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Mutational and gene expression analysis of *mtrDEF*, *omcA* and *mtrCAB* during arsenate and iron reduction in *Shewanella* sp. ANA-3

Carolina Reyes, Julie N. Murphy, and Chad W. Saltikov*

Department of Microbiology and Environmental Toxicology, 1156 High Street, Santa Cruz, CA 95064, USA

Summary

Arsenate respiration and Fe(III) reduction are important processes that influence the fate and transport of arsenic in the environment. The goal of this study was to investigate the impact of arsenate on Fe(III) reduction using arsenate and Fe(III) reduction deficient mutants of *Shewanella* sp. strain ANA-3. Ferrihydrite reduction in the absence of arsenate was similar for an arsenate reduction mutant (*arrA* and *arsC* deletion strain of ANA-3) compared with wild-type ANA-3. However, the presence of arsenate adsorbed onto ferrihydrite impeded Fe(III) reduction for the arsenate reduction mutant but not in the wild-type. In an Fe(III) reduction mutant (*mtrDEF*, *omcA*, *mtrCAB* null mutant of ANA-3), arsenate was reduced similarly to wild-type ANA-3 indicating the Fe(III) reduction pathway is not required for ferrihydrite-associated arsenate reduction. Expression analysis of the *mtr/omc* gene cluster of ANA-3 showed that *omcA* and *mtrCAB* were expressed under soluble Fe(III), ferrihydrite and arsenate growth conditions and not in aerobically grown cells. Expression of *arrA* was greater with ferrihydrite pre-adsorbed with arsenate relative to ferrihydrite only. Lastly, *arrA* and *mtrA* were simultaneously induced in cells shifted to anaerobic conditions and exposed to soluble Fe(III) and arsenate. These observations suggest that, unlike Fe(III), arsenate can co-induce operons (*arr* and *mtr*) implicated in arsenic mobilization.

Introduction

Microbial reductive dissolution of arsenic and iron(III)-bearing minerals impacts arsenic fate and transport in aquifers (Nickson *et al.*, 1998; 2000; Harvey *et al.*, 2002; Smedley and Kinniburgh, 2002). Under anaerobic conditions, certain bacteria can reduce arsenate to arsenite; the latter arsenical has greater hydrologic mobility compared with arsenate (reviewed in Oremland and Stolz, 2005). Although a variety of arsenic and Fe(III)-reducing bacteria have been isolated from arsenic impacted sediments (Islam *et al.*, 2004) (reviewed in Reyes *et al.*, 2008), a detailed understanding of how arsenic and iron effects the metabolism of these microbes is needed.

*For correspondence. saltikov@etox.ucsc.edu; Tel. (+1) 831 459 5520; Fax (+1) 831 459 3524.

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Model metal-reducing bacteria have been used to work out the molecular mechanisms for iron and arsenate reduction. One such model, *Shewanella* sp. ANA-3, has detoxifying (*arsC*) and respiratory (*arrA*) arsenate reductases. The respiratory pathway (encoded by *arrAB*) allows the cell to couple the oxidation of an electron donor to the reduction of arsenate resulting in cell growth (Saltikov and Newman, 2003). The arsenate detoxification pathway is encoded by the *ars* operon, which comprises a cytoplasmic arsenate reductase (ArsC) and an ATP driven pump arsenite efflux pump (ArsAB) that extrudes arsenite out of the cell (Saltikov *et al.*, 2003). Both arsenate and iron reduction in *Shewanella* sp. ANA-3 are dependent on the membrane bound tetraheme *c*-type cytochrome, CymA (Murphy and Saltikov, 2007; Zargar and Saltikov, 2009). The mechanism for iron reduction has been extensively investigated (reviewed in Shi *et al.*, 2007; Fredrickson *et al.*, 2008). In *Shewanella*, certain genes within the *mtr/omc* cluster are necessary for iron reduction. This cluster includes genes encoding for three outermembrane decaheme cytochromes (MtrF, OmcA, MtrC), two periplasmic decaheme cytochromes (MtrD and MtrA), and two outer membrane noncytochrome proteins (MtrE, MtrB) (Pitts *et al.*, 2003). The gene synteny and sequence homologies within the *mtr/omc* cluster are highly conserved in many *Shewanella* species (Fredrickson *et al.*, 2008). Most studies have focused on the outer membrane cytochromes MtrC and OmcA because they are required for the majority of iron oxide reduction activity in *Shewanella* (Shi *et al.*, 2007). However, various mutations in *mtrCAB* can still exhibit Fe(III) reductase activity (Beliaev *et al.*, 2001).

We previously generated a seven-gene deletion strain (*mtrDEF*, *omcA*, *mtrCAB*, called 'FERM') of ANA-3 that was used to investigate how the absence of iron reduction affected the release of arsenic from columns packed with ferrihydrite-coated sand (Tufano *et al.*, 2008). The results showed that under hydrodynamic (with flow) conditions, columns inoculated with the iron reduction deficient strain (FERM) mobilized the most arsenic compared with wild-type and iron reduction-only strains (*arrA*, *arsC*, called ARM1). Here, we further characterize the FERM strain for its ability to reduce arsenate-ferrihydrite minerals in batch conditions as well as other electron acceptors. We also investigated the impact of arsenate on the expression of the *mtr/omc* genes and how *arrA* and *mtrA* gene expression responded to a shifting from aerobic to anaerobic iron and arsenate-reducing conditions.

Results

Growth rate analysis of ANA-3 mutant strains

The first part of our study was to generate a series of mutants that (i) lacked the ability to reduce arsenate (called 'ARM1', arsenate reduction mutant), (ii) lacked the ability to reduce soluble and mineral forms of Fe(III) (called 'FERM', Fe(III) reduction mutant) and (iii) lacked both iron and arsenate reduction abilities (called 'FARM', Fe(III)/As(V) reduction mutant). The latter strain served as a biological control strain for background iron and arsenate reduction activities. ARM1 and FERM were generated in previous reports for investigating arsenic dependent gene expression of *arr* (Saltikov *et al.*, 2005) and arsenic mobilization under hydrodynamic conditions (Tufano *et al.*, 2008). In this report, we performed a thorough examination of the growth response of each strain to different electron

acceptors. Growth rate analyses were done to characterize the phenotypes of the various *Shewanella* strains listed in Table 1 and are summarized in Table 2 and Fig. S1. In general, the growth phenotypes of the FERM, FARM and ARM1 strains were similar to that of the wild-type ANA-3 strain when grown on several terminal electron acceptors: fumarate, oxygen, nitrate and trimethyl-N-amine oxide (TMAO) (Table 2, Fig. S1). These results indicate that deleting the *mtr/omc* and/or *arrA/arsC* genes specifically affected growth on iron and arsenate respectively.

Membrane cytochrome content of ANA-3

After characterizing the phenotypes of the *mtr/omc* and *arrA/arsC* mutant strains, we next examined the total cytochrome content in these strains. Table 3 summarizes the pyridine-hemochrome analysis of total membrane prepared from anaerobically and aerobically grown ANA-3 and FERM cells. In general, the wild-type had 1.4- to 2-fold higher *c*-type haem content compared with total membranes prepared from the FERM strain. Haem staining of total membrane protein preparations from FERM cells, grown under aerobic and anaerobic conditions, showed very little to no cytochromes when compared with the wild-type ANA-3 samples (Fig. S2). These results indicate that the *mtr/omc* gene cluster encode nearly 50% of the total membrane-associated cytochromes.

Incubations of arsenate-ferrihydrite with ANA-3 strains

Next we determined the iron and arsenate reducing capabilities of the mutant *Shewanella* sp. ANA-3 strains. This was carried out using batch growth conditions and ferrihydrite (8 mM) presorbed with 1 mM arsenate. Based on an Fe(III) surface site density of 2.2 sites nm⁻² we calculate 5.8% of the iron mineral was covered by arsenate and 4.1% by phosphate. Approximately 300 µM of arsenate was detected in solution at the initial time point, indicating that arsenic desorption had occurred prior to inoculation with cells (Fig. 1A). Within 48 h, 300 µM of the dissolved arsenate was reduced to arsenite in the incubations with the wild-type and FERM strains (Fig. 1A and B). Conversely, no dissolved arsenite was detected for the arsenate reduction mutants (ARM1 and FARM) and no-cell control (Fig. 1B). Solid-phase arsenic was determined at the final time point, 144 h, for incubations of each strain (Fig. 1C). Approximately 90% of the adsorbed arsenic was arsenite for the wild-type and FERM strains (Fig. 1C). Conversely, 95% of the adsorbed arsenic remained as arsenate in the ARM1, FARM and no cell incubations (Fig. 1C). It is concluded that deleting the *mtr/omc* genes had no observable effect on mineral-associated arsenate reduction.

Because the ARM1 strain reduces Fe(III) and not arsenate, we were interested in determining if arsenate would affect its ability to reduce ferrihydrite presorbed with arsenate. In the absence of arsenate, the wild-type and ARM1 strains showed similar trends in ferrihydrite (Fig. 2A) and Fe(III)-citrate (Fig. 2C) reduction. In cultures containing ferrihydrite and the FERM strain, only minor increases in Fe(II) were observed, which was similar to the FARM strain and the no-cell control (Fig. 2A). In addition to being deficient in reduction of ferrihydrite, the FERM (and FARM) strain was completely deficient in soluble Fe(III)-citrate reduction (Fig. 2C). The iron reduction phenotype for ARM1 and the wild-type were different when grown with arsenate presorbed onto ferrihydrite. Fe(III)-reduction by the wild-type appeared to be unaffected by the presence of arsenate (Fig. 2B).

However, the ARM1 strain reduced 50% less Fe(III) than the wild-type ANA-3. These results demonstrate that the presence of arsenate negatively affected iron reduction in strains that cannot reduce arsenate.

Quantitative expression of *omc/mtr* iron reduction genes relative to *gyrB*

To further investigate the effects of arsenate on the iron reduction, we used quantitative RT-PCR (qRT-PCR) to monitor the expression of the *omc/mtr* cluster relative to the housekeeping gene, *gyrB* (DNA gyrase) in the wild-type under several growth conditions. Total RNA was extracted from cultures grown to mid-log phase (OD₆₀₀, 0.1) aerobically or anaerobically with either 10 mM Fe(III) oxide, 10 mM Fe(III)-AsV oxide, 10 mM Fe(III) citrate or 10 mM arsenate as the terminal electron acceptor. Expression of *mtrC* and *mtrB* was greater than the other *mtr/omc* genes in samples grown on Fe(III) oxide followed by *mtrA* and *omcA* (Fig. 3). Little to no expression was detected for the *mtrDEF* genes in RNA prepared from samples grown with oxygen, arsenate, Fe(III) citrate and Fe(III) oxide (Fig. 3). Expression of the *omcAmtrCAB* genes was very low when samples were grown on oxygen. Surprisingly, *omcAmtrCAB* genes were expressed in samples grown on arsenate and showed a two- to fourfold increase in expression over samples grown on Fe(III) citrate (Fig. 3). Expression of *mtrA* and *arrA* were also determined in cells grown on arsenate presorbed onto ferrihydrite. Expression of *mtrA* was similar to cells grown either on arsenate or ferrihydrite. Transcriptional patterns of *arrA* showed its expression was highest under arsenate only conditions compared with samples grown on ferrihydrite and arsenate or ferrihydrite alone. These results show that the highest expression of *omcAmtrCAB* genes occurs in the presence of iron oxide or arsenate. Additionally, these results imply that the *omcAmtrCAB* genes are expressed in response to anaerobic conditions and that growth on oxygen represses the expression of these genes.

Expression analysis of *arrA* and *mtrA* genes relative to 16S rRNA gene

Having determined that arsenate, soluble and insoluble Fe(III) induces the expression of the *omcAmtrCAB* and given that arsenate triggers the expression of the *arr* genes, we were interested in knowing the relative order of expression of these genes when ANA-3 encounters both arsenate and Fe(III). To examine the induction of iron and arsenic reduction pathways, upon iron and arsenic exposure, we performed an aerobic-anaerobic shift experiment with ANA-3. We selected *mtrA* as a target for expression of the iron cluster genes under soluble iron-reducing conditions because MtrA is involved in reducing Fe(III) citrate (Pitts *et al.*, 2003). The wild-type ANA-3 was grown aerobically to mid-log growth phase, shifted to anaerobic conditions, and induced with 10 mM Fe(III) citrate and 10 mM arsenate. The presence of *arrA*- and *mtrA*-specific mRNA was analysed by RT-PCR at several time points (0, 2, 4 and 8 h) post-shift to anaerobic conditions (Fig. 4). At the start of the shift with Fe(III) citrate and arsenate, *arrA* expression and *mtrA* expression were nearly undetectable (Fig. 4A). At 2, 4 and 8 h, *arrA* and *mtrA* expression increased (Fig. 4A, lanes 3–8 of the top and middle gels respectively). The 16S rRNA gene was detectable by RT-PCR with similar intensities for each strain and throughout the time-course (Fig. 4, bottom gel, lanes 1–8). The *arrA* and *mtrA* genes were induced approximately fivefold within 8 h post shifting into anaerobic-Fe(III) citrate/arsenate reducing conditions (Fig. 4B). Gene expression also coincided with increased arsenite (Fig. 4C) and Fe(II) concentrations (Fig.

4D). At 2 h post induction, arsenite was detectable at 2 mM. Background levels of Fe(II) were detectable at the initial time point because of its presence in the Fe(III) citrate stock solution. Nevertheless, Fe(III) reduction occurred within the 2–4 h time interval, which was a slight delay compared with the onset of arsenate reduction.

Discussion

The overarching goal of this work is to address the hypothesis that arsenic contaminated groundwater is caused by Fe(III) reduction and dissolution of arsenic-bearing iron oxides (Smedley and Kinniburgh, 2002; McArthur *et al.*, 2004; Zheng *et al.*, 2004). However, an alternative hypothesis is emerging that arsenate reduction alone can lead to arsenic release from solid-phase arsenate-bearing minerals (Zobrist *et al.*, 2000; Kocar *et al.*, 2006; Tufano *et al.*, 2008). Moreover, abiotic studies have demonstrated that arsenite can strongly adsorb to iron minerals (Dixit and Hering, 2003). Such observations raise questions about the relative role of arsenate versus iron-reducing bacteria in processes that lead to arsenic contamination of drinking water. Because most arsenate respiratory reducers can also reduce Fe(III), it is difficult to conclude which metal-reduction pathway is most influential in controlling arsenic transport.

Additional laboratory studies with model metal-reducing bacteria have shown that simultaneous Fe(III) and arsenate reduction can promote arsenic retention into a solid phase (Zobrist *et al.*, 2000; Kocar *et al.*, 2006). These studies were carried out with either different genera of metal-reducers or a bacterium capable of Fe(III) and arsenate reduction. As an improvement over using different genera with varying arsenate or Fe(III) reduction abilities, we used a single species, *Shewanella* sp. ANA-3, to generate specific mutant strains with limited capacities to reduce either Fe(III), arsenate, none or both. These strains were used to address which metal-reduction pathway was most dominant over arsenic transport (Tufano *et al.*, 2008). When these strains (ARM1 and FERM) were examined in more detail, we found that the presence of arsenate impeded ferrihydrite reduction in the arsenate reduction deficient ANA-3 strain (deletion of *arrA* and *arsC*, ARM1 strain). Compared with the wild-type ANA-3, the arsenate reduction mutant (ARM1) reduced twofold less Fe(III) in both batch (Fig. 2B) and hydrodynamic conditions (Tufano *et al.*, 2008). When we repeated the batch experiment with ferrihydrite in the absence of arsenate, the ARM1 strain was able to reduce Fe(III) comparable to the wild-type (Fig. 2A). We envisage several possible reasons that may contribute to this difference. First, the adsorbed arsenate may have altered the reactivity of the iron oxide surface. Studies with *Shewanella putrefaciens* strain CN-32, an Fe(III) and arsenate reducing bacterium, showed that Fe(III) reduction rates decreased with increasing phosphate concentrations (Borch *et al.*, 2007). Because phosphate and arsenate have similar adsorption properties to ferrihydrite (Manning and Goldberg, 1996; Jain and Loeppert, 2000; Dixit and Hering, 2003), then the presence of arsenate on an iron oxide surface should have a similar negative impact that phosphate has on Fe(III) oxide reduction. This effect would be dependent on arsenate and phosphate surface coverage. In our batch experiments, we estimated that ~90% of the reactive Fe(III) sites should be unoccupied by phosphate or arsenate. Therefore, it is unlikely that the arsenate surface coverage was a significant factor in the decreased Fe(III) reduction observed for the ARM1 strain relative to wild-type ANA-3. Second, ARM1 may be more sensitive to arsenate compared with the

wild-type and FERM strain. The lack of an internal arsenate reductase could lead to accumulation of toxic levels of arsenate in the cytoplasm, which would impede growth of the strain. However, ARM1 and wild-type have similar growth phenotypes with fumarate containing 10 mM arsenate (Saltikov *et al.*, 2005), indicating that arsenate toxicity may not be a factor. Third, because the ARM1 strain is not able to reduce and grow on arsenate, lower cell numbers relative to wild-type might have been present in the ARM1 batch cultures. This could result in less Fe(III) reduction compared with the wild-type and FERM strains. Since we did not determine the cell densities throughout the time-course, we cannot rule out this third possibility.

Because single gene deletions in *mtrC* or *omcA* (as shown in *Shewanella oneidensis* MR-1) are not completely deficient in Fe(III) reduction (Beliaev *et al.*, 2001), we needed a strain that would be completely deficient in iron reduction. We constructed FERM, a strain lacking *mtrDEFomcAmtrCAB*, with the expectation to obtain a complete Fe(III) reduction deficient strain. As expected, this strain exhibited very little Fe(III) reduction abilities both for soluble and hydr(oxide) forms of iron. When originally tested under hydrodynamic conditions, FERM showed the greatest release of arsenic (as arsenite) from ferrihydrite-coated sands compared with the wild-type and ARM1 strain (Tufano *et al.*, 2008). However, we did not know if deleting the *mtrDEF omcA mtrCAB* caused secondary growth defects or altered its arsenate reduction abilities. Using batch growth conditions, we showed that the FERM strain reduced arsenate similarly to the wild-type ANA-3 strain. In the absence of iron reduction under batch conditions, arsenate reduction alone is sufficient to reduce adsorbed and dissolved arsenate. However, under flow conditions, simultaneous iron and arsenate reduction can increase arsenic retention on ferrihydrite-coated sands, probably as a secondary iron-arsenic mineral.

In our batch incubations, ferrihydrite reduction by the FERM strain, although extremely low, was not completely abolished. The low background Fe(III)-oxide reduction may have involved other *c*-type cytochromes. *Shewanella* species are known to have over 40 genes encoding for various *c*-type cytochromes (Meyer *et al.*, 2004; Hau and Gralnick, 2007). Haem profiles for FERM showed that the strain still expressed some membrane-associated cytochromes (Fig. S1). Two additional genes are present in ANA-3 that may encode for outer membrane multiheme *c*-type cytochromes (Shewana3_1409 and Shewana3_2787). However, we did not test if these genes were expressed under iron-reducing conditions. Background Fe(III) reduction could also be due to secretion of organic ligands and electron shuttling molecules such as flavins (Taillefert *et al.*, 2007; von Canstein *et al.*, 2008; Marsili *et al.*, 2008). Decreased Fe(III) reduction activity in FERM is consistent with nearly 50% diminished cytochrome content within membrane preparations of the FERM (and FARM) strain relative to the wild-type. Although the FERM has lower cytochrome content, the mutations of *mtr/omc* appeared to be specific to Fe(III) reduction because FERM can grow on other terminal electron acceptors similarly to the wild-type.

Expression analysis of each gene within the *mtr/omc* gene cluster showed that insoluble and soluble Fe(III) as well as arsenate and fumarate can cause increased expression of the *omcA mtrCAB*, but not *mtrDEF*. This result is consistent with a past study showing that an *mtrC* promoter fusion to *lacZ* exhibited similar LacZ activity for nitrate, fumarate and iron-citrate

grown cells of *Shewanella oneidensis* MR-1. Interestingly, a transcriptome study with *Shewanella oneidensis* MR-1 showed that *mtrCAB* mRNA was two- to eightfold lower in cells grown under metal-reducing conditions compared with fumarate-grown cells (Beliaev *et al.*, 2005). Although no comparisons to oxygen-grown cells were made, it is likely that the *mtrCAB* genes in MR-1 were induced under anaerobic conditions relative to aerobic conditions. The mechanism for this aerobic-anaerobic regulation likely involves cAMP-CRP. Previously, we showed that cAMP-CRP binds upstream (~75 bp) of the *arrA* gene and that several CRP binding motifs are present upstream of several *mtr/omc* genes (Murphy *et al.*, 2009). Moreover, a *crp* null mutant in ANA-3 cannot reduce Fe(III) or arsenate. Based on this observation it is highly likely that *omcA* and/or *mtrCAB* are part of the cAMP-CRP regulon. Further work will be needed to determine how *omcA* and *mtrCAB* genes are regulated.

It has been hypothesized that due to their high degree of sequence homology to *mtrCAB*, *mtrDEF* could be involved in Fe(III) reduction. Interestingly, we found no increase in the expression of the *mtrDEF* gene cluster in cells grown on Fe(III) and other electron acceptors. A previous study by Mclean and colleagues (2008), however, showed that the *mtrDEF* genes were expressed in MR-1 grown under highly aerobic chemostat conditions (50% oxygen saturation). Addition of Ca²⁺ to cells grown under this condition caused aggregation. The physiological relevance for this is not known. Further work is needed to determine the functional roles for *mtrDEF*.

We also addressed the temporal changes in *arrA* versus *mtrA* transcription from aerobic to anaerobic Fe(III)-citrate/arsenate conditions. It is possible that the *mtr/omc* and *arr* operons are differently expressed such that one is induced prior to the other. However, we found that both genes were induced within 2 h post-anaerobic shift into arsenate and Fe(III)-reducing conditions requiring nearly 8 h to reach fivefold induction compared with *arrA* and *mtrA* expression determined in aerobic grown cells (Fig. 4). It appeared that arsenate reduction preceded Fe(III) reduction. Compared with solid-phase arsenate-Fe(III) substrates, ANA-3 appeared to simultaneously reduce arsenate and Fe(III) (Campbell *et al.*, 2006). In light of these results, the timing of Fe(III) reduction (whether soluble or solid) might be constrained by the bioavailability of arsenate and Fe(III).

In summary, our findings indicate that deleting the *mtr/omc* genes (*mtrDEFomcAmtrCAB*) dramatically lowered the ability of ANA-3 to reduce insoluble and soluble Fe(III), but the strain is still capable of reducing arsenate and mobilizing arsenite from Fe(III) oxides. This observation demonstrates that arsenate respiration pathway alone is sufficient for reducing arsenate associated with ferrihydrite. However, deleting the arsenate reduction pathway lowers the amount of ferrihydrite ANA-3 can reduce in the presence of arsenate. Expression of the *mtr/omc* genes under arsenate respiring conditions is consistent with the ability of *Shewanella* sp. to adapt to anoxic metal-reducing conditions in redox stratified anaerobic sediments. The order in which the arsenate respiration and Fe(III) reducing genes are expressed, along with the relative order that arsenate and Fe(III) reduction occurs, may depend on the mineralogy, total surface area on the mineral and arsenic surface coverage of the Fe(III) oxide. Additional work is needed to determine if varying the Fe(III)-oxide and

arsenic conditions will lead to changes in the expression of Fe(III) and arsenate respiration genes.

Experimental procedures

Strains and plasmids

All *Escherichia coli* and *Shewanella* sp. ANA-3 strains used in this study are described in Table 1.

Mutagenesis

In-frame non-polar deletions of *mtrDEF omcA mtrCAB* (Shewana3_2672-Shewana3_2678; FERM; iron reduction mutant) and *arrA3, arsC1* (Shewana3_2341, Shewana3_2345; ARM1; arsenate reduction mutant) (Saltikov *et al.*, 2005) were generated using previously published methods (Saltikov and Newman, 2003). The primers used to generate the FERM mutant are listed in Table 1 as *mtrD-A*, *mtrD-B*, *mtrB-C* and *mtrB-D*. The *arrA3, arsC1* mutations were introduced into the FERM mutant by conjugation to generate the FARM mutant (iron and arsenate reducing mutant; Shewana3_2341, Shewana3_2345, Shewana3_2672-Shewana3_2678).

Growth conditions

Standard culturing of *E. coli* was done in Luria–Bertani (LB) medium. Culturing of ANA-3 was done in a basal salts medium [called ‘TME’ (Saltikov *et al.*, 2005)] with the following composition per litre: 1.5 g of NH₄Cl, 0.6 g of NaH₂PO₄, 0.1 g of KCl, 0.5 g of yeast extract, 10 mM Hepes, 10 ml each of trace elements and vitamin solution (Kostka and Nealson, 1998), the media was adjusted to pH 7. Lactate was included in the medium as the electron donor at 20 mM final concentration. For the batch experiments yeast extract was excluded from the medium and the phosphate decreased to 500 µM. ANA-3 strains were grown at 30°C in liquid cultures shaken at 250 r.p.m. Preparation of anaerobic media and anaerobic culturing was done as previously described (Saltikov *et al.*, 2005). The following electron acceptors were used at 10 mM: fumarate, nitrate, arsenate, TMAO, ferric citrate and ferrihydrite, unless otherwise indicated.

Growth experiments

Aerobic cultures were grown overnight in basal salts medium. The optical densities (OD) at 600 nm of each culture were adjusted to below 0.6 and standardized to each other by the addition of medium to ensure that inoculation levels for each strain were equal. Cells were inoculated at 1/100 dilution into anaerobic medium. Growth was monitored using a Spectronic 20+. Control cultures were also grown and monitored in anaerobic medium without added electron acceptor. For the analysis of aerobic conditions 20 ml cultures were grown in 250 ml Erlenmeyer flasks shaken at 250 r.p.m. All growth experiments were done in triplicates unless otherwise indicated.

Preparation of ferrihydrite

Ferrihydrite was prepared by rapid drop-wise addition of 1 N NaOH to 27 g of Fe(III) chloride ($\text{Cl}_3\text{Fe}_6\text{H}_2\text{O}$) in 500 ml of water and the pH brought up to 7.5. The precipitated iron was washed four times with sterile water and resuspended in 1.9 l of Hepes buffer pH 7. The ferrihydrite was sonicated to prevent aggregation of the ferrihydrite and 100 ml of a 1 M arsenate filter sterilized solution was added. An aliquot of the supernatant was taken for arsenic measurement and the ferrihydrite-arsenate slurry incubated overnight while stirring. Following overnight incubation, the ferrihydrite-arsenate slurry was washed with Nanopure water several times and aliquots taken of each wash. Aliquots before and after the incubation as well as the washes were analysed for total arsenic with inductively coupled plasma optical emission spectrometry (ICP-OES).

Arsenate surface coverage on the synthetic ferrihydrite was determined from the estimated surface area and surface site density as described by Dzombak and Morel (1990). Based on the concentration of arsenate adsorbed to Fe(III) at the start of the experiment (700 μM) the concentration of total iron (6 g l^{-1}), a surface area of 600 $\text{m}^2 \text{g}^{-1}$ and a surface site density of 2.2 sites nm^{-2} , we calculated 5.8% of the reactive Fe(III) sites were covered by arsenate at the start of the experiment. Using the same parameters and a phosphate concentration of 500 μM , we calculated 4.1% of reactive Fe(III) sites were covered by phosphate.

Incubations with ANA-3

Ten grams of ferrihydrite adsorbed arsenate was dissolved in 1.5 l of modified TME and distributed (75 ml each) into anaerobic serum bottles. ANA-3, FERM, ARM1 and FARM strains were inoculated into these anaerobic bottles. Lactate was included in all bottles as the electron donor. Bottles were inoculated to 10^6 cells ml^{-1} initially. Aliquots were taken at 30, 48, 72 and 144 h. All manipulations were carried out in a Coy anaerobic glovebox.

Iron and arsenic analyses

At each time point aliquots were filtered through a 0.2 μm filter and stored for arsenic analysis at -80°C . Aliquots of 100 μl were acidified in 900 μl 1 N Trace Metal HCl, filtered through a 0.2 μm filter and Fe(II) measured using the Ferrozine assay (Stookey, 1970). Aliquots of 100 μl were acidified in 900 μl 6 N Trace Metal HCl for total Fe measurements. For dissolved arsenic speciation, 1 ml of filtered sample was diluted in 4 ml of Nanopure water, and processed as previously described (Langner and Inskeep, 2000; Kocar and Inskeep, 2003). Arsenic samples were measured using ICP-OES and hydride generation. Prior to the ICP-OES analysis, 5 ml samples were acidified with 1.7 ml of 5% potassium iodide in 12 N trace metal-grade HCl and mixed with 0.6% NaBH_4 in 0.5% NaOH. Total dissolved arsenic was determined by adding 1 ml of filtered sample to 4 ml of 1 N trace metal-grade HCl, processed and measured as described above. Concentrations of dissolved arsenite were determined as the difference between total arsenic and dissolved arsenate. To determine arsenic species bound by the ferrihydrite solid phase, samples taken at the final time point (144 h) were prepared using previously published methods (Langner and Inskeep, 2000) and measured using ICP-OES as described above.

Semi-quantification of *arrA* and *mtrA* transcription

For the analysis of *arrA* and *mtrA* transcription, 20 ml duplicate cultures were grown aerobically in 500 ml Erlenmeyer flasks shaken at 250 r.p.m. to an OD₆₀₀ of 0.1 (exponential phase of growth). Cultures were then transferred to an anaerobic chamber and amended with 10 mM arsenate and ferric citrate. Samples (1.5 ml) were removed for RNA extraction (described below) at several time points. Arsenic analysis was done on culture filtrates and total Fe(II) monitored using ferrozine assay as described above.

RNA was extracted from the cell pellets using the QIAGEN RNeasy miniprep kit according to the manufacturer's instructions, with a final elution volume of 30 µl. DNA contamination was removed by DNase (Promega RQ1 DNase) digestion. cDNA was synthesized as previously described (Saltikov *et al.*, 2005). RT-PCR and agarose gel electrophoresis on a 2.5% gel were used to monitor *arrA*, *mtrA* and 16S rRNA gene expression at time points 0, 2, 4 and 8 h post shift to anaerobic conditions. The efficiency of DNase treatment of RNA extracts was assessed from control cDNA reaction lacking reverse transcriptase enzyme; no RT-PCR products were detected in the control cDNA samples (data not shown). The intensities of the RT-PCR DNA bands were quantified with the ImageJ software (<http://rsb.info.nih.gov/ij/>). For semi-quantitative analysis, the background intensity of the negative control lane from of the *arrA*, *mtrA* and 16S rRNA gels was subtracted from the DNA band intensities for *arrA*, *mtrA* and 16S rRNA. The resulting pixel intensities for *arrA* and *mtrA* were normalized to the pixel intensities for the corresponding 16S rRNA gene.

Quantitative reverse transcriptase PCR analysis (qRT-PCR)

RNA from arsenate-grown cells was prepared as previously described (Saltikov *et al.*, 2005). For cells grown with iron oxide, 5 ml aerobically grown ANA-3 cultures (OD₆₀₀ ~0.6) were transferred to 50 ml serum bottles containing anaerobic TME with 10 mM ferrihydrite or 10 mM ferrihydrite presorbed with arsenate as the electron acceptors. Cultures were incubated at 30°C in the anaerobic chamber. Upon detection of increased Fe(II), cells were harvested from 3 ml of culture under anaerobic conditions. The cell pellets were placed on ice; resuspended in 1 ml of 0.3 M oxalic acid pH 3; and centrifuged again at 4°C to collect the cells. RNA extraction and cDNA synthesis were carried out as described above.

The real-time PCRs (30 µl) consisted of 15 µl 2× Syber-Green *Taq Mix* (Applied Biosystems), 300 nM of primers listed in Table 1 and 4 µl of RNA sample diluted 1/4 in nuclease-free water. Each sample was run in duplicate using the MJ Research Opticon2. The thermocycle profile consisted of 95°C for 10 min, 40 cycles of 95°C for 30 s and 60°C for 1 min, and a final denaturing cycle to examine the DNA melting curves of PCR products. Quantification of expression and normalization to DNA gyrase gene *gyrB* have been described elsewhere (Saltikov *et al.*, 2005; Murphy and Saltikov, 2007; Murphy *et al.*, 2009).

Cell fractionation and membrane preparation

ANA-3 cells were grown to an O.D. of ~0.16 for haem quantification or overnight for SDS-PAGE analysis on 10 mM fumarate, arsenate, TMAO, nitrate or aerobically on TME. Cells

were harvested and centrifuged at 6000 r.p.m. for 30 min, the supernatant discarded and the pellet resuspended in 30 ml of 0.25 g l⁻¹ sodium bicarbonate buffer pH 7.5. Cell pellets were lysed by sonicating cells at 30 s cycles at 70% amplitude with 1–2 min breaks in an ice bath at 4°C. To pellet the cell debris, the cell lysate solution was centrifuged at 10 000 r.p.m. for 30 min. To obtain the total membrane fraction, the cell lysate solution was centrifuged at 45 000 r.p.m. for 1 h in a Sorvall Ultraspeed Centrifuge with rotor T-865 at 4°C. The cellular fraction was separated from the pellet containing the total membrane fraction. This pellet was resuspended in 400 µl of 0.1% Triton X-100, 0.25 g l⁻¹ sodium bicarbonate buffer solution pH 7.5.

Protein quantification

The Bradford Assay was used to determine total protein concentrations (Sambrook *et al.*, 1989; Ausubel, 1999).

Haem quantification

Reduced minus oxidized spectra of total membrane preparations of ANA-3 were obtained on a Beckman DU650 spectrophotometer. Samples were analysed for haem content by a pyridine haemochrome assay (Berry and Trumpower, 1987). Briefly, 200 µl of total membrane sample was combined with 800 µl of 40% pyridine made in 200 mM NaOH in a 1 ml quartz cuvette. Total membrane samples were oxidized by adding 3 µl of potassium ferricyanide [K₃Fe(CN)₆] (Sigma). Samples were mixed and scanned within 1 min of adding the potassium ferricyanide, between 400 and 600 nm and the oxidized absorbance recorded. Samples were transferred to a Coy anaerobic chamber and reduced with 10 µl of a 500 mM stock of sodium dithionite in 50 mM Tris pH 10, mixed and the reduced absorbance measured within 1 min. Haem content was determined using the extinction coefficient of 21.84 mM⁻¹ cm⁻¹ at 550 nm for the pyridine reduced minus oxidized haemochrome spectra using the Beer–Lambert law equation. The haem concentration was normalized to the total protein content of the sample.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Measured concentrations of arsenate and arsenite in incubations with wild-type FERM, FARM and ARM1 null mutants incubated with arsenate-equilibrated ferrihydrite

A. Dissolved concentrations of arsenate.

B. Dissolved concentrations of arsenite.

C. Concentrations of solid-phase arsenate and arsenite determined at 144 h. Symbols correspond to: $\circ$, No cell; $\times$, FARM; $\blacktriangle$, ARM1; $\bullet$, ANA-3; $\square$, FERM. The data points and error bars represent the means and standard deviations of triplicate samples respectively.

Fig. 2.

Measured total concentration of Fe(II) in incubations with wild-type, FERM, FARM and ARM1 null mutants in batch cultures containing: (A) ferrihydrite, (B) arsenate presorbed onto ferrihydrite and (C) Fe(III) citrate. Symbols correspond to: $\square$, No cell; $\blacklozenge$, FARM; $\circ$, ARM1; $\bullet$, ANA-3; $\blacktriangle$, FERM. The data points and error bars represent the means and standard deviations of triplicate samples respectively.

Fig. 3.

Expression of *mtrDEFomcAmtrCAB* and *arrA* genes relative to that of housekeeping gene *gyrB* in wild-type *Shewanella* sp. strain ANA-3 as determined by quantitative RT-PCR. Transcriptional patterns of *mtrDEFomcAmtrCAB* and *arrA* were done for ANA-3 grown aerobically and anaerobically with different terminal electron acceptors. Relative expression refers to the ng genomic equivalent of mRNA for each gene normalized to the ng equivalent of mRNA for the DNA gyrase gene, *gyrB*. Data points represent the averages and standard deviations of mRNA extracts from triplicate cultures. Expression of *arrA* was not measured under oxygen or Fe(III) citrate conditions.

Fig. 4.

Reverse transcription PCR (RT-PCR) analysis (A) of *arrA* (top), *mtrA* (middle) and 16S rRNA genes (bottom) in *Shewanella* sp. ANA-3 after shift from aerobic to anaerobic conditions. Duplicate cultures of ANA-3 were grown aerobically with vigorous shaking until OD₆₀₀ of 0.1. The cultures were placed in an anaerobic chamber and supplemented with arsenate (10 mM) and Fe(III)-citrate (10 mM). RT-PCR for 16S rRNA gene, *arrA* and *mtrA* were carried out with RNA extracts from duplicate cultures sampled at the initial anaerobic shift (lanes 1 and 2); 2 h post shift (lanes 3 and 4); 4 h post shift (lanes 5 and 6); and 8 h post shift (lanes 7 and 8). ANA-3 genomic DNA and no DNA controls are shown in lanes 9 and 11 respectively. 'L' indicates 100 bp region of the DNA ladder.

B. Semi-quantitative analysis of *arrA* (black bars) and *mtrA* (white bars) gene expression was determined by subtracting the gel background from the band intensities and normalizing the difference in intensity of the *arrA* [RT-PCR product (top) and *mtrA* RT-PCR product (middle)] to the corresponding 16S rRNA gene RT-PCR product (bottom).

C and D. (C) Arsenate (white bars) and arsenite (black bars) and (D) Fe(II) concentrations determined for each time point. Data points and error bars for (C) and (D) represent the average and range for the concentrations of the duplicate cultures respectively.

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Table 1

Bacterial strains, plasmids and primers used in this study.

Strain or plasmid	Genotype or markers; characteristics and uses	Source or reference
<i>E. coli</i> strains		
UQ950	<i>E. coli</i> DH5 α λ (pir) host for cloning; F- (<i>argF-lac</i>)169 ϕ 80d <i>lacZ</i> 58(- M15) <i>glnV44</i> (AS) <i>rfbD1</i> <i>gyrA96</i> (NalR) <i>recA1 endA1 spoT1 thi-1 hsdR17 deoR</i> λ pir+	Saltikov and Newman (2003)
WM3064	Donor strain for conjugation: <i>thrB1004 pro thi rpsL hsdS lacZ</i> M15 RP4-1360 (<i>araBAD</i>)567 <i>dapA1341::[erm pir(wt)]</i>	Saltikov and Newman (2003)
<i>Shewanella</i> strains		
<i>Shewanella</i> sp. ANA-3	Isolated from an As-treated wooden pier pilling in a brackish estuary (Eel Pond, Woods, Hole, MA)	Saltikov <i>et al.</i> (2003)
ARMI	<i>Shewanella</i> sp. ANA-3; <i>arrA</i> (Shewana3_2341), <i>arsC</i> (Shewana3_2345)	Saltikov <i>et al.</i> (2005)
FERM	<i>Shewanella</i> sp. ANA-3; <i>mtrD</i> (Shewana3_2672), <i>mtrE</i> (Shewana3_2673), <i>mtrF</i> (Shewana3_2674), <i>omcA</i> (Shewana3_2674), <i>mtrC</i> (Shewana3_2676), <i>mtrA</i> (Shewana3_2677), <i>mtrB</i> (Shewana3_2678)	This study
FARM	<i>Shewanella</i> sp. ANA-3; <i>arrA</i> , <i>arsC</i> , <i>mtrD</i> , <i>mtrE</i> , <i>mtrF</i> , <i>omcA</i> , <i>mtrC</i> , <i>mtrA</i> , <i>mtrB</i>	This study
Plasmid		
pSMV10	9.1 kb mobilizable suicide vector; <i>oriR6K</i> , <i>mobR4</i> , <i>sacB</i> , Km ^r , Gm ^r	Saltikov and Newman (2003)
Deletion primers		
X- <i>mtrD</i> -A	GG <u>ACTAGT</u> GCCCGTCAAATAGAGGT	
X- <i>mtrD</i> -B	[CCCATCCAGCATGCTTAAACA]JTCCCGAGTGCGTTTTAT	
X- <i>mtrB</i> -C	[TGTTTAAGCATGCTGGATGGG]GATAGTCAAAAGCCCGCACTG	
X- <i>mtrB</i> -D	GG <u>ACTAGT</u> GCAATTCGGCCTGACACAGTT	
qPCR Primers		
q <i>mtrD</i> -F2	AACGCTCATCCCATCCATT	
q <i>mtrD</i> -R1	CGTCATTTGCTTCAGACT	
q <i>mtrE</i> -F1	CCGTTATTCACCTGCTTGCT	
q <i>mtrE</i> -R1	AAGGGTTGCTCGGACTTTG	
q <i>mtrF</i> -F1	TCTATGCTAACTGGGGAACC	
q <i>mtrF</i> -R1	AGTGTAAGTGCCGTTGCTG	
q <i>omcA</i> -F1	ATCCACGCTATCCACAAAGG	
q <i>omcA</i> -R2	TGTAATGAACCACCGTAACCAA	
q <i>omcB</i> -F1	TCACCAACGAAGACGATTTG	
q <i>omB</i> -R2	GAGGCATTACCAGCACCTGT	
q <i>mtrA</i> -F1	CTTGCCACGGACCTATGG	
q <i>mtrA</i> -R1	ACGCTGTTTTGCTTGTCG	
q <i>mtrB</i> -F2	GCAACAGCCTTCTACAACCA	
q <i>mtrB</i> -R2	CCAACCACATCCACTTTGTC	

SpeI sites are underlined; the bracketed areas are the 21 bp PCR linkers with SphI sites underlined.

Table 2
Growth rates of ANA-3 wild-type and mutants for different terminal electron acceptors.

Strain	TMAO (h ⁻¹)	Fumarate (h ⁻¹)	Nitrate (h ⁻¹)	Arsenate (h ⁻¹)	Oxygen (h ⁻¹)
ANA-3	0.7 ± 0.05	0.7 ± 0.03	0.3 ± 0.07	0.8 ± 0.06	0.4 ± 0.04
FERM	0.8 ± 0.02	0.7 ± 0.04	0.3 ± 0.03	0.9 ± 0.07	0.4 ± 0.03
ARM1	0.7 ± 0.03	0.6 ± 0.11	0.2 ± 0.03	–	0.4 ± 0.00
FARM	0.7 ± 0.02	0.7 ± 0.06	0.3 ± 0.07	–	0.4 ± 0.01

Data represent averages and standard deviations of quadruplicate cultures.

Table 3

Haem content of wild-type and FERM strains of ANA-3 grown on different terminal electron acceptors.

Terminal electron acceptor	Wild-type ($\mu\text{mol haem per g protein}$)	FERM ($\mu\text{mol haem per g protein}$)
Fumarate	3.1 ± 0.27	1.7 ± 0.46
Arsenate	2.5 ± 0.11	1.3 ± 0.18
TMAO	1.4 ± 0.09	1.0 ± 0.04
Nitrate	2.0 ± 0.65	1.4 ± 0.02
Oxygen	1.7 ± 0.46	0.9 ± 0.17

Data represent averages and standard deviations of triplicate samples.