Characterization of freshwater and seawater Fe(III)-reducers from Salt Pond

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Salt Pond is a seasonally stratified coastal marine basin. Anaerobic sediments from the shallow edge of this pond were used as the inoculum for enrichments of anaerobic Fe(III)-reducing bacteria. Enrichments were performed in both freshwater and seawater media containing acetate and $Fe(OH)_3$. After three successive liquid enrichments, the enrichment cultures were transferred to agar shakes containing Fe(III)-NTA with acetate. Total DNA was extracted from liquid enrichments as well as from select individual colonies in agar shakes. Polymerase chain reaction was used to amplify the 16S small subunit rRNA genes and these products were analyzed by RFLP and sequencing. One of the Fe(III)-reducing isolates is most closely related to organisms in the genus *Cytophaga*, which is the first report of metal reduction in this group.

The biogeochemical cycling of iron is an important processes in the environment. Under aerobic conditions, above pH 6, the spontaneous abiotic oxidation of soluble Fe(II) to insoluble Fe(III) oxides and oxyhydroxides occurs quite rapidly. The highly charged surfaces of these precipitates are capable of adsorbing many metals and organics. In recent years, much attention has been focused on organisms capable of reducing the insoluble oxidized forms of iron to soluble forms. In particular, dissimilatory metal-reducers, coupling anaerobic carbon oxidation to metal reduction, have been isolated from many environments (Lovley, 1991). Although the reductive dissolution of iron minerals often results in the concomitant release of phosphate and trace metals into the environment, Fe(III)-reducers are also capable of mineralizing organic pollutants, making them potentially useful for bioremediation applications.

The two groups of dissimilatory metal-reducing organisms that have been studied most extensively are as follows: (1) facultative anaerobes in the genus *Shewanella* and (2) strict anaerobes in the group *Desulfuromonas acetoxidans* and the closely related *Geobacter metallireducens* (Kostka & Nealson, 1998). Most likely, only a small proportion of the bacteria capable of metal reduction have been cultured or characterized. The goal of this study was to enrich for and isolate Fe(III)-reducing organisms from sediments of the Salt Pond, a shallow seasonally-stratified marine

basin, in both seawater and freshwater media, as well as to use molecular techniques to compare the organisms present in these respective enrichment cultures.

Materials and Methods

Sample collection and enrichments

Sediments were collected from the eastern shore of the Salt Pond, a shallow marine basin just north of Woods Hole, Massachusetts. A 500 ml screwcap bottle was used to collect the top 10 cm of nearshore sediment at a depth of approximately 0.5 m. A gram (wet wt) of homogenized sediment was used to inoculate standard anaerobic freshwater or saltwater medium (Widdel and Pfennig) containing 5 mM sodium acetate and 40 mM $Fe(OH)_3$. Reduction of Fe(III) was monitored visually by observing the change in color of the iron from orange to dark brown/black, or by using the ferrozine assay for measuring Fe(II) production. Liquid cultures demonstrating Fe(III)-reduction were shaken and then used to inoculate subsequent liquid enrichments at 1/20 to 1/50.

Agar shakes for obtaining individual colonies

In order to obtain isolated colonies for further purification and molecular analysis, dilution series of FW and SW 3° enrichments were made in agar shakes. Dilutions were made ranging from 10^{-1} to 10^{-6} in freshwater and seawater media containing a final concentration of 10 mM acetate, 10 mM Fe(III)-NTA, and 1% agar. The agar (Difco Bacto-Agar) was washed extensively with dH2O, prior to making shakes, in order to remove contaminants. Agar shakes were incubated at 30°C and were monitored daily for the presence of colonies and reduction of the orange Fe(III)-NTA. Colonies were removed from agar shakes, using a Pasteur pipette, and transferred to fresh acetate/$Fe(OH)_3$ liquid media or to Eppendorf tubes for DNA extraction.

Molecular analysis

DNA was extracted from single colonies and liquid enrichments using a bead beater protocol. Bacterial pellets were resuspended in 10 mM Tris buffer, and an equal volume of phenol/chloroform/isoamyl alcohol was added; after adding a microspatula of glass beads, the sample was placed in a beadbeater for 1 min; samples were centrifuged at 13,000 x g for 5 min, and the aqueous phase was removed; this phase was extracted again with phenol/chloroform/isoamylalcohol followed by a chloroform extraction; DNA was precipitated by the addition of 0.1 vol 3M Na acetate and 1

volume of 2-propanol, for 10 min on ice; DNA was pelleted, washed w/ 70% ethanol, repelleted, and allowed to air-dry; the DNA was dissolved in a small volume (20 µl) of sterile dH20. Dilutions were made and used directly in PCR.

PCR was carried out using universal primers (8f and 1492r) with TaqGold (Perkin Elmer) and a standard program with a 55°C annealing temperature. In addition, a 12 minute "hot" start at 94°C was added for the TaqGold enzyme, as well as a 10 minute extension at 72°C at the end of the cycle for subsequent TOPO-TA (Invitrogen) cloning. PCR products (2 µl) were cloned directly into the pCR2.1/topoisomerase (Invitrogen) vector according to the manufacturer's instructions, without further purification of PCR products. White colonies, putatively containing inserts, were picked from LB(ampicillin)/X-gal plates and used directly in PCR reactions with primers specific for the flanking regions of the insert in pCR2.1. PCR products were run in 1% agarose gels containing the nucleic acid stain GelStar (FMC BioProducts) and were visualized on the UV transilluminator. Restriction Fragment Length Polymorphism (RFLP) analysis, using the enzymes *Msp*1 and *Hin*P1, was used to screen the TOPO-PCR products for unique sequences. After 3 hr, digests were run in 1.5% MetaPhor agarose gels. Unique 16S PCR products were sent to the Forsythe Dental Institute (Boston, MA) for sequencing. Sequences were analyzed by BLAST and by using various programs for generating phylogenetic trees.

Results and Discussion

Fe(III)-reducing enrichments

Anaerobic Fe(III)-reducers were successfully enriched from the Salt Pond sediments in both freshwater (FW) and seawater (SW) anaerobic media containing 5 mM acetate/40 mM $Fe(OH)_3$. The reduction of Fe(III) was clearly evident as a zone of dark brown/black which moved down through the solid orange $Fe(OH)_3$ precipitate over time. For some reason, iron reduction always occurred much more rapidly in the freshwater enrichments. It generally took 2 to 4 days for the Fe(III) to become completely brown/black in FW enrichments, whereas it took at least twice as long for SW enrichments to become brown.

Magnetite (Fe_3O_4) could be detected in freshwater enrichments, at least 2 weeks after inoculations, by placing a magnetic stirbar next to the bottom of the Pfennig bottle and dragging the iron up the side. The formation of magnetite was only observed in FW enrichments, suggesting that some component of the artificial seawater may have prevented its formation. Alternatively, it is possible that the marine bacteria themselves

were unable to produce this form of iron. Microscopic examination of the enrichments revealed a wide variety of both motile and non-motile rods of different sizes.

Molecular analysis of FW and SW enrichments

In an effort to assess the diversity of organisms within the FW and SW enrichments, a molecular approach was employed. Total DNA was extracted from 10 ml pellets of FW and SW 3° enrichment cultures. After bead-beating, the aqueous phase appeared pinkish/red, possibly indicating the release of cytochromes, heme, etc. from the cells. This might be a useful way of monitoring of monitoring cell lysis. Dilutions of purified DNA were used directly in PCR and these 16S amplicons were cloned into pCR2.1 using the TOPO-TA (Invitrogen) cloning kit. After clones with 16S inserts were identified using PCR with TOPO primers, these PCR products were subjected to RFLP analysis. This allowed the identification of unique sequences among the clones. In the FW clones (Fig 1a), there were 4 different banding patterns, but only one dominant pattern (11/15). Alternatively, there were 10 different banding patterns in the SW clones (Fig. 1b), one of which was present 3 times. Thus, the SW enrichment appeared to contain a greater diversity of organisms than the FW enrichment.

PCR products from all 14 unique clones (4 FW, 10 SW) were sent off for sequencing and only one of the sequencing reactions (a FW clone) did not work. The results of the BLAST analysis of these sequences are listed in TABLE 1. As expected, the closest known relatives of all of the sequences were either strict or facultative anaerobes. In addition, most of the sequences were significantly different from the closest sequence in the database, indicating that they are probably novel organisms. Several of the sequences were closest to well known Fe(III)-reducers, such as *Desulfuromonas* and *Shewanella* species. Many of the other sequences were most closely related to other sulfate- and sulfur-reducing organisms, which often are capable of Fe(III)-reduction as well. Sequences related to *Desulfuromonas* (Fe-reducers) and *Azoarcus* (nitrate- and chlorate-reducing organisms) were found in both the FW and SW enrichments, but the FW and SW sequences, themselves, were not identical.

The 16S sequence corresponding to the most common RFLP pattern of the FW clones (11/15) was the *Desulfuromonas*-related organism, suggesting that it may be the most numerically abundant Fe(III)-reducer in the FW enrichment. The strongest match of the three FW sequences was a 100% match with *Pseudomonas stutzeri*, a common denitrifying organism. Several of the SW clones had strong matches (97% and 99% identity) to *Marinobacterium georgiensii*, an organism isolated from a lignin enrichment. This is interesting in light of the fact that the surface of these Salt Pond sediments was

composed mainly of decaying greenish/brown plant-like organic material. Further screening of clones from both the FW and, especially, the SW enrichment would most likely reveal even greater diversity.

Isolation of Fe(III)-reducing isolates

In order to obtain individual Fe(III)-reducing isolates for further analysis, 3° enrichments were transferred (1/10) to agar shakes containing soluble Fe(III)-NTA and acetate. Distinct individual colonies could be clearly seen in the dilution series of the FW enrichment. In addition to the numerically-abundant, small, white, colonies found even in the highest dilution (10^{-6}), two larger, faster-growing, colony types were found in dilutions up to 10^{-5}: orange, lens-shaped colonies and yellow, spherical colonies. These colonies could be easily removed from agar shakes with a Pasteur pipette and were placed into 50 ml of liquid media, containing 5 mM acetate/40 mM $Fe(OH)_3$, and into Eppendorf tubes for DNA extraction. These liquid enrichments were monitored to confirm whether these colonies were actually Fe(III)-reducers or simply contaminating anaerobic bacteria. After 4 days, the orange colony was visibly reducing the $Fe(OH)_3$ to a dark brown color, while the yellow colony showed no signs of Fe(III)-reduction. The yellow colony was either killed/stressed during the transfer from agar to liquid, or it is not actually an Fe(III)-reducer and somehow lives anaerobically within these enrichments.

Molecular analysis of isolates

DNA was extracted from these 2 colony types and the 16S rRNA genes were amplified. Sequence analysis of approximately 500 bp of these 16S rRNA genes revealed that both organisms are most closely related to bacteria in the genus *Cytophaga*. Neither of these sequences were obtained from the molecular analysis of the total FW enrichment described above, although only four 16S PCR products were sequenced, and one of them did not work. It is also quite possible that these Cytophaga-like organisms thrive in the agar shakes, but are not dominant members of the liquid FW enrichments. This is consistent with the fact that several other people in our class also isolated Cytophaga-like organisms from the Salt Pond in agar shakes.

A phylogentic tree was constructed (by Scott Dawson) containing these 2 organisms organisms as well as many other members of the Cytophaga-Flexibacter-Bacteroides (CFB) group of bacteria (Fig. 2). The other new Cytophaga-like organisms from the Salt Pond sediments were also included in this tree, which revealed that there appears to be 2 distinct clusters of Salt Pond Cytophaga-like sequences. The 16S

sequence of the orange colony, designated "chrisWH94", is identical to the Fe(III)-reducing organism independently isolated by Andreas Kappler, "andreWH68". The sequence of the yellow colony, designated "chrisWH96", is found in the second cluster of Salt Pond isolates and is most similar to "milvaWH99", which was isolated from a H2/CO2 enrichment.

The phylogenetic clustering of these anaerobic Salt Pond isolates is quite striking, particularly considering the range of salinities (FW vs. SW media) and growth conditions (e.g., TMA w/ BES, H2/CO2, H2/CO2 w/ sulfate, acetate/AQDS, acetate/Fe(OH)3, etc.) employed in the different enrichments. In terms of Fe(III)-reducing organisms, the isolation of a Cytophaga-like organism is quite intriguing. To my knowledge, this is the first report of a Cytophaga-like organism capable of metal reduction. It is also possible that this metal-reducing capacity is shared by other *Cytophaga* and simply has not been explored in these organisms. Further molecular and physiological characterization of these Cytophaga-like organisms from the Salt Pond is underway.

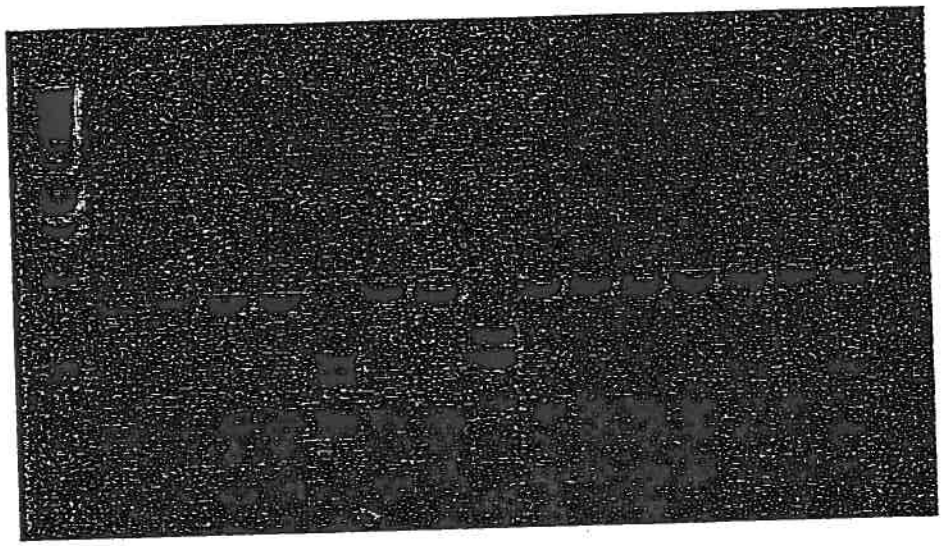
References

Kostka and Nealson (1998) "Isolation, cultivation, and characterization of iron and manganese-reducing bacteria" in Techniques in Microbial Ecology (ed. Burlage et al.) New York: Oxford University Press, pp. 58-78.

Lovley (1991) Dissimilatory Fe(III) and Mn(IV) reduction. Microbiol. Rev. 55: 259-287.

Figure 1

a.



RFLP Patterns

b.

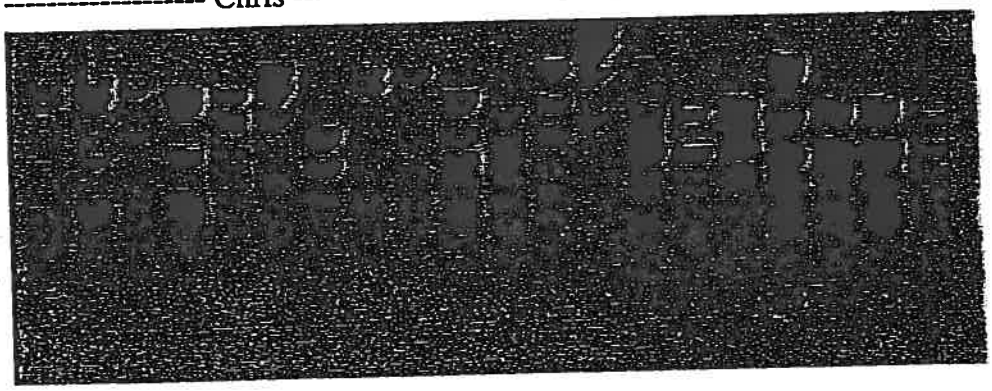


TABLE 1. Comparison of 16S rRNA sequences from FW and SW Fe(III)-reducing enrichments. FW = WH126-128; SW = WH130-139

Clone	Closest Relative	% Identity	Accession #
WH126	Desulfuromonas sp.	91%	U28172
WH127	Azoarcus sp. BS5-8	91%	AF011350
WH128	Pseudomonas stutzeri	100%	U64001
WH130	Marinobacterium georgiensii	97%	U58339
WH131	Azoarcus sp. BS5-8	95%	AF011350
WH132	Marinobacterium georgiensii	99%	U58339
WH133	Acidaminobacter hydrogenoformans	89%	AF016691
WH134	Shewanella alga (FeRed)	98%	X81622
WH135	Desulfovibrio sapovorans	92%	M34402
WH136	Desulfuromusa bakii	93%	X79412
WH137	Desulfuromonas palmitatis	88%	U28172
WH138	Unidentified δ-proteo	93%	AB015242
WH139	Unidentified bacterium	93%	Z73447

Figure 2

Cytophaga Flexibacter Bacteroides (CFB) Division

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