Report

Liposoluble quinone promotes the reduction of hydrophobic mineral and extracellular electron transfer of *Shewanella oneidensis* MR-1

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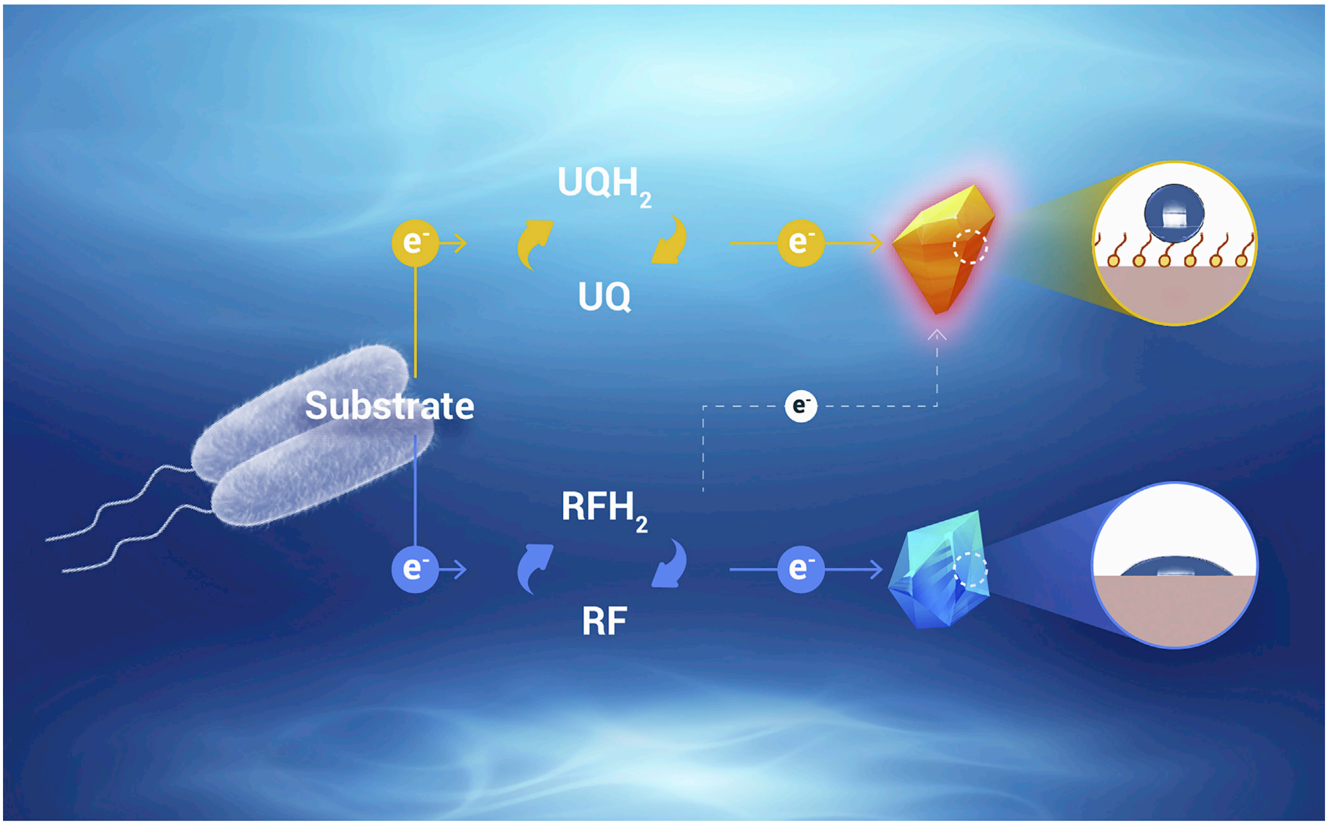
Graphical abstract



Public summary

- Extracellular electron transfer can be regulated by wettability of mineral surface
- Hydrophobic surface hinders the transport of water-soluble mediator riboflavin
- Ubiquinone can mediate extracellular electron transfer at the hydrophobic interface

Liposoluble quinone promotes the reduction of hydrophobic mineral and extracellular electron transfer of *Shewanella oneidensis* MR-1

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A large number of reaction systems are composed of hydrophobic interfaces and microorganisms in natural environment. However, it is not clear how microorganisms adjust their breathing patterns and respond to hydrophobic interfaces. Here, *Shewanella oneidensis* MR-1 was used to reduce ferrihydrite of a hydrophobic surface. Through Fe(II) kinetic analysis, it was found that the reduction rate of hydrophobic ferrihydrite was 1.8 times that of hydrophilic one. The hydrophobic surface of the mineral hinders the way the electroactive microorganism uses the water-soluble electron mediator riboflavin for indirect electron transfer and promotes MR-1 to produce more liposoluble quinones. Ubiquinone can mediate electron transfer at the hydrophobic interface. Ubiquinone-30 (UQ-6) increases the reduction rate of hydrophobic ferrihydrite from 38.5 ± 4.4 to 52.2 ± 0.8 $\mu M \cdot h^{-1}$. Based on the above experimental results, we propose that liposoluble electron mediator ubiquinone can act on the extracellular hydrophobic surface, proving that the metabolism of hydrophobic minerals is related to endogenous liposoluble quinones. Hydrophobic modification of minerals encourages electroactive microorganisms to adopt differentiated respiratory pathways. This finding helps in understanding the electron transfer behavior of the microbes at the hydrophobic interface and provides new ideas for the study of hydrophobic reactions that may occur in systems, such as soil and sediment.

Keywords: ubiquinone; wettability; hydrophobic; *Shewanella oneidensis*; riboflavin; extracellular electron transfer

INTRODUCTION

Extracellular electron transfer (EET) is the basic reaction for microbial respiration.[1–3] Microbial EET was first discovered in the Earth's hydrosphere which is rich in solid-phase Fe(III) (hydr)oxides.[4] The microbial metabolism of iron probably drove the carbon cycle and the formation of massive sedimentary ore deposits called banded iron, and thus affect the biogeochemical cycle.[3] It is critical to clarify the various mechanisms of interface process as EET is ubiquitous and deeply influences the biogeochemical cycle of iron and carbon, etc.[5,6]

As one of the most studied electrochemically active bacteria, *Shewanella oneidensis* MR-1 is widely distributed in mineral-rich sediments.[7,8] It has been reported that MR-1 has a well-developed respiratory network and releases electrons to extracellular acceptors via below pathways: (1) MR-1 undergoes a metal reduction pathway, transferring electrons through the quinone pool in the cell membrane and realizing direct electron transfer through the cytochromes on the inner membrane, periplasmic space, and outer membrane cytochromes.[9,10] There are at least three electron conduits (MtrABC, MtrDEF, and DmsE-FAB) across the inner and outer membrane;[11] (2) MR-1 was reported to have conductive bacterial plus-like structures, which was demonstrated as chains of inter-connected outer membrane vesicles containing cytochromes for direct EET;[12,13] (3) MR-1 release flavins to act as electron mediators to achieve indirect electron transfer.[14–16] Flavins are small, water-soluble molecules and can transfer electrons to minerals that contain Fe(III).[17,18] Through a powerful respiratory strategy, these endogenous electron mediators allow bacteria to use extracellular substrates that cannot be physically contacted (e.g., solid-phase electron acceptors with hydrophilic interfaces).[19,20]

There are many hydrophobic surfaces in the natural environment. For example, complexes formed by natural organic matters and hydrated minerals are common in environment.[21] Natural organic matters easily coat on the surface of solid minerals[22,23] and also participate in mineral synthesis.[24,25] Among them, humus have both hydrophobic and hydrophilic groups, they are easily adsorbed on the surface of minerals and the hydrophobic ends expose outward.[24] This combination significantly enhances the hydrophobic property and the adsorption capacity of liposoluble organics on its surface.[26,27] Ferrihydrite in environment mostly exists in the form of a co-precipitation complex with natural organic matters, including humic acid, while pure ferrihydrite is rare.[28] The co-precipitation significantly affects the wettability of mineral surface.[26,29] As shown in Figure S3A, the increase in C content can enhance the contact angle more than 80° from the original 20°. Luan et al. analyzed the reaction kinetics of simultaneous reduction of nitrobenzene and goethite, and proposed the possibility that electroactive bacteria, such as *Shewanella putrefaciens* CN32 extracellularly reduce nitrobenzene, a hydrophobic organic compound, through EET.[30] Subsequently, the reduction of Nitroaromatic compounds by *Shewanella oneidensis* MR-1 was also proved to be closely related to the Mtr respiratory pathway.[31,32] Polycyclic aromatic hydrocarbons are refractory organic pollutants with two or more fused benzene rings.[33,34] Their high hydrophobicity makes them easily adsorbable on the surface of soil mineral particles or water sediment particles;[33] they are then degraded and mineralized by polycyclic-aromatic-hydrocarbon-degrading bacteria in the environment.[34] Microorganisms in anaerobic reservoir environments also exhibit extracellular respiration. Microbes existing in petroleum, oil sands[35] and asphalt lakes[36] can utilize these hydrophobic carbon sources. The methane-producing degradation of petroleum is considered to be the main degradation pathway in crude oil.[36] The above-mentioned substrates with a hydrophobic surface usually possess the characteristics of large molecular weight and low affinity with bacteria, making it difficult for them to reach the cell interior. Microorganisms with EET ability has a natural advantage to interact with heterogeneous substances. It does not require the pollutants to enter the microbial cells and therefore has limited toxic effects on the microbes, which is beneficial for the microbes to adapt to different environments. All the above-mentioned substrates have been proven to be utilized by microorganisms, but less is known about the electron transfer mechanism between the hydrophobic interface and the microorganisms. Currently known extracellular electron mediators are mostly small, water-soluble molecules, and their diffusion at the hydrophobic interface is limited by mass transfer resistance.[14,17] Whether electroactive microorganisms can actively respond to changes in interface wettability, and how

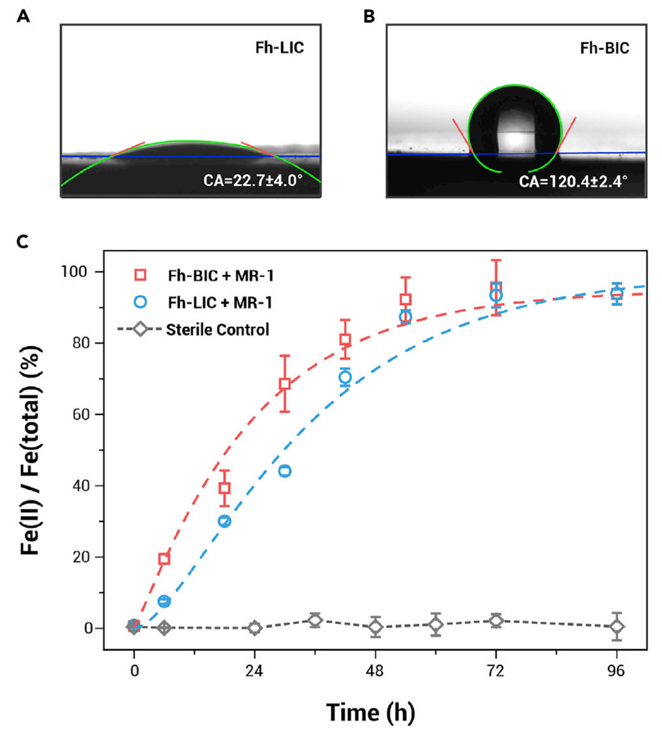

Figure 1. Reduction of hydrophobic ferrihydrite by *Shewanella oneidensis* MR-1 (A) Contact angle measurement of Fh-LIC. (B) Contact angle measurement of Fh-BIC. (C) Dynamics of accumulation of Fe(II) in experimental mixtures, along with negative controls. Error bars represent standard deviations of values measured in three biological replicates.

the hydrophobic interface regulates the extracellular electron transport of electroactive microorganisms is still unclear.

Here, we prepared ferrihydrite with hydrophobic surface. *S. oneidensis* MR-1 was used as the model microorganism. We measured the reaction kinetics of MR-1 reducing hydrophilic and hydrophobic ferrihydrite under anaerobic conditions and examined the secretion of riboflavin and quinone compounds during the respective reduction processes. The study found that the biological reduction rate of hydrophobic minerals was 1.8 times that of hydrophilic minerals, but the effect of the water-soluble electron mediator riboflavin on the hydrophobic interface was almost half of that on hydrophilic interface. Liposoluble ubiquinone could mediate the transfer of extracellular electrons at the hydrophobic interface and play a leading role in this process.

RESULTS AND DISCUSSION

The hydrophobicity of ferrihydrite affects the rate of Fe(III) reduction by *Shewanella oneidensis* MR-1

Two kinds of ferrihydrite with contact angles of 22.7° ± 4.0° and 120.4° ± 2.4° were prepared (Figures 1A, 1B, and S1). The two types of ferrihydrite are referred to as hydrophilic ferrihydrite (Fh-LIC) and hydrophobic ferrihydrite (Fh-BIC) in the following text. No significant change was found in particle size after modification (Figure S2A). The zeta potential increased positively by approximately 10 mV (Figure S2B) which is consistent with the situation of ferrihydrite-humus complex in the environment (Figure S3).[22] The entire Fourier transform infrared spectrum of ferrihydrite shows typical iron oxide characteristics. The Fh-BIC sample covered with oleic acid was detected significant CH_2 bands at 2,921.5 (νa CH), 2,851.7 (νs CH), and 1,521.0 (δs CH) cm^{-1}, corresponding to the CH stretching vibration on the carbon chain.[37] These bands belong to the typical unsaturated carbon chain CH_2 characteristic bands in oleic acid, which proves that oleic acid molecules are successfully coated on the surface of ferrihydrite (Figure S4). The test results show that the BET-specific surface area of Fh-BIC (250.2 m^2/g) was slightly reduced compared with Fh-LIC (269.7 m^2/g). This may be due to the modifier covering part of the adsorption sites.

The Fe(III) reductions of hydrophilic (Fh-LIC) and hydrophobic ferrihydrite (Fh-BIC) by *S. oneidensis* MR-1 were measured. Results showed that the reduction rate of Fh-BIC was 38.51 ± 4.38 $\mu M \cdot h^{-1}$, which was about 1.8 times that of the hydrophilic control group (21.60 ± 7.64 $\mu M \cdot h^{-1}$) (Figure 1C). Using ion chromatography, lactate (as an electron donor) was detected. The average consumption rate of lactate in the Fh-BIC group was 0.086 ± 0.017 $mg \cdot (L \cdot h)^{-1}$, which was approximately 1.9 times that of the Fh-LIC control group (0.046 ± 0.005 $mg \cdot (L \cdot h)^{-1}$) (Figure S5). We did not observe a significant difference in biomass between the experimental groups (Figure S6), which proves no relationship was found between the rate increment and biomass change. The addition of hydrophobic modifiers did not promote the biological reduction of ferrihydrite because it has no electron mediator capacity (Figure S7). The mineral products after 120 h of biological reduction were detected by X-ray diffraction (Figure S8), The results show that the reduction products of hydrophilic and hydrophobic ferrihydrite are both Vivianite, syn. The molecular formula of the product is $Fe_3(PO_4)_2 \cdot (H_2O)_8$. Scanning electron microscopy images showed no significant difference in the morphology of the mineral reduction products (Figure S9).

Previous studies have found that the EET capacity of cells on hydrophobic electrodes is lower than that of hydrophilic electrodes.[38–40] A possible reason is that the hydrophobic surface causes higher solid-liquid interfacial tension, which is not conducive to bacterial colonization, and thus hinders biofilm formation.[41] However, in our experiment, electroactive bacteria were detected to have a higher electron transport capacity at the hydrophobic interface. The difference in the wettability of the mineral interface did not cause the difference in biomass, which can be derived from the biomass measurement data (Figure S6). The mechanism needs to be further clarified.

Effect of riboflavin on the surface of hydrophobic minerals

It was reported that ferrihydrite reduction in *Shewanella* relies on secreted redox mediators.[14,42] To explore whether the flavin-based electron mediator acts on the surface of hydrophobic minerals, we measured the secretion of water-soluble riboflavin in supernatants. In the Fh-BIC group, riboflavin accumulates rapidly at the beginning. Within the first 36 h, the content of riboflavin in supernatant reached 65.9 ± 1.7 $mg \cdot L^{-1}$ which is 1.9 times that of the Fh-LIC group (34.9 ± 1.3 $mg \cdot L^{-1}$). Before the end of the 72 h ferrihydrite reduction, the riboflavin in the supernatant of the Fh-BIC group was maintained at a higher level than that of the Fh-LIC group. After the reduction, the two groups reached the same level (Figure 2A). Interestingly, when we measured the riboflavin eluted from sediments, although the concentration was low, the level of riboflavin eluted in the hydrophilic mineral was higher than that in the hydrophobic group (Figure 2A). Previous research proposed that the mediator effect of riboflavin is controlled by mass transfer.[14] The water solubility of riboflavin makes it have a better affinity with the hydrophilic interface. Therefore, we hypothesized that the existence of the hydrophobic surface hindered the diffusion of flavin to the mineral surface,[15] thus affecting the electron donation and uptake of riboflavin on the mineral surface.

To verify our hypothesis, 531.4 μM riboflavin was added to the above reduction system, which is equivalent to the final cumulative amount of riboflavin in the above test. The results of Fe(II) accumulation kinetics showed that when riboflavin was added to the Fh-BIC group, the reaction rate reached 66.9 ± 3.8 $\mu M \cdot h^{-1}$ and increased by 74% (Figure 2B). When riboflavin was added to the Fh-LIC group, the reaction rate was 59.6 ± 3.8 $\mu M \cdot h^{-1}$ and increased by 176% (Figure 2C). The effect of riboflavin on the reduction of ferrihydrite on the hydrophilic surface is much higher than that of the hydrophobic surface. The cumulative kinetic fitting correlation constants of Fe(II) are given in the supplemental information (Table S3). Compared with that of hydrophilic surfaces, the effect of riboflavin on hydrophobic interfaces was weakened. Therefore, we tried to explore whether there is a hydrophobic electronic mediator to break through the barriers of the hydrophobic layer.

Lipophilic quinones mediate EET on hydrophobic surfaces

Quinone compounds, including naphthoquinone, menaquinone, and ubiquinone, exist in the exogenous environment and can also be synthesized by microorganisms.[43] In recent years, artificial polymers containing

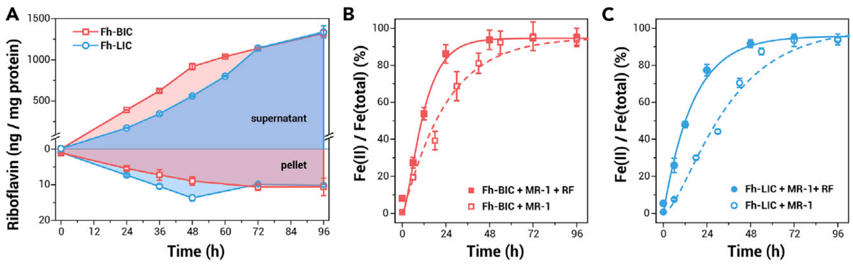

Figure 2. The effect of riboflavin on the hydrophobic interface (A) The accumulation dynamics of riboflavin (RF) in the ferrihydrite reduction experiment.
(B and C) The accumulation dynamics of Fe(II) in experiments involving RF. Error bars represent standard deviations of values measured in three biological replicates.

hydrophobic redox-active mediators have been used for electrode modification to enhance electron transfer.[44–46] Previous studies have reported that there are different kinds of ubiquinone UQ-n on the inner membrane and periplasm of *Shewanella*. The main quinoids of *Shewanella* are ubiquinone-30 (UQ-6), ubiquinone-35 (UQ-7), and ubiquinone-40 (UQ-8).[47] The long isoprene side chain makes ubiquinone hydrophobic and lipophilic, and the electron transfer process in the cell membrane almost entirely revolves around the quinone cycle.[48] Based on this, we speculate that under the action of the hydrophobic interface, microorganisms may use the quinones synthesized by themselves to mediate electron transfer.

We first explored the behavior of ubiquinone in the anaerobic respiration of hydrophobic minerals by examining the content of liposoluble quinones during the reduction of hydrophobic minerals. Liposoluble products were extracted using organic solvents. High-performance liquid chromatography (HPLC) was used to detect liposoluble quinones extracted from hydrophobic ferrihydrite reduction experiments. Three quinones with relatively high content were detected at an excitation wavelength of 275 nm. We added a standard to verify that the chromatographic peak with a retention time of 5.47 min belongs to ubiquinone-30 (UQ-6) synthesized by MR-1 (Figure 3A). Use LC-MS for verification, the molecular weight of ubiquinone-30 is 590.88. As shown in Figure S11, we extracted the chromatographic peak corresponding to m/z = 613.4, which corresponds to [UQ-6+Na^+]. The content of ubiquinone-30 in the hydrophobic group is higher than that in the hydrophilic group (Figure 3B). Besides, several other substances were also detected in the experiment, which indicates that there may be synergistic effects of other quinoids (Figure S9). Previous studies have shown that *Shewanella putrefaciens* (later renamed as *Shewanella oneidensis*) contains only 1%–3% UQ-6 in all quinones.[49] UQ-8 and MK-7, the most abundant quinone, were not detected in our test, which indicates that UQ-6 may have been released outside the cell.

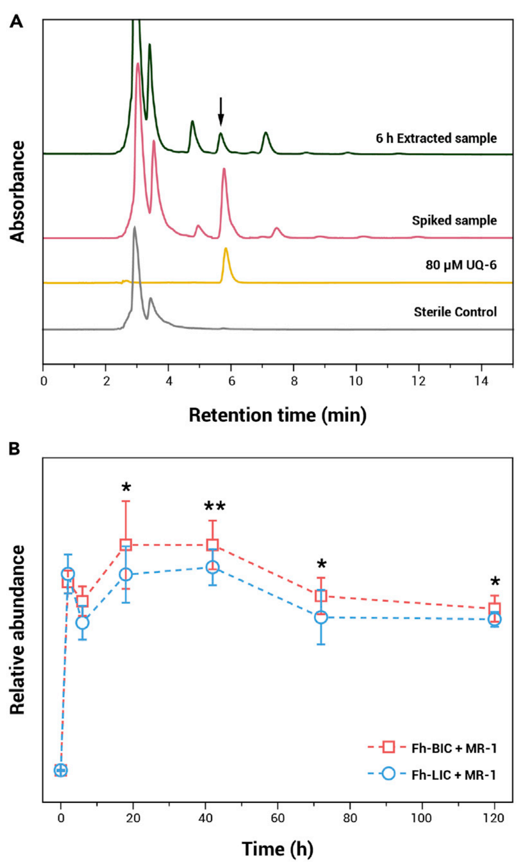

Figure 3. Detection of liposoluble quinone components (A) Chromatogram of liposoluble quinone compounds, along with negative control, standard, and a spiked sample of ubiquinone-30 (UQ-6). (B) The relative abundance of ubiquinone-30. Error bars represent standard deviations of values measured in three biological replicates. Two-tailed Student's t test: *$p < 0.05$, **$p < 0.01$.

To verify whether liposoluble quinone acts on the hydrophobic interface of the environment to mediate the reduction of the solid-phase electron acceptor by microorganisms, 80 μM ubiquinone-30 was added to the hydrophobic ferrihydrite reduction test system. It was found that the reduction rate of the hydrophobic ferrihydrite increased from 38.5 ± 4.4 to 52.2 ± 0.8 $\mu M \cdot h^{-1}$, although it did not significantly promote the reduction of hydrophilic ferrihydrite (Figure 4). Compared with typical water-soluble redox mediators, ubiquinone has a wider redox potential window, a larger molecular weight, and a higher affinity for hydrophobic interfaces.[43] It has a common electrochemically active group, C=O, with natural humus, and therefore, plays an important role in the electrochemical reactions of microorganisms.[23]

Electrochemical analyses revealed the cause of EET at the hydrophobic mineral interface mediated by lipophilic ubiquinone

Two kinds of ferrihydrite, ubiquinone, and riboflavin standards were measured by cyclic voltammetry in a three-electrode electrochemical system (Figures 5A–5C). The reference electrodes used below were all Ag/AgCl. Ubiquinone undergoes a two-electron and two-proton redox process in cyclic voltammetry.[48,49] The voltammogram shows that ubiquinone-30 has two oxidation peaks at +26 and +402 mV and two reduction peaks at −489 and −206 mV (Figure 5B). The peak shapes are consistent with those previously reported.[48] Comparing the cyclic voltammograms of the two ferrihydrites (Figure 5A), the reduction peaks of ubiquinone-30 (−489 mV) are more negative than the reduction peak of ferrihydrite. This result indicates that ubiquinone can theoretically mediate ferrihydrite to obtain electrons. The open-circuit potentials of *Shewanella* MR-1, ubiquinone-30, riboflavin, Fh-BIC, and Fh-LIC were determined to be −42, +85, +35, +146, and +135 mV (versus Ag/AgCl), respectively. Electrons flow from low to high potentials, so these values indicate that *Shewanella oneidensis* MR-1 can transfer electrons to ferrihydrite directly or indirectly through ubiquinone or riboflavin (Figure 5D).

Conclusion

Our research found that the reduction rate of hydrophobic ferrihydrite was 1.8 times that of hydrophilic ferrihydrite, indicating that the hydrophobic interface of minerals could make *S. oneidensis* MR-1 regulate extracellular respiration. The hydrophilic interface was more beneficial to the mediation effect of water-soluble small-molecule riboflavin. *S. oneidensis* MR-1 may rely on liposoluble quinone active substances for indirect electron transfer, increasing the reduction rate of hydrophobic ferrihydrite. Liposoluble quinone ubiquinone-30 was detected in the extracellular environment. Through the Fe(III)

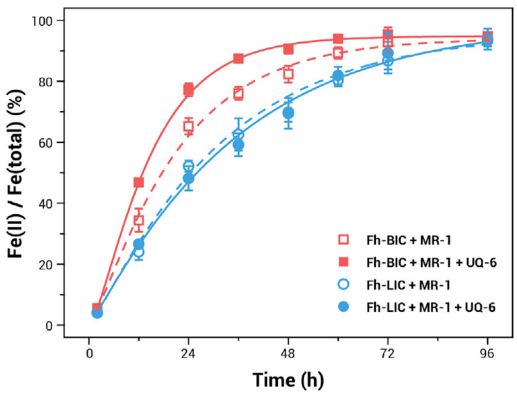

Figure 4. The accumulation dynamics of Fe(II) in experiments involving ubiquinone-30 (UQ-6) Error bars represent standard deviations of values measured in three biological replicates.

mineral reduction test, we verified that ubiquinone-30 can mediate ferrihydrite reduction, especially at the hydrophobic interface, where it can improve the electron transfer efficiency and promote the reduction of metal oxides. This may be related to its lipophilic and hydrophobic properties. The six-unit isoprene side chain of ubiquinone-30 helps it to affinity with the hydrophobic interface. Although in the past it was generally believed that ubiquinone had a high redox potential[3] and considered to be the electron carrier of the inner membrane in aerobic respiration,[50] our ferrihydrite reduction experiments showed that even in an extracellular anaerobic environment, liposoluble ubiquinone has the effect of promoting the electron transfer process at the hydrophobic interface, which has not been reported in previous studies.

Microorganisms in the environment of hydrophobic substrates may use lipophilic and hydrophobic quinones to assist EET to improve substrate utilization. These results reveal the complexity of the extracellular electron transport process of electroactive microorganisms and broaden the existing understanding. In the future, the various EET mechanisms of electroactive microorganisms need to be further studied, and more electronic intermediaries under complex environmental conditions need to be discovered and explored.

MATERIAL AND METHODS

Synthesis and hydrophobic modification of ferrihydrite

The synthesis of ferrihydrite was based on the previous studies.[51] The modification of ferrihydrite was completed in a sterile environment, and the preparation process is shown in Figure S1. More details are provided in the supplemental information.

Contact angle and BET surface area measurement

The mineral sample was centrifuged and collected for freeze drying. For testing of contact angle a 300 mg ferrihydrite sample was pressed into a sheet and placed on a goniometer. Droplets (2 μL) of water were dropped onto the sample to measure the right and left angles of the droplet with the sample surface. An average of at least three droplets was reported. The specific surface area was measured with a fully automatic specific surface area and porosity analyzer (Micromeritics, USA/ASAP, 2020M + C).[52]

Culture conditions and Fe(III) reduction assays

Shewanella oneidensis MR-1 cells were cultured in fresh Luria-Bertani medium (50 mL) at 30°C and 150 rpm for approximately 18 h. Cells in the late logarithmic phase

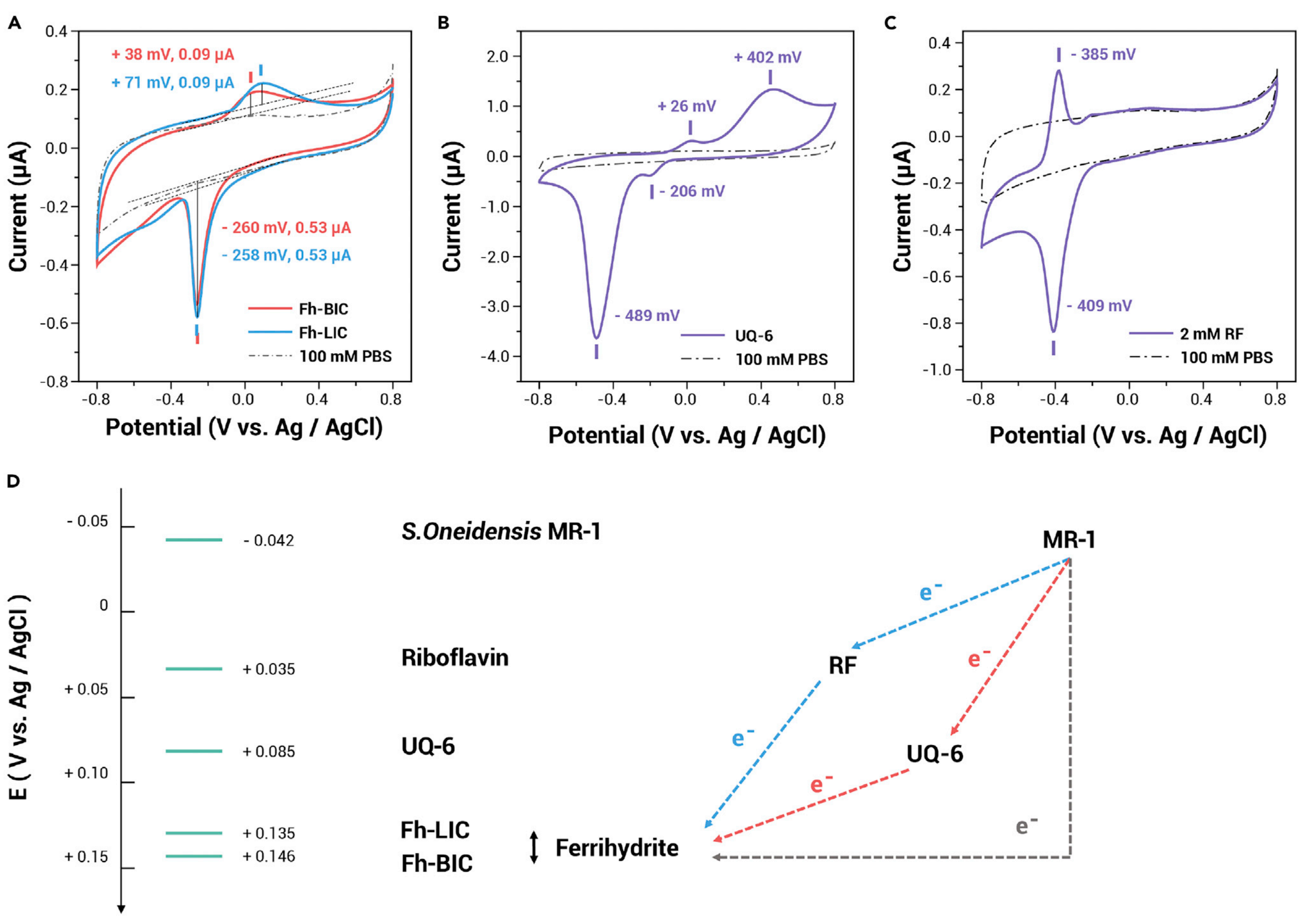

Figure 5. Electrochemical analyses of ferrihydrite, ubiquinone, riboflavin and *Shewanella oneidensis* MR-1 (A) Cyclic voltammetry of ferrihydrite. (B) Cyclic voltammetry of ubiquinone-30 (UQ-6). (C) Cyclic voltammetry of riboflavin. (D) Schematic of reduction potentials of ferrihydrite reduction electron transfer pathway. The red arrow indicates the ubiquinone-30-mediated indirect electron transfer that may occur at the interface of hydrophobic minerals. All electrochemical tests were performed in 100 mM PBS (pH 7.0).

of growth (optical density at 600 nm [OD_{600}] = 1.3) were collected by centrifugation (5,000 × *g*, 5 min) and washed twice with mineral salts (AMS) medium (details in supplemental information). The washed cells were resuspended in AMS medium (50 mL) in 100 mL anaerobic bottles (OD_{600} = 0.9) and purged with high-purity nitrogen (for 30 min under liquid, 15 min capped with a butyl rubber stopper, and 15 min in the headspace) to remove oxygen. Hydrophobic ferrihydrite (Fh-BIC) was added to the cell suspension to a final concentration of 5 mM. Hydrophilic ferrihydrite (Fh-LIC) without hydrophobic modification was used as a control. Concentrations of Fe(total) and Fe(II) were quantified using the phenanthroline method, as described previously.[51] The iron reduction rate was calculated by the first-order reaction kinetic equation fitting:[17]

$$C_t = C_e*(1-\exp(-k_1 t))$$

C_t represents the Fe(II) content in the reaction, C_e represents the Fe(II) content in the initial reaction, and k_1 is the reaction rate constant. We compare the reaction rate constants to reflect the production rate of Fe(II) per unit concentration (Table S3).

Electrochemical measurements

The electrochemical measurements were conducted in a three-electrode system using a CHI 832 electrochemical workstation. Cells samples were prepared as a thick suspension, and 3 μL of the sample was dropped onto the glassy carbon electrode for electrochemical measurements. Mix 0.5 mL of ferrihydrite (2.5 mol/L) and ethanol 1:1 and ultrasonic for 0.5 h. Add 20 μL of 5% Nafion solution, ultrasonic for 0.5 h. Drop 2.5 μL of the above suspension on the glassy carbon electrode, and test after drying. More details are provided in the supplemental information.

Extraction and analysis of quinones

Quinones were extracted from the cell suspension in the Fe(III) reduction assay according to a previously described method with modifications.[50] In brief, 3 mL of the cell suspension was injected into an amber glass vial. The cell suspension was mixed with the same volume of ice-cold quenching buffer (0.2 M $HClO_4$ in methanol). Then, 3 mL of petroleum ether (60°C–90°C) was added as the extractant. The mixture was placed on a magnetic stirrer and stirred for 1 min. The mixture was transferred to a centrifuge tube and centrifuged at 900 × *g* for 1 min. The upper organic phase was collected. The extraction process was carried out three times, and the extracts were combined and dried with nitrogen. The residual solid was dissolved in 120 μL of chromatographic grade absolute ethanol before detection.

The extracted quinone/quinol mixture was analyzed by an HPLC system (Hitachi DL2000) equipped with a C18 column (5 μm, 4.6 mm I.D. × 250 mm). Isocratic elution of quinones was achieved by chromatography using a mobile phase consisting of 25 mM $NaClO_4$ dissolved in ethanol-methanol-$HClO_4$ (750:350:1). The elution flow rate was 1.0 mL·min^{-1}. The wavelength of the UV detector was set at 275 nm. The injection volume was 10 μL and the column temperature was set to 40°C.

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DECLARATION OF INTERESTS

The authors declare no competing financial interest.

SUPPLEMENTAL INFORMATION

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