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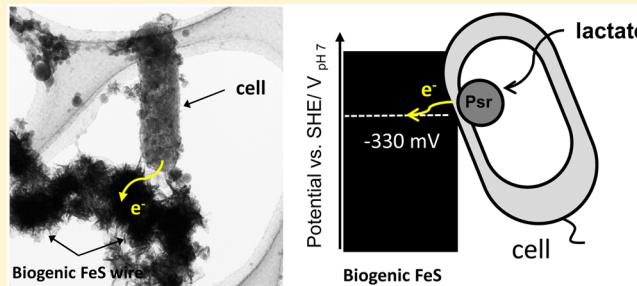
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Supporting Information

ABSTRACT: In addition to serving as an energy source for microbial growth, iron sulfides are proposed to act as naturally occurring electrical wires that mediate long-distance extracellular electron transfer (EET) and bridge spatially discrete redox environments. These hypothetical EET reactions stand on the abilities of microbes to use the interfacial electrochemistry of metallic/semiconductive iron sulfides to maintain metabolisms; however, the mechanisms of these phenomena remain unexplored. To obtain insight into EET to iron sulfides, we monitored EET at the interface between *Shewanella oneidensis* MR-1 cells and biomineralized iron sulfides in an electrochemical cell. Respiratory current steeply increased with the concomitant formation of poorly crystalline mackinawite (FeS) minerals, indicating that *S. oneidensis* has the ability to exploit extracellularly formed metallic FeS for long-distance EET. Deletion of major proteins of the metal-reduction (Mtr) pathway (*OmcA*, *MtrC*, *CymA*, and *PilD*) caused only subtle effects on the EET efficiency, a finding that sharply contrasts the majority of studies that report that the Mtr pathway is indispensable for the reduction of metal oxides and electrodes. The gene expression analyses of polysulfide and thiosulfate reductase suggest the existence of a sulfur-mediated electron-shuttling mechanism by which HS^- ions and water-soluble polysulfides (HS_n^- , where $n \geq 2$) generated in the periplasmic space deliver electrons from cellular metabolic processes to cell surface-associated FeS. The finding of this Mtr-independent pathway indicates that polysulfide reductases complement the function of outer-membrane cytochromes in EET reactions and, thus, significantly expand the number of microbial species potentially capable of long-distance EET in sulfur-rich anoxic environments.



INTRODUCTION

Iron sulfides, such as mackinawite, greigite, marcasite, and pyrite, are ubiquitous and abundant minerals in anoxic marine environments and are formed as byproducts of microbial metabolism or through geothermal activities.^{1–6} A number of biogeochemical processes are induced by microbial interactions with iron sulfides,² which function as important energy sources for microbial activities in environments isolated from solar irradiation. Recently, beside the energy sources, iron sulfides have been highlighted to act as naturally occurring electrical wires^{7–14} and electrocatalysts^{12–14} by virtue of their metallic and/or semiconductive properties and, thus, facilitate microbial metabolism and electron-transfer reactions. For example, the chemolithotrophic microbial communities in hydrothermal mounds appear to directly use electrons transported from hydrothermal fluids via iron sulfides as energy sources for carbon assimilation.^{12,13,15–17} An electrical current passing across iron sulfides and Fe oxides may also facilitate intercellular and interspecies energy transfer^{8,9,18–22} and bridge spatially discrete redox environments.^{9,10,12,23} An iron-sulfide-mediated electrical current may also be generated in microbial communities involved in microbial fuel cells,^{24–26} bioremedia-

tion of industrial acid mine water,^{27,28} pipeline corrosion,^{29,30} and direct and indirect bioleaching.³¹

In these hypothetical electron transfer, certain species of microbes are speculated to donate or accept electrons generated during the course of cellular metabolism to/from metallic and/or semiconductive iron sulfides through extracellular electron transfer (EET) processes. Several mechanisms may mediate microbial EET, including indirect electron transfer via redox-active electron shuttles^{32,33} and direct electron transfer by c-type cytochromes (c-Cyts) located in the outer membrane (OM)^{34–36} or on nanometer-scale filaments or membrane vesicles,^{37–40} known as the metal-reduction (Mtr) pathway that acts as the primal conduit for microbial EET to metal oxides and electrodes.^{41–44} The Mtr pathway also reduces secreted flavins,^{32,45,46} such as riboflavin (RF) and flavin mononucleotide (FMN), which function as electron shuttles and, thereby, greatly facilitate the microbial reduction of Fe(III) and Mn(IV) oxides and anodes. To date, however,

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most studies have focused on the mechanisms of electron transport to metal oxides, particularly Fe(III) and Mn(IV) oxides, and electrodes in microbial fuel cells, and the processes underlying microbial EET to iron sulfide interfaces remain unclear.

To understand EET to iron sulfides and its significance in microbially driven redox chemistry in sulfur-rich environments, we have examined the biominerization of iron sulfides by the Fe-reducing bacterium *Shewanella loihica* PV-4, which is able to use both ferric ions and thiosulfate as terminal electron acceptors for anaerobic respiration.⁸ Our analyses have revealed that PV-4 cells use metabolically generated metallic FeS (mackinawite) as a long-distance EET conduit of Fe 3d electrons and facilitate intercellular energy transfer.⁸ The involvement of biogenic FeS in electrical conduction and long-range EET with PV-4 cells was confirmed in experiments with nanoelectrode platforms.⁴⁷ However, the specific cellular components and molecular details of EET to biogenic FeS have not been determined. To identify the electron conduit for FeS-mediated EET processes, here, we electrochemically cultured a series of Mtr pathway mutants of *Shewanella oneidensis* MR-1, which is a model strain for the study of microbial EET. Using mutants lacking major Mtr pathway proteins, including outer- and inner-membrane *c*-Cys ($\Delta omcA/\Delta mtrC$ and $\Delta cymA$) and a pili secretion protein ($\Delta pilD$), we present the first evidence that the dissimilatory sulfur respiratory system rather than the Mtr pathway delivers electrons from a cellular metabolic system to extracellular biogenic FeS.

MATERIALS AND METHODS

Cell Preparation. *S. oneidensis* strain MR-1 was grown aerobically in 10 mL of Luria–Bertani (LB) medium (20 g L⁻¹) at 30 °C for 16 h. The cells were collected by centrifugation at 6000 rpm for 10 min and were then resuspended in 10 mL of modified defined medium (DM), as previously reported by Roh et al.⁴⁸ The cells were further cultivated aerobically at 30 °C for 10 h using 10 mM lactate as a carbon source. The cell suspension was centrifuged at 6000 rpm for 10 min, and the collected cells were washed with DM prior to use in electrochemical experiments. Mutant strains lacking MtrC/OmcA, CymA, and PilD were previously constructed by allele replacement using a two-step homologous recombination method.^{49,50} The mutants were cultivated and prepared for electrochemical measurements using the same procedures as those described for wild-type (WT) MR-1 cells.

Electrochemical Measurements. A single-chamber three-electrode system equipped with the working electrode on the bottom surface of the reactor was used for the electrochemical analysis of intact cells. Tin-doped In₂O₃ (ITO; surface area = 3.1 cm²) was used as the working electrode. The reference and counter electrodes were Ag/AgCl (KCl saturated) and a platinum wire, respectively. For *in situ* generation of iron sulfide nanominerals, DM containing 5 mM iron citrate, 5 mM thiosulfate, and 10 mM lactate was added to the electrochemical cell as an electrolyte and was deaerated by bubbling with N₂ for >15 min until a dissolved O₂ concentration of 0.1 ppm was reached (Microx TX3 trace, PreSens). Freshly prepared cell suspension (0.1 mL) was then injected into the electrochemical cell under potentiostatic conditions of 0.2 V (versus Ag/AgCl, KCl saturated). As control experiments, deaerated DM containing either 5 mM iron citrate and 10 mM lactate or 5 mM thiosulfate and 10 mM lactate was used as an electrolyte.

Scanning Electron Microscopy (SEM). At the end of each experiment, the ITO electrodes were fixed in 2.5% glutaraldehyde, sequentially dehydrated with ethanol [25, 50, 75, 90, 95, and 100% (v/v) ethanol, 15 min for each treatment], and then freeze-dried using a vacuum device (VFD-21S, MAKER). The electrodes were fixed on aluminum stubs using conductive carbon tape, coated with platinum, and imaged by SEM (Hitachi SU-8000).

Transmission Electron Microscopy (TEM). The cell suspension within the electrochemical cells was collected and diluted to a proper concentration. A solution of 2.5% glutaraldehyde was added to the diluted cell suspension, which was then added dropwise onto a copper mesh. After 15 min, the cell suspension was removed by dabbing with filter paper, and the surface of the copper mesh was gently washed with double-distilled water. The adhered cells were stained with 0.2 wt % phosphotungstic acid and dried at room temperature. Images of sections were obtained by TEM at 300 kV.

Qualitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). Cells were harvested from the electrode surface when the current reached 6.7 μ A cm⁻². For qRT-PCR analysis, cells incubated in the presence and absence of FeS were harvested and centrifuged at 15 000 rpm for 15 min at 4 °C. The resulting supernatant was discarded, and the cell pellets were resuspended in 1 mL of Triazol solution and then treated with 200 μ L of chloroform to isolate total RNA. After centrifugation at 15 000 rpm for 15 min at 4 °C, the obtained supernatant was mixed with 600 μ L of isopropanol, incubated at 4 °C for 10 min, and then centrifuged at 15 000 rpm for 15 min at 4 °C. The resultant pellets were resuspended in 1 mL of 70% ethanol, centrifuged again using the same conditions, and resuspended in 300 μ L of RNase-free H₂O (Qiagen). Extracted RNA was further purified with a RNeasy Mini Kit (Qiagen) according to the instructions of the manufacturer. All samples had A_{260}/A_{280} ratios between 1.8 and 2.0, indicating that they were of high purity. The RNA samples were then analyzed by quantitative RT-PCR.

RESULTS AND DISCUSSION

Microbial Current Generation with Biogenic FeS. To examine the influence of cell-surface-associated iron sulfide

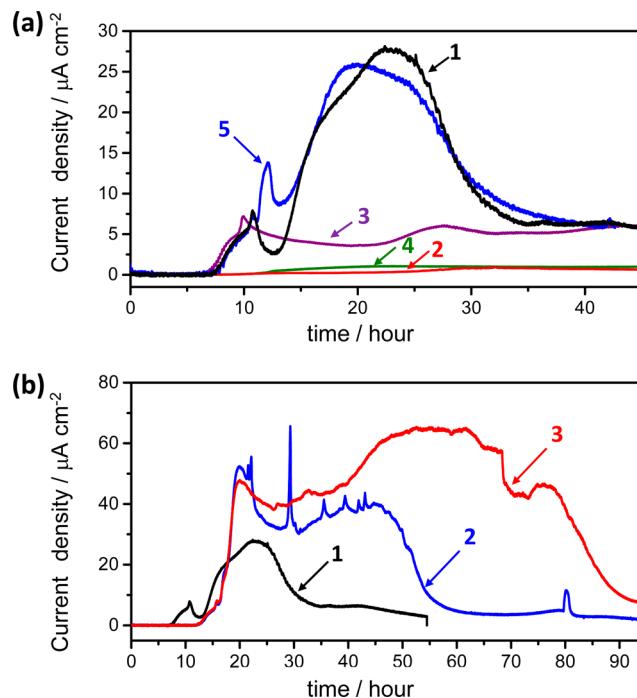


Figure 1. (a) Current density versus time measurements of current generation by *S. oneidensis* MR-1 on an ITO electrode in the presence of 5 mM iron citrate, 5 mM thiosulfate, and 10 mM lactate (trace 1). Current generation by MR-1 cultures lacking both iron and sulfur sources (trace 2) and cultures containing either 5 mM iron citrate (trace 3) or 5 mM thiosulfate (trace 4) are also shown. Trace 5 is the same condition as trace 1, except for the addition of 1 μ M riboflavin. (b) Long-term measurements of current generation for MR-1 cultures containing 10 mM (trace 1), 20 mM (trace 2), and 30 mM (trace 3) lactate.

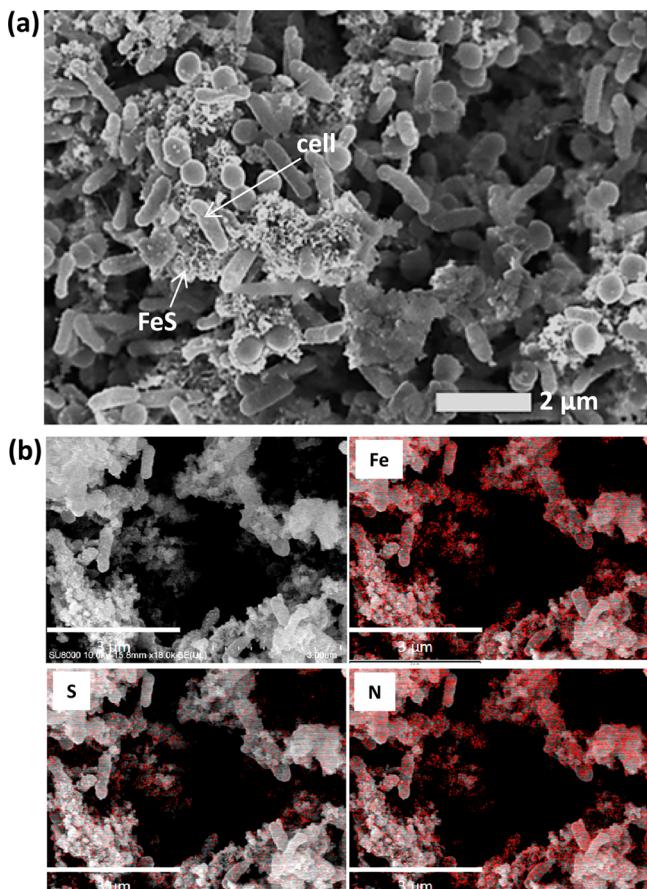


Figure 2. (a) SEM images of FeS-associated biofilms of MR-1 cells obtained after 50 h of cultivation. (b) SEM image and corresponding EDX elemental mapping of a MR-1 cell associated with biogenic FeS nanoparticles.

nanominerals on the EET efficiency of strain MR-1, FeS was biominerilized in an electrochemical cell (EC) containing WT MR-1 cells. In the EC system, an ITO substrate poised at 0.2 V

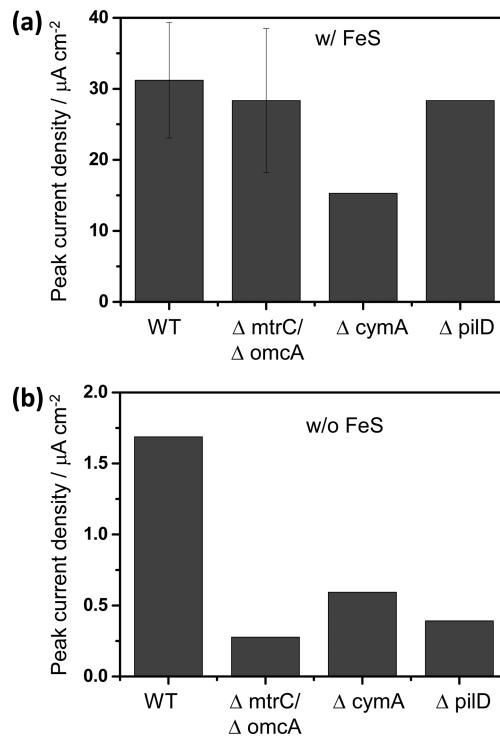


Figure 4. Comparison of the maximum current density of microbial current generation for WT and mutant cells ($\Delta mtrC/\Delta omcA$, $\Delta cymA$, and $\Delta pilD$) of *S. oneidensis* MR-1. Cells cultured in the (a) presence and (b) absence of both iron citrate and thiosulfate were examined.

versus an Ag/AgCl (KCl saturated) electrode served as the anode and deaerated DM containing 5 mM iron citrate, 5 mM thiosulfate, and 10 mM lactate was used as the electrolyte. After approximately 7 h of injection of WT cells with an OD_{600} of $\sim 10^{-4}$ in the EC, the yellow cell suspension turned brown in color (see Figure S1 of the Supporting Information). After approximately 11 h of electrochemical cultivation, a black precipitate was clearly visible on the bottom of the EC, which is

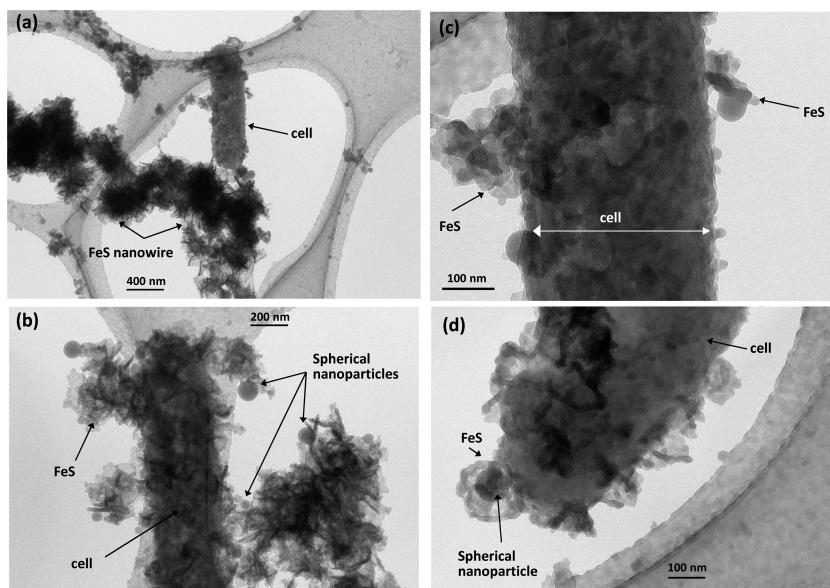


Figure 3. High-resolution TEM images of the cellular surface of MR-1 cells cultured in the presence of 5 mM iron citrate and 5 mM thiosulfate for 25 h.

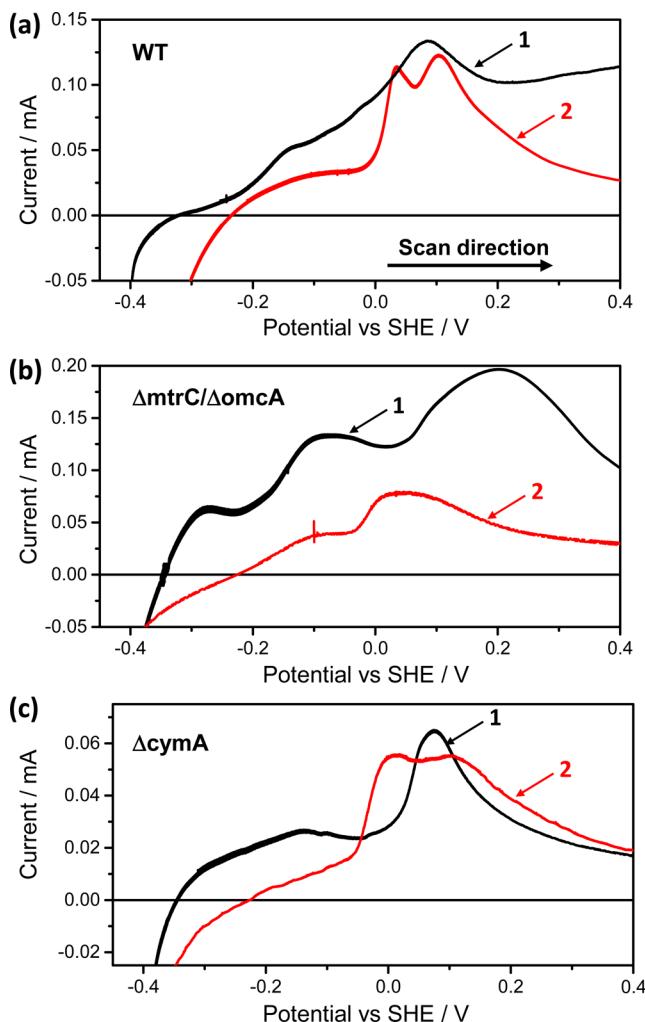


Figure 5. LSVs of WT and mutant cells ($\Delta mtrC/\Delta omcA$ and $\Delta cymA$) of *S. oneidensis* MR-1. LSVs were measured before (trace 1) and after (trace 2) the depletion of lactate: (a) WT, (b) $\Delta mtrC/\Delta omcA$, and (c) $\Delta cymA$.

assigned to poorly crystalline mackinawite (FeS) by X-ray diffraction (XRD) analysis (see Figure S2 of the Supporting Information).

Although initially undetectable in the EC, the respiratory current from strain MR-1 showed a steep increase that closely corresponded with the appearance and accumulation of the FeS precipitate. After reaching the maximum current density of $25 \mu\text{A cm}^{-2}$ at approximately 24 h, the current rapidly decreased, which correlated with the decrease of the lactate concentration in the reactor (trace 1 in Figure 1a). When the initial concentration of lactate was increased from 10 to 30 mM, the cell culture generated a respiratory current for a markedly longer duration and the maximum current density reached $60 \mu\text{A cm}^{-2}$ (Figure 1b). This degree of current increase sharply contrasted with current production in the EC lacking ferric ions and thiosulfate, which maintained a current density of only $1.5 \mu\text{A cm}^{-2}$ (trace 2 in Figure 1a). Importantly, in contrast to the amount of current generated with biosynthesized FeS, current densities of 5 and $2 \mu\text{A cm}^{-2}$ were obtained when the cell was inoculated in DM containing either 5 mM iron citrate or 5 mM thiosulfate (traces 3 and 4 in Figure 1a, respectively). Taken together, these results demonstrate that biogenic FeS was primarily responsible for facilitating the EET process.

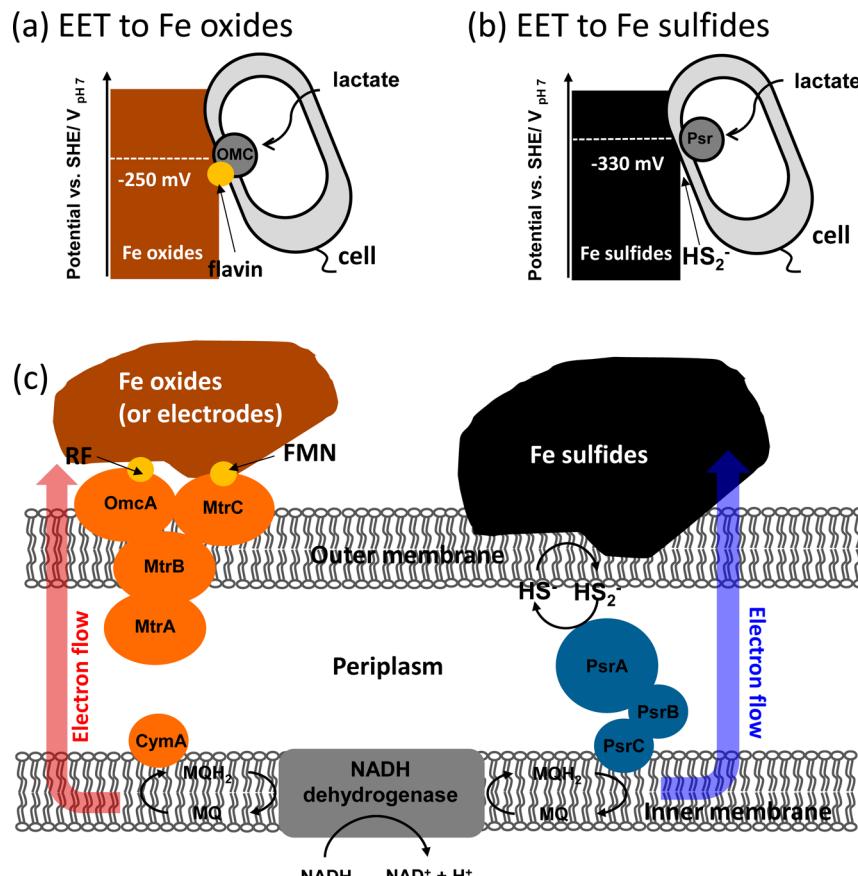
SEM/TEM Analyses of a Cell/Colloidal Network. SEM analysis of the ITO electrode surface after 50 h of current production showed that MR-1 cells formed a colloidal assemblage (Figure 2a). In these assemblages, clusters of rod-shaped cells were surrounded by nanosized FeS particles that appeared to interconnect the cells. Elemental mapping using energy-dispersive X-ray spectroscopy confirmed that FeS was uniformly distributed on the cell surface (Figure 2b). High-resolution TEM observations of the colloidal assemblages revealed that the rod-shaped FeS particles were directly associated with the OM and formed a complex extracellular network (Figure 3a). Spherical shape nanoparticles with a diameter of approximately 100 nm were also observed in the cellular assemblages with FeS (Figure 3b–d). These sphere nanoparticles were not directly associated with cells but were linked to cells via biogenic FeS wires.

Poorly crystalline mackinawite synthesized by *Shewanella* species was previously confirmed to have a metallic electron conduction.^{8,47} Ultraviolet photoemission spectroscopic analysis of biogenic mackinawite revealed the existence of extensively delocalized 3d electrons in the tetrahedral layers of Fe and S atoms as the origin of metallic conduction.⁸ Moreover, $I-V$ characterization using nanopatterned electrodes confirmed the metal-like electron conduction, in which the conductance of cell–FeS assemblages, $\sim 2.2 \text{nS}$, was over 100-fold higher than *Shewanella* biofilm alone.⁴⁷ Therefore, the substantial increase in the microbial current in EC cells containing biogenic FeS can be attributed to the exploitation of the metallic conduction properties of cell-surface-associated FeS wire by MR-1 cells for facilitating long-distance EET.

EET to FeS in $\Delta omcA/\Delta mtrC$, $\Delta cymA$ and $\Delta pilD$ Mutants. To examine that the Mtr pathway component function as the primary conduit between the cellular metabolic system and extracellular biogenic FeS wires, we cultivated four MR-1 mutant strains ($\Delta omcA/\Delta mtrC$, $\Delta cymA$, and $\Delta pilD$) in an EC system containing ferric ions, thiosulfate, and lactate. As a control, respiratory current generation of the mutants was also measured in the absence of biogenic FeS. In the control EC, respiratory current reflects electrons delivered to the ITO electrode via the Mtr pathway, similar to microbial EET to Fe and Mn oxides, and graphite electrodes in microbial fuel cells.^{50–54} Contrary to our expectation, all four mutant strains showed a steep increase in respiratory current at approximately 10 h of cultivation in the presence of biogenic FeS, together with the formation of black precipitate. The $\Delta omcA/\Delta mtrC$ mutant, which lacks the major OM decaheme *c*-Cysts OmcA and MtrC, and the $\Delta pilD$ mutant, which lacks a gene important for the secretion of OM *c*-Cysts, generated similar respiratory currents as that of WT (Figure 4a). The $\Delta cymA$ mutant, which lacks an IM-bound tetraheme *c*-Cyt that functions as a menaquinone oxidase,^{41–44} maintained approximately 40% of the respiratory current relative to WT cells.

It is noted that the reduction in microbial current production by Mtr pathway mutants with biogenic FeS (Figure 4a) sharply contrasts with that for the EET reaction to the ITO electrode in the absence of biogenic FeS. As shown in Figure 4b, the double deletion of OmcA and MtrC ($\Delta omcA/\Delta mtrC$) resulted in an approximately 85% reduction in microbial current production. Cells lacking either CymA or PilD showed approximately 70% reduction in microbial current generation. Such a significant impairment of EET ability by the $\Delta omcA/\Delta mtrC$, $\Delta cymA$, and $\Delta pilD$ mutants was previously reported for the microbial reduction of Fe(III) and Mn(IV) oxides and anodic current

Scheme 1. Energy Diagram for the EET Processes of *S. oneidensis* MR-1 Cells to (a) Fe Oxides and (b) Fe Sulfides and (c) Bifurcated EET Pathway for Electron Transfer to Fe Oxides and Fe Sulfides^a



^aFor Fe oxides, EET is mediated by outer-membrane cytochromes (OMCs) associated with flavin at -200 mV versus standard hydrogen electrode (SHE).^{36,45,46} For Fe sulfides, water-soluble polysulfides (HS_n^- , where $n \geq 2$) generated in the periplasmic space deliver electrons from the cellular metabolic processes to cell-surface-associated iron sulfides at -330 mV versus SHE. On the basis of a Pourbaix diagram of polysulfides at pH 5–10, protonated disulfide (HS_2^-) is the most plausible candidate for the electron shuttle in the periplasmic region. The cellular location of PsrABC was assumed on the basis of a previously reported homologue.^{55–58}

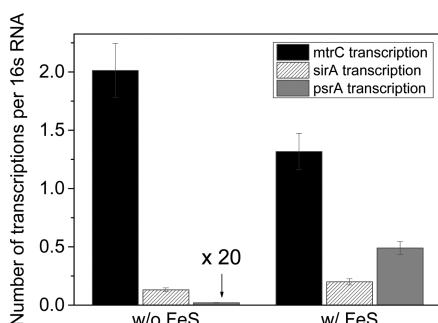


Figure 6. Number of mRNA transcripts of *mtrC* (black bars), *sirA* (hatched bars), *psrA* (gray bars) in the absence (left) and presence (right) of FeS. The values were normalized to the expression of the 16S RNA gene. The number of *psrA* transcripts was increased 20-fold in the absence of FeS.

production on graphite electrodes.⁴⁵ The results presented in Figure 4 demonstrate that the Mtr pathway is not crucial for EET to biogenic FeS.

To further confirm this conclusion, we examined the effect adding $1.0\ \mu\text{M}$ RF on current generation by WT cells in the FeS-associated biofilms that form on the ITO electrode. Respiratory current increased only in the early stage of EC

operation, in which the black precipitates of mackinawite had not yet formed (see Figure S1 of the Supporting Information), and was almost identical with the current generated in the absence of RF (trace 5 in Figure 1a). These results support the hypothesis that EET to biogenic FeS is mediated by an alternative pathway to the Mtr pathway.

Energetics of EET to FeS. To identify the EET pathway operating at the interface between MR-1 cells and biogenic FeS, we performed linear sweep voltammetry (LSV) with an FeS-associated MR-1 biofilm formed on an ITO electrode (Figure 5). For the LSV measurements, a slow scan rate of $0.1\ \text{mV s}^{-1}$ was applied from -0.4 to $0.0\ \text{V}$ in the presence of lactate to monitor respiratory current production as a function of electrode potential. The ITO electrode with an FeS-associated biofilm had an onset potential of $-330\ \text{mV}$, which is approximately $100\ \text{mV}$ more negative than that observed for the EC cell system lacking biogenic FeS (trace 1 in Figure 5a).⁴⁶ After the depletion of lactate, the onset potential shifted from -330 to $-220\ \text{mV}$ (trace 2 in Figure 5a), indicating that high-energy electrons were generated at $-330\ \text{mV}$ as a consequence of microbial metabolism. These results also confirmed that the Mtr pathway is not involved in the high-energy EET process, because neither deletion of *OmcA/MtrC* nor *CymA* affected the onset potential in the presence of FeS

precipitates (panels b and c of Figure 5). Taken together, the LSV results indicate that high-energy electrons generated by microbial metabolism are injected into biogenic FeS via a Mtr-independent EET pathway.

Sulfur-Mediated Electron Shuttling. We noted that the onset potential for the anodic current in FeS-associated MR-1 biofilm (Figure 5a) was located in the potential region of a polysulfide and sulfide redox couple at neutral pH.⁴ Although the molecular mechanisms of sulfur respiration in *Shewanella* species have not been well studied, a polysulfide and thiosulfate reductase (Psr) was identified in strain MR-1.⁵⁵ Psr family proteins are well-studied molybdenum- or tungsten-containing enzymes and include the Mo/W bis-molybdopterin (MGD) oxidoreductases. Psr is anchored on the periplasmic side of the inner membrane and mediates the two-electron reduction of thiosulfate and polysulfide.⁵⁶ Because the present findings indicate that a sulfur respiration system operates in MR-1 independently from the Mtr pathway, HS⁻ ions generated in the periplasmic space of MR-1 cells are predicted to mediate the transfer of respiratory electrons to membrane-associated FeS surfaces, as schematically illustrated in Scheme 1.

In this proposed model, microbially generated HS⁻ ions are oxidized at the surface of FeS, yielding elemental sulfur (S⁰) and free electrons in the tetrahedral layers of Fe and S atoms in mackinawite. The free electrons are transported to distant electron acceptors, such as anodically poised electrodes, via the extracellular FeS matrix, while S⁰ formed on the FeS surfaces reacts with HS⁻ to form water-soluble polysulfides (HS_n⁻, where n ≥ 2). The generated polysulfides are then reduced by polysulfide reductase (Psr) to form HS⁻ in the periplasmic space.⁵⁷ According to a Pourbaix diagram of polysulfides at pH 5–10,⁴ protonated disulfide (HS₂⁻) is the most plausible candidate for the electron shuttle from FeS to Psr. Because HS⁻ ions chemically react with ferric citrate and/or Fe(III) oxides to form poorly crystalline mackinawite, extracellularly formed biogenic FeS is synthesized without involvement of Mtr pathway components, meaning that even mutants lacking major proteins of the Mtr pathway are able to generate electrical current at comparable levels to WT cells (Figure 4).

To confirm the validity of the proposed EET model, we examined the expression of the *mtrC*, *sirA*, and *psrA* genes of strain MR-1 by qRT-PCR. RNA samples for the qRT-PCR analysis were harvested in the logarithmic growth phase, and the expression level of each gene was determined relative to the 16S rRNA gene. SirA is an octahaem *c* reductase that functions as the terminal sulfite (SO₃²⁻) reductase in the form of HS⁻ and is predicted to be a periplasmic protein.⁵⁸ As depicted in Figure 6, the culture of WT cells in the presence of ferric citrate and thiosulfate resulted in an approximately 30% down-regulation of *mtrC* gene expression, whereas that of *sirA* was almost unchanged (Figure 6). In contrast, the expression level of the *psrA* gene was increased 500 fold under conditions that promoted the biomimetication of FeS. Because the specific upregulation of the *psrA* gene corresponds with the delivery of high-energy electrons via the redox cycling of HS⁻/HS₂⁻, it is likely that the Psr-mediated sulfur respiratory system operates as an alternative pathway to that of the Mtr pathway for EET to the extracellular FeS matrix.

CONCLUSION

We demonstrated that *S. oneidensis* MR-1 is able to generate extracellularly FeS wires as a consequence of cellular metabolic processes and use these structures as an electron conduit for

long-distance EET. Using MR-1 mutants that lack major proteins of the Mtr pathway and examining the effects of the electron shuttle RF on microbial current generation, we demonstrated that EET to biogenic FeS occurs in a Mtr-independent manner and propose that sulfur-mediated electron shuttling functions as the predominant mechanism. In the proposed model, the redox couple of HS⁻/water-soluble polysulfides (S_n⁻, where n ≥ 2) acts as an electron shuttle in the periplasm to deliver electrons from the cellular metabolic processes to cell-surface-associated FeS. Although the biomimetication of metallic and/or semiconductive iron sulfides is a well-known phenomenon, only a few previous studies have investigated EET reactions at the interface between the cell surface and iron sulfides, likely because of the commonly accepted notion that OM c-Cyts are needed for the direct transfer of electrons to solid electron acceptors. Thus, the Mtr-independent pathway for microbial EET found in this study significantly expands the number of microbial species that can exploit electrochemistry of FeS minerals to maintain and promote metabolisms and provides new insight into microbially driven redox chemistry in sulfur-rich anoxic environments.^{6,59}

ASSOCIATED CONTENT

Supporting Information

Photographs of an electrochemical reactor during iron sulfide generation (Figure S1) and XRD patterns of biogenic iron sulfides (Figure S2). The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.5b01033.

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Notes

The authors declare no competing financial interest.

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