Chapter 4

An excreted 1,4-dihydroxy-2-naphthoic acid derivative is converted to menaquinone by *Shewanella oneidensis men* mutants but is not an electron shuttle

* Adapted from Hernandez, Lies & Newman, *J. Bact.* (submitted).

4.1 Introduction

The reduction of ferric [Fe(III)] minerals is an important microbial process in anaerobic environments that has broad biogeochemical significance (14, 22, 34) and may be useful for the bioremediation of organic and metal contaminants (16, 17) corrosion control (9), and harvesting electrical current from marine sediments (4). At near neutral pH, Fe(III) exists in the form of very poorly soluble minerals (6), thus Fe(III)-reducing bacteria face the challenge of delivering electrons to an external and solid electron acceptor. Despite the importance of this process, its molecular mechanisms are not well understood. For years it was believed that direct contact between the cell and the mineral was required for reduction (1, 5, 7, 19, 28), and consistent with this idea, several outer membrane proteins are involved in Fe(III) reduction by different bacterial species (2, 3, 10, 14, 20, 23-27). More recently, it has become accepted that Fe(III) reduction can proceed at a distance mediated by a variety of exogenous organic and inorganic compounds that serve either as chelators or electron shuttles (13, 18, 32). Among these

compounds are humic substances, a class of macromolecular redox-active organic compounds that contain quinone moietiesm, and anthraquinone-2,6-disulfonate (AQDS), a commonly used functional surrogate for humic substances (36).

The idea that the Fe(III)-reducing bacterium, *Shewanella oneidensis* strain MR-1, might excrete a quinone-like molecule into culture fluids as an extracellular electron shuttle was first proposed by Newman and Kolter (33).This was based on several facts: 1.) A mutant defective in the *menC* gene (open reading frame [ORF] SO4575 in the MR-1 genome sequence) that encodes o-succinylbenzoic acid synthase (required for menaquinone (MQ) biosynthesis) can be complemented with respect to its ability to reduce AQDS by an extracellular diffusible factor released by the wild-type (WT). 2.) The *menC* mutant does not make this factor under any condition, and 3.) Semi-purified fractions of culture fluids from the WT with maximal complementation activity have spectrophotometric properties reminiscent of quinones. However, it was not clear from this study whether the observed complementation of the *menC* mutant was due to the restoration of menaquinone biosynthesis or the provision of an extracellular electron shuttle, nor was it demonstrated that the excreted factor could shuttle electrons to Fe(III) minerals. Subsequent work by Nevin and Lovley suggested that electron shuttling, as well as Fe(III) chelation, occurred in *Geothrix fermentans* and *Shewanella algae* (30, 31) but not in *Geobacter metallireducens* (29), leading to the conclusion that different species have different strategies for reducing Fe(III) minerals. The genetic and biochemical details of how any of these organisms reduces Fe(III) at a distance, however, are poorly understood.

Desiring to determine whether the unknown small molecule (USM) excreted by *S. oneidensis* strain MR-1 that complemented the *menC* mutant is also an extracellular electron shuttle, we undertook the present study. We define "extracellular electron shuttle" in this context to be any molecule that is present in culture fluids and can stimulate Fe(III) reduction from a distance.

4.2 USM permits AQDS reduction by restoring MQ biosynthesis

To elucidate the nature of the complementation of the *menC* mutant by cross-feeding from the WT, we extended our cross-feeding analysis to other *men* mutants downstream of *menC* in the menaquinone (MQ) biosynthetic pathway (Figure 1). The *menC* and *menB* mutants (strains H2 and SR536, respectively) have been described previously (33, 34). The *menE* mutant (strain Ard20, with an insertion in ORF SO4576 of the MR-1 genome) was isolated following transposon mutagenesis of MR-1 with Tn*phoA*'-1 (40) during a screen for mutants defective in AQDS reduction (D. Malasarn and D. Newman, unpublished data). The Δ*menA* mutant (strain DSP101) was constructed by first generating a cloned copy of the *menA* gene (ORF SO1910 of the MR-1 genome) with an in-frame deletion using crossover PCR (15) with external primers MenAFS (5'-GGACTAGTCTACGGTCGCGGTTAGCG-3')and MenARS (5'-GGACTAGTATGTCAGTCTCATGCCCG-3') and internal primers MenADelN (5'-CCCATCCACTAAATTTAAATAGGCTAAAATCCAAGGATTC-3') and MenADelC (5'-TATTTAAATTTAGTGGATGGGCTGGTCTTTACCGCTTAG-3'). The amplified fragment was cloned, together with a gentamicin resistance cassette, into pWM91 (21) using the *Spe*I restriction sites encoded by the first six nucleotides of the external primers.

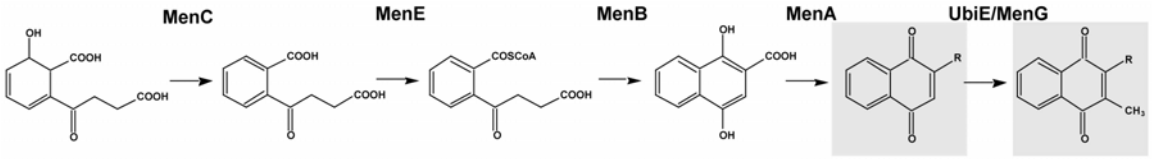
A kanamycin resistance cassette was then cloned into the Δ*menA* fragment at the *Swa*I restriction site encoded in the 21-base pair complementary region at the 5' end of the internal primers (underlined). The resulting plasmid, pSONE20 carrying the Δ*menA1001*::*aphA* allele, was transferred into strain MR-1 by conjugation using *E. coli* donor strain β2155 (8) and kanamycin-resistant *S. oneidensis* colonies were selected and then screened for gentamicin sensitivity. Kanamycin-resistant and gentamicin-sensitive colonies were confirmed for incorporation of the Δ*menA1001*::*aphA* mutation by PCR with the MenAFS and MenARS primers. We did not make mutations in any genes that are involved in subsequent modification of demethylmenaquinone (DMQ) to MQ because our goal was simply to determine whether membrane-bound electron carriers vs. a soluble electron shuttle derived from the *men* pathway were required for AQDS reduction (**Fig. 1**).

A quantitative bioassay was developed to measure complementation by cross-feeding. Culture fluids were concentrated from stationary phase cultures of different *men* mutants or the WT grown aerobically in a defined minimal medium (35). A concentrated extract was generated by passing 5 ml of culture fluid through a commercially available mixed-mode resin cartridge (OASIS MAX, Waters), washed with 2 ml of 50 mM sodium acetate/methanol (95/5) pH 7 buffer, washed again with 2 ml of methanol, and finally eluted by a 2% formic acid-methanol solution (2 ml). The eluent was concentrated in a speed-vac and resuspended in 100 μl of a 50:50% methanol:water solution. The bioassay was performed by adding 5 μl of the concentrated extract to 140 μl of minimal medium containing 5 μl of a washed overnight LB-grown culture (~10^9 cell/ml) and 5 mM AQDS. This mixture was incubated 9-24 hours in a microtiter plate in

an anaerobic COY glove box (80:15:5% N_2:CO_2:H_2 atmosphere), and AQDS reduction to AHDS was measured spectrophotometrically at 450 nm on a Opsys microtitre plate reader. Under these conditions, the absorption of reduced AQDS (AHDS) is linearly correlated with the amount of the complementing factor in the concentrated extract. The extent of AQDS reduction thus provides a quantitative proxy for the amount of complementing factor in the added culture fluid.

If MQ and not a biosynthetic intermediate or derivative thereof is required for AQDS reduction, culture fluids from the WT and all *men* mutants downstream in the biosynthetic pathway of a given *men* mutant should restore AQDS reduction to the mutant in the bioassay. This assumes that the accumulated intermediate in the blocked pathway is released into culture fluids and is concentrated by our extraction protocol. The AQDS reduction bioassay shows that the *menC* mutant is cross-fed by culture fluids from *menE, menB* and Δ*menA* mutants; the *menE* mutant is cross-fed by the Δ*menA* mutant but not by the *menB* mutant (likely due to a lack of recovery of OSB-CoA due to breakdown to OSB, see **Fig. 1**); and the *menB* mutant is cross-fed by the Δ*menA* mutant (**Fig. 1**). Similar results are observed in cross-feeding assays performed by streaking *men* mutant and WT strains in close proximity on Kligler Iron Agar (Difco) plates containing thiosulfate (another low potential soluble electron acceptor), where thiosulfate reduction can be scored by the appearance of black precipitates in the cell mass (data not shown). It thus appears that the complementing factors excreted by these different mutants are likely soluble intermediates in the MQ biosynthesis pathway whose uptake restores the ability of their respective upstream mutant(s) to synthesize MQ.

Because all of the mutants with the exception of the Δ*menA* mutant are cross-fed by the WT, we reason that the USM in the WT culture fluids is DHNA or a derivative of DHNA. Assuming that the WT does not excrete every soluble MQ biosynthetic pathway intermediate, then we would expect the last soluble intermediate of the pathway to be the only one capable of cross-feeding *menC*, *menE*, and *menB* mutants. Previous work has shown that the USM has a different thin layer chromatographic profile than DHNA (Newman & Kolter); we therefore infer that the USM is a derivative of DHNA. This is consistent with a recent report where an excreted derivative of DHNA has been implicated in CCl_4 reduction by strain MR-1 (39). That the Δ*menA* mutant is the only *men* mutant not to be cross-fed by the WT demonstrates that MQ is required for AQDS reduction: *menA* encodes for DHNA-octaprenyltransferase, an enzyme that attaches a hydrophobic isoprenoid tail to DHNA, the penultimate step in MQ biosynthesis (38). This is supported by the fact that the Δ*menA* mutant is complemented in the bioassay by a commercially available MQ (Vitamin K2, Sigma) (data not shown).



menC	-	-	-	-	nd
menE	+	-	-	-	nd
menB	+	-	-	-	nd
menA	+	+	+	-	nd
WT	+	+	+	-	nd
Vit K2	+	+	+	+	nd

Figure 4.1 Schematic of the menaquinone (MQ) biosynthesis pathway and cross-feeding results in the AQDS reduction bioassay. The names of the enzymes catalyzing the conversion of MQ precursors are given above the arrows showing the steps in the MQ biosynthetic pathway. A plus or a minus is assigned to a given position in the table based on whether a strain deficient in the production of the enzyme listed at the top of the figure can be complemented in the AQDS bioassay by concentrated supernatant fluids from *men* strains, the WT or purified Vitamin K2, respectively (listed in the left-most column of the figure). The compounds shown in the pathway are: SHCHC, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate; OSB, o-succinylbenzoate; OSB-CoA, o-succinylbenzoyl-CoA; DHNA, 1,4-dihydroxy-2-naphthoic acid; DMQ, demethylmenaquinone; MQ, menaquinone. DMQ and MQ are shaded to indicate that they are membrane-bound electron carriers. Nd=not determined. Vit K2 = MQ-4 (Sigma).

4.3 Production of the USM by other strains and conditions of production by MR-1.

To determine the relative amounts of USM produced by different strains, we used the quantitative bioassay to measure AQDS reduction by cross-feeding of strain H2. In addition to MR-1, all wild-type *Shewanella* species tested (i.e., *S. algae* BrY, *S. putrefaciens* CN32, and *Shewanella* sp. ANA-3) produced the USM, with BrY, CN32 and ANA-3 producing more USM than MR-1 (data not shown). Other MQ-producing strains (i.e., *Vibrio cholera* strain El Tor and *Escherichia coli* strain ZK126) are able to cross-feed strain H2, although not as well as MR-1; strains unable to produce menaquinones (i.e., *Pseudomonas fluorescens* strain WCS365*, P. chlororaphis* strain 1391, and a *menD* mutant of *E. coli* strain ZK126) do not cross-feed H2 (data not shown). This suggests that organisms that make MQ produce a soluble intermediate that can be

taken up and processed into MQ by H2, as has been observed for *E.coli* strain K12 (11). Whether this intermediate has an additional function or is just a leaky by-product of the MQ biosynthetic pathway is unknown at this time.

The USM is produced by the tested *Shewanella* strains under both aerobic and anaerobic conditions, and its concentration in culture fluids positively correlates with cell density (data not shown). Addition of 25% and 50% filtered spent-supernatants from high-density cultures back to low-density cultures of MR-1 does not lead to a measurable increase in its production earlier in the growth phase when measured by a 9 hour bioassay. This suggests that quorum-sensing does not play a role in inducing synthesis of the USM.

4.4 USM is not an electron shuttle to Fe(III) minerals.

Having explained the AQDS reduction phenotype of the H2 mutant, we next sought to determine whether USM was required for Fe(III) mineral reduction at a distance. To do this, we made use of an assay involving Fe(III)-(hydr)oxide-coated porous glass beads [Fe-beads]. Briefly, the majority of the iron is sequestered inside pores within the beads and is not available to the bacteria for reduction by "direct" contact. Accordingly, if significant Fe reduction is observed, it can be attributed to an indirect mechanism. Using this assay, we have shown that MR-1 can reduce Fe at a distance and that the addition of AQDS greatly stimulates such reduction (12). If the USM were an extracellular electron shuttle we would expect to observe similar stimulation by the addition of concentrated supernatants from the WT but not from a *men* mutant strain blocked in USM biosynthesis (e.g., strain H2). To test this hypothesis,

MR-1 cells were prepared for the shuttling assay as previously described (12), resuspended at a total cell density of $2*10^8$ cells/3 ml in minimal medium with 20 mg of Fe-beads, and incubated under anaerobic conditions. After 3 days, the experiments were stopped by the addition of 0.75 ml of 12N HCl to extract all the Fe in the system. Fe(II) was determined in the acidified culture samples by the ferrozine assay and total iron was quantified after a reduction step with NH_2OH (37). To determine the sensitivity of our shuttling assay, we used AQDS as a representative electron shuttle, and found that we could detect the presence of as little as 1 μM by measuring enhanced Fe-bead reduction relative to the amount of Fe-bead reduction achieved in the absence of an exogenous shuttle.

When concentrated extracts of WT and H2 culture fluids processed as described above were provided to MR-1 cells in the presence of the beads, no difference in the amount of Fe(III) reduction was observed (data not shown). Similarly, the addition of 66% filtered culture fluids (not concentrated) from the WT or H2 mutant to cultures of MR-1 did not affect the rate of Fe-bead reduction (**Fig. 2a**). This suggests that there were no redox-active molecules in either the WT or H2 fluids or concentrated culture fluids at a concentration higher than 1 μM. To insure that toxic compounds from the culture fluids were not poisoning the shuttling assay, we added 5 μM AQDS to concentrated culture fluids from H2 and observed enhanced Fe(III) reduction in the shuttling assay with MR-1 cells. The addition of 10 μM Vitamin K2 or 10 μM DHNA did not stimulate Fe-bead reduction by MR-1 either (data not shown). In addition, the rate of poorly crystalline Fe(III)-(hydr)oxide reduction was not enhanced by the concentrated extracts of WT fluids over a period of 2 days in a microtiter plate assay similar to that described for the AQDS

reduction bioassay in LM basal medium (22) (**Fig. 2b**). We therefore conclude that the USM is not an electron shuttle.

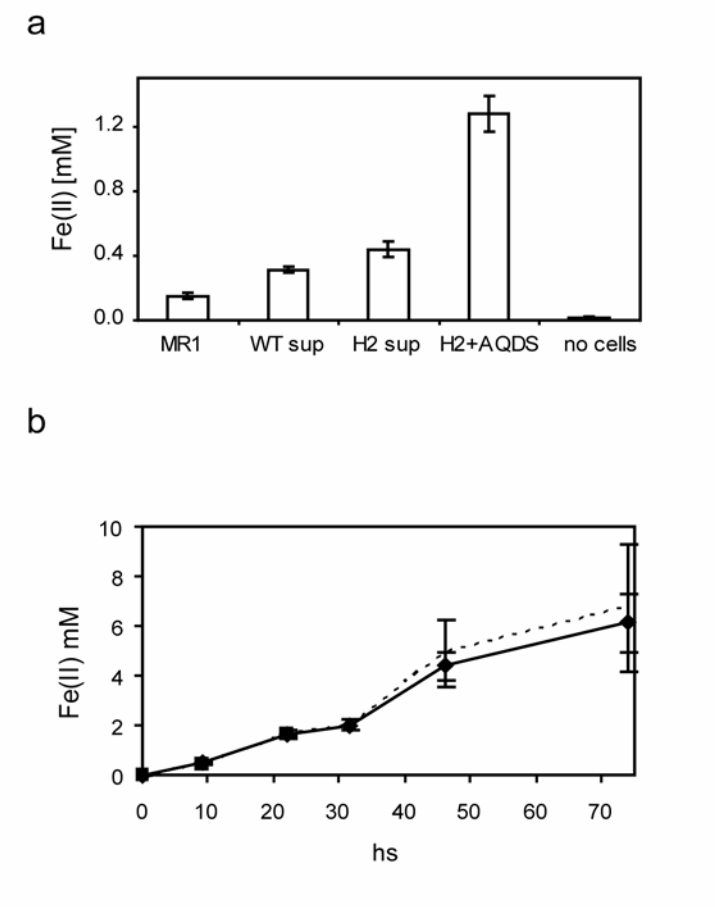


Figure 4.2 USM is not an electron shuttle to minerals. **(a).** Fe(II) produced by *Shewanella oneidensis* MR-1 after 3 days in the presence of Fe-beads without any addition (MR-1) or with the addition of 66% of filtered supernatants from the WT (WT sup) or the *menC* mutant (H2 sup). The positive control contains 5 μM AQDS added to the *menC* supernatants (H2+AQDS). The negative control is uninoculated. Data represent the average of duplicate experiments, with error bars indicating the data range. **(b).** Poorly crystalline Fe(III)-(hydr)oxide reduction by *Shewanella oneidensis* MR-1 without (diamonds) or with the addition of WT supernatant extracts

(squares/dashed line). Points represent the average of triplicate experiments, with error bars indicating the data range.

In summary, our results demonstrate that the small molecule released by *S. oneidensis* strain MR-1 that complements H2 in the AQDS reduction bioassay is a derivative of DHNA, and functions as a MQ precursor; it is not an extracellular electron shuttle to Fe(III) minerals. How MR-1 reduces Fe(III) at a distance remains an intriguing open question, as does whether the excretion of a DHNA derivative serves a physiological function for MR-1.

4.5 References

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