Chapter 4
Extracellular Electron Uptake Mechanisms in Sulfate-Reducing Bacteria

Xiao Deng and Akihiro Okamoto

4.1 Overall Introduction

The ubiquitous sulfate-reducing bacteria (SRB) participate in significant biogeological processes such as the cycling of sulfur (Jørgensen 1982; Berner and Raiswell 1983) and carbon and anaerobic oxidation of methane (AOM) (Boetius et al. 2000). Moreover, since hydrogen sulfide (H_2S), a metabolite of SRB, is a metal-corroding chemical, SRB induce anaerobic corrosion of metal infrastructure such as underground oil and gas pipelines. This results in significant economic losses amounting to 0.4% of the gross domestic product of an industrial nation (Koch et al. 2001; Beavers and Thompson 2006). SRB are one of the most ubiquitous bacteria in anaerobic environments, especially the marine environments that are abundant with sulfate (~28 mM in seawater) (Christensen 1984; Parkes et al. 1989; Muyzer and Stams 2008; Fichtel et al. 2012). These bacteria reduce sulfate as the electron acceptor to hydrogen sulfide, coupled with the oxidation of diverse organics, hydrocarbon (So and Young 1999), and hydrogen (H_2) as the electron donors. In addition to these soluble or gaseous energy sources, recent studies demonstrated that some SRB use solids as electron donor via extracellular electron uptake (EEU) processes (Deng et al. 2015, 2018; Beese-Vasbender et al. 2015; Deng and Okamoto 2018; Deng et al. 2020). Direct electron transport process from metal iron specimens into the cell interior was first proposed in two marine sedimentary SRB strains, *Desulfovibrio ferrophilus* strain IS5 and *Desulfobacterium corrodens* strain IS4.

X. Deng
CSIRO Land and Water, Floreat, WA, Australia

A. Okamoto (✉)
National Institute for Materials Science, Tsukuba, Ibaraki, Japan

Graduate School of Chemical Sciences and Engineering, Hokkaido University, Sapporo, Hokkaido, Japan
e-mail: okamoto.akihiro@nims.go.jp

M. Ishii, S. Wakai (eds.), *Electron-Based Bioscience and Biotechnology*,
https://doi.org/10.1007/978-981-15-4763-8_4

This was based on the observation of significantly faster anaerobic corrosion in the absence of organic electron donors and at rates higher than other conventional SRB strains which corrode iron by producing H_2S or depleting accumulated H_2 on the iron surface (Dinh et al. 2004). The direct EEU process from solids in SRB was shown by excluding the involvement of H_2 (generated as a result of proton reduction on the surface of a solid electron donor) as an electron mediator between the solids and SRB cells in whole-cell electrochemical measurements. It has also been proposed that EEU by SRB from solid electron donors is an important mechanism in mediating the interspecies electron transfer process from methane-oxidizing archaea to SRB in the AOM consortia (McGlynn et al. 2015; Wegener et al. 2015; Scheller et al. 2016) as well as the bacterial energy acquisition in energy-scarce subsurface environments (Deng et al. 2018). While the importance and ubiquity of EEU mechanism for the biogeological and biophysical processes and anaerobic iron corrosion have been suggested, the number of microbial species with experimental evidences is still limited.

The present chapter reviews the methodology and background physiochemistry for measuring and calculating the H_2-evolution overpotential of an electrode, bioelectrochemical analyses on electrodes poised at potentials in the potential window for avoiding H_2 evolution, and the temperature and electrode potential dependency of cell activity analyses for investigating whether H_2 is involved in the EEU process. Finally, the limitations of current studies are discussed and critical questions that need to be clarified in future studies are addressed.

4.2 Experimental Background and Methods to Characterize the EEU Process

Since the oxidation of iron [$Fe^0 \rightleftharpoons Fe^{2+} + 2e^-$; $E^0 = -0.44$ V versus standard hydrogen electrode (SHE)] can proceed with proton reduction ($2H^+ + 2e^- \rightleftharpoons H_2$, $E^0_{pH\ 7} = -0.413$ V), H_2 spontaneously forms on the surface of iron under neutral pH condition ($Fe^0 + 2H^+ \rightleftharpoons Fe^{2+} + H_2$, $\Delta G^0_{pH\ 7} = -5.2$ kJ/mol). Therefore, to confirm whether SRB conduct EEU from solids without using H_2 as an electron mediator, artificial electrodes with large overpotentials for H_2 evolution such as indium-tin doped oxide (ITO) and graphite electrodes have been used instead of iron for tracking EEU processes in three-electrode electrochemical reactors.

To determine the onset potential ($E_{on\text{-}set}$) of H_2 evolution for an electrode material under a specific experimental condition, one can apply linear sweep voltammetry (LSV), in which the electrode current is measured as scanning the electrode potential in the negative direction. Because the standard potential of H_2 evolution is $E = -0.059$ pH (V) at 298 K, the H_2 evolution can be assigned to the current that exhibits a shift of $E_{on\text{-}set}$, according to the pH of the electrolyte. A previous study determined that the $E_{on\text{-}set}$ for H_2 evolution on an ITO electrode in artificial seawater medium at neutral pH was approximately -0.9 V (Deng et al. 2015). Based on the

$E_{\text{on-set}}$, the potential window suitable for tracking EEU processes for a certain electrode material can be determined, in which the electrode serves as the sole electron donor providing sufficient energy for cell metabolism while inhibiting the production of H_2.

Results of LSV measurement also enable the calculation of the H_2 evolution current on the electrode at any potential by using the cathodic part of the Butler-Volmer equation as described in Eq. (4.1), provided the H_2 evolution current is small enough and the rate-limiting step is not shifted to the H^+ diffusion. The anodic part is omitted because oxidation reactions on the negatively poised electrode are negligible.

$$j_{H_2} = -j_0 \exp\left[-\frac{2a_c F\left(E - E_{eq}\right)}{RT}\right] \tag{4.1}$$

where j_{H_2} is the H_2-evolution current density on the electrode, j_0 is the exchange current, a_c is cathodic charge transfer coefficient, F is Faraday constant, E is electrode potential, E_{eq} is the equilibrium potential of H_2 evolution, R is the universal gas constant, and T is the temperature. a_c is first calculated based on two current values (j_1 and j_2), measured at different electrode potentials, E_1 and E_2, respectively, as described in the following equation:

$$a_c = -\frac{RT}{2F(E_1 - E_2)} \ln\left(\frac{j_1}{j_2}\right) \tag{4.2}$$

j_0 is then calculated by inserting j_1 and E_1 (or j_2 and E_2) into Eq. (4.1). With the calculated values of a_c and j_0, the j_{H_2} at any electrode potential E can be determined based on Eq. (4.1), unless H^+ diffusion limits the reaction rate. By this method, the j_{H_2} on an ITO electrode in artificial seawater electrolyte with a neutral pH at -0.4 V was found to be ~ -0.11 nA/cm^2. Chronoamperometry measurement which detects currents on an electrode poised at a constant potential showed that the SRB cells capable of EEU produced -0.2 to 20 μA/cm^2 currents (Deng et al. 2015, 2018; Beese-Vasbender et al. 2015; Deng and Okamoto 2018), which are 10^3 to 10^5-fold larger than the j_{H_2}, on electrodes poised at -0.4 V, strongly suggest in the EEU capabilities of SRB cells.

Furthermore, because Eq. (4.1) also describes the relationship between j and T, the theoretical value of H_2-evolution current density at temperature T_1, $j_{H_2,\text{theoretical},T_1}$, can be approximately calculated by using a_c and j_0 values obtained at a known temperature (different from T_1). If the calculated $j_{H_2,\text{theoretical},T_1}$ value is consistent with the measured value j_{H_2,T_1}, it can be considered that the observed current is caused by abiotic reactions (e.g., H_2 production). In contrast, if the $j_{H_2,\text{theoretical},T_1}$ deviates largely from j_{H_2,T_1}, it strongly suggests that the observed current is directly associated with microbial metabolic activities, because the cell activity is largely affected by temperature and can be severely hampered at a suboptimal temperature

only slightly different from the growth temperature. For example, when the reaction temperature decreases from 30 to 4 °C, in theory, the $j_{H_2,\text{theoretical},4°C}$ would be 95% of the $j_{H_2,30°C}$; however, the measured current at 4 °C with SRB cells was less than 20% of that at 30 °C, indicating that the current was attributable to the hampered metabolic activity of SRB cells at suboptimal temperature (Pietzsch et al. 1999) rather than to the inorganic H_2 formation.

Another electrochemical measurement method, differential pulse voltammetry (DPV), is used to examine whether H_2 is involved in the EEU process by microbes. In DPV, a small potential pulse (pulse height ΔE = 10–100 mV, pulse width P_w = ~50 ms level) is repetitively applied to the working electrode (with an interval time Δt = 0.5–5 s), while the potential of the working electrode is slightly changed for several mV after each pulse toward the negative (reduction) direction or the positive (oxidative) direction. Currents at timings right before (before faradic reaction, I_1) and near the end of the pulse (in the middle of faradic reaction; I_2) are measured. By recording the difference of I_1 and I_2 as ΔI, the charging current can be largely eliminated. Therefore, DPV detects the redox potential and relative amount of active species on the electrode surface with a high sensitivity (as low as 10^{-8} M). The DPV analysis of *D. ferrophilus* IS5 cells on the ITO electrode surface revealed that its OMCs had a redox potential of ~−0.46 V and a half width of ~130 mV (Deng et al. 2018). Because the redox potential of OMCs is much more positive compared to the H_2 evolution potential, the result indicates that OMCs solely mediate EEU in IS5 cells without the involvement with H_2. Additionally, the exclusion of H_2 in the EEU process of SRB was also achieved by observing the unchanged current by introducing H_2-metabolizing SRB (e.g., *Desulfobacterium vacuolatum* and *Desulfovibrio* sp. strain HS3) on the electrode surface (Beese-Vasbender et al. 2015; Deng et al. 2018).

4.3 EEU Pathways Across the Cellular Outer Membrane of SRB

Mechanisms of EEU in SRB have been studied using electrochemical, microbiological, and bioinformatic methods. Outer-membrane cytochromes (OMCs), biosynthesized iron sulfide nanoparticles (FeS NPs), and soluble redox mediators are proposed to mediate EEU in cells and solid surfaces (Fig. 4.1). These mechanisms are similar to those identified in iron-reducing bacteria (IRB) that export metabolically generated electrons to the extracellular solid electron acceptors.

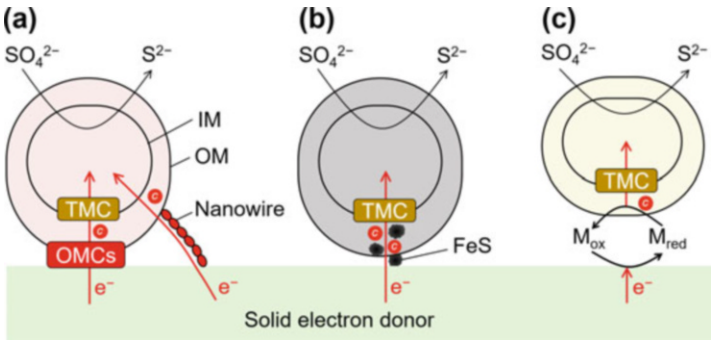

Fig. 4.1 Schematic illustration of identified mechanisms of extracellular electron uptake from solid electron donors coupled with sulfate reduction in sulfate-reducing bacteria (SRB). Electrons (e^-) were transferred across the outer membrane (OM) via (**a**) outer-membrane cytochromes (OMCs) and nanowire structure that is an extension of the OM, (**b**) electrically conductive iron sulfide nanoparticles (FeS NPs) biosynthesized on cell surface, and (**c**) diffusive redox mediators (M_{ox}/M_{red}). Periplasmic cytochromes (*c* in red circles) and/or periplasmic FeS NPs may facilitate the extracted electrons to be further transported to the cellular electron transport chain. *IM* inner membrane, *TMC* transmembrane complex

4.3.1 Outer-Membrane Cytochromes (OMCs) and Nanowires

OMCs are cytochrome proteins located on the cell outer membrane (OM) and were first identified in *Shewanella oneidensis* MR-1 (Myers and Myers 1992), an iron-reducing bacterium which respires oxide minerals (e.g., MnO_2 and Fe_2O_3) as electron acceptors when other soluble electron acceptors (e.g., oxygen) become scarce (Myers and Nealson 1988). The OMCs of *S. oneidensis* MR-1 consist of extracellularly localized decaheme cytochromes OmcA and MtrC, periplasmic decaheme MtrA, and an OM porin MtrB. The extracellular OmcA and MtrC interact with the periplasmic MtrA through MtrB (Myers and Myers 1992). These heme-porin complexes enable extracellular electron transport (EET) in *Shewanella* cells (Bretschger et al. 2007; Bucking et al. 2010; Jensen et al. 2010). Protein crystal structures (Clarke et al. 2011; Edwards et al. 2012, 2014, 2017), binding cofactors (Okamoto et al. 2013, 2014a; Hong and Pachter 2016; Tokunou et al. 2016), and electron transport mechanisms (Breuer et al. 2014) of OMCs have been studied extensively using *Shewanella* OMCs. Meanwhile, similar heme-porin complexes have been just recently identified in *D. ferrophilus* IS5 by gene and protein identification methods (Deng et al. 2018).

The IS5 genome harbors 26 genes encoding cytochromes with four or more heme-binding motifs [CX_nCH (n=2–5)], 7 of which are predicted to be localized in the OM region (Table 4.1). Furthermore, some of the protein products encoded by the potential OMCs gene clusters were detected on the isolated OM. The presence of OMCs in IS5 cells was confirmed by spectroscopic absorption measurement of the isolated OM fraction and was visualized by transmission electron microscopic (TEM) of cell cross-sections stained by a cytochrome-reactive diaminobenzidine

Table 4.1 Potential gene clusters encoding OMCs complex in the genome of *D. ferrophilus* IS5

Genes no.	Amino acids	Heme-binding motifs (most commonly CX_2CH)	Subcellular localization prediction
DFE_448	268	12	Periplasm
DFE_449	301	12	Periplasm
DFE_450	**340**	**6 (including one CX_5CH)**	**Extracellular**
DFE_451	343	N.A. (β-propeller protein)	Unknown
DFE_461	330	14	Periplasm
DFE_462	413	16	Periplasm
DFE_463	299	N.A. (β-propeller protein)	Unknown
DFE_464	**378**	**7 (including one CX_4CH)**	**Unknown but most likely extracellular**
DFE_465	386	7 (including one CX_3CH)	Periplasm

Bold characters indicate cytochromes with predicted extracellular localization

(DAB)-H_2O_2 method (McGlynn et al. 2015; Graham and Karnovsky 1966). Additionally, it was found that IS5 not only expressed OMCs on the cell surface but also the segmented nanowires with diameters ranging from 30 to 50 nm, which were most likely the extensions of OM and periplasmic space in a form of aligned OM vesicles (Fig. 4.1a) (Deng et al. 2018). Similar nanowire structures were also observed in *Shewanella* cells (Deng et al. 2018; Pirbadian et al. 2014; Subramanian et al. 2018). The capability of OMCs in mediating EEU was confirmed by IS5 cells upon controlling the expression of OMCs. The redox potential of IS5 OMCs was approximately −0.46 V (Deng et al. 2018), which is slightly more negative compared to that of the OMCs in *Shewanella* cells (−0.39 V) under EEU conditions. However, much needs to be explored about the IS5 OMCs, for instance, their protein crystal structure, potential binding cofactors, and whether they participate in mediation of the EET process.

The identified genes encoding OMCs in IS5 are widely found in the genomes of bacteria belonging to the phyla Aquificae, Thermodesulfobacteria, and Proteobacteria, which respire various sulfur compounds (e.g., thiosulfate, polysulfide, and sulfite) and primarily inhabit sedimentary environments (Deng et al. 2018). In contrast, the genes encoding OMCs in IRB strains, *Shewanella* and *Geobacter*, were found in the genomes of a few highly similar IRB strains living in oxic-anoxic water column and surface sediments. Because abundant minerals (e.g., iron-copper-sulfides) with adequately negative redox potentials can serve as potential electron donors for microbes, EEU mediated by OMCs may be potential energy acquisition strategy for a wide range of bacteria in these environments to surpass the competition for organic and gaseous electron donors (Deng and Okamoto 2017).

4.3.2 Conductive FeS Nanoparticle (NPs)-Mediated EEU

Electrically conductive NPs, such as palladium precipitated on the cell surface, were reported to function similarly to OMCs by mediating the transportation of

respiratory electrons to solid electron acceptors in some Gram-negative bacteria (Wu et al. 2011). The model of biosynthesized FeS NPs-mediated EEU in SRB was recently verified by a study using *Desulfovibrio vulgaris* Hildenborough, because many SRB like *D. vulgaris* lack a known EEU pathway, including OMCs and secretion of redox mediators (Heidelberg et al. 2004), but produce FeS.

D. vulgaris cells, cultivated in its growth medium supplemented with 2 mM Fe^{2+}, biosynthesized conductive FeS NPs (e.g., mackinawite) extracellularly, intracellularly, and on the cell surface. Electrochemical studies revealed that cells with biosynthesized FeS NPs became capable of direct EEU on the surface of −0.4 V-poised ITO electrodes, serving as the sole electron donor (Deng et al. 2020). By controlling the cell activity on the electrodes (e.g., by adding antibiotic, removing/re-supplementing sulfate or by maintaining a suboptimal growth temperature), it was revealed that the EEU of *D. vulgaris* was associated with its active cell metabolism. Notably, the electron uptake rate of FeS NPs-mediated EEU was approximately sevenfold faster in *D. vulgaris* than that mediated by OMCs in *D. ferrophilus* IS5, indicating that biosynthesized FeS NPs function as an efficient EEU conduit in SRB.

This finding significantly expands the ubiquity of EEU, which was previously considered to be a specific microbial process limited to those bacteria that possess OMCs, to a wider range of bacteria capable of self-synthesis of FeS NPs or have FeS NPs precipitated on the cell surface. FeS NPs are ubiquitously identified in nature, such as marine sediments, because they are predominantly produced by the ubiquitous SRB (Fortin et al. 1994; Donald and Southam 1999; Watson et al. 2000; Williams et al. 2005; Picard et al. 2016) and also in geological processes, e.g., the eruption of hydrothermal fluids and earthquakes (Findlay et al. 2019). These FeS NPs can be transported via water/ocean flow and precipitated on the cell surface of other bacteria. Based on the Pourbaix diagram, mackinawite and pyrrhotite that have been identified as EEU pathways in SRB are stable at conditions with pH $>$ 4, potentially more negative than −0.2 V, and a wide temperature range. The stable pH and potential ranges for mackinawite and pyrrhotite slightly shift to the acidic and reduced direction in accordance with elevated temperatures (Fig. 4.2) (Ning et al. 2015). This indicates that the EEU mechanism mediated by mackinawite and pyrrhotite can be exploited by a wide range of psychrophilic, mesophilic, and thermophilic bacteria for energy acquisition in slightly acidic, neutral, or alkaline reductive environments. In comparison, the semiconductive pyrite and greigite stay stable in lower pH even at strongly oxidative potentials. To further elucidate the distribution of FeS NPs-mediated EEU process, it would be important to explore whether the semiconductive greigite and pyrite can mediate EEU in SRB because they are also found in acidic environments with pH $<$ 4 (Sen and Johnson 1999; Meier et al. 2012; Sanchez-Andrea et al. 2013).

Given that SRB play a primary role in carbon mineralization in marine sedimentary environments (Jørgensen 1982; Berner and Raiswell 1983), the identified FeS NPs-mediated EEU brings novel insights into the biogeochemical cycles of carbon, iron, and sulfur. In addition, FeS NPs-mediated EEU may also provide a pathway for iron oxidation by SRB. A previous corrosion study has observed persistent corrosion of carbon steel by *D. vulgaris* after organic electrons depletion for up to 55 days

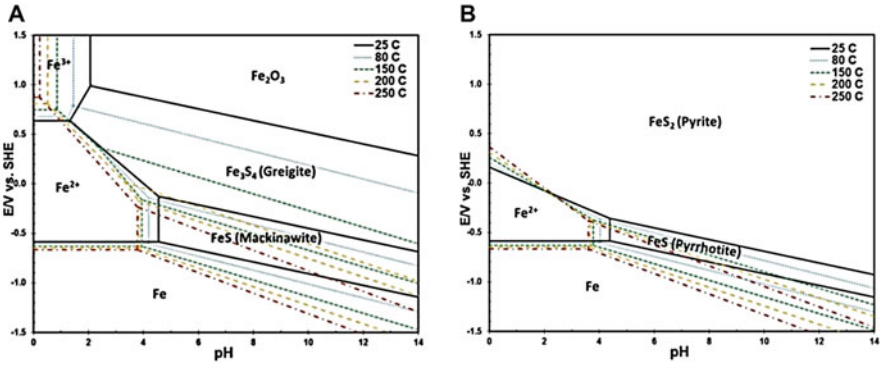

Fig. 4.2 Pourbaix diagrams for H_2S–H_2O–Fe system showing step changes in temperature up to 250 °C (T = 25–250 °C, $[H_2S]_{aq} = 9.4 \times 10^{-3}$ M, $[Fe^{2+}] = 10$ ppm, $[Fe^{3+}] = 10^{-6}$ M): (**a**) mackinawite/greigite and (**b**) mackinawite/greigite/pyrrhotite/pyrite. (Adapted from J. Ning *et al.*, Materials Science, 2015)

(Chen et al. 2015). Therefore, future identification of the mechanism underlying the synthesis of long, electrically conductive pathways via FeS NPs would contribute to the development of an effective strategy for inhibiting microbial iron corrosion.

4.3.3 Soluble Redox Electron Shuttles to Mediate EEU

Soluble redox mediators have been demonstrated to mediate the electron transportation process from cell interior to cell exterior in various microbes. So far, endogenous redox mediators, e.g., riboflavin (RF; $E^{\circ\prime} = -260$ mV), flavin mononucleotide (FMN; $E^{\circ\prime} = -205$ mV), and phenazine derivatives (e.g., phenazine-1-carboxylic acid, phenazine-1-carboxamide, and procyanin, with $E^{\circ\prime} = -275$, -150, and -32 mV, respectively), were identified to be secreted by *Geobacter sulfurreducens*, *S. oneidensis* MR-1, and *Pseudomonas aeruginosa* during cell growth (von Canstein et al. 2008; Marsili et al. 2008; Okamoto et al. 2014b). Meanwhile, exogenous redox mediators derived from the degradation of microbial and plant matter, such as humic acids (HA, $E^{\circ\prime} = -314$ to 430 mV) (Lovley et al. 1996; Jiang and Kappler 2008) artificial mediators [e.g., anthraquinone-2,6-disulfonate (2,6-AQDS; $E^{\circ\prime} = -186$ mV), and potassium ferricyanide ($E^{\circ\prime} = 436$ mV at pH 7)], were also identified (Nevin and Lovley 2000). The redox potentials of these membrane-permeable redox mediators are compatible to microbial cell metabolic processes, and some of these mediators, such as RF and FMN, can function as specific binding cofactors for OMCs (Okamoto et al. 2013, 2014b). Therefore, soluble mediators enable or accelerate electron transfer processes between cells and extracellular solids.

Although information about the capability of biosynthesizing and utilizing redox mediators remains very limited in SRB, it was reported that *Desulfotomaculum*

reducens MI-1, a Gram-positive SRB strain, secreted flavins during cell growth and used the flavins to facilitate the reduction of extracellular Fe^{3+} compounds (e.g., solid-phase hydrous ferric oxide). This suggested that this SRB strain may conduct EET mediated by flavins (Dalla Vecchia et al. 2014). Moreover, it was reported that the addition of redox mediators, RF and flavin adenine dinucleotide (FAD, $E^{\circ\prime} = -340$ mV), accelerated the corrosion of carbon steel and stainless steel by *D. vulgaris*, thereby suggesting that redox mediators may accelerate the EEU from metal iron in this SRB strain (Fig. 4.1c) (Li et al. 2015; Zhang et al. 2015). Further examination of the roles and mechanisms of redox mediators in the OMCs by SRB would contribute to our understanding of EEU mechanisms in SRB.

4.4 Microbial Physiology Coupled with EEU in SRB

4.4.1 Single Cell Activity Measurement for EEU Process

The surface-based cell activity analysis method, nanoscale secondary ion mass spectrometry (NanoSIMS), can be used to measure cell isotopic ratios (e.g., $^{13}C/C_{total}$ and $^{15}N/N_{total}$) at nanometer scale (Nana et al. 2018). It has been observed that when incubated on electrodes with isotopic C and nitrogen (N) sources (e.g., [1-^{13}C] acetate and $^{15}NH_4Cl$), the cells which obtain more energy from the electrode will assimilate more C and N, resulting in higher $^{13}C/C_{total}$ and $^{15}N/N_{total}$. NanoSIMS analysis has been commonly applied to analyze the distribution of cell activity on electrodes (Deng and Okamoto 2018; Saito et al. 2017).

NanoSIMS measurement of the cell activity of *D. ferrophilus* IS5 cells on electrodes at varied potentials (−0.2, −0.3, −0.4, and −0.5 V) demonstrated that cells, which conducted EEU at −0.4 and −0.5 V, obtained energy for C and N assimilation (Deng and Okamoto 2018). In contrast, cells at −0.2 and −0.3 V, which did not conduct EEU, had only $^{13}C/C_{total}$ and $^{15}N/N_{total}$ of natural abundances. Additionally, NanoSIMS measurement of cell activity at varied potentials also revealed the $E_{on\text{-}set}$ for EEU was consistent with the LSV measurement result. Therefore, NanoSIMS measurement of the potential dependency of cell activity could be applied to detect slow microbial EEU process and determine the $E_{on\text{-}set}$ of EEU process where the microbial signals are comparable to or smaller than the background signals.

4.4.2 Cell Growth of SRB During EEU

Electrochemical and NanoSIMS analyses on various SRB species with different EEU pathways, including *D. ferrophilus* IS5, *Desulfobacterium corrodens* IS4, and *D. vulgaris* Hildenborough, showed that their $E_{on\text{-}set}$ was similar and was

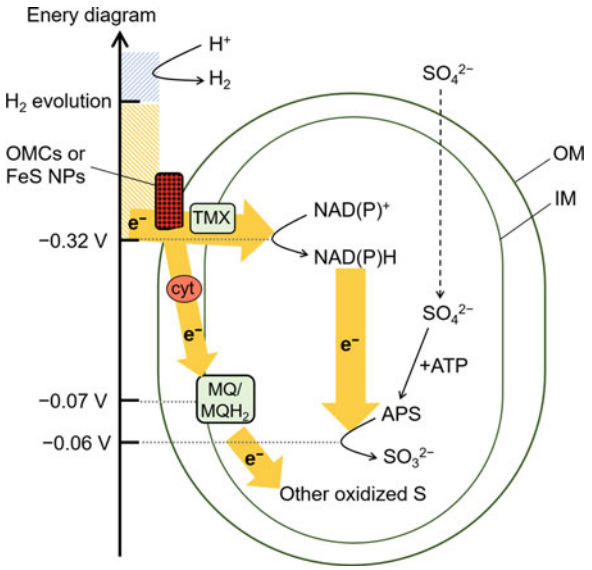

Fig. 4.3 Energy diagram of the extracellular electron uptake from extracellular solids via OMCs or FeS NPs in SRB. The onset potential for EEU is more negative than −0.3 V, which allows sufficient energy for generating nicotinamide adenine dinucleotide (phosphate) [NAD(P)H] and/or reduced menaquinone (MQH_2) which drives the reduction of oxidized sulfur (S) compounds (e.g., sulfate, SO_4^{2-}; sulfite, SO_3^{2-}) and intermediates, such as adenosine phosphosulfate (APS). If the solid has a redox potential negative enough for the proton (H^+) reduction, hydrogen (H_2) is formed on the electrode surface serving as an alternative electron donor for SRB. *cyt* periplasmic cytochrome, *OM* outer membrane, *IM* inner membrane, *TMX* transmembrane complex. Unit, V versus standard hydrogen electrode

approximately −0.3 V (Beese-Vasbender et al. 2015; Deng and Okamoto 2018). Therefore, EEU thermodynamically enables the generation of NAD(P)H ($E^{\circ\prime} = -0.32$ V) by reduction reactions and fuels cell growth (Fig. 4.3). However, in electrochemical studies using single-chamber three-electrode reactors equipped with −0.4 V-poised electrodes as the sole electron donor for incubating *D. ferrophilus* IS5 cells, cell activity was too slow to allow replication in a time span up to 66 days (Deng et al. Under review). The lack of cell growth on electrode surface in single-chamber reactors was also reported for the IRB strain *S. oneidensis* MR-1, which couples EEU with the reduction of oxygen or fumarate (Rowe et al. 2018). In contrast, some iron-oxidizing bacteria (IOB), e.g., *Acidithiobacillus ferrooxidans*, could grow in single-chamber reactors by coupling EEU with oxygen (O_2) reduction (Summers et al. 2013; Ishii et al. 2015). Therefore, although factors that differentiate SRB and IRB cells from IOB for growing on electrodes are unclear, EEU was proposed to supply limited energy for cell maintenance but not cell growth.

A recent study revealed factors specific to the electrochemical reactors that restricted cell growth on the electrodes by focusing on the generation of oxidative stress in the electrochemical reactors (Deng et al. Under review). By incubating IS5 cells in H-type reactors in which the counter electrode (CE) at which oxidation

reactions occur associated with EEU process is separated from cells using a proton-exchange membrane, the first evidence of the growth of *D. ferrophilus* IS5 cells on −0.4 V-poised electrodes was obtained by NanoSIMS analysis. Moreover, in addition to the exogenous oxidative stress, IS5 cell activity was also likely to be restricted by the endogenous oxidative stress produced by the reduction of trace amount of O_2 by the reduced cellular electron transport chain. Therefore, these results demonstrated that the EEU process is intrinsically coupled with the production of exogenous and endogenous oxidative stress and clarified that EEU is an important mechanism supporting cell growth under energy-limited conditions, rather than a mere support for cell maintenance.

However, it is possible that factors specific to the electrochemical systems that inhibit cell growth on the electrode surface. For example, the oxidizing species produced at the counter electrode or an unsuitable working electrode potential may supress cell acitity on the working electrode. These possibilities are currently under examination by Deng et al.

4.4.3 *Gene Expression of* D. ferrophilus *IS5 During EEU*

Transcriptome analysis of IS5 cells which generated currents on electrodes demonstrated upregulated expressions of genes encoding central energy metabolism, including ATP synthesis, sulfate-reducing pathway, and cell division and cell wall synthesis, compared to cells that generated lower currents or cells incubated without an electrode (Deng and Okamoto 2018). The analysis further revealed the significant upregulation of genes encoding OMCs but not those encoding periplasmic hydrogenases required for H_2 utilization in cells that produced higher currents. This in turn strongly suggested that OMCs are the pathway mediating the EEU process without the requirement of oxidizing H_2 as an electron mediator. Moreover, the upregulated levels of antioxidative genes were also observed in cells that produced higher current on an electrode poised at −0.5 V, compared to cells that produced lower currents at −0.4 V. This implied that cells that obtained more energy during EEU had to deal with more antioxidative stress, most likely originating endogenously from the reduction of trace O_2 in the system via the electron transport chain (Deng et al. Under review).

4.5 Future Perspective of EEU by SRB

4.5.1 *Exploration of New Genes for EEU Mechanism in SRB*

OMCs, FeS NPs, and redox mediators have been identified as different pathways mediating EEU in SRB. However, there is a possibility of the presence of new pathways. Therefore, the EEU potential should be explored in SRB living in various

environments. For example, a different EET pathway was recently identified in the Gram-positive bacterium *Listeria monocytogenes*, whereby a novel membrane-attached cytoplasmic NADH dehydrogenase mediated the transportation of respiratory electrons to membrane-localized quinone pool that was further transported to extracellular flavoprotein and/or flavin shuttles to reach extracellular electron acceptors (Light et al. 2018).

4.5.2 Proof of EEU Process in AOM and Iron Corrosion Processes

Although the capability of EEU from electrode has been identified in SRB (Dinh et al. 2004), electron extraction by cells from iron still remains unverified, largely due to the fact that H_2 spontaneously generates on the iron surface and serves as a potential electron donor. To exclusively identify EEU process in iron corrosion by SRB, approaches such as comparison of corrosion rates in wild-type, OMC-deficient, and hydrogenase-deficient strains would be useful. In addition, comparative gene expression analyses of OMCs and hydrogenases of SRB in the presence of iron and H_2 as the sole electron donor could provide insights into the corrosion mechanism.

EEU in SRB was also reported to be important for the activity of syntrophic consortia performing AOM. An interspecies electron transport model has been proposed for AOM consortia. This model states that SRB likely receive electrons directly from the methanotrophic archaea via OMCs and/or conductive pili structures (McGlynn et al. 2015; Wegener et al. 2015; Scheller et al. 2016) rather than using soluble intermediating compounds such as a methane-derived organic carbon compound (Moran et al. 2008). To directly prove this model, electrochemical analysis using archaeal and bacterial isolates that are put in separate anodic and cathodic chambers, respectively, would be required.

4.5.3 Electrical Incubation to Isolate Uncultivated SRB

It is estimated that more than 99.9% of subsurface microbes are uncultivated by conventional methods using soluble electron donors (Short 1997). Because minerals with sufficiently negative potentials, such as iron-copper-sulfides, and microbes potentially possessing an EEU pathway, are widespread in anoxic subsurface environments, electrical incubation methods using electrochemical reactors may enable the enrichment and isolation of novel uncultivated EEU-capable strains from these environments. Moreover, since EEU is not limited to cell-mineral interactions but also intercellular/interspecies interactions, EEU-capable microbes may also be found in non-mineral environments, such as animal guts. Gut microbial strains, such as *Desulfovibrio piger* and *Faecalibacterium prausnitzii*, have been reported to

conduct EET (Khan et al. 2012; de Campos Rodrigues and Rosenbaum Miriam 2014). Testing the capability and significance of EEU in these microbes related with host health may contribute to the development of new technologies for treating EEU-capable pathogens. Because previous studies suggested that different EEU-capable microbes may prefer different electrode potentials and electrode physical properties, using different electrode materials with different range of potential windows may enable the isolation of different EEU-capable microbes.

4.5.4 Proof of Ubiquity of EEU in Environments

Given that EEU-mediating pathways, including OMCs, FeS NPs, and redox mediators, are potentially widespread in sediment bacteria and electric current flow on the mineral surface of sediment hydrothermal vents has been reported (Nakamura et al. 2010), EEU likely plays important role in the energy acquisition by a wide range of bacteria in sediment environments. However, such a model describing EEU-supported subsurface biosphere would require identification of EEU-capable microbes in different sites and the comparison of cell metabolism in situ and ex situ. If EEU capability and the pathways can be identified in abundant microbial lineages, rigorous analysis of gene heritage in the phylogenetic tree would be possible and reveal the origin of EEU biomarker. If the biomarker is conserved vertically in numerous strains living in closely related habitat, it would signify the importance of EEU mediated by the biomarker in the environment. Furthermore, because hydrothermal vent systems like those on Earth are also present in extraterrestrial planets, such as on the icy surface of Europa (Gaidos et al. 1999; McCollom 1999; Chyba 2000; Zolotov and Shock 2003) and on ancient Mars (Michalski et al. 2017), identification of the capability of EEU via non-enzymatic pathways in SRB as well as other microbes with an ancient origin from minerals of hydrothermal vents would provide insights on the origin of life on Earth as well as possible life on extraterrestrial planets.

4.6 Conclusion

In this chapter, we introduced electrochemical methods and studies for identifying direct EEU process in SRB, the most ubiquitous and ancient bacteria on Earth (Shen and Buick 2004). The evolution of H_2 on the surface of solid electron donors has been a major obstacle in the research progress of the EEU mechanisms. By using stable electrodes with large overpotential for H_2 evolution and controlling the physicochemical parameters (e.g., temperature) during the EEU process, the possibility of H_2 involvement in EEU is finally excluded. So far, the identified EEU pathways in SRB include OMCs, FeS NPs, and possibly electron shuttles and can be ubiquitously found in subsurface environments. EEU might be an ancient energy conservation mechanism for supporting the subsurface ecosystems. These findings

contribute to our understanding of SRB physiology and have broad implications in other critical processes associated with SRB, such as anaerobic iron corrosion, AOM, and biogeochemical cycles of iron, sulfur, and carbon. In the future, we anticipate that new EEU pathways in SRB and the related electrosynthetic and biomedical applications will be established and an increasing number of microbes capable of EEU will be identified.

References

Beavers JA, Thompson NG (2006) External corrosion of pipelines in soil. In: Oil and gas pipelines. https://doi.org/10.1002/9781119019213.ch20

Beese-Vasbender PF, Nayak S, Erbe A, Stratmann M, Mayrhofer KJJ (2015) Electrochemical characterization of direct electron uptake in electrical microbially influenced corrosion of iron by the lithoautotrophic SRB Desulfopila corrodens strain IS4. Electrochim Acta 167:321–329

Berner RA, Raiswell R (1983) Burial of organic-carbon and pyrite sulfur in sediments over phanerozoic time—a new theory. Geochim Cosmochim Acta 47:855–862

Boetius A et al (2000) A marine microbial consortium apparently mediating anaerobic oxidation of methane. Nature 407:623–626

Bretschger O et al (2007) Current production and metal oxide reduction by Shewanella oneidensis MR-1 wild type and mutants. Appl Environ Microbiol 73:7003–7012

Breuer M, Rosso KM, Blumberger J (2014) Electron flow in multiheme bacterial cytochromes is a balancing act between heme electronic interaction and redox potentials. Proc Natl Acad Sci 111:611–616

Bucking C, Popp F, Kerzenmacher S, Gescher J (2010) Involvement and specificity of Shewanella oneidensis outer membrane cytochromes in the reduction of soluble and solid-phase terminal electron acceptors. FEMS Microbiol Lett 306:144–151

Chen YJ et al (2015) Long-term survival of Desulfovibrio vulgaris on carbon steel and associated pitting corrosion. Corros Sci 90:89–100

Christensen D (1984) Determination of substrates oxidized by sulfate reduction in intact cores of marine-sediments. Limnol Oceanogr 29:189–192

Chyba C (2000) Energy for microbial life on Europa (vol 403, p 381, 2000). Nature 406:368–368

Clarke TA et al (2011) Structure of a bacterial cell surface decaheme electron conduit. Proc Natl Acad Sci U S A 108:9384–9389

Dalla Vecchia E, Suvorova EI, Maillard J, Bernier-Latmani R (2014) Fe(III) reduction during pyruvate fermentation by Desulfotomaculum reducens strain MI-1. Geobiology 12:48–61

de Campos Rodrigues T, Rosenbaum Miriam A (2014) Microbial electroreduction: screening for new cathodic biocatalysts. ChemElectroChem 1:1916–1922

Deng X, Okamoto A (2017) Energy aquisition via electron uptake by the sulfate-reducing bacterium *Desulfovibrio ferrophilus* IS5. J Jpn Soc Extremophiles 16:67–75

Deng X, Okamoto A (2018) Electrode potential dependency of single-cell activity identifies the energetics of slow microbial electron uptake process. Front Microbiol 9:2744

Deng X, Nakamura R, Hashimoto K, Okamoto A (2015) Electron extraction from an extracellular electrode by Desulfovibrio ferrophilus strain IS5 without using hydrogen as an electron carrier. Electrochemistry 83:529–531

Deng X, Dohmae N, Nealson KH, Hashimoto K, Okamoto A (2018) Multi-heme cytochromes provide a pathway for survival in energy-limited environments. Sci Adv 4:eaao5682

Deng X, Dohmae N, Kaksonen AH, Okamoto A (2020) Biogenic iron sulfide nanoparticles to enable extracellular electron uptake in sulfate-reducing bacteria, Angewandte Chemie-International Edition. https://doi.org/10.1002/anie.201915196

Deng X, Saito J, Kaksonen A, Okamoto A (Under review) Enhancement of cell growth by uncoupling extracellular electron uptake and oxidative stress production in sediment sulfate-reducing bacterial

Dinh HT et al (2004) Iron corrosion by novel anaerobic microorganisms. Nature 427:829–832

Donald R, Southam G (1999) Low temperature anaerobic bacterial diagenesis of ferrous monosulfide to pyrite. Geochim Cosmochim Acta 63:2019–2023

Edwards MJ, Fredrickson JK, Zachara JM, Richardson DJ, Clarke TA (2012) Analysis of structural MtrC models based on homology with the crystal structure of MtrF. Biochem Soc Trans 40:1181–1185

Edwards MJ et al (2014) The X-ray crystal structure of Shewanella oneidensis OmcA reveals new insight at the microbe-mineral interface. FEBS Lett 588:1886–1890

Edwards MJ, Gates AJ, Butt J, Richardson DJ, Clarke TA (2017) Comparative structure-potentio-spectroscopy of the Shewanella outer membrane multiheme cytochromes. Curr Opin Electrochem 4:199–205

Fichtel K, Mathes F, Könneke M, Cypionka H, Engelen B (2012) Isolation of sulfate-reducing bacteria from sediments above the deep-subseafloor aquifer. Front Microbiol 3:65

Findlay AJ et al (2019) Iron and sulfide nanoparticle formation and transport in nascent hydrothermal vent plumes. Nat Commun 10:1597

Fortin D, Southam G, Beveridge TJ (1994) Nickel sulfide, iron-nickel sulfide and iron sulfide precipitation by a newly isolated desulfotomaculum species and its relation to nickel resistance. FEMS Microbiol Ecol 14:121–132

Gaidos EJ, Nealson KH, Kirschvink JL (1999) Biogeochemistry—life in ice-covered oceans. Science 284:1631–1633

Graham RC, Karnovsky MJ (1966) The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. J Histochem Cytochem 14:291–302

Heidelberg JF et al (2004) The genome sequence of the anaerobic, sulfate-reducing bacterium Desulfovibrio vulgaris Hildenborough. Nat Biotechnol 22:554–559

Hong G, Pachter R (2016) Bound flavin–cytochrome model of extracellular electron transfer in Shewanella oneidensis: analysis by free energy molecular dynamics simulations. J Phys Chem B 120:5617–5624

Ishii T, Kawaichi S, Nakagawa H, Hashimoto K, Nakamura R (2015) From chemolithoautotrophs to electrolithoautotrophs: CO2 fixation by Fe(II)-oxidizing bacteria coupled with direct uptake of electrons from solid electron sources. Front Microbiol 6:994

Jensen HM et al (2010) Engineering of a synthetic electron conduit in living cells. Proc Natl Acad Sci U S A 107:19213–19218

Jiang J, Kappler A (2008) Kinetics of microbial and chemical reduction of humic substances: implications for electron shuttling. Environ Sci Technol 42:3563–3569

Jørgensen BB (1982) Mineralization of organic matter in the sea bed—the role of sulphate reduction. Nature 296:643–645

Khan MT et al (2012) The gut anaerobe Faecalibacterium prausnitzii uses an extracellular electron shuttle to grow at oxic–anoxic interphases. ISME J 6:1578–1585

Koch GH, Brongers MPH, Thompson NG, Virmani YP, Payer JH (2001) Corrosion cost and preventive strategies in the United States. NACE International, Dublin

Li HB et al (2015) Extracellular electron transfer is a bottleneck in the microbiologically influenced corrosion of C1018 carbon steel by the biofilm of sulfate-reducing bacterium Desulfovibrio vulgaris. PLoS One 10:e0136183

Light SH et al (2018) A flavin-based extracellular electron transfer mechanism in diverse Gram-positive bacteria. Nature 562:140

Lovley DR, Coates JD, BluntHarris EL, Phillips EJP, Woodward JC (1996) Humic substances as electron acceptors for microbial respiration. Nature 382:445–448

Marsili E et al (2008) Shewanella Secretes flavins that mediate extracellular electron transfer. Proc Natl Acad Sci U S A 105:3968–3973

McCollom TM (1999) Methanogenesis as a potential source of chemical energy for primary biomass production by autotrophic organisms in hydrothermal systems on Europa. J Geophys Res Planets 104:30729–30742
McGlynn SE, Chadwick GL, Kempes CP, Orphan VJ (2015) Single cell activity reveals direct electron transfer in methanotrophic consortia. Nature 526:531–535
Meier J, Piva A, Fortin D (2012) Enrichment of sulfate-reducing bacteria and resulting mineral formation in media mimicking pore water metal ion concentrations and pH conditions of acidic pit lakes. FEMS Microbiol Ecol 79:69–84
Michalski JR, Dobrea EZN, Niles PB, Cuadros J (2017) Ancient hydrothermal seafloor deposits in Eridania basin on Mars. Nat Commun 8:15978
Moran JJ et al (2008) Methyl sulfides as intermediates in the anaerobic oxidation of methane. Environ Microbiol 10:162–173
Muyzer G, Stams AJM (2008) The ecology and biotechnology of sulphate-reducing bacteria. Nat Rev Microbiol 6:441–454
Myers CR, Myers JM (1992) Localization of cytochromes to the outer-membrane of anaerobically grown Shewanella-Putrefaciens Mr-1. J Bacteriol 174:3429–3438
Myers CR, Nealson KH (1988) Bacterial manganese reduction and growth with manganese oxide as the sole electron-acceptor. Science 240:1319–1321
Nakamura R et al (2010) Electrical current generation across a Black Smoker Chimney. Angew Chem Int Ed 49:7692–7694
Nana GYG et al (2018) Division-based, growth rate diversity in bacteria. Front Microbiol 9:849
Nevin KP, Lovley DR (2000) Potential for nonenzymatic reduction of Fe(III) via electron shuttling in subsurface sediments. Environ Sci Technol 34:2472–2478
Ning J, Zheng Y, Brown B, Young D, Nesic S (2015) Construction and verification of Pourbaix diagrams for hydrogen sulfide corrosion of mild steel. In: NACE—International corrosion conference series 2015
Okamoto A, Hashimoto K, Nealson KH, Nakamura R (2013) Rate enhancement of bacterial extracellular electron transport involves bound flavin semiquinones. Proc Natl Acad Sci U S A 110:7856–7861
Okamoto A et al (2014a) Cell-secreted flavins bound to membrane cytochromes dictate electron transfer reactions to surfaces with diverse charge and pH. Sci Rep 4:5628
Okamoto A et al (2014b) Uptake of self-secreted flavins as bound cofactors for extracellular electron transfer in Geobacter species. Energy Environ Sci 7:1357–1361
Parkes RJ, Gibson GR, Muellerharvey I, Buckingham WJ, Herbert RA (1989) Determination of the substrates for sulfate-reducing bacteria within marine and estuarine sediments with different rates of sulfate reduction. J Gen Microbiol 135:175–187
Picard A, Gartman A, Girguis PR (2016) What do we really know about the role of microorganisms in iron sulfide mineral formation? Front Earth Sci 4:68
Pietzsch K, Hard BC, Babel W (1999) A Desulfovibrio sp capable of growing by reducing U(VI). J Basic Microbiol 39:365–372
Pirbadian S et al (2014) Shewanella oneidensis MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components. Proc Natl Acad Sci U S A 111:12883–12888
Rowe AR et al (2018) Tracking electron uptake from a cathode into Shewanella cells: implications for energy acquisition from solid-substrate electron donors. mBio 9:e02203-17
Saito J, Hashimoto K, Okamoto A (2017) Nanoscale secondary ion mass spectrometry analysis of individual bacterial cells reveals feedback from extracellular electron transport to upstream reactions. Electrochemistry 85:444–446
Sanchez-Andrea I, Stams AJM, Amils R, Sanz JL (2013) Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments. Environ Microbiol Rep 5:672–678
Scheller S, Yu H, Chadwick GL, McGlynn SE, Orphan VJ (2016) Artificial electron acceptors decouple archaeal methane oxidation from sulfate reduction. Science 351:703–707

Sen AM, Johnson B (1999) Acidophilic sulphate-reducing bacteria: candidates for bioremediation of acid mine drainage. In: Biohydrometallurgy and the environment toward the mining of the 21st century, pt A, vol 9, pp 709–718

Shen YN, Buick R (2004) The antiquity of microbial sulfate reduction. Earth Sci Rev 64:243–272

Short JM (1997) Recombinant approaches for accessing biodiversity. Nat Biotechnol 15:1322–1323

So CM, Young LY (1999) Isolation and characterization of a sulfate-reducing bacterium that anaerobically degrades alkanes. Appl Environ Microbiol 65:2969

Subramanian P, Pirbadian S, El-Naggar MY, Jensen GJ (2018) Ultrastructure of Shewanella oneidensis MR-1 nanowires revealed by electron cryotomography. Proc Natl Acad Sci U S A 115:E3246–E3255

Summers ZM, Gralnick JA, Bond DR (2013) Cultivation of an obligate Fe(II)-oxidizing lithoautotrophic bacterium using electrodes. mBio 4:e00420-12

Tokunou Y, Hashimoto K, Okamoto A (2016) Acceleration of extracellular electron transfer by alternative redox-active molecules to riboflavin for outer-membrane cytochrome c of Shewanella oneidensis MR-1. J Phys Chem C 120:16168–16173

von Canstein H, Ogawa J, Shimizu S, Lloyd JR (2008) Secretion of flavins by Shewanella species and their role in extracellular electron transfer. Appl Environ Microbiol 74:615–623

Watson JHP et al (2000) Structural and magnetic studies on heavy-metal-adsorbing iron sulphide nanoparticles produced by sulphate-reducing bacteria. J Magn Magn Mater 214:13–30

Wegener G, Krukenberg V, Riedel D, Tegetmeyer HE, Boetius A (2015) Intercellular wiring enables electron transfer between methanotrophic archaea and bacteria. Nature 526:587–590

Williams KH et al (2005) Geophysical imaging of stimulated microbial biomineralization. Environ Sci Technol 39:7592–7600

Wu XE et al (2011) A role for microbial palladium nanoparticles in extracellular electron transfer. Angew Chem Int Ed 50:427–430

Zhang PY, Xu DK, Li YC, Yang K, Gu TY (2015) Electron mediators accelerate the microbiologically influenced corrosion of 304 stainless steel by the Desulfovibrio vulgaris biofilm. Bioelectrochemistry 101:14–21

Zolotov MY, Shock EL (2003) Energy for biologic sulfate reduction in a hydrothermally formed ocean on Europa. J Geophys Res Planets 108:5022