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Tang, Yinjie

Publication Date
2009-07-22

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Invariability of central metabolic flux distribution in *Shewanella oneidensis* MR-1 under environmental or genetic perturbations

Running title: flux distribution in *Shewanella oneidensis*

Yinjie J. Tang[1], Hector Garcia Martin[2], Adam Deutschbauer[3,4], Xueyang Feng[1], Rick Huang[1], Xavier Llora[5], Adam Arkin[3,4,6], Jay. D. Keasling[2,3,4,6,7*],

[1]Department of Energy, Environmental and Chemical Engineering, Washington University, St. Louis, MO 63130

[2] Joint BioEnergy Institute, 5885 Hollis, Emeryville, CA 94608.

[3] Virtual Institute of Microbial Stress and Survival, Lawrence Berkeley National Laboratory, Berkeley, USA.

[4]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, USA.

[5]National Center for Supercomputing Applications, University of Illinois at Urbana-Champaign, 1205 W. Clark Street, Urbana, IL 61801.

[6]Department of Bioengineering, University of California, Berkeley, USA.

[7]Department of Chemical Engineering, University of California, Berkeley, USA

*Corresponding author:

Email: Keasling@berkeley.edu

Abstract

An environmentally important bacterium with versatile respiration, *Shewanella oneidensis* MR-1, displayed significantly different growth rates under three culture conditions: minimal medium (doubling time ~ 3 hrs), salt stressed minimal medium (doubling time ~ 6 hrs), and minimal medium with amino acid supplementation (doubling time ~1.5 hrs). ^{13}C-based metabolic flux analysis indicated that fluxes of central metabolic reactions remained relatively constant under the three growth conditions, which is in stark contrast to the reported significant changes in the transcript and metabolite profiles under various growth conditions. Furthermore, ten transposon mutants of *S. oneidensis* MR-1 were randomly chosen from a transposon library and their flux distributions through central metabolic pathways were revealed to be identical, even though such mutational processes altered the secondary metabolism, for example, glycine and C1 (5,10-Me-THF) metabolism.

Keywords: *growth rate; ^{13}C-based; transposon mutants; transcript; metabolite profiles; secondary metabolism*

Introduction

Shewanella oneidensis MR-1 has versatile respiration and can engage in co-metabolic bioremediation of a diverse number of environmental contaminants, such as chromium, radionuclides, and halogenated organic compounds [1-3]. In addition, its ability to transfer electrons to solid metals indicates its potential application in microbial fuel cells [1]. Previous studies on *S. oneidensisis* MR-1 have examined its transcript and metabolite profiles in response to various growth conditions [4-7]. However, cell physiology might not be accurately reflected by the annotated genome or by the transcript, protein, and metabolite profiles [8-10]. For example, *E. coli*'s transcript profile can have little relationship to the metabolic flux profile due to post-transcriptional regulation of protein synthesis and enzymes activity [10, 11]. Since one of the most physiologically relevant descriptions of a cell's metabolism is the set of metabolic fluxes (a key determinant of cellular physiology) [8], we investigated *S. oneidensisis* MR-1's flux distributions in response to environmental and genetic perturbations, and thus its metabolic robustness (a recently recognized microbial phenotype [12-14]) in the face of environmental uncertainty and genetic perturbations. This study improves our understanding of *Shewanella* phenotypes and gene regulation attributed to the nature of adaptation in their environment. The variation of flux distributions in the MR-1 transposon mutants can reveal the possible impact of genetic modification on central metabolism, i.e., from an evolutionary robustness viewpoint[15], whether the random nature of mutational processes (e.g., genetic drift or horizontal gene transfer) in the environment can have a significant effect on central flux distributions in *Shewanella* species.[16]

Materials and Methods

Culture conditions and analytical methods for metabolites. *Shewanella oneidensis* MR-1 was obtained from The American Type Culture Collection (ATCC 700550). Ten MR-1 mutants were provided by Adam Arkin's Group at University of California, Berkeley (Table 1), which were obtained via Tn5 transposon mutagenesis using plasmid pRL27 [17]. In brief, mutagenesis was carried out by conjugation of the *E. coli* donor strain WM3064 harboring pRL27 with *Shewanella oneidensis* MR-1; transposon mutants were selected on Luria Broth (LB) medium with kanamycin (10 μg/mL); the insertion location of transposon mutants was mapped via a two-step degenerate PCR protocol as described previously [18]. From over 1000 thousand mutants, 10 strains were randomly picked and investigated how natural mutation could affect the central flux distribution.

All MR-1 strains were grown in the defined [3-^{13}C] sodium L-lactate (98%, Cambridge Isotope, USA) minimal medium (30mM, pH=7, buffered with 20mM PIPES) and duplicate experiments were performed (n=2) [19]. For salt stress experiments, 330 mM NaCl was added to the medium. To enhance growth, a standard amino acid mix (containing 17 unlabeled amino acids, without tryptophan, glutamine, and asparagine, Cat#AA-S-18, Sigma, USA) was added to the medium for a final concentration of 25 μM for each amino acid. The inoculum was prepared in LB medium and incubated overnight; the cultures were started with a 0.09% inoculation volume. All cultures (12 mL) were incubated in glass tubes at 30°C and 200 rpm shaking speed. Total biomass growth was monitored by measuring the OD_{600} and converting it to the corresponding dry weight (specifically, the harvested culture was centrifuged at 4,800 x g for 20 min and lyophilized overnight; then the dried biomass was weighed to obtain a correlation curve

between dried biomass and its corresponding OD_{600}). The concentrations of lactate, acetate, and pyruvate in the medium were measured using enzyme kits (r-Biopharm, Darmstadt, Germany).

The GC-MS protocol for isotopomer measurement has been described previously [9, 20, 21]. In brief, the protein in biomass from the early exponential growth phase (OD_{600}=0.3~0.4) was hydrolyzed to free amino acids in 6 M HCl at 100°C for 24 hours. GC-MS samples were prepared in 100 μl tetrahydrofuran (THF) and 100 μl N-(tert-butyldimethylsilyl)-N-methyl-trifluoroacetamide (Sigma-Aldrich, USA) and baked at 65-80°C for one hour. GC-MS analysis was carried out using a gas chromatograph (DB5 column, HP6890 series, Agilent Inc, USA) equipped with a mass spectrometer (5973 Network, Agilent Inc, USA). Two types of positively charged species were used in flux calculation: unfragmented amino acids, $[M-57]^+$, and fragmented amino acids without α carboxyl group, $[M-159]^+$ [22].

Algorithm for isotopomer analysis and flux calculation. The metabolic network used to calculate the metabolic flux profile of *S. oneidensisis* MR-1 included the tricarboxylic acid (TCA) cycle (including the glyoxylate shunt), C_1 metabolism, the Entner-Doudoroff (ED) pathway, gluconeogenesis, and the pentose phosphate (PP) pathway (total 43 reactions, Supplementary materials Table S-1). The algorithm for metabolic flux calculation has been described in detail in our previous paper [15, 23]. In brief, the flux profile was represented through an independent fluxes vector $\boldsymbol{v_{ind}}$, which comprised 16 independent fluxes plus the unlabeled fractions of CO_2 and C1, for a total of 18 independent variables. The carbon labeling expected from a flux profile $\boldsymbol{v_{ind}}$ was calculated via the cumomer method [24] and the best fit for the data was calculated by minimizing the error function (i.e. average difference between the calculated and the experimental data) through the use of genetic algorithms[25]. The independent fluxes to biomass pools were not fixed by measurement values, but were loosely constrained by MR-1

biomass compositions[15, 23], which were optimized by the model using isotopomer information. The lower and upper limits for the fluxes to biomass synthesis in the model calculations are listed in Supplementary Table S-1. Confidence intervals were obtained using a Monte Carlo Method [26]: the GC-MS data were changed randomly within the measurement error, and simulated annealing was performed [27] until the error function did not decrease any further.

Amino acid isotopomer noise correction. The incorporation of unlabeled amino acids from the rich medium into proteins affects the isotopomer distribution determined for each amino acid and therefore the flux calculation. A mathematical algorithm was used to correct for noise in the amino acid measurements by assuming that the fraction of completely unlabeled proteinogenic amino acids was only determined by uptake of unlabeled amino acids supplemented in the medium and unlabeled lactate. This assumption was based on the fact that lactate was labeled in the third position, and this labeled carbon was not lost before it entered TCA cycle and gluconeogenesis (note: the first carbon of lactate is lost as CO_2 in the step pyruvate→acetylCoA). Hence, there were no unlabeled metabolites produced from lactate labeled in the third position. In the algorithm for correcting the isotopomer distributions, M_0 of the GC-MS data (completely unlabeled proteinogenic amino acids) was thus assumed to derive only from unlabeled amino acids and unlabeled lactate (see Supplementary Material for details):

$$M_O^{'} = 0.02\,;\; M_i^{'} = \frac{0.98 \cdot M_i}{1 - M_O} \forall i \neq 0 \qquad \text{(A)}$$

where M is the original GC-MS data, M' is the corrected GC-MS data; *i* is the number of labeled carbon atoms (0,1, 2, 3…); and 0.02 refers to 2% non-labeled carbon substrate (i.e., 2% of lactate is not labeled) in the medium.

Results and Discussion

Shewanella oneidensis MR-1 had very different exponential phase growth rates in the various culture media: in minimal medium, the doubling time was approximately 3 hrs, whereas in the salt stress medium, the doubling time was approximately 6 hrs (Figure 1). Under salt stress, the lactate consumption and metabolite production (pyruvate and acetate) rates were significantly slower compared to those rates measured from cultures grown under normal conditions (Figure 2). The flux distributions of the normal growth and stressed cultures were calculated based on the fitting of isotopomer data (Supplementary Figures S1-S3). Despite significant change in the growth rate under salt stress (half the rate), the calculated relative intracellular fluxes in the early exponential phase were very similar between salt stressed and normal growth conditions (within the standard deviation) (Figure 3). In the exponential growth phase, MR-1 had limited flux through the TCA cycle (36~37% of lactate uptake), resulting in lactate not being fully oxidized and acetate and pyruvate accumulating in the medium due to overflow metabolism (up to ~50% of lactate uptake) [9]. Gluconeogenesis, the pentose phosphate pathway, and the ED pathway were mainly used for biomass production, and as such their fluxes were very small (<3% of lactate uptake). Two anapleurotic reactions (pyruvate to malate; oxaloacetate to phosphoenolpyruvate) and the glyoxylate shunt appeared to be active (10~20% of the lactate uptake) in both culture conditions. Although salt stress reduced the specific growth rate of cells, it did not change the relative flux distribution for most pathways. This observation does not correlate with microarray data obtained from elevated salt conditions [5], which indicated that the expression of most of the TCA cycle-related genes were up-regulated under salt stress. A similar lack of correlation between fluxome and transcriptome data had been recently reported for *E. coli* grown on lactate [11].

Addition of amino acids to minimal medium significantly enhanced the growth rate and biomass production (without amino acids the doubling time was ~3 hrs; with amino acids the doubling was ~1.5 hrs) (Figure 1). Similarly, addition of amino acids improved MR-1's growth rate under salt stress conditions (without amino acids the doubling time was ~6 hrs; with amino acids the doubling was ~3 hrs) (Figure 1). The percentages of unlabeled proteogenic amino acids provided information on the relative ratios of endogenous and exogenous amino acid utilization [28]. In a defined rich medium containing an amino acid mix (17 amino acids, 25 μM each), the labeling patterns of Ala, Asp, Glu, Gly, Ser (group 1 ♦, Figure 4a) did not change (<2%); those of Val, Leu, Iso, Pro, Thr, and Lys (group 2 □) changed moderately (<10%); and those of Met, Phe, His, Tyr (group 3 ○) changed significantly (>10%). The three groups of amino acids correlated with an increasing number of biochemical steps away from central metabolism and the amount of energy required for synthesis (e.g., group 1 is closest to central metabolism and requires the least energy for production). Hence, the first group was mostly endogenously synthesized, whereas the last group was mainly imported from the medium. After a suitable correction using Equation A, the labeling data from the amino acid supplemented medium was very close to the labeling data from a completely minimal medium (Figure 4b). This suggested that the relative flux distribution through the central pathways did not change significantly even though the addition of certain amount of amino acids doubled the growth rate, i.e., the additive effect on biomass synthesis with extra building blocks did not dramatically change the reaction ratios of overall enzymatic reactions in central pathways.

The robustness of flux distributions to genetic perturbations was also investigated by comparing isotopomer distributions in amino acids between mutants and the wild-type strain. Ten mutants were randomly picked from the MR-1 mutant bank (>1000 strains) to study the

possible change in central metabolic flux distribution under genetic perturbations. The growth rates of the ten mutants (Table 1) are similar to the growth rate of the wild type in the minimal growth medium (within the measurement noise). The isotopomer data for all ten mutants were mostly identical to those for the wild type, and very few of the mutated genes appeared to have a specific impact on the relative flux through central carbon metabolism (Figure 5). However, perturbation of certain amino acid metabolisms was observed, i.e., the SO0781 mutant (lacking glycine dehydrogenase: glycine→C1+CO_2) showed increased ^{13}C abundance in histidine. This was due to the fact that the pool of C1 used in histidine biosynthesis is derived mainly from two reactions: serine degradation and glycine degradation (Figure 3)[23]. Since glycine displayed a relatively low level of ^{13}C labeling compared to serine, eliminating the glycine degradation pathway (i.e., altered C1 metabolism) increased the amount of histidine produced from serine degradation and resulted in a higher abundance of ^{13}C in histidine. The observed invariability in the relative flux distributions to genetic perturbations agrees with previous reports on the robustness of central metabolism in *Bacillus subtilis* and *E. coli* [12, 14]. However, it must be mentioned that the ten randomly chosen mutants in this study had no obvious phenotypic change. Based on the "neutral mutation theory" [16], the vast majority of single-nucleotide changes (such as genetic drift) have little or no biological effect. Through the transposon mutants, this study improves our understanding of the general effect of molecular evolution on the robustness of central metabolic flux distributions in *Shewanella*. Indeed, the mutants with the most perturbed central metabolism would have grown very poorly compared to the wild type strain in the same environment. Such deleterious MR-1 mutants, of course, would be rejected through natural selection.

Conclusion

In spite of versatile respiration metabolism, the relative flux distribution for *Shewanella oneidensis* MR-1 in the early exponential phase is robust with respect to amino acid addition, salt stress, and genetic perturbation, The rigidity of MR-1 metabolism under the studied conditions provides further evidence that microbial metabolism is not solely geared towards growth rate maximization when carbon sources are sufficient [12]. Since metabolic fluxes are the final functional output of the interaction of all molecular machinery, such rigidity of the metabolic flux distributions in microorganisms indicates that the functional output of combined regulatory activity (e.g. transcription, translation, post-translational regulation, etc.) leads to a stable relative flux profile despite the large differences in growth rates. Although it is easy to change a cell's secondary metabolism or biosynthesis pathways (e.g., MR-1 glycine dehydrogenase mutant), the relative fluxes through central metabolic reactions are not easily altered and this may be the key mechanism for organism to survive and evolve in the environments.

Acknowledgements

This work is part of the Virtual Institute for Microbial Stress and Survival (http://VIMSS.lbl.gov) supported by the U.S. Department of Energy, Office of Science, Office of Biological and Environmental Research, Genomics:GTL Program through contract DE-AC02-05CH11231 between the Lawrence Berkeley National Laboratory and the US Department of Energy. This work is also supported by the Joint BioEnergy Institute to JDK, and I-CARES (International Center for Advanced Renewable Energy and Sustainability) at Washington University in St Louis to YJT.

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Figure Caption

Figure 1. Growth kinetics of *Shewanella oneidensis* MR-1 under salt stress condition and with amino acid supplements (n=2). ♦: minimal medium with amino acids supplement, no salt; ◊: minimal medium, no salt; ■: minimal medium with salt (0.33 M NaCl supplemented into minimal medium) and amino acids (17 amino acids, 25 μM each), □: minimal medium with salt (0.33 M NaCl supplementation into minimal medium), no amino acids.

Figure 2. Lactate consumption and metabolite production by *Shewanella oneidensis* MR-1 under normal growth or salt stress conditions in minimal medium (n=2). Normal growth conditions: ■, lactate; ♦, acetate; ●, pyruvate. Salt stress (0.33 M NaCl supplemented): □, minimal medium; ◊, acetate; ○, pyruvate.

Figure 3. Fluxes in central metabolic reactions of *S. oneidensis* MR-1 under normal growth conditions (upper number) and salt stress conditions (bottom number). The flux distributions through central metabolic pathways in ten selected mutants were identical to those under normal growth conditions. The estimated fluxes for serine and glycine metabolism in mutant SO0781 are in parentheses. Abbreviations: ACoA, acetyl-coenzyme A; CIT, citrate; E4P, erythrose-4-phosphate; C1, 5,10-Me-THF; F6P, fructose-6-phosphate; G6P, glucose-6-phosphate; 6PG, 6-phosphogluconate; ICT, isocitrate; MAL, malate; OAA, oxaloacetate; OXO, 2-oxoglutarate; PEP, phosphoenolpyruvate; PGA, 3-phosphoglycerate; C5P, ribose-5-phosphate (or ribulose-5-phosphate or xylulose-5-phosphate); S7P, sedoheptulose-7-phosphate; SUC, succinate; T3P, triose-3-phosphate; PYR, pyruvate.

Figure 4. Effect of unlabeled amino acids on central metabolism in MR-1 (illustrated by a change in GC-MS data in key amino acids). (a) Comparison of GC-MS data for proteinogenic amino acids from cells grown on minimal medium (control) and those grown on rich medium (addition of amino acid mixtures, 17 amino acids, 25 μM each). ♦: Ala, Asp, Glu, Gly, Ser; □: Val, Leu, Iso, Pro, Thr, Lys; ○: Met, Phe, His, Tyr. (b) GC-MS data for the amino acid-supplemented medium were corrected for incorporation of unlabeled amino acids into protein using equation A.

Figure 5. Effect of transposon mutagenesis on central metabolism. The GC-MS data were from 10 different mutants (Table 1). Nine key amino acids were included (Ala, Asp, Glu, Gly, Ser, Val, Leu, Phe, His). The open dots were GC-MS data from SO0781 (lack of glycine cleavage system P protein). The outliers M2[-57] and M3[-57] are histidine labeling data. The increased ^{13}C abundance in histidine (M3[-57] was above the diagnal line) was due to the knockout of the glycine dehydrogenase (see main text). The measurement noise for isotopic data from independent tracer experiments should be below 5%.

Figure 1

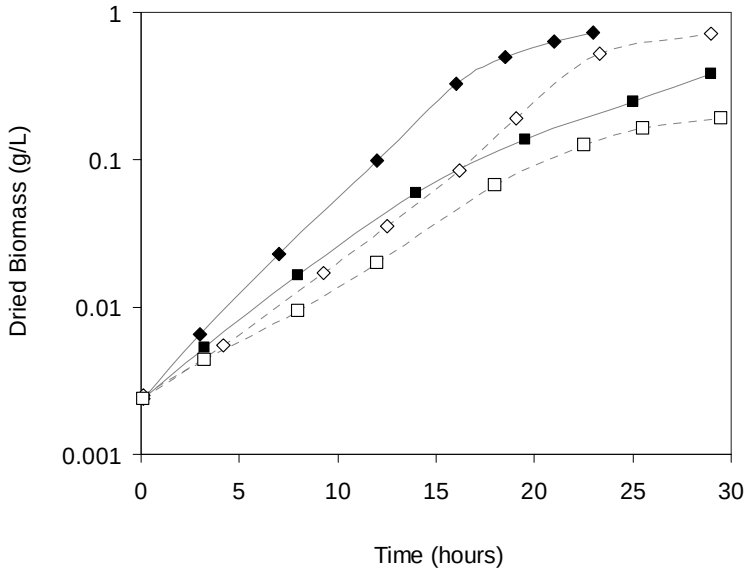


Figure 2

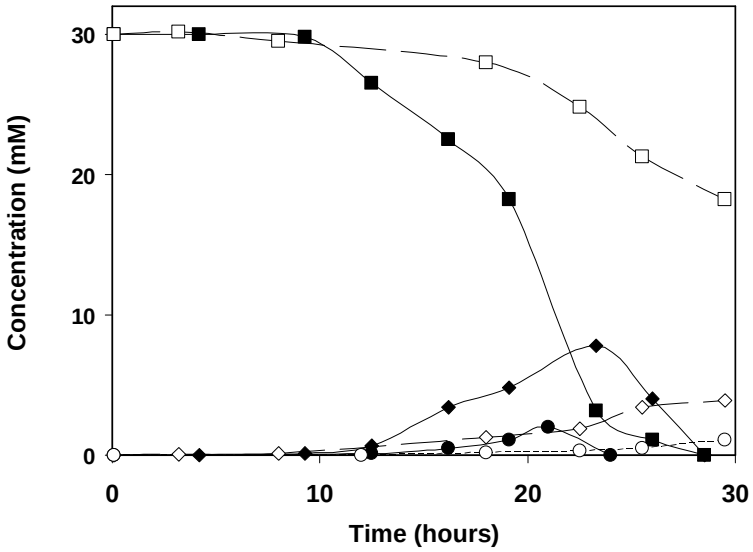
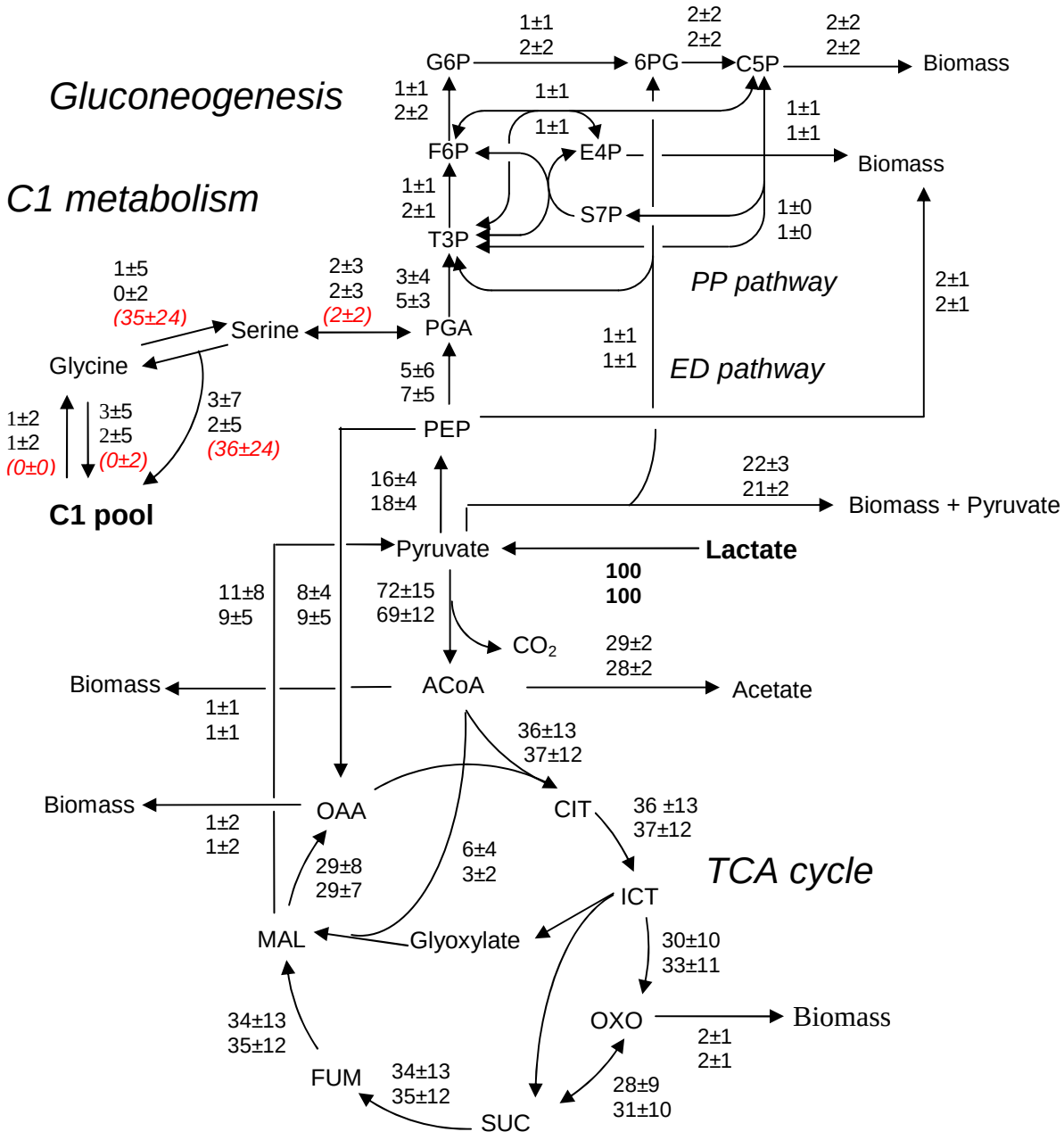


Figure 3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C5P
Biomass
F6P
E4P
S7P
T3P
PP pathway
ED pathway
Serine
Glycine
PGA
PEP
C1 pool
Biomass + Pyruvate
Pyruvate
Lactate
CO2
ACoA
Acetate
Biomass
OAA
CIT
TCA cycle
ICT
MAL
Glyoxylate
OXO
Biomass
FUM
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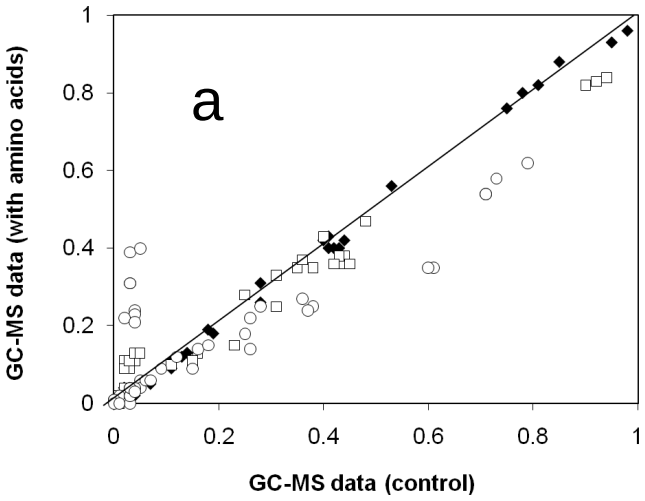
Figure 4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GC-MS data (control)
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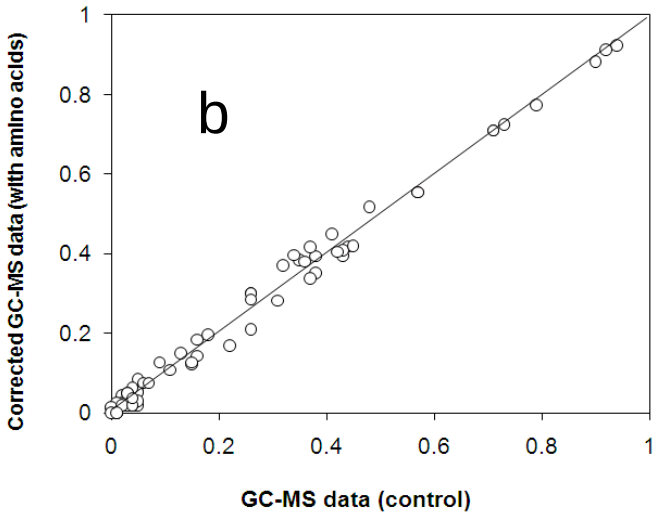
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GC-MS data (control)
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0.4
0.6
0.8
1

Figure 5

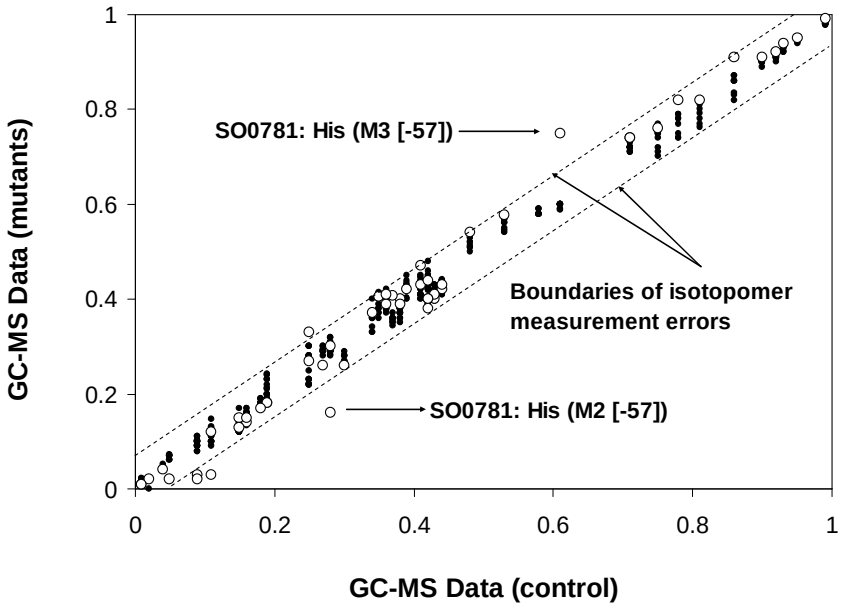
GC-MS Data (mutants)
GC-MS Data (control)
SO0781: His (M3 [-57])
SO0781: His (M2 [-57])
Boundaries of isotopomer measurement errors
0
0.2
0.4
0.6
0.8
1

Table 1. *Shewanella oneidensis* MR-1 mutants tested in this study.

Mutant	Growth rate,hr^{-1}	Gene	Description
MR-1	0.23±0.04	Wild type	Minimal medium (no stress)
MR-1	0.10±0.02	Wild type	Salt stress
1	0.20±0.03	SO0781	glycine cleavage system protein P; gcvP
2	*	intergenic	none
3	*	SO3925	biotin synthase family protein
4	*	SO_A0184	conserved hypothetical protein
5	*	SO1420	outer membrane porin, putative
6	*	SO2867	permease PerM, putative
7	*	SO1143	conserved hypothetical protein
8	*	SO3230	flagellar regulatory protein C; flrC
9	*	SO4529	RNA methyltransferase, TrmH family, group 2
10	*	SO3106	cold-active serine alkaline protease; aprE

*: Growth rates for mutant 2 to 10 were same as the MR-1 wild type.