

# Constraint-based *in silico* modelling of the Fe(III)-reducing bacteria *Geobacter sulfurreducens*: insights into the subsurface microbial activity

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## 1. Abstract

Here we describe the application of the constraint-based modeling approach, coupled in an iterative fashion with experimental studies, to further elucidate the physiology of *Geobacter sulfurreducens*, a well-studied representative of the Geobacteraceae, which play a critical role in organic matter oxidation coupled to Fe(III) reduction, bioremediation of groundwater contaminated with organics or metals, and electricity production from waste organic matter. The completed reconstructed metabolic network of *G. sulfurreducens* contained 588 genes (or 17% of a total of 3,467 ORFs), 522 biochemical reactions, and 541 unique metabolites. Examination of the reconstructed metabolic network revealed that *G. sulfurreducens* has multiple reactions for acetate utilization, the main electron-donor for these bacteria in the subsurface. Simulations fit well with experimental data obtained from chemostat studies, predicting different flux rates and growth yield under a number of growth rates. Evaluation of the rates of proton production and consumption in the extracellular and cytoplasmic compartments revealed the energy conservation with extracellular electron acceptors as Fe(III), was limited compared to intracellular acceptors as fumarate. These results demonstrate that iterative modeling coupled with experimentation can accelerate the understanding of the physiology of poorly studied but environmentally relevant organisms and may help optimize their practical applications.

## 2. Introduction

The constraint-based approach to modeling microbial metabolism has proven to be an effective strategy for predicting the physiological responses of microorganisms (Price et al, 2003). This approach relies on implementing a

series of physico-chemical constraints including thermodynamic directionality, and enzymatic capacity constraints and reaction stoichiometry constraints arising from the requirement that fluxes consuming and producing both metabolites and protons are balanced. This systems approach to microbial physiology has the ability to predict the metabolic response of organisms to various environmental conditions without the need for information on kinetic parameters for each of the individual reactions (Edwards and Palsson, 2000). Substrates that can be metabolized and the nutrients that are required from the environment to support growth can be successfully predicted, as can growth rates under various conditions.

Although all those models have been limited to *Escherichia coli* and pathogens (Edward and Palsson, 1999; Edward and Palsson, 2000; Schilling et al., 2002), this methodology should be able to predict the behaviour of microorganisms in more remote environments where they are of geomicrobiological relevance. Here we describe the application of this constraint-based modeling approach, coupled in an iterative fashion with experimental studies, to further elucidate the physiology of *Geobacter* species (Mahadevan et al., 2006), the first organisms found to have the ability to conserve energy for growth by completely oxidizing organic compounds to carbon dioxide with Fe(III) serving as the electron acceptor (Lovley et al., 1987; Caccavo et al., 1994). In addition to transferring electrons to <sup>\*</sup>Fe(III), *Geobacter* species can also reduce a variety of toxic and radioactive metals (Lovley et al., 1991; Lloyd et al., 2000; Ortiz-Bernad et al., 2004). Moreover, stimulating the activity of *Geobacter* species in the subsurface is an effective strategy for removing such contaminants from groundwater (Lovley et al. 1994). Another practical application of *Geobacter* species is their ability to oxidize organic compounds with an electrode serving as the electron acceptor (Bond et al., 2002; Bond and Lovley, 2003), which makes it possible to harvest electricity from waste organic matter.

To develop this kind of model, a complete sequenced genome of the microorganism is required, thus *Geobacter sulfurreducens* is the best candidate to be modelled because it is closely related to the environmental strains isolated from the subsurface and its genome had been recently sequenced (Methe et al., 2003). In addition, a chemostat system has been developed (Esteve-Núñez et al., 2005), to further evaluate *in silico* predictions with well established growth conditions. Modelling growth and metabolism under relevant environmental conditions could provide an insight into the factors that might be limiting the rate and extent of bioremediation processes at contaminated sites.

### 3. Theoretical

This section provides a brief introduction to the constraint-based modelling approach that has been extensively reviewed elsewhere (Price et al., 2004). In this work, we have used the flux balance analysis approach which assumes that

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the cellular objective is growth maximization, to calculate the flux distribution in the metabolic network given the input and output fluxes of substrates exchanged across the membrane.

### 3.1. Flux Balance Analysis (FBA):

FBA is an analysis tool to quantitatively investigate the systemic properties of a metabolic network. It is based on material balances for each of the internal metabolites and the assumption of optimal growth as the objective of the cell. The details of FBA and the significance of the objective function have been reviewed earlier . The FBA formulation includes a series of linear equations (material balances) and a linear objective function with flux through the reactions as the independent variables as shown below:

$$\begin{aligned} \text{Max } & c^T v \\ S \cdot v &= 0 \\ \alpha \leq v &\leq \beta \end{aligned}$$

These equations (see symbols section) are solved along with constraints on the fluxes and an objective defined in terms of the biomass growth rate (based on the biomass composition) using Linear Programming (LP) techniques in the SimPheny platform.

## 4. Experimental

**4.1. Strain and Culturing Conditions:** Wild type *Geobacter sulfurreducens* (ATCC 51573) was obtained from our laboratory collection. *G. sulfurreducens* was grown in a chemostat under continuous culture and strict anaerobic conditions at 30 °C using previously described method (Esteve-Núñez et al., 2005). Sodium acetate (5.5mM) was used as sole electron donor, and either sodium fumarate (30mM) or Fe(III)-citrate (60mM) were used as electron acceptor in a bicarbonate-buffered freshwater medium. Organic acids content in culture supernatant were monitored by HPLC as previously described (Esteve-Núñez et al., 2005), and Fe(II) was determined as described by Lovley and Phillips (1982).

**4.2. The genome-scale metabolic model** for *G. sulfurreducens* was developed using the constraint-based modeling approach (Bonarius et al., 1997) and the SimPheny<sup>TM</sup> (Genomatica, San Diego, CA) platform (Mahadevan et al. 2006). BLAST searches of publicly available databases (Overbeek et al., 2000) resulted in the identification of 588 genes (or 17% of a total of 3467 ORFs). The completed reconstructed metabolic network contained 522 biochemical reactions, and 541 unique metabolites. These reactions were further refined using published biochemical and physiological information. To allow full stoichiometric balancing, all reactions were entered into the model database as balanced reactions, including the net charge of each metabolite or cofactor and the localization (cytoplasmic or extracellular) of reactants and products. For all simulations presented in this report, all genes included in the network were

assumed to be expressed and their associated reactions functional. Maximization of biomass production (growth) was the objective for all the simulations. The complete list of genes, reactions, applied constraints, and confidence scores is available at the following website (<http://www.geobacter.org/>).

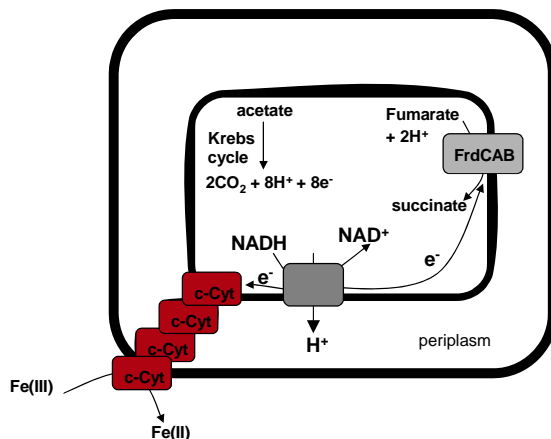
## 5. Results and Discussion

### 5.1. Evaluation of the proton translocation stoichiometry give insights into the growth yield during Fe(III) and fumarate reduction.

*Geobacter sulfurreducens* is able to use either the metal Fe(III) or the dicarboxylate acid fumarate to conserve energy from acetate oxidation. However, these two respiratory mechanism are quite different, fumarate reduction is an intracellular and well characterized process catalyzed by the enzyme FrdCAB (Butler et al., 2006), while the biochemical mechanism responsible of Fe(III) reduction is much more complex with a high number of cytochromes c involved in electron transport (Leang et al., 2001; Lloyd et al., 2000; Butler et al., 2004; Kim et al., 2005, 2006).

Fe(III) reduction was first modelled as a reaction that occurred outside the cell, consistent with the fact that insoluble Fe(III) oxides are the predominant form of Fe(III) in most soils and sediments (Lovley 1991). Under Fe(III)-reducing conditions, the TCA cycle operated as a closed loop (Galushko and Schink, 2000) and produced 8 electrons per mole of acetate oxidized. However, model simulations using this electron transport scheme indicated that cells would not be capable of growth (*in silico*) under Fe(III)-reducing conditions.

The inability of a single  $2\text{H}^+/2\text{e}^-$  NADH dehydrogenase coupling site to support simulated Fe(III)-dependent growth was traced to the fact that the site of Fe(III)-reduction was extracellular. The cytoplasmic protons that were produced from each mole of acetate oxidized in the cytoplasm were consumed in the cytoplasm when fumarate was the electron acceptor (Figure 1). In contrast, during Fe(III) reduction, electrons were transported outside the cell, while leaving protons in the cytoplasm, effectively dissipating the membrane potential and acidifying the cytoplasm (Figure 1). In order to generate sufficient energy to compensate for the production of protons in the cytoplasm, an additional coupling step was required.



**Figure 1.** A model for proton and electron consumption in fumarate and Fe(III) reduction by *Geobacter sulfurreducens*

The most likely mechanism for additional membrane potential generation during Fe(III)-reduction was during transfer of electrons into the periplasmic cytochrome pool. Based on the fact that cytochromes implicated in Fe(III) reduction have midpoint potentials in the range of  $-190$  mV (*omcB*) (Magnuson et al., 2000) and  $-136$  to  $-155$  mV (*ppcA*) (Lloyd et al., 2002), the energy available for coupling at this site could support translocation of  $1\text{H}^+/2\text{e}^-$ . This reaction was modeled as the release of menaquinol protons back to the cytoplasm by a protein capable of translocating  $1\text{H}^+$  per pair of electrons transferred to the cytochrome pool. Inclusion of this reaction and accounting for all the protons produced and consumed during metabolism, resulted in a theoretical maximum yield with Fe(III) as the electron acceptor of  $0.5$  mol ATP/mol acetate as compared to the  $1.5$  mol ATP/mol acetate during fumarate reduction. This output of the model provides an explanation for the experimental finding that growth yields of *G. sulfurreducens* are ca. three-fold higher when fumarate ( $E_h=0.03\text{V}$ ) serves as the terminal electron acceptor versus growth with Fe(III)-citrate ( $E_h=0.37\text{V}$ ) (Esteve-Núñez et al., 2004), in spite of the higher redox potential of the metal. This result is unexpected because it is generally accepted that those electron acceptors with higher redox potential show a more negative Gibbs free energy and subsequently support higher yield (Unde and Bongaerts, 1997). These results suggest that reducing extracellular electron acceptors such as Fe(III) oxides, Fe(III)-citrate, elemental sulfur ( $\text{S}^0$ ), or electrodes will result in the generation of less biomass per electron transferred than growth with intracellularly reduced electron acceptors. This may be an important consideration for applications such as bioremediation and electricity harvesting from waste organic matter, in which electron transfer to metals or electrodes, rather than production of biomass, is the primary goal.

## 5.2. Growth yield predictions fit with the experimental data.

The metabolic reaction network, combined with demand reactions for biomass synthesis, correctly predicted growth yields and acetate consumption rates for growth in standard acetate-limited chemostats with Fe(III)-citrate or fumarate as the electron acceptor (Table 1).

**Table 1:** *in silico* prediction and experimental values for growth parameters of *G. sulfurreducens* growing under Fe(III)/fumarate-respiring conditions.

Growth parameter with Fe(III) as TEA	<i>in silico</i> ( $0.05\text{h}^{-1}$ )	experimental ( $0.05\text{h}^{-1}$ )
$Y_{\text{acetate}}$ (gdw /mol acetate)* $10^3$	4.5	3.5
$q_{\text{electron}}$ (mol/g dw h) * $10^3$	83.2	107.61

Growth parameter with Fumarate as TEA	<i>in silico</i> ( $0.05\text{h}^{-1}$ )	experimental ( $0.05\text{h}^{-1}$ )
$Y_{\text{acetate}}$ (gdw /mol acetate) * $10^3$	11.5	11.5
$q_{\text{fumarate}}$ (mol/g dw h) * $10^3$	16.425	19.21

Perturbations in variables used to construct the model, such as the biomass composition, which was derived from batch cultures of fumarate grown cells, had minimal effect on predicted acetate consumption for *G. sulfurreducens* under a number of growth rates. For instance, when a range of biomass composition equations (e.g., reflecting a range from 0.40 g protein/ g dw to 0.55 g protein/ g dw), were incorporated into the model, predicted yields were not significantly affected (1.5-2.5 % differences) (Fig. 2). This revealed that the model was robust to changes in biomass composition and nutrient availability, and was consistent with other work showing that variations in biomass composition produce only subtle effects on predicted growth yields or fluxes through central metabolic pathways (Pramanik and Keasling, 1998; Daae et al., 1999). Hence, it is possible to assume that even significant changes (10-20 %) in biomass composition would not affect the nature of metabolic predictions.

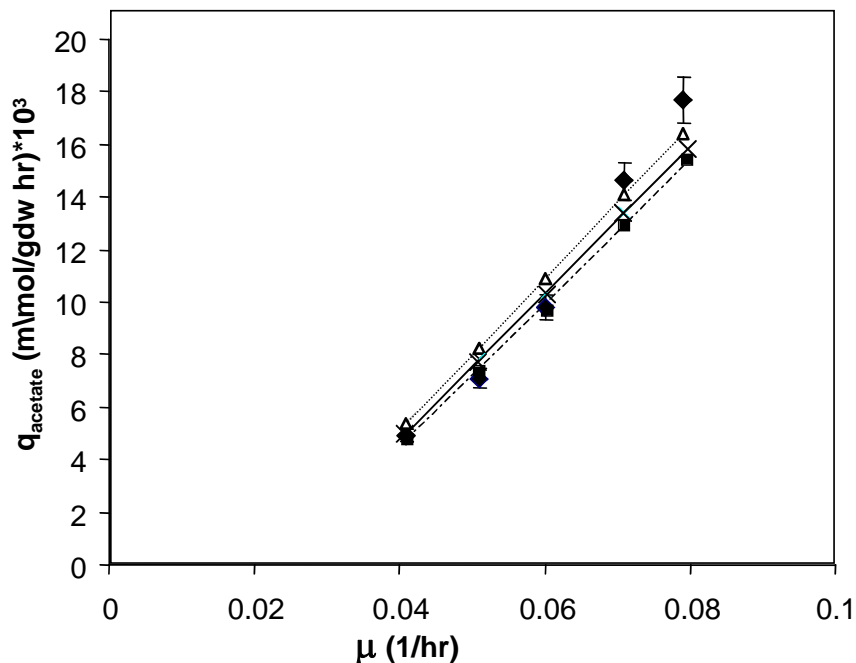


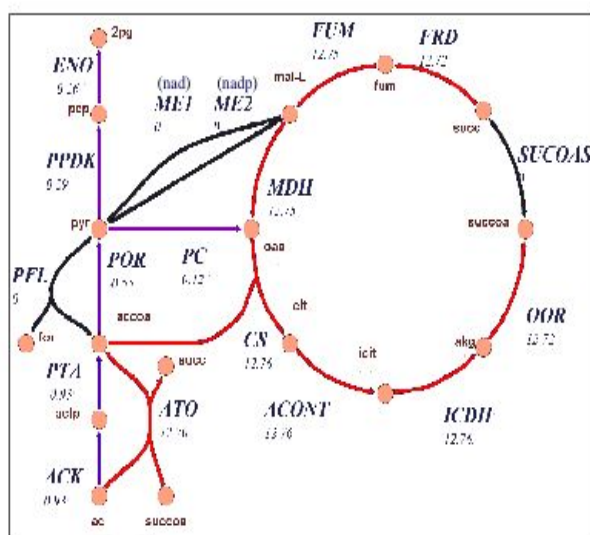
Figure 2. Acetate uptake predictions under different growth rate with Fe(III) as TEA. Experimental data: (◆), model prediction 40% protein (×), model prediction 46% protein (■), model prediction 55% protein (△),

### 5.3. Model-based characterization of acetate metabolism in *Geobacter sulfurreducens*

The ability to oxidize acetate is important because acetate is the central intermediate in the anaerobic degradation of organic matter in sedimentary environments (Lovley and Chapelle, 1995). *Geobacter* species metabolize acetate via the tricarboxylic acid cycle (TCA) cycle (Champine and Goodwin, 1991; Galushko and Schink, 2000). In addition, it has been found that injection of acetate into groundwater to stimulate the uranium bioremediation activity of

*Geobacter* results in this microbial genus becoming the most abundant one in those environments (Anderson et al., 2003; Vrionis et al., 2005). Thus, an extensive analysis of acetate metabolism in *Geobacter* is desired.

Examination of the reconstructed metabolic network revealed that *G. sulfurreducens* has multiple pathways for acetate utilization (acetyl CoA transferase, acetate kinase, and phosphotransacetylase), interconversion of pyruvate to acetyl-CoA (pyruvate formate lyase, pyruvate ferredoxin oxidoreductase, and pyruvate dehydrogenase), and anapleurotic reactions (phosphoenolpyruvate carboxykinase and pyruvate carboxylase), as shown in Figure 3.



**Figure 3.** Predicted flux distribution (mmol/gdw h) through central metabolism in *G. sulfurreducens* during *in silico* growth with limiting acetate and excess Fe(III)-citrate. Ac, acetate; fum, fumarate; succ, succinate; succoA, succinyl-CoA; actp, acetylphosphate; for, formate; acCoA, acetyl-CoA; pyr, Pyruvate; pep, phosphoenolpyruvate; cit, citrate; icit, isocitrate; akg, alpha-ketoglutarate; mal, malate; oaa, oxalacetate; ACK, acetate kinase; ATO, acetyl-CoA transferase; PTA, phosphate transacetylase; PFL, pyruvate formate lyase; POR, pyruvate oxidoreductase; PC, pyruvate kinase; PPDK, pyruvate phosphate dikinase; ENO, enolase; CS, citrate synthase; ACONT, aconitase; ICDH, isocitrate dehydrogenase; OOR, oxoglutarate oxidoreductase; SUCOAS, succinyl-CoA synthetase; FRD, fumarate reductase; FUM, fumarase; ME, malic enzyme.

Flux from acetyl-CoA to pyruvate via pyruvate-ferredoxin oxidoreductase was predicted to be the sole source of carbon fixation in *G. sulfurreducens*, and *in silico*, 4% of consumed acetate (0.55 mmol/g dw/h) was utilized in this fixation reaction when Fe(III)-citrate was the electron acceptor. Simulations predicted that during acetate-limited growth with Fe(III)-citrate (acetate uptake rate of 13.63 mmol/gdwh for a growth rate of 0.06 hr<sup>-1</sup>), 93.6 % of all acetate transported into the cell was utilized for oxidation and ATP generation via the TCA cycle which fit well with experimental data from chemostat cultures (Esteve-Núñez et al., 2005).

#### 5.4. Functional analysis of *G. sulfurreducens* mutant phenotypes

The availability of a genome scale model also enabled the characterization of systems level properties of the metabolic network. One such property is the set of genes and reactions that are essential to support growth in a defined medium. This information is important for genetic investigations as it can provide insight into which mutations may or may not have an observable phenotype.

*In silico* deletion analysis (Edward and Palsson, 2000) for growth with acetate as the electron donor and Fe(III)-citrate or fumarate as the electron acceptor indicated that most mutations were predicted to have either lethal (139 for fumarate, 143 for Fe(III)) or silent phenotypes (440 for fumarate, 437 for Fe(III)) (Table 2). Lethal mutations (*e.g.*, deletion of acetyl-CoA transferase and pyruvate carboxylase) reflected the inability of the perturbed network to synthesize essential components from acetate, a relatively simple two-carbon compound, or the fact that a non-fermentable substrate such as acetate presents few alternative energy-yielding oxidative mechanisms.

Some silent phenotypes predicted by this analysis corresponded to reactions associated with seemingly redundant enzymes. The presence of functionally similar (but non-orthologous) enzymes could be due to selection for genetic robustness, in order to protect against mutations in essential reactions. Alternatively, this redundancy could reflect a need for metabolic robustness, where different enzymes are needed to favor flux in opposite directions, or are optimized for oxidation of different substrates. For instance, model simulations indicated that a mutation in any component of pyruvate-ferredoxin oxidoreductase would be compensated by activity of pyruvate dehydrogenase or pyruvate-formate-lyase. However, as pyruvate-formate-lyase strongly favors function in the oxidative direction, it is unlikely that this enzyme can substitute for pyruvate-ferredoxin oxidoreductase *in vivo*, and the redundancy at this node likely reflects the presence of enzymes specialized for different tasks. Mutational and biochemical investigations are underway to test these hypotheses.

**Table 2.** Impact of *in silico* deletion of entire reactions, on predicted growth rate of *G. sulfurreducens*.

<b>Growth conditions</b>	<b>% lethal deletions</b>	<b>% intermediate deletions</b>	<b>% silent deletions</b>
Acetate&fumarate	40	4	58
Acetate&Fe(III)	41	3	58

## 6. Conclusion

These results suggest that genome-based *in silico* modelling can provide important insights into the physiology of environmentally relevant organisms, such as *Geobacter* species. Not only may such *in silico* models aid in understanding the likely physiological responses of *Geobacter* species in environments in which they are important, but the models can serve as a guide for evaluating the likely outcome of various possible strategies for genetically engineering *Geobacter* species in order to improve practical applications such as bioremediation and electricity production. Furthermore, the coupling of genome-based *in silico* models with hydrological/geochemical models may make it possible to predictively model subsurface bioremediation strategies prior to implementation (Lovley 2003) and coupling such models with electrochemical models is likely to enhance the development of microbial fuel cells (Lovley, 2006).

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## Symbols

TEA	Terminal Electron Acceptor	
$q_{\text{electron}}$	Respiration rate	mol/g dw h
$q_{\text{acetate}}$	Acetate consumption rate	mol/gdw h
$Y_{\text{acetate}}$	Growth Yield	g dw/ mol acetate
S	Stoichiometric matrix	
v	Vector of the reaction fluxes	

## GREEK LETTERS

$\mu$	Growth rate	$\text{h}^{-1}$
$\alpha$	Lower bound	
$\beta$	Upper bound	

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