Vol. 38: 81–91, 2005 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published January 21

Metal reduction at cold temperatures by *Shewanella* isolates from various marine environments

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ABSTRACT: Members of the genus *Shewanella* capable of reducing metals and forming minerals under cold-temperature conditions were isolated from 3 distinct marine habitats (the coast of Washington State, the Puget Sound, and an iron-rich microbial mat off Hawaii). Cultures of microorganisms were isolated at 8°C on nutrient agar medium prepared in artificial seawater. Isolates in this study could use a wide variety of electron acceptors such as oxygen, nitrate, and metals, and reduce various metals coupled to the oxidation of several organic acids, glucose or hydrogen at temperatures down to 0°C. Akaganeite was reduced to either magnetite or siderite, depending on the test conditions. The geochemical profiles at the sample sites from which these strains were isolated spanned a temperature range of 1.8 to 11°C, and all showed active oxygen and nitrate reduction as well as metal reduction. This confirms previous reports that sediment microorganisms participating in biogeochemical cycles remain active at low temperatures.

KEY WORDS: Biogeochemistry · Geomicrobiology · Iron reduction · Cobalt reduction · Magneite

INTRODUCTION

Marine sediments have long been known as sites of active biogeochemical processes, including nitrate reduction, manganese/iron reduction, sulfate reduction and methanogenesis. Sedimentary biogeochemical activities have been documented to continue at temperatures around 0°C (Kostka et al. 1999). Organic matter mineralization by dissimilatory iron-reducing bacteria (DIRB) has been shown to be important in marine environments (Lovley & Phillips 1988). Fe- and Mn-oxides may often represent the electron acceptors available at the highest concentrations in marine sediments (Canfield et al. 1993, Aller et al. 1998, Thamdrup et al. 2000). Early reports demonstrated that the oxidation of organic material could be coupled with the reduction of Fe(III) (Sørenson 1982, Lovley & Phillips 1986). Sediment magnetism has also been linked to iron cycling in marine environments (Kostka & Nealson 1995).

Sedimentary microbial communities are complex (Bowman et al. 2000). The recovery of pure cultures for laboratory experimentation is a tedious process, often fraught with biases. However, pure cultures of microorganisms isolated from specific environments provide laboratory models for investigators to examine. Since most of the global marine environment exists at low temperatures, recovery of microbes capable of reducing metals, such as iron and cobalt, at low temperatures would provide useful models to further the understanding of sedimentary biogeochemistry.

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While their quantitative importance in marine sediments and water columns is not well understood, members of the genus *Shewanella* are commonly encountered in marine environments (Perry et al. 1993, Thamdrup et al. 2000, Brettar et al. 2002). These isolates have provided useful models for laboratory investigations on metal reduction (Nealson & Saffarini 1994, Kostka & Nealson 1995), food spoilage, barophilic growth (Kato & Nogi 2001) and, more recently, psychrophilic enzymes (Irwin et al. 2001a,b). The present study describes the isolation of members of the genus *Shewanella* from 3 marine environments. Specifically, strains W3-6-1 and PS-7 were used as models to investigate microbial metal reduction and mineral formation at low temperatures.

MATERIALS AND METHODS

Site description and sampling. Marine sediment samples were collected from a transect off the coast of Washington State (USA), at 2 locations in Puget Sound (USA) and from an iron-rich microbial mat associated with the Naha Vents on the South Rift of Loihi Seamount near Hawaii. With the exception of the vent sites, sediments were collected by box core sampling. The microbial mat sample was collected during DSRV Pisces V dive number 342 (Emerson & Moyer 2002). Each box core was sub-cored for sectioning using 7.5 and 10 cm cast-acrylic core tubes. Core sectioning took place within approximately 1 h of coring in a glove-box at ~5°C in a N_2 atmosphere. Cores were stored at ~5°C to minimize effects due to warming. Details of sample location, including geographic coordinates, are listed in Table 1.

The geochemistry of the sediments was inferred from sediment pore-water distributions. Pore-water was separated from the sediments by centrifugation at ~7000 rpm (>10 000 × *g*) for 20 min; supernatants were filtered through pre-combusted GF-F filters before sub-sampling for various analyses. Whole-core squeezing at ~5°C was used to obtain high-resolution pore-water profiles of O_2 and NO_3^- (Bender et al. 1987). Pore-water samples were expressed through an in-line, radiometer-style oxygen electrode before collection for nutrient analysis (Brandes & Devol 1995). The oxygen electrode was standardized against air-saturated seawater and N_2-purged seawater.

For the Naha Vents sample, temperature was measured using a thermistor probe on the submersible, while for the Puget Sound and coast of Washington State sites the bottom water temperature was determined with a SeaBird CTD.

Bacterial isolation. Anaerobic culturing techniques were used throughout the study following standard protocols (Hungate 1969, Miller & Wolin 1974). The culture medium (g l^{-1} dH_2O: sodium chloride, 30.0; sodium bicarbonate, 2.5; MOPS (3-{N-morpholino} propanesulfonic acid), 2.0; ammonium chloride, 1.0; yeast extract, 0.5; magnesium chloride heptahydrate, 0.2; calcium chloride dihydrate, 0.1; trace vitamins and minerals; Phelps et al. 1989) was boiled and prepared under an anaerobic mixed gas atmosphere of N_2/CO_2 (80/20 %) or H_2/CO_2 (80/20 %). The medium was cooled under the mixed gas atmosphere, dispensed into anaerobic pressure tubes, capped with butyl rubber stoppers, and autoclaved. Stocks of electron donors, electron acceptors, and pH adjustment reagents were sterilized individually, and then added to the autoclaved basal medium as required.

Table 1. Sources and growth characteristics at different temperatures of bacterial isolates described in this study. Sample depth: depth (m) of water column over sediment location where core was recovered; Core depth: depth (cm) within a sample core from which the sediment sample was recovered. PMS: Pacific marine sediment; PS: Puget Sound; nd: not determined or not applicable

Isolate	Origin	Location	*In situ* temp. (°C)	Sample depth	Core depth	Growth at 0°C	Growth at 37°C	Optimal growth range (°C)
W3-4-1	PMS Stn 307	Lat 46°26′61″, Long 124°47′22″	3.4	997	5–6	Yes	No	15–18
W3-6-1	PMS Stn 307	Lat 46.26.61 Long 124.47.22	3.4	997	9–10	Yes	No	15–18
W3-7-1	PMS Stn 304	Lat 46.45.05 Long 126.00.58	1.8	2530	0.5–1	Yes	No	15–18
W3-7-2	PMS Stn 304	Lat 46.45.05 Long 126.00.58	1.8	2530	0.5–1	Yes	No	14–17
W3-11-1	PMS Stn 301	Lat 46.48.60 Long 124.37.20	8.1	119	3–4	Yes	No	15–18
W3-11-2	PMS Stn 301	Lat 46.48.60 Long 124.37.20	8.1	119	3–4	Yes	No	14–17
W3-14-2	PMS Stn 301	Lat 46.48.63 Long 124.37.23	8.1	119	7–8	Yes	No	15–18
PV-4	Naha Vent mat		11.0	nd	nd	Yes	Yes	≥37[a]
PS-3	PS sediments	Lat 47.43.5 Long 122.23.9	8.1	220	nd	Yes	No	17–20
PS-5	PS sediments	Lat 47.43.5 Long 122.23.9	8.1	220	nd	Yes	No	14–17
PS-7	PS sediments	Lat 47.43.5 Long 122.23.9	8.1	220	nd	Yes	No	17–20

[a] Highest growth for PV-4 occurred at the 37°C, the highest temperature that was checked for this organism

Enrichment cultures were initiated using Fe(III)-citrate (20 mM) and either acetate (10 mM), formate (10 mM), pyruvate (10 mM), or hydrogen (80/20% hydrogen/carbon dioxide mix), and incubated at 8°C in the dark (Zhang et al. 1999). Pure cultures of microorganisms were obtained by spread-plating the enrichment cultures on nutrient agar medium prepared in artificial seawater (g l^{-1} dH_2O: sodium chloride, 30.0; magnesium sulfate heptahydrate, 6.0; calcium chloride dihydrate, 1.0; potassium chloride, 1.0; potassium nitrate, 0.5; nutrient agar, 23.0; pH 7.2). The plates were incubated aerobically at 8°C. Individual colonies were transferred at least 4 times prior to assessing the culture purity by either Enterobacterial Repetitive Intergenic Consensus (ERIC)-PCR or Repetitive Extragenic Palindromic (REP)-PCR (de Bruijn 1992). Once a culture was determined to be pure, it was evaluated for its ability to reduce ferric iron. Cultures of freshly isolated bacteria were maintained in the dark at 8°C in 9.0 ml medium with ferric citrate (20 mM) as the electron acceptor and either lactate (10 mM) or hydrogen as the electron donor.

Isolates recovered from Pacific Ocean sediments were given the prefix W3, followed by an enrichment culture number, and then an isolate number. For example, isolate W3-6-1 was isolated from enrichment culture 6 and it was the first organism recovered. Isolates recovered from the Pacific vent iron-rich mat sample were given the prefix PV, and bacteria from Puget Sound sediments were given the prefix with PS (Table 1).

Phylogenetic analyses of bacterial isolates. Genomic DNA was recovered from the isolates as described previously (Zhou et al. 1995). SSU rRNA genes were amplified from genomic DNA using modified primers fD1 and rP1 (Weisberg et al. 1991, Zhou et al. 1995). Amplification, sequencing, and sequence analysis were performed as previously described (Smith et al. 1994, Strunk et al. 1996, Zhou et al. 1997, 2001).

Physiological and analytical analyses. The ability of the pure cultures to use alternate electron acceptors and electron donors was examined by visually monitoring growth (e.g. changes in culture turbidity) or reduction (e.g. changes in medium color) in anaerobic medium (Coates et al. 1998a). Potential electron donors (Table 1) were evaluated in anaerobic medium containing 20 µM Fe(III)-citrate at concentrations of 10 µM (lactate, formate, acetate, pyruvate, succinate, and citrate) except for glycerol (5 µM), buyrate (5 µM), toluene (1 µM), and hydrogen (1 atm in the headspace). Potential electron acceptors (Table 2) were evaluated individually at concentrations of 0.5 to 70 µM (Table 2) in anaerobic basal medium with 10 µM lactate. Control tubes without cells were monitored in all experiments. Studies of the effects of sodium chloride concentration on Fe(III)-citrate (20 mM) reduction were performed in the basal anaerobic medium amended with 10 mM lactate.

Growth of the isolates at various temperatures was evaluated aerobically in nutrient broth medium with artificial seawater. The number of generations was calculated following the equation by Prescott et al. (1990). Cell enumeration was performed using fluorescence microscopy (Hobbie et al. 1977). HCl-extractable Fe(II) was evaluated using ferrozine (Zhang et al. 1996). Reduction of Co(III) to Co(II) was evaluated with a spectrophotometer by measuring absorbance at 535 nm (Zhang et al. 1996). Depletion of nitrate was measured using the Szechrome Reagent following manufacturer's instructions (Polysciences). Evolution of N_2O gas was performed by gas chromatography.

Table 2. Additional growth characteristics of bacterial isolates described in this study. No isolates were capable of using butyrate, succinate, or citrate. Only the Puget Sound isolates were tested for the ability to use toluene and glycerol and none of them were able to utilize these compounds. Standard set: all used oxygen (air in headspace), Fe(III) citrate at 20 mM, Fe(III) EDTA at 10 mM, akaganeite at 70 mM, Mn(IV) at 20 mM (as amorphous manganese oxide-MnO_2), Co(III)-EDTA at 1 mM; none were able to use Cr(IV) at 0.5 mM (as the potassium salt). Nitrate support weak growth. No isolates grew at 10% salt

Isolate	Electron donors	Electron acceptors	Salinity range (%)
W3-4-1	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
W3-6-1	Lactate, formate, pyruvate, hydrogen	Standard set, Nitrate	0.1–5
W3-7-1	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
W3-7-2	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
W3-11-1	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
W3-11-2	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
W3-14-2	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
PV-4	Lactate, formate, pyruvate, hydrogen	Standard set	0.1–5
PS-3	Lactate, formate, pyruvate, hydrogen, glucose	Standard set, Nitrate	1–3
PS-5	Lactate, formate, pyruvate, hydrogen	Standard set	1–3
PS-7	Lactate, formate, pyruvate, hydrogen, glucose	Standard set, Nitrate	1–3

The mineralogical composition of the transformed phases during microbial Fe(III) reduction was determined using X-ray diffraction (XRD; Zhang et al. 1997). Scanning electron microscopy (SEM) with energy dispersive X-ray analysis was also used for the analysis of morphology and mineralogy of the iron mineral phases formed by the isolates (Zhang et al. 1997). The akaganeite used in this study was prepared as described elsewhere (Roh et al. 2003).

RESULTS

Sedimentary geochemical environment

The greatest geochemical differences were observed between deep ocean and shallow stations. Dissolved Mn(II) concentrations were near the analytical detection limit throughout the sediment profile at the 3 shallowest stations from the coast of Washington State. Conversely, at these stations Fe(II) concentrations were low at the surface but all profiles exhibited a subsurface maximum that varied in concentration between 40 and 65 mM. At the deep ocean station the situation was reversed, with Fe(II) being near the limit of detection and Mn(II) showing the subsurface maximum. Fe(II) and Mn(II) profiles in the Puget Sound showed maxima at the same depth interval. Also, both Mn(II) and Fe(II) reached much higher concentrations in the Puget Sound core. The peaks in the metal distributions were clearly due to reduction at those depths and subsequent diffusion both upward and downward Although sulfate reduction is active within microsites at all locations sampled, the sulfide produced is either quickly oxidized or precipitated as metal sulfides. Thus, free sulfide and highly reducing conditions are not observed in the sediment layers sampled (Hartnett & Devol 2003).

Pore-water profiles of dissolved O_2, NO_3, and NH_4 were very similar at all the stations from the coast of Washington State and in the Puget Sound, with some differences apparent in nitrate penetration at the deepest station (Fig. 1). No profiles were available for the vent sample due to the differences in sampling techniques employed at that site. Dissolved oxygen decreased rapidly within the sediments at all stations sampled. The shallowest penetration depth was about 0.5 cm at the shallowest station and the deepest penetration at the deepest station was about 1.8 cm (no oxygen data were available from the 997 m station). Similarly, nitrate decreased rapidly, and the nitrate penetration depth was indistinguishable from the oxygen penetration depth at all stations except at the deepest Washington coast station. There, the nitrate penetration depth (about 3.5 cm) was approximately twice as deep as the oxygen penetration depth. At all stations, ammonium was low at the surface but accumulated to significant concentrations between 40 and 80 mM at depth in the sediments.

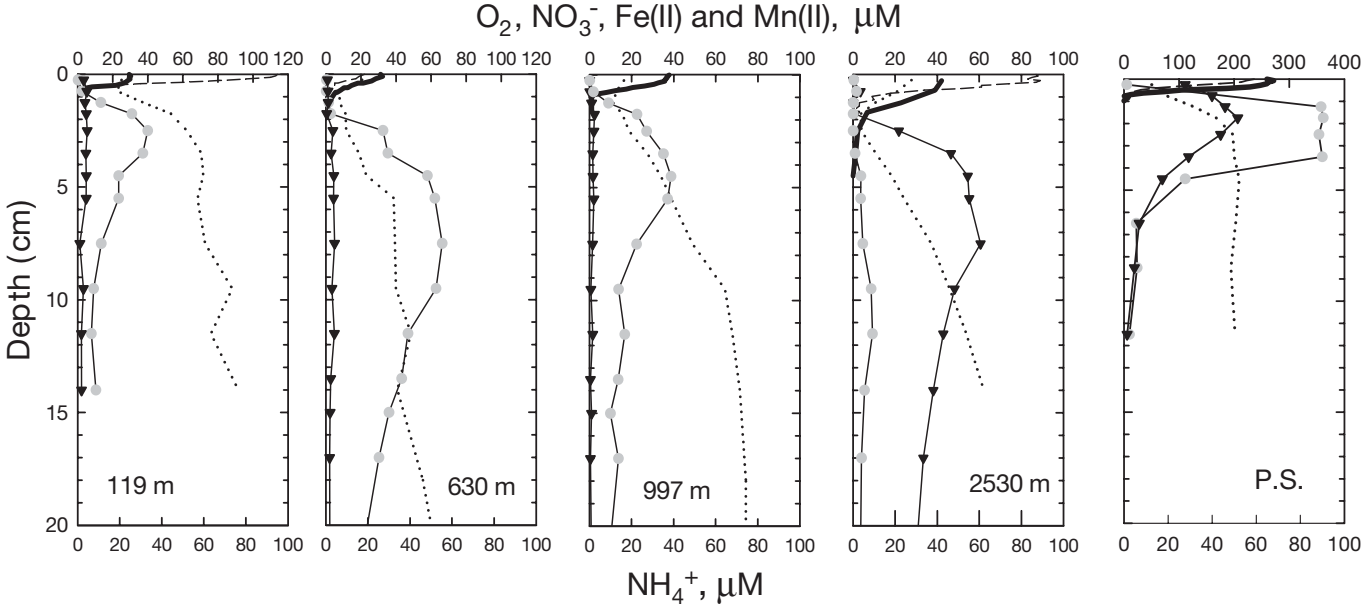

Fig. 1. Pore water chemistry, including O_2 (– – –), NO_3^- (——), Fe(II) (●), Mn(II) (▼) and NH_4 (·····), for Pacific Ocean and Puget Sound (P.S.) sediment sample stations. Depth: depth within the sample core. Pacific Ocean samples were collected at the following depths: Stn 301 at 119 m, Stn 306 at 630 m, Stn 307 at 997 m, and Stn 304 at 2530 m. The Puget Sound sample was collected at a depth of 220 m

Isolation and identification of bacteria

Bacteria capable of coupling anaerobic growth at low temperatures typical of open ocean sediment environments with the utilization of various forms of Fe(III) and other metals were successfully recovered from the 3 marine environments (Table 1). They were all Gram negative, rod-shaped, facultative anaerobes that grew well either aerobically in the presence of oxygen or anaerobically coupled to the reduction of various metals. Studies of electron donor and electron acceptor utilization and of temperature and salinity limitations on metal reduction were done on all strains.

Maximum likelihood analysis of SSU rRNA gene sequences indicated that the isolates were all closely related to members of the well-studied facultative-anaerobe genus *Shewanella* (Fig. 2) and that location appeared to play a role in the group of *Shewanella* isolated. A similar topology for the phylogenetic tree was obtained with the neighbor-joining distance method (data not shown). One cluster of isolates from the coast of Washington State, consisting of W3-14-2, W3-4-1, W3-7-1, and W3-11-2, as well as PS-3, PS-5, and PS-7 from the Puget Sound, grouped closely to Shewanellae associated with reproductive glands of the Pacific Ocean squid *Loligo pealei* (Leonardo et al. 1999). Isolates W3-11-1 and W3-7-2 were 91 to 92% similar to the species *Shewanella gelidimarina*, a bacterium isolated from Antarctic Sea ice (Bowman et al. 1997). Isolate W3-6-1 was most closely related to *S. oneidensis* MR-1, showing a similarity of 98.4% (Ventkateswaren et al. 1999). PV-4 was found to be most similar to *Shewanella algae* BrY. However, based on a complete SSU gene sequence similarity of only 92% and the fact that it grows well at 4°C, PV-4 may represent a novel species within the genus *Shewanella*. Detailed taxonomic studies will be required for confirmation.

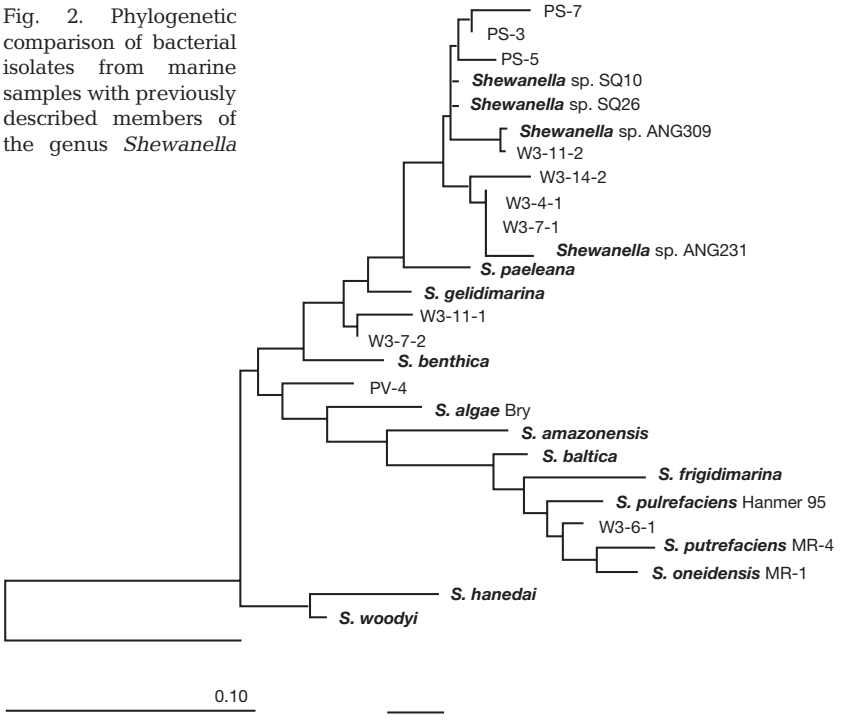

Fig. 2. Phylogenetic comparison of bacterial isolates from marine samples with previously described members of the genus *Shewanella*

Utilization of electron acceptors and donors

All isolated bacteria were able to utilize Co(III)-EDTA, manganese oxide, and various forms of iron as electron acceptors for growth coupled to the oxidation of lactate under anaerobic conditions at 8°C (Table 2). All the isolates were able to use chelated forms of iron, including Fe(III)-citrate and Fe(III)-EDTA, as well as poorly crystalline iron hydroxide as terminal electron acceptors. Iron reduction did not occur in anaerobic medium in the absence of the isolates with only the addition of the electron acceptors Fe(III)-citrate, Fe(III)-EDTA or amorphous iron. The organisms also did not reduce iron when the above forms of iron were supplemented with 10 mM citrate, indicating that neither the chelate (citrate) nor the minimal amount of yeast extract present in the medium were able to serve as electron donors for growth. All isolates reduced brown, non-magnetic Fe(III) oxyhydroxides (akaganeite; FeOOH) to black magnetic minerals using lactate as an electron donor under a N_2 or an N_2/CO_2 (80/20%) headspace. Magnetite was not formed in the absence of lactate. W3-6-1, PS-3, and PS-7 were capable of reducing nitrate but the other isolates were not (Table 2). PS-3 and PS-7 were able to couple the oxidation of 10 mM glucose with the reduction of Fe(III) and the other isolates were not (Table 2). All of the isolates were able to use lactate, formate, pyruvate, and hydrogen as electron donors for Fe(III) reduction, with lactate and hydrogen producing the fastest rates of reduction. The Puget Sound isolates were also tested for the ability to use toluene and glycerol but could not use them as electron donors (Table 2).

Temperature limits for growth and salt requirements were similar for the 10 bacteria isolated from the coast of Washington State and from the Puget Sound, but not the vent isolate PV-4 (Tables 1 & 2). The

Puget Sound and Pacific Ocean isolates demonstrated the ability to grow at both 0 and 37°C, with the temperature optimum falling between 14 and 20°C. PV-4 exhibited a wider range of temperature tolerance, including exhibiting very good growth at 37°C. The Puget Sound isolates required 1 to 3% for growth and iron reduction. In contrast, the isolates from the coast of Washington State and PV-4 grew and produced magnetite at 0.1 to 5% salt concentration. None of the isolates grew or produced iron at a salt concentration of 10%.

Detailed studies with W3-6-1 and PS-7

Strains W3-6-1, isolated from deep Pacific Ocean marine sediments, and PS-7, isolated from Puget Sound, were evaluated for iron reduction coupled to hydrogen at 8°C. Hydrogen was chosen for the detailed studies due to its environmental relevance as an electron donor in aquatic environments (Lovley 2001). Cell numbers increased over time in association with the reduction of Fe(III), which indicated that they obtained energy to support growth (Coates et al. 1996). No increase in cell numbers was observed in medium without the addition of the electron acceptor, indicating that growth was dependent on the presence of the supplemented metals. After 1 wk of incubation, Fe(II) concentration reached a level 19-fold or higher than controls without cells for both W3-6-1 (Fig. 3A) and PS-7 (data not shown).

There is little information detailing the reduction of Co(III)-EDTA at low temperatures. Co(III)-EDTA reduction coupled to the oxidation of hydrogen was examined in W3-6-1 and PS-7. After 30 h at 8°C, Co(II) reached a level approximately 16-fold higher than controls for both W3-6-1 (Fig. 3B) and PS-7 (data not shown). Growth and Co(III) removal from solution appeared to be somewhat uncoupled (Fig. 3), perhaps due to more rapid uptake of cobalt by the bacteria than of reduction. W3-6-1 was also studied in greater detail to determine the effects of still lower temperature on the rate of Co(III)-EDTA reduction (Fig. 4) with hydrogen as the electron donor. Although rates were much slower that at 8°C, at both 4°C (Fig. 4A) and 0°C (Fig. 4B), W3-6-1 was capable of substantial reduction of Co(III)-EDTA coupled to the production of new cells after 10 d.

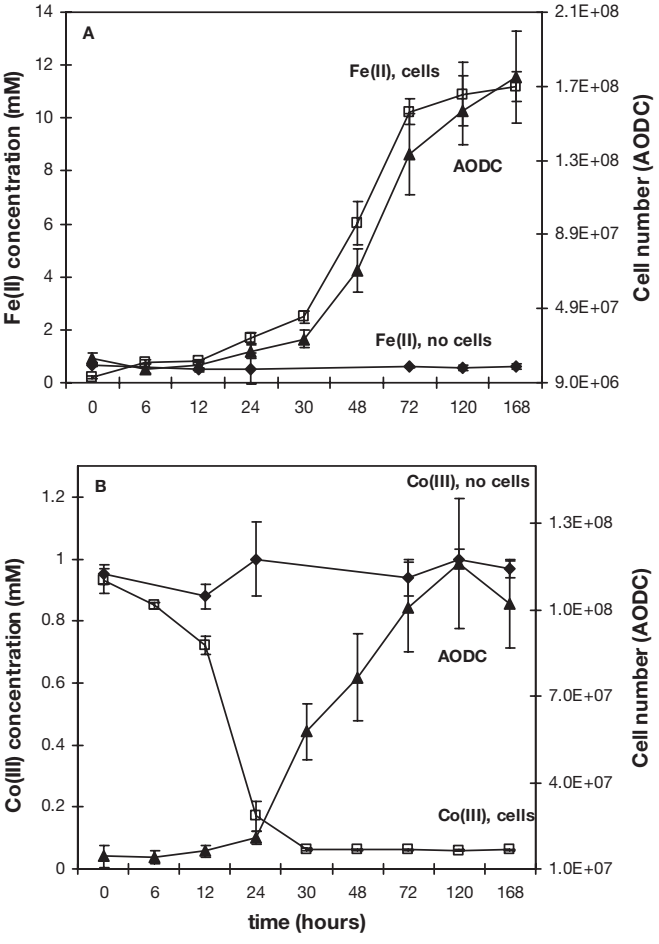

Fig. 3. Reduction of (A) ferric citrate and (B) Co(III)-EDTA at 8°C by W3-6-1. Results are the average of 3 replicate samples. Cell growth (▲) was evaluated in triplicate using acridine orange direct counts (AODC) and fluorescence microscopy. Fe(II) (with added cells: □; without added cells: ◆) was quantified in triplicate using the ferrozine method

Mineral formation

W3-6-1 was able to reduce non-magnetic Fe(III) oxyhydroxides (akaganeite; FeOOH) to black magnetic minerals at a wide range of temperatures using either hydrogen or lactate as an electron donor under a N_2 or N_2/CO_2 (80/20%) headspace. XRD analysis of the black magnetic minerals revealed that magnetite was mainly formed under a N_2 headspace and a mixture of magnetite and siderite were formed under the N_2/CO_2 headspace after the microbial reduction of Fe(III) oxyhydroxide (e.g. Fig. 5). SEM with EDX (Energy Dispersive X-Ray Spectroscopy) analysis of iron minerals formed under a N_2/CO_2 headspace showed that siderite particles were disk-like or ball-shaped crystals with diameters between 10 and 20 µm. The siderite crystals co-existed with microcrystalline magnetite crystals. Siderite formation did not occur when carbon dioxide was not included as a headspace gas. The magnetic minerals were not observed if the culture medium was not inoculated with the isolates.

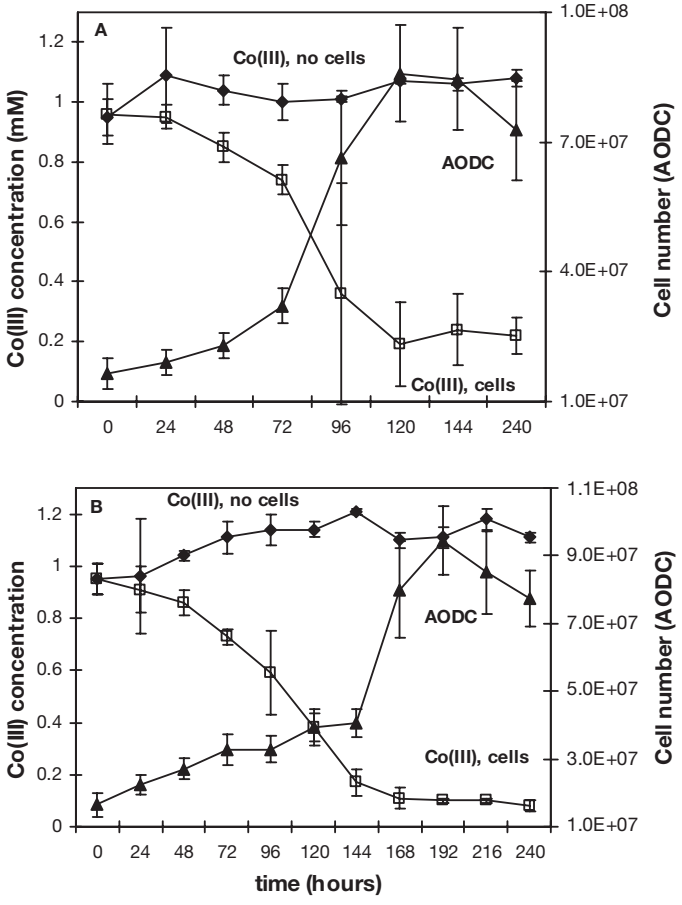

Fig. 4. Reduction of Co(III)-EDTA coupled to the oxidation of hydrogen by W3-6-1 at (A) 4°C and (B) 0°C. Results are the average of 3 replicate samples. Cell growth (▲) was evaluated in triplicate using acridine orange direct counts (AODC) and fluorescence microscopy. Co(III) (with added cells: □; without added cells: ◆) was quantified in triplicate by spectroscopy

DISCUSSION

The geochemical profiles of the sample sites from which these strains were isolated confirm previous reports that sediment microorganisms participating in biogeochemical cycles remain active at low temperatures (Kostka et al. 1999). The sediment pore-water chemistry (e.g. absence of oxygen below the upper few mm and the peaks in the metal profiles at depth) indicates that the environments from which we isolated the bacteria represent active metal reduction zones. It is generally assumed that the various electron acceptors are used in their respective order of thermodynamic energy yield, $O_2 > NO_3^- > MnO_2 > FeOOH > SO_4^-$ (Froelich et al. 1979, Luther et al. 1997). All sediments from the coast of Washington State and those from the Puget Sound show that oxygen and nitrate are consumed before metal reduction, higher up in the sediment column, and that metal reduction follows. However, for the coast of Washington State samples, it is not totally obvious from the profiles that Mn(III,IV) is reduced before Fe(III) because in all but one of the profiles there is an Fe(II) peak with no Mn(II) peak above it. However, the lack of reduced manganese in the shallow Washington State stations is likely due to near complete reduction and mobilization out of the sediments of available manganese oxides as the unpublished data on solid-phase manganese suggests very low concentrations at the shallow water sites (Devol unpubl., J. W. Murray pers. comm.). Previous reports have indicated that microbial metal respiration influences not only the speciation of iron and manganese in anoxic marine sedimentary environments, but also the carbon cycle and the fate of a variety of trace metals and nutrients (Lovley 1993, 2001).

Although these isolates are closely related to previously described members of the genus *Shewanella*, they exhibited slightly different physiological growth patterns. In particular, growth on acetate and glucose while reducing iron, salt concentration, and maximum growth temperature provide useful physiological tests for separating species. Previous reports for *S. putrefaciens*, *S. algae*, *S. frigidimarina*, and *S. gelidimarina* (Bowman et al. 1997, Vogel et al. 1997) demonstrate their ability to utilize acetate as an electron donor in order to reduce ferric iron. Neither *S. frigidimarina* nor *S. gelidimarina* grow above 30°C. Several, but not all, *Shewanella* species isolated from marine environments require salt for growth. *S. pealeana*, *S. amazonensis*, *S woodyi*, and *S. gelidimarina* have optimal growth in medium containing at least 1% sodium chloride (Bowman et al. 1997, Makemson et al. 1997, Ventkateswaran et al. 1998, Leonardo et al. 1999). However, there have been reports of strains of *S. algae* and *S. putrefaciens* growing in salt concentrations ranging from 1 to 10% sodium chloride (Caccavo et al. 1992, Vogel et al. 1997). The recently described *Shewanella* species isolated from Antarctica, *S. frigidimarina*, was shown to not require sodium ions for growth, but was able to tolerate a maximum NaCl concentration of up to 8% (Bowman et al. 1997). The Puget Sound strains required 1 to 3% NaCl for iron reduction, while the other strains did not. Of all the isolates, only PS-3 and PS-7 from the Puget Sound sediments are able to grow by coupling the oxidation of glucose to the reduction of ferric iron. Glucose utilization during iron respiration has been proposed to represent a novel mode of metabolism for the Shewanellae, but it is not widespread (Coates et al. 1998b).

Except for the vent isolate, the strains isolated are not distinguishable by growth temperature response. Since the optimum growth temperatures are above 15°C and strains grew well above 20°C, these isolates

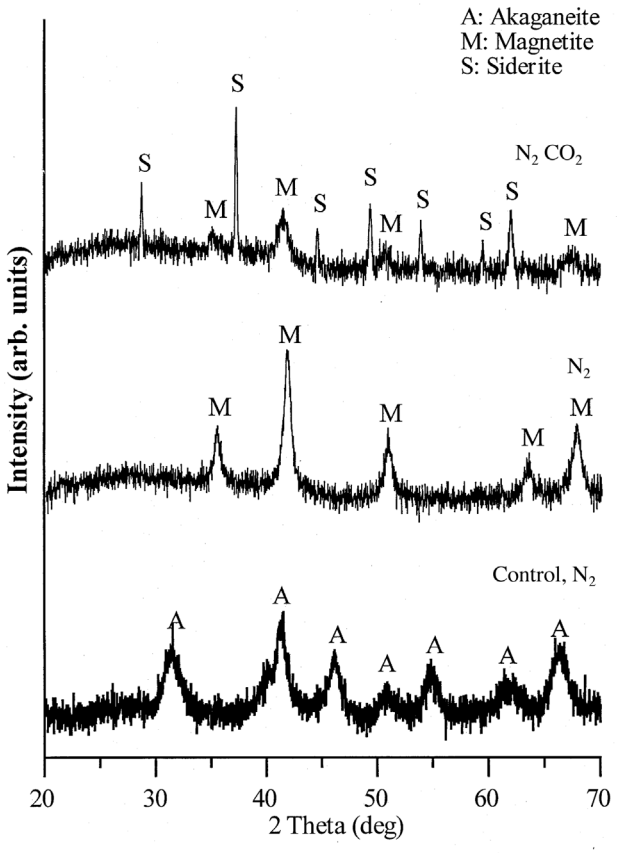

Fig. 5. Differences in X-ray diffraction (XRD) analysis of iron minerals formed by W3-6-1 at 18°C under a N_2 and a N_2/CO_2 headspace

are classified as psychrotolerant according to the definition presented by Morita & Moyer (2001). It is interesting that the optimum growth temperature for each isolate is above the *in situ* temperature of the environment from which it was recovered, indicating that these organisms have adapted to their current living conditions. Brettar et al. (2003) recently reported on the isolation of *Idiomarina baltica*, a marine bacterium from the Baltic Sea that demonstrated growth over a wide range of temperatures, with the optimum growth occurring between 20 and 44°C. The higher temperature tolerance of PV-4 and its ability to reduce metal is interesting in view of its origin. The location near the vent may indicate that it originated in a higher temperature environment and was adapted to temperatures higher than the 4°C typical of marine bottom waters. Similarly, the mat may have been subject to temperature variations that would favor bacteria with a wide temperature tolerance. Temperatures measured at the vents with a thermistor probe from a submersible have been both higher than those in bottom waters and variable, dropping from 23°C in 1996 to 11°C in 1997 (when sample was collected) and 10°C in 1998 (Moyer unpubl. data). Hydrothermal vents are well known as centers of intense geochemical and biological extremes (Lutz et al. 1994, Duennebier et al. 1997, Sievert et al. 1999).

Several characteristics identified the *Shewanella* isolates described in this study as being useful low temperature models for studying the possible effects of metal reduction on pore-water chemistry and for examining mineral formation. The *Shewanella* isolates use a wide variety of electron acceptors such as oxygen, nitrate, and metals, and reduce various metals coupled to the oxidation of several organic acids and hydrogen at temperatures down to 0°C. Results from W3-6-1 and PS-7 demonstrated that in pure culture microbial iron reduction at low temperatures (e.g. 8°C) could occur at rates similar to mesophilic bacteria. Even though the experiments were conducted at 8°C, the rate of Fe(II) evolution coupled to the oxidation of hydrogen demonstrated by W3-6-1 was very similar to those published for the well-studied mesophilic, metal-reducing *Geobacter* organisms (Coates et al. 1996) and the recently described thermophilic *Thermus* isolate (Kieft et al. 1999). W3-6-1 was also capable of iron reduction down to 0°C but at reduced rates.

Because the significance of Co(III) reduction in marine sediments remains unclear, the importance of the ability of these iron-reducing bacteria to reduce Co(III) is also uncertain. Cobalt is present at relatively low concentrations in marine systems water (0.3 µg l^{-1}) and is most often in the form of $CoCl_2$, $Co(OH)_2$, or CoO (Richardson 1993). It is unlikely that Co(III) oxides represent a significant means of bacterial respiration during carbon oxidation. More likely, Co(III) reduction will impact the cycling of this trace element, which is an essential co-factor for cobalamine (vitamin B12). Although open ocean cobalt concentrations are very low, localized enrichments of Co(III) due to adsorption may be found associated with manganese oxides or ferromanganese nodules (Lee & Tebo 1994, and references within). Bacterial reduction of insoluble Co(III) may release soluble Co(II), which can then be taken up by either micro- or macroorganisms living nearby. The practical importance of Co(III) reduction may lie in the terrestrial environment. ^{60}Co has been identified as a priority pollutant by the United States Department of Energy, and its migration in contaminated subsurface environments is influenced by the presence of chelating agents, as well as local mineralogy (Szecsody et al. 1994, Brooks et al. 1999). W3-6-1 indicates that the cobalt cycle should remain active at low temperatures, similar to iron cycling in marine sediments.

Metal reduction by the isolates resulted in formation of the minerals magnetite and siderite, depending on the presence of headspace CO_2 (e.g. Figs. 5 & 6).

Microorganisms often directly and indirectly influence mineral formation. This magnetite formation indicates that dissimilatory iron-reducing bacteria may contribute to sediment magnetism by magnetite formation during anaerobic respiration (Karlin et al. 1987, Lovley et al. 1987). Our results are consistent with those of Fredrickson et al. (1998) and Roh et al. (2003), who found siderite formation by microbial iron reduction in high carbonate systems and minerals including magnetite under other conditions. The siderite globules formed by the these Fe(III)-reducing bacteria have a surface structure resembling flakes of crystals rather than a single rhombohedral crystal formed by *Geobacter metallireducens* (Mortimer & Coleman 1997). Fredrickson et al. (1998) also show that pH has a strong influence on the minerals formed, and that presence of electron shuttles strongly influences rates of reduction. Although not presently common, formation of siderite in marine sediments may present a unique potential for the sequestration of CO_2 (Postma 1981, Pye et al. 1990) in high iron environments or with the addition of iron.

Marine sediments represent sites of active biogeochemical processes. The microorganisms indigenous to these habitats are well adapted to their environment and continue to remain active at temperatures at or below 0°C (Kostka et al. 1999). We described bacteria isolated from 3 distinct marine habitats that were capable of reducing metals and forming minerals under cold-temperature conditions. W3-6-1 demonstrated that the reduction of iron and cobalt, as well as the formation of magnetite and siderite, could be studied in pure culture at temperatures as low as 0°C. This serves well as a model for future investigations into the biogeochemisty of marine sediments.

Acknowledgements. This research was funded by The United States Department of Energy's Biotechnology Initiative-Ocean Marine and Microbial Genome programs, and by the Laboratory Directed Research and Development Program through Oak Ridge National Laboratory. R.D.S. was supported by the Hollaender Distinguished Postdoctoral Fellow Program administered by Oak Ridge Institute for Science and Education and Oak Ridge Associated Universities. Cobalt-EDTA was kindly provided by Drs. P. Jardine and S. Brooks of Oak Ridge National Laboratory. Oak Ridge National Laboratory is managed by The University of Tennessee-Battelle LLC (limited liability company) for The United States Department of Energy under contract DE-AC05-00OR22725.

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Editorial responsibility: Jed Fuhrman, Los Angeles, California, USA

Submitted: February 15, 2003; Accepted: June 28, 2004
Proofs received from author(s): January 13, 2005