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Article

Influence of Bacterial Strains and Cell Numbers on the Reduction of Fe(III)-Citrate and Ferrihydrite with and without an Electron Shuttle

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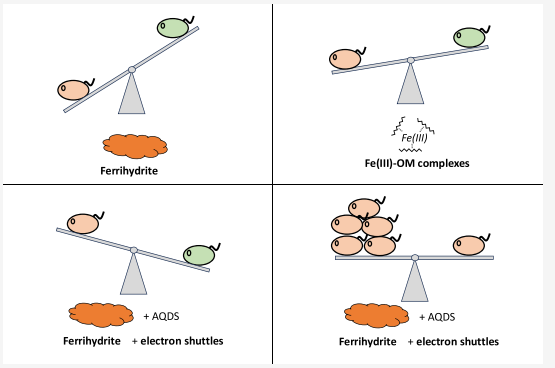
ABSTRACT: Fe(III)-reducing bacteria, consisting of different species with varying cell numbers in the environment, are present, together with redox-active electron shuttles and both dissolved and solid Fe(III) species. However, the effect of electron shuttles and Fe(III)-organic-matter(Fe(III)-OM) complexes on microbial Fe(III) reduction is mainly based on a few early isolated model strains. Due to variations in experimental methods among different researchers, it remains unclear whether these two types of compounds influence different Fe(III)-reducing bacteria differently at varying cell numbers. To address this question, we conducted cell suspension experiments with *Shewanella oneidensis* MR-1, *Aeromonas* sp. CD and *Aeromonas* sp. XH, and evaluated reduction rates of Fe(III)-citrate and ferrihydrite with or without AQDS at three different inoculum concentrations. Our results showed that electron shuttles promoted Fe(III) reduction to different extents among these bacteria, along with varying ratios of the rates of Fe(III)-OM reduction and ferrihydrite reduction. Furthermore, cell numbers also influenced the impact of electron shuttle on Fe(III) reduction; ferrihydrite reduction rates were not increasing proportionally with increasing inoculum concentrations when electron shuttles were present. Comparative genomics suggested that differences in the identity of the Fe(III) reductase and the type of Fe(III)-chelators potentially synthesized probably led to these varied promotional effects. These results highlight the significant differences between model and nonmodel Fe(III)-reducing bacteria, suggesting that the presence of Fe(III)-OM complexes and electron shuttles may determine the presence and abundances of certain Fe(III)-reducing bacteria in different environments.



KEYWORDS: *Fe(III) reduction, electron shuttle, Fe(III)-OM complexes, ferrihydrite, cell numbers, AQDS*

1. INTRODUCTION

Iron (Fe) is one of the most abundant metals on earth, distributed throughout aquatic and terrestrial ecosystems.[1] In natural environments, the majority of Fe compounds exist in two distinct oxidation states: + 3 (Fe(III)) and +2 (Fe(II)). These two oxidation states of Fe differ in several critical physical and chemical parameters, such as water solubility, redox activity, and formation of minerals with unique capacities for adsorption or binding other elements and compounds.[2] The reduction of Fe(III) to Fe(II) is not only coupled to the oxidation of organic carbon but also the oxidation of many other important compounds, such as methane and ammonium, and plays an important role in regulating global warming and wastewater treatment.[2−4] Additionally, the reduction of Fe(III) also influences the behavior of numerous hazardous materials in the environment, including arsenic,[5] cadmium,[6] and antibiotics[7] through various chemical processes, such as adsorption and redox reactions.

The process of Fe(III) reduction to Fe(II) is primarily driven by Fe(III)-reducing bacteria in anoxic environments. Fe(III)-reducing bacteria affiliating to different genera have been identified from various habitats.[8−11] Among them, genera *Shewanella* and *Geobacter*, which were shown to have Fe(III)-reducing capacities in the 1980s, have been extensively studied. In contrast, other genera such as *Aeromonas*, whose Fe(III)-reducing abilities were confirmed much later, have received comparatively less attention, despite their ecological importance. For instance, recent studies have shown that under specific environmental conditions, nonmodel bacteria can outcompete *Shewanella* and *Geobacter* due to differences in carbon sources, Fe(III) mineral types, and other environmental parameters.[12,13]

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In contrast to other bacterial types, such as aerobes and nitrate-reducing bacteria of which the respiratory chains are contained within the cell wall,[14] Gram-negative Fe(III)-reducing bacteria have evolved a specialized porin−cytochrome system located in their outer membrane.[8,15−17] This specialized electron transfer system enables Fe(III)-reducing bacteria to efficiently transfer electrons from the inner membrane to external electron acceptors outside of the cells. Some Fe(III)-reducing bacteria within the same genus may possess a common type of porin-cytochrome system.[18] However, it is important to note that even among bacteria with the same type of porin-cytochrome system, there can be variations in the proteins involved in electron transfer.[19,20]

Even though the concentration of total Fe is often high in many environments, Fe(III) is typically found in the form of poorly soluble minerals due to its low solubility at neutral pH.[2] Fe(III)-reducing bacteria have evolved two distinct mechanisms to utilize Fe(III) indirectly. First, Fe(III)-reducing bacteria can not only utilize naturally occurring Fe(III)-OM complexes (chemical associations between Fe(III) and organic matter (OM), where Fe(III) is often chelated by OM) but also synthesize and secrete Fe(III)-chelators to form Fe(III)-OM complexes.[21−23] These Fe(III)-chelators are different from the siderophores. Although siderophores also chelate Fe(III), they are typically produced for iron assimilation under iron-limited conditions.[24] In contrast, the Fe(III)-chelators are produced during dissimilatory Fe(III) reduction.[21−23] Despite this distinction, they both have a strong affinity for Fe(III), can dissolve solid Fe(III) minerals, and form Fe(III)-OM complexes. The Fe(III)-OM complexes can subsequently be reduced by Fe(III)-reducing bacteria. Afterward, the organic ligands are liberated from the complexes and can then bind to another Fe(III).[21,25] Although the complexation of Fe(III) by many chelators typically lowers the redox potential of the Fe(III)/Fe(II) redox pair theoretically making the reduction of Fe(III) thermodynamically less favorable,[2,26] bacterial reduction of most Fe(III)-OM complexes is generally faster compared to the reduction of solid Fe(III) minerals. This is because the Fe(III) dissolution process improves access to the Fe(III) and allows cells to efficiently reduce Fe(III) without the need for direct contact with the mineral surface.[3,27,28] Second, many Fe(III)-reducing bacteria have the ability to utilize redox-active electron shuttles in order to transfer electrons from the cells to the Fe(III) minerals.[29,30] Many different types of electron shuttles have been identified in the environment, including dissolved and solid-phase redox-active natural organic matter (NOM).[31] Additionally, some bacteria are capable of synthesizing and secreting their own electron shuttles, such as flavins and phenazines.[32−36] These electron shuttles serve as intermediates for electron transfer between Fe(III)-reducing bacteria and Fe(III) minerals. The reduction of electron shuttles by Fe(III)-reducing bacteria can occur at either the outer membrane or inner membrane of the cell, depending on the chemical properties of the electron shuttles.[37] The reduced electron shuttles are then oxidized by the Fe(III) minerals transferring electrons from the reduced shuttles to the Fe(III) which becomes reduced to Fe(II). The distance over which electron transfer can occur can span several centimeters.[38] Previous studies have shown that both Fe(III)-OM complexes and electron shuttles play a crucial role in facilitating the reduction of Fe(III) by most Fe(III)-reducing bacteria.[39] It has been observed that even for Fe(III)-reducing bacteria that do not produce their own electron shuttles, the presence of external electron shuttles can significantly increase their rates of Fe(III) mineral reduction.[40]

In different environments, Fe(III)-reducing bacteria vary in both cell numbers and species.[13,41,42] Previous research also indicated that nonmodel Fe(III)-reducing bacteria from various genera such as *Aeromonas* and *Desulfovibrio* were potentially more dominant than the commonly studied *Geobacter* and *Shewanella* under certain conditions of carbon and iron sources.[13] In addition, different environments have also been found to contain distinct Fe(III) minerals, electron shuttles, and dissolved Fe(III)-OM complexes at varying concentrations.[27,43,44] Previous research has indicated that environmental parameters can affect both the kinetics, products, and populations of microbial Fe(III) reduction.[45] Besides the mineralogy of Fe(III),[13,46] DOM was also suggested to be a strong factor that determines the relative population of different Fe(III)-reducing bacteria.[47] This suggests that various types of Fe(III)-reducing bacteria may exhibit preferences for specific forms of Fe(III) or utilize distinct pathways for reducing different Fe(III) minerals. Investigating the functional capacities of nonmodel strains could uncover new strategies for optimizing microbial Fe(III) reduction in environmental systems.

To date, most research on the impacts of Fe(III)-OM complexes and electron shuttles on microbial Fe(III) reduction has focused on model Fe(III)-reducing bacterial strains with strong Fe(III)-reducing capacities, such as *Shewanella oneidensis* MR-1. However, numerous nonmodel or weaker Fe(III)-reducing bacterial strains have also been identified and play roles in the biogeochemical cycling of iron.[9] Despite this, the extent to which Fe(III)-OM and electron shuttles affect the Fe(III)-reducing capabilities of these strains, as compared to model strains, still remains unclear due to variability in cell numbers, mineral types, and methodological differences in the synthesis and concentration of ferrihydrite.[48,49]

In order to gain a deeper understanding of the microbial Fe(III) reduction processes driven by different Fe(III)-reducing bacteria, we conducted a comparative analysis of the Fe(III) reduction rates and Fe(III)-reducing functional genes of three distinct Fe(III)-reducing bacterial species. This included both well-studied and lesser-known bacterial strains as well as strong and weak Fe(III) reducers. Our aim was to investigate whether certain Fe(III)-reducing bacteria exhibit a stronger preference to reduce Fe(III)-OM complexes or utilize electron shuttles for ferrihydrite reduction as well as examine how variations in cell numbers influence the Fe(III)-reducing rates and mechanisms among different bacterial species.

2. MATERIALS AND METHODS

2.1. Source of Bacteria. Two Fe(III)-reducing bacteria, named strains CD and XH, were isolated from an agricultural field and Xihe river sediment near the China West Normal University campus (30°49′0″ N, 106°3′47″ E for strain CD, and 30°49′29″ N, 106°3′45″ E for strain XH), respectively. Environmental samples were collected using sterile plastic core tubes in June 2021, which served as the initial inocula for enrichments. A modified anoxic freshwater medium[50] supplemented with $MgSO_4{\cdot}7H_2O$ (0.025 g·L^{-1}), $MgCl_2$ (0.187 g·L^{-1}), $CaCl_2{\cdot}2H_2O$ (0.1 g·L^{-1}), KH_2PO_4 (0.6 g·L^{-1}), NH_4Cl (0.3 g·L^{-1}), $NaHCO_3$ (1.85 g·L^{-1}), 1 mL/L vitamin solution, trace element solution SL-10, and selenite-tungstate solution was utilized for the enrichments. Ten mM freshly synthesized ferrihydrite[51] was added into the medium

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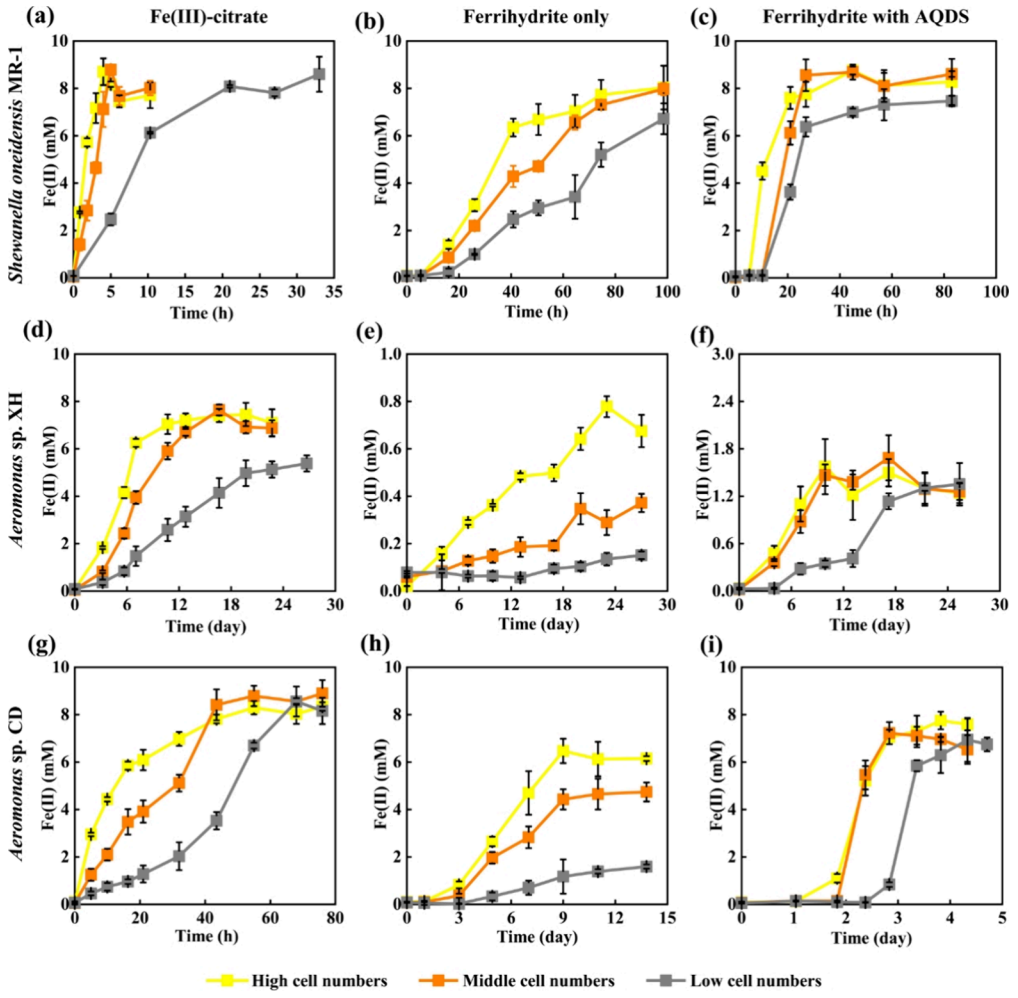

Figure 1. Reduction of 10 mM Fe(III)-citrate, ferrihydrite with and without 0.1 mM AQDS by *Shewanella oneidensis* MR-1 (a–c), *Aeromonas* sp. XH (d–f), and *Aeromonas* sp. CD (g–i) with 10 mM lactate as electron donors. The experiments were performed with three different cell numbers: high, middle, and low cell numbers stand for 3.75×10^9, 1.5×10^9, and 0.375×10^9 cells/mL for *Shewanella oneidensis* MR-1, 1.9×10^9, 0.76×10^9, and 0.19×10^9 cells/mL for *Aeromonas* sp. XH, and 1.2×10^9, 0.48×10^9, and 0.12×10^9 cells/mL for *Aeromonas* sp. CD. Error bars represent standards errors of three biological triplicates.

as an electron acceptor. Additionally, 10 mM sodium lactate or 10 mM sodium acetate were added into the medium as electron donors for strain CD and XH, respectively. The enrichments were incubated at 28 °C and transferred into a fresh medium every 3–5 weeks. After the fourth successive transfer, the enrichments were plated on agar plates containing the same freshwater medium with the addition of 1.5% agar. Ferrihydrite was replaced with 10 mM Fe(III)-citrate in these plates. The plates were incubated anoxically at 28 °C in an anoxic box (Oxoid, USA) equipped with an oxygen scavenger and indicator. Single colonies that emerged on the plates were picked and transferred into a liquid LB medium. The cultured cells were preserved using a 50% glycerin solution and stored at −80 °C. The purity of the isolated strains was confirmed through microscopic observation and 16S rRNA sequencing, which revealed that the two Fe(III)-reducing strains belonged to the genus *Aeromonas*.

2.2. Preparation of Cell Suspensions. *Shewanella oneidensis* MR-1 and the two isolated Fe(III)-reducing strains were cultivated individually in LB liquid medium under aerobic conditions at 28 °C until they reached the late exponential growth phase. Subsequently, the cells were harvested through centrifugation (3020*g*, 5 min), followed by a washing process using anoxic 20 mM PIPES buffer (pH 7), repeated three times. The cells were then resuspended in an anoxic 20 mM PIPES buffer. A portion of the cell suspension was preserved at 4 and −20 °C for cell number quantification via most probable number (MPN) analysis and subsequent genome sequencing, respectively.

2.3. Setup of Fe(III) Reduction Experiments. To determine whether some Fe(III)-reducing bacteria have a stronger preference for utilizing electron shuttles or Fe(III)-chelators to reduce Fe(III), we conducted cell suspension experiments using a modified basal medium. In this modified medium, the bicarbonate buffer was substituted with 20 mL of PIPES buffer, maintaining a pH of 7. Additionally, the medium consisted of 10 mM sodium lactate to ensure an adequate supply of electron donors and either 10 mM Fe(III)-citrate alone, 10 mM ferrihydrite alone, or 10 mM ferrihydrite along with 0.1 mM anthraquinone-2,6-disulfonic acid (AQDS). To assess Fe(III) reduction rates at different cell numbers, we added the washed cells into the medium at three different inoculum concentrations: $3.75 \times 10,^9$ 1.5×10^9, and 0.375×10^9 cells/mL for strain MR-1; $1.9 \times 10,^9$ 0.76×10^9, and 0.19×10^9 cells/mL for strain XH; $1.2 \times 10,^9$ 0.48×10^9, and 0.12×10^9 cells/mL for strain CD, which were within the range of

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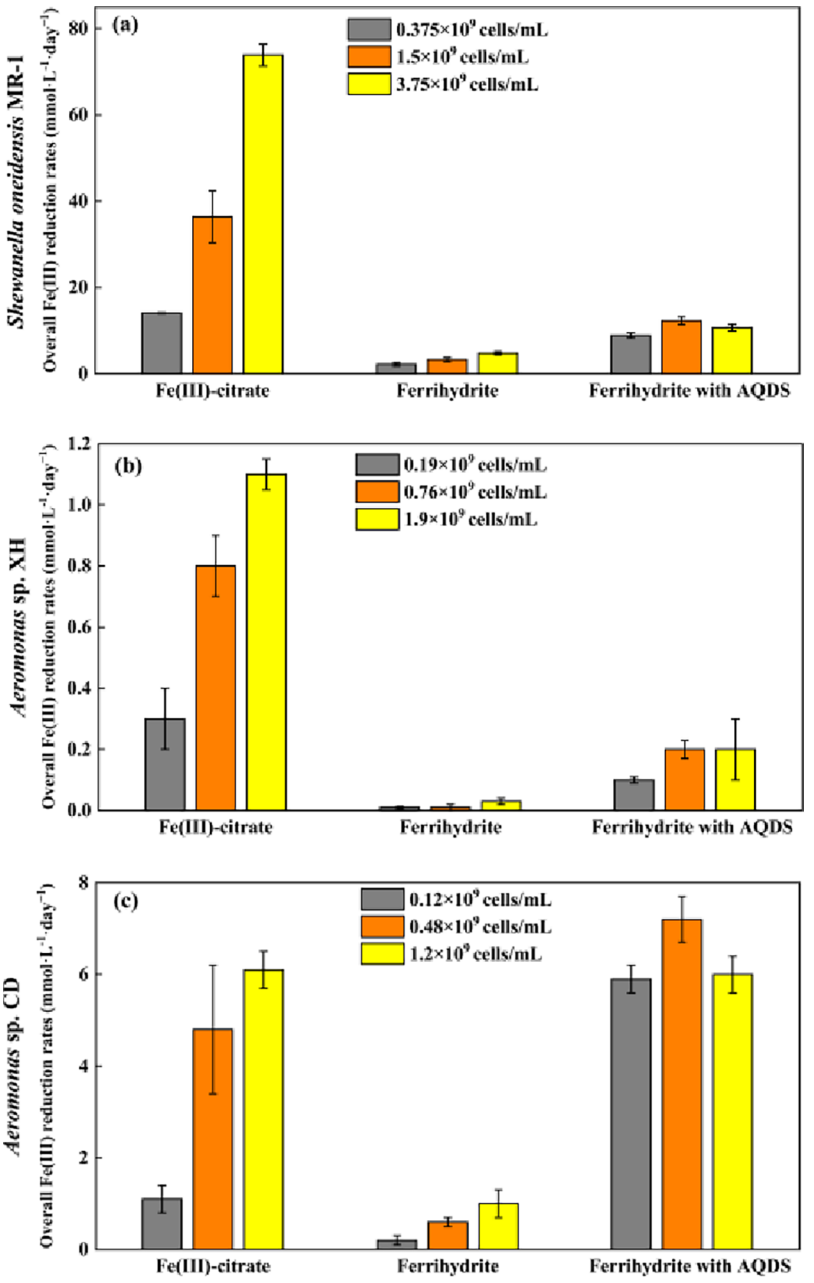

Figure 2. Overall reduction rates of Fe(III)-citrate, ferrihydrite with and without AQDS at three different inoculate concentrations for *Shewanella oneidensis* MR-1, *Aeromonas* sp. XH, and *Aeromonas* sp. CD. Error bars represent standards errors of three biological triplicates.

those used in many previous research.[52−54] These cell suspensions were incubated in Balch-type tubes sealed with airtight butyl stoppers in the dark and at a temperature of 28 °C. No Fe(III) reduction was observed in the abiotic controls carried out in the same way as the biotic experiments but without cells. All experiments were carried out in triplicate, and all the glassware and butyl stoppers were sterilized using an oven at 180 °C or an autoclave, respectively.

2.4. Complete Genome Sequencing and Assembly. The complete genomes of strains CD and XH were sequenced using a combination of Illumina NovaSeq6000 and Nanopore PromethION sequencing platforms from Shanghai Majorbio Biopharm Technology Co., Ltd. (Shanghai, China). The clean short and long reads were coassembled to construct complete genomes using Unicycle v0.4.8. Details of the DNA extraction procedure, sample preparation, sequencing parameters, and genome assembly settings are provided in the Supporting Information.

2.5. Analytical Methods. *2.5.1. Fe(III) Reduction Kinetics.* Samples for determining Fe(III) reduction rates were collected using anoxic sterile syringes that were preflushed with N_2. The samples were incubated with 1 M HCl in the dark for 2 h. The concentrations of Fe(II) were quantified utilizing a solution of 1 M HCl and ferrozine (0.1% w/v) dissolved in ammonium acetate ($C_2H_7NO_2$, 50% w/v).[55] The resulting purple ferrozine-Fe(II) complex was quantified at 562 nm using a microplate reader (Multiskan GO, USA). To calculate Fe(III) reduction rates and the corresponding standard deviations, we performed linear regression analysis using the steepest slopes derived from the reduction curves obtained from the three individual triplicates.

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2.5.2. Cell Number Quantification. Cell numbers of strains MR-1, CD, and XH were quantified using a plate dilution plate count method. We chose this method because we discovered that some cells may die during the cell-washing process to remove noncellular components from the LB medium. To quantify cell numbers, the cell suspension was diluted in 20 mM anoxic PIPES buffer to a final density approximately in the order of 10^4 cells/mL and then spread onto LB agar plates in triplicate. These plates were incubated aerobically at 25 °C, and after 1 day of incubation, the number of colony-forming units (CFU) was manually counted.

2.5.3. Genome Comparisons and Phylogen. The assembled genomes were uploaded to the Joint Genome Institute's Integrated Microbial Genome and Microbiome Expert Review (IMG/MER) pipeline for annotation and comparison with the genome of *Shewanella oneidensis* MR-1 and other finished *Aeromonas* genomes. Genomes of other strains belonging to the genera *Shewanella* and *Aeromonas*, which harbor both *mtrC* and Fe(III)-chelator synthesis genes, were downloaded from NCBI. Additionally, to compare iron-relevant genes, such as those involved in the synthesis of Fe(III)-chelators, FeGenie[56] was utilized. The genes identified using FeGenie were subsequently double-checked and confirmed to be the same in the IMG database. MEGA7 was employed to construct phylogenetic trees using the maximum likelihood method with 1,000 bootstrap replications.

3. RESULTS

3.1. Effect of Bacterial Strains on Fe(III) Reduction Rates. First, we determined and compared the reduction rates of Fe(III)-citrate and ferrihydrite, with and without AQDS, by three Fe(III)-reducing bacteria (in this study, "reduction" refers specifically to the chemical process of Fe(III)-to-Fe(II) conversion unless otherwise stated). The strains were incubated with either Fe(III)-citrate or ferrihydrite with and without AQDS. The results showed that Fe(III) reduction was strongly enhanced for all strains when they were incubated with Fe(III)-citrate or ferrihydrite with AQDS. However, the extent of enhancement varied among the different bacterial species.

For strain XH, the difference in reduction rates between Fe(III)-citrate and ferrihydrite was particularly notable compared to those of strains CD and MR-1 (Figures 1 and 2). Minimal Fe(III) reduction by strain XH was observed after 25 days of incubation with ferrihydrite, even at high cell densities. However, when strain XH was incubated with Fe(III)-citrate, almost all of the Fe(III) was reduced within 10 days, approximately 30- to 80-fold higher than the rate observed with ferrihydrite alone. In contrast, the rates of Fe(III)-citrate reduction by strains MR-1 and CD were only 6- to 15-fold and 6- to 8-fold higher (depending on inoculum concentrations) than their ferrihydrite reduction rates. This demonstrates that strain XH had a stronger preference for utilizing dissolved Fe(III)-complexes over solid-phase Fe(III) compared to strains CD and MR-1.

The effect of AQDS on Fe(III) reduction also varied among the different strains (Figure 1c,f,i). Compared to the reduction of ferrihydrite alone, the addition of AQDS increased the Fe(III)-reducing rates of strain MR-1 only by 2- to 4-fold. In contrast, for strains XH and CD, the presence of AQDS led to a much more significant increase in Fe(III) reduction rates, ranging from 6- to 20-fold for strain XH and 6- to 30-fold for strain CD (Figure 2, Table S1).

3.2. Effect of Cell Numbers on Fe(III) Reduction Rates. To examine the impact of cell numbers on the Fe(III) reduction rates, we evaluated the Fe(III) reduction rates at three different inoculum concentrations for each strain. Our findings demonstrate that, in addition to the influence of Fe(III)-citrate and AQDS, the cell numbers are controlling the Fe(III) reduction rates per inoculated cell across all tested conditions.

The overall Fe(III) reduction rates generally increased with increasing inoculum concentrations when cells were incubated with Fe(III)-citrate and ferrihydrite alone (Figure 2). However, when AQDS was present in addition to ferrihydrite, the increase in inoculum concentrations did not always correspondingly increase the overall Fe(III) reduction rate for all tested strains (Figure 2). The ranking of Fe(III) reduction rates for the three conditions (Fe(III)-citrate, ferrihydrite only, and ferrihydrite with AQDS) also varied with inoculum concentrations. For strain CD at the middle inoculum concentrations, the Fe(III) reduction rates followed the order: ferrihydrite with AQDS > Fe(III)-citrate > ferrihydrite. However, at inoculum concentrations and for strains XH and MR-1, the order was Fe(III)-citrate > ferrihydrite with AQDS > ferrihydrite (Figure 2).

Additionally, we observed that while the overall Fe(III) reduction rates increased with increasing inoculum concentrations, the Fe(III) reduction rates per individual inoculated cells decreased in almost all tested cases (Figure 3, Table S2). This decrease was particularly notable when the bacteria were incubated with ferrihydrite in the presence of AQDS, and it was less pronounced when the strains were incubated with Fe(III)-citrate. Specifically, as the inoculum concentrations of strains MR-1, XH, and CD increased by 10-fold, the Fe(III) reduction rates of ferrihydrite with AQDS per inoculated cells decreased by 8.2-fold, 6-fold, and 9.9-fold, respectively, for strains MR-1, XH, and CD. However, the reduction rates of Fe(III)-citrate per inoculated cell decreased only 1.9-fold, 2.5-fold, and 1.7-fold, and the reduction rates of ferrihydrite without AQDS per inoculated cell decreased by 4.5-fold, 1.5-fold, and 1.75-fold for strains MR-1, XH, and CD, respectively.

It has to be noted, however, that although the reduction rates of ferrihydrite per inoculated cell in the presence of AQDS decreased with increasing inoculum concentrations, the higher inoculum concentrations led to a shorter lag time before the onset of Fe(III) reduction (Figure 1c,f,i). For example, the reduction of ferrihydrite with AQDS by strain CD began 2 days after the start of incubation with the lowest inoculum concentration (Figure 1i), while it started within the first day when the inoculum concentration was increased 10-fold. This trend was also evident for strains MR-1 and XH, irrespective of their Fe(III) reduction rates. In the setup with the lowest inoculum concentrations, strain MR-1 initiated the reduction of ferrihydrite with AQDS after 21 h of incubation, while strain XH started after 7 days (Figure 1c,f). However, with a 10-fold increase in inoculum concentrations, noticeable Fe(III) reduction was already observed within only 10.3 h for strain MR-1 and within 4 days for strain XH.

3.3. Genome Analysis. Genomic analysis and comparison of the three Fe(III)-reducing strains revealed variations in genes associated with Fe(III) reduction. Among the three strains, only strains CD and MR-1 contain the *mtrCAB* gene cluster (Table 1), which facilitates electron transfer across the nonconductive lipid outer membrane. While there is a significant divergence in the amino acid sequences of the

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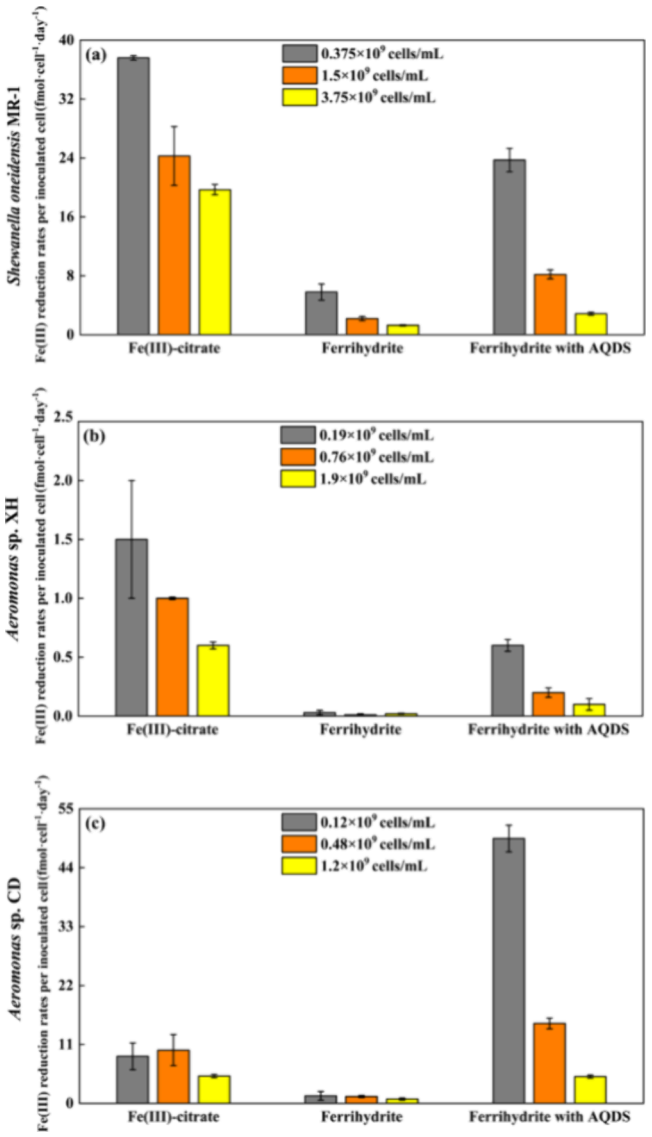

Figure 3. Fe(III) reduction rates per inoculated cell under different conditions for *Shewanella oneidensis* MR-1, *Aeromonas* sp. XH, and *Aeromonas* sp. CD. Error bars represent standards errors of three biological triplicates.

Table 1. Genes Related to Fe(III) Reduction and Synthesis of Fe(III)-Chelators in the Genomes of *Shewanella oneidensis* MR-1, *Aeromonas* sp. XH, and *Aeromonas* sp. CD[a]

bacteria	Fe(III) reduction	Fe(III)-chelator synthesis
Shewanella oneidensis MR-1	*mtrD, mtrE, mtrF, omcA, mtrC(omcB), mtrA, mtrB*	*pubA, pubB, pubC, putA, putB*
Aeromonas sp. XH	no previously identified genes for Fe(III) reduction	*vabC, vabE, vabB, vabF, vabA*
Aeromonas sp. CD	*mtrC, mtrA, mtrB*	*vabC, vabE, vabB, vabF, vabA, rhbB, rhbE, pchH*

[a]These genes were identified, and their functions were predicted using FeGenie.

mtrC, *mtrB*, and *mtrA* genes between strains CD and MR-1 (25.0, 56.4, and 27.5%, respectively) (Figure 4b, Figures S1 and S2), it is notable that within their respective genera, these genes display high similarity (Figure 4b). This indicates a pronounced genetic differentiation in the *mtrC*, *mtrB*, and *mtrA* genes between the two genera. Moreover, strain MR-1 also possesses the *omcA* gene, which is absent in strains CD and XH (Table 1). Furthermore, phylogenetic analysis based on the 16S rRNA sequence showed that strains CD and XH were grouped into two distinct clusters within the genus *Aeromonas* (Figure 4a, Figure S3). The majority of isolated strains clustered with strain CD possessed the *mtrCAB* gene cluster, while the bacterial strains clustered with strain XH lacked this gene cluster (Figure S1). In addition, the terminal Fe(III) reductase gene *mtrC* also appeared to be vertically transferred when comparing the phylogeny of 16S rRNA genes with those encoding *mtrC* (Figure 4).

Further examination of the genomes of strain CD and XH showed that only strain CD contains the gene clusters *netBCD* and *pdsA*, which are responsible for electron transfer from the inner membrane and periplasm to the outer membrane.[19] The genes involved in the synthesis of Fe(III)-chelators also differed between the two *Aeromonas* strains CD and XH and strain MR-1. Both strains CD and XH contain *vabABCEF* genes putatively responsible for the production of catechol-type Fe(III)-chelators.[57] Strain MR-1 exclusively contains *pubABC* and *putAB* genes predicted to be responsible for the production of hydroxamate-type chelators[58] (Table 1).

4. DISCUSSION

4.1. Promotion of Fe(III) Reduction by Fe(III)-Chelators and Electron Shuttles Is Bacterial Strain-Dependent. Natural organic matter has the capacity to chelate Fe(III) and shuttle electrons, promoting the reduction of solid Fe(III) minerals.[59] Previous studies indicated that organic matters that are redox-active or have the capacity to chelate Fe(III) can stimulate microbial Fe(III) reduction by the same bacterial species to varying extents.[28,31,60] In this study, we conducted a comparison of the reduction rates at the three different conditions (Fe(III)-citrate and ferrihydrite with and without AQDS) with three different Fe(III)-reducing bacteria. The results showed variability in the ratios of the rate of Fe(III)-citrate and the rate of ferrihydrite reduction, indicating that the impact of Fe(III)-OM formation on different bacteria varies in extent. Furthermore, the results also revealed that the promotional effects of the identical electron shuttle(AQDS) differ among various Fe(III)-reducing bacteria. The reduction rates of Fe(III)-citrate were up to 30−80-fold (strain XH) higher or as little as 6−15-fold higher (strain MR-1) than the reduction rates of ferrihydrite. Additionally, the reduction of ferrihydrite with AQDS, was as high as 6−30-fold faster or as little as 2−4-fold faster than the reduction of ferrihydrite for strains CD and MR-1, respectively (Figure 2).

These findings indicate that in addition to the previously reported pathway linking the fate of iron and organic carbon,[3,61−63] the cycling of organic matter and iron is interconnected through the modulation of Fe(III) reduction rates by various bacterial species. The concentration of electron shuttles and dissolved Fe(III)-OM complexes also varies with the changing concentration of organic matter (OM) in different environments.[64,65] Therefore, environments characterized by higher concentrations of Fe(III)-chelators and electron shuttles may provide favorable conditions for specific Fe(III)-reducing bacteria that preferentially reduce Fe(III)-OM complexes or Fe(III)-containing minerals through

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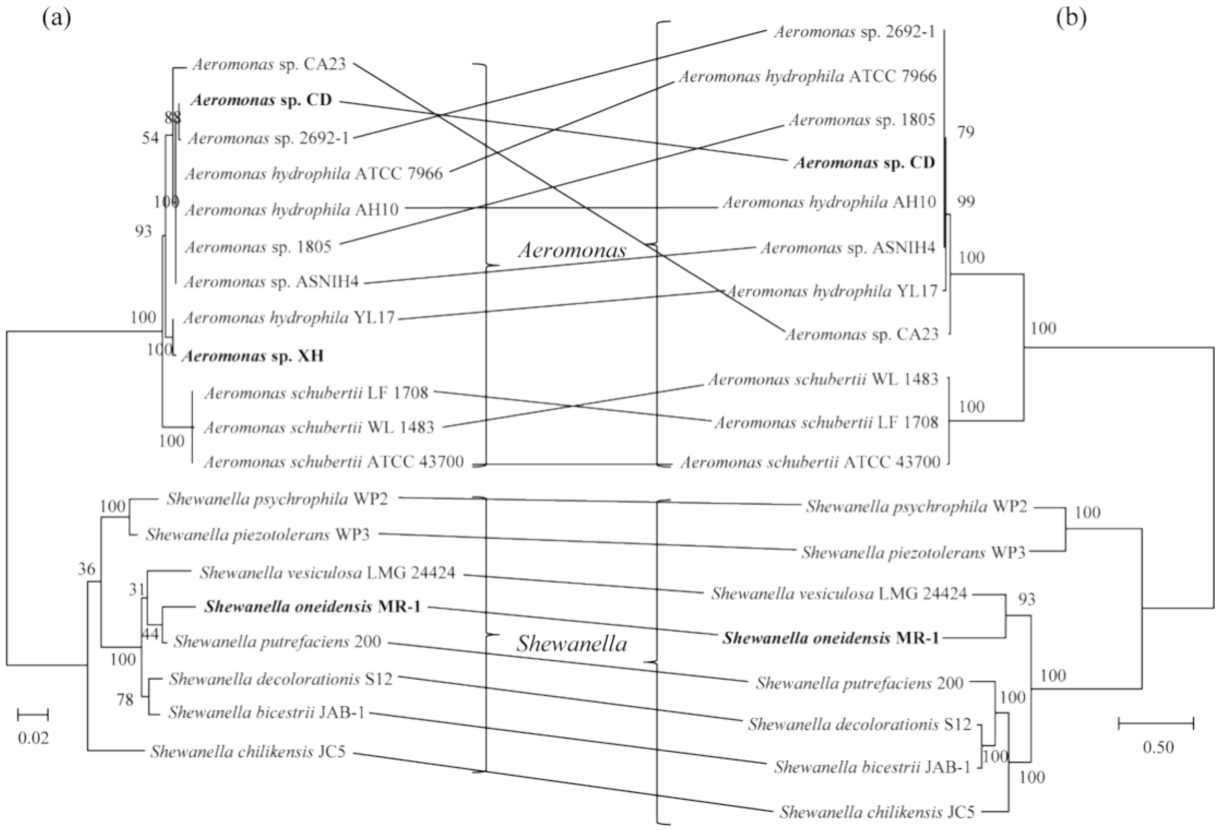

Figure 4. Phylogenetic comparison between 16S rRNA genes (a) and deduced amino acid sequences of *mtrC* genes (b). These maximum likelihood trees contain *Shewanella* and *Aeromonas* strains which possess *mtrC* genes. Bootstrap values are indicated along branch points.

electron shuttles. Consequently, this phenomenon may also result in a diverse array of Fe(III)-reducing bacteria across various biogeochemical settings.[41,42,66−68]

4.2. Genetic Evidence for Bacterial Strain-Dependent Reduction of Fe(III). By comparing genomic sequences, we identified variations in the genes related to Fe(III) reduction among different Fe(III)-reducing bacteria. These differences may provide an explanation for their varying dependencies on electron shuttles and dissolved Fe(III)-OM complexes. First, strain XH, which was unable to reduce ferrihydrite but able to reduce dissolved Fe(III)-OM complexes or ferrihydrite in the presence of AQDS, does not possess any previously identified iron-reducing genes. Conversely, strain MR-1 and CD, which displayed rapid Fe(III) reduction in this study, both contain the *mtrCAB* gene cluster, a set of genes encoding proteins that facilitate electron transfer across the bacterial outer membrane (Table 1). This finding aligns with earlier studies and underscores the significance of this gene cluster in the reduction of solid Fe(III) compounds.[18,19] Second, despite both strain CD and MR-1 harboring the *mtrCAB* gene cluster, there was a noticeable disparity in the amino acid sequence similarities of *mtrC*, *mtrA*, and *mtrB* between the two strains. These similarities ranged from 25 to 50%, indicating significant differences in their encoded proteins. Prior research has emphasized the importance of heme side chains within the MtrCAB complexes in influencing electron transfer rates by several orders of magnitude.[20,69,70] Therefore, the preference for utilizing dissolved Fe(III)-OM complexes and electron shuttles might be attributed to the dissimilarities in the terminal Fe(III) reductases of these strains. Third, although possibly less direct, in contrast to the *Aeromonas* sp. CD, strain MR-1 exhibited dissimilarities in genes associated with Fe(III)-chelator synthesis, which are responsible for solubilizing solid Fe(III) minerals as Fe(III)-complexes.[71] Although it remains unclear whether and to what extent Fe(III)-chelators contributed to the Fe(III) reduction in these strains, it is also possible that the bacteria are efficient in reducing Fe(III)-OM complexes formed with their own Fe(III) chelators. One might suspect that variations in the type of Fe(III)-chelator potentially synthesized could also influence the protein structure of their own Fe(III) reductase. Consequently, this could affect the bacteria's reliance on dissolved Fe(III) and electron shuttles. Specifically, strain MR-1 possessed genes that facilitated the production of hydroxamate-type Fe(III)-chelators,[72] but lacked the capability to produce catecholic-type Fe(III)-chelators. On the other hand, strain CD and XH harbored genes for the synthesis of both hydroxamate and catecholate-type Fe(III)-chelators (Table 1). Given the significant disparity in binding capacity between the catecholate-type chelators produced by the two *Aeromonas* strains (typical binding constants $K = 10^{-52}$ M^{-1}) and the hydroxamate-type chelators produced by strain MR-1 (typical binding constants $K = 10^{22}-10^{32}$ M^{-1}),[58,73] which may lead to differences in the redox potential of Fe(III)/Fe(II),[2,60] it is reasonable to hypothesize that their differing preferences for reducing dissolved Fe(III)-OM complexes versus solid ferrihydrite could be attributed to their adaptation to the specific Fe(III)-chelators they produce.

Interestingly, strain XH, which is a slow Fe(III)-reducer and does not possess known Fe(III)-reducing functional genes (such as *mtrCAB*), demonstrated a remarkable ability to reduce either dissolved Fe(III)-citrate or ferrihydrite amended with

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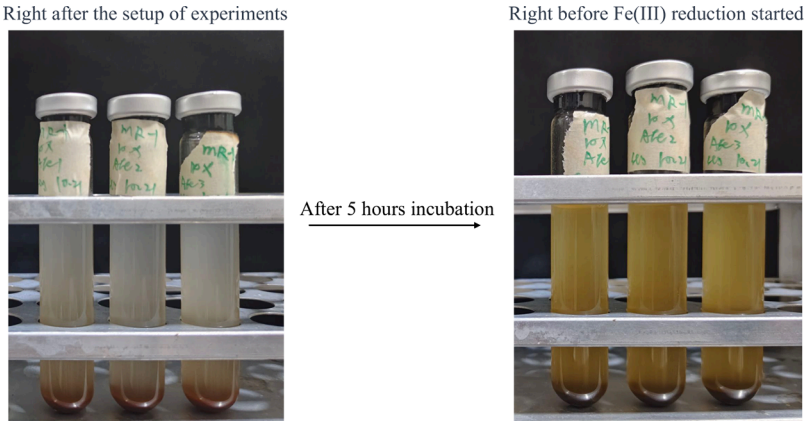

Figure 5. After 5 h of incubation, the supernatant in the media changed from transparent to yellow. The images were taken from an Fe(III) reduction experiment involving strain MR-1, ferrihydrite and AQDS. 3.75×10^9 cells/mL were inoculated. This picture indicates the potential transformation of AQDS to AH_2QDS before Fe(III) reduction begins.

AQDS. It is worth noting that AQDS and chelated Fe(III) were previously believed to ineffectively diffuse through the outer membrane of the cells.[74] The capability to reduce dissolved Fe(III) or solid Fe(III) using electron shuttles has also been observed in the Gram-positive fermentative Fe(III)-reducing bacterium *Clostridium acetobutylicum*.[75] However, since strain XH was incubated with lactate instead of sugars or other complex organic matter, it is unlikely that the reduction observed in strain XH is attributed to the chemical reaction of fermentation products.[71] There are several possible explanations for this phenomenon. One possibility is genetic compensation,[15] wherein the bacteria possess alternative genetic mechanisms that allow them to transfer electrons to dissolved Fe(III)-citrate and AQDS, giving them a survival advantage even under thermodynamically limiting conditions. These findings highlight that Fe(III)-reducing bacteria are not limited to those capable of reducing solid Fe(III) minerals. Bacteria with the ability to reduce dissolved Fe(III)-OM complexes and solid Fe(III) using electron shuttles may be more abundant and diverse than those previously recognized.

4.3. Dual Effects of Electron Shuttles and Cell Numbers on Fe(III) Reduction Rates. Another intriguing discovery from this study is that AQDS has a species-dependent influence on the overall reduction rate of ferrihydrite, but this influence seems to be unrelated to the cell numbers of the bacteria. The overall reduction rates of ferrihydrite in the presence of AQDS were notably higher, with an increase ranging from 6- to 20-fold for strain XH, 6- to 30-fold for strain CD, and 2- to 4-fold for strain MR-1, compared to the reduction of ferrihydrite alone (Figure 2). Given that microbial reduction of electron AQDS coupled to lactate oxidation rather than abiotic reduction of Fe(III) oxide by AH_2QDS was identified as the rate-limiting step during microbial Fe(III) reduction,[76] it can be inferred that various bacteria may display varying rates and extent of AQDS reduction.

Additionally, the presence of an electron shuttle, such as AQDS, seemed to mitigate the variations in Fe(III) reduction rates observed among the bacterial strains with the *mtrCAB* gene cluster. Without AQDS, strain MR-1 exhibited a significantly higher rate of ferrihydrite reduction per inoculated cell compared with strain CD. However, in the presence of AQDS, the reduction rate of ferrihydrite per inoculated cell by strain CD exceeded that of strain MR-1 (Figure 3). These results are also consistent with a previous study,[77] which highlighted the importance of the Mtr pathway in AQDS-mediated Fe(III) reduction by MR-1. In our study, strain XH, which lacks the *mtrCAB* gene cluster, showed very low Fe(III) reduction rates for ferrihydrite even in the presence of AQDS. However, strain XH reduced soluble Fe(III)-citrate at a much faster rate compared to ferrihydrite reduction (Figure 2), further supporting the critical role of the Mtr pathway in the reduction of solid-phase ferrihydrite and electron transfer involving AQDS, though with a relatively smaller effect on reducing soluble Fe(III). Moreover, in contrast to the reduction of Fe(III)-citrate and ferrihydrite that accelerated with increasing cell numbers, the overall reduction rates of ferrihydrite in the presence of AQDS remained relatively constant irrespective of the number of cells inoculated. However, it was also found that a higher number of microbial cells inoculated can accelerate the onset of Fe(III) reduction in the presence of AQDS, whereas the onset of Fe(III)-citrate reduction remained largely unaffected by variations in cell numbers (Figure 1). There are several factors that may contribute to the observed "lag phase" in ferrihydrite reduction, which was observed only in the presence of AQDS. One of the factors may be the initial role of AQDS as an electron sink, especially during the early stages. In setups with high cell numbers, more microbial cells are available to transfer electrons to the AQDS, rapidly filling this electron sink. This can be seen through the color change in the liquid media, which goes from transparent (AQDS) to slightly yellow (AH_2QDS) before the onset of Fe(III) reduction (Figure 5). This delay in Fe(III) reduction due to AQDS was rarely observed in previous studies, possibly because earlier studies had different sampling points and often maintained a constant cell number. Furthermore, in certain previous studies that investigated the role of biochar, a solid material with electron shuttle properties, it was observed that biochar could actually decrease the rate of Fe(III) reduction under certain conditions. The authors proposed that biochar acted as an additional electron sink, diverting electrons away from the Fe(III)-reducers and slowing down the overall reduction process,[78,79] highlighting the complex interactions between electron shuttles, microbial activity, and Fe(III) reduction kinetics in different environmental settings. Another potential reason for this "lag phase" could be that cells preincubated with LB medium require some time to synthesize essential enzymes for

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Fe(III) reduction with AQDS, such as a candidate for the terminal AQDS reductase MmcA found in *Methanosarcina acetivorans*.[80] Another possible explanation could be that the bacteria require time to generate sufficient reducing power, as they were incubated aerobically in an LB medium. However, since the onset of Fe(III)-citrate reduction was largely unaffected by variations in cell numbers, this explanation seems less likely.

Additionally, although the overall reduction rates of Fe(III)-citrate and ferrihydrite were positively correlated with cell numbers, similar to the reduction of ferrihydrite with AQDS, the reduction rate of Fe(III)-citrate and ferrihydrite per inoculated cell exhibited a decreasing trend with increasing cell numbers (Figure 3). It is worth mentioning that at lower inoculum concentration (1.2×10^8 cells/mL), strain CD exhibited even higher Fe(III) reducing rates per cell for ferrihydrite compared to strain MR-1 at higher inoculum concentration (3.75×10^9 cells/mL). This result could be attributed to several factors, including intercellular competition,[81] limited reactive surface area of the mineral,[82] and other chemical reactions involved in the process.[49] Although it remains unclear which factor contributed the most, this result suggested that the capacity of bacterial Fe(III) reduction is influenced not only by the diversity of Fe(III)-reducing bacteria and environmental parameters but also by the number of cells themselves.

5. CONCLUSIONS

In natural environments, a variety of distinct Fe(III)-reducing bacteria exist at differing cell numbers together with redox-active electron shuttles and a diverse range of dissolved and solid Fe(III) species.[83,84] First, the findings of this study suggest that the role of Fe(III)-OM and electron shuttles in microbial Fe(III) reduction in natural environments may be underestimated. This is highlighted by the observation in this study that certain Fe(III)-reducing bacteria exhibited a stronger response to dissolved Fe(III)-OM and electron shuttles than the widely used model strain, *Shewanella oneidensis* MR-1. Differences in the genes related to Fe(III) reduction might account for these bacteria's varied responses to Fe(III)-OM and electron shuttle. This finding may explain the diverse presence of Fe(III)-reducing bacteria in different environments and highlights the importance of considering nonmodel strains when evaluating Fe(III) cycling processes. Habitats that are rich in electron shuttles and Fe(III)-OM complexes may provide favorable conditions for the growth of specific nonmodel Fe(III)-reducing bacteria that have a stronger preference for utilizing dissolved Fe(III) and electron shuttles.

Second, our study demonstrates that an increase in cell numbers may enhance the overall rates of Fe(III) reduction for both dissolved and solid-phase Fe(III) but not necessarily when electron shuttles are present. This suggests that the rates of Fe(III) reduction may not primarily rely on the number of Fe(III)-reducing bacteria in the presence of electron shuttles. Other chemical factors, such as the availability and concentration of electron shuttles, play a more crucial role in regulating Fe(III) reduction rates in some environments. Furthermore, environments enriched with electron shuttles may exhibit more efficient Fe(III) cycling, even in the presence of lower bacterial cell densities.

Overall, the results of this study unveil that organic matter that can chelate Fe(III) or shuttle electrons exert a significant influence not only on the iron biogeochemical cycle but also on its key drivers—the Fe(III)-reducing bacteria. These findings underscore the importance of accounting for the natural diversity of Fe(III)-reducing bacteria when designing or optimizing these technologies for real-world applications, such as those used in soil remediation and pollution management.

■ ASSOCIATED CONTENT

Data Availability Statement

The complete genomes are available through Integrated Microbial Genomes & Microbiomes (IMG) (https://img.jgi.doe.gov/), with the taxon identification (IMG Genome ID) 8023730922 and 8023735616 for the strain CD and XH respectively. Complete sequencing data have been deposited with links to BioProject accession number PRJNA1029467 and PRJNA1029364 in the NCBI BioProject database.

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsearthspacechem.4c00260.

Detailed methods for DNA extraction, library preparation, sequencing parameters, and genome assembly; tables summarizing Fe(III) reduction rates under various conditions for different bacterial strains; phylogenetic relationships of MtrC amino acid sequences; sequence alignments of MtrC homologues; and phylogenetic analyses of 16S rRNA gene and functional genes involved in Fe(III) reduction (PDF)

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Notes

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