

QUANTIFYING THE EXPRESSION OF DISSIMILATORY SULFITE REDUCTASE AS A
METRIC FOR SULFATE REDUCTION RATES

BY

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THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Geology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

We tested whether the gene expression of dissimilatory sulfite reductase (*dsr* mRNA), a critical enzyme in the sulfate reduction pathway, can serve as an indicator of the rate of sulfate reduction in natural systems. We grew *Desulfovibrio vulgaris* in fed-batch reactors under electron-donor limiting conditions. To simulate conditions characteristic of oligotrophic environments such as anoxic aquifers, we constrained the rates of sulfate reduction from 0.1 $\mu\text{M h}^{-1}$ to 20 $\mu\text{M h}^{-1}$ (0.89 – 85.9 $\text{fmol cell}^{-1}\text{d}^{-1}$) by controlling the rate of formate addition into the system. We used quantitative-PCR to measure the number of *dsr* mRNA transcripts per cell from biomass sampled over the course of these experiments. We observed a well-defined relationship between the rate of sulfate reduction and the number of *dsr* mRNA transcripts per cell. Cells from reactors maintained with the highest rate of sulfate reduction contain 315 times more *dsr* mRNA per cell than those in reactors with the lowest reduction rate. These results suggest we might be able to infer rates of sulfate reduction in the field by measuring the amount of *dsr* mRNA per cell in biomass samples. Such estimates are difficult to make directly because the rate at which bacteria consume reactants and generate products cannot be observed readily in many environments, such as aquifers open to groundwater flow.

To S.T.E.

ACKNOWLEDGEMENTS

I would first like to thank my advisors Craig M. Bethke and Robert A. Sanford for trusting in my project and providing outstanding acumen in both writing and research matters. I extend many thanks to Theodore M. Flynn for his laboratory assistance and writing suggestions. Also, I would like to thank Brian Farrell and Matt Kyrias for their keen observations and support throughout these experiments. This work was made truly possible with Bruce Fouke's aid in providing critical laboratory space and machinery for the RNA work and insightful comments. Finally, I thank my family and friends for their support throughout this experience. Funding for this project was provided by Department of Energy grant DE-FG02-02ER15317.

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CHAPTER 1

INTRODUCTION

Sulfate reducing bacteria are ubiquitous in geochemical environments depleted in dioxygen (Pedersen 1993; Fredrickson and Balkwill 2006) and the reactions they catalyze control the chemistry of anoxic natural waters (Banfield and Hamers 1997; Nealson and Stahl 1997; Chapelle 2000). The organisms live, for example, in deep aquifers (Baker, Moser et al. 2003), heavy metal mines (Chang, Peacock et al. 2001; Nakagawa, Hanada et al. 2002), deep-sea vents (Cottrell and Cary 1999; Dhillon, Teske et al. 2003; Fukuba, Ogawa et al. 2003), and microbial mats (Canfield and Marais 1993; Teske, Ramsing et al. 1998; Minz, Flax et al. 1999). Sulfate reducers play a critical role in the global sulfur cycle (Castresana and Moreira 1999) and, by affecting the rates of mineral weathering, the carbon cycle (Gorham 1991; Ehrlich 2002; Park, Sanford et al. 2009). The bacteria consume aromatic hydrocarbons, such as naphthalene and benzene, so stimulating their growth can be an effective strategy in environmental remediation (Lovley, Coates et al. 1995; Coates, Woodward et al. 1997; Galushko, Minz et al. 1999; Anderson and Lovley 2000; Annweiler, Materna et al. 2000; Sublette, Peacock et al. 2006). Geochemists and geomicrobiologists are keenly interested, therefore, in understanding the activity of sulfate reducing bacteria in the natural environment.

The molecular revolution in biology has in recent decades revealed in unprecedented detail the phylogeny of the thousands of strains of bacteria and archaea that make up natural microbial communities (Devereux, He et al. 1990; Chang, Peacock et al. 2001; Castro, Reddy et al. 2002; Nakagawa, Hanada et al. 2002; Baker, Moser et al. 2003; Dhillon, Teske et al. 2003; Liu, Bagwell et al. 2003; Perez-Jimenez and Kerkhof 2005; Leloup, Loy et al. 2007).

Geomicrobiologists as a routine matter derive data about the populations of bacteria known to, or suspected of catalyzing sulfate reduction in an environment of interest. In stark contrast, few methods are available to observe the *activity* of the sulfate reducing community, especially in environments open to mass fluxes. This point is a critical distinction because the sulfate reducing bacteria may be fully active, partly active, or completely dormant (Price and Sowers 2004). Knowing the phylogenetic composition of a community of sulfate reducers, then, or even the size of the community, gives little information that can be used with confidence to gauge the extent to which bacterial sulfate reduction occurs in an environment, or even if it does at all.

Given the limitations of phylogenetic analysis, geomicrobiologists are increasingly interested in deriving information about bacterial activity directly, by observing gene expression (Neretin, Schippers et al. 2003; Chin, Esteve-Nunez et al. 2004; Holmes, Nevin et al. 2004; Holmes, Nevin et al. 2005; Lee, Johnson et al. 2006; Chin, Sharma et al. 2008; Villanueva, Haveman et al. 2008). A sulfate reducing cell controls the rate at which it catalyzes the sulfate reduction reaction by regulating the production of enzymes within its cytoplasm. One enzyme, dissimilatory sulfite reductase, or DSR, is used by all known sulfate reducing bacteria (Karkhoffschweizer, Huber et al. 1995; Wagner, Roger et al. 1998; Klein, Friedrich et al. 2001). DSR catalyzes the reduction of bisulfate to sulfide, the final step in the sulfate reduction pathway (Odom and Peck 1984).

When a cell expresses the gene that codes for DSR, it produces a messenger RNA transcript, *dsr*. The *dsr* transcript carries to the cell's ribosomes the information necessary to produce a molecule of DSR enzyme. The number of *dsr* transcripts present in a sulfate reducer's cytoplasm, then, provides a direct measure of the cell's activity. Several studies of sulfate reducers growing in pure culture (Neretin, Schippers et al. 2003; Chin, Sharma et al. 2008;

Villanueva, Haveman et al. 2008) have targeted the *dsrA* transcript, which encodes the alpha subunit of the DSR gene. The studies showed the number of *dsrA* transcripts contained in a cell is closely related to rate the cell catalyzes sulfate reduction. Specifically, the studies related reduction rate to the number of *dsrA* transcripts observed in biomass retrieved from a laboratory experiment, expressed per unit mass of total RNA in the bulk genetic material.

Inferring sulfate reducing activity in nature by this method may prove difficult. As much as 98% of the bulk RNA in a cell's cytoplasm is composed of stable RNA's, which are not directly related to the expression of *dsrA* (Ruimy, Breittmayer et al. 1994; Gourse, Gaal et al. 1996; Deutscher 2003; Huggett, Dheda et al. 2005). Stable RNA's, comprised mostly of rRNA plus tRNA, are protected from degradation in the cell in most physiological states and only degraded under special conditions such as starvation. In contrast, messenger RNA has a high turnover rate and is more directly related to the production of enzymes in the cells' cytoplasm (Deutscher 2006). The ratio of *dsrA* to bulk RNA, for this reason, may vary depending on factors not directly related to sulfate reduction. As well, the bulk RNA in a natural sample contains genetic material from a gamut of microbes, including many incapable of reducing sulfate (Fey, Eichler et al. 2004). The amount of *dsrA* in a natural sample taken relative to the bulk RNA likely reflects poorly the actual rate of of sulfate reduction.

In this paper we consider the relationship of DSR gene expression to sulfate reducing activity in a way that might be applied more readily to interpret the dynamics of natural environments. Specifically, we study in the laboratory the anaerobic oxidation of formate



by the sulfate reducer *Desulfovibrio vulgaris* in reactors designed to tightly control the rate of sulfate reduction. In contrast to previous studies, we compare the number of *dsrA* transcripts to

the number of DSR gene copies in the bulk DNA. The number of DSR gene copies per genome is known and invariant for a given sulfate reducing organism (Talaat, Howard et al. 2002). The ratio of *dsrA* transcripts to DSR gene copies, therefore, reflects the sulfate reduction rate more directly than comparing the *dsrA* count to total RNA. We further conduct our experiments at reduction rates considerably slower than achieved in previous studies, in order to more closely approximate conditions encountered in common natural environments. And unlike previous studies, we use formate as the electron donor in our reactors, rather than lactate, which is seldom found in natural environments (McMahon and Chapelle 1991).

CHAPTER 2

METHODOLOGY

2.1 Continuous-fed reactor experiments

We grew *Desulfovibrio vulgaris* sr. Hildenborough (ATCC 29579) in a series of continuous-fed batch reactors configured to supply formate, the electron donor, at pre-set rates. We constructed the reactor array from 160 ml serum bottles fitted to a 10 channel syringe pump (KD Scientific, Holliston, MA). The bottles were connected to the pump with 4-inch stainless-steel canulas (Popper and Sons, New Hyde Park, NY), PEEK tubing (Sigma-Aldrich, St. Louis, MO), and 2.5 ml sterile glass syringes (SGE, Austin, TX) via a luer-lock assembly. The canulas passed into the sample bottles so the tip remained submerged in the growth medium.

We filled each serum bottle with 100 ml of degassed sulfate-free mineral salt medium, as described by (He and Sanford 2004), before closing the bottle with a butyl rubber stopper and an aluminum seal. To each bottle we added formate, acetate, and sulfate at various concentrations, as shown in Table 1. The formate served as the electron donor for cellular respiration, the acetate was the carbon source for growth, and the sulfate was the electron acceptor. Before initiating an experiment, we cultured the bacteria on the corresponding medium for at least three consecutive passages for 48 to 72 h. We added 10 mls (10% v/v) of this active culture as an inoculum to all the bottles (usually eight) in the reactor array.

We set the syringe pump to inject formate (or pyruvate) solutions of different concentrations into the serum bottles at a volumetric delivery rate of $.083 \text{ ml hr}^{-1}$ (Table 1). The mass addition rate of formate thus varied among the bottles, depending on the formate concentration of the feed solution in the corresponding syringe. We confirmed the delivery rate

by adding a bromide tracer (5mM) to several of the syringes. The use of butyl rubber stoppers insured that the reactors were maintained under strictly anaerobic conditions, in an atmosphere with a 90:10 volume ratio of N₂ to CO₂. After inoculation, we incubated the culture array in the dark at 30 °C while continuously mixing on a table shaker at 48 rpm. Each reactor array consisted of duplicate bottles fed the same formate (or pyruvate) mass addition rate.

To evaluate metabolism in the absence of sulfate, we grew *D. vulgaris* by pyruvate fermentation as a control. Cultures were pregrown on pyruvate alone for three passages prior to the start of the experiment when a 10% (v/v) inoculum was used as above with the sulfate fed cultures. We used a 2 mM pyruvate injectate at a volumetric addition rate of .083 ml hr⁻¹ (Table 1).

2.2 Sampling and analysis

We withdrew liquid samples over the course of the experiments through the butyl rubber stoppers with a nitrogen-flushed syringe. We collected cells by filtering 4 ml of a liquid sample through a 0.22 µm filter (Nitrocellulose, Millipore, Ireland) placed inside a reusable filter holder. The filtrate was stored in 2 ml tubes at -20 °C for later analysis of formate, acetate, sulfate, and bromide concentrations. To preserve the RNA, 2 ml of fresh RNAlater (Ambion, Austin, TX) was flushed through the filter, then the filter was stored in the individual filter holders at 4 °C for about 24 hours. The filters were then taken from the filter holders and stored in Whirl-Pak® bags (NASCO, Atkinson, WI) at -80 °C, pending nucleic acid extraction.

We measured formate and acetate concentrations using a Shimadzu Prominence high-pressure liquid chromatograph (Shimadzu Scientific Instruments, Columbia, Maryland) with a BioRad Aminex HPX-87H column. We analyzed for sulfate and bromide concentrations with a Metrohm Peak Advanced ion chromatograph equipped with a Metrosep A Supp 7 -

250/4.0 Anion column (Metrohm Park Inc., Houston, TX).

2.3 Flow cytometry analysis and biomass concentrations

We quantified the number of cells in the inoculum and reactors using flow cytometry. Cells were preserved in an 8% formaldehyde solution (3 parts cells: 1 part formaldehyde solution) and stored at 4°C until analysis. We added 1 µl SYTO BC Bacteria Stain and 10 µL resuspended microsphere standard (Bacteria Counting Kit, Molecular Probes, Eugene, OR) to 500 µl preserved cell mixture. Fluorescence detection at 480 nm, forward scatter, and side scatter were collected until a set number of the microspheres were detected, from which we were able to calculate the volume of cell mixture counted, and ultimately the concentration of cells per sample.

We also measured biomass gravimetrically at the termination of the experiments to determine the mass of cells in each reactor. We then pre-rinsed, dried, and pre-weighed glass microfiber filters (Fisher, 47mm diameter, 0.7µm) to filter approximately 100 ml of the reactor fluid. We massed the serum bottles before and after filtering the cell suspensions to quantify the actual volume filtered. Filters were heated at 110°C in a muffle furnace for three hours to drive off all water (thus obtaining a mass of cells plus precipitates) and then at 500°C for 1 hour (leaving only precipitates). After each heating step the filters were allowed to cool to room temperature and then massed. We calculated biomass per ml of suspension by subtracting the mass of precipitate (per ml suspension) from the mass of cell precipitate plus cell biomass (per ml solution).

2.4 Nucleic acid extraction and reverse transcription

We extracted high molecular weight genomic DNA from cells trapped on the filters using a procedure modified from (Tsai and Olson 1991; Flynn, Sanford et al. 2008) We began with direct enzymatic lysis of the cells by incubating the filter at 37 °C in 2 ml of lysis solution (0.15 M NaCl, 0.1 M EDTA, pH 8) containing 15 mg ml⁻¹ lysozyme. We added an equal volume of STS solution (0.1 M NaCl, 0.48 M Tris, 10% sodium dodecyl sulfate, pH 8) and incubated the filter again for 30 minutes. To ensure complete lysis we exposed the cells to freeze-thaw cycling, passing three times from liquid nitrogen to a 55 °C water bath and back. Afterwards, we added 10 µL of proteinase K (50 µg ml⁻¹) to each tube and the filters were incubated at 37 °C for 30 minutes. We centrifuged the tubes (Sorvall Legend RT, Thermo Fisher Scientific) at 9,000 rpm for 20 minutes at 16 °C.

We split the supernatant into equal volumes (~2ml) and transferred them into two 15 ml conical tubes (Ambion, Austin, TX), one for extracting RNA and one for DNA. For the RNA extraction, proteins were removed by successive organic extractions with equal volumes of phenol (pH 4.3), phenol:chloroform:isoamyl alcohol (125:24:1, pH 4–5), and chloroform:isoamyl alcohol (24:1). The RNA was precipitated from solution by adding 10.5 M ammonium acetate to achieve a final concentration of 2.5M, 50 µg/ml glycogen, and an equal volume of isopropanol. The nucleic acids were resuspended in 100 µl of molecular-grade water (Ambion, Austin, TX).

For the DNA extraction, proteins were removed by successive organic extractions with equal volumes of phenol (pH 8), phenol:chloroform:isoamyl alcohol (25:24:1, pH = 7.7-8.3), and chloroform:isoamyl alcohol (24:1). Genomic DNA was precipitated and resuspended in the same manner as stated above. The DNA was stored at –80 °C until further analysis. An RNase

decontaminated hood was used for all extractions, DNase treatment, and reverse transcription reactions. All reagents, tubes, and pipettes were RNase-free.

We removed DNA from the RNA extract samples using a Turbo DNA-free kit (Ambion, Austin, TX). Samples were treated with DNase and incubated at 37 °C for 40 min. The DNase Inactivation Reagent was pelleted by centrifugation so as not to introduce contamination to downstream applications. We checked the purity of the RNA using agarose gel electrophoresis. We stored the RNA at -80 °C for later reverse transcription and quantitative PCR analysis.

For reverse transcription and quantitative PCR of the DSR mRNA, we used the primers DSR1R (5'-TTA TCT CAG GTG TCT CTT GCG GT-3') and DSR1F (5'-AAG GAA CCC CGC ACC AAC-3') (position 1 to 102, *dsrA* gene) as previously described (Villanueva, Haveman et al. 2008). For reverse transcription and quantitative PCR of the 16S rRNA, we used the primers 341F (5'-CCT ACG GGA GGC AGC AG-3') and 534R (5'-ATT ACC GCG GCT GCT GG-3'). The primers were synthesized by Integrated DNA Technologies (Eugene, OR).

For the reverse transcription, we used 2µl RNA template, 5µl molecular-grade water, and 5µl DSR1R primer (1µM working concentration), or 2µl of diluted (1:10) RNA template with 10µl 534R primer (2µM working concentration). We denatured the RNA at 80 °C for 3 minutes, followed by an annealing step at 61 °C for 30 seconds, after which the samples was removed to ice. We added to the RNA and primers a reaction mixture of 1µl 100 U MMLV-RT, 2µl 10X RT buffer, 4µl dNTP's (2.5mM), and 1µl 10 U RNase Inhibitor (Retrosript Kit, Ambion, Austin, TX), then incubated mixture at 43 °C for 1 hour, followed by an enzyme inactivation step at 92 °C for 10 minutes, and finally rapid cooling to 4 °C. The cDNA samples were stored at -20 °C for PCR analysis.

2.5 Real-time PCR

We used a Stratagene MX3000P analyzer for quantitative PCR measurements. The assays consisted of 1 μ l cDNA template, 12 μ l SYBR Green Master Mix and 2.5 μ l of each primer (100 nM) to a final volume of 25 μ l, prepared in 96-well optical reaction plates (Midsco, St. Louis, MO) and sealed with optical caps (Applied Biosystems, Foster City, CA). The thermal profile consisted of an initial denaturation step of 10 min at 95 °C, 40 cycles of denaturation at 95 °C for 30 seconds, an annealing and elongation step at 61 °C for 1 minute, and a final melting curve analysis after 40 cycles. We performed two negative DNA controls (no RT performed) and three negative controls (no template) with each quantitative PCR run. We gathered and analyzed the results with the MxPro software v4.10 (Stratagene).

For qPCR calibration we used dilution series of DNA extracted from pure cultures of *D. vulgaris*. The calibration curve consisted of gene copy number, calculated based on the known molecular mass of the genome (see equation below), plotted against the threshold cycle number (CT) (Figure 1). The DNA concentration of the standard was quantified by fluorometry with the PicoGreen dsDNA quantification kit (Invitrogen, Carlsbad, CA). We calculated the number of gene copies in our DNA standards using an approach previously described (Ritalahti, Amos et al. 2006) The number of target genes per ml

$$\text{Copies} = \left(\frac{\text{ng DNA}}{\mu\text{l}} \right) \left(\frac{1 \text{ g}}{10^9 \text{ ng}} \right) \left(\frac{1 \text{ mol bp}}{660 \text{ g DNA}} \right) \times \left(\frac{6.023 \times 10^{23} \text{ bp}}{\text{mol bp}} \right) \left(\frac{1 \text{ copy}}{\text{genome bp}} \right) (\mu\text{l template}) \quad (0.2)$$

of sample was similarly determined, as described in this reference.

CHAPTER 3

RESULTS

Formate concentrations in the reactors decreased to below the quantitative detection limit within about 100 hours when formate was added at relatively slow rates ($0.42 \mu\text{M hr}^{-1}$ to $9.16 \mu\text{M hr}^{-1}$) (Figure 2). This indicates that the *D. vulgaris* cells in the reactors were consuming the injected formate as quickly as it was supplied. Based on the stoichiometry shown in reaction (1), the rate of sulfate reduction at this time was fixed at one quarter of formate addition rate, or $.10 \mu\text{M hr}^{-1}$ to $2.29 \mu\text{M hr}^{-1}$ (Table 1).

When formate was supplied at higher rates ($41.5 \mu\text{M hr}^{-1}$ and $80.3 \mu\text{M hr}^{-1}$), formate concentrations remained above the detection limit, however they remained relatively constant and did not show accumulation until the sulfate in the reactors had been depleted (Figure 3). In these cases, formate was supplied at rates that exceeded the capacity of the culture in the reactor to deplete to non-detectable levels. Based on the steady state formate concentrations at 96 hours, the sulfate reduction rates calculated were $10.4 \mu\text{M hr}^{-1}$ and $20.1 \mu\text{M hr}^{-1}$ for the formate addition rates of $41.5 \mu\text{M hr}^{-1}$ and $80.3 \mu\text{M hr}^{-1}$, respectively. In these reactors with the highest sulfate reduction rates, $10.4 \mu\text{M hr}^{-1}$ and $20.1 \mu\text{M hr}^{-1}$, the concentration of sulfate was calculated to deplete completely after 110 hours and 170 hours, respectively. This was confirmed by observing the formate concentration increasing at the rate it was supplied to the reactors after the sulfate was gone (Figure 3). Efforts to measure sulfate concentrations in the reactors were inconclusive due to interferences with co-eluting anions during IC analysis.

Cell concentrations in the reactors were quantified using two approaches, total biomass by gravimetric analysis and cell counts using flow cytometry. Using the relationship between the

mg/L biomass and the cells/L counts we found a quantitative relationship of 3.80×10^{11} cells g^{-1} . Using this relationship we determined the cell per ml in all the reactors. The experiment 2 inoculum contained 1×10^7 cells ml^{-1} which was distributed to each reactor at a starting cell concentration of 8.9×10^5 cells ml^{-1} (Table 2). Total cell counts at the 96 h ranged from 2.0×10^6 to 5.6×10^6 cells ml^{-1} for the no-feed control and the 20.1 μM sulfate per hour culture (Table 2). This indicated that some growth continued in the inoculum as the residual formate was consumed even without added formate. For sulfate reduction rates from $0.1 - 2.29 \mu M hr^{-1}$ the time 96 hour cell counts ranged from $2.7 - 3.4 \times 10^6 ml^{-1}$, indicating that less than a doubling of the cells had occurred during the experiment. For the higher sulfate reduction rates the cell concentration increase indicated at least one biomass doubling had occurred during the experiment (Table 2).

Using cells collected from each reactor, we extracted both DNA and RNA at the same time. A common lysis protocol was used on all the cells prior to splitting the lysate into two fractions for both extractions. This minimized the variability between the RNA and DNA extraction efficiencies and allowed quantification of the mRNA *dsr* transcripts relative to the *dsr* gene copies in the DNA, which corresponds to the number of cells. To compare variations in *dsrA* mRNA transcripts per cell across the two sets of experiments, we normalized to the value measured in cells from reactors poised at a sulfate reduction rate of $2.29 \mu M hr^{-1}$ (Table 1). At the end of an initial 48 hour growth phase for the *D. vulgaris* culture, when the experiments were started, we detected an average of 2.3 *dsrA* mRNA transcripts per cell for all reactors (Figure 4). After 96 hours the normalized *dsrA* mRNA transcripts per cell ranged from 0.04 to 12.6 for sulfate reduction rates ranging from 0.1 to 20.1 $\mu M h^{-1}$, respectively (Figure 4, Table 2). When sulfate was completely depleted in the reactors fed with the highest formate feed rates (i.e. after

110 h), corresponding to $0 \mu\text{M h}^{-1}$, the normalized *dsrA* mRNA transcripts per cell decreased 48-fold to 0.25. Pyruvate control reactors fed at $1.64 \mu\text{M h}^{-1}$ showed a constitutive expression of DSR with an average normalized *dsrA* mRNA transcripts per cell of 5.0.

As an additional metric of cell physiology, we monitored the rRNA transcript level in the reactors maintained at different sulfate reduction rates during Experiment 1. Adjusting for extraction efficiency, the transcripts detected per ml of culture ranged from 3.4×10^{10} to 2.0×10^{11} for sulfate reduction rates ranging from 0.18 to $20.1 \mu\text{M h}^{-1}$, respectively (data not shown). The normalized *dsrA* mRNA transcripts per rRNA ranged from 16 – 18 for the highest sulfate reduction rate ($20.1 \mu\text{M h}^{-1}$) or a cell-specific sulfate reduction rate (csSRR) of $85.9 \text{ fmol cell}^{-1}\text{d}^{-1}$, which corresponds fairly closely the *dsrA* mRNA transcripts per cell values measured (data not shown). In contrast, at the lower bulk sulfate reduction rates of 0.1 and $2.3 \mu\text{M h}^{-1}$ (csSRR = 1.9 and $16.4 \text{ fmol cell}^{-1}\text{d}^{-1}$) it was not possible to differentiate the *dsrA* mRNA transcripts per rRNA values for each feed rate, however these were clearly differentiated when using the normalized *dsrA* mRNA transcripts per cell values (Figure 5).

CHAPTER 4

DISCUSSION

We interpret these results as reflecting the activity of *D. vulgaris*, specifically the relationship between the rate of sulfate reduction and the production of *dsrA* transcripts per cell. From these results, we show that the number of *dsrA* mRNA transcripts per cell increase by a factor of 315 in reactors with the lowest to the highest sulfate reduction rates (Figure 4). When we plot the bulk sulfate reduction rate or cell specific sulfate reduction rate (csSRR) verses the normalized *dsrA* mRNA transcripts per cell, we observe a linear relationship corresponding to a regression curve with an $r^2 > 0.95$ (Figures 6 and 7). This linear relationship demonstrates that under electron donor limiting conditions at low rates of sulfate reduction, levels of *dsrA* per cell correspond to increasing levels of sulfate reduction rates. This result contrasts previous findings by Villanueva et al., 2008. and Chin et al., 2008, where they determined that the number of *dsrA* transcripts per cell did not parallel increasing rates of sulfate reduction. This in part may be due to their using bulk sulfate reduction rates in chemostats orders of magnitude higher than we used and that lactate was used as an electron donor.

By comparing *dsrA* per cell to bulk rates in a lab setting, our results suggest that *dsrA* mRNA per cell levels can be used as a proxy for determining the bulk rate of sulfate reduction in the environment. Biogeochemists can gain information about rates of sulfate reduction by monitoring the reduction [³⁵S]-labeled sulfate to [³⁵S] sulfide (Fossing and Jorgensen 1989) or by measuring [³⁴S] fractionation in the sediments (Habicht and Canfield 1997). However, the latter method does not indicate which populations of bacteria control the rate of sulfate reduction (Detmers, Bruchert et al. 2001). A critical question that needs to be addressed is how can we gain

information about the rates of sulfate reduction in the environment in conjunction with the metabolic state of microbes.

Electron donor choice in any study of sulfate reducing activity impacts the results that are obtained. The predominant electron donors that exist in pristine, anoxic aquifers are formate, acetate, and H₂ (McMahon and Chapelle 1991). In contrast, despite being the electron donor choice in previous studies (Neretin, Schippers et al. 2003; Villanueva, Haveman et al. 2008), lactate does not likely exist in pristine oligotrophic environments such as aquifers. Growth yields for *Desulfovibrio vulgaris* are higher when lactate is used as the electron donor because bacteria gain an ATP for every acetate generated during oxidation in addition to respiration of sulfate (Magee, Ensley et al. 1978; Voordouw 2002). Lactate even supports growth of *Desulfovibrio vulgaris* via fermentation in the absence of sulfate (Pankhania, Spormann et al. 1988; Voordouw 2002). These issues are not of concern with formate and acetate. It has been shown, furthermore, that cells growing in electron donor limiting conditions have a lower maintenance energy requirement than cells growing in electron acceptor limiting conditions (Esteve-Nunez, Rothermich et al. 2005). This result suggests that levels of *dsrA* per cell will vary according to the type of electron donor available in a natural environment, consistent with the different metabolic pathways for each electron donor in *D. vulgaris* (Heidelberg, Seshadri et al. 2004).

Extraction efficiency and normalization techniques can impact laboratory results that need to be correlated with environmental samples (Bustin 2000). To minimize these effects, we carried out separate DNA and RNA extractions on each sample. This method is independent of efficiency errors because the DNA and RNA extraction efficiencies are roughly equal for each sample. Conversely, total RNA extraction methods are efficiency dependent and can results can

be impacted when using different samples (von Wintzingerode, Gobel et al. 1997). Our method can be applied directly to field samples because primers can be constructed to target a specific species in the environment. Thus, we do not need to count cells nor normalize *dsr* to total RNA. We normalized *dsrA* per cell across multiple experiments by comparing the levels of *dsrA* per cell in each reactor to the number of *dsrA* per cell in reactors that were fed with formate at a rate of $9.2 \mu\text{M hr}^{-1}$.

CHAPTER 5

CONCLUSIONS

To estimate rates of sulfate reduction in environmental samples, we would first need to understand the total range, both in numbers and phylogenetic distribution, of sulfate reducing bacteria in both the sediment and groundwater (Flynn, Sanford et al. 2008). The diversity of sulfate reducers in an aquifer would be estimated by sequencing the DSR genes from a sample. Additionally, the number of gene copies of DSR per genome is variable for different organisms, but a fixed value for a given species. We could then quantify the number of *dsr* transcripts for each group of sulfate reducer that is determined to be the most abundant in a sample. By normalizing this ratio (mRNA:DNA) across various studies, we could estimate the rate of sulfate reduction from studies of sulfate reducers grown in pure culture.

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FIGURES

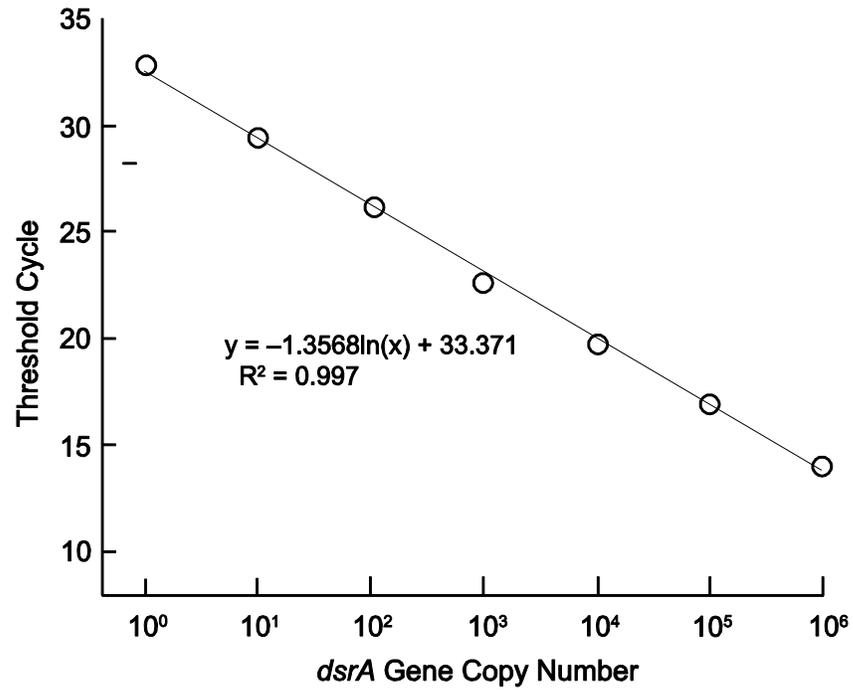


Figure 1: Calibration curve from qPCR analysis used to calculate copy numbers of *dsrA* gene. Each symbol represents the average threshold cycle (CT) of three replicate standard samples \pm standard deviations. In each case, the data range is smaller than the symbol shown.

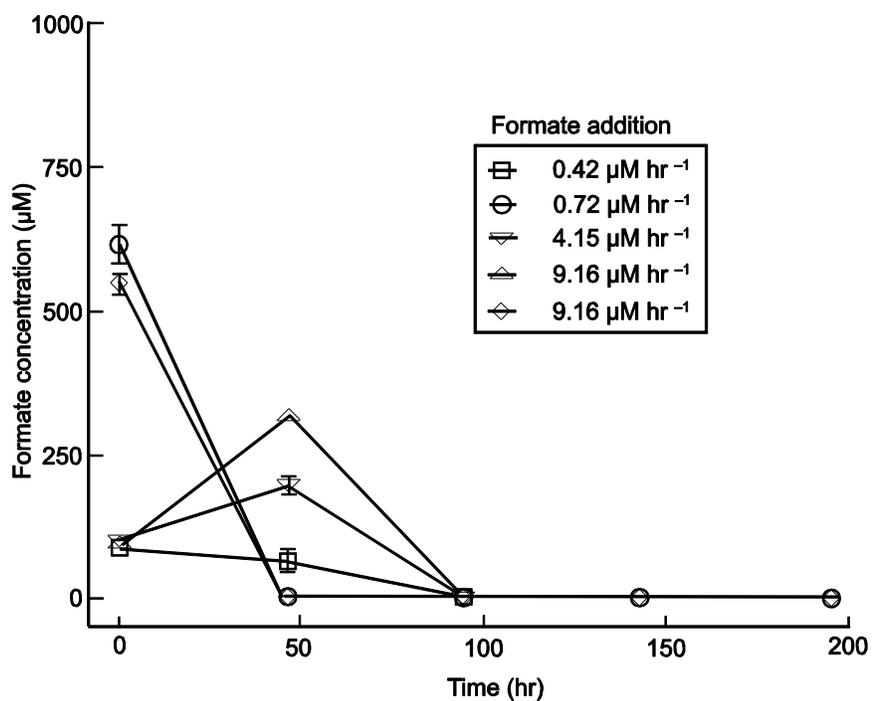


Figure 2: Variation in formate concentrations in duplicate reactors for different feed rates. Formate was supplied continuously at rates shown from $0.42 \mu\text{M hr}^{-1}$ to $9.16 \mu\text{M hr}^{-1}$. Bottle sets fed $9.16 \mu\text{M hr}^{-1}$ were run in two separate experiments. Each symbol represents the average of samples taken from duplicate reactors. By 96 hours, the steady-state formate concentration fell below the detection limit, indicating the cells consumed formate as rapidly as it was supplied.

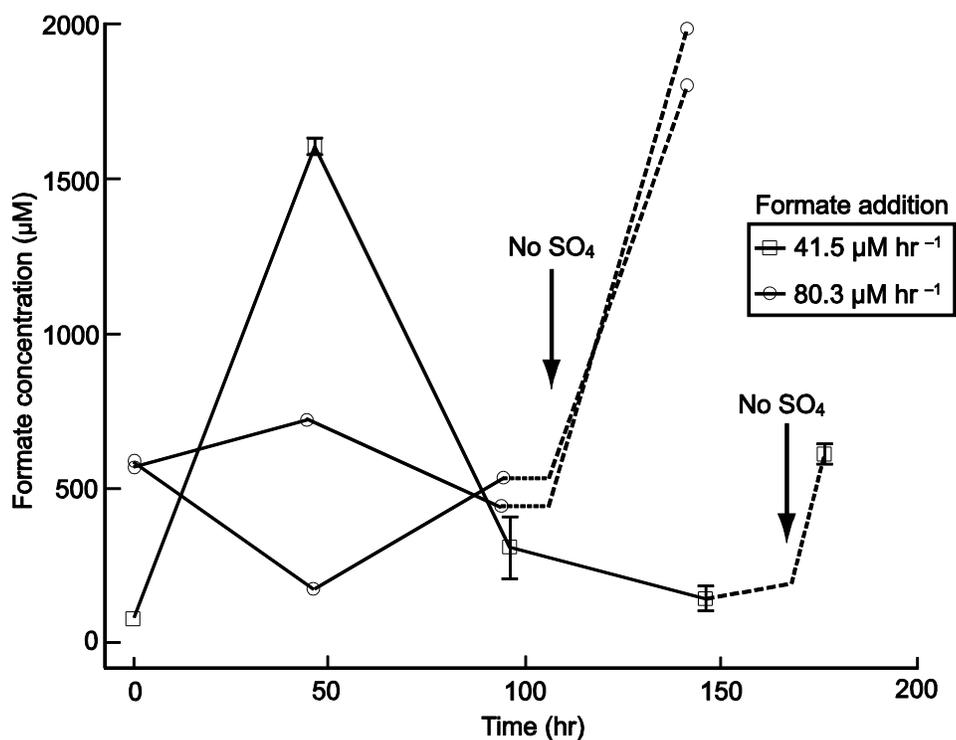


Figure 3: Variation in formate concentration in duplicate reactors in which formate was supplied continuously at $41.50 \mu\text{M hr}^{-1}$ and $80.28 \mu\text{M hr}^{-1}$. After 96 hours formate concentrations remained relatively constant indicating a steady-state rate of consumption. For the reactors fed $41.25 \mu\text{M hr}^{-1}$ formate, the corresponding sulfate reduction rate is sufficient to consume all the sulfate by 158 hours, as shown by the dotted line. Reactors fed formate at $80.28 \mu\text{M hr}^{-1}$, were predicted to consume all the sulfate by 103 hours, as shown by the dotted line. Note the last formate analysis in each set of reactors reflects rapid accumulation of formate due to the absence of the electron acceptor.

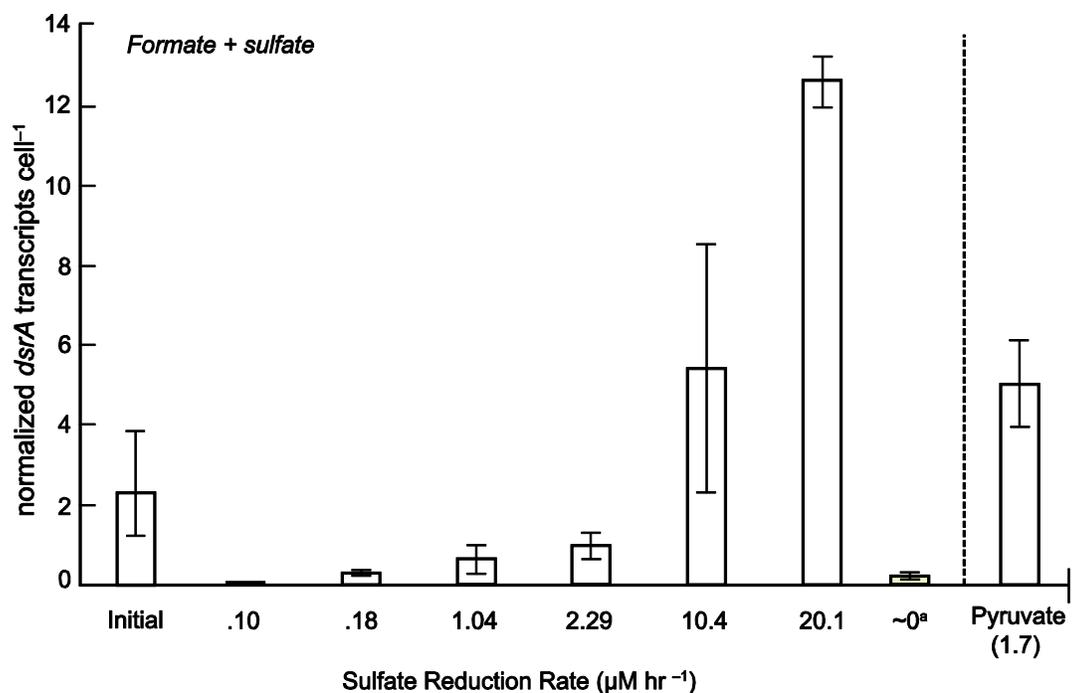


Figure 4: Relative *dsrA* mRNA transcripts expressed per cell by *D. vulgaris* grown for duplicate reactors under six sulfate reduction rates after 96 hours of continuous feeding. All of the values have been normalized to the 9.16 $\mu\text{M hr}^{-1}$ formate feed rate. Initial reaction conditions represent samples taken at time=0 in 6 reactors. The bars labeled as 0 $\mu\text{M hr}^{-1}$ sulfate shows the number of *dsrA* per cell after sulfate had been exhausted from the 20.1 $\mu\text{M hr}^{-1}$ sulfate bottles at 145 h. All bar graphs represent the means \pm standard deviations of samples taken from duplicate cultures, two reverse transcription reactions, and triplicate qPCR reactions per sample.

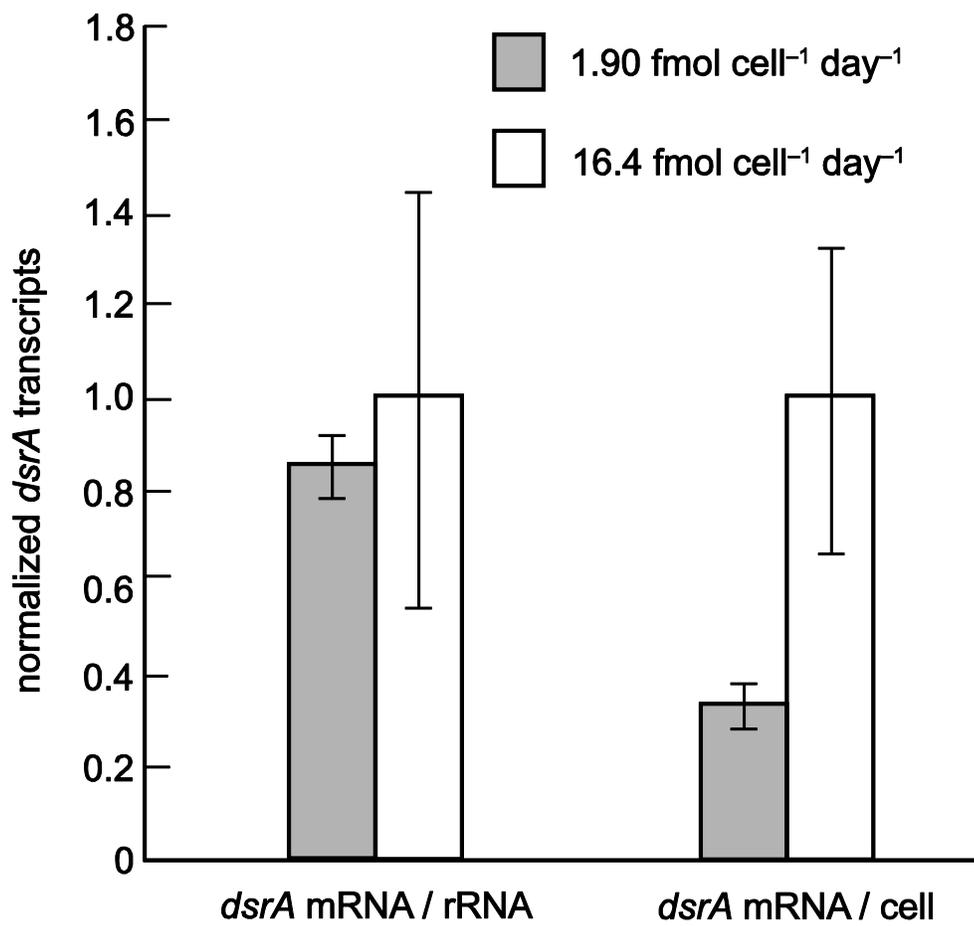


Figure 5: Relative amount of *dsrA* transcripts expressed as a ratio between rRNA and per cell and the sulfate reduction rates for 1.90 fmol cell⁻¹ day⁻¹ maintained in the reactors.

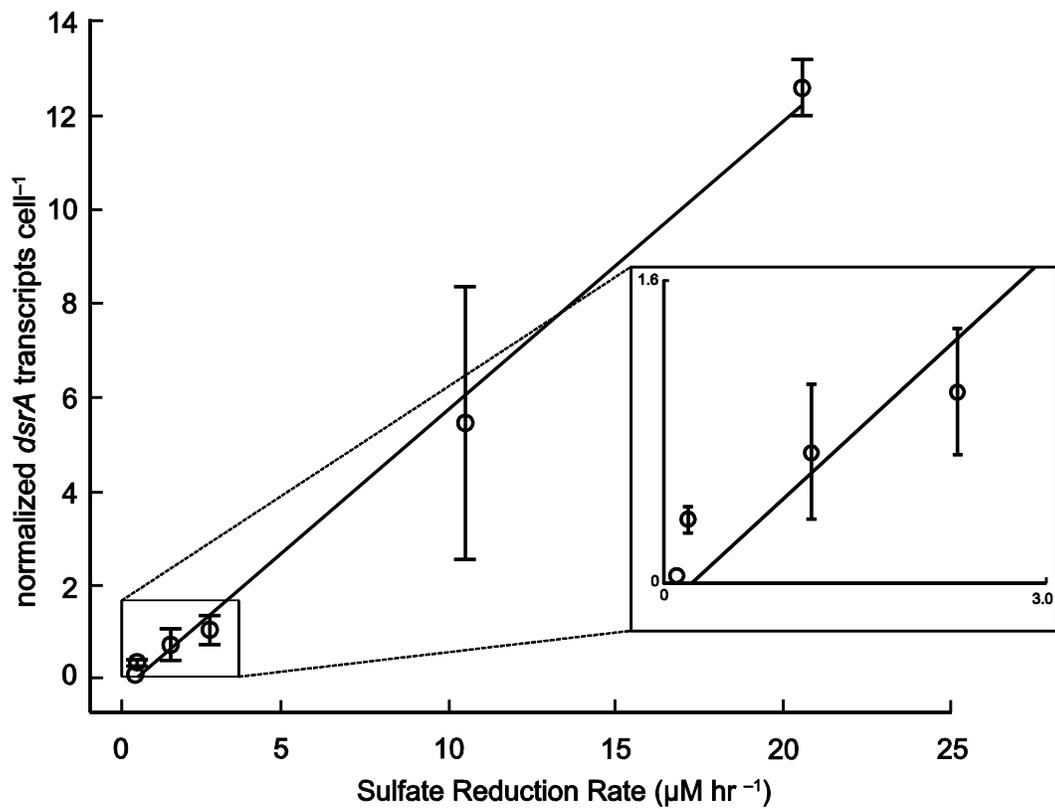


Figure 6: Relationship between the relative *dsrA* mRNA transcripts expressed per cell and the sulfate reduction rate maintained in the reactors. The symbols represent the means \pm standard deviations of samples taken from duplicate cultures, two reverse transcription reactions, and triplicate QPCR reactions per sample. The regression equation is $y = 0.6097x - 0.125$ ($R^2 = 0.99$).

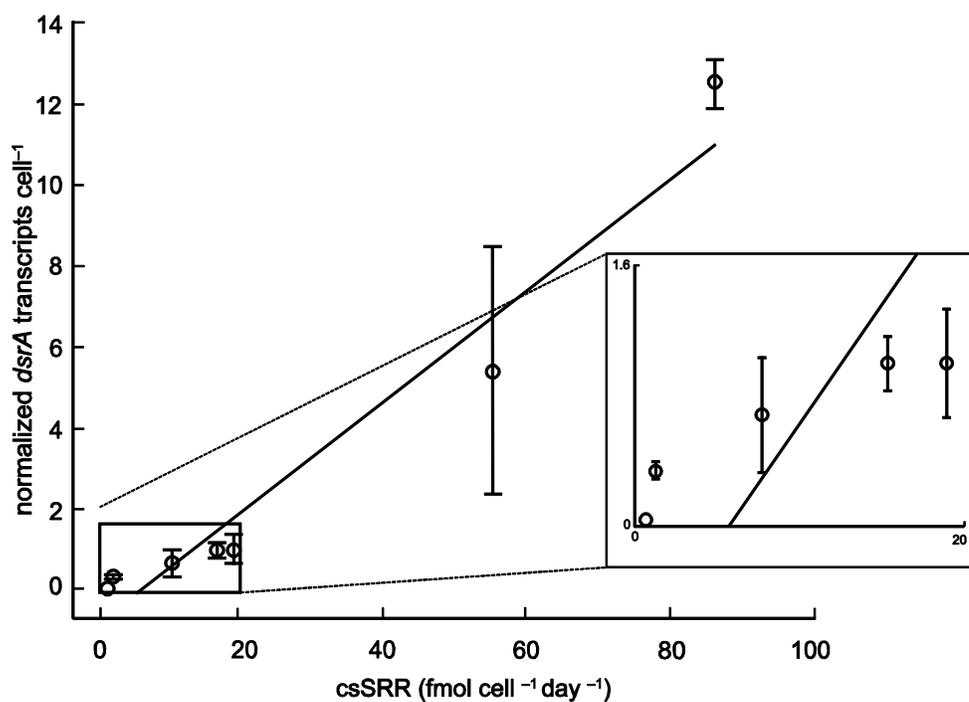


Figure 7: Relationship between the relative amount of *dsrA* mRNA expressed per cell and the cell specific sulfate reduction rate (csSRR). The symbols represent the means \pm standard deviations of samples taken from duplicate cultures, two reverse transcription reactions, and triplicate QPCR reactions per sample. The regression equation is $y = 0.1413x - 0.8077$ ($R^2 = 0.95$).

Table 1: Conditions at the start of two continuous-fed reactor experiments. Data are shown for duplicate culture bottles (A, B) for each mass addition rate. The concentration of formate or pyruvate in the injectate, the mass addition rate corresponding to a volumetric injection rate of 0.083 ml hr^{-1} , and the initial concentrations of electron donor, electron acceptor, and carbon source in each bottle are shown.

Experiment	Substrate concentration (mM) ^a	Delivery rate ($\mu\text{M hr}^{-1}$)	Initial conditions (mM)			
			Formate or pyruvate	Sulfate	Acetate	
<i>Formate respiration</i>						
1	0.929	0.72	A	.64	1.7	.48
			B	.59	1.6	.43
1	11.9	9.16	A	.58	$\sim 1.7^b$.43
			B	.54	1.7	.39
1	104	80.3	A	.57	$\sim 1.6^b$.42
			B	.58	1.5	.42
2	0.5	0.42	A	.10	1.0	.55
			B	.09	1.0	.53
2	5	4.15	A	.10	1.1	.54
			B	.10	1.0	.53
2	11.9	9.16	A	.10	0.9	.54
			B	.10	1.1	.54
2	50	41.5	A	.57	$\sim 1.0^b$.53
			B	.58	$\sim 1.0^b$.54
<i>Pyruvate fermentation</i>						
1	2.0	1.68	A	0	—	—
			B	0	—	—

^a Substrate concentration in injectate.

^b By formulation.

Table 2: Normalized *dsrA* transcripts per cell, number of cells per ml in the reactors, and the estimated nucleic acid extraction efficiency for reactors maintained at different sulfate reduction rates or fed pyruvate.

Sample Type	Sulfate reduction rate $\mu\text{M hr}^{-1}$ ($\text{fmol cell}^{-1}\text{d}^{-1}$)	Average Initial Cells ml^{-1} (^d)	Average Final Cells ml^{-1} (^d)		<i>dsrA</i> cell ⁻¹ (<i>dsrA</i> /DSR)	Extraction efficiency ^e
Sulfate	.10			A	0.03	23%
96h	(0.89)	8.9×10^5	2.7×10^6	B	0.05	23%
	.18	ND	2.7×10^6	A	0.3	100%
96h	(1.90)			B	0.4	97%
	1.04	8.9×10^5	2.8×10^6	A	1.0	19%
96h	(10.1)			B	0.3	31%
	2.29	8.9×10^5	2.9×10^6	A	0.7	40%
96h	(18.8)			B	1.3	28%
	2.29	ND	3.4×10^6	A	.84	37%
96h	(16.4)			B	1.2	49%
	10.4 ^a	8.9×10^5	4.4×10^6	A	8.5	82%
96h	(54.9)			B	2.4	43%
	20.1 ^a	ND	5.6×10^6	A	11.9	58%
96h	(85.9)			B	13.2	52%
	$\sim 0^b$	ND	9.0×10^6	A	0.3	n/a
145h				B	0.2	n/a
No Formate Added	0	8.9×10^5	2.0×10^6	A	.025	23%
				B	.086	19%
Pyruvate^c	n/a	ND	2.4×10^6	A	3.9	51%
				B	6.1	89%

^a Sample taken before sulfate was exhausted.

^b Sampled after exhaustion.

^c Fermentation control, growing on pyruvate.

^d Cell concentrations obtained from dry weight and flow cytometry.

^e Efficiency calculated from qPCR and cell concentrations.