Report Number: WUCSE-2011-5

2011

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Flux Balance Analysis of Dynamic Metabolism in *Shewanella oneidensis* MR-1 Using A Static Optimization Approach

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Running title: Dynamic Metabolism of *Shewanella*

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Abstract

Background

Shewanella oneidensis MR-1 is an environmentally important bacterium for metal reduction, carbon cycling, bioremediation, and microbial fuel cells. Depending on substrate concentrations, MR-1 can sequentially utilize a carbon source (lactate) and its waste products (pyruvate and acetate) during batch culture. Therefore, MR-1 demonstrates a dynamic mixed-substrate metabolism which is an important regulatory mechanism for microbial competition in nutrient-limited ecosystems [1].

Results

We developed a dynamic flux balance analysis (dFBA) framework by linking two metabolic models: 1) a Monod-based kinetic model for biomass growth and metabolite production, and 2) a flux balance (FBA) model (incorporating 236 key reactions and 213 metabolites) with the maximal biomass production rate as the objective function. The Monod kinetics provided the time-dependent model inputs (i.e., substrate uptake rate and metabolite secretion rates) for flux balance analysis so that the intracellular metabolism can be resolved via a static optimization approach. During the entire growth period, the model revealed that the TCA cycle and anaplerotic pathways had two metabolic peaks: a strong peak during lactate metabolism and a weak peak during acetate metabolism. Moreover, pyruvate decarboxylation was active only in the early growth phase, while the glyoxylate shunt was active in the late growth phase. Gene expression study and enzyme activity detection qualitatively confirmed the variation of intracellular metabolism during growth. Compared to measured biomass curves, the dynamic

FBA with the objective functions for maximal production of biomass or ATP/flux predicted 30~50% higher growth rates in the late growth stage, which indicated the cellular metabolism became "sub-optimal" when switching its carbon source to acetate, a less energy-favorable substrate.

Conclusions

The dFBA model integrates the *Shewanella* growth kinetics with conventional flux profiling to quantify the metabolic status during different growth periods. The model links dynamic regulation of global carbon metabolism with knowledge from gene expressions and enzyme activities. Such an approach can be a routinely-used strategy to quantitatively study unsteady-state cellular metabolism in other biological systems.

Key words: mixed-substrate, kinetic model, metabolic peak, objective functions, sub-optimal

Background

Shewanella oneidensis MR-1 is an environmentally important and facultative anaerobic bacterium which has a versatile metabolism for carbon utilization and metal reduction [2]. MR-1 can play important role in ecological nutrient cycling, bioremediation, and microbial fuel cells [3]. To understand MR-1 metabolism, transcription analysis and proteomic profiling have often been used to reveal the dynamic physiological responses to different cultural conditions (such as pH, oxygen, and genetic modifications) [4-6]. On the other hand, metabolic flux analysis reflects the *in vivo* enzymatic reaction rates and reveals the functional output of genomic, transcriptional, and post-transcriptional regulation inside the cell [7]. Metabolic flux analysis has been performed to solve a simplified MR-1 metabolic network via isotopomer tracing [8,9]. For a cell-wide analysis of MR-1 carbon metabolism, an FBA model (774 reactions and 783 genes) has been developed, based on reaction stoichiometry in addition to a series of physical, chemical, and biological characteristics [10]. Such a genome-scale model allows systematic estimation of cellular ATP requirements, identification of metabolic cycles, prediction of biomass yields, and quantification of the metabolic fluxes.

Traditional flux analysis is based on a steady metabolic state, in which the intracellular flux distribution remains approximately stable [11-13]. This assumption avoids difficulties in developing kinetic models and measuring intracellular metabolite pools. However, MR-1 metabolism changes with time during batch cultivation with lactate as the initial carbon substrate. When the medium contains sufficient lactate, MR-1 uses it for growth and produces extracellular metabolites (acetate and pyruvate). Then, when the lactate concentration has become insufficient, it uses less energy-favorable pyruvate and acetate [14]. To model this metabolic behavior through the entire growth phase, we integrated growth kinetics with FBA

to illustrate the non-stationary metabolic behavior and the intracellular regulation mechanisms (Figure 1). A multiple-substrate Monod model was developed first to quantify the kinetics of cell growth and the dynamic transition between the primary carbon source (lactate) and the secondary carbon sources (pyruvate and acetate). The kinetics of carbon sources were then incorporated as time-dependent model inputs for dynamic FBA (dFBA). The dFBA was solved based on a Static Optimization Approach (SOA) [15], in which the growth period was divided into numerous time intervals so that a steady-state flux could be assumed at each time interval. The dFBA model can quantify the metabolic regulations in key pathways and link the dynamic metabolic status to gene expression and enzyme activity analysis. It has played an important role in analyzing the metabolic flux of industrial fermentation processes and revealing metabolic regulations [16,17]. Such an approach can avoid a long cultivation period to achieve a metabolic steady-state condition and can reduce analytical efforts for intracellular metabolite measurement. Thus it can be a widely applicable tool for analyzing other unsteady-state biological systems to gain novel biological insights into less well-characterized microorganisms.

Methods

Culture conditions and analytical methods for extracellular metabolites

MR-1 (ATCC 70050) was first grown in LB medium in shake flasks overnight. A 0.1% inoculum volume was then cultured into the modified MR-1 defined medium [18] in shaking flasks (150 mL, shaking 150 rpm) at 30°C. The initial carbon source was 30 mM lactate. The growth curve was monitored by dried biomass weight. The concentrations of lactate and acetate in the medium were measured using enzyme kits (r-Biopharm, Darmstadt, Germany). The concentration of pyruvate in the medium was measured with the enzyme assay developed by

Marbach and Weli [19]. The concentrations of all carbon sources were below detection limits after 30 hours.

Monod model and parameter estimation

For the FBA model, the inputs (measured fluxes) were obtained by analysis of the metabolite concentrations. The slope of extracellular metabolite concentrations during the cultivation captures the measured fluxes. To avoid measurement of metabolites at a short time scale, we developed a model, by which one could take the derivative at each time point to obtain the time-dependent measured fluxes. These "simulated" measured fluxes were used as model inputs for calculation of intracellular flux distributions. Recently, empirical polynomial fitting has been used to approximate the experimental data for dynamic flux analysis of *E.coli* during transition from carbon to nitrogen limitation conditions [20]. In this study, a multiple-substrate Monod model was developed to describe the cell growth, lactate consumption, and acetate and pyruvate secretion and simultaneous reuse (Figure 2). We assumed the pattern of mixed substrate utilization was generally dependent on their concentrations [21]. The culture displayed an apparent lag phase after inoculation, which can be described by implementing a unit-step time delay function in the biological system.

(1)

(2)

(3)

(4)

where X was biomass (g DCW/L); LACT, ACT, and PYR were lactate, acetate, and pyruvate concentrations (mmol/L), respectively; μ_L, μ_A, and μ_P were the specific growth rates (h^{-1}) on lactate, acetate, and pyruvate, respectively; k_e was endogenous metabolism rate constant (h^{-1}) [1]; $Y_{X/L}$, $Y_{X/A}$, and $Y_{X/P}$ were the biomass yield coefficients (g DCW/mol substrate) of lactate, acetate, and pyruvate respectively; $r_{P,L}$ and $r_{A,L}$ were the production rates (mmol/L/h) of acetate and pyruvate from lactate, respectively. $r_{ng,L}$, $r_{ng,A}$, and $r_{ng,P}$ were the non-growth-associated substrate consumption rates (mmol/L/h) for lactate, acetate, and pyruvate, respectively, which reflected non-growth-associated cell metabolism (e.g., maintenance metabolism) during aerobic respiration [22]. Introduction of the non-growth associated terms to the kinetic model allowed a better "fitting" of observed experimental data. $S(t-t_L)$ was the dimensionless unit-step time delay function ($S=0$ when $t < t_L$; $S = 1$ when $t = t_L$). The specific cell growth rate was described by Monod equations:

(5)

(6)

(7)

where $\mu_{max,L}$, $\mu_{max,A}$, and $\mu_{max,P}$ were the um specific growth rates (h^{-1}) for fully aerobic growth on lactate, acetate, and pyruvate, respectively; $K_{s,l}$, $K_{s,a}$, and $K_{s,p}$ were Monod constants (mmol/L) for lactate, acetate, and pyruvate, respectively. The acetate and pyruvate production rates were assumed to be proportional to the biomass and lactate concentrations, as indicated by previous *Shewanella* kinetic model [21].

(8)

(9)

(9)

(9)

where k_a and k_p were acetate and pyruvate production rate constants ($L\cdot (h\cdot g\ DCW)^{-1}$).

In initial tests, the rate of non-growth associated substrate consumption was assumed to be proportional only to the biomass. However, it was found that such a model was not able to closely predict acetate formation and biomass curves, especially during the late growth phase when lactate was depleted. Therefore, the non-growth associated substrate consumptions were subsequently assumed to be proportional to both biomass and substrate concentrations.

(10)

(11)

(12)

where $k_{ng,L}$, $k_{ng,a}$, and $k_{ng,p}$were non-growth substrate consumption coefficients ($L\cdot(hr\cdot gDCW)^{-1}$) for lactate, acetate, and pyruvate, respectively. These coefficients reflected that the maintenance metabolism was dependent upon both substrate availability and biomass.

The parameters of the kinetic model in Table 1 were obtained by model fitting to experimental data (Figure 3a and b). The aforementioned kinetic model (Equations 1–12) contains a total of 16 kinetic parameters. The maximum specific growth rate using lactate ($\mu_{max,L}$) and apparent biomass yield coefficient from lactate ($Y_{X/L}$) were taken from our previous research [21]. The lag time for growth was measured directly (10 hrs). The remaining 13 parameters were determined by minimizing the inverse-variance weighted least squares between the model's predictions and the experimentally observed growth and metabolite profiles, as described before [21]. The "ode23" command and Systems Biology Toolbox 2 [23] in MATLAB (R2009a) were used to solve differential equations, fit parameters, and obtain model standard deviations.

Dynamic flux balance model and sensitivity analysis

The pathway map for *S. oneidensis* MR-1 was derived from the KEGG database (www.genome.jp/kegg), which included the TCA cycle (with the glyoxylate shunt), the ED pathway, gluconeogenesis pathway, anaplerotic pathway, and the pentose phosphate pathway. The preliminary FBA model included 236 key reactions and 213 metabolites (Supplementary Table S1). The solution to the dynamic metabolism for sequential utilization of multiple carbon sources was based on a static optimization approach, where the entire growth phase was divided into multiple pseudo-steady-state intervals with instantaneous transitions between the two adjacent intervals [15]. The time profiles of lactate, acetate, and pyruvate simulated from Monod model were used as time-dependent inputs for the overflow fluxes in the dFBA model:

—

where Obj was the objective function for FBA in each time interval (note: maximization of growth rate μ was set as the default objective function if not otherwise specified). v and v_{ext} were intracellular and extracellular fluxes, respectively; S and S_{ext} were the stoichiometry matrices for intracellular and extracellular fluxes, respectively; lb and ub were the lower and upper boundaries for each flux. In the dFBA model, three types of objective function were chosen to describe the dynamic intracellular metabolism: 1) maximizing optimal growth rate μ; 2) maximizing ATP production per flux; 3) maximizing ATP production [24]. The dynamic flux balance model was formulated using AMPL (A Modeling Language for Mathematical Programming) and solved by IPOPT (Interior Point Optimizer, https://projects.coin-or.org/

Ipopt), which is a software package for large-scale nonlinear optimization. The entire time lapse (30 hours) was divided into 360 segments, with 5 minutes in each segment. Each individual optimization problem was solved by IPOPT in less than 1s.

The analysis of confidence intervals was performed based on the Monte Carlo method [25]. The confidence intervals for calculated fluxes during the dynamic utilization of carbon sources were generated by adding the normally distributed measurement noises (assuming 15% as an average) to the six substrate associated specific growth rates (i.e. $\mu_{max,L}$, $\mu_{max,A}$, $\mu_{max,P}$, $K_{s,l}$, $K_{s,a}$, and $K_{s,p}$). The perturbation of parameters in the Monod model led to different simulation time profiles for lactate, acetate and pyruvate, which provided different constraints for overflow fluxes in the dFBA model. Confidence limits for each flux value were obtained from the probability distribution of calculated fluxes resulting from the simulated data sets (n=50).

Quantitative Reverse Transcription PCR (qRT-PCR) and Enzyme Activity Measurement

To analyze the dynamic change of expressions in eight key genes (aceE, gltA, aceB, ppsA, sfcA, ppc, pckA, and icd) for carbon metabolisms, qRT-PCR was performed for the culture samples at early-log (13 hr), mid-log (19 hr), late-log (23 hr), and stationary phase (27 hr) (Figure 3c). RNA extraction was performed using a PureLink™ RNA Mini Kit (Invitrogen). cDNA was synthesized from 1~2 μg RNA and random primers using Superscript III reverse transcriptase (Invitrogen). The qRT-PCR reactions were performed via ABI 7500 Real-Time PCR System (Applied Biosystems). qRT-PCR used the primers shown in Table 2. The SYBR Green Master Mix (Promega) was used for amplifying DNA. The cycle threshold (Ct) was determined as the cycle number at which the fluorescence threshold crossed the baseline. Data were normalized by analyzing:

ΔCt = Ct of the target gene – Ct of the internal control gene (16S rRNA)

Each relative gene expression value was calculated with $2^{-(\Delta Ct)}$. Three biological replicates, with six technical replicates for each biological sample were performed. The PCR products were verified by 2% agarose gel electrophoresis.

To analyze the enzyme activity as a function of time, the samples were taken at early-log (13 hr), mid-log (19 hr), late-log (23 hr), and in the stationary phase (27 hr) (Figure 3d). The harvested cells were centrifuged and re-suspended in 100 mM Tris buffer. The samples were then ultra-sonicated for 5 min to release the enzymes. The activity of isocitrate dehyrogenase was detected by Kornberg's assay [26], which measured the enzyme activity based on the increase in absorbance at 340 nm associated with the reduction of $NADP^+$ to NADPH. The activity of citrate synthase was detected by quantification of the CoA production, using the increase in absorbance associated with DTNB (5,5'-Dithio-bis(2-nitrobenzoic acid)) at 412 nm [27]. The malate synthase activity was detected based on reaction of CoASH with DTNB, as described by Dixon and Kornberg [28]. The activity of malic enzyme was detected based on increased absorbance at 340 nm due to the reduction of NAD^+ to NADH [29]. For all samples, three biological replicates were performed.

Results

Growth kinetics and dynamic intracellular fluxes

Model fitting of kinetic parameters (Table 1) was based on comparing the assumed model with experimental data. Figure 3a and b showed that the kinetic model well described the growth

and metabolite curves: initially MR-1 showed minimal growth during the lag phase, followed by a rapid population increase after 15 hrs; in parallel with this, the acetate and pyruvate rapidly produced and reached their maxima around 20 hrs. The kinetic model estimated model inputs for dynamic FBA at each discrete time interval. Figure 4 shows the metabolism profile of MR-1 at four different growth phases (13, 19, 23, 27 hrs). In the first and second sub-phase, lactate was quickly consumed, with overflows of pyruvate and acetate as waste products. In the 3rd and 4th sub-phase, the carbon source switched from lactate to a mixture of substrates (lactate, pyruvate and acetate). A recent report indicates that *E.coli* uses available substrates from a mixture of five carbon sources (glucose, galactose, maltose, glycerol, and lactate) either preferentially or simultaneously depending on the growth stages [30]. Similarly, in this study the growth of *S. oneidensis* MR-1 in multiple carbon substrates consumed its metabolic "overflow" products. During the periods of sequential utilization/production of carbon sources, the fluxes between intracellular and extracellular pyruvate and acetate were bi-directional (Figure 4).

During the entire growth phase, fluxes into the pentose phosphate pathway and Entner-Doudoroff (ED) pathway were below 0.1 mmol/g DCW/hr and were used for biomass synthesis (Figure 4). Fluxes into central pathways including the TCA cycle, anaplerotic pathways, and the gluconeogensis pathway demonstrated similar dynamic patterns. In general, two peaks can be observed in the time profile of intracellular fluxes through the TCA cycle and anaplerotic pathways. The first peak was achieved in the early-log phase, followed by a slow decrease of intracellular fluxes into a pseudo steady-state (Figure 5), concurrent with the consumption of lactate and accumulation of waste products (pyruvate and acetate). Then the activity of central pathways was slightly up-regulated, and forming the second flux peak (much smaller than the

first one) when waste products became utilized as carbon sources. The glyoxylate shunt bypass was inactive (i.e., flux reached the lower bound in dFBA) until the late growth phase when lactate was depleted. Such characteristics of the glyoxylate shunt agreed with the prediction of genome-scale FBA [10] and observations from ^{13}C-assisted metabolic flux analysis [14]. In the early growth phase, the pyruvate dehydrogenase pathway became highly active, generating the acetyl-CoA from lactate. In the late growth phase, acetyl-CoA was completely replenished from extracellular acetate, and thus pyruvate decarboxylation was inactive (Figure 5).

In the dynamic metabolism, more ATP was produced than needed for cell growth, thus sustaining the non-growth associated metabolism (e.g., maintenance). This non-growth associated ATP production was dependent on the growth rates (Figure 6). The ATP over-production increased with the elevation of growth rate μ. However, the slope for ATP-μ was not identical in the slow-growth phase ($0.1<\mu<0.3$ h^{-1}, slope = 206.9 mmol ATP/g DCW) and the high-growth phase ($0.5<\mu<0.9$ h^{-1}, slope = 678.9 mmol ATP/g DCW). This observation indicates that high growth cells could over-produce ATP for their maintenance or other non-growth associated activities, while reducing the "optimal" biomass yield and product synthesis [31].

Temporal gene expression and enzyme activity

The temporal expression levels of several key genes in the central metabolism also demonstrated dynamic patterns during the growth period (Figure 3c). The expressions of tested genes were first down-regulated during the transition of carbon sources in the mid-log phase and then up-regulated when pyruvate and acetate were utilized in the late growth phase. With the depletion of carbon sources, the genes' expressions were again down-regulated in the stationary phase. In general, the transcriptional level of genes in the central metabolism was one

order of magnitude higher in the late growth phase than in the early growth phase. This observation could be explained by the gene expressions for the central pathways becoming enhanced to adapt to conditions when the favorable carbon source (lactate) was insufficient and "over-flow" products were used as substitute carbon sources. The temporal gene expressions of key pathways in the central metabolism were changed with time, which were qualitatively consistent with the dynamic flux distributions simulated by dFBA (Figure 5), but there were still discrepancies. For example, the transcriptional analysis indicated the presence of pyruvate dehydrogenase expression during early and late growth phases, respectively. In contrast, the dFBA showed that such a metabolic reaction was not active in the late growth phase when substrate (pyruvate or lactate) was unavailable (even associated genes had high expression levels). The temporal activities of TCA cycle enzymes (citrate synthase, isocitrate dehydrogenase, malate synthase, and malic enzyme) are also shown in Figure 3d. One interesting observation is that *in vitro* activity of isocitrate dehydrogenase was relative stable in the late growth phase (acetate metabolism), in contrast to up-regulation of malate synthase activity (acetyl-CoA+H_2O+glyoxylate→malate). This observation indicated that malate synthase controls the split ratio of the glyoxylate shunt to the isocitrate dehydrogenase pathway (oxidative TCA branch).

Discussion

The multiple-substrate Monod model described the physiological behavior in dynamic growth of *S. oneidensis* MR-1with the kinetic transition of carbon sources. Based on the kinetic

model of providing the extracellular fluxes as the function of time, a steady-state flux balance model was conducted to investigate the dynamics of intracellular flux at individual short-time intervals. The integration of the Monod model and dFBA provides a general platform for characterizing the unsteady-state microbial systems.

Different FBA objective functions can describe the intracellular metabolism under different cultivation modes [24]. We compared three objective functions for predicting dynamic biomass growth in Figure 7. In the early growth phase when lactate was sufficient, objective functions (optimal biomass production or maximal ATP production per flux) predicted biomass rates relatively close to the measured growth data, while the biomass yield was underestimated when assuming the maximization ATP production in the intracellular dynamic metabolism. In the late growth phase, both maximizing growth rate and ATP production per flux led to overestimation of biomass yield by 30~50%. This differences indicates that the metabolisms might be under significantly "suboptimal" condition during the switching of carbon sources from energy-favorable lactate to less energy efficient acetate [32]. Consistent with previous report [24], the maximization of the ATP yield per flux best characterized the dynamic intracellular metabolism for batch cultures of MR-1, though it is still difficult for a single objective function to precisely predict the biomass production through cultivation period by dFBA. When growing in mixed substrates, the bacteria may display clear stress responses during the change of metabolic status from one carbon substrate to another [30]. Such suboptimal operation of cellular metabolism may give difficulties for FBA model to describe the actual metabolism. Therefore, ^{13}C-based dynamic metabolic flux analysis needs to be integrated in future research to understand sub-optimal metabolism.

The kinetics of intracellular fluxes in central pathways was qualitatively confirmed

by comparing gene expression levels and enzyme activities at different time intervals. Post-transcriptional regulation could explain the adjustment of intracellular metabolism in adapting to the dynamic availability of substrates. The pseudo steady-state that was normally chosen for metabolic flux analysis (e.g., ^{13}C-MFA or FBA) was observed only in the early log phases (12-20 h, Figure 8) during MR-1 growth, when most key intracellular fluxes remained the same. Furthermore, energy metabolism in MR-1 is also dynamic regulated. The over-production of non-growth associated ATP was associated with the growth rates (Figure 6), which could be explained by the fact that the metabolic dormant cell population was higher under slow-growth phase (e.g. lag phase or stationary phase) so that the ATP requirement for maintaining active metabolism was not as strong as for high-growing cells [33].

List of Abbreviations:

3PG: 3-phosphoglycerate; **6PG:** 6-phosphogluconate; **ACCOA:** acetyl-CoA; **ACT:** acetate; **AKG:** α-ketoglutarate; **C5P pool:** xylulose 5-phosphate, ribose 5-phosphate and ribulose 5-phosphate; **CIT:** citrate; **E4P:** erythrose 4-phosphate; **F6P:** Fructose 6-phosphate; **FBA:** flux balance analysis; **FBP:** Fructose 1,6-bisphosphate; **FUM:** fumarate; **G6P:** Glucose 6-phosphate; **GAP:** glyceraldehyde 3-phosphate; **GLY:** glyoxylate; **ICIT:** isocitrate; **LACT:** lactate; **MAL:** malate; **OAC:** oxaloacetate; **PEP:** phosphoenolpyruvate; **PYR:** pyruvate; **S7P:** sedoheptulose 7-phosphate; **SOA:** Static Optimization Approach; **^{13}C-MFA:** ^{13}C-assisted metabolic flux analysis.

Authors' contributions

XF and YJT conceived the study, developed the Monod model, prepared figures and tables, and wrote the manuscript. XF, YX and YC integrated the Monod model with dynamic flux balance analysis and implemented the analysis of confidence intervals. XF, YX, YC, and YJT revised the manuscript. All authors read and approved the final manuscript.

Acknowledgements

We appreciate Jing Jiang's for help on qRT-PCR. This study is supported by an NSF Career Grant (MCB0954016) to YJT.

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Figure Legends

Figure 1. Flowchart of integrated growth kinetics and dynamic flux balance analysis.

Figure 2. Monod model for growth kinetics of *S. oneidensis* MR-1. The mixed substrates are used for both biomass synthesis and non-growth associated maintenance metabolisms (i.e., CO_2 production).

Figure 3. Physiological study for *S. oneidensis* MR-1. (**A**) Carbon source consumption: ● lactate; ■ acetate; ▲pyruvate. The simulated curves based on revised dFBA model for each carbon source are plotted in solid lines respectively; (**B**) Growth curve of *S. oneidensis* MR-1: ● experimental observed cell growth; the simulated growth curve by revised dFBA is plotted in solid line; (**C**) Temporal expression levels of key genes in the central carbon metabolism. Gene name (encoding enzyme, EC number): *gltA* (citrate synthase, EC 2.3.3.1), *icd* (isocitrate dehydrogenase, EC 1.1.1.42), *sfcA* (malic enzyme, EC 1.1.1.38), *aceB* (malate synthase, EC 2.3.3.9), *aceE* (pyruvate dehydrogenase E1 component, EC 1.2.4.1), *pckA* (phosphoenolpyruvate carboxykinase (ATP), EC 4.1.1.49), *ppc* (phosphoenolpyruvate carboxylase, EC 4.1.1.31), *ppsA* (phosphoenolpyruvate synthase, EC 2.7.9.2). Black column, sample time at 13 hr; red column, sample time at 19 hr; green column, sample time at 23 hr; yellow column, sample time at 27 hr; (**D**) Activity of key enzymes in central metabolic pathways. Black column, sample time at 13 hr; red column, sample time at 19 hr; green column, sample time at 23 hr. CS, citrate synthase; IDH, isocitrate dehydrogenase; MS, malate synthase; ME, malic enzyme.

Figure 4. Flux distribution (mmol/gDCW/hr) of *S. oneidensis* MR-1 at (**A**) T=13 hr; (**B**) T=19 hr; (**C**) T=23 hr and (**D**) T=27 hr.

Figure 5. Time profiles of selected intracellular fluxes. The shaded area indicates the confidence intervals of intracellular fluxes (flux±SD).

Figure 6. ATP production for non-growth associated usage.

Figure 7. Comparison of growth curve simulated by different objective functions applied in dynamic flux balance analysis. Red line, simulated growth curve by maximizing μ; blue line, simulated growth curve by maximizing ATP production per flux; green line, simulated growth

curve by maximizing ATP production; circles, measured data.

Figure 8. Compare of key intracellular fluxes at different time points. Black bar, fluxes at T=13 hr; red bar, fluxes at T=19 hr; green bar, fluxes at T=23 hr; blue bar, fluxes at T=27 hr.

Table.1 Parameters of Monod model for S. oneidensis MR-1 growth

Symbols	Notation	Unit	Value
μmax,L	Maximum specific growth rate using lactate	h-1	0.47[21]
μmax,P	Maximum specific growth rate using pyruvate	h-1	0.43±0.02
μmax,A	Maximum specific growth rate using acetate	h-1	0.37±0.04
YX/L	Apparent biomass yield coefficient from lactate	g DCW/mol lactate	19.1[21]
YX/P	Apparent biomass yield coefficient from pyruvate	g DCW/mol pyruvate	17.8±2.3
YX/A	Apparent biomass yield coefficient from acetate	g DCW/mol actate	12.4±1.6

Ks,l	Monod lactate saturation constant	mM	8.3±1.2
Ks,p	Monod pyruvate saturation constant	mM	8.1±1.4
Ks,a	Monod actate saturation constant	mM	9.3±1.5
kng,l	Non-growth lactate consumption coefficient	L· (h·g DCW)-1	1.0±0.2
kng,p	Non-growth pyruvate consumption coefficient	L· (h·g DCW)-1	2.1±0.3
kng,a	Non-growth acetate consumption coefficient	L· (h·g DCW)-1	1.1±0.2
ka	Acetate production coefficient	L· (h·g DCW)-1	1.7±0.3
kp	Pyruvate production coefficient	L· (h·g DCW)-1	0.9±0.2
ke	Endogenous metabolism rate constant	h-1	0.009±0.010
tL	Lag time in growth	h	10.0

Table.2 Primers used in qRT-PCR

Enzyme	Gene	Forward Primer/Reverse Primer (5'→3')
pyruvate dehydrogenase, E1 (EC 1.2.4.1)	aceE	TATGGTGCTTCGTGGTTC CCTGATAAATCGCTTGGA
citrate synthase (EC 2.3.3.1)	gltA	TTAGAGGCTTCCGTCGTG AATACCCGCAGCGATACA
malate synthase (EC 2.3.3.9)	aceB	AAGCGTAAAGATAGACAAGC CAAACCTCCAGGGATAGA

PEP synthase (EC 2.7.9.2)	ppsA	CTGCCGTTGTAGGTTGTG TTCTTGTAAAGCCGCATC
Malic enzyme (EC 1.1.1.38)	sfcA	AAGGAACGCTACTGCTGC AACGGTCAACCATACAAACT
PEP carboxylase (EC 4.1.1.31)	ppc	ATTCTGTCGCAACCACCC TGACTCCTCAGCAATACGC
PEP carboxylase (ATP) (EC 4.1.1.49)	pckA	CGGCTTACCACTTCCTCT CACTTGGCTACCGAATGAC
isocitrate dehydrogenase (EC 1.1.1.42)	icd	TGCGTGACTATTTGACTGA TACCCACTGTTTGGCTTA
16S rRNA	16S rDNA	GGGAGCAAACAGGATTAGA CACAACACGAGCTGACGA

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8