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Manganese oxidation by bacterial isolates from the Indian Ridge System

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Abstract

The abundance and activity of culturable manganese-oxidizing bacteria were assessed from near-bottom water samples of the tectonically active Carlsberg Ridge. Retrievable counts as colony forming units (CFU) on dilute nutrient agar medium (dilNA = 2 gm l^{-1} nutrient broth + 2% agar) and on dilNA supplemented with 1, 2 and 3 mM $MnCl_2{\cdot}4H_2O$ were in the order of 10^6 CFU l^{-1}. Retrievability of heterotrophs ranged from non-detectable levels (ND) to 2.82×10^6 CFU l^{-1}. The retrievable counts on Mn amended dilNA ranged from ND to 3.21×10^6, 1.47×10^6 and 1.45×10^6 CFU l^{-1} on 1, 2 and 3 mM, respectively. About 87% of the Mn tolerant isolates ($n = 39$) showed taxonomic affinities to *Pseudomonas* I and II sp. Two representative strains CR35 and CR48 (CR–Carlsberg Ridge) isolated on manganese-supplemented media were tested for their ability to tolerate a range of Mn amendments from 1 nM to 100 mM in terms of growth and respiration. CR35 represents 66% of the total CFU (3.04×10^6 CFU l^{-1}), while CR48 represented only 6% of the total CFU (1.05×10^6 CFU l^{-1}). The colonies of these two isolates were dark brown in color suggesting precipitation of Mn as oxide. Tests for the effect on growth and respiration were conducted in media simulating heterotrophic (amended with 0.01% glucose) and lithotrophic (unamended) conditions. Maximum stimulation in growth and respiration of CR35 occurred at 100 μM Mn both in unamended and amended media. At levels of Mn greater than 100 μM the counts decreased steadily. Total respiring cells of CR48 were stimulated to a maximum at 1 μM Mn in unamended medium and 1 nM in amended medium. Total cells counts for the same decreased beyond 100 μM Mn in unamended and 1 nM in amended medium. The isolates were tested for their ability to oxidize Mn ammendments from 1 μM to 10 mM Mn. At the end of a 76-day incubation period, there was evidence of manganese oxide precipitation at high Mn concentrations (≥1 mM) as a dark brown coloration on the sides of culture tubes. Highest Mn oxidation rates were observed at 10 mM Mn(II) concentration with CR35 oxidizing 27 and 25 μM Mn day^{-1} in unamended and amended condition, respectively. CR48 oxidized Mn at the rate of 26 μM Mn day^{-1} in unamended medium and 35 μM Mn day^{-1} in amended medium. Scanning electron microscope (SEM) observations of both isolates revealed free-living cells in clustered matrices ≈2 μm diameter. Energy dispersive spectrum of the cell matrix of CR35 cultured in 1 mM Mn detected 30% Mn, while the cell aggregates of CR48 harbored 7–10% Mn. The relatively high specific activity of these mixotrophic bacteria under relatively oligotrophic conditions suggests that they may be responsible for scavenging dissolved Mn from the Carlsberg Ridge waters and could potentially participate in oxidation.

Introduction

A 55,000 Km long network of Mid-Oceanic Ridges (MOR) traverses the bottom of the world's oceans. MORs are the constructive margins of oceanic plates where new oceanic lithosphere is continually generated due to rifting and are characterized by high heat flow and marked seismicity (Iyer *et al.* 2003). One such feature is the submarine Carlsberg Ridge in the Arabian Sea,

which forms a part of the Indian mid-oceanic ridge system. The ridge marks the boundary between the Indian and African plates trending NW–SE between 2° S and 10° N. It is characterized by a slow spreading rate of 1.2–1.3 mm yr−1 (Ramana *et al.* 1993). This tectonically active plate boundary provides an excellent opportunity for researchers to study the biogeochemical interactions occurring at spreading centers.

Mid-Oceanic Ridges are known to be an excellent habitat, supporting chemosynthesis. Microbes derive energy (Jannasch 1985; McCollom & Shock 1997) from the chemical flux of metals such as Fe, Mn, Zn, Cu and reduced sulfur spewing out from hydrothermal solutions at vents on mid-ocean spreading centers. Also, the hydrothermally influenced oceanic crust at mid-ocean spreading centers host diffuse fluids (Summit & Baross 2001). This diffusion of fluids maybe responsible for the trace element mobility into ambient seawater. A quantitative estimation of total deep-sea chemosynthesis has been based on high Mn concentrations (Jannasch 1995). Primary source of manganese in the deep oceans is that injected by hydrothermal solutions at vents on mid-ocean spreading centers. Bacteria are key players in several redox reactions involving metal ions such as Fe and Mn. Thus, the autotrophic microbial community contributes substantially to the primary production (Dover & Fry 1994) forming the basis of an inimitable deep-sea food chain. Though no reports of manganese concentration in the Carlsberg Ridge waters have so far been cited, concentrations of 9.8 nM have been reported within plumes near active vents at the Rodriguez triple junction on the Central Indian Ridge (Gamo *et al.* 1996). Manganese content of ferromanganese nodules recovered from the Southern part of Carlsberg Ridge was found to be between 15 and 21% (Karisiddaiah 1985). Plüger *et al.* (1990) reported manganese concentrations as high as 23 nmol kg−1 at the 'Sonne plume' site along the Central Indian Ridge segment.

Much of the research on microbial oxidation of manganese has been focused to evaluate the involvement of biological processes in the geochemical cycling of manganese (Nealson 1983; Greene & Madgwick 1991; Ehrlich 1999; Nelson *et al.* 1999; Emerson 2000; Francis & Tebo 2002). Ehrlich (1983) and Jannasch (1995) have been successful in the isolation of Mn-oxidizing microbes from deep-sea vents. Chandramohan *et al.* (1987) have investigated heterotrophic bacteria capable of mobilizing and immobilizing manganese from ferromanganese nodules recovered from the Indian Ocean. Bacterially produced manganese oxides have been characterized in several prior investigations by Villalobos *et al.* (2003) and Mandernack *et al.* (1995). Jakubovics *et al.* (2002) examined the oxidative stress tolerance regulation induced by manganese in microbes. Francis *et al.* (2001) and Tebo *et al.* (2004) have contributed to more recent studies in understanding mechanisms of biogenic manganese oxide formation. Buatier *et al.* (2004) have characterized the minerals formed along the flanks of Juan de Fuca Ridge and suggest that Mn-oxides are present as small particles that aggregate in a matrix or around biogenic remains.

The present work was carried out as a part of plume tracing studies from potential sites on the Indian Ridge ecosystem. The work aims to assess the abundance of culturable manganese tolerant bacteria from water samples of Carlsberg Ridge area and estimate their Mn immobilizing activity. Though these forms could participate in reducing and oxidizing interactions of the metal, this study is restricted to the latter. Besides, this study suggests that the bacteria could potentially participate in Mn scavenging and possibly oxidation in the Carlsberg Ridge waters.

Methodology

Sample collection, culture isolation and characterization

Near-bottom water samples were retrieved with Niskin samplers mounted onto a CTD rosette from the Carlsberg Ridge waters during the cruise SK194 onboard *ORV Sagar Kanya* (July, 2003). A total of 25 samples along the ridge axis and flanks were collected in 100 ml sterile polypropylene bottles and analyzed immediately onboard. An inoculum of 100 μl was used for the enumeration of culturable manganese tolerant bacteria by surface plating. The ridge waters were also checked for total retrievable heterotrophic bacterial population. Media were prepared using diluted nutrient agar in seawater [dilNA = 2 gm l^{-1} nutrient broth in sea water + 2% agar (Qualigens, India)] for heterotrophs and dilNA supplemented with 1, 2 and 3 mM $MnCl_2 \cdot 4H_2O$ for manganese tolerant

bacteria (1 mM Mn l^{-1} has 54.9 mg Mn l^{-1} and hereafter referred as 1 mM Mn). Retrievable counts in the form of colony forming units (CFU) were recorded after incubating the plates at 18 °C ($\pm$ 1 °C) for 15 days. Representative isolation was done based on the variability in colony morphology. Each isolate represented a percentage of colonies bearing similar morphologies recorded in the plate counts. Well-isolated colonies were checked for purity by microscopic examination after Gram staining and then further characterized using standard biochemical tests (Oliver 1982).

Effect of Mn on growth & respiration

A preliminary assay to assess the growth of two representative isolates (CR – Carlsberg Ridge) CR35 and CR48 in presence of Mn was carried out. They were selected based on their abundance and colony characteristics. Tests were done at Mn ammendments of 1, 10 and 100 nM; 1, 10 and 100 μM; and 1, 10 and 100 mM. Exponentially growing cells were harvested from Mn supplemented dilNA plates in physiological saline. The suspension was centrifuged at 5000 rpm for 10 min. Cell pellets were washed twice with sterile saline and resuspended by vortexing. The inoculum size was calculated by direct cell counts in a cell counting chamber. Triphenyl tetrazolium chloride (TTC; 0.025%) was added to every tube prior to incubation. For heterotrophic or amended medium, 0.01% glucose was added while the same was excluded for lithotrophic or unamended medium. The experiments were done in triplicates at pH 7.6–7.8 and incubated in dark for a period of 76 days. Over the incubation period, respiring cells convert TTC to triphenyl formazan (Lenhard 1956) that gives a deep pinkish appearance. As measurements were carried out spectrophotometrically, a longer incubation period was necessary to facilitate formazan production by the slow growing bacteria. Cell counts (respiring and total) were carried out in a cell counting chamber and observed with a bright field (Olympus BHF-342).

The amount of formazan produced corresponds to the respiring activity of cells. Hence to measure the amount of TTC reduced to formazan, samples were centrifuged at room temperature at 8000 rpm for 10 min with an Eltek TC 4100D centrifuge. Three milliliters of methanol was added to the cell pellet and the tubes were vortexed. The tubes were kept overnight for complete extraction of formazan by methanol and centrifuged again. The supernatant was measured spectrophotometrically at 520 nm to estimate the formazan formed.

Mn immobilization study

The two isolates CR35 and CR48 were simultaneously tested for their ability to oxidize manganese ammendments of 1, 10 and 100 μM; and 1, 10 mM. The experiment was done in triplicates and the cultures were incubated in dark.

To measure the rate of Mn(II) oxidized at the end of a 76-day incubation period, the cultures in both amended and unamended media were spinned at 8000 rpm for 10 min. A 5 ml aliquot of the supernatant was analyzed spectrophotometrically at 560 nm with a Shimadzu UV-1601 spectrophotometer to estimate dissolved residual manganese. Dissolved manganese was estimated as described by Chin *et al.* (1992). The method is sensitive over a concentration range of 300 nM–30 μM. Culture tubes bearing higher Mn concentrations ($>$ 30 μM Mn) were diluted with distilled water prior to measuring manganese. Mn concentration in the distilled water was also recorded to minimize error. All measurements were done in triplicates. Control tubes with sterile media were also maintained for each set of culture tubes to measure the levels of auto oxidation of manganese.

SEM/EDS analysis

Suspensions of Mn oxidizing colonies of CR35 and CR48 cultured on 1 mM Mn amended dilNA medium were prepared in 0.9% sterile saline. Clean glass pieces were immersed in the suspensions and left overnight for the cells to form a biofilm on the glass surface. The glass pieces were retrieved and the biofilm was then subjected to dehydration by running it through a series of increasing acetone concentrations from 10, 30, 50, 70, 90 and 100%. The samples were then air-dried, mounted on a stub and sputter coated with Au/Pd. The specimens were then visualized with a JEOL JSM-5800 Scanning electron microscope (SEM). The presence of Mn-oxide deposits in the culture was corroborated using an energy dispersive X-ray spectrometer (EDS) in conjunction with the SEM.

Results

Retrievability and culture characteristics

Retrievable counts on 1, 2 and 3 mM Mn supplemented dilNA ranged from non-detectable (ND) to 3.21×10^6, 1.47×10^6 and 1.45×10^6 CFU l^{-1}, respectively. Heterotrophic retrievability ranged between ND and 2.82×10^6 CFU l^{-1}. Though the Mn tolerant retrievable bacterial population along the Carlsberg Ridge bottom waters ranged in the same order, the diversity of colony morphotypes on 1 mM (Figure 1) and 2 mM Mn amended plates was higher than on 3 mM Mn amended plates with more of Mn oxidizers.

A total of 39 Mn tolerant isolates were biochemically characterized and identified. Twenty-eight percent of the colonies showed brown precipitation (Figure 2) on prolonged incubation. There were 15 morphologically different colony types. All the isolates tested were gram-negative, short rods ($\cong 0.67 \times 0.43$ μM). Most of them were pigmented and motile. Eighty-seven percent isolates showed taxonomic affinities to *Pseudomonas* I & II sp. and 13% comprised of *Pseudomonas* IV sp. Both the isolates CR35 and CR48 used in this study resembled *Pseudomonas* I & II sp. The colony morphotype CR35 represents 66% of the total CFU (3.04×10^6 CFU l^{-1}), while CR48 represented only 6% of the total CFU (1.05×10^6 CFU l^{-1}). Intense brown color observed possibly due to deposition of oxide (Figure 3) by the bacterial colonies of the two strains was the major criteria for the selection of strains CR35 and CR48 for a detailed study.

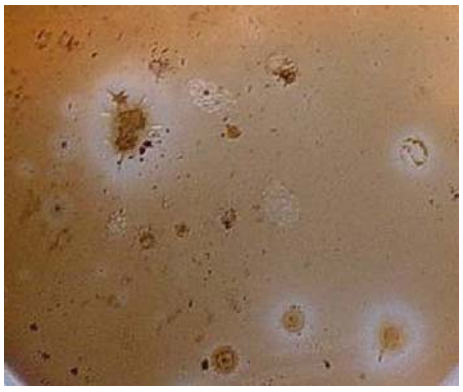

Figure 1. Colony forming units (CFUs) of manganese tolerant bacteria on 1 mM Mn amended dilNA. Manganese oxidizing bacteria turn brown on prolonged incubation. Halos around some of the CFU show Mn clearance zones.

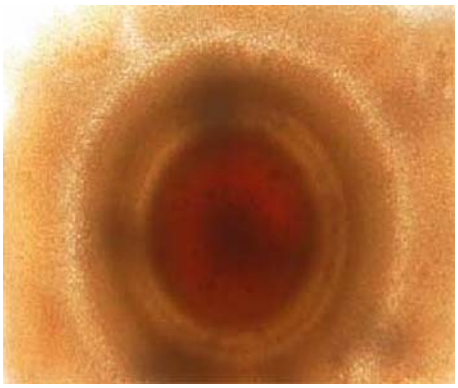

Figure 2. Close-up of a manganese tolerant bacterial colony.

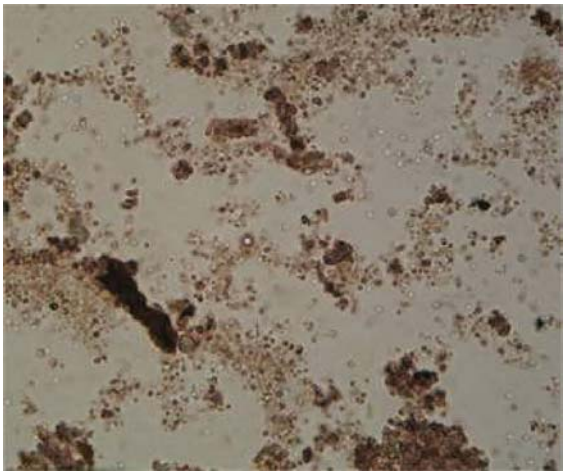

Figure 3. Manganese-oxidizing bacterial cells with extracellular oxide precipitation (40×).

Growth & respiration

Growth and respiration experiments with two representative isolates showed varying results. At the end of a 76-day incubation period, the respiring cell counts in control tubes of unamended incubations for strains CR35 and CR48 were 3.84×10^8 and 4.8×10^8 cells ml^{-1}, respectively. Control tubes of seawater amended with 0.01% glucose showed 8.4×10^7 and 5.72×10^8 cells ml^{-1} for CR35 and CR48, respectively.

Maximum stimulation in growth and respiration of CR35 occurred at 100 μM Mn in both media. Respiring cell counts were observed to

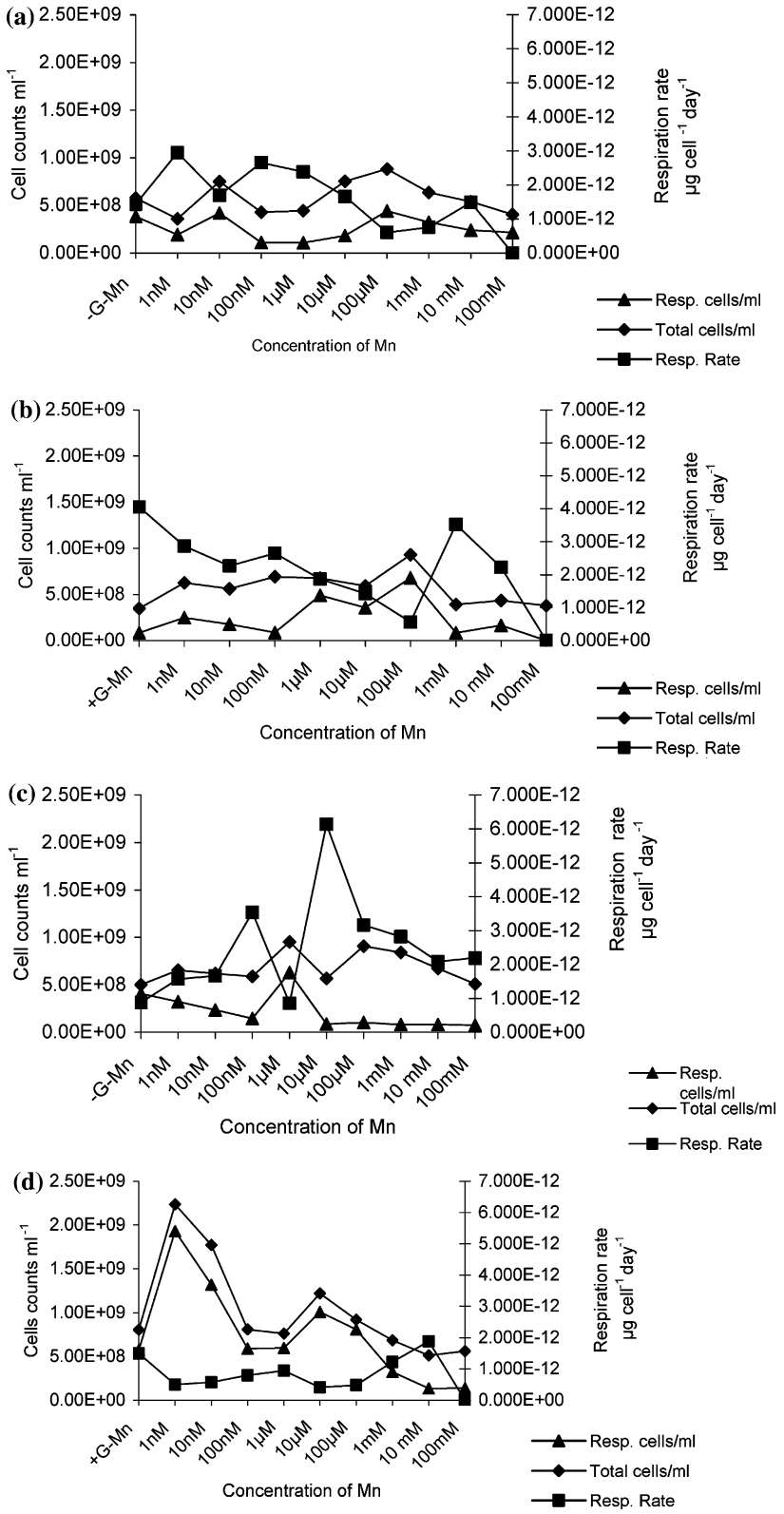

Figure 4. (a) Growth of CR35 in unamended medium; (b) growth of CR35 in amended medium; (c) growth of CR48 in unamended medium; (d) growth of CR48 in amended medium.

be stimulated to a maximum of 4.41×10^8 cells ml^{-1} in unamended medium (Figure 4a) in contrast to 6.8×10^8 cells ml^{-1} under amended condition (Figure 4b). A steady decline in cell counts was seen beyond 100 μM Mn. Maximum cell growth was observed to be 8.83×10^8 and 9.32×10^8 cells ml^{-1} at 100 μM Mn in unamended and amended media, respectively. Respiration rate in unamended medium was 2.95×10^{-12} μg cell^{-1} day^{-1} and in amended medium it was 4.06×10^{-12} μg cell^{-1} day^{-1}. There was a steady decline in the respiration rate in the presence of Mn followed with a peak of 3.53×10^{-12} μg cell^{-1} day^{-1}at 1 mM Mn.

In CR48, respiring cells were stimulated to a maximum of 6.3×10^8 cells ml^{-1} at 1 μM Mn (Figure 4c) in unamended medium and 1.93×10^9 cells ml^{-1} at 1 nM Mn in amended medium (Figure 4d). Respiring cells of CR48 decreased at Mn concentration of 100 μM in unamended medium while the decline was beyond 10 μM in amended medium. Cell growth maxima coincided at the same Mn concentrations with cell counts at a high of 2.24×10^9 cells ml^{-1} in unamended medium and 9.52×10^9 cells ml^{-1} in amended medium. Respiration rates peaked at 10 μM Mn (6.14×10^{-12} μg cell^{-1} day^{-1}) in unamended incubations where as it was 1.88×10^{-12} μg cell^{-1} day^{-1} at 10 mM under amended conditions.

Mn oxidation

Manganese oxide precipitation was evident as dark brown stains on the sides of culture tubes (Figure 5) of CR35 and CR48 at higher Mn concentrations (≥1 mM). Both the strains were able to oxidize manganese in amended and unamended media. At 10 mM Mn, CR35 oxidized it at a rate of 27 μM day^{-1} (Figure 6a) with a specific activity of 188fM cell^{-1} day^{-1} in unamended media. In amended medium the oxidizing capacity of the strain was found to be 25 μM Mn day^{-1} (Figure 6b) with a specific activity of 148fM cell^{-1} day^{-1}. Strain CR48 had a similar oxidizing capacity of 26 μM Mn day^{-1} in unamended media (Figure 6c) but with a higher specific activity of 973fM cell^{-1} day^{-1}. The immobilizing capacity of cells incubated in amended medium was higher at 35 μM Mn day^{-1} (Figure 6d) with a lower specific activity of 262fM cell^{-1} day^{-1} in unamended medium.

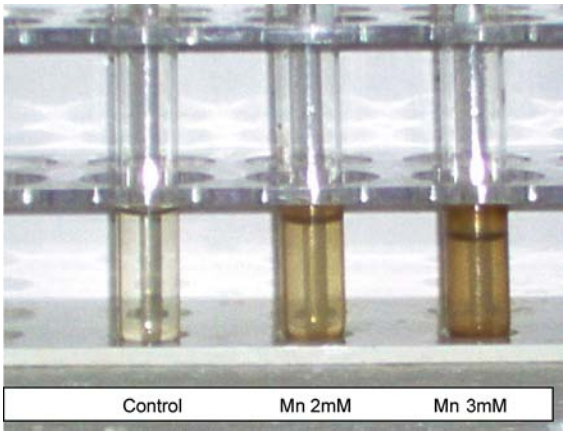

Figure 5. Evidence of oxidation in culture tubes with increasing Mn concentration after a 76-day incubation period.

Scanning electron microscope analysis of the experimental cultures revealed rod-shaped bacteria (0.27–0.95 μM size) in exopolymer giving a clustered appearance (Figure 7). EDS could not detect Mn oxide on individual bacterial cells since the oxides were only found to be present within the bacterial exopolymeric secretions. This was confirmed by X-ray diffraction patterns of the amorphous matrix of CR35 in Mn amended lithotrophic medium which displayed peaks of manganese oxides showing 30% Mn (Table 1). CR48 also had a similar appearance of its cells clustered and enveloped by exopolymeric material. Mn content in its extracellular material ranged from 7 to 10% (Table 2; Figure 8). The oxide was assumed to be Mn(IV) since it is most likely to be deposited in the exopolymer on prolonged incubation (Ehrlich 1999). Besides, the amorphous nature of the oxide as evidenced by its insolubility in water suggests that it could be Mn(IV). An insufficient quantity of dry sample prevented further confirmation by electron diffraction.

Discussion

In this experimental study, we have focused on the growth of two *Pseudomonas* sp. isolated from the Indian Ridge environment to varying concentration of Mn (1 nM–100 mM) and its subsequent removal from the liquid phase at concentrations from 1 μm to 10 mM. The results are in accordance with studies by Mandernack *et al*. (1995) on oxidation of manganese by marine bacterial spores

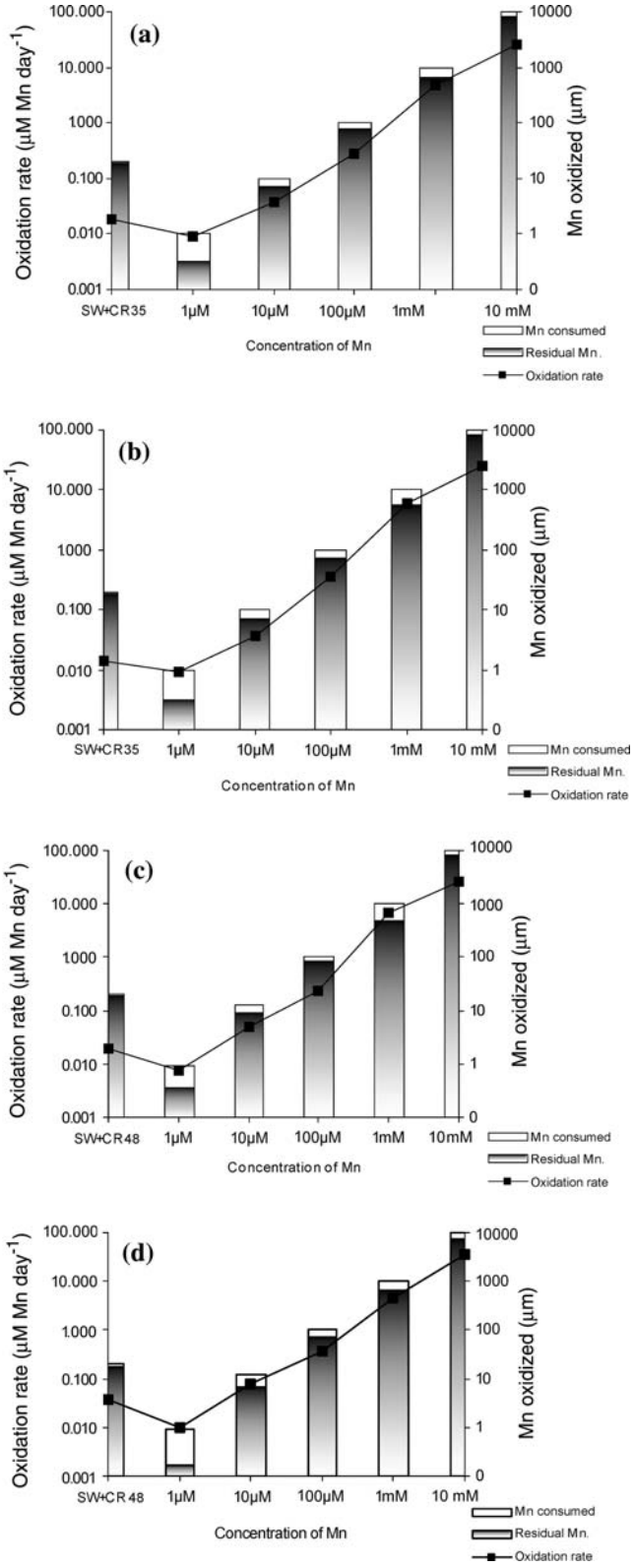

Figure 6. (a) Mn oxidation by CR35 in unamended medium; (b) Mn oxidation by CR35 in amended medium; (c) Mn oxidation by CR48 in unamended medium; (d) Mn oxidation by CR48 in unamended medium.

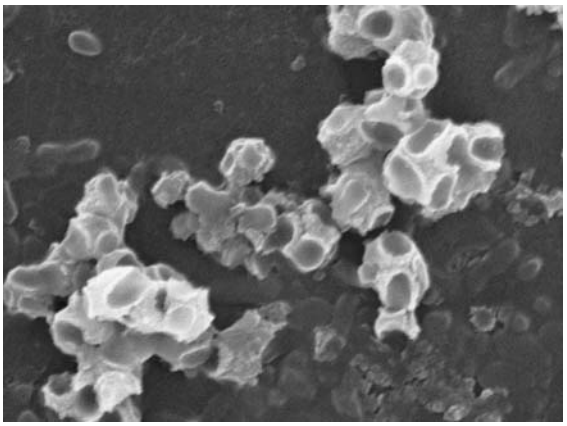

Figure 7. Electron micrograph of clustered manganese-oxidizing bacteria held together by its exopolymeric secretions.

Table 1. EDS result for CR35 matrix.

Element	Element (%)	Atomic (%)
Na	8.69	10.04
Mg	2.35	2.57
Si	0.98	0.93
P	9.55	8.19
Cl	13.22	9.90
K	2.34	1.59
Ca	10.35	6.85
Mn	23.12	11.18
O	29.38	48.75
Total	100.00	100.00

over a wide range of Mn concentrations (< 1 nM to > 25 mM) wherein precipitation of Mn(IV) minerals occurred in seawater amended with ≥1 mM Mn(II). Greene & Madgwick (1991) have demonstrated bacterially mediated manganese oxidation at 25 mM Mn concentration.

Table 2. EDS result for CR48 matrix.

Element	Element (%)	Atomic (%)
Na	7.98	7.44
Mg	2.19	1.93
Al	0.92	0.73
Si	31.68	24.17
Cl	1.17	0.71
Ca	4.15	2.22
Mn	7.06	2.75
Total	100.00	100.00

The present experiment brings out the stimulatory effect of manganese at lower Mn concentrations (1 nM–100 μM). The marginal difference in cell numbers in both amended and unamended media suggests the organic compound requirements of the bacteria used in this study are minimal therefore suggestive of lithotrophic mode of nutrition. Hence, Mn could serve as a potential source of energy for these strains, which were isolated from relatively oligotrophic waters. It is also evident that the bacterial cells are capable of oxidizing at higher Mn concentration (≥ 1 mM). Spectrophotometric analysis of residual dissolved manganese in the incubated tubes revealed maximum removal of Mn from the dissolved phase occurred at 10 mM Mn(II) concentration. As evident by SEM/EDS analysis, the bacterial exopolymer was responsible for immobilizing the manganese oxide. The tolerance of gram-negative bacteria to elevated manganese concentrations appears to be due to enzymatic periplasmic oxidation of manganese (Ehrlich 1999; Emerson 2000). Toner *et al*. (2005) report that Mn-precipi-

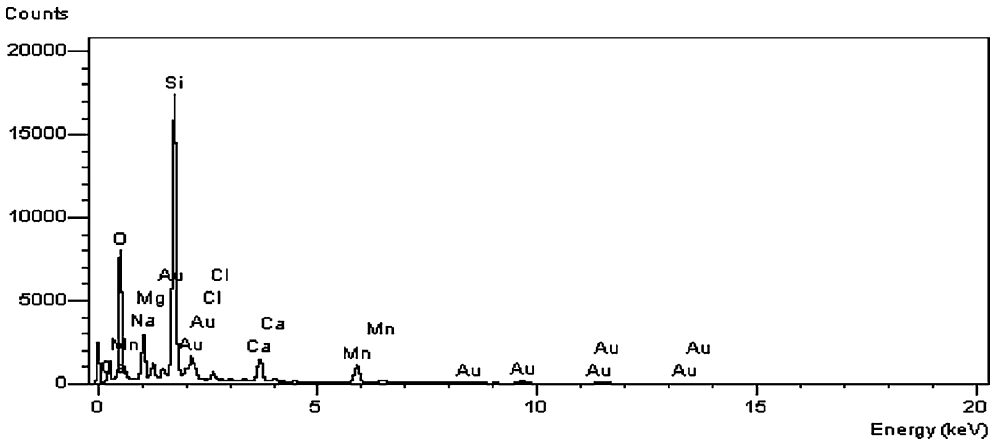

Figure 8. EDS spectrum of CR48.

tate gets completely enveloped by bacterial biofilm material in *Pseudomonas putida* strain MnB1.

The fall in formazan at Mn concentration ≥ 1 mM suggests stress to the organisms to which they respond by precipitating Mn as an insoluble oxide. The microbial mineral formation could be a form of immobilization for detoxification (Ehrlich 1999). Moreover, immobilizing capacity of the isolates could be dependent on the concentration of manganese and the tolerance-threshold of the strain. Though both the test strains were able to oxidize comparable amounts of manganese, the higher specific activity of CR48 under lithotrophic condition is indicative of its chemosynthetic potential and suggests that it could play a significant role in controlling Mn chemistry of the Carlsberg Ridge waters.

Acknowledgements

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