

Mobilization of manganese by basalt associated Mn(II)-oxidizing bacteria from the Indian Ridge System

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Abstract

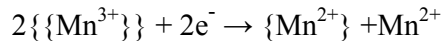
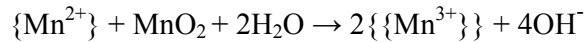
The Indian Ridge System basalt bearing Mn-oxide coatings had todorokite as the major and birnesite as the minor mineral. We posit that microorganisms associated with these basalts participate in the oxidation of Mn and contribute to mineral deposition. We also hypothesized that, the Mn-oxidizing microbes may respond reversibly to pulses of fresh organic carbon introduced into the water column by mobilizing the Mn in Mn-oxides. To test these two hypotheses, we enumerated the number of Mn-oxidizers and -reducers and carried out studies on the mobilization of Mn by microbial communities associated with basalt. In medium containing 100 μ M Mn²⁺, 10³ colony forming units (CFU) were recovered with undetectable number of reducers on Mn-oxide amended medium, suggesting that the community was more oxidative. Experiments were then conducted with basalt fragments at 4 \pm 2°C in the presence ‘G⁺’ and absence ‘G⁻’ of glucose (0.1%). Controls included set-ups, some of which were poisoned with 15mM azide and the others of which were heat-killed. The mobilization of Mn in the presence of glucose was 1.76 μ g g⁻¹ d⁻¹ and in the absence, it was 0.17 μ g g⁻¹ d⁻¹ after 150d. Mn mobilization with and without added glucose was 13 and 4 times greater than the corresponding azide treated controls. However, rates in ‘G⁺’ were 16 times and ‘G⁻’ 24 times more than the respective heat killed controls. The corresponding total counts in the presence of added glucose increased from 1.63 \times 10⁶ to 6.71 \times 10⁷ cells g⁻¹ and from 1.41 \times 10⁷ to 3.52 \times 10⁷ cells g⁻¹ in its absence. Thus, the addition of glucose as a proxy for organic carbon changed the community’s response from Mn(II)-oxidizing to Mn(IV)-reducing activity. The results confirm the participation of Mn oxidizing bacteria in the mobilization of Mn. Identification of culturable bacteria by 16S rRNA gene analysis showed taxonomic affiliations to *Bacillus*, *Exiguobacterium*, *Staphylococcus*, *Brevibacterium* and *Alcanivorax* sp.

Key words: Manganese, Basalt, Mobilization, Microbial community, 16S rDNA, Carlsberg Ridge

1. Introduction

Sea floor ridges are elevated underwater chain of mountains formed by geological processes associated with floor spreading and plate tectonics. The release of hydrothermal fluids associated with venting at these locations act as a source of elevated Mn(II) and Fe(II) concentrations. These elements exert an influence on the geochemical characteristics of the water column and thereby support unique forms of microbial life that catalyse the oxidation/reduction of metal cations leading to the precipitation or dissolution of minerals. The microbial interactions could, however, change the balance between oxidizing and reducing processes, in which one process dominates the other depending on the key environmental conditions. Under oxidizing conditions with ambient dissolved organic carbon (DOC) concentrations of 42.8 μM as in most deep waters (Hansell and Carlson, 1998), microbial interactions lead to the formation of ferromanganese (Fe-Mn) mineral deposits. The bacterial members that partake in Fe-Mn oxidation comprise the known, non-photosynthetic lineages of organisms like the *Firmicutes*, *Verrucomicrobia*, *Thermales* and the *Cytophaga–Flexibacter–Bacteroides* groups in mid-ocean ridge systems (Takai et al., 2006). In addition, Mn and sulphur-oxidizing bacterial communities belonging to class α -, β - and γ -*Proteobacteria*, *Actinobacteria* and *Bacilli* occupy the oceanic crust basalts and sediments along the ridge axis (Rathsack et al., 2009).

In the event of an episodic supply of organic carbon that may arise from an earthquake, a phytoplankton bloom due to aeolian or fluvial input, anthropogenic activity, etc., the Mn(II)-oxidizing activity of the basalt associated community may change to Mn(IV)-reducing activity. The Mn so released may play an essential role in the biogeochemical cycling of carbon, nitrogen, phosphorous, and other metals in the ridge ecosystem. The dissolution of Mn could result from one of the two processes. In one, direct contact between the surface of a Mn(IV)-reducing cell and Mn-oxide leads to Mn solubilization. In the other, the Mn(IV)-reduction occurs indirectly through microbially mediated changes in reducing conditions (Baglin et al., 1992; Sand et al., 2001). In some cases, Mn may be reduced to fulfil a nutritional need for soluble Mn(II) (de Vrind et al., 1986) or to scavenge excess reducing power as in the case of nitrate and iron reduction (Robertson et al., 1988; Lovley, 1991). The key steps in the reduction of Mn(IV) to Mn(II) involve the reactions of strongly bound Mn^{3+} $\{\{\text{Mn}^{3+}\}\}$ and weakly bound Mn^{2+} $\{\text{Mn}^{2+}\}$ on the surface of Mn-oxide (Ehrlich, 2002; Sujith and Loka Bharathi, 2011). The reactions proposed to occur in the presence of Mn-reducing bacteria are



In contrast, the abiotic reduction of Fe and Mn-oxides could occur through the involvement of inorganic and organic reductants like H_2S , Fe^{2+} , catechol, ascorbate and humic substances (Thamdrup, 2000). The metal reducing bacteria that participate in Mn-oxide reduction could use oxidized Mn as a terminal electron acceptor for anaerobic respiration (Myers and Nealson, 1988). They could be obligately aerobic, obligately anaerobic, facultatively anaerobic (Madgwick, 1987) and strictly autotrophic, heterotrophic, or mixotrophic (Enrich-Prast et al., 2009). Glucose, lactate, succinate, acetate or hydrogen may serve as suitable reductants for those organisms that participate in metal oxide reduction (Ehrlich, 1988).

Carlsberg Ridge (CR) in the Arabian Sea supports oxidation as the dominant process with ambient DOC concentration of $42.8 \mu\text{M}$ at water depths of 1000 to 4300 m (Hansell and Carlson, 1998). Hence, we hypothesize that the microorganisms, associated with these basalts participate in the oxidation of Mn and contribute to mineral deposition. However, this area experiences seasonal changes in DOC concentration due to both physical and biological processes. Concentrations of DOC of the region are reported to vary from $70\text{-}80 \mu\text{M}$ during the northeast monsoon and $75\text{-}90 \mu\text{M}$ during the spring inter-monsoon period (Hansell and Peltzer, 1998). Therefore, we further hypothesize that Mn-oxidizing microbes could respond reversibly to pulses of fresh organic carbon introduced into the water column by mobilizing Mn in Mn-oxides. Further, we also identified culturable Mn(II)-oxidizing bacteria and other heterotrophic bacteria that could have an interactive role in the mobilization of Mn in the ridge ecosystem.

2. Materials and methods

2.1. Study area and sampling

The Carlsberg Ridge in the Arabian Sea is a segment of the Mid-Indian Ridge. It extends from near Rodrigues Island to the Gulf of Aden, trending Northwest to Southeast and separates the Arabian Sea in the Northeast from the Somali Basin in the Southwest (Fournier et al., 2008). The mean elevation of the Carlsberg Ridge crest is ~ 2100 m and the water depth is 1800 to 3600 m (Mudholkar et al., 2000). A rock sample bearing oxide coating was collected at $3^\circ 39.718$ N (lat) and

63°49.922 E (long) (Supplementary Fig. 1) during the Akademik Boris Petrov cruise 36 (2009), using a chain-bag dredge at a water depth of 3390 m.

2.2. Characterisation of the natural Mn oxides coating on basalt

Basalt fragments bearing oxide coatings of dimensions $0.54\text{-}3.93 \times 0.61\text{-}3.91$ mm ($n = 15$) were aseptically removed using a sterile scalpel and rinsed with phosphate buffered saline (300 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.7 mM NaH_2PO_4 , pH 7.0) before microscopic analyses. They were air-dried, mounted on metal stubs using conductive adhesive tape and then sputter-coated with Au using a SPI-Module sputter coater. The edges were coated with conductive silver to eliminate any possible charging. Using a JEOL JSM-5800 scanning electron microscope (SEM) the surface texture and microbial load on basalt surfaces were observed at different magnifications. Oxford INCA 5431 Energy Dispersive Spectrophotometer (EDS) in conjunction with SEM was done on randomly chosen points of the fragments at an accelerating voltage of 15 kV for determining the composition of the rock.

The solid phase concentration of Mn was determined from 50 mg of powdered rock fragments by closed vessel digestion method (Roy et al., 2007). The determination of Mn was carried out by adsorptive stripping voltammetry in interface with 797 VA computrace (Metrohm, Switzerland) in a differential pulse mode according to Colombini and Fuoco (1983) and as modified and described by Sujith et al. (2011). The analysis was quantified with two standard additions of appropriately diluted 1 ppm concentrations of Mn^{2+} standard ($\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$). The concentration of Mn in solution was then normalized per gram dry weight of rock after correcting for the blank.

The mineralogy of natural Mn-oxide was characterized by X-ray diffraction analysis (XRD) as described by Villalobos et al. (2003). For XRD analysis, oxide coatings were removed from a single large rock fragment using scalpel and then finely powdered using an agate mortar and pestle. The XRD patterns of the Mn-oxides were recorded in a Rigaku X-ray powder diffractometer using a monochromatic $\text{Cu K}\alpha_1$ radiation (operating at 40 kV and 20 mA) and a scintillation detector. All samples were run in scan mode over a 2θ range of $0\text{-}80^\circ$ with a scanning rate of $1.2^\circ \text{ min}^{-1}$. The interpretation of the peaks was done according to Burns and Burns (1977).

2.3. Characterisation of chemically synthesized Mn-oxides for enumeration of reducers

The synthesis of manganese dioxide was carried out by the oxidation of manganous ion by permanganate according to [Kostka and Nealson, \(1998\)](#). Briefly, the steps involved 1) gradual addition of NaOH and MnCl₂·4H₂O solution to the hot, stirring KMnO₄ solution maintained at 90 °C followed by 2) cooling of the mixture with continuous stirring for 30 min to room temperature (28 ± 2 °C). Subsequently, the precipitate was washed, dried and allowed to age for at least 24 h. The mineralogy of the chemically synthesized Mn-oxide was characterized by XRD as described above for the natural samples.

2.4. Plate counts of bacteria associated with basalt

For the enumeration of bacteria, surface fragments were removed from a single large rock fragment with a sterile scalpel. About 1 g of the rock fragments of dimensions 0.62-1.7 × 0.52-1.25 mm (n =15) was suspended in 9 ml of sterile seawater. The resulting suspension was dispersed by sonication. A Vibra Cell™ generator Model VC50 (Sonics and Materials Inc., Danbury, C.T. USA) with a high power level ultrasonic probe (Model ASI) with a standard 3.2 mm diameter titanium tip was used for ultrasonication. The volumes of media containing basalt fragments were kept constant at 5 ml in sterile glass vials, and the probe tip was kept 1 cm above the bottom of the tube. These tubes were pre-cooled at 4±1 °C and then packed in crushed ice during sonication to dissipate heat. Sonication was carried out at 15 Hz for 15 s in bursts of 5 s with intermittent cooling for 10 s in crushed ice, allowed to settle and then 0.1 ml aliquots were used to inoculate respective media for the growth of heterotrophic, Mn-oxidizing and -reducing bacteria. The heterotrophic bacteria were enumerated by standard spread plate method in 1% nutrient agar medium (Himedia, Mumbai, India) and Mn-oxidizing bacteria in 0.01% nutrient agar medium amended with 100 µM final concentration of Mn²⁺. The Mn-reducing bacteria were enumerated by agar overlay method ([Bratina et al., 1998](#)). The media for their growth consisted of the following composition per litre of seawater: 0.5 g peptone, 2.48 g glucose, 0.5 g K₂HPO₄ and 0.24 g KH₂PO₄ with a 0.75 % agar overlay containing chemically synthesized Mn-oxide (ca. 17 g l⁻¹). The inoculated and control media plates were incubated at 28 ± 2 °C for 7-10 d. The colony forming units (CFU) of Mn-oxidizing bacteria were to be recognized by their brown colour of the colony due to the formation of Mn-oxides. Mn-reducers were to be recognized by clear zones around their colonies. The numbers of CFU in each plate were counted and then normalized per gram dry weight of the rock.

2.5. Experiment on the basalt associated microbial community

2.5.1. Mobilisation of manganese

Though reduction refers to dissolution by change in oxidation state, we have used the word mobilization to depict both reduction and dissolution. The experiment to demonstrate the mobilization of Mn from oxide coatings on basalt by bacteria resident on it was conducted in 25 ml screw-capped tubes containing rock fragments of dimensions $0.86\text{-}9.07 \times 0.85\text{-}5.91$ mm and 20 ml of liquid medium as given in [Table 1](#). The experiment was incubated in triplicate at 4 ± 2 °C in the dark for 30, 60, 90, 120 and 150 d. After every 30 d of incubation, tubes from experiment and respective controls were sacrificed for the analyses. The dry weights of the rock fragments were determined after drying to constant weight at

105 °C.

2.5.2. Microbial growth upon incubation

Diluted samples were sonicated as described in section 2.4 and total counts (TC) were determined both before and after incubation. The cells were counted from 20 µl aliquots of the sonicated samples immediately using the Neubauer counting chamber or were preserved with 2% final concentration of buffered formalin prior to counting. For every sample, 50 small squares at 400X magnification were counted using a Nikon 50i bright field microscope (Tokyo, Japan) for determining the average number of cells.

2.5.3. pH and Eh

The pH measurements were carried out using Thermo Orion Triode 3-in-1 pH electrode and Eh (mV) using Thermo Orion Epoxy body sure flow, combination redox/ORP electrode. The above electrodes were calibrated using reference solutions prior to measurements to ensure accurate readings. The calibration of the pH meter (Thermo Orion 3-star benchtop pH/mV/Temp. Meter) was as per the protocol given in the user's guide, Thermo Electron Corporation (2005). The calibration for pH was done using buffer solutions of known pH 4, 7, and 10. For Eh, the potential of the platinum (Pt) electrode versus the Ag/AgCl reference electrode with KCl electrolyte in ZoBell's solution was measured as a function of temperature. The Eh (mV) readings of the samples were taken for 60 s followed by pH, in triplicate. Eh values (mV) were calculated as described in standard methods [APHA AWWA WEF \(2005\)](#).

2.5.4. Determination of manganese in solution

The Mn in supernatant was analyzed after centrifugation of samples at $5009 \times g$ for 10 min at 4 °C (Sigma 3-k). The procedure for Mn analysis was as described under section 2.2. However, the digestion in this case was under UV instead of acid. To deduce the change in metal concentration 1 ml supernatant from each tube was diluted with 9 ml of Milli-Q water (18.2 Ω resistance) and then acidified to pH < 2 with 30% Suprapur HCl (Merck). The acidified samples were UV digested using high-pressure mercury lamp (705 UV Digester, Metrohm) in quartz cuvettes at 90 °C for one hour. The UV irradiated samples were analysed immediately after cooling or were stored in the dark at 4 °C until analysis. The concentration of Mn in solution was then normalized for the volume of media used and per gram dry weight of rock fragments after correcting for the respective blanks.

2.5.5. Scanning electron microscope and X-ray analysis

The increase in bacterial numbers, cell morphology and details like the presence of exopolysaccharides (EPS) and cell aggregates were visualized under SEM at different magnifications both before and after 150 d of incubation. EDS in conjunction with SEM was done to observe the changes in composition of the surfaces during incubation. The details of the procedures followed are described in section 2.2.

2.6. Screening of Mn(II)-oxidizing bacterial isolates for Mn(IV)-mobilizing activity

Mn-oxidizing bacterial isolates designated R1, R2, R3 and R4 that produced brown colonies on Mn^{2+} amended media plates were screened for Mn-mobilizing activity after growth in 0.01% nutrient broth. Bacterial biomass was harvested by centrifugation, washed with 0.22 μm filter sterilized and autoclaved seawater before mixing with an equal volume of 1.5% agar media containing nutrients (section 2.4) and Mn-oxide (17 g l^{-1}). The melted soft agar was held at 45°C in a water bath, mixed, poured in to sterile screw capped tubes and then allowed to solidify. For controls without inocula, the same procedure was followed. The tubes were incubated at 28 ± 2 °C in the dark for 45 d and constantly observed for the disappearance of Mn-oxides. Mn-reduction was evidenced by comparing the results between experiment and control.

2.7. Phenotypic and genotypic characterization

Cultures were examined for gram-stain reaction, and tested for motility, catalase, oxidase and DNase activity according to [Gerhardt et al. \(1981\)](#). The MOF (Marine oxidation fermentation

medium) test was done with 1% glucose as carbon source. Results from the MOF test were interpreted based on the colour change of the pH indicator bromothymol blue after incubation at 28 ± 2 °C for 72 h (Hugh and Leifson, 1953). The screening of bacterial isolates for amylase and lipase enzymes was done on plates of nutrient agar supplemented with 1% starch and 1% tributyrin respectively (Bairagi et al., 2002). The plates were flooded with Lugol's iodine (1%) after growth for detecting amylase activity. The hydrolysis of starch and tributyrin was observed as clear halos surrounding the colony. Similarly, screening for cellulase producers was on carboxymethylcellulose (CMC) agar (0.2% NaNO₃, 0.1% K₂HPO₄, 0.05% MgSO₄, 0.05% KCl, 0.02% peptone and 1.7% agar) with 1% CMC sodium salt as substrate. Enzyme activity was detected by the use of Gram's iodine reagent (Kasana et al., 2008). For the extracellular caseinase production, bacterial isolates were spot inoculated on soybean-casein digest agar medium (Himedia, Mumbai, India). Regions of enzyme activity were detected as clear halos after the addition of 0.1% amido black in methanol-acetic acid-water mixture in the ratio 30:10:60 (Vermelho et al., 1996).

The procedure followed for genomic DNA isolation, PCR amplification, purification of PCR products and sequencing was as described in Krishnamurthi et al. (2009), except that the sequencing chemistry utilized for 16S rRNA gene was as per recommendations from the manufacturer for ABI 3130 XL analyzer. The universal bacterial primers used for sequencing were 27F (5' AGAGTTTGATCCTGGCTCAG 3'), 685R (3' CATCGCCACTTTACGCAT

CT 5'), 1100R (3' GTTGCTCGCGTTGGG 5'), 533F (5' GTGCCAGCMGCCGCGGTAA 3') and 1492R (3' TTCAGCATTGTTCCATYGGCAT 5'). The 16S rRNA gene sequence of the strains was used as a query to search for similar sequences in the EzTaxon database. The sequence was aligned with 16S rRNA gene sequences of all the related species with validly published names using the CLUSTAL X software (Thompson et al., 1997). The alignment was edited manually. The 16S gene sequence similarities were calculated from the alignment. Gaps at the 5' and 3' ends of the alignment were omitted from further analysis. Evolutionary distance matrices were calculated by using the algorithm of Jukes and Cantor (1969) with the DNADIST program. A phylogenetic tree was constructed using the neighbour-joining method (Saitou and Nei, 1987) available within the TREECON software package (Van de Peer and De Wachter, 1997). Bootstrap analysis was done to assess the confidence limits of the branching. The 16S rRNA gene sequences of the manganese oxidizing (R1, R2, R3 and R4) and heterotrophic (NA1, NA2, NA3 and NA5) bacterial isolates were deposited in the European Molecular Biology Laboratory (EMBL) data library under accession numbers HE681807, HE681809, HE681810, HE681812 for *Bacillus* sp (R1, R3, R4 and NA2

respectively), HE681808 for *Exiguobacterium* sp (R2), HE681811 for *Alcanivorax* sp (NA1), HE681813 for *Staphylococcus* sp (NA3) and HE681814 for *Brevibacterium* sp (NA5).

3. Results

3.1. Microscopic and X-ray characterization

Physical characteristics of the natural basalts mostly showed black coloured coatings with rough surface, numerous pits, fissures and fractures. EDS spot analyses indicated heterogeneity in chemical composition with rough surfaces particularly high in Mn-oxides (Supplementary Table 1). The thickness of the coatings vary from >1-3 mm with greater thickness on irregular surfaces and vice versa on regular surfaces. The solid phase concentration of Mn in the rock bearing Mn-oxide varied from 59.12 to 287.59 mg g⁻¹. Mineralogical investigations showed todorokite [(Na, Ca, Mn)₂Mn₅O₁₂. 3H₂O] as the major and birnesite (Na₄Mn₁₄O₂₇.9H₂O) as the minor Mn mineral. Halite (NaCl) was due to the background of seawater (Fig. 1A). Analysis of chemically synthesized oxide of Mn showed broader peaks corresponding to the mineral vernadite (δMnO₂) (Fig. 1B).

3.2. Culturability of Mn-oxidizers,- reducers and heterotrophs

All CFU formed on Mn-oxidizing media were brown and their average number was 5.47×10^3 CFU g⁻¹. However, none of the CFU (3.18×10^3 CFU g⁻¹) in Mn-reducing medium showed halos. The average yield of heterotrophic bacteria was 7.92×10^3 CFU g⁻¹. The isolates from Mn-reducing plates were unable to reduce Mn-oxide but those from Mn-oxidizing plates were able to reduce Mn-oxide. Mn-oxidizing bacterial isolates R1, R2, R3 and R4 constituted 22, 25, 7 and 46% respectively of the total CFU in Mn²⁺ amended media. Whereas, isolates NA1, NA2, NA3 and NA5 representing the heterotrophs constituted 30, 25, 19 and 25% respectively of the total CFU in non-metal amended media. Bacterial colonies on 1% nutrient agar medium for heterotrophs were larger (3-5 mm) compared to colonies on 0.01% nutrient agar medium amended with Mn²⁺ and the other medium for reducers containing Mn-oxides (>1-3 mm). The colonies were mostly circular, convex and opaque with smooth edges on the different types of media used. Bacterial colonies developing on Mn(II) supplemented medium were however brown, suggesting that they promoted Mn-oxidation.

3.3. Experiment on the basalt associated microbial community

3.3.1. Mobilization of manganese

The mobilization rate of Mn was maximum at the end of 150 d incubation. In the experiment, the rate of Mn mobilization in 'G⁺' was 1.76 $\mu\text{g g}^{-1} \text{d}^{-1}$ and 0.17 $\mu\text{g g}^{-1} \text{d}^{-1}$ in 'G⁻' media (Fig. 2). The experimental rates in 'G⁺' were 13 times and in 'G⁻', 4 times more than the respective azide poisoned controls. Compared to heat killed controls, the respective experimental rates in 'G⁺' were 16 times and in 'G⁻', 24 times more.

3.3.2. Bacterial growth

TC increased by more than an order of magnitude in the presence of added glucose and less than an order of magnitude in the absence of added glucose (Fig. 3). It ranged from 1.63×10^6 to 6.71×10^7 cells g^{-1} in 'G⁺' and 3.52×10^7 cells g^{-1} to 1.41×10^7 cells g^{-1} in 'G⁻' incubations. The maximum cell counts in the presence of added glucose occurred on the 120 d of incubation (6.71×10^7 cells g^{-1}) and in the absence of added glucose (1.41×10^7 cells g^{-1}) on the 30 d. In the azide poisoned control the counts ranged from 4.59×10^6 to 2.01×10^7 cells g^{-1} in the 'G⁺' and 4.27×10^6 cells g^{-1} to 2.61×10^7 cells g^{-1} in the 'G⁻'.

3.3.3. Variation in pH and Eh

Notable difference in pH and Eh was not apparent between the experiments and the corresponding controls. The pH decreased from 8.1 to 7.2 in the 'G⁺' and from 7.9 to 7.2 in the 'G⁻' (Supplementary Fig. 2A). The Eh shifted from positive to negative redox potentials toward the end of incubation (150 d). It varied from 117 to -116 mV in the 'G⁺' and 137 to -118 mV in the 'G⁻' incubations (Supplementary Fig. 2B).

3.3.4. SEM and EDS analysis

Compared to the natural samples (Fig. 4 A, B), the textural and chemical composition of laboratory incubated basalt fragments showed numerous microbial cells adhering on the surface. The cells in medium with added glucose varied in morphology after incubation. The size of the cells increased from $0.5 \pm 0.1 \mu\text{m}$ to $5.0 \pm 1 \mu\text{m}$. The shape of the cells varied from circular to oval on the initial day to long slender rods at the end of 150 d (Fig. 4F). The cells attached to the rock surfaces produced

EPS in 'G⁺' medium (Supplementary Fig. 3). In contrast, in 'G⁻' medium, there was no EPS production, variation in cell size or change in morphology (Fig 4D). Compositional analyses of basalt fragments after incubation showed the loss of Mn and K and gain of Na and Mg in the experimental tubes (Supplementary Table 1). On the other hand, the basalt fragments from the controls appeared unchanged and seemed similar in texture and composition to the natural samples (Fig. 5). These observations strongly suggest the bacterial community's participation in the mobilization of Mn.

3.4. Experiment on the Mn(IV)-mobilizing activity of Mn(II)-oxidizing bacterial isolates

Demonstration of Mn(IV)-mobilizing activity with selected isolates of Mn(II)-oxidizing bacteria showed isolate R2 to be positive for Mn-oxide mobilization/reduction. Mobilization was evident from the disappearance of the Mn-oxides (Supplementary Fig. 4).

3.5. Characterization and identification of bacterial isolates

Of the 8 representative bacterial isolates identified, all were Gram-positive except isolate NA1. Tests for extracellular enzyme activity showed isolate R1 to be positive for 6/7 different enzymes that were screened. Positive enzyme test included those for catalase, oxidase, amylase, caseinase, lipase and cellulase. Isolates R2, R3, R4 and NA1 showed activity for 5 different enzymes. Tests for oxidative fermentative reaction showed isolate R1 as fermentative and the rest oxidative in the metabolism of glucose (Supplementary Table 2).

Based on phylogenetic analysis, each of the above bacterial isolates could be assigned to one of three bacterial phyla: *Firmicutes*, *Actinobacteria* and *Proteobacteria*. Half of the isolates were identified as *Bacillus* sp., with the rest, one each belonged to the genus *Exiguobacterium*, *Staphylococcus*, *Brevibacterium* or *Alcanivorax* sp. A neighbor-joining phylogenetic tree obtained with Jukes-Cantor one-parameter model (Fig. 6) showed that strain NA1 forms a separate cluster with *Alcanivorax dieselolei* B-5^T with maximum sequence similarity of 99.7%. On the other hand, strains NA2 and NA3 showed 100% sequence similarity with *Bacillus flexus* IFO 15715^T and *Staphylococcus sciuri subsp. sciuri* DSM 20345^T respectively. Isolate NA5 fell in 99.6% sequence similarity with *Brevibacterium epidermidis* NCDO 2286^T. Isolates R1 and R4 resembled to *Bacillus firmus* NCIMB 9366^T with sequence similarity of 93.6%. Isolates R2 and R3 formed clusters with *Exiguobacterium indicum* HHS31^T and *Bacillus aryabhatai* B8W22^T with maximum sequence similarity of 99.4% and 99.2% respectively.

4. Discussion

The deep-sea hydrothermal plumes at CR (Quadfasel et al., 1997; Ray et al., 2012) exert an influence on the geochemical characteristics of the water column and support unique forms of microbial life. They thereby offer favourable conditions for the growth and activity of metal immobilizing/mobilizing microorganisms. The concentration of Mn in basalt used in the present study varied between 5.9 to 28.76%. These values agree with the range of those reported (25.5 to 65.2%) from the Galapagos spreading axis (Moore and Vogt, 1976). The lower enrichment of Mn in the CR samples compared to that found at the Galapagos could be attributed to the greater distance of the sampling point from the potential volcanic sources. Also, we observed a large number of Mn-oxidizing bacteria associated with the Mn-oxide coatings on basalt surfaces. The culturability of Mn-oxidizing bacteria from these samples equaled that of general heterotrophic bacteria. Their colony characteristics except for the colour were similar on medium amended and unamended with Mn. These results agree with the reports of Ehrlich et al. (1972); Schutt and Ottow (1978) on culturability of Mn-oxidizing bacteria from manganese nodules. The mobilization of Mn was evident despite the non-detectable number of reducers on Mn-oxide plates. Of the four representative Mn(II)-oxidizing bacterial isolates one was positive for Mn-mobilization. These observations suggest that mobilization of Mn from the basalt surfaces could be mediated by bacteria that could either oxidize Mn(II) or reduce Mn-oxide, depending on the ambient conditions. The current results agree with the report of Bromfield and David (1976) on mobilization of Mn from Mn-oxide by *Arthrobacter* sp. that had the ability to either mobilize or immobilize Mn, depending on conditions. Furthermore, Thamdrup (2000) reported on different strains of Mn and Fe oxidizing *Bacillus* sp. that were able to reduce oxidized forms of Fe and Mn under anaerobic conditions. Similarly, de Vrind et al. (1986) reported on Mn-oxide reduction by the vegetative cells of Mn-oxidizing *Bacillus* SG-1. The different bacterial genera so far known to reduce Mn-oxides include *Bacillus*, *Deferribacter*, *Thermoanaerobacter*, *Fervidobacterium*, *Desulfovibrio*, *Shewanella*, *Achromobacter*, *Enterobacter* and *Salmonella* sp (Ehrlich, 1980; Pak et al., 2002; Das et al., 2011).

The bacterial communities living in basaltic environment may therefore shift to high affinity transport systems (Geesey and Morita, 1979) as they contain no apparent energy reserves (lipids and poly- β hydroxyl butyrate) required for the maintenance of life (Morita, 1988). The nutritional requirements of such microbes are met by the production of suitable exoenzymes or depend upon syntrophism for their energy needs. In our study, the bacterial isolates expressed multiple exoenzymes with different growth substrates and also oxidized Mn. It suggests that bacteria

associated with the basalt surfaces are highly efficient in adapting to the nutrient poor environment and in meeting the metabolic requirements. Our results agree with that of [Lorenz et al. \(2006\)](#) and [De Souza et al. \(2006\)](#) on the tolerance of bacteria to heavy metals and their ability to express multiple enzymes.

Under low nutrient availability, bacteria lose the ability to multiply but energy-yielding mechanism tends to remain intact ([Novitsky and Morita, 1978](#)). Such an adaptation to nutrient concentrations was observed in the current study under experimental conditions. The onset of growth was faster in 'G⁻' and slower in 'G⁺' media. The faster growth in 'G⁻' could be because the laboratory conditions simulated the natural conditions more in terms of temperature and ambient organic carbon concentration (glucose). In contrast, the delay in the onset of growth with 'G⁺' could be the adaptation period required by those organisms in a non-energized state. The higher mobilization of Mn in the presence of glucose could have been because the active cells were chemotactically more active than the non-energized cells ([Torrella and Morita, 1981](#)). The oxidation of glucose by cultured bacteria from basalts further confirms that microbial cells indigenous to the rock surfaces couple the oxidation of glucose to the reduction of Mn-oxide. Our results agree with [Roh et al. \(2002\)](#) on the utilization of glucose by heterotrophic bacteria as an electron donor for the reduction of Mn oxide and [Madgwick \(1987\)](#) and [Baglin et al. \(1992\)](#) for the solubilisation of Mn from ores.

Although the pH of seawater is typically in the range of 7.5 to 8.4, a decrease in pH may occur during bacterial growth under experimental conditions. The drop in pH is strongly influenced by the growth of bacteria ([Li et al., 2006](#)) and facilitates the release of metal cations by promoting an increase in the solubility of metal oxides ([Burkhardt et al., 2011](#)). Following incubation, the pH of the media in the present study decreased from 8.1 to 7.2 in the 'G⁺' and from 7.9 to 7.2 in the 'G⁻'. Our results agree with [Rusin and Ehrlich \(1995\)](#) on Mn-oxide reduction by bacteria in the pH range of 6 to 8. This reduction could be significant because the bacterial groups associated with basalt participate in the cycling of Mn and vary in their tolerance to different pH levels and metal concentrations. The redox cycling of Mn could also be bacterially mediated under optimal Eh conditions ([Webb et al., 2005](#)) because of the general relationship between pH and Eh ([Pareuil et al., 2008](#)) wherein the growth and metabolic activity of bacteria either increases or decreases the pH of the surrounding. The increase in H⁺ activity (drop in pH) leads to Eh decrease and consequently to the increased mobilization of metallic elements and vice versa with decrease in H⁺ and an increase in

Eh (DeLaune and Reddy, 2005; Pareuil et al., 2008). In our study, the Eh varied between +117 to -116 mV in media with added glucose and +137 to -118 mV in media without added glucose. The Eh values indirectly show that some portions of Mn and/or Fe compounds are present in their reduced state and large portion in their oxidized or bound state. The results most likely suggest that bacteria present in the optimal pH and Eh range are responsible for Mn-oxide reduction.

Bacteria exhibit morphological variation in response to heavy metal stress, nutrient deprivation and excess nutrient availability (Shehata and Marr, 1971; Amy and Morita, 1983; Antony et al., 2011). Low metal concentrations i.e., <10 μ M stimulate bacterial growth (Sujith et al., 2010) but exert a toxic effect on them at higher concentrations (Antony et al., 2011). Some bacteria adapt to high metal concentrations just by merely decreasing their cell surface area, as, for instance, by increasing their cell size and by forming cell aggregates (Sujith et al., 2010; Antony et al., 2011). In the present study, the cells that were tiny and coccoidal in shape on initial day of incubation became long slender rods in medium with added glucose at the end of incubation. However, there was no variation in size or change in morphology of cells in medium without added glucose. These observations agree with those by Shehata and Marr (1971) with respect to an increase in bacterial size and numbers during growth in the presence of 0.1% glucose. Further, the presence of EPS associated with the bacterial cells on medium amended with glucose explains the ability of bacteria to withstand heavy metal stress and promote Mn dissolution. The EPS or slime layers have been shown earlier to help bacteria in attaching to the surfaces, alleviating the toxic effect of metals and in the dissolution of minerals (Geesey and Jang, 1989; Vandevivere and Kirchman, 1993). Following incubation, EDS analysis of the basalt surfaces showed the loss and gain of elements in addition to colonization by abundant rod-shaped bacteria in the experiment relative to the controls. It thus indicates the direct participation of bacteria in the mobilization of Mn from the basalt surfaces. The mineralogy of the Mn-oxide coatings on basalt showed characteristic peaks for Mn minerals todorokite and birnessite apart from background peak for halite. The present results agree with the reported mineralogy of Mn-oxides from the mid-ocean ridge systems (Rao and Pattan, 1989).

The identification of culturable bacteria by 16S rDNA method showed taxonomic affinities to *Bacillus*, *Exiguobacterium*, *Staphylococcus*, *Brevibacterium* and *Alcanivorax* sp. Among them, *Bacillus* represents the predominant genus, which signifies its importance in the redox cycling of Mn in the CR ecosystem. Our results agrees with the reports of Rusin et al. (1991), Ehrlich (1993) and Boone et al. (1995) on the capacity of *Bacillus* to reduce Mn and Fe under aerobic and anaerobic conditions. The induction of MnO₂-reductase system in *Bacillus* by Mn²⁺ (Trimble and Ehrlich,

1970; Chandramohan et al. 1987) further explains its importance in controlling the redox speciation of Mn. The lack of detection of other known Mn reducing bacterial groups may not indicate their absence in our samples but may have been due to lesser abundance or lack of some required nutrients in the culture medium employed. However, *Exiguobacterium* sp a member of the *Firmicutes* was found associated with the Mn-oxide surfaces of basalt. Apart from Mn(II)-oxidation this strain exhibited the property of Mn-oxide reduction under laboratory conditions in the present study. Their occurrence has also been reported from the oligotrophic seas to the deep-sea hydrothermal vents (Vishnivetskaya et al., 2009). They could be candidate organisms for catalyzing the oxidation/reduction of Mn in the ridge ecosystem because of their potential for tolerating and reducing heavy metals in solution (Okeke, 2008).

Members of the genus *Staphylococcus* show a diverse spectrum of metabolic activities, which is indicative of the ability to adapt to various environmental niches. They are efficient members in the mobilization of Mn from low grade ores (Das et al., 2012). They also contain the enzymes manganese superoxide dismutase and urease with Mn and Ni as the cofactors respectively (Horsburgh et al., 2002). On the other hand, *Brevibacterium* has specific requirement of Mn for the activity of the enzyme ribonucleotide reductase (Willing et al. 1988). It is efficient in energy dependent Mn uptake (Schmid and Auling, 1987). The metabolic flexibility and the versatile nature of these bacterial isolates therefore could contribute to the biogeochemical cycling of redox sensitive elements like Mn/Fe and thereby the fate of several other elements in the ridge ecosystem. The occurrence of *Alcanivorax* sp a marine petroleum oil-degrading bacterium known for alkane metabolism (Sabiroya et al., 2006) in the CR environment may be due to their role in Mn cycling as was reported in Arctic sediments (Xuezheng et al., 2008).

The results of the present study suggest that the Mn(II)-oxidizing bacteria associated with basalt from the CR actively participate in promoting Mn-accretion at ambient concentrations of organic carbon and Mn-mobilization under conditions of organic carbon enrichment. This could be biogeochemically significant because the redox speciation of Mn controls the fate of several other associated elements in the ridge ecosystem by consuming or releasing electrons or protons. Experiments conducted under microaerophilic and anaerobic conditions would throw more light on particle associated bacterial contribution from the water column above to the redox changes in Mn chemistry in ridge ecosystems.

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Tables and Figures

Table 1. Experimental set up to measure rate of Mn mobilization by indigenous bacterial

Communities on crust at 1atm and 4±1°C

	Blank*				Heat killed* controls		Azide treated* controls		Experiments*	
	B1	B2	B3	B4	Hk1	Hk2	Az1	Az2	E1	E2
Glucose 10% (ml) (final 0.1%)	-	0.2	-	0.2	-	0.2	-	0.2	-	0.2
Azide 1M (ml) (final 15 mM)	-	-	0.3	0.3	-	-	0.3	0.3	-	-
Crust (g)	-	-	-	-	~1	~1	~1	~1	~1	~1
Sterile seawater (ml)	20	19.8	19.7	19.5	20	19.8	19.7	19.5	20	19.8
Total volume without crust (ml)	20	20	20	20	20	20	20	20	20	20

*Tubes incubated in triplicate. Heat killed refers to rock fragments that were steam-sterilized at 15 lbs per in² and 121°C for 15 min.

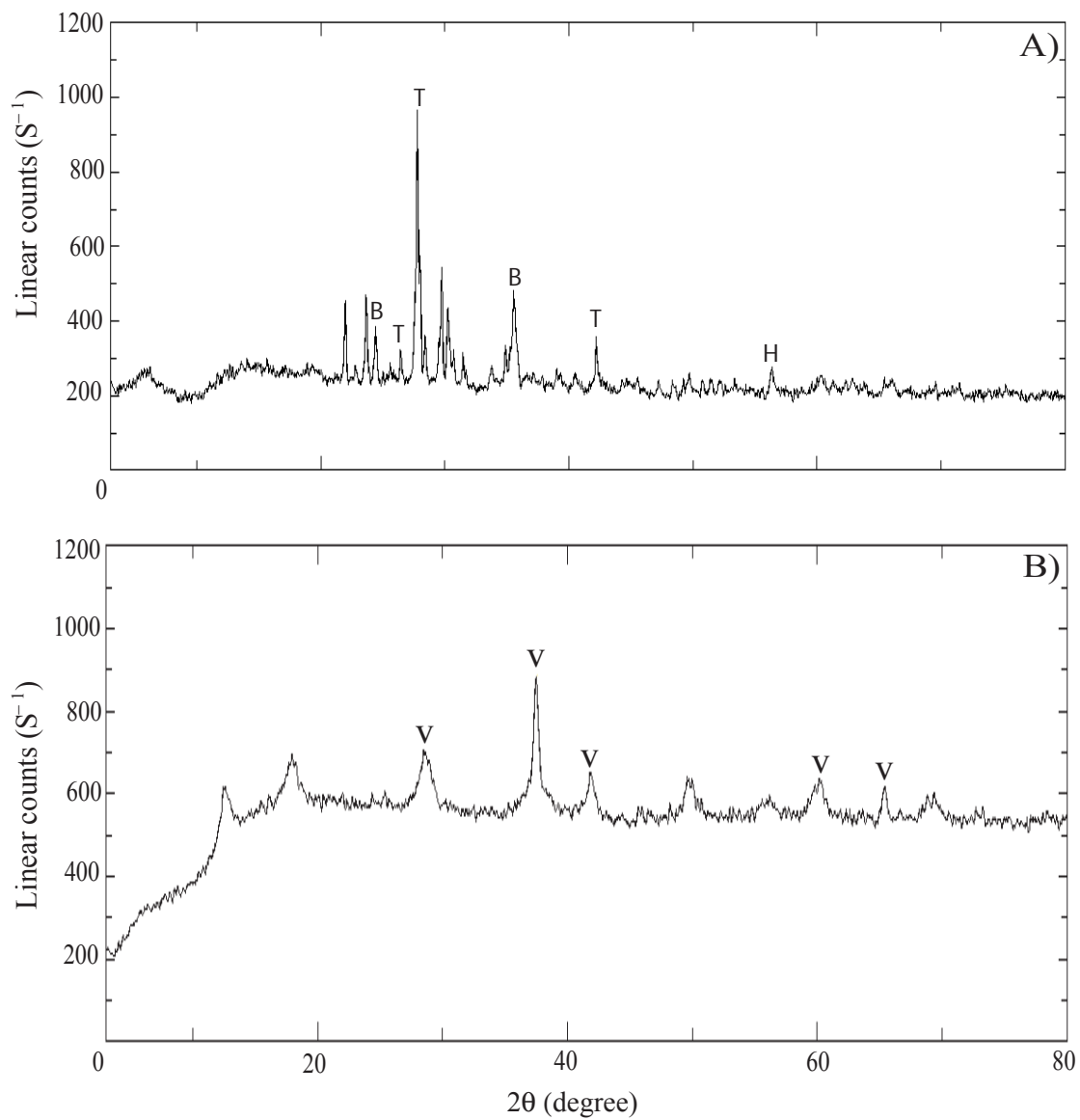


Fig. 1. X-ray diffraction analysis results. A) Mn-oxide coatings removed from basalt surface and B) chemically synthesized Mn oxide (Peak labels B: birnessite, H: halite, T: todorokite and V: vernadite).

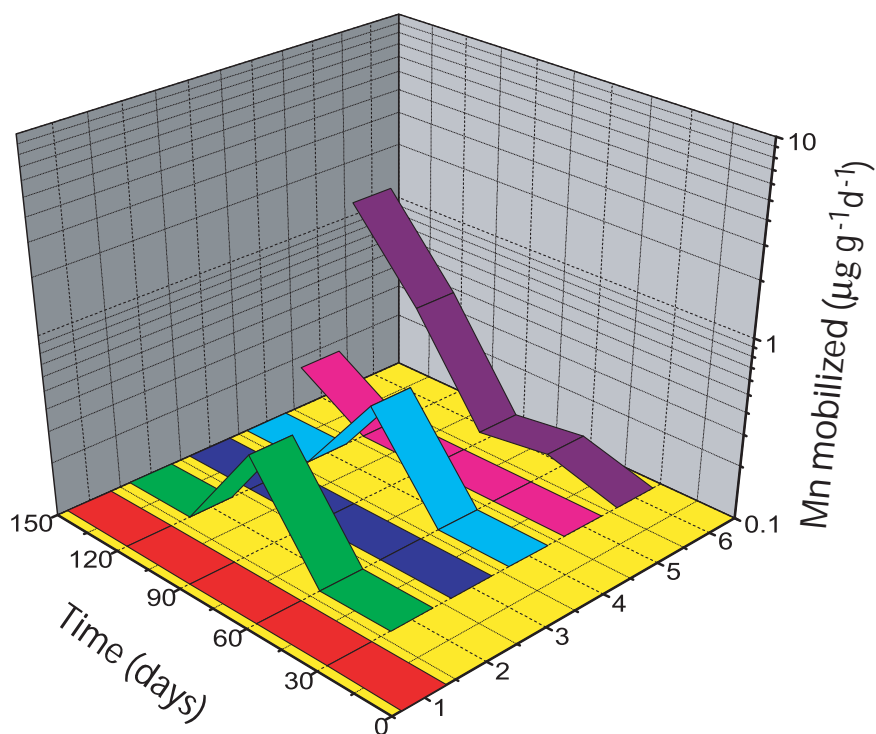


Fig. 2. Mobilization rate of Mn from basalt by indigenous microbial communities. Numbers 1-2 in the secondary X-axis indicates heat killed controls, 3-4 azide poisoned controls and 5-6 experimental samples without and with added glucose respectively. The values in the figure are mean values \pm SD, $n = 3$.

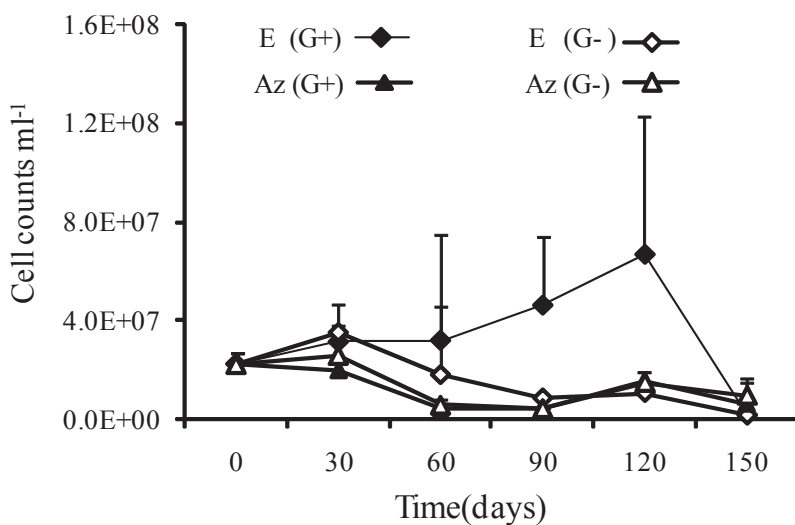


Fig. 3. Variation in cell numbers in relation to the mobilization of Mn (E: experiment, Az: azide poisoned control, Hk: heat killed control, G^+ : with added glucose and G^- : without added glucose).

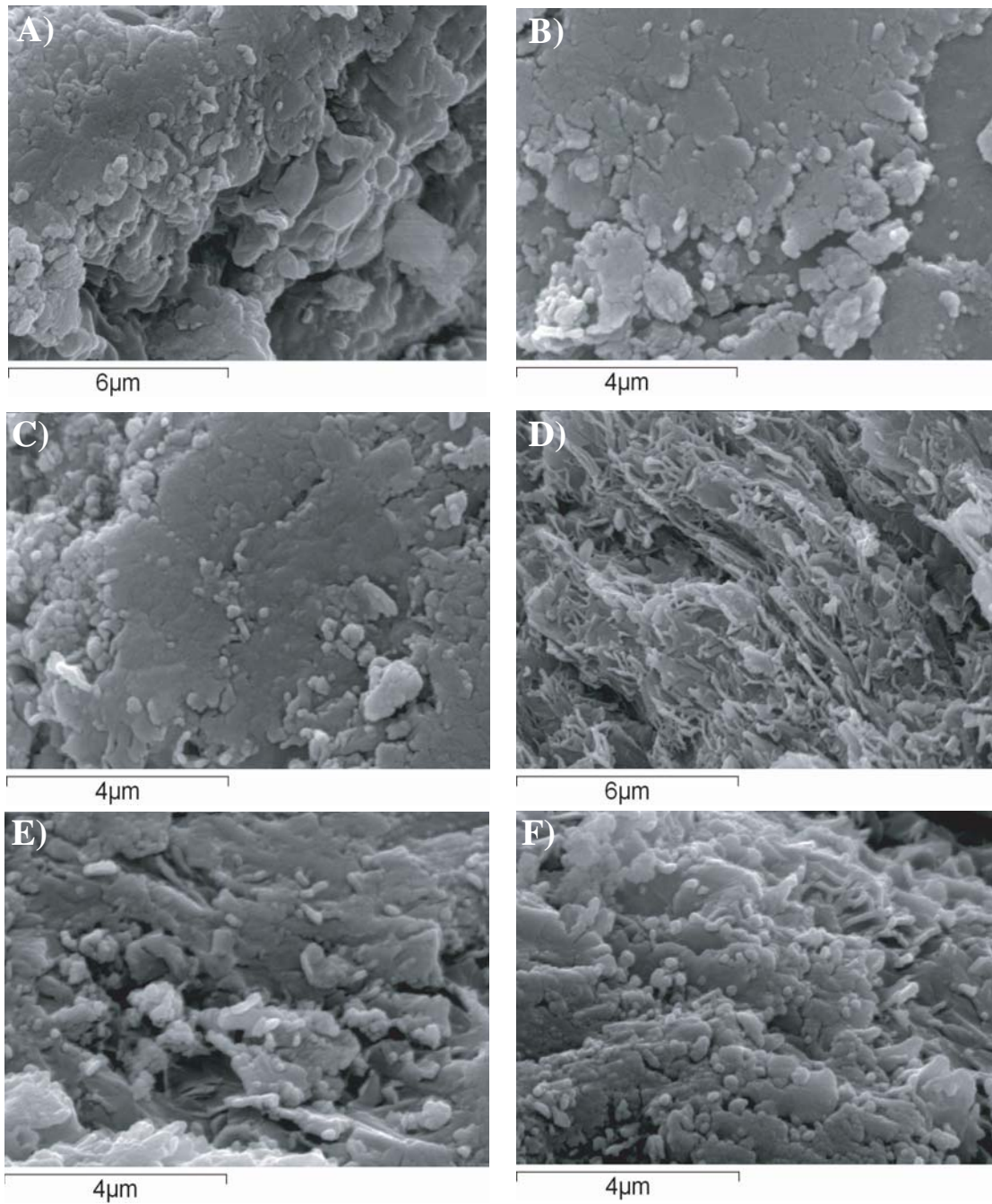


Fig. 4. SEM images of bacterial cells associated with basalt. Image initial day A) control and B) experiment. SEM final day C) control (G⁻) D) experiment (G⁻), E) control (G⁺), F) experiment (G⁺). (Legend G⁺: with added glucose and G⁻: without added glucose).

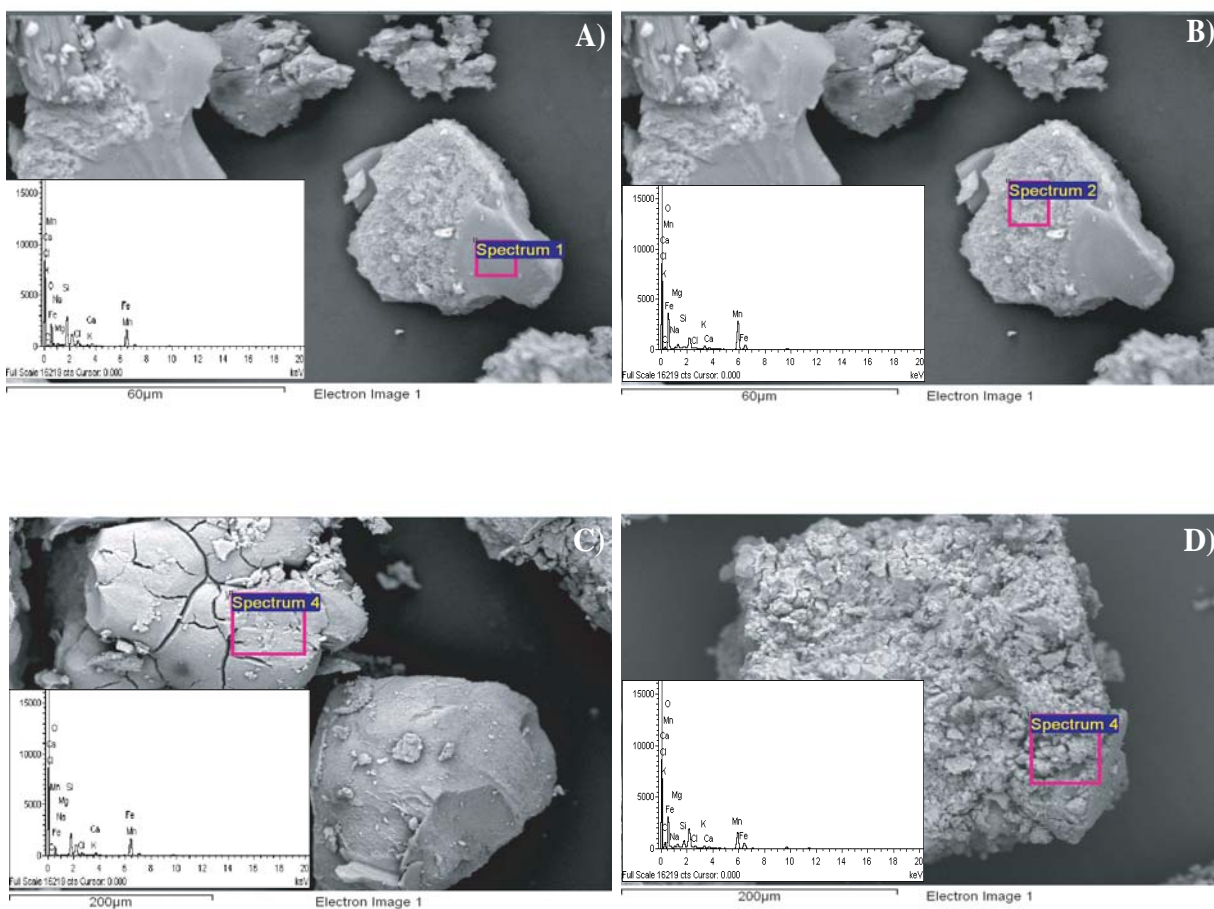


Fig. 5. SEM images show the surface texture and EDS spectra the chemical composition of basalt fragments before (A, B) and after incubation (C, D) for 150 d. The rough surface show higher concentration of Mn relative to smooth surface and tend to decrease with time on rough surface.

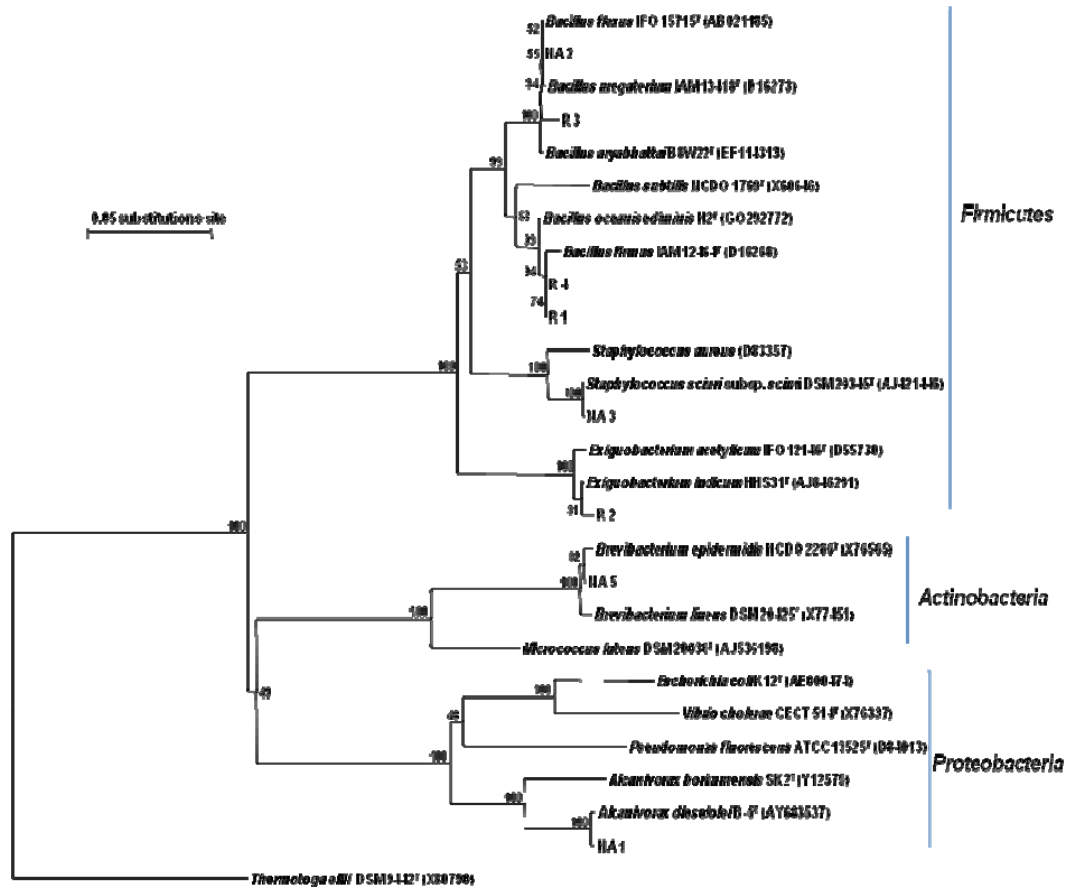


Fig. 6. Neighbor-Joining phylogenetic tree of bacterial isolates constructed using Treecon software showing phylum-level diversity. The bootstrap values were calculated as percentage of 1000 replicates. The bar indicates 0.05 substitutions per site. The sequence from *Thermotoga elfii* was taken as outgroup.

SUPPLEMENTARY FILES

Table S1. EDS point analysis of Mn oxide coatings on basalt.

Element (Wt %)	Random EDS points				
	Before incubation			After incubation	
	1	2	3	4	5
Na	5.86	4.52	5.47	7.45	6.23
Mg	2.44	4.48	3.82	2.75	3.54
Si	0.45	9.63	5.6	5.02	3.01
Cl	8.46	8.21	7.3	9.02	9.66
K	1.40	0.99	1.02	0.84	0.69
Ca	0.81	1.53	0.98	1.17	0.61
Mn	32.62	40.87	29.87	23.0	16.13
Fe	1.82	4.03	3.60	4.81	4.97
O	46.14	25.74	42.34	45.94	55.16
Total	100	100	100	100	100

Table S2. Phenotypic characteristics of culturable bacteria associated with basalt

Isolates	Gram ±	KOH	Motility	OF Test	Catalase	Oxidase	Amylase	Dnase	Protease	Lipase	Cellulase
R1	+	NT	Motile	F	+	+	+	-	+	+	+
R2	+	NT	Motile	O	+	+	+	-	+	+	-
R3	+	NT	Motile	O	+	+	-	-	+	+	+
R4	+	NT	Motile	O	+	-	+	-	+	+	+
NA1	-	T	Non-motile	O	+	-	+	-	+	+	+
NA2	+	NT	Non-motile	O	+	+	-	-	-	-	-
NA3	+	NT	Motile	O	+	+	-	-	-	-	-
NA5	+	NT	Motile	O	+	+	-	-	-	-	-

Isolate numbers R1 to R4 represent Mn oxidizing bacteria and NA1 to NA3 and NA5 represent heterotrophic bacteria. OF = oxidation fermentation, O= oxidative, F= fermentative. NT= no thread formation and T = thread formation in KOH test.

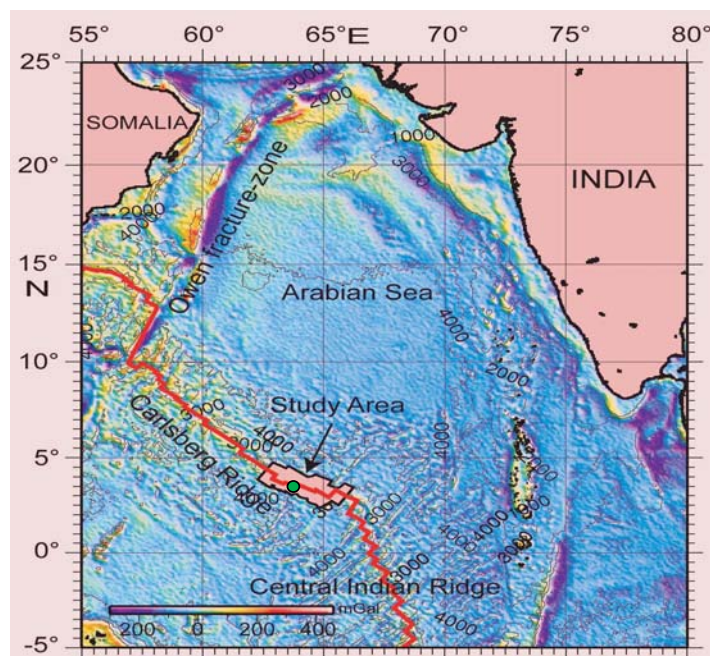


Fig. S1. Map of the study area (courtesy Kamesh Raju et al. 2008). The filled circle in green depicts the location of basalt sampling.

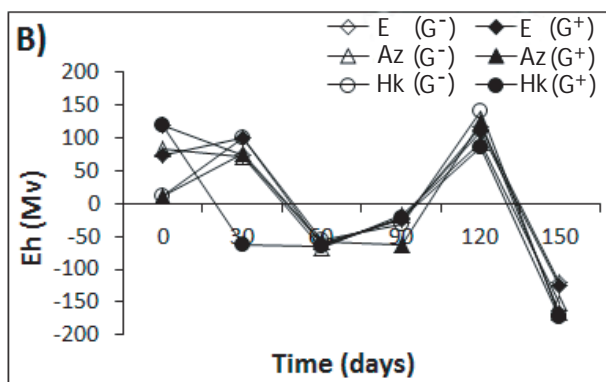
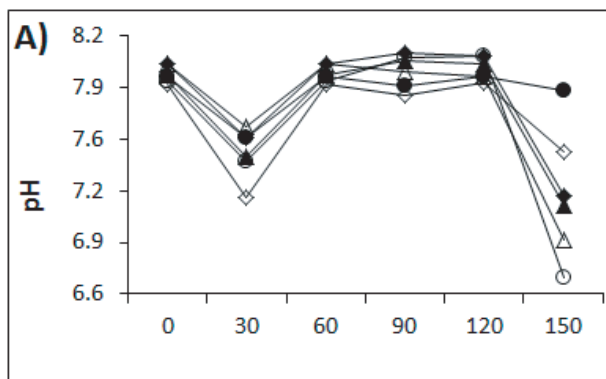


Fig. S2. A) Variation in pH and B) Eh (mV) in relation to the mobilization of Mn (E: experiment and Az: azide poisoned control, G⁺: with added glucose and G⁻: without added glucose).

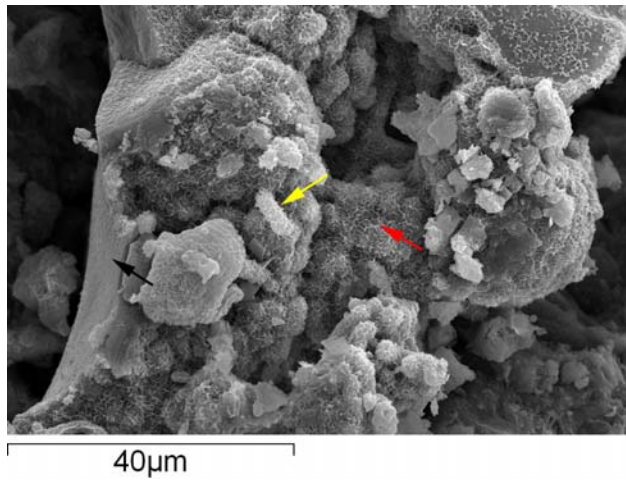


Fig.S3. Exopolysaccharides associated with the bacterial cells on basalt surfaces. Arrow in yellow indicates the bacterial cell, red the exopolysaccharide and black the basalt.

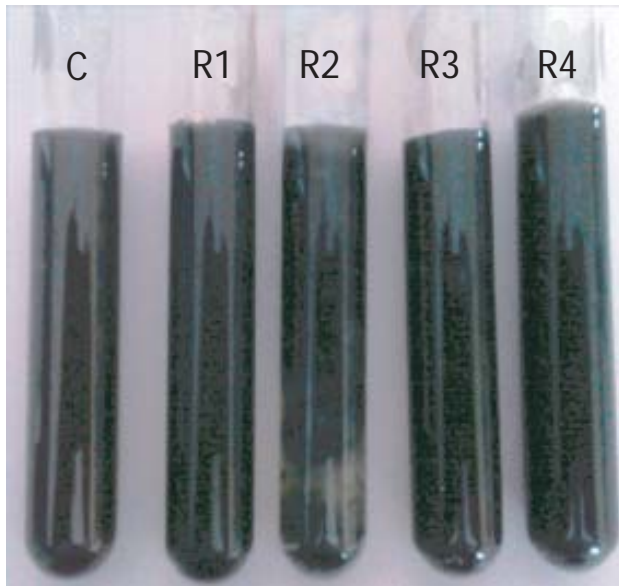


Fig.S4. Reduction of synthetic Mn-oxide by Mn(II)-oxidizing bacterial isolates (C: control, R1, R3 and R4: negative for Mn reduction, R2: positive for Mn reduction).