Chapter 3

Shewanella oneidensis MR-1 reduces Fe(III) at a distance under conditions relevant for biofilms

* Adapted from Hernandez, Lies, Kappler, Mielke & Newman, *A&EM* (submitted).

3.1 Introduction

Microbial Fe(III) reduction plays an important role in a variety of biogeochemical cycles (24, 37) and holds promise for the bioremediation of organic and metal contaminants (23), corrosion control (12), and harvesting electrical current from marine sediments (4). To better predict and/or stimulate the activity of Fe(III) reducing organisms in the environment, it is essential to be able to quantify their numbers, as well as to understand the mechanisms whereby any given microbial community catalyzes Fe(III) reduction. Several groups in recent years have begun to probe the distribution of metal-reducing organisms in the environment as well as study the mechanistic basis of Fe(III) reduction. Three different Fe(III) reducing bacterial genera have been the primary objects of investigation: *Geobacter* species (8, 26, 29, 48), *Geothrix fermentans* (7, 39) , and *Shewanella* species (17, 34, 51). These organisms adopt different strategies for reducing Fe(III), including those that appear to require "direct" contact between the cell and the mineral surface (6, 9, 13, 21, 28, 38, 41) to those that occur at a distance, mediated by endogenous/exogenous electron shuttles (18, 25, 39, 40, 42, 50) and/or Fe(III) chelators (10, 27) .

Although we now have a reasonably good idea of the types of electron carriers involved in the direct contact pathways (e.g., menaquinone (33, 44), periplasmic and outer-membrane *c*-type cytochromes (3, 22, 30, 32)), we have very little information about the types of molecules that support Fe(III) reduction at a distance. Moreover, for organisms that are capable of both direct and indirect Fe(III) reduction, the conditions that favor the expression of these pathways are ill-defined, and the extent to which they differ from one another is not well understood. Several years ago, our group suggested that extracellular quinones might play a role in Fe(III) reduction by *Shewanella oneidensis* strain MR-1 but did not demonstrate this directly (42). Nevin and Lovley later reported evidence for indirect Fe(III) reduction by *Shewanella algae* strain BrY (40) and *Geothrix fermentans* (39), but not by *Geobacter metallireducens* (38). Although no electron shuttling molecules were isolated from these studies, in the case of *G. fermentans*, preliminary thin layer chromatograms of culture filtrates suggested the presence of (a) hydrophilic quinone(s) (39); additional data in support of the presence of Fe(III)-chelating molecules was also provided (40). More recently, iron reduction was demonstrated to occur at a distance in iron-reducing-enrichment cultures but the mechanism of the reduction was unclear (49). To-date, the most chemically well-defined study of iron reduction at a distance involves electron shuttling by phenazines and other redox active antibiotics to iron minerals by *Pseudomonas, Shewanella, and Geobacter* species (18).

Desiring to understand the mechanism of iron reduction at a distance by *S. oneidensis* strain MR-1 in more detail, we undertook the present study. Here we describe a new method for measuring Fe(III) reduction at a distance based on Fe(III)-(hydr)oxide-

coated porous glass beads [Fe-beads], and show that MR-1 can reduce Fe(III) at a distance using a diffusible mediator compound that acts locally. Our data support the hypothesis we advanced previously (20) that Fe(III) reduction occurs at a distance under conditions relevant for biofilms.

3.2 Materials and methods

3.2.1 Bacterial strains and culture conditions

Shewanella oneidensis MR-1 was isolated from Oneida Lake, New York (34). It was grown in either Luria-Broth (LB) medium, a minimal medium (pH 7.2) (45) or LM-lactate (LML) medium (31) at 30 °C. A *fliI* mutant was isolated following transposon mutagenesis of MR-1 with Tn*phoA*'-1 (53) during a screen for mutants defective in biofilm formation (12). *MtrB* and *tolC* mutants were isolated in a similar way during a screen for mutants defective in AQDS reduction (strain AQ-38, (46)) and *omcB* was isolated during a screen for mutants defective in $Fe(OH)_3$ reduction (J. Gralnick and D. Newman, unpublished data). The *menB* mutant (strain SR536) was provided by D. Saffarini (44). Other strains used in this study were *Shewanella algae* BrY (5), *Shewanella putrefaciens* CN32 (54), and *Shewanella* sp. strain ANA-3 (45) and were cultured in the same conditions. Vitamin K2 (menaquinone MK-4), and anthraquinone-2,6-disulfonate (AQDS) were purchased from Sigma. Phenazine methosulfate (PMS) was purchased from Fluka.

3.2.2 Synthesis of Fe-beads

Fe-beads were prepared by adding 25 g porous glass beads (Prime Synthesis Inc., Aston, PA, ~100 μm particle size, 108 m^2/g surface area, 1.4 ml/g pore volume, average

pore size 50 nm) to 500 ml of a 50 mM $FeCl_3$ solution in a 2 liter Erlenmeyer flask. Poorly crystalline ferric (hydr)oxide [$Fe(OH)_3$] was precipitated on the inner and outer glass bead surface by dropwise addition of 2M KOH in small portions within several hours (the flask was shaken by hand while the KOH was added). After the pH reached 6.5-7 the suspension was allowed to equilibrate for several hours before the pH was readjusted to pH 7. Excess $Fe(OH)_3$ was decanted after gentle shaking (the coated glass beads are slightly heavier than the excess $Fe(OH)_3$). The Fe-beads were washed ten times with 50 mM KCl, five times with H2O, collected in a paper filter and freeze-dried or air-dried before storage at room temperature. The poorly crystalline Fe(III)-mineral produced during this procedure was identified by X-Ray diffraction (XRD) of excess Fe(III)-precipitates isolated from the supernatant. XRD spectra were obtained on a Scintag Pad V X-ray Powder Diffractometer using Cu-Kα radiation operating at 35 kV and 30 mA and a θ-2θ geniometer equipped with a germanium solid-state detector. Each scan used a 0.04º step size from 10º to 80º with a counting time of 2 seconds per step.

3.2.3 Scanning electron microscopy (SEM)

Fe-beads (~50 mg) were fixed on double-sticking carbon tape mounted on a sample holder and coated with a few-nm-thick layer of carbon using a carbon evaporator (Edwards E306A, Edwards, UK). The samples were examined by a LEO 1550VP Field Emission SEM with an Oxford INCA energy dispersive X-ray spectroscopy (EDS). The system was operated at 1-15 kV for high resolution secondary electron imaging and elemental analysis.

3.2.4 Transmission electron microscopy (TEM)

Per sample, a volume of culture was fixed with 2% (v/v) glutaraldehyde and kept

at 4 ºC until the following embedding procedure. Fixed cultures were washed 3 times in water by centrifugation and the final pellet was dispersed in Noble agar worms. Half the sample was stained with 2% osmium tetroxide and 2% uranyl acetate (1 hr. each), while the other half was left unstained. Both stained and unstained specimens were put through an ethanol dehydration series: 25%, 50%, 75%, and 2x 100% EtOH steps, 15 minutes each. This was followed by 15 min. washes with 50:50 EtOH:acetone and 100% acetone before incubating in a 50:50 acetone:EPON resin overnight. The next day, culture worms were orientated in embedding moulds with 100% EPON resin and placed in an oven. After 24 h at 60 ºC, sample blocks were trimmed and sectioned (60nm) on a MT-X Ultramicrotome with a 55º Diatome diamond knife. Ultrathin sections were placed on 200 mesh copper grids with a formvar/carbon coating. The pre-stained ultrathin section samples were subsequently post-stained with 2% uranyl acetate before final imaging. Thin sections were examined with an Akashi EM-002B LaB_6 transmission electron microscope operating at 100 kV, equipped with an Oxford EDS unit. The selected area sampled by the Oxford spectrum analyzer was approximately 500 to 300 nm in diameter. Acquisition rates were maintained at 10-20% deadtime with 60 s of livetime at 83 kX. The electron beam was defocused at the condenser lens to maintain counting rates of 1-2 kcps.

3.2.5 Confocal microscopy

Samples were fixed with 2 % glutaraldehyde and stained with the Live-Dead dye (L-7012, Molecular Probes) for imaging on an inverted Zeiss LSM 510 confocal microscope with a 40x water immersion lens in the Biological Imaging Center at Caltech.

. All cells and the beads were visualized using laser excitation at 543 nm and a LP560 emission filter.

3.2.6 Fe(III) reduction assays

MR-1 (and other strains) were initially grown under oxygen-limited conditions in LB medium (10 ml in 18 mm test tubes, incubated shaking aerobically at 30 ºC), harvested in early stationary phase (OD=2-3), and then washed three times with LML medium. The washed cells were diluted to an OD_{600}=0.1 in LML with 1mM fumarate as an electron acceptor and pre-incubated for 4-5 hours at 30 ºC in a Coy anaerobic chamber (80:15:5% $N_2/CO_2/H_2$ atmosphere). These cells were then washed three times in the anaerobic chamber with minimal medium, inoculated with the Fe-beads (0.02 g) or freshly prepared poorly crystalline ferric (hydr)oxide (1 mM final concentration) in 3 ml of minimal medium and incubated in capped tubes without shaking at 30 ºC in the anaerobic chamber. The final cell densities used in the experiments were $2*10^9$ cell/3 ml or tube unless otherwise noted. The incubations were stopped after 3 days by the addition of 0.75 ml of 12N HCl to extract the total amount of iron present in the tubes. Fe(II) was determined in the acidified culture samples with the ferrozine assay (47) and total iron [Fe(II) + Fe(III)] was quantified after a reduction step with hydroxylamine hydrochloride ($NH_2OH{\cdot}HCl$). Supernatants from Fe(III)-coated bead reduction experiments were prepared from high cell density tubes ($2*10^9$ cells/tube) where 80% of the iron was reduced and were either vortexed and centrifuged in the anaerobic chamber (reduced sups) or aerobically (oxidized sups). For experiments with supernatant additions, samples were taken and added at 66% without a filtration step (to avoid losing organic molecules by adsorption to the filter) to new tubes containing Fe(III)-coated beads with a

final cell density of $4*10^8$ cells/tube. Cell lysates were prepared by concentrating 10 ml of a LB-grown stationary phase culture in 1ml HEPES buffer (pH=7.2), sonicating for 10 second pulses for 1 minute (5 cycles on ice), and centrifuging to remove the unlysed pellet. Lysates were frozen at –20 °C until use. 200µl were added to 3ml Fe-bead tubes.

3.2.7 Comparison of aerobically- versus anaerobically-grown inocula.

Aerobically-grown cells of strain MR-1 were prepared by first inoculating 25 µl of an aerobic overnight culture grown in LB medium into 25 ml of LB medium in a 250 ml Erlenmeyer flask and incubating this culture shaking aerobically at 250 rpm at 30 ºC until the culture reached an OD_{600}=0.05-0.1. The cells were harvested by centrifugation, washed three times with LML medium, then diluted into 250 ml of LML medium in a 2-liter Erlenmeyer flask and incubated shaking aerobically at 250 rpm at 30 ºC until the culture reached an OD_{600}=0.07-0.1. The cells were then harvested, washed, and incubated with the Fe(III)-coated beads as described above.

Anaerobically-grown cells were prepared by inoculating 500 µl of oxygen-limited cells prepared as for the typical Fe(III)-coated bead experiment into 250 ml of LML medium containing 10 mM fumarate as electron acceptor. This culture was incubated at 30 ºC in the Coy anaerobic chamber to an OD_{600}=0.1. The cells were then harvested, washed, and incubated with the Fe(III)-coated beads as described above. Chloramphenicol was added to cells when necessary at a final concentration of 105 µg/ml from a 30 mg/ml stock in ethanol to inhibit further protein synthesis.

3.3 Results

3.3.1 Method of measuring Fe(III) reduction at a distance

To study iron reduction at a distance we synthesized Fe(III)-coated porous glass beads. SEM micrographs (**FIG1a**) show their characteristic surface structure. In general, Fe(III)-coated glass beads look similar to the glass beads before $Fe(OH)_3$ precipitation; EDS analysis showed iron in all spots tested after $Fe(OH)_3$ precipitation whereas no Fe was detected in the raw beads. We could not distinguish $Fe(OH)_3$ particles on the surface of the beads, except for one micrograph where an Fe-replete aggregate was found in a depression on the bead surface (data not shown). Because the beads have a very high surface area (108 m^2/g), it is likely that Fe(III) is adsorbed at multiple sites and the growth of $Fe(OH)_3$ crystals from each of those seeds is small and therefore not distinguishable by this form of microscopy. TEM micrographs (**FIG1b**) show that interconnected pores with an average diameter of 50 nm comprise the bulk of the bead's interior. The diameter of a typical MR-1 cell is about 10 times bigger than the diameter of a pore. Although in these pictures the ferrihydrate coating around the pores is not obvious either, EDS analysis of thin sections of the beads in randomly selected circular areas of 0.3 μm diameter showed that iron is homogenously distributed and constitutes an average of 0.41 atomic % (stdev=0.11; # spots analyzed (n)=50) of the core and 1.6% (stdev=0.69, n=50) of the cortex ($\%Fe_{cortex}$=1.6) of the beads (See **FIG1c**). We also determined that 0.1% iron is left in the core of the beads after the extraction procedure we routinely perform to measure the iron in the experiments and thus its oxidation state is not known ($\%Fe_{core}$=0.41-0.1= 0.31). Given these values, assuming a spherical shape for the beads (radius=50 μm) and that the cortex is the 0.3 μm thick outer layer of the bead to

calculate the volumes of cortex (V_{cortex}) and the core (V_{core}) (**FIG1c**), we calculated the percentage of the total Fe we can measure that was originally in the cortex ($Fe_{\%\,from\_cortex}$), i.e., that would be available for direct contact by the cells, (Equation 1) and the uncertainty of this number (Equation 2):

$$Fe_{\%\,from\_cortex} = \frac{\%Fe_{cortex}V_{cortex}}{\%Fe_{cortex}V_{cortex} + \%Fe_{core}V_{core}} * 100\% \quad (1)$$

$$uFe_{\%\,from\_cortex} = \sqrt{\left(\frac{dFe_{\%\,from\_cortex}}{d\%Fe_{cortex}}u\%Fe_{cortex}\right)^2 + \left(\frac{dFe_{\%\,from\_cortex}}{d\%Fe_{core}}u\%Fe_{core}\right)^2} * 100\% = \sqrt{\frac{\left(\%Fe_{cortex}{}^2 u\%Fe_{core}{}^2 + u\%Fe_{cortex}{}^2 \%Fe_{core}{}^2\right)V_{cortex}{}^2 V_{core}{}^2}{\left(\%Fe_{cortex}V_{cortex} + \%Fe_{core}V_{core}\right)^4}} * 100\% \quad (2)$$

We estimate that a maximum of 8.9 ± 4.6 % of the total iron we can measure in the system is present in the cortex. This implies that *S. oneidensis* MR-1 is reducing at least 91.1 % of the iron in the system by a mechanism that does not involve direct contact. This is supported by TEM-EDS examination of the beads at the end of the experiments showing that the beads maintain their structure and integrity (**FIG1b**) and that the iron in the core of the beads gets depleted during microbial Fe(III) reduction (**FIG1d**).

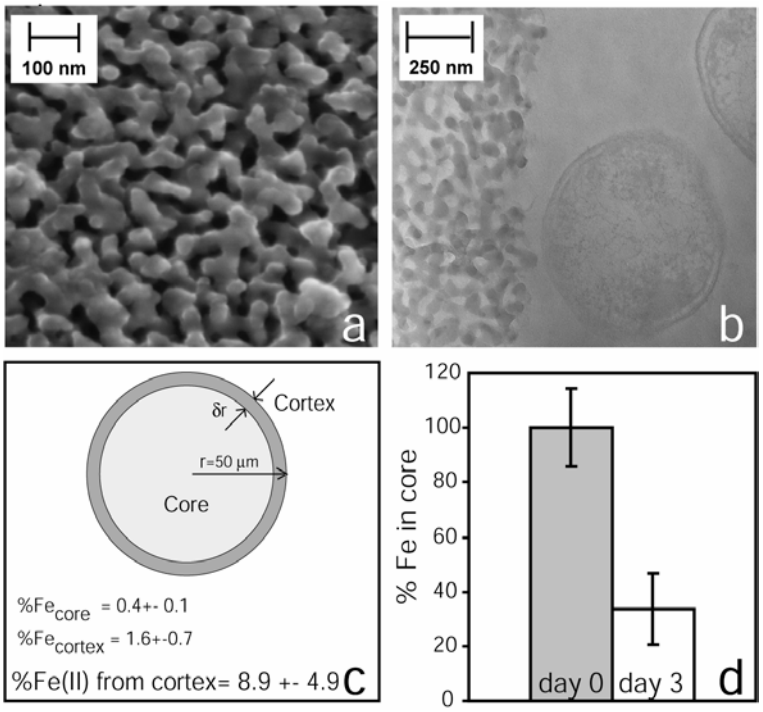

Figure 3.1 Bead characterization. (a) SEM and (b) TEM image of the Fe-beads showing surface structure, pores of 50 nm diameter and its size relation with a S. oneidensis MR-1 cell (on the right hand side of the image) (c) Schematic map of iron concentration (% atomic weight by TEM-EDS) within the beads (d) Percent iron in the core of the beads at day 0 and at day 3 by TEM-EDS.

3.3.2 Optimal conditions for Fe(III) reduction at a distance

When $2*10^9$ fumarate-preincubated *S. oneidensis* MR-1 cells were inoculated anaerobically in 3 ml of minimal media with 20 mg of the beads, most of the Fe(III) in the system (1.1 mM) was reduced within 3 days. This is the standard condition we utilized in the experiments that followed. Moreover, the amount of Fe(III) reduced in 3 days decreased linearly with cell density in two independent experiments (**FIG2**).

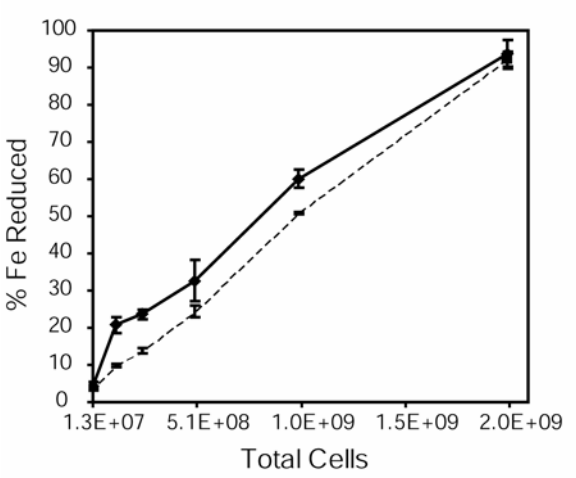

Figure 3.2 Reduction of Fe-beads as a function of total cell number in a 3-day assay. Data represent the average of two independent experiments (connected by a dashed or a solid line, for the different data sets), with error bars indicating the data range for duplicate samples.

The reduction process could be visually followed by the change of color of the beads, from bright orange to dark brown and finally to transparent. Beads incubated with bacteria clumped in the tubes and were part of a biofilm matrix that stayed at the bottom when the tubes were inverted (**FIG3a**). Clumping did not occur in tubes without cells or in tubes with killed cells. Confocal microscopy of stained samples showed that MR-1 cells first attached to the bead surface and then formed microcolonies. Although the biofilms were unavoidably disrupted during the sampling procedure, characteristic microcolonies were observed on the beads (**FIG3b**). The beads were clearly distinguishable with the red filter. Planktonic cells as well as cells in an organic matrix of unknown composition were also observed in the sample. Other *Shewanella* strains tested, e.g., BrY, CN32 and ANA-3 were also able to reduce the iron within the beads under the same conditions (data not shown).

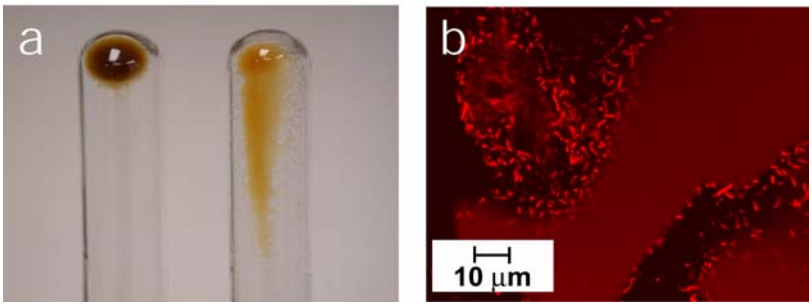

Figure 3.3 Biofilm formation on Fe-beads by MR-1. a. Inverted tube showing clumping of beads (left); uninnoculated control (right). b. Confocal image of MR-1 microcolonies on the bead surface.

To investigate the physiological conditions that are optimal for Fe reduction at a distance, we pre-incubated the cells with different electron acceptors as well as challenged the cells with a protein synthesis inhibitor (chloramphenicol). Cells that had been pre-incubated with ferric citrate or fumarate as the electron acceptor reduced 90% of the total iron provided in 3 days, whereas cells grown overnight under oxygen-limiting conditions and inoculated directly without the fumarate pre-incubation step reduced 75% of the total iron in 3 days (data not shown). Cells grown under conditions of high aeration (i.e. , oxygen-grown) reduced only 50% of the total iron reduced by fumarate pre-incubated cells in 3 days (**FIG4**). The addition of chloramphenicol to fumarate-grown cells decreased the amount of iron reduced to 40% of the untreated control, whereas the addition of chloramphenicol to oxygen-grown cells reduced the amount to only 10%, which is indistinguishable from the amount observed in control experiments with formaldehyde-killed cells. Antibiotic-treated cells still clumped at the bottom of the tubes.

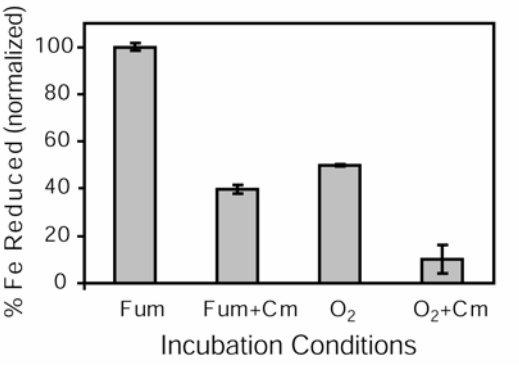

Figure 3.4 Effect of an inhibitor of protein synthesis (chloramphenicol) on iron reduction within ferrihydrate-coated porous glass beads using MR-1 cells pre-incubated with either fumarate or oxygen as electron acceptors. Data are normalized to the amount of reduction achieved by fumarate-grown cells (defined as 100%, see text). Data represent the average of duplicate experiments, with error bars indicating the data range.

Because the medium used for our experiment contains 64 μM NTA in the mineral solution and a vitamin solution that could be involved in either mobilizing Fe(III) from the beads to the bacteria or transferring electrons from the bacteria to Fe(III), we did a control with the same medium without adding the vitamins and mineral solution, and saw no differences in the rate of iron reduction at the highest cell density (data not shown). This suggests that the bacteria are not growing under these conditions.

3.3.3 Role of specific gene products in facilitating Fe(III) reduction at a distance

Having determined that iron reduction at a distance occurs in pure cultures of MR-1 under conditions that are relevant for biofilms (e.g., high cell density, low oxygen tension) and where biofilm behaviors are observed (e.g., microcolony formation, clumping due to production of exopolymeric substances), we tested whether genes known

to be required for mineral reduction (*omcB, mtrB* and *menB)* and attachment *(fliI)* were required for reduction of iron within the beads. An *omcB* mutant, defective in a decaheme cytochrome that is loosely attached to the outer membrane (35), exhibited 45% of WT levels (**FIG5)** The same was observed for a *mtrB* mutant (data not shown), defective in a different outer membrane protein that is required for localization and insertion of outer membrane cytochromes (2, 32). The *fliI* mutant, defective in assembling a functional flagellum, showed 64% of WT Fe(III) reduction levels. A *tolC* mutant, defective in an outer-membrane protein involved in the transport of anthraquinone-2,6-disulfonate (AQDS), a commonly-used artificial electron shuttle, was able to reduce as much iron as the wild-type (data not shown). On the other hand, a menaquinone deficient mutant (*menB*) showed only ~10% reduction (indistinguishable from the amount of reduction catalyzed by dead-cell controls). When provided with exogenous menaquinone (Vitamin K2 (MK4), Sigma), however, the *menB* mutant was almost completely rescued.

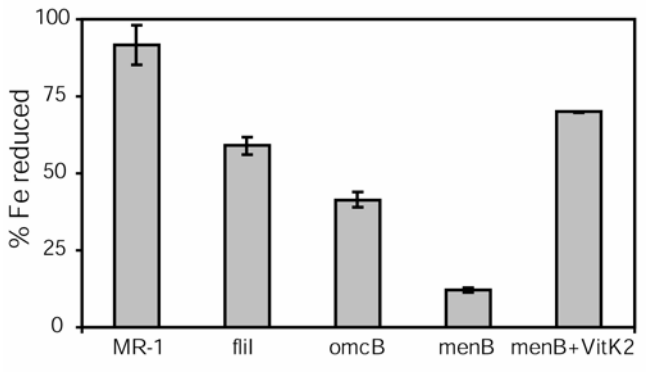

Figure 3.5 Reduction of Fe-beads by MR-1, *fliI*, *omcB,* and *menB* mutants, and an *menB* mutant in the presence of exogenously supplied menaquinone (VitK2). Data represent the average of duplicate experiments, with error bars indicating the data range.

3.3.4 Comparison of Fe-bead reduction to free $Fe(OH)_3$ particle reduction

Iron reduction rates were compared for Fe-beads or free $Fe(OH)_3$ particles using three different cell densities (total cell number per tube: $2*10^9$, $2*10^8$ and $2*10^7$) to determine whether there are kinetic differences between mineral and bead reduction. In general, the cells reduce $Fe(OH)_3$ particles faster than they reduce the Fe-beads; but the greater the cell density, the faster the overall rate, regardless of the form of iron (**FIG6**). The rates per cell for the different cell densities reducing the free $Fe(OH)_3$ particles are lower for the highest cell densities ($9.5*10^{-9}$, $1.6*10^{-8}$, $2.6*10^{-8}$ uM/h*cell, respectively) , while they are similar for the cells reducing the beads ($6.7*10^{-9}$, $7.7*10^{-9}$, $6.9*10^{-9}$ uM/h*cell, respectively). Tubes with a total cell number of $2*10^7$ did not achieve more than 10% reduction of the beads in 18 days.

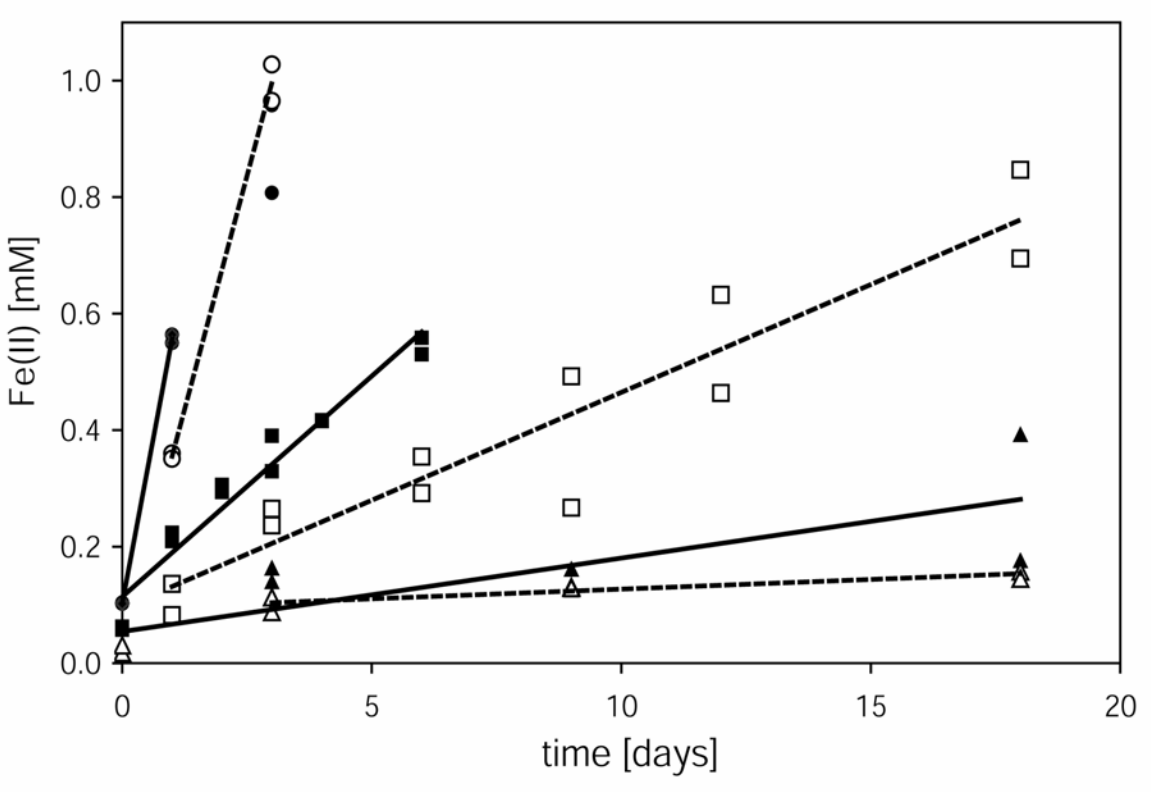

Figure 3.6 Time-course of Fe(III) reduction by $2*10^9$ (circles), $2*10^8$ (squares) and $2*10^7$ (triangles) total MR-1 cells (in 3 ml) with Fe-beads (empty symbols) or free $Fe(OH)_3$ particles (solid symbols) as the electron acceptor. Each data point represents a separate tube sacrificed at a given time-point for the measurement. Regression lines were calculated by the least square method. The initial amount of iron reduced (first day) is not included in the regressions for the Fe-beads because we cannot exclude direct contact reduction of the iron in the bead cortex.

3.3.5 Mechanism of Fe(III) reduction at a distance

If chelators or shuttles were being produced and were accumulating in the culture fluids the addition of "conditioned" supernatants should accelerate rates of iron reduction within the beads. However, the addition of either reduced or oxidized supernatants to high cell density Fe-bead cultures did not stimulate iron reduction at a distance, but the addition of 5 μM AQDS did (**FIG7**). The small difference between the amount of

reduced iron in tubes with reduced supernatants added and the WT control can be explained by carry over of Fe(II) added with the supernatants. In oxidized supernatants, the iron had precipitated during the oxidation treatment and therefore did not carry over. Added cell lysate did not enhance iron reduction either (data not shown). Supernatants contain about 10% of dissolved Fe(II), the rest is presumably bound to the cells, their EPS, the beads or precipitated as Fe(II) minerals. We did not observed dissolved Fe(III) in detectable concentrations.

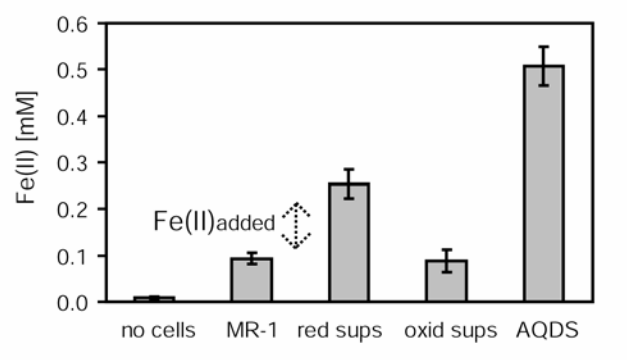

Figure 3.7 Effect of conditioned supernatants. The amount of Fe(II) produced by MR-1 (total cell number $4.5*10^8$) after 3 days in the presence of Fe-beads without any addition (MR-1), or with the addition of 66% of reduced (unprocessed) or oxidized supernatants from high cell density tubes ($2*10^9$ cells/tube) that had reduced 80% of iron within the beads, is shown. The amount of Fe(II) carried over by the addition of the reduced supernatants is indicated. The positive control contains 5 μM AQDS added to the reduced supernatants (AQDS). The negative control is uninoculated. Data represent the average of duplicate experiments, with error bars indicating the data range.

3.4 Discussion

Previous studies employing Fe(III) sequestered in alginate beads provided evidence suggesting that Fe(III) can be reduced at a distance by *Shewanella* and *Geothrix* species (39, 41). In an effort to reproduce these results, and to define the conditions where Fe(III) reduction at a distance occurs for *S. onediensis* strain MR-1 in particular, we developed an assay that makes use of porous glass beads coated with $Fe(OH)_3$. This system has advantages over the alginate bead system in that it is inert, non-toxic, stable under a wide range of pH, well-characterized (e.g., the location of iron is precisely defined, as are the sites of its release) and amenable for microscopy. Because our calculations indicate that a maximum of 9.1% of the iron in the system is available for reduction by direct contact, the vast majority of the iron (90.9%) can only be reduced by an indirect mechanism. Thus, the Fe(III) beads provide an effective means of assaying for Fe reduction at a distance, via a mechanism that either involves mobilization of Fe(III) from the inside of the beads to the cells for reduction, or electron transfer from the cells to the Fe(III) inside the beads by a diffusible electron carrier. Using this system, we have demonstrated that *S. oneidensis* MR-1 can reduce iron at a distance under conditions relevant for anaerobic biofilms.

Fe(III) reduction at a distance is an active process that occurs under anaerobic conditions. This is evidenced by the fact that killed cells show only minor reduction (20%) and new protein synthesis is required for maximal Fe(III) reduction over the course of our experiments for cells that are not pre-adapted to anaerobic conditions. Although cells that are pre-grown in anaerobic conditions (e.g., with fumarate or ferric citrate as the electron acceptor) prior to trial in the Fe-bead assay reduce iron at a distance

faster than aerobically-grown cells, the later can reduce iron at a distance if given time to adapt to anaerobic conditions. The fact that anaerobically-grown cells still require new protein synthesis to achieve maximal Fe(III) bead reduction over the time course of the assay, suggests either that some new components of the electron transfer pathway must be synthesized under these conditions or that some of the components are degraded and/or lose their activity and must therefore be regenerated. The complete inhibition of Fe reduction at a distance observed for aerobically-grown cultures exposed to chloramphenicol suggests that (a) key constituent(s) of the electron transfer pathway is/are missing, and only expressed when the cells are allowed to adapt to anaerobic conditions and induce the expression of anaerobically-regulated genes. Evidence in support of this comes from the fact that the *menB* mutant cannot reduce Fe(III) at a distance above the level measured for the killed-cell control.

We previously hypothesized that Fe(III) reduction at a distance would be most likely to occur in biofilms (20), and our results are consistent with this idea. Indeed, microscopic examination of the beads revealed that MR-1 colonizes the surfaces of the beads, forming a matrix with characteristic microcolonies. On a macro scale, biofilm formation was obvious in tubes inoculated with Fe-beads and cells due to the establishment of a compact aggregate that would readily reform after physical disruption. Not only do biofilms form under the same conditions where Fe(III) bead reduction is achieved, but they appear to play a role in enabling the process (possibly by trapping the diffusible mediator). The results with the *fliI* mutant support this conclusion, as significantly less iron was reduced at a distance with this mutant as compared to the WT. Why the *fliI* mutants are impaired in Fe(III) reduction at a distance is not yet known, but

may be related to: 1.) flagellar-mediated induction of genes (1, 52) that are required for Fe(III) reduction at a distance or, 2.) the need to attach to a substrate in order to build a biofilm that will retain and recycle electron shuttles or Fe(III) chelators. These different possibilities remain to be tested.

Iron reduction in the Fe-bead system does not appear to be limited by availability of reduction sites (Fe(III)) at high cell density, in contrast to previous findings that the rate of ferric mineral reduction by *S. algae* BrY and *Shewanella putrefaciens* MR-4 plateaued at high cell density (11, 43). In the case of ferric minerals, this plateau was attributed to the saturation of reduction sites on the mineral (11, 43). In our comparative experiments with minerals and Fe-beads rates per initial cell number go down at higher cell densities in the $Fe(OH)_3$ system, as previously observed, but the rates of Fe reduction at a distance per cell are similar for all cell densities. Although most of the iron in the bead system is inside the pores and therefore further from the cells, unlike in the mineral phase any given Fe atom is likely reactive in this matrix because it is exposed to the water interface and therefore accessible for reduction or chelation. Not only does this suggest that surface saturation is not limiting in this system, but it indicates that quorum sensing does not control Fe reduction at a distance. The overall rates of $Fe(OH)_3$ reduction are faster than the rates of Fe-bead reduction. The rate limiting step in the bead system is thus likely to be the diffusion of chelates, shuttles, or iron within the pores. The cells interaction with the Fe-beads is completely different than with poorly crystalline $Fe(OH)_3$ particles, since mineral particles are much smaller than the cells and can attach to the cell surface and sometimes even intrude into the cell (14-16). The fact that a flagellar mutant has impaired ability to reduce iron at a distance and not $Fe(OH)_3$ (5,12)

might be explained by this size difference, since small mineral particles will naturally attach and cover the bacterial cell surface, but the cells will presumably need flagella to colonize and attach to the beads.

In comparing the ability of *omcB* and *mtrB* mutants to reduce Fe at a distance (relative to the WT) to their ability to reduce $Fe(OH)_3$ "directly", it appears that these proteins are not absolutely required in either case (2, 3, 32, 36). This contrasts with the results for the *menB* mutant, which indicate that menaquinone is an essential carrier in the electron transfer pathway to iron, be it at a distance as shown in this study, or directly (33, 44). Together, these results imply that the pathway of electron transfer from the cell to the mineral flows through menaquinone in all cases, but diverges, and probably overlaps at later steps, and that these outermembrane reductases may not be essential under some conditions.

In light of these observations, the question arises: what is the mechanism of Fe reduction at a distance? Our data shows that iron is being depleted inside the beads. We cannot determine at this point whether the iron is leaving as Fe(III), presumably by the aid of chelators, or as dissolved Fe(II) after being reduced inside the pores. We did not observe detectable concentrations of dissolved Fe(III) in the Fe-bead system (in contrast to was reported for BrY in the presence of alginate beads (40)), as would be expected if a chelator were present in significant amounts. Moreover, we could not find evidence in spent supernatants from Fe-bead cultures for any factor (e.g., an electron shuttle) that stimulates Fe reduction at a distance under the conditions of our experiments. We can rule out a role for an excreted 1,4-dihydroxy-2-naphthoic acid (DHNA) derivative stemming from the *men* pathway in Fe reduction as a distance given that the *menB*

mutant, which cannot make DHNA, could be rescued by the addition of menaquinone, consistent with our findings that an electron shuttling compound is not excreted into the medium (19). The cells may still be producing electron shuttles or chelators that partition into the biofilms and therefore are not recovered in spent supernatants in appreciable concentrations, or they may be at high enough concentrations in the local microenvironment close to the bead surface, but too dilute to be detected when the biofilms forming around the beads are disrupted and they are released to the culture fluids. We therefore conclude that the mediator MR-1 uses to reduce iron at a distance acts locally within the biofilm-bead environment.

3.5 References

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