Reductive Precipitation of Sulfate and Soluble Fe(III) By *Desulfovibrio vulgaris*: Electron Donor Regulates Intracellular Electron Flow and nano-FeS Crystallization

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ABSTRACT

Fully understanding the metabolism of SRB provides fundamental guidelines for allowing the microorganisms to provide more beneficial services in water treatment and resource recovery. The electron-transfer pathway of sulfate respiration by *Desulfovibrio vulgaris* is well studied, but still partly unresolved. Here we provide deeper insight by comprehensively monitoring metabolite changes during *D. vulgaris* metabolism with two electron donors, lactate and pyruvate, in presence or absence of citrate-chelated soluble Fe^{III} as a competing electron acceptor. H_2 was produced from lactate oxidation to pyruvate, but pyruvate oxidation produced mostly formate. Accumulation of lactate-originated H_2 during lag phases inhibited pyruvate transformation to acetate. Sulfate reduction was initiated by lactate-originated H_2 , but MQ-mediated e^- flow initiated sulfate reduction without delay when pyruvate was the donor. When H_2 -induced electron flow gave priority to Fe^{III} reduction over sulfate reduction, the long lag phase before sulfate reduction shortened the time for iron-sulfide crystallite growth and led to smaller mackinawite (Fe_{1+x}S) nanocrystallites. Synthesizing all the results, we propose that electron flow from lactate or pyruvate towards SO_4^{2-} reduction to H_2S are through at least three routes that are regulated by the e^- donor (lactate or pyruvate) and the presence or absence of another e^- acceptor (Fe^{III} here). These routes are not competing, but complementary: e.g., H_2 or formate production and oxidation were necessary for sulfite and disulfide/trisulfide reduction to sulfide. Our study suggests that the e^- donor provides a practical tool to regulate and optimize SRB-predominant bioremediation systems.

Key Words: *D. vulgaris*; SO_4^{2-} reduction; Soluble Fe^{III} reduction; Iron-sulfide; Crystallization

Introduction

In nature, sulfate (SO_4^{2-}) reduction by sulfate-reducing bacteria (SRB) significantly influences the biogeochemistry of anaerobic environments and plays a crucial role in global sulfur cycling (Fredrickson and Gorby 1996, Fredrickson et al. 2000, Sani et al. 2004, Zachara et al. 2001). In practice, their capacity for concomitant SO_4^{2-} and Fe^{III} reduction has been widely exploited to remediate acid mine drainage (AMD) (Bai et al. 2013, Sánchez-Andrea et al. 2014), and the consequent iron-sulfide production is attracting greater attention for its potential application in long-term uranium remediation (Abdelouas et al. 1999, Bi et al. 2013, Moyes et al. 2000) – for example, nanocrystalline iron-sulfide (nano-FeS) can be applied to effectively retard oxidative dissolution of immobilized U(IV) (Bi and Hayes 2014) or accelerate microbial dechlorination of chlorinated organics (Huo et al. 2016). On the other hand, SO_4^{2-} reduction needs to be minimized to avoid generation of hydrogen sulfide that is corrosive, toxic, and inhibitory to other desired processes such as denitrification (Zhao et al. 2013a, Zhao et al. 2013b, Zhao et al. 2014). Thus, fully understanding the metabolism of SRB provides fundamental guidelines for allowing the microorganisms to provide more beneficial services in water treatment and resource recovery.

Numerous studies of the electron-transfer pathway in respiratory sulfate reduction have focused on *Desulfovibrio* spp., a group of mesophilic SRB belonging to the Gram-negative *Deltaproteobacteria* class. Nevertheless, electron transfer in sulfate reduction remains incompletely understood and even controversial.

Earlier models (Elias et al. 2004, Heidelberg et al. 2004, Pankhania et al. 1988, Pieulle et al. 1997, Voordouw 2002) feature lactate ($\text{CH}_3\text{CHOHCOO}^-$), an exogenous electron donor and

carbon source, first being fermented to pyruvate ($\text{CH}_3\text{COCOO}^-$) by lactate dehydrogenase (Ldh), releasing protons and electrons. These protons and electrons react with membrane-bound hydrogenases (Coo Hase) to form molecular hydrogen (H_2). Pyruvate is then fermented to acetate (CH_3COO^-) through soluble pyruvate:ferredoxin oxidoreductase (Por) in the cytoplasm, also forming either H_2 through Coo Hase, formate (CHOO^-) through cytoplasmic pyruvate formate lyase (Pfl), or both. As intracellular electron carriers, H_2 and CHOO^- diffuse to the periplasm; there, they can be oxidized back to protons and electrons by periplasmic hydrogenases (hyn) and formate dehydrogenases (fdh), respectively. The electrons from oxidation of H_2 and CHOO^- are transported to Type I cytochrome c_3 (TpIc₃) and then transported through the cytochrome network back to the cytoplasm, where SO_4^{2-} is activated by the ATP generated through lactate fermentation, and the consequent adenosinephosphosulfate (APS) accepts the electrons and is gradually reduced to sulfide. In this so-called “hydrogen cycling model (Peck et al. 1987)”, lactate oxidation and SO_4^{2-} reduction are independent processes that can be linked only by the intracellular electron carriers. When pyruvate, H_2 , or formate is an exogenous electron donor, it can enter into this pathway of electron transfer.

This original model has been challenged by recent studies (Keller and Wall 2011, Keller et al. 2014, Noguera et al. 1998, Venceslau et al. 2010, Walker et al. 2009) that propose that sulfate also can accept electrons directly from lactate and pyruvate oxidations mediated by menaquinone (MQ) and ferredoxin redox loops, respectively; both electron flows are catalyzed by the membrane-bound quinone-interacting oxidoreductases (Qmo) complex (Ramos et al. 2012). In this new model, the role of H_2 cycling in sulfate reduction is diminished by half (Noguera et al. 1998) or even to almost nothing (Rabus et al. 2006). However, the latest studies (Keller and Wall 2011, Oliveira et al. 2008, Santos et al. 2015) propose that DsrMKJOP, a membrane-bound

dissimilatory sulfite reductase complex, catalyzing the last step of sulfate reduction (disulfide/trisulfide to free sulfide), may carry electrons exclusively from TpIc_3 ; in this scenario, electron transfer indirectly through H_2 cycling and directly through MQ mediation are complementary pathways instead of alternative pathways. Furthermore, some of these new models (Keller and Wall 2011, Pereira et al. 2011) propose that, during sulfate-reduction, H_2 production is attributed only to pyruvate oxidation to acetate, while electrons from lactate oxidation to pyruvate are exclusively involved in the MQ redox loops.

In this study, we provide deeper insight into some of the controversial steps of electron transfer by *D. vulgaris*, including 1) whether sulfate reduction can directly utilize electrons from lactate oxidation to pyruvate; 2) whether lactate oxidation to pyruvate exclusively produces H_2 in absence of SO_4^{2-} reduction; 3) whether pyruvate oxidation to acetate generates electron carriers for indirect/transmembrane SO_4^{2-} reduction; and, if so, 4) whether pyruvate oxidation to acetate produces formate alone. In order to differentiate combined disulfide/trisulfide from free sulfide, we introduced soluble iron, which combines only with free sulfide to form iron-sulfide precipitates. In addition, Fe^{III} is able to be reduced in the periplasm by TpIc_3 , which can carry electrons from H_2 or formate (Elias et al. 2004); thus, it can be used to retard indirect sulfate reduction featuring membrane-crossing electron transfer. Consistent with previous studies (Zhou et al. 2015a, Zhou et al. 2014a), amorphous iron sulfide formed initially by *D. vulgaris* was crystallized into mackinawite nanoparticles, but to different degrees depending on the electron donor. In our study, differences in electron flows provide new interpretations about the underlying mechanisms of iron-sulfide crystallization indirectly affected by *D. vulgaris*.

Material and Methods

Strain, growth medium, and culturing conditions

D. vulgaris was purchased from American Type Culture Collection (ATCC #29579) and grown in 160-ml serum bottles with 100 ml sterile standard ATCC 1249 medium and a headspace of N₂. The serum bottles were sealed with rubber stoppers and aluminum caps and incubated in a shaker (200 rpm) at 30°C right after inoculation. The cells were transferred to new culturing bottles every four to seven days by injecting 2-3 ml inoculum into new culturing bottles containing freshly prepared medium.

Preparation of biomass suspension

Right before the electron-flow tests, we prepared a fresh biomass suspension from culturing bottles in which bacterial growth had reached the late log phase after a 48-hour incubation (Zhou et al. (2014a). We followed the procedures of Zhou et al. (2014b) for preparing biomass suspensions. In brief, we transferred the liquid part from the culturing bottles into centrifuge tubes, centrifuged the tubes, discarded the supernatants, and re-suspended the remaining pellets in the basic assay solution (described below). We repeated this cycle three times and then distributed 5 mL of the biomass suspension into each test bottle. This gave 4-5 mg/L protein as the initial biomass concentration in each test bottle.

Experimental conditions

The basic assay solution in all the electron-flow tests was modified from the standard ATCC 1249 medium. It featured 5 mM sulfate (as Na₂SO₄, the respiratory electron acceptor), 15 mM citrate (as Na₃C₆H₅O₇, the primary pH buffer and soluble Fe stabilizer), 2 mM phosphate (as

K₂HPO₄, the P source and part of pH buffer), 7.5 mM calcium (as CaCl₂, the calcium source), 15 mM magnesium (as MgCl₂, the magnesium source), and 20 mM ammonium (as NH₄Cl, the N source). In particular, yeast extract was replaced with 5 mL of trace metal stock solution described by Chung et al. (2006) for all the electron-flow tests.

In the primary study, we tested four conditions based on different combinations of electron donor and iron source supplemented into the basic assay solution: sodium L-lactate plus ferrous chloride (Lac+Fe^{II}); sodium L-lactate plus ferric chloride (Lac+Fe^{III}); sodium pyruvate plus ferrous chloride (Pyr+Fe^{II}); and sodium pyruvate plus ferric chloride (Pyr+Fe^{III}). We added the same electron equivalent concentration of lactate (12 mM) and pyruvate (24 mM) before autoclaving and the same mole concentration of iron sources (5 mM) after autoclaving. The ratios of electron donors to acceptors were matched so that electrons released from lactate or pyruvate would be almost completely consumed for reductions of all SO₄²⁻ and Fe^{III} and for biomass growth. We prepared two bottles as duplicates for each condition.

In a supplementary study, we tested the four conditions with the same liquid matrix and incubation conditions as in the primary study, except that we continuously purged the headspaces with filtered UHP N₂ gas at a constant pressure of 2 psig (1.14 atm absolute pressure) throughout incubation in order to remove endogenously produced H₂ gas.

Analytical methods

In the primary study, we routinely collected 3-ml liquid samples from the bottles using sterile syringes and needles. We used unfiltered samples for protein and pH measurement, and we filtered samples through 0.22-μm membrane filter for other chemical analyses. Protein, sulfide, soluble Fe(III), and total soluble Fe concentrations were assayed using a UV–Visible

spectrophotometer (Cary 50 Bio, Varian, Inc., Santa Clara, CA). In particular, soluble Fe species were analyzed by the colorimetric 5-sulfosalicylic acid (SSA) method (Karamanev et al. 2002). Concentrations of anions – including sulfate, sulfite, thiosulfate, and phosphate – were analyzed using ion-exchange chromatography (IC, Dionex ICS2000; U.S. EPA, 1993). Volatile fatty acids (acetate, lactate, pyruvate, and formate) were identified and quantified using high-performance liquid chromatography (HPLC; Shimadzu 20AT) with a refractive index detector (RID). H_2 in the headspace was analyzed using a gas chromatograph (GC 2010, Shimadzu) equipped with a thermal conductivity detector (TCD). Elemental sulfur was extracted in tetrachloroethylene solvent and analyzed using an ultra-performance liquid chromatography (UPLC; Waters, Milford, MA, USA). Further details of sample preparation procedures and instrument conditions were described in previous studies (Zhou et al. 2014a, Zhou et al. 2014b).

In the supplementary study, we only analyzed concentrations of S and Fe species in the beginning and the end of the incubation period. In addition, iron-sulfide precipitates were quantified by digesting unfiltered samples in 0.5 M HCl solution for iron extraction (details described in Carpenter et al. (2015) and calculating the difference of measured total soluble iron before and after HCl treatment. The unidentified S species in each bottle were calculated by subtracting the total concentration of all detectable S species – SO_4^{2-} , SO_3^{2-} , $S_2O_3^{2-}$, S^0 , S^{2-} (including aqueous and gaseous H_2S), and FeS – in the end of incubation from the measured total SO_4^{2-} concentration in the beginning of incubation.

Mathematical modeling

Experimental results of substrate utilizations were fit to logistic, first-order, or Monod models (as appropriate) using SigmaPlot 12.5 software (SPSS, Inc., Chicago, IL, USA). The

models used for different purposes are introduced in the Results section, and details are provided in Electronic Supplementary Material (ESM). Kinetic and stoichiometric parameters were calculated from the experimental data using the equations described in Rittmann and McCarty (Rittmann and McCarty 2001). Electron balances were determined based on reaction stoichiometry modified from our previous study (Zhou et al. 2015b) and is detailed in ESM.

Separation of solids from culture medium

After the 90-hour incubation, we opened up all bottles in the primary study in an anaerobic glove box after 15-min centrifugation at 3,000 g using a benchtop centrifuge (VWR Clinic 200) located inside the glove box, removed supernatant, and transferred the precipitates and residual liquid (~10 mL) into sterile 50-ml centrifuge tubes. We repeated water addition + centrifugation + supernatant removal three times, and then we freeze dried the solids at -50°C under 10 Pa vacuum for 2 days; under this cold and O₂-free scenario, FeS oxidation was negligible. The dried solids were ground and stored anaerobically in sealed bottles in the -20°C freezer for further analyses.

Solid characterizations

We examined the powders using X-ray diffractometer (XRD; Rigaku D/Max-IIIB with monochromated Cu K_α radiation) and transmission electron microscopy (TEM; FEI Corp., Eindhoven, The Netherlands). We estimated average particle sizes of the mackinawite in those samples by visually measuring lattice fringe dimensions in TEM images and by mathematically calculating crystallite plane thickness in XRD spectra on the basis of the Scherrer equation:

$$t = \frac{0.9\lambda}{B \cos \theta_B} \quad (1)$$

188 where t is thickness of the particles ($\text{\AA}$) in a direction perpendicular to the reflection (001) planes,
189 λ is wavelength of the incident X-ray ($\text{\AA}$), B is full width at half maximum (FWHM) of the (001)
190 peak (radian), and θ_B is the Bragg angle (degrees) at the (001) peak (Cullity 1978; Hyun and
191 Hayes, 2009).

Results

Figures 1 and 2 profile the changes of substrates, metabolites, pH, and biomass (represented by protein concentration) during the 90-hour incubations of *D. vulgaris* utilizing lactate or pyruvate as the sole electron donor, respectively. Table 1 summarizes kinetic parameters estimated from these experimental results.

Conversion of SO_4^{2-} and soluble Fe to iron-sulfide by *D. vulgaris* utilizing lactate or pyruvate

Within 90 hours, most SO_4^{2-} was reduced for all conditions. Fe^{III} was first converted to Fe^{II} , and Fe^{II} was depleted along with SO_4^{2-} reduction, as FeS was formed. The measured concentrations of other S species – SO_3^{2-} , $\text{S}_2\text{O}_3^{2-}$, S^0 , and S^{2-} – were below detection limits (<0.02 mM, or $<0.4\%$ of initial SO_4^{2-} ; data not shown) throughout the incubation period; this confirms that the lost SO_4^{2-} was completely reduced to sulfide and rapidly precipitated with iron.

The lag phase before SO_4^{2-} reduction was longer during lactate utilization ~~was lagged more~~ than during pyruvate utilization

Once launched, SO_4^{2-} reduction was rapid for all conditions. The maximum rates were moderately higher for pyruvate (3.4-3.7 mM/d) than for lactate (3.1-3.2 mM/d), as quantified by the logistic decay model, which is summarized in Fig. 3A. However, the duration of lag-phases and lag patterns prior to the rapid takeoff of SO_4^{2-} reduction varied considerably for the different conditions.

In Lac+ Fe^{II} bottles (A-1 in Fig. 1), the SO_4^{2-} concentration did not change significantly ($<3\%$ change) during the first 30 hours, but then was rapidly reduced to <0.1 mM in the following 40 hours; the approximate zero-order rate was 3.1 mM/d at around the 50th hour. In contrast, the

Pyr+Fe^{II} bottles (A-1 in Fig. 2) launched SO₄²⁻ reduction along with pyruvate consumption without obvious delay; the maximum rate was 3.7 mM/day at the 30th hour.

In the presence of Fe^{III}, rapid SO₄²⁻ reduction occurred only after most Fe^{III} was reduced to Fe^{II}. In Lac+Fe^{III} bottles (B-1 in Fig. 1), SO₄²⁻ reduction was not detectable until 94% of Fe^{III} was reduced to Fe^{II} at the 56th hour; SO₄²⁻ reduction then was initiated with an approximate zero-order rate of 3.2 mM/d. In Pyr+Fe^{III} bottles (B-1 in Fig. 2), SO₄²⁻ reduction was retarded, but not entirely inhibited at first: By the 56th hour and with 88% Fe^{III} reduction, 0.9 mM (16%) of the starting SO₄²⁻ was reduced, and 1.0 mM total Fe was lost as a result of FeS precipitation.

Retardation of Fe^{III} reduction for pyruvate

When lactate was the added electron donor, Fe^{III} reduction exhibited first-order kinetics ($R^2 = 0.9992$) with a rate constant of 1.2 d⁻¹ (details in Fig. 3B), as SO₄²⁻ reduction was not detectable (B-1 in Fig. 1). When pyruvate was the added electron donor, Fe^{III} reduction was initially retarded, along with slowed SO₄²⁻ reduction (B-1 in Fig. 2). After the initial 40 hours, when 57% of Fe^{III} and 10% of SO₄²⁻ had been depleted, the Fe^{III}-reduction rate reached its maximum, a first-order rate of 1.9 d⁻¹ (Fig. 3B).

H₂ and pyruvate accumulations and contribution to Fe^{III} reduction in lactate bottles

We detected transient H₂ accumulation before rapid SO₄²⁻ reduction in all lactate bottles, but no H₂ above the detection limit (0.001 atm) throughout the incubation in pyruvate bottles.

All H₂ production was derived from lactate fermentation. In each Lac+Fe^{II} bottle (A-2 in Fig. 1), 0.16 mmole lactate was consumed during the first 30 hours, with minimal SO₄²⁻ reduction and bacterial growth (consuming <1% electrons from lactate), and nearly equal amounts (totally 0.17 mmole in a single 160-mL serum bottle containing 100 mL liquid) of H₂

and pyruvate were generated. For pyruvate, 53% was detected directly, and 47% was detected indirectly from its fermentation to acetate. An electron balance on the experimental data (Fig. 4A) suggests that >99% of the H₂ that was produced came from lactate fermentation to pyruvate; in other words, the H₂ produced from pyruvate transformation to acetate was negligible.

Contribution of H₂ and pyruvate to Fe^{III} reduction. In Lac+Fe^{III} bottles (B-2 in Fig. 1), H₂ and pyruvate accumulated up to 0.09 and 0.14 mmole during the first 56 hours, which had no SO₄²⁻ reduction; this represented only 41% of the electrons released from lactate (2% was in biomass). As the electron balance (Fig. 4B) reveals, H₂ was linked to 61% of the measured Fe^{III} reduction, while pyruvate transformation to acetate was responsible for reducing the other 39% Fe^{III}.

Subsequent H₂/pyruvate depletion for SO₄²⁻ reduction. In all lactate bottles, once rapid SO₄²⁻ reduction was initiated, H₂ and pyruvate concentrations began to decrease gradually. Most electrons released from depleted H₂ and pyruvate were targeted to SO₄²⁻ reduction, the exclusive electron-accepting process at that time. Thus, the accumulated H₂ was responsible for at least 9% and 5% of SO₄²⁻ reduction in Lac+Fe^{II} and Lac+Fe^{III} bottles, respectively.

Formate buildup and contribution to Fe^{III} reduction

We observed formate accumulation in all bottles. Electron flow calculation for lactate bottles (Fig. 4) suggests that formate was the exclusive product from pyruvate transformation to acetate, as H₂ production through this process was negligible (<1%). Prior to rapid SO₄²⁻ reduction, formate slowly accumulated along with acetate; when Fe^{III} was present, the detected formate concentrations were always lower than the total formate production, as reflected by the acetate concentration according to the stoichiometry (Eqn. S2-2); this confirms that partial

utilization of formate was responsible for 39% and 100% of Fe^{III} reduction in Lac+ Fe^{III} and Pyr+ Fe^{III} bottles, respectively.

Depriving H_2 resulted in incomplete SO_4^{2-} reduction

In the supplementary study, we observed an obvious discrepancy between the metabolic activities with lactate versus pyruvate, as shown in the final S speciation chart in Figure 5. On the one hand, continuous N_2 purging removed most biogenic H_2 , depriving *D. vulgaris* of at least one-half of the electrons released from lactate for H_2 -mediated SO_4^{2-} reduction (according to stoichiometry). In the lactate bottles, however, SO_4^{2-} depletion was still over 60%, which indicates that *D. vulgaris* partially bypassed the fermentation pathway and directly delivered electrons through MQ mediation for SO_4^{2-} reduction. Furthermore, *D. vulgaris* accomplished complete Fe^{III} and ~90% SO_4^{2-} reductions with H_2 stripping, which demonstrated the capacity of *D. vulgaris* to route electrons for Fe^{III} exclusively through formate.

According to the S mass balance in Figure 5, the lactate bottles accumulated more than 40% of the depleted SO_4^{2-} as sulfite plus some unknown intermediate, probably bisulfide- and/or a trisulfide-DsrC complex that cannot be detected by IC; these metabolites were minimal in the pyruvate bottles. The accumulation of partially reduced S metabolites with lactate indicates that some S-reducing pathways may rely exclusively on H_2 oxidation. Full S reduction with pyruvate means that formate was able to fully take over the responsibility of S reduction when H_2 was not normally generated.

Ambient pH and solid growth duration affected crystallization of biogenic mackinawite

Figure 6 presents the XRD patterns and TEM images of the biogenic solids collected from the bottles in the primary study. They confirm that mackinawite (Fe_{1+x}S) nanoparticles were the

predominant solid products. The lateral thicknesses of the mackinawite crystallites estimated using XRD and TEM were consistent (Table 1), and they reflected poorer crystallization of biogenic mackinawite with lactate as the electron donor or Fe^{III} as the iron source. Pyruvate transformation to acetate decreases the pH more than does lactate transformation to acetate, because it produces more protons for reducing the same quantity of SO_4^{2-} (stoichiometry in Eqns. S1 and S2 and experimental confirmation in Figs. 1 and 2). Thus, the observed greater crystallization with pyruvate is consistent with a previous study (Zhou et al. 2014a) that revealed that lower pH accelerated mackinawite crystallization. In addition, lactate experiments with a lag period before SO_4^{2-} reduction shortened the time for mackinawite crystallite growth.

Figure 7 presents a 3-dimensional graph to visualize the correlation of biogenic mackinawite size with pH and the time for crystal growth on basis of 20 sets of experimental data in this study and previous studies (Zhou et al. 2015a, Zhou et al. 2014a). The simulation clearly identifies the synergetic effect of low pH and longer crystal-growth time for promoting mackinawite crystallization. The underlying mechanism probably is associated with Ostwald ripening that, as illustrated in Figure S1A, states that smaller particles dissolve and re-precipitate on the larger particles as a means to minimize the overall energy (Guilbaud et al. 2010, Luther). Based on Ostwald ripening, lower pH speeded up mackinawite dissolution (Fig. S1B), while longer growth time allowed more re-precipitation.

Discussion

We propose Figure 8 as a conceptual framework for understanding electron flow from lactate and pyruvate for dissimilatory SO_4^{2-} and Fe^{III} reductions by *D. vulgaris*. In short, electron flow from lactate or pyruvate towards SO_4^{2-} reduction is not exclusively governed by only direct, membrane-bound MQ mediation or by indirect, membrane-crossing H_2 /formate cycling; rather, electron flow is regulated by the e^- donor and the presence of another e^- acceptor (i.e., Fe^{III} in our study). Thus, our framework reconciles earlier models that appeared to contradict each other. The following sections and Figures S2 – S6 systematically explain how the experimental results lead to the framework in Figure 8.

Endergonic formation of H_2 from lactate for SO_4^{2-} reduction: redundancy or strategy?

Lactate fermentation to pyruvate has a higher half-reaction reduction potential (-0.19 mV (Rabus et al. 2006); Table 2) than reduction of potential e^- acceptors, including proton to H_2 (-0.414 mV (Langmuir 1997)) and SO_4^{2-} to sulfide (-0.22 mV (Rabus et al. 2006)); thus, the processes having electron flow counter to the half-reaction redox potential need to be fueled by pyruvate oxidation to acetyl-CoA, which generates energy that can be coupled with reduction of protons (-0.414 mV (Langmuir 1997)), CO_2 (-0.498 mV (Rabus et al. 2006)), or SO_4^{2-} (-0.432 mV (Meyer et al. 2013)).

The additional energy cost for fermenting lactate to pyruvate became a thermodynamic barrier reflected by longer lag phase, lower biomass yield, and lower substrate utilization rate than pyruvate metabolism (Table 1). Taking the “Ockham's Razor” principle to the thermodynamic perspective (Schönheit et al. 2016, Schoepp-Cothenet et al. 2013), we conclude that, compared to directly delivering electrons through MQ pool for SO_4^{2-} reduction, it is less

efficient for bacteria to invest more energy to form H_2 and then recover the energy from H_2 for SO_4^{2-} reduction. The reason is that H_2 diffusion or electron transport through membrane-bound carriers between the cytoplasm and the periplasm costs additional energy.

The fulcrum of the regulation is the H_2 concentration, which has to be very low to allow fermentation to proceed. Any “friction” to H_2 diffusion to its sink raises the H_2 concentration near its point of generation. In some models (Meyer et al. 2013, Walker et al. 2009), H_2 is produced only after SO_4^{2-} is depleted or with sulfate-free conditions; H_2 accumulation immediately inhibited further metabolism unless the H_2 was scavenged by syntrophic partners like methanogens. In contrast, we observed H_2 accumulation up to 8% of the total available electron equivalent in lactate (or 0.06 atm, twice of the proposed inhibitive threshold as reported by Noguera et al. (1998)) during the lag phase of our primary study. We saw that H_2 buildup partially inhibited subsequent pyruvate oxidation during the first 20 hours (the first 2/3rd of the lag phase), but not lactate-induced H_2 production itself, as indicated by accumulation of pyruvate (Figs. 1A-2 and 3A), which otherwise would be rapidly transformed to acetate due to its faster metabolism (Table 1). The underlying inhibition mechanism may be associated with H_2 -regulated deactivation of pyruvate formate lyase (Pfl) and/or pyruvate:ferredoxin oxidoreductase (Por), as proposed by Figure S4. As SO_4^{2-} reduction provides higher biomass yield than CO_2 and/or proton reduction (Noguera et al. 1998), this “long-term-investment” strategy would limit the too-rapid diversion of pyruvate-generated electrons towards CO_2 or proton reductions before rapid and more energetically profitable SO_4^{2-} reduction was able to be initiated; thus, delaying pyruvate transformation would maximize bacterial growth.

Direct versus indirect e^- flow for SO_4^{2-} reduction is regulated by e^- donor

In addition to inhibiting pyruvate metabolism, lactate-originated H_2 also may regulate the initiation of SO_4^{2-} metabolism: in our study, rapid SO_4^{2-} reduction in lactate bottles was not initiated until dissolved H_2 in the liquid phase accumulated up to over 10 fold of the reported H_2 half-saturation constant at 1.4 μM for *D. vulgaris* (Noguera et al. 1998); when H_2 was less, SO_4^{2-} reduction was greatly retarded. The metabolic patterns in presence of soluble Fe^{III} reduction supported further insight on the pathway regulation. As an e^- acceptor for *D. vulgaris* metabolism, soluble Fe^{III} is more favorable than SO_4^{2-} because: 1) soluble Fe^{III} reduction has a higher half-reaction reduction potential (Table 2); and 2) Fe^{III} is able to accept electrons carried by periplasmic TpIc_3 (Fig. 8 and Fig. S3). Periplasmic Fe^{III} reduction shortcuts electron flow and eliminates electron delivery back to the cytoplasm for indirect SO_4^{2-} reduction (i.e., the membrane-crossing H_2 cycling process). In contrast, the route of direct SO_4^{2-} reduction through membrane-bound MQ mediation is independent from periplasmic Fe^{III} reduction; thus, it is able to compete for electrons with Fe^{III} . For this scenario, complete inhibition of SO_4^{2-} reduction until Fe^{III} depletion in Lac+ Fe^{III} bottles (B-1 in Fig. 1) suggests that the initiation of SO_4^{2-} reduction was controlled predominantly by the indirect cycling of H_2 produced from lactate (as proposed in Fig. S3). In contrast, concomitant Fe^{III} and SO_4^{2-} reduction in Pyr+ Fe^{III} bottles (B-1 in Fig. 2) underscores that the initiation of SO_4^{2-} reduction was regulated by direct electron flow from pyruvate (as proposed in Figs. S4 and S5).

Although the metabolic significance underlying this electron-donor-dependent regulation is not completely clear, its impact on biogenic iron-sulfide was apparent: H_2 -induced electron flow initiated SO_4^{2-} reduction after long lag phase (B-1 in Fig. 1) or gave full priority to Fe^{III} reduction (B-1 in Fig. 2). The consequence was a shortened time for iron-sulfide crystallite growth.

The TpIc₃ pool was also essential for completing SO₄²⁻ reduction to S²⁻.

For SO₄²⁻ reduction, indirect electron flow through H₂ or formate cycling was thermodynamically less favorable than through direct MQ mediation. Ideally for energy conservation, the indirect electron flow should have been spontaneously shut off upon initiation of rapid SO₄²⁻ reduction. However, formate accumulated along with electron donor consumption and subsequently was depleted during rapid SO₄²⁻ reduction in all conditions of our study (A-2 and B-2 in Figs. 1 and 2). This implies that formate-involved electron transfer was responsible for at least 10% of SO₄²⁻ reduction. The metabolic significance of maintaining part of TpIc₃-mediated H₂/formate oxidation was further documented in the supplementary study, where H₂ was stripped out of the headspace. Incomplete SO₄²⁻ reduction when H₂ was stripped implies that part of the SO₄²⁻ respiratory pathway relied on TpIc₃ activities. For instance, our observation of considerable sulfite accumulation in the absence of H₂ (Fig. 5) identifies that H₂-induced electron transfer involving the TpIc₃ pool is necessary for full reduction. In addition, previous research (Pereira et al. 2011, Santos et al. 2015) proposes that membrane-bound sulfite reductases (DsrMKJOP) transfer electrons from TpIc₃ for catalyzing disulfide or trisulfide reduction to sulfide. In our supplementary study, unidentified S species -- possibly the disulfide- and/or trisulfide-DsrC complex -- accumulated in the absence of lactate-originated H₂; the phenomenon indicates the indispensable role of TpIc₃-involved electron transfer in completing SO₄²⁻ reduction (as proposed in Fig. S6).

Conclusions

Our study emphasizes the significant effect of the e^- donor in directly stimulating SO_4^{2-} and Fe^{III} reduction and in indirectly controlling the production of desired iron-sulfide nanoparticles. In particular, when H_2 -induced periplasmic electron flow gave greater priority to Fe^{III} reduction over membrane-crossing electron acceptance by SO_4^{2-} as the main route of lactate-mediated SO_4^{2-} reduction, the longer lag phase before sulfate reduction shortened the time for iron-sulfide crystallite growth and led to smaller mackinawite (Fe_{1+x}S) nanocrystallites. Overall, the study suggests that the e^- donor provides a practical tool to regulate and optimize SRB-predominant bioremediation systems. For example, FeS can be used to retard re-oxidation of immobilized uraninite. Having FeS with controllable quality is an advantage, and our results point out that Fe^{III} is a better iron source to maintain FeS nanoparticles with higher redox reactivity. A second example is bioremediating AMD containing high concentrations of Fe^{III} and SO_4^{2-} . Pyruvate is a better electron donor for bio-stimulation for two reasons. First, pyruvate accelerates the overall process by enabling SO_4^{2-} reduction concomitant with Fe^{III} reduction. Second, pyruvate saves operating costs because the same amount of sulfide is produced with 45% the chemical cost compared to lactate (details in ESM) to immobilize hazardous soluble metals (e.g., Pb^{2+} , Cd^{2+} , and Mn^{2+}).

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TABLES

Table 1. Kinetic and stoichiometric parameters estimated from the experimental data, and mackinawite crystallite size estimated from XRD and TEM characterization

	Kinetics					Mackinawite (XRD)		Mackinawite (TEM)	
	Y_{obs}^a (g/ e^- -eq)	$f_{\text{s, obs}}^b$	μ_{max}^c (d $^{-1}$)	$r_{\text{max, sulfate}}^d$ (mM/day)	q_{max}^e (e^- -eq/g/d)	$FWHM^f$ (radiance)	$thickness$ (nm)	$d\text{-spacing}^g$ (Å)	$Dimension$ (nm)
Lac+Fe ^{II}	1.2±0.5	0.20±0.03	3.8±0.7	3.1±0.1	1.3±0.1	0.8±0.0	10.1±0.6	-	14.1±7.8
Lac+Fe ^{III}	1.0±0.2	0.20±0.02	3.4±0.8	3.2±0.2	1.5±0.2	2.7±0.1	3.1±0.3	5.2±0.1	3.6±0.7
Pyr+Fe ^{II}	1.5±0.9	0.28±0.03	4.0±1.2	3.7±1.0	1.5±0.3	0.4±0.0	20.6±0.9	-	19.9±4.4
Pyr+Fe ^{III}	1.6±0.3	0.29±0.01	4.2±1.6	3.4±0.8	1.0±0.0	1.5±0.1	5.4±0.6	5.3±0.2	4.1±0.8

^a Y_{obs} is the apparent yield coefficients. $Y_{\text{obs}} = \frac{\rho}{f_s} (M_c \text{ g cells/mol cells}) / (n_e e^- \text{-eq /mol cells})$, where M_c is the empirical formula weight of cells, n_e is the number of electron equivalents in an empirical mole of cells. ^b $f_{\text{s, obs}}$ is the apparent proportion electrons initially transferred into microbial cells. ^c μ_{max} is the maximum specific growth rate. $\mu = dx/(xdt) \approx \ln(x_2/x_1)/(t_2-t_1)$, where x is the biomass concentration and t is the time. ^d $r_{\text{max, sulfate}}$ is the maximum utilization rate of sulfate. $r = -qSx/(K+S)$, where q is the maximum specific rate of sulfate of sulfate utilization, K is the concentration giving one-half the maximum rate, and S is the sulfate concentration. ^e q_{max} is the maximum specific rate of sulfate of sulfate utilization. $q \approx (S_2-S_1)/(x_2-x_1)/(t_2-t_1)$. ^f $FWHM$ is the full-width at half-maximum. ^g $d\text{-spacing}$ is the crystal plane spacing of mackinawite crystallite.

Table 2. Values of theoretical redox potential (E_h) at neutral pH and 25°C for coupled reduction-oxidation reactions involved in this study.

Coupled Reaction	E_h (V)	Reference
acetyl-CoA + CO ₂ + 2e ⁻ → pyruvate	-0.498	Rabus et al. (2006)
acetyl-CoA + formate → pyruvate	-0.432	Meyer et al. (2013)
Fd _{ox} + e ⁻ → Fd _{red}	-0.420	Thauer et al. (2007)
acetyl-CoA + CO ₂ + H ₂ → pyruvate	-0.414	Meyer et al. (2013)
H ⁺ + e ⁻ → 1/2 H ₂	-0.414	Langmuir (1997)
cytochrome <i>c</i> ₃ ox/red	-0.290	Yagi and Maruyama (1971)
SO ₄ ²⁻ + 8e ⁻ → S ²⁻	-0.220	Rabus et al. (2006)
Pyruvate + 2e ⁻ → lactate	-0.190	Rabus et al. (2006)
Fe ^{III} -Cit + e ⁻ → Fe ^{II} -Cit	+0.372	Thamdrup (2000)
Fe ³⁺ + e ⁻ → Fe ²⁺ (pH<2)	+0.772	Brock et al. (1994); Thauer et al. (1977)

FIGURES

Figure 1. Profiles of pH, substrate, metabolite, and protein concentrations in the main study with lactate as the sole electron donor. Error bars represent standard deviation values.

Figure 2. Profiles of pH, substrate, metabolite, and protein concentrations in the main study with pyruvate as the sole electron donor. Error bars represent standard deviation values.

Figure 3. (A) Fitting of experimental sulfate concentration changes in Lac+Fe^{II}, Lac+Fe^{III}, Pyr+Fe^{II}, and Pyr+Fe^{III} using the logistic decay model ($R^2 = 0.9959, 0.9971, 0.9995$, and 0.9971 , respectively). (B) Fitting of experimental Fe^{III} concentration changes in Lac+Fe^{III} using the exponential decay model ($R^2 = 0.9992$); and in Pyr+Fe^{III} using the logistic and the Gompertz decay models for all data ($R^2 = 0.9984$ and 0.9988 , respectively) and the exponential decay model for the data during the last 50 hours ($R^2 = 0.9960$).

Figure 4. Fates of electron flow from lactate before SO_4^{2-} reduction during the first 30 hours in Lac+Fe^{II} bottles (A) and first 56 hours in Lac+Fe^{III} bottles (B). Details of the calculation steps are provided in the SI.

Figure 5. Mole distribution of sulfur species for the four different conditions at the end (90 hours) of the supplementary study.

Figure 6. XRD patterns (A1-D1) and TEM images (A2-D2) of solids separated from bottles in the main study identified in the graph. The red lines in the TEM images represent examples of lattice fringes featuring average spacing of $5.3 \pm 0.2 \text{ \AA}$ corresponding to d -spacing of (001) planes of mackinawite.

Figure 7. 3D simulation of biogenic mackinawite crystallite thickness as a function of pH and growth time, based on experimental data from this and previous studies. The four balls highlight the mackinawite produced by *D. vulgaris* under the four conditions in this study. The growth time was measured from the beginning of rapid sulfate reduction to the end of incubation (solids separation).

Figure 8. Comprehensive model of electron flow from lactate or pyruvate for dissimilatory sulfate and Fe^{III} reductions by *D. vulgaris*. Red arrows represent electron flow, and black arrows represent substrate transformation. APS, Adenosine-5'-phosphosulfate; *ApsAB*, APS hydrogenases; *Coo Hase*, CO-induced membrane-bound hydrogenase; *DsrAB* and *DsrC*, cytoplasmic sulfite reductases; *DsrMKJOP*, membrane-bound sulfite reductases; *Fd^{2-red}* and *Fd^{ox}*, reduced and oxidized ferredoxin, respectively; *FdhAB*, formate dehydrogenases; *Hase*, periplasmic hydrogenases; *Ldh*, lactate dehydrogenase; *MQ*, menaquinone; *MQH₂*, menaquinol; *Pfl*, pyruvate formate lyase; *Por*, pyruvate:ferredoxin oxidoreductase; *QmoABC*, quinone-interacting membrane-bound oxidoreductases; *QrcABCD*, TplC₃:MQ oxidoreductases; *TplC₃*, type I tetraheme cytochrome *c*₃. The separate electron-flow pathways are illustrated in Figures S2 – S6 in ESM.