)	0.02 ± 0.01	0.90 (0.89)	0.89 ± 0.01
	M1	0.98 (0.97)	0.97 ± 0.01	0 (0.01)	0 ± 0
	M2	0 (0.01)	0.0 ± 0	0.01 (0)	0.01 ± 0
Val	M0	0.01 (0.01)	0 ± 0	0.79 (0.81)	0.81 ± 0
	M1	0.03 (0.02)	0.02 ± 0.01	0.01 (0.01)	0.01 ± 0
	M2	0.92 (0.95)	0.92 ± 0.02	0.10 (0.09)	0.10 ± 0.01
	M3	0.01 (0.01)	0.01 ± 0.01	0.09 (0.09)	0.08 ± 0
Asp/Asn	M0	0 (0)	0 ± 0	0.75 (0.76)	0.77 ± 0.02
	M1	0.20 (0.18)	0.20 ± 0.02	0.15 (0.13)	0.13 ± 0.02
	M2	0.40 (0.40)	0.39 ± 0.01	0.07 (0.09)	0.05 ± 0.02
	M3	0.40 (0.41)	0.41 ± 0.02	0.03 (0.02)	0.06 ± 0.02
Met	M0	0.01 (0)	0.01 ± 0	0.64 (0.65)	0.66 ± 0.03
	M1	0.04 (0.05)	0.05 ± 0.01	0.21 (0.21)	0.22 ± 0.02
	M2	0.22 (0.24)	0.24 ± 0.02	0.12 (0.10)	0.09 ± 0.02
	M3	0.39 (0.40)	0.38 ± 0.03	0.03 (0.04)	0.03 ± 0.01
Ser	M0	0.07 (0.06)	0.06 ± 0.02	0.86 (0.87)	0.85 ± 0
	M1	0.72 (0.72)	0.74 ± 0.03	0.07 (0.04)	0.06 ± 0.01
	M2	0.16 (0.16)	0.15 ± 0.02	0.03 (0.02)	0.03 ± 0
Gly	M0	0.68 (0.69)	0.69 ± 0.02	0.88 (0.87)	0.90 ± 0.01
	M1	0.21 (0.23)	0.22 ± 0.02	0.04 (0.06)	0.01 ± 0

Glu/Gln	M0	0 (0)	0 ± 0	0.70 (0.71)	0.72 ± 0.03
	M1	0.01 (0.01)	0.01 ± 0	0.15 (0.14)	0.14 ± 0.01
	M2	0.31 (0.30)	0.32 ± 0.02	0.12 (0.13)	0.12 ± 0
	M3	0.48 (0.49)	0.48 ± 0.01	0.02 (0.02)	0.02 ± 0
	M4	0.18 (0.19)	0.20 ± 0.01	0 (0)	0 ± 0
Phe	M0	0 (0)	0 ± 0	0.57 (0.55)	0.61 ± 0.05
	M1	0 (0)	0 ± 0	0.17 (0.18)	0.12 ± 0.03
	M2	0.02 (0.03)	0.01 ± 0.01	0.09 (0.10)	0.08 ± 0.02
	M3	0.45 (0.38)	0.61 ± 0.11	0.12 (0.12)	0.14 ± 0.04
	M4	0.30 (0.32)	0.19 ± 0.06	0.03 (0.03)	0.02 ± 0.01
	M5	0.14 (0.18)	0.09 ± 0.03	0.01 (0.01)	0.02 ± 0.01
His	M0	0.01 (0)	0 ± 0	0.71 (0.68)	0.73 ± 0.03
	M1	0.04 (0.03)	0.05 ± 0.01	0.07 (0.13)	0.04 ± 0.03
	M2	0.26 (0.27)	0.27 ± 0.02	0.11 (0.10)	0.11 ± 0
	M3	0.50 (0.50)	0.49 ± 0.02	0.09 (0.08)	0.11 ± 0.01
	M4	0.11 (0.14)	0.06 ± 0.03	0.01 (0.01)	0 ± 0
¹³ CO ₂ percentage in total CO ₂ (based on the off gas composition)					
	$\frac{^{13}\text{CO}_2}{\text{CO}_2}$	0.13 (0.14) ³	ND	0.04 (0.03) ³	ND

* Mass distributions of tyrosine and threonine were identical to phenylalanine and aspartate, respectively.

¹ Shake-flask culture at exponential phase with 98% [3-¹³C] L-lactate (30mM), standard deviation of isotopomer data were based on three replicates.

² Shake-flask culture at exponential phase with a mixture of 10% fully-labeled L-lactate and 90% unlabeled lactate (30mM), n=3.

³ ¹³CO₂ fraction is based on off-gas composition.

Table 4. NMR measurement and model prediction (in parenthesis) ¹³C isotopomer distribution of key amino acids from *S. oneidensis* MR-1 hydrolysates.

Amino Acid	α -carbon fragments	Chemostat, carbon-limited ¹	Chemostat, oxygen-limited ¹
α-Asp	C α	0.23 (0.18)	0.37 (0.33)
	C α -C β	0.47 (0.49)	0.11 (0.06)
	C α -C=O	0.05 (0.02)	0.42 (0.47)
	C β -C α -C=O	0.25 (0.30)	0.10 (0.13)
α-Ser	C α	0.17 (0.17)	0.21 (0.19)
	C α -C β	0.52 (0.51)	0.06 (0.03)
	C α -C=O	0.07 (0.02)	0.26 (0.28)
	C β -C α -C=O	0.25 (0.29)	0.47 (0.50)
α-Gly	C α	0.70 (0.68)	0.41 (0.41)
	C α -C=O	0.29 (0.32)	0.59 (0.59)
α-Glu	C α	0.25 (0.32)	0.31 (0.32)
	C α -C β	0.45 (0.43)	0.15 (0.13)
	C α -C=O	0.06 (0.04)	0.54 (0.51)
	C β -C α -C=O	0.24 (0.22)	0.01 (0.04)
α-Phe	C α	0.18 (0.17)	0.14 (0.10)
	C α -C β	0.51 (0.51)	0.01 (0.01)
	C α -C=O	0.04 (0.02)	0.04 (0.11)
	C β -C α -C=O	0.27 (0.29)	0.83 (0.77)
β-Ser	C β	0.81 (0.82)	0.50 (0.49)
	C β -C α	0.19 (0.18)	0.50 (0.51)
β-Asp	C β	0.26 (0.32)	0.36 (0.33)
	C β -C α	0.41 (0.41)	0.15 (0.13)
	C β -C γ	0.07 (0.04)	0.40 (0.47)
	C α -C β -C γ	0.26 (0.23)	0.09 (0.07)
	C β	0 (0)	0.72 (0.73)
β-Glu	C β -C α or C β -C γ	0.26 (0.21)	0.28 (0.27)
	C α -C β -C γ	0.73 (0.79)	0 (0.01)
	C β	0.96 (0.98)	0.11 (0.10)
β-Ala	C β -C α	0.04 (0.02)	0.89 (0.90)
	C β	NA ²	0.10 (0.15)
β-His	C β -C α	NA ²	0.90 (0.85)
	C β -C γ	NA ²	0 (0)
	C α	ND ³	0.11 (0.10)
α-Ace	C α -C β	ND ³	0.89 (0.90)

¹ The standard deviation for NMR measurement was 4-5%.

² NA, natural abundance.

³ ND, not determined.

Table 5. Sensitivity test of predicted mass distribution signals (GC-MS [M-57]⁺ and NMR [α or β carbon]) for aspartate and glutamate upon changes in the futile flux, v_8 .

		GC-MS Data		NMR Data		
Amino acids	Sensitivity coefficient	3 rd carbon labeled ¹	Fully labeled ²	Sensitivity coefficient	3 rd carbon labeled ¹	Fully labeled ²
	$\left \frac{\Delta M_0}{\Delta v_8} \right $	0.0001	0.0041	$\left \frac{\Delta C\alpha}{\Delta v_8} \right $	0.013	0.015
Asp	$\left \frac{\Delta M_1}{\Delta v_8} \right $	0.0100	0.0057	$\left \frac{\Delta C\alpha - C\beta}{\Delta v_8} \right $	0.005	0.008
	$\left \frac{\Delta M_2}{\Delta v_8} \right $	0.0089	0.0001	$\left \frac{\Delta C\alpha - C = O}{\Delta v_8} \right $	~0	0.002
	$\left \frac{\Delta M_3}{\Delta v_8} \right $	0.0179	0.0011	$\left \frac{\Delta C\beta}{\Delta v_8} \right $	0.016	0.019
	$\left \frac{\Delta M_0}{\Delta v_8} \right $	~0	0.0024	$\left \frac{\Delta C\alpha}{\Delta v_8} \right $	0.019	0.018
Glu	$\left \frac{\Delta M_1}{\Delta v_8} \right $	0.0006	0.0039	$\left \frac{\Delta C\alpha - C\beta}{\Delta v_8} \right $	0.012	0.016
	$\left \frac{\Delta M_2}{\Delta v_8} \right $	0.0132	0.0016	$\left \frac{\Delta C\alpha - C = O}{\Delta v_8} \right $	~0	0.003
	$\left \frac{\Delta M_3}{\Delta v_8} \right $	0.0053	0.0003	$\left \frac{\Delta C\beta - C\alpha - C = O}{\Delta v_8} \right $	0.006	0.003
	$\left \frac{\Delta M_4}{\Delta v_8} \right $	0.0082	0.0001	$\left \frac{\Delta C\beta}{\Delta v_8} \right $	~0	0.016
Sum of squares		0.00077	0.00007		0.00082	0.00128

¹ Data from the carbon-limited chemostat

² Data from the oxygen-limited chemostat

Table 6. Flux distribution reliability test: predicted and measured fragment mass [M-57]⁺ distribution of key amino acids when [1-¹³C] labeled lactate medium was used for shake-flask culture (n=2)*.

Related Pathway	Amino acids	Ion, m/z	Model prediction	Measured value	
Pyruvate synthesis route	Ala	M0	0.02	0.03 ± 0.01	
		M1	0.98	0.98 ± 0.01	
		M2	0	0 ± 0	
	Val	M0	0.02	0.05 ± 0.01	
		M1	0.96	0.94 ± 0.01	
		M2	0	0 ± 0	
	Serine oxidation route	Ser	M0	0.24	0.21 ± 0.03
			M1	0.76	0.79 ± 0.03
			M2	0.0	0 ± 0
Serine oxidation route	Gly	M0	0.25	0.34 ± 0.03	
		M1	0.75	0.66 ± 0.03	
		M2	0	0 ± 0	
TCA cycle	Asp	M0	0.78	0.75 ± 0.04	
		M1	0.12	0.14 ± 0.02	
		M2	0.10	0.10 ± 0.01	
	Glu/Gln	M0	0.89	0.86 ± 0.03	
		M1	0.09	0.07 ± 0.02	
		M2	0.01	0.03 ± 0.02	
PP pathway	Phe	M0	0.01	0.01 ± 0	
		M1	0.13	0.09 ± 0.03	
		M2	0.42	0.36 ± 0.05	
		M3	0.42	0.52 ± 0.08	
	His	M0	0.23	0.18 ± 0.05	
		M1	0.76	0.75 ± 0.06	
		M2	0.01	0.04 ± 0.02	

* The same flux rate distribution as listed in Figure 3 (the carbon-limited condition) was used for model prediction.