

Immobilization of Nickel by Bacterial Isolates from the Indian Ridge System and the Chemical Nature of the Accumulated Metal

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Bacterial isolates from Carlsberg-Ridge waters were tested for their ability to immobilize Ni. For this, test cultures were suspended for 60 d in seawater with and without added Ni at concentrations of 10 to 10,000 μM . At an initial concentration of 100 μM , isolates CR35 and CR48 caused a 89.8 and 6.95 μM decrease in dissolved Ni at $28 \pm 2^\circ\text{C}$ and a 14.75 and 6.38 μM at $3 \pm 1^\circ\text{C}$. Analyses showed the contribution of hydroxyl, carbonyl, sulfide and phosphoryl groups in Ni binding. The study proves that Ni immobilization can occur even in the absence of Mn and suggests their plausible role in the ridge ecosystem.

Keywords Carlsberg Ridge, energy dispersive x-ray, immobilization, infrared spectroscopy, nickel, scanning electron microscope, X-ray powder diffraction

INTRODUCTION

Marine bacteria are indigenous to an environment characterized by a small amount of organic matter and a large amount of inorganic salts (Gonye and Jones 1973). Like any complex system, the marine environment also serves as a unique niche for many specialized life forms and supports a diverse array of microorganisms (Prescott et al. 1999). The hydrothermal vent fields and the nearby water columns are a source for reduced inorganic ions. Some of the microorganisms in these habitats may oxidize certain reduced form of metals (Jannasch and Wirsén

1979; Jannasch 1984; Mandernack and Tebo 1993) and are able to derive energy in oxidizing relatively large amounts of these reduced metal species to fulfil their metabolic needs (Ehrlich 1976, 1978).

The tectonically active Carlsberg Ridge in the Arabian Sea is such a habitat known to support chemosynthesis and can serve as sink for various metal ions (Mn, Fe, Co, Ni, etc). Besides the ridge sources, mining and metallurgical activities also contribute to an increase in the level of trace metals in the sediment and aquatic environments (Gonzalez et al. 1997). The average global concentration of Ni in the oceans is 0.6 ppb ($\mu\text{g}/\text{kg}$), in soils 16 ppm ($\mu\text{g}/\text{g}$), in lakes and in rivers 1.0 ppb (Nriagu 1980), and in sediments from southwest Carlsberg Ridge 21–251 ppm (Pattan and Higgs 1995).

Metal ion homeostasis is very crucial for optimum growth and survival of microorganisms. In order to meet the physiological needs for these nutritionally required trace elements, microorganisms have special uptake mechanisms for some of them. Once in the cell, the elements may be sequestered. Excess concentrations of these elements in ionic form in cells may, however, be toxic (Schmidt and Schlegel 1989). The mechanisms such as intracellular sequestration of metals by proteins and polyphosphate granules may be a means of metal storage or detoxification (Keasling 1997). However, intracellular toxicity of metals is mainly overcome by metal efflux.

Excess metal uptake may also be controlled in some organisms by binding of metal ions to slime consisting of exopolysaccharide (Helmann et al. 2007). This in turn limits the direct effect of metal ion on cells. As a result, the cells survive and continue to perform their normal metabolic activities in the presence of excess metal concentration. In the present study, we attempted to understand the nature of interaction between bacteria and nickel and their ability to immobilize the metal.

Our observation is applicable to sediments and other organically depleted natural ecosystems where the metal concentrations vary from a few ppm to ppb levels. This is the first study to show that, bacterial isolates from a ridge system capable of immobilizing Mn can also immobilize Ni. The oxides of Mn are otherwise generally known to stimulate the immobilization of other metals including Ni. The study demonstrates how immobilization of Ni can be effected even in the absence of manganese.

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METHODS AND MATERIALS

Organism and Growth

The Mn oxidizing bacterial isolates CR35 and CR48 employed in the present study were retrieved from Carlsberg Ridge waters (Fernandes et al. 2005). The isolate CR35 was obtained from a water depth of 3040 m at 3°35'N Lat and 64°07'E Long and CR48 from a water depth of 3510 m at 4°31'N Lat and 62°33'E Long. The isolate CR35 was capable of growing in media prepared in distilled water with 0.5% NaCl, whereas, seawater was mandatory for the growth of CR48. The isolates when grown in the presence of Ni in seawater showed no additional growth requirement. Therefore, the incubation experiment for Ni immobilization was done only with metal salt (NiCl₂ · 6H₂O, Merck) in aged seawater (pH 7.8).

Inocula of the bacterial isolates were grown in 0.01% nutrient broth (Merck) containing 15g peptone, 3g yeast extract, 6g sodium chloride and 1g D(+) glucose in a litre of seawater at 3 ± 1°C. They were harvested by centrifugation at 8000 rpm for 10 min at 4°C when the cell density reached an OD₆₀₀ of 1.5. Cell pellets were washed twice with sterile saline, re-suspended and used for the inoculation of experiment tubes. The stock cultures were maintained on 25% nutrient agar slants at 4°C.

Immobilization of Nickel

To quantify immobilization of Ni by whole cells, the experiment was carried out in 15 ml screw capped test tubes with Ni amendments of 10, 100, 1000 and 10000 μM in seawater containing triphenyl tetrazolium chloride at a final concentration of 0.025%. Controls for each Ni concentration, but without cells were included to correct for chemical oxidation and loss of metal due to adsorption on glassware. The experimental tubes containing 11 ml media were inoculated in triplicate to give an initial cell count of 2.97 × 10⁷ cells ml⁻¹ for CR35 and 3.52 × 10⁷ cells ml⁻¹ for CR48. The tubes were incubated at 28 ± 2°C and 3 ± 1°C in the dark. Samples (1 ml) were removed immediately after inoculation and after 60d of incubation for determining the total count, respiring cell count and Ni concentration in the liquid phase. The total count and the number of respiring cells (pink) were determined after segregation of cell aggregates by sonication at 15 Hz for 3s.

The cells were counted in a Neubauer counting chamber at 400-x magnification with a bright field microscope (Nikon 80i). The formazan in experimental tubes was determined and the values were corrected for the 0d absorbance at the end of the experiment (Fernandes et al. 2005). The OD values for formazan in culture were converted to mg ml⁻¹ formazan using the equation for the line of best fit derived from a standard curve of formazan (Himedia) prepared in methanol. Ni was estimated from the supernatant by a spectrophotometric method (Chester and Hughes 1968) at 460 nm after centrifugation at 8000 rpm for 10 min. For higher concentration >10 μM Ni, supernatant was suitably diluted with distilled water. The experimental

readings were corrected for control to estimate the rate of Ni immobilization.

Effect of Added Ni on Bacterial Growth Rate

The isolates CR35 and CR48 were grown at 100 μM concentration of Ni in seawater together with controls lacking added Ni to study the effect of added Ni on growth of strains CR35 and CR48. The inoculum for the experiment was prepared as above and 0.5 ml of the culture was inoculated in triplicate and was incubated at 28 ± 2°C for 6 d. Daily 20 μl of samples from individual tubes were withdrawn and loaded into Neubauer counting chamber for bright field microscopic (Nikon 80i) count. The cells were examined under 400-x magnification and the counts were expressed as numbers per ml of sample.

Microscopy

The bacterial cells grown in the presence and absence of Ni were harvested by centrifugation at 8000 rpm for 10 min at 4°C after 60 d of incubation. The cell pellet was suspended in sterile 0.85% saline and a drop was placed on small pieces of cover slip and air-dried. The cells were dehydrated by passing the cover slips carrying the cells through 10, 30, 50, 70, 90 and 100% acetone. The cover slips containing the dehydrated sample smears were mounted on an electron microscope stub, sputter coated with Au/Pd. The cells were then examined under a JEOL JSM-5800 scanning electron microscope (SEM). For light microscopy, wet mounts of bacterial cells were made using acid cleaned glass slides and cover slips. Bacterial morphology and cell aggregation were observed at 400-x magnification and photographed with a digital camera and the images processed using software image pro 6.2.

X-ray Diffraction (XRD) and Energy Dispersive X-ray (EDX) Analysis

The bacterial isolates CR35 and CR48 were grown at 28 ± 2°C for 7d in seawater with and without added 1000 μM NiCl₂ · 6H₂O. The respective cultures were then harvested and washed three times with sterile 0.85% saline and once with acetone by centrifugation at 8000 rpm for 15 min at 4°C and air-dried. The samples were powdered with a micro pestle in 2 ml eppendoff tubes.

The XRD pattern (Deplanche and Macaskie 2008) were recorded in a Rigaku x-ray powder diffractometer using monochromatic Cu Kα₁ radiation (λ = 1.54050 Å). The diffraction spectra were recorded over the range of 10° to 100° (2θ) with a step length of 0.02° (2θ). The identification of Ni salts was based on comparison with the joint committee on powder diffraction standards file (1981). An EDX analysis was done on a small portion of intact cells from the above cultures on JEOL JSM-5800 at an accelerating voltage of 15 kV.

Infrared (IR) Analysis

The CR35 and CR48 bacterial isolates grown at room temperature (28 ± 2°C) for 2 d in seawater in the presence of

100 μM added Ni and in its absence were separated by centrifugation at 8000 rpm for 15 min at 4°C (Sigma 3-18K), washed three times with sterile 0.85% saline and once with distilled water and then lyophilized (Heto LL1500 freeze dryer). A known quantity of lyophilized bacterial sample (0.8 mg) and dry potassium bromide (1.75 mg) was placed in a smooth agate mortar and mixed thoroughly. The powder was filled in a micro-cup of 2 mm internal diameter (Naumann et al. 1991). The diffuse reflectance infrared spectrum of the samples was recorded in a Shimadzu FTIR-8201PC spectrophotometer with 60 scans at a resolution of 4 cm^{-1} in the transmission mode (mid infrared region 4000–400 cm^{-1}).

Plasmid Screening

The isolates CR35 and CR48 were grown in 0.1% nutrient broth in tubes with and without Ni (100 μM) amendment and were incubated at $28 \pm 2^\circ\text{C}$. The cells of the early exponential phase (OD_{600} of 1.2) were harvested by centrifugation at 8000 rpm for 10 min at 4°C. The cell pellets were washed twice with physiological saline (0.85%) and were immediately used for the preparation of cell lysates (Kado and Liu 1981). The bacterial lysates were analyzed by agarose gel electrophoresis with agarose concentration of 0.6 and 1% according to the procedure of Meyers et al. (1976). Supermix DNA ladder (0.5 to 33.5/24.5 kb) obtained from Bangalore Genie was used as the reference marker. The gel was stained with ethidium bromide (0.5 $\mu\text{g ml}^{-1}$) for 30 min and photographed after destaining using the gel documentation and analysis system (Biorad).

RESULTS

Nickel Immobilization

The Ni immobilization by both the isolates enhanced was with increasing Ni concentration (Figure 1). CR35 showed a maximum Ni immobilization of 120.7 μM at $28 \pm 2^\circ\text{C}$ (Figure 1A) and 20.3 μM at $3 \pm 1^\circ\text{C}$ (Figure 1B) with 1000 μM test Ni concentration. In parallel, CR48 showed maximum Ni immobilization of 309.5 μM at $28 \pm 2^\circ\text{C}$ and 38.53 μM at $3 \pm 1^\circ\text{C}$ with 10,000 μM test Ni concentration. Nevertheless, at 100 μM added Ni concentration, CR35 immobilized 89.8% of the Ni, at $28 \pm 2^\circ\text{C}$ and 14.75% at $3 \pm 1^\circ\text{C}$, which was the maximum removal observed with this strain. With CR48, similar trend was not observed, rather immobilization was maximum of 19.27% at $28 \pm 2^\circ\text{C}$ at 1000 μM added Ni concentration and 11.9% at $3 \pm 1^\circ\text{C}$ at 10 μM added Ni concentration. The study showed that temperature has an effect on Ni immobilization and was faster at $28 \pm 2^\circ\text{C}$ with both of the test isolates.

Bacterial Growth

The bacterial growth was monitored with Ni to determine the effect of the metal cation on bacterial cells. The bacterial number decreased with increase in Ni concentration. The maximum cell number was $6.63 \pm 4.89 \times 10^7$ cells ml^{-1} with CR35

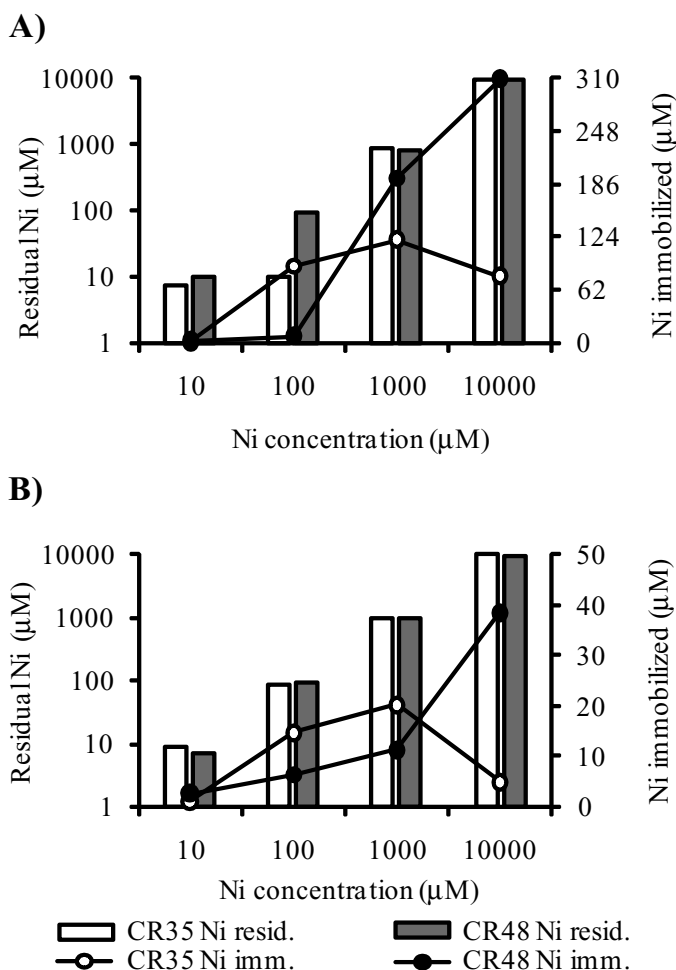


FIG. 1. Effect of temperature on Ni immobilization by bacterial isolates CR35 and CR48. A) Immobilization at $28 \pm 2^\circ\text{C}$ and B) Immobilization at $3 \pm 1^\circ\text{C}$. The legend “Ni imm” represents Ni immobilization and “Ni resid” represents Ni residual. The values are \pm mean, $n = 3$.

at $28 \pm 2^\circ\text{C}$ (Figure 2A) and $6.19 \pm 5.09 \times 10^7$ cells ml^{-1} at $3 \pm 1^\circ\text{C}$ (Figure 2B) in the presence of 10 μM added Ni. However, with CR48, the cell number was maximum at 100 μM and it was $9.32 \pm 2.97 \times 10^7$ cells ml^{-1} at $28 \pm 2^\circ\text{C}$ (Figure 2C) and $3.27 \pm 9.63 \times 10^7$ cells ml^{-1} at $3 \pm 1^\circ\text{C}$ (Figure 2D). The difference in cell number between $28 \pm 2^\circ\text{C}$ and $3 \pm 1^\circ\text{C}$ was 74.7% with CR35, and 85% with CR48. Beyond 100 μM Ni concentration, no increase in cell number was observed, either at $28 \pm 2^\circ\text{C}$ or at $3 \pm 1^\circ\text{C}$, with both the isolates.

The cell counts were comparatively smaller at $3 \pm 1^\circ\text{C}$ than at $28 \pm 2^\circ\text{C}$. The formazan estimation of respiration was maximum (0.18 \pm 0.01 mg ml^{-1}) at 10 μM Ni with CR35 and 0.15 \pm 0.016 mg ml^{-1} with CR48 at 100 μM Ni concentration at room temperature (Figure 2). These results suggest that 100 μM Ni is the threshold concentration for tolerance in both the isolates. The number of respiring cells at 100 μM Ni concentration was $4.26 \pm 4.33 \times 10^7$ cells ml^{-1} at $28 \pm 2^\circ\text{C}$ (Figure 2A) and $9.89 \pm 3.33 \times 10^6$ cells ml^{-1}

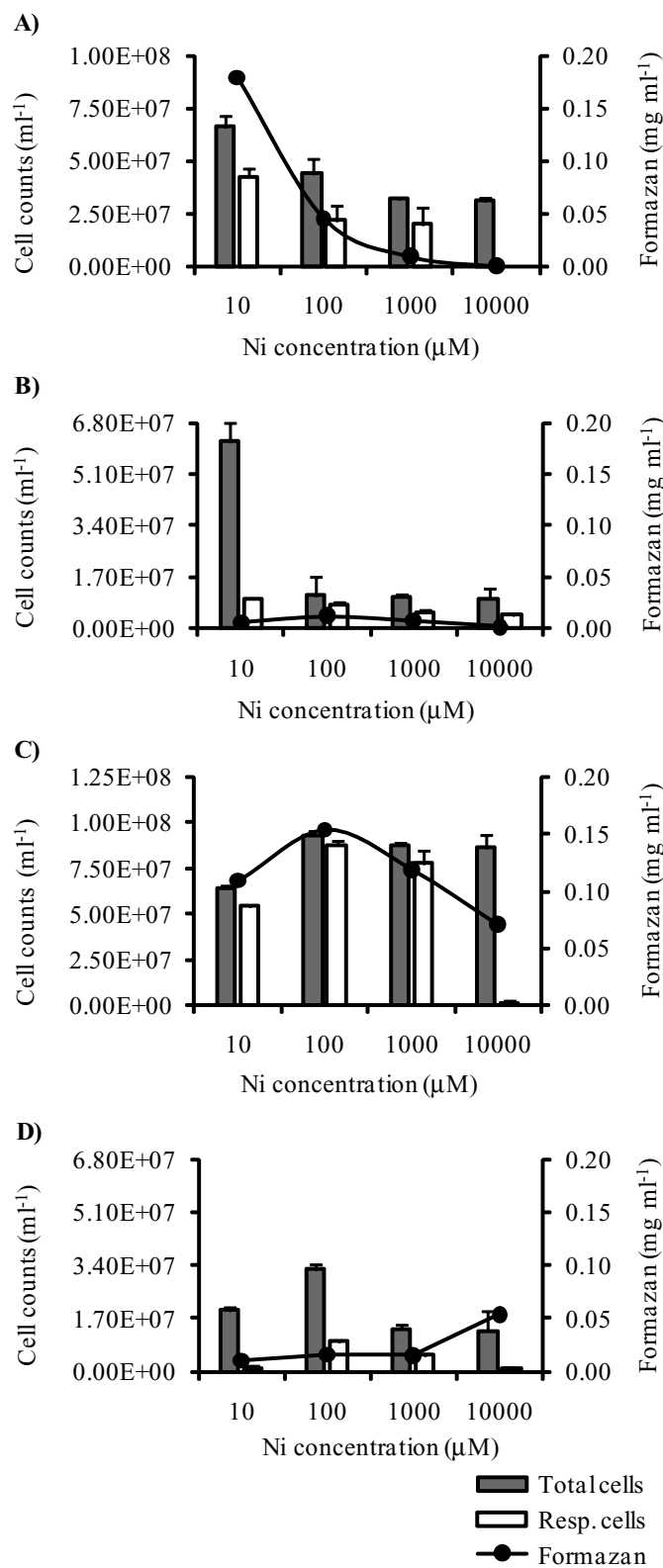


FIG. 2. Total cell count, respiring cell count (resp. count) and formazan concentration in Ni amended seawater medium. A) CR35 ($28 \pm 2^\circ\text{C}$), B) CR35 ($3 \pm 1^\circ\text{C}$), C) CR48 ($28 \pm 2^\circ\text{C}$) and D) CR48 ($3 \pm 1^\circ\text{C}$). Note that the scale for figures 'A' and 'C' is different from 'B' and 'D'.

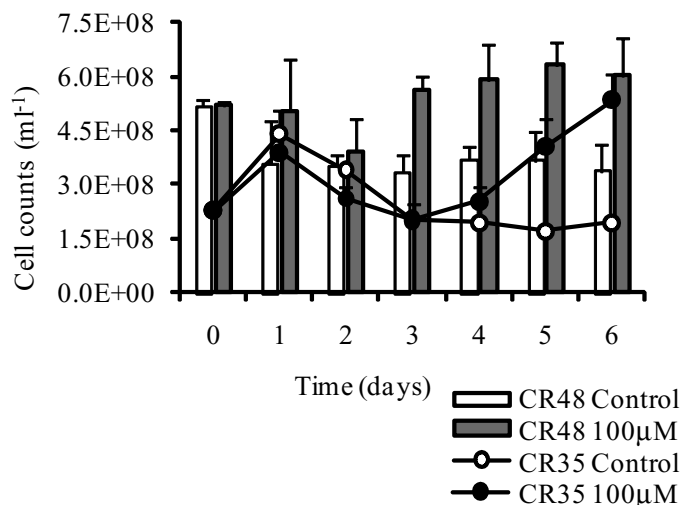


FIG. 3. Growth rate of CR35 and CR48 in the presence ($100 \mu\text{M}$) and absence of NiCl_2 at $28 \pm 2^\circ\text{C}$.

(Figure 2B) at $3 \pm 1^\circ\text{C}$ for CR35 and $8.80 \pm 2.56 \times 10^7$ cells ml^{-1} at $28 \pm 2^\circ\text{C}$ (Figure 2C) and $9.63 \pm 2.77 \times 10^6$ cells ml^{-1} at $3 \pm 1^\circ\text{C}$ (Figure 2D) for CR48.

Stimulatory Effect of Nickel on Bacteria

The growth rate of bacteria in the presence and absence of Ni is shown in Figure 3. It is clear from the figure, the bacterial count increased with Ni ($100 \mu\text{M}$) and decreased in its absence with time. The cell count for CR35 showed a marginal increase with Ni from $2.28 \pm 9.8 \times 10^8$ to $5.34 \pm 1.28 \times 10^8$ cells ml^{-1} at the end of 6d incubation. With CR48, the cell count attained a maximum ($6.3 \pm 6.56 \times 10^8$ cells ml^{-1}) on 5d of incubation and thereafter showed a marginal decrease on 6d.

Microscopy and EDX Analysis

The Ni-exposed and -unexposed bacterial cells were observed under SEM to investigate morphological changes induced by the metal after 60 d of incubation. Control cells grown in seawater without any nutrient or Ni amendment did not show any morphological variation and appeared normal (Figure 4A, D). The average cell size ($l \times w$) of CR35 was from $1.72 \pm 0.2 \times 1.20 \pm 0.14 \mu\text{m}$ and that of CR48 was $1.16 \pm 0.28 \times 0.96 \pm 0.17 \mu\text{m}$. Bacterial cells exposed to $100 \mu\text{M}$ Ni showed healthy dividing cells (Figure 4B, E). The average size of CR35 ranged from $4.02 \pm 0.38 \times 1.58 \pm 0.12 \mu\text{m}$ and CR48 from $5.2 \pm 0.77 \times 1.83 \pm 0.33 \mu\text{m}$. At $10,000 \mu\text{M}$ Ni concentration, increase in cell size and subsequent rupture was observed (Figure 4C&F). The average size of CR35 ranged from $4.37 \pm 0.47 \times 3.01 \pm 0.34 \mu\text{m}$ and CR48 from $6.89 \pm 0.34 \times 2.08 \pm 0.13 \mu\text{m}$.

Overall, the cell size increased to $2.65 \times 1.81 \mu\text{m}$ with CR35 and to $5.73 \times 1.12 \mu\text{m}$ with CR48 at $10,000 \mu\text{M}$ Ni concentration. EDX analysis of CR35 and CR48 isolates grown in the absence of Ni showed peaks for phosphorus, potassium,

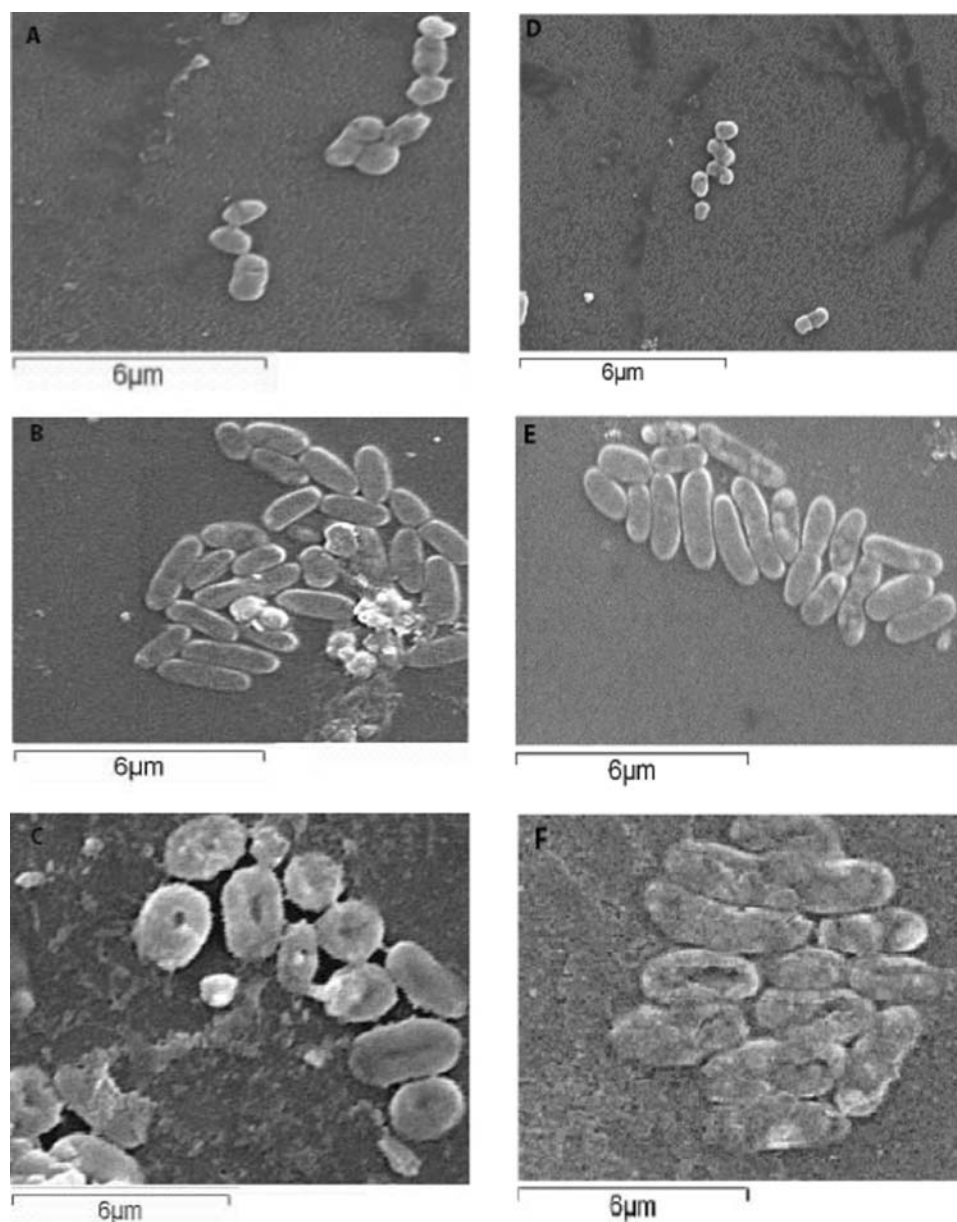


FIG. 4. Scanning electron microscope images showing morphological changes of CR35 cells grown in seawater medium A) with no Ni added, B) 100 μM and C) 1000 μM added NiCl_2 . CR48 cells grown in seawater medium D) with no Ni added, E) 100 μM and F) 1000 μM added NiCl_2 .

magnesium, calcium, silicon and oxygen (Figure 5a, c). The bacterial cells exposed to the metal showed peaks for Ni with decrease in intensity of the peak for Mg in both the isolates and loss of K peak in CR35 indicating cation efflux (Figure 5b, d). The wet mount observation under bright field microscope showed the occurrence of cell aggregates. The size of the aggregates increased and their number decreased with higher concentration of Ni. It was vice versa with lower test concentrations. Cells grown in the absence of Ni showed discrete cells (Figure 6). In an another attempt it was observed that, above 10,000 μM Ni concentration both cell aggregate size and their number decrease with no further increase in cell growth (data not shown).

X-ray Diffraction Analysis

The XRD analysis of bacterial cells showed that Ni was sequestered as both a diphosphide and a sulfide mineral in the bacterial cells. Diffractograms of control cells CR35 (Figure 7a) and CR48 (Figure 7c), lacked any distinct peak, and showed the characteristic amorphous nature of the samples, while distinct peaks for Ni loaded biomass from the experimental flasks indicated the deposition of crystallized Ni compounds. Based on the D values (1.42, 1.300, 1.140 and 1.01), peaks for the Ni-loaded CR35 cells (Figure 7b) at $2\theta = 65.96, 72.68, 85$ and 99.56° could be assigned to the existence of Ni sulfides. In contrast, for CR48 isolate (Figure 7d) most of the peaks were at $2\theta = 28.2,$

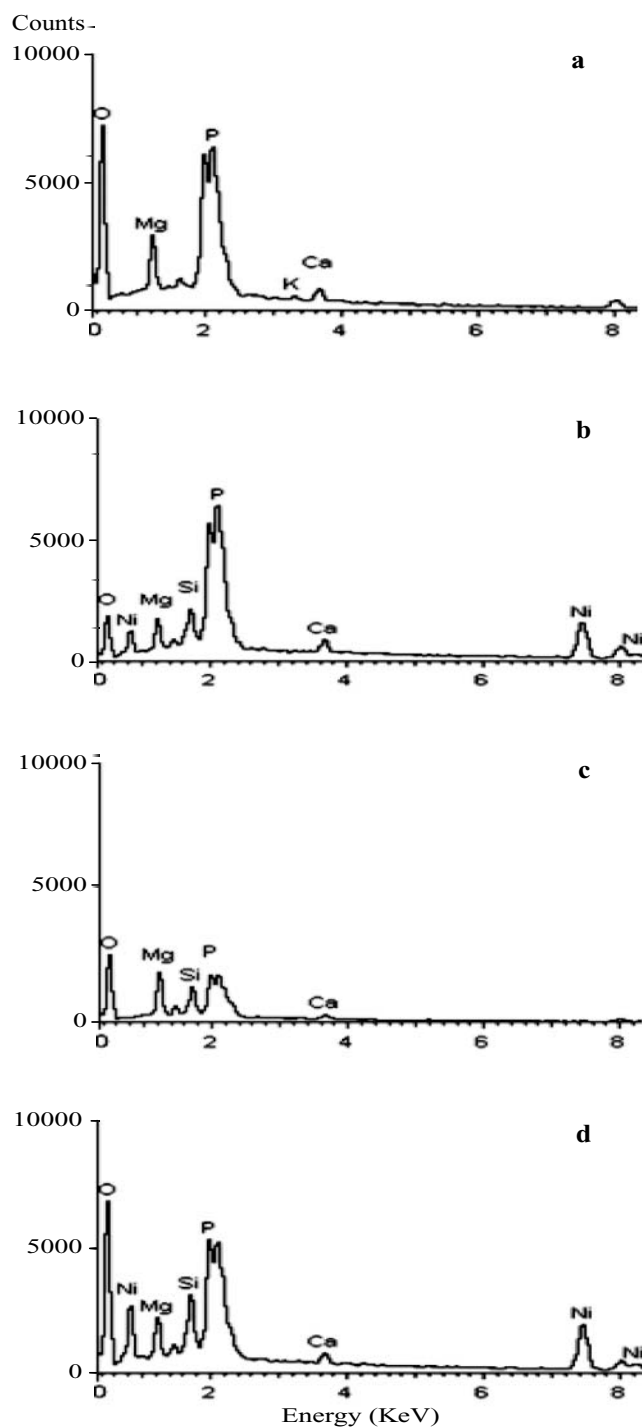


FIG. 5. Energy dispersive x-ray spectra of bacterial cells (a) CR35 in the absence of Ni, (b) CR35 exposed to 1000 μM Ni, (c) CR48 in the absence of Ni, (d) CR48 exposed to 1000 μM Ni.

34.66 and 59.96 with D values of 3.162, 2.586 and 1.541 which correspond to Ni diphosphides. However, peaks at $2\theta = 64.7$ and 98.08 with D values 1.44 and 1.09 could be related to Ni sulfides. Though EDX confirmed the presence of phosphorus in the cells, we were unable to interpret some XRD peaks, which

could correspond to phosphide mineral in CR35. The present study suggests that both CR35 and CR48 could accommodate Ni as both phosphide and sulfide minerals.

IR Spectral Analysis

The IR analysis of bacterial isolates CR35 and CR48 were examined in the presence and absence of Ni to understand the Ni induced changes in bonding characteristics of the functional moieties in the bacteria. The characteristic bands of CR35 biomass were at 435.9, 1064.6, 1238.2, 1647.1 and 2925.8 cm^{-1} (Figure 8A) and CR48 biomass were at 420.5, 1068.5, 1240.1, 1645.2 and 2923.9 cm^{-1} (Figure 8C). The biomass exposed to Ni indicated discernable changes in the intensities of the bands. The band at frequency 435.9 cm^{-1} in CR35 increased in intensity on Ni exposure to 451.3 cm^{-1} (Figure 8B).

In contrast, with CR48 the frequency at 420.5 cm^{-1} decreased in intensity to 416.6 cm^{-1} (Figure 8D). This shows that $\delta(\text{O-H})$ group could participate in Ni chelation. The stretching vibration at frequency 1064.6 cm^{-1} in CR35 decreased in intensity on Ni exposure to 1004.8 cm^{-1} , while in CR48 the frequency at 1068.5 cm^{-1} increased to 1083.9 cm^{-1} . This shows that (C-O), (C-C), (C-O-C), (C-O-P) of cell wall polysaccharides participate in Ni chelation. But, stretching vibration at 1645.2 cm^{-1} showed no change in intensity on Ni exposure with CR48 while the frequency at 1647.1 cm^{-1} decreased to 1645.2 cm^{-1} after Ni exposure in CR35 indicating the change in peptide bond characteristic of (C=O) group. Similar changes in frequency intensities were observed for (P=O) group in CR35 on Ni exposure and authenticate the binding of Ni to hydroxyl, carbonyl and phosphoryl functionalities.

Plasmid

Lysates of bacterial cells grown in the presence and absence of Ni examined by agarose gel electrophoresis showed no plasmids.

DISCUSSION

The present study shows the Ni immobilizing ability of Mn oxidizing bacterial isolates CR35 and CR48 retrieved from Carlsberg Ridge bottom waters under ambient ($28 \pm 2^\circ\text{C}$) and cold temperature ($3 \pm 1^\circ\text{C}$) (Fernandez et al. 2005). The sequestration of Ni as NiS , NiS_1 and NiP_2 was revealed by X-ray diffraction analysis. Though the cells showed active division at 100 μM Ni concentration, the cells grew larger and even formed aggregates at increasing Ni concentrations. The study highlights the stimulatory effect of Ni (Figure 3) at lower concentration and accumulation of the metal as either sulphides or diphosphides at higher concentration. This is the first report that gives an insight into the cellular changes and Ni accumulation within bacteria of the Indian ridge ecosystem.

Spectroscopic analysis of Ni showed a decrease of dissolved Ni in the aqueous phase in the experimental tubes. The decrease in the concentration of dissolved Ni was highest at the lower

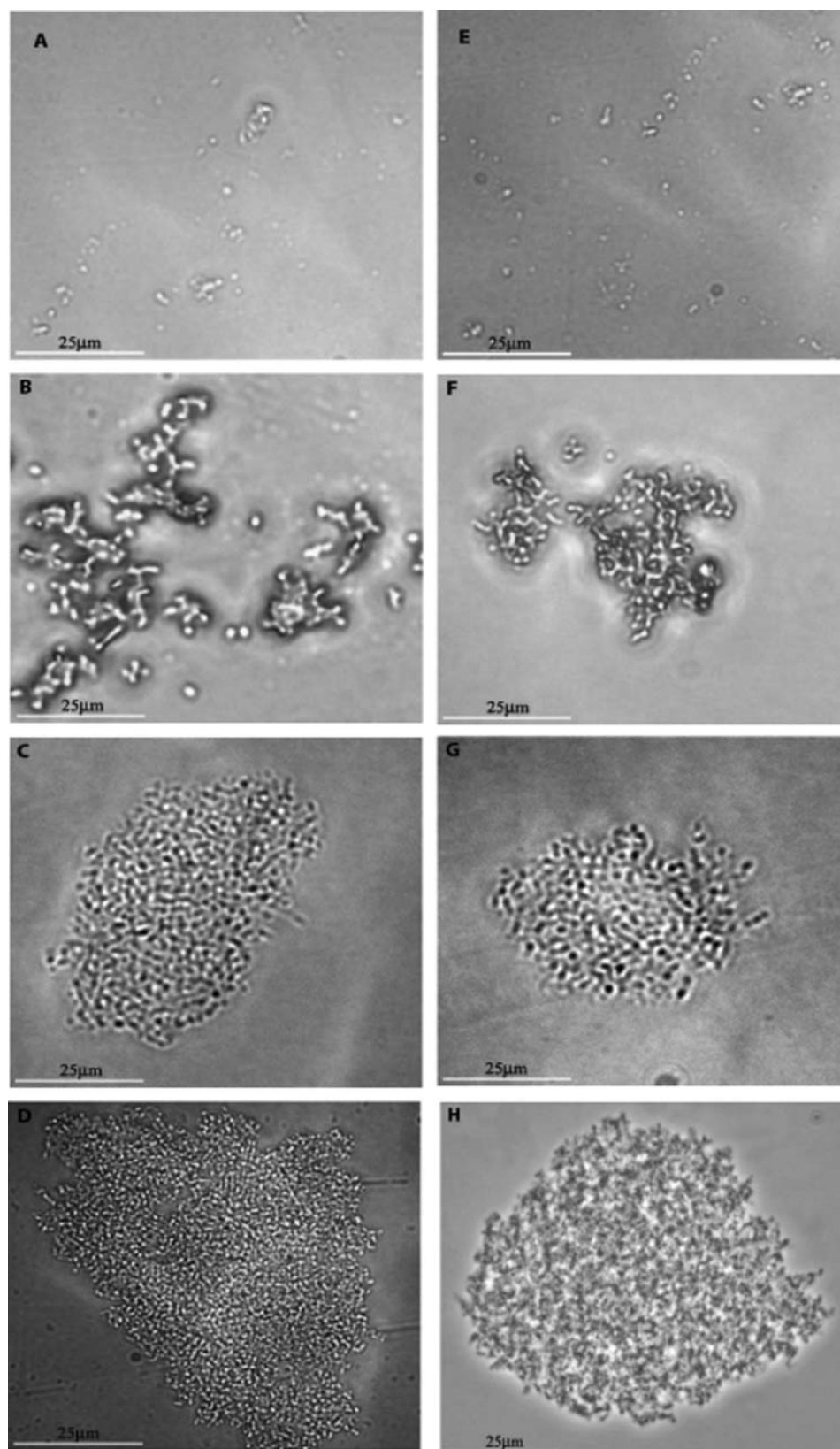


FIG. 6. Bright field images showing bacterial cell aggregates and their size difference respect to concentration of Ni in seawater medium. A) CR35 in the absence of Ni, B) 10 μM , C) 100 μM and D) 1000 μM added NiCl_2 . E) CR48 in the absence of Ni, F) 10 μM G) 100 μM and H) 1000 μM added NiCl_2 .

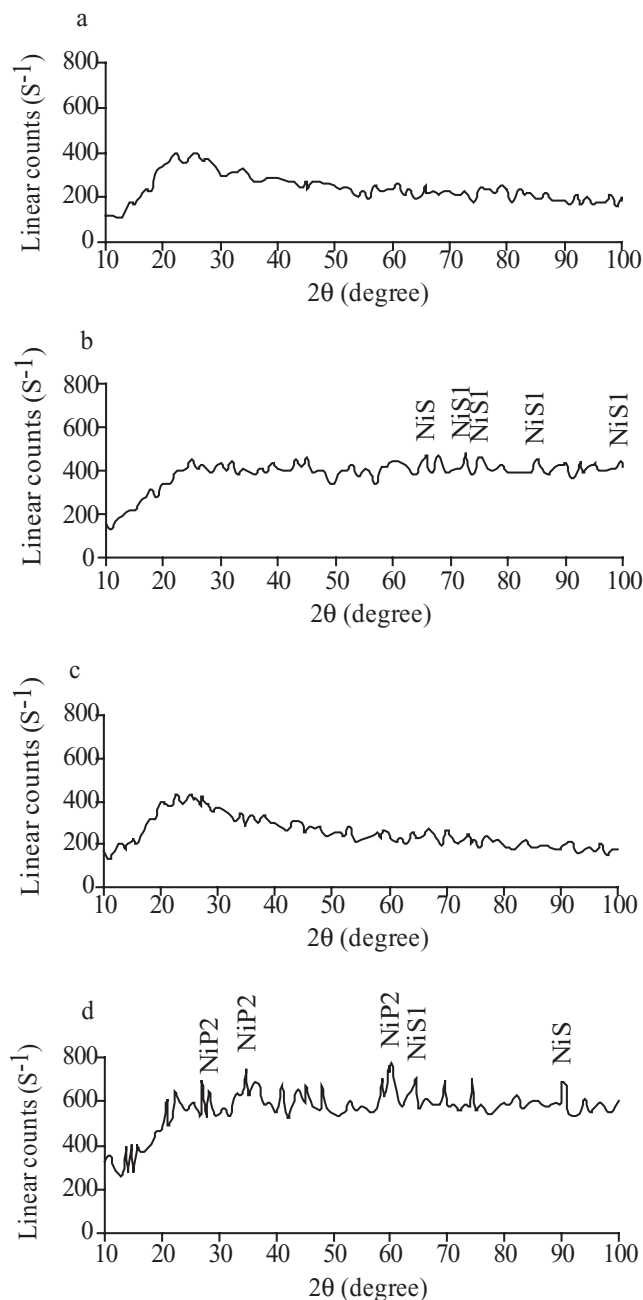


FIG. 7. X-ray diffraction spectra of dried and powdered bacterial biomass (a) CR35 in the absence of Ni, (b) CR35 exposed to $1000 \mu M$ Ni, (c) CR48 in the absence of Ni, (d) CR48 exposed to $1000 \mu M$ Ni.

Ni concentrations and smallest at the higher Ni concentration at both $28 \pm 2^\circ C$ and $3 \pm 1^\circ C$. However, the extent of Ni immobilization at $3 \pm 1^\circ C$ was comparatively smaller than at $28 \pm 2^\circ C$. The lower immobilization at $3 \pm 1^\circ C$ corresponds to the slower growth rate at low temperature (Figure 1B). The results of the study showed an increase in bacterial number at 10 and $100 \mu M$ Ni, which suggests that the 10 and $100 \mu M$ Ni concentrations were stimulatory for bacterial growth.

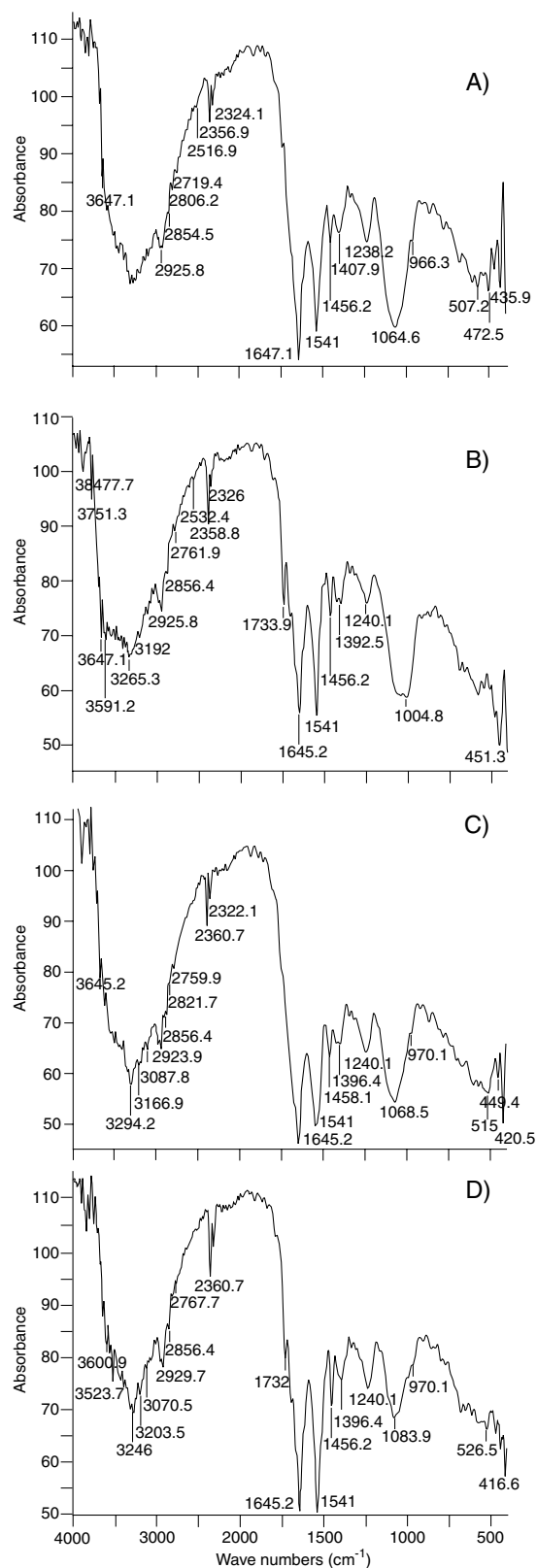


FIG. 8. Fourier transform infrared (FTIR) spectra of bacterial biomass. (A) CR35 in the absence of Ni, (B) CR35 exposed to $100 \mu M$ Ni, (C) CR48 in the absence of Ni, (D) CR48 exposed to $100 \mu M$ Ni.

The studies of Bartha and Ordal (1965) on *Hydrogenomonas* strains, Partidge and Yates (1982) on *Azotobacter chroococcum* and Stults et al. (1987) on *Bradyrhizobium japonicum* support the previously presented evidence of growth stimulation by Ni. However, excessive concentration of Ni was found to inevitably disorder cell metabolism followed by decrease in cell count. The results agree with those of Sigeo and Al-Rabae (1986), who reported cell wall damage and subsequent reduction in growth and cell metabolism of *Pseudomonas tabaci* in excess concentration of Ni in the growth medium. We observed in our study that, the amount of formazan produced was maximum at $28 \pm 2^\circ\text{C}$ and occurred below $100 \mu\text{M}$ Ni concentration with both isolates (Figure 2). Our findings suggest that our strains suffered stress above a threshold concentration of Ni, some cells surviving and others perishing.

The SEM observation of bacterial morphology showed a loss in cell wall rigidity above the minimum Ni threshold concentration. Nonetheless, microscopic observation and EDX analysis suggested that Ni was sequestered by the bacterial cells (Figure 4 and 5). The decrease in the level of Mg and K in cells exposed to Ni indicated that Ni immobilization involved ion exchange. The acquisition of Ni and the cell rupture are consistent with the observed microscopic cell counts. The results are in agreement with Sigeo and Al-Rabae (1986) with respect to exchange of Ni ions in cells and consequent inhibition of cell division in excess concentration of Ni.

Our findings are in agreement with a report by Nies (1999) with respect to a need for heavy metal uptake to cause any physiological or toxic effects. The observation by Cobet et al. (1970) of a morphological change of *Arthrobacter marinus* grown in the presence of an excess of Ni, lends support to our findings. Nickel availability in excess of that required and tolerated by an organism may impair its growth. The rate of Ni uptake by strains CR35 and CR48 was slower comparatively than the rate of Mn oxidation by these two strains (Fernandes et al. 2005). The Mn oxidizing activity, which was monitored maximum at $100,000 \mu\text{M}$ concentration, was 10 times higher than at $10,000 \mu\text{M}$ Ni concentration. Although the cultures failed to oxidize Mn at $100,000 \mu\text{M}$, the inhibitory effect of Ni occurred at a lower Ni concentration of $10,000 \mu\text{M}$. Ni appeared to have been sequestered within the cells in the strains CR35 and CR48, whereas oxidized Mn was deposited on the exterior of the cells.

The precipitation of Ni as NiS (Millerite) has been suggested as the crystalline phase of biogenic nickel sulfides (Waybrant et al. 1998; Southam 2005). The degradative metabolism of assimilated sulfur-containing amino acids by bacteria could lead to Ni precipitation as NiS (Freeman et al. 1975). Microbial conversion of sulfate (SO_4^{2-}) to sulfide (S^{2-}) in waters with reducing conditions could also lead to NiS formation (Babich and Stotzky 1983). Our XRD analysis of Ni grown strains CR35 and CR48 showed the immobilized Ni as NiS and NiP_2 . Our result agree with the report of Kazy et al. (1999) on accumulation of copper as CuS and Sar et al. (2001) on Ni as NiP_2 by *Pseudomonas*

aeruginosa. The reports of Aickin et al. (1979) and Macaskie et al. (1987) on sequestration of lead and cadmium as phosphate minerals in *Citrobacter* sp also support the present findings.

Our IR analysis of bacterial cells showed a band at frequency 1064.6 cm^{-1} with a shoulder at 966.3 cm^{-1} for CR35 (Figure 8A) and 1068.5 cm^{-1} with a shoulder at 970.1 cm^{-1} for CR48 (Figure 8B). This was interpreted as a stretching vibration of C-O, C-C, C-O-C and C-O-P groups (Wolkers et al. 2004; Yee et al. 2004). The intensity of the band in the presence of Ni decreased with CR35 and increased with CR48 (Figure 8C and 8D) suggesting that Ni immobilization was accompanied by a decrease/increase in polysaccharide and phosphate. Parikh and Chorover (2005) also observed a similar stretching vibration in relation to manganese oxidation. The stretching vibration characteristic of C=O of peptides was observed at 1647.1 cm^{-1} in CR35 and 1645.2 cm^{-1} in CR48.

The intensity of the band decreased to 1645.2 cm^{-1} with CR35 and remained unchanged with CR48 in Ni exposed cells, which could indicate an interaction of Ni with peptide bonds of protein in CR35. Backmann et al. (1996) explained a similar stretching vibrations of C=O specific for peptide bond in the $1700\text{--}1600 \text{ cm}^{-1}$ region. The other stretching vibration specific for P=O decreased in intensity from 1407.9 cm^{-1} to 1392.5 cm^{-1} in Ni treated cells of CR35 indicating a role of phosphate in binding of Ni. A report by Fiona et al. (2003) is consistent with our results that suggested a crucial role of sugar phosphate esters in metal chelation. The bands in the lower frequencies $<700 \text{ cm}^{-1}$ are an indication of stretching vibration of the $\delta(\text{O-H})$ group (Kamnev et al. 1997).

In the present study we observed stretching vibration of $\delta(\text{O-H})$ group and an increase in intensity at the lower frequencies of bands in Ni-exposed cells of CR35 and CR48. This could indicate an intense formation of $\gamma(\text{M-O})$ complex (M=metal ion). The study of Kamnev et al. (1997) on Cu-loaded *Azospirillum brasilense* biomass suggested that a relatively higher absorption at lower frequencies could be assigned to the formation of $\gamma(\text{M-O})$ and $\delta(\text{O-M-O})$ bands.

Bacterial resistance to Ni in heavy-metal contaminated ecosystems and in natural systems through which Ni percolates is inducible (Stoppel and Schlegel 1995). Screening for plasmids in CR35 and CR48 isolates from the metal rich ridge ecosystem showed no plasmids. However, an uptake of Ni was evident in these isolates, which could be through the activation of dormant genes for metal uptake. However, further study is required to prove the existence of chromosome mediated genes in these isolates. The genes for Ni resistance in *Alcaligenes denitrificans* 4a-2 (Kaur et al. 1990) and *Klebsiella oxytoca* CCUG 15788 (Stoppel and Schlegel 1995) were found to reside on chromosomal DNA.

CONCLUSIONS

This study provides evidence of Ni sequestration and of bacterial resistance to excess dissolved Ni under conditions of

nutrient starvation frequently encountered in natural seawater. Ni immobilization was faster at $28 \pm 2^\circ\text{C}$ than at $3 \pm 1^\circ\text{C}$. The present observations help us to infer that the two bacterial isolates differ in the mechanism that permits toleration of high concentrations of Ni and that they respond differently in respect to induction of growth at different Ni concentrations. This is the first report to show Ni immobilization as NiP_2 , NiS , and NiS_1 by bacterial isolates from the Indian ridge system as determined by X-ray diffraction analysis. The IR analysis also showed the involvement of phosphate group in addition to carboxyl and hydroxyl group as probable-binding sites occupied by Ni in CR35 and CR48 biomass. The study suggests the plausible role of the isolates in the ridge ecosystem. Because these ridge isolates are capable of tolerating and immobilizing high concentration of Ni, they could be candidate organisms for scavenging Ni ions from Carlsberg Ridge waters.

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