

Effect of heavy metals on marine *Bacillus* sp. and *Flavobacterium* sp.

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Responses to varying concentrations of Hg, Cd, and Zn were studied in two strains of bacterial isolates (*Flavobacterium* sp. and *Bacillus* sp.) from Indian coastal waters. Growth responses showed inhibition (in terms of percentage reduction with respect to control) and the order of inhibition was Hg>Zn>Cd in the case of *Bacillus* sp. and Hg>Cd>Zn in the case of *Flavobacterium* sp. With prolonged incubation *Bacillus* sp. overcame the inhibitory effect whereas *Flavobacterium* sp. did not. Higher concentrations of glucose in the growth medium increased the inhibitory effect of the metal. At lower concentrations ($15 \mu\text{g ml}^{-1}$) there was a stimulatory effect on [^{14}C] glucose uptake on *Flavobacterium* sp. Our studies showed that the Gram positive isolate was less adaptable to metals than the Gram negative.

Keywords: inhibition; metal; marine; bacteria

Introduction

Studies to date have shown an increased frequency of occurrence of antibiotic and drug resistant bacteria among the pigmented forms in estuarine and coastal waters (Nair *et al.*, 1992). This high frequency of resistance may be due to the adaptability of the microorganisms to heavy metals (Azam *et al.*, 1977; Mergeay, 1991). Moreover, bacteria originating from metal contaminated water show greater endurance to heavy metals than those from uncontaminated environments (Mills and Colwell, 1977). This study describes the short term response of two marine species of bacterium, isolated from coastal waters, to ppm levels of cadmium, mercury and zinc.

Materials and methods

The influence of zinc, cadmium and mercury on *Bacillus* and *Flavobacterium* sp. have been followed using growth and heterotrophic potential studies. The former was carried out by monitoring the change in optical density and the latter by following the change in the incorporation and respiration of labelled glucose. It is noted here that the measured effects in these organisms are related to the theoretical and not the actual dose of the metals.

Organisms and growth conditions

The microorganisms were taken from the stock culture collection of the Institute and had been isolated from the coastal waters. They were both rods, and were identified based on the following test results. The light orange coloured bacteria were Gram

negative, oxidase positive, glucose oxidative, motile, and polymixin B sensitive, and therefore characterized as *Flavobacterium* sp. (Oliver and Smith, 1982). The other isolate was identified as *Bacillus* sp. based on Gram positive, spore forming and proteolytic traits.

The cells were grown at $28 \pm 2^\circ\text{C}$ on a complete medium composed of nine salt solutions (NSS) amended with the following organic constituents in g l^{-1} : peptone, 0.1; yeast extract, 0.05; glucose, 0.05; soluble starch 0.05. The pH was 7.8. NSS consisted of (in g per l distilled water): NaCl, 17.6; Na_2SO_4 , 1.47; NaHCO_3 , 0.08; KCl, 0.25; KBr, 0.04; $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 1.87; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.41; $\text{SrCl}_2 \cdot 6\text{H}_2\text{O}$, 0.01; H_3BO_3 , 0.01 (Hermanson *et al.*, 1987). The cultures were harvested by centrifugation ($8000 \times \text{g}$ for 6 min at 4°C) in the middle of exponential growth (1×10^8 cells ml^{-1}). The cells were washed twice in NSS. They were resuspended in NSS and used as inocula (10^3 ml^{-1}) for all experimental studies.

Chemicals

Stock solutions of CdCl_2 , ZnSO_4 and HgCl_2 were made in distilled water, sterilized by filtration through $0.22 \mu\text{m}$ pore membrane filters (Nucleopore Corp.) into sterile flasks, and stored in the dark at 4°C . All chemicals were analytical reagent grade.

Inhibition study

The effect of the three metals on the two species of bacterium was studied by monitoring growth in liquid medium and also by using a heterotrophic potential technique (^{14}C glucose).

Growth

The effect of metals at several concentrations on bacterial growth was determined by adding filter-sterilized metal stock solution to the complete medium. Growth without metal solution was used as control. Growth was measured as the increase in optical density at 600 nm on a Beckman DU-6 spectrophotometer. The concentrations of metal used were 10, 100 and $500 \mu\text{g ml}^{-1}$ for Zn, 10 and $50 \mu\text{g ml}^{-1}$ for Cd, and 1 and $10 \mu\text{g ml}^{-1}$ for Hg. The concentrations of mercury used were much lower for both growth and heterotrophic studies (see below). Lower orders were chosen because this metal had a greater inhibitory effect than the other two (Nair *et al.*, 1992). Also, under certain circumstances, a lower concentration of the metal tends to have a stimulatory effect (Loka Bharathi *et al.*, 1990). The experiments were carried out in triplicate and the average value is given.

Heterotrophic potential technique

For heterotrophic studies the techniques used were essentially those of Hobbie and Crawford (1969) and Gocke (1977), along with the modifications given by Chandramohan and Ramaiah (1987). Due precautions of cleaning all glassware, by soaking overnight in $\text{HNO}_3:\text{H}_2\text{O}$ (1:3) and rinsing with distilled water, were taken. The methodology involved the addition of varying amounts of $\text{U-}^{14}\text{C}$ labelled substrate ($\text{D-}[\text{U-}^{14}\text{C}]\text{glucose}$ with a specific activity of 53 mCi mmol^{-1} (BARC, Bombay)] to identical subsamples. Labelled substrate was pipetted into 25 ml of artificial sea water containing 1×10^3 cells per ml of the bacterial culture. Experiments were carried out in two major sets. With set I, $30 \mu\text{g}$ glucose per l was used. In set II, a concentration lower, viz. $15 \mu\text{g}$ glucose

per l and a concentration higher, viz. 45 μg glucose per l, were used. To the control, 4% HCHO was added before incubation in order to record the amount of adsorbed radioactive substance. The samples were incubated with respective metals (1 $\mu\text{g ml}^{-1}$ for Hg and 10 $\mu\text{g ml}^{-1}$ for both Cd and Zn) for 24 and 48 h at $28 \pm 2^\circ\text{C}$. In each flask, a vial containing a Whatman filter paper strip soaked with 0.2 ml ethylene glycol was kept suspended near the neck before closing with a rubber bung. At the end of incubation the samples were fixed with HCHO. The fixed samples were acidified to pH 2.5 with 0.5 N HCl, gassed for 15 min to blow off any residual CO_2 , and then filtered on 0.2 μm Millipore filters. The radioactivity was assayed in replicates as disintegration per minute (dpm) using a liquid scintillation counter (LKB Wallac Model 1214). The amount of ^{14}C in the Whatman filter paper represented the amount respired, whereas the amount on the Millipore filter represented the uptake, i.e., the concentration within the cells. The 0 h corrections were made with the control values.

$$\% \text{ respired} = \frac{\text{dpm respired}}{\text{dpm activity added}} \times 100$$

$$\% \text{ uptake} = \frac{\text{dpm uptake}}{\text{dpm activity added}} \times 100$$

Results

The response of *Bacillus* sp. to different metals is shown in Fig. 1a–c. Growth declined with increasing concentrations of each metal. Percentage inhibition was calculated as % reduction in exponential growth, and varied with the metal tested (Zn, 65%; Cd, 60%; Hg, 87%). There was also a delay in the peak attainment. With prolonged incubation, *Bacillus* sp. could adapt to the inhibitory effect of the metals, resistance to the three metals being in the order Cd>Zn>Hg. In the case of *Flavobacterium* sp., the inhibition was less (Zn, 38%; Cd, 79%; Hg 73%). Unlike *Bacillus* sp. prolonged incubation did not increase the adaptability (Fig. 1a–c) of *Flavobacterium* sp.

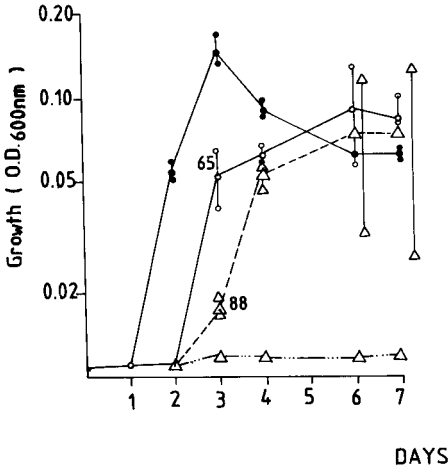
Metal toxicity at 30 μg glucose per l

Mercury was the most inhibitory of the three metals to *Flavobacterium* sp., causing only 45% ^{14}C to be respired, compared with 67% for Zn and 72% for Cd (Table 1). Hg caused the greatest inhibition. This was in spite of Hg being one order less in concentration than the other metals. However, there was not much difference in the amount of [^{14}C]glucose incorporated in the case of Zn (50%) and Cd (57%). *Bacillus* sp. showed 45 and 69.5% inhibition in respiration, and incorporation of ^{14}C in the presence of Zn; Cd and Hg almost completely inhibited both the processes, accounting for only 3.1 and 4.7% of the control respiration, respectively (Table 1).

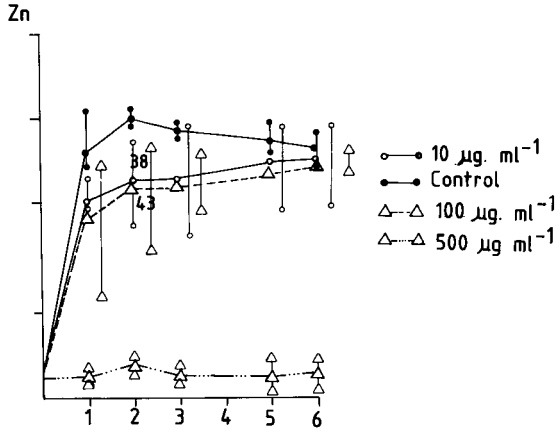
Metal toxicity at 15 and 45 $\mu\text{g l}^{-1}$

As inhibition was noted at the initial concentration of glucose tested, a set of experiments was carried out at higher and lower concentrations, viz., 15 and 45 μg per l glucose. Increase in glucose concentration brought about a general increase in inhibition of the metal for both the isolates. With *Bacillus* sp., increase in glucose concentration did not bring appreciable decrease in respiration in the presence of Zn. In fact, the difference

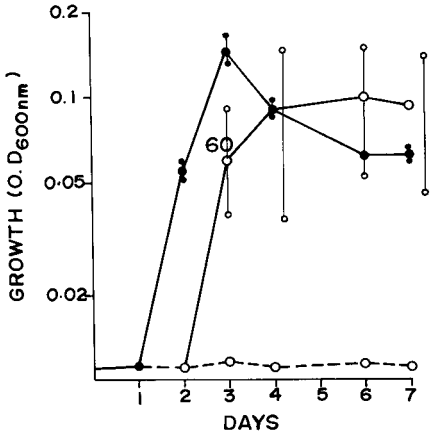
Bacillus sp.



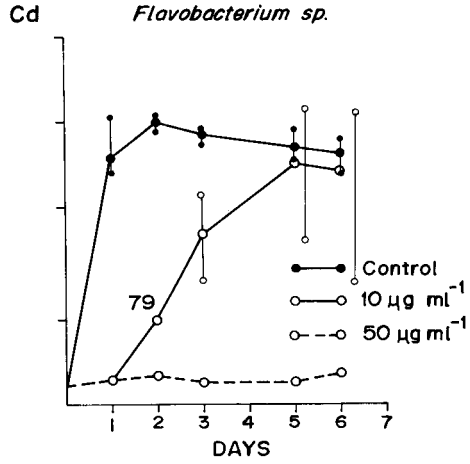
Flavobacterium sp.



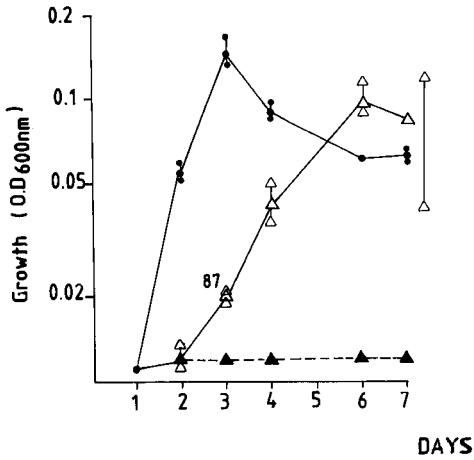
Bacillus sp.



Flavobacterium sp.



Bacillus sp.



Flavobacterium sp.

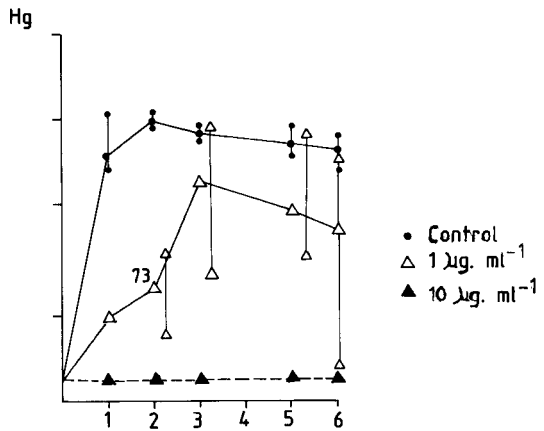


Table 1. Influence of Zn, Cd and Hg on ^{14}C respiration and incorporation at 30 μg glucose per l^a

	Zn (10 $\mu\text{g ml}^{-1}$)		Cd (10 $\mu\text{g ml}^{-1}$)		Hg (1 $\mu\text{g ml}^{-1}$)	
	Incorp.	Resp.	Incorp.	Resp.	Incorp.	Resp.
<i>Flavobacterium</i> sp.	50.0 \pm 2.8	67.0 \pm 8.5	57.0 \pm 1.4	71.5 \pm 8.8	50.5 \pm 5.01	45.0 \pm 4.2
<i>Bacillus</i> sp.	69.5 \pm 14.1	45.0 \pm 12.7	13.6 \pm 9.2	4.7 \pm 1.6	9.1 \pm 1.2	3.1 \pm 1.7

^aThe values are % of control (mean value \pm standard deviation, $n=3$).

in inhibition was hardly discernible. In the presence of Cd and Hg, however, there was a marked decline in both glucose uptake and respiration (Fig. 2). Increased glucose concentration caused a drastic reduction in its uptake and respiration in the case of all three metals in *Flavobacterium* sp. Thus, at 45 $\mu\text{g l}^{-1}$ concentration there was total inhibition of ^{14}C respired in both the organisms with Cd and Hg, whereas with Zn it amounted to 50%. At 15 ξg glucose per l the ^{14}C uptake was increased to 118% with respect to the control in the case of Cd (Fig. 2). The trend was the same with respiration. Zn and Hg showed higher percentages of uptake, but the respiration was inhibited, showing about 82% and 57% of the control, respectively.

Growth was also monitored during the experimental period. The trend was similar at both fixed and varying concentrations of glucose (data not shown).

Discussion

The pH and composition of the medium have considerable effect on the speciation and, therefore, the toxicity of the metals (Babich and Stotzky, 1984). At pH 8 and below, in a pure aqueous solution, cadmium occurs mostly as Cd^{2+} although $\text{Cd}(\text{OH})_2$ begins at pH 7.5. At high Zn concentrations, a considerable part of Zn would hydrolyse and even precipitate as $\text{Zn}(\text{OH})_2$ at a pH above 5.7 (Gadd and Griffiths, 1978). At pH 8.1, the major Zn species were Zn^{2+} (17%), $\text{Zn}(\text{OH})_2$ and ZnCl^+ (6.4%) (Zirino and Yamamoto, 1972). Mercury as Hg(II) hydrolyses at pH 2 with $\text{Hg}(\text{OH})_2$ being the final species in the absence of chloride (Babich and Stotzky, 1978). However, in a 0.1 M Cl^- medium as in this study, HgCl_2 would dominate.

Mg limited organisms have been resistant to Cd and Zn (Pickett and Dean, 1976). The medium in this study had a low concentration of 10^{-5} Mg. Under growth limiting conditions, microbial uptake systems tend to have high affinities (Silver, 1978). Therefore, it is possible that the Mg uptake system has a specificity that is too high to permit the uptake of Cd and Zn (Brynildsen *et al.*, 1988).

Jeanthon and Prieur (1990) have indicated clearly the necessity for standardizing methods for metal-microbe interaction studies. Attention has also been drawn by a

Fig. 1. Effect of heavy metals on the growth of *Bacillus* sp. and *Flavobacterium* sp.: (a) Zn; (b) Cd; (c) Hg. Bars represent \pm standard deviation of the mean ($n=3$), which are omitted when smaller than the symbol.

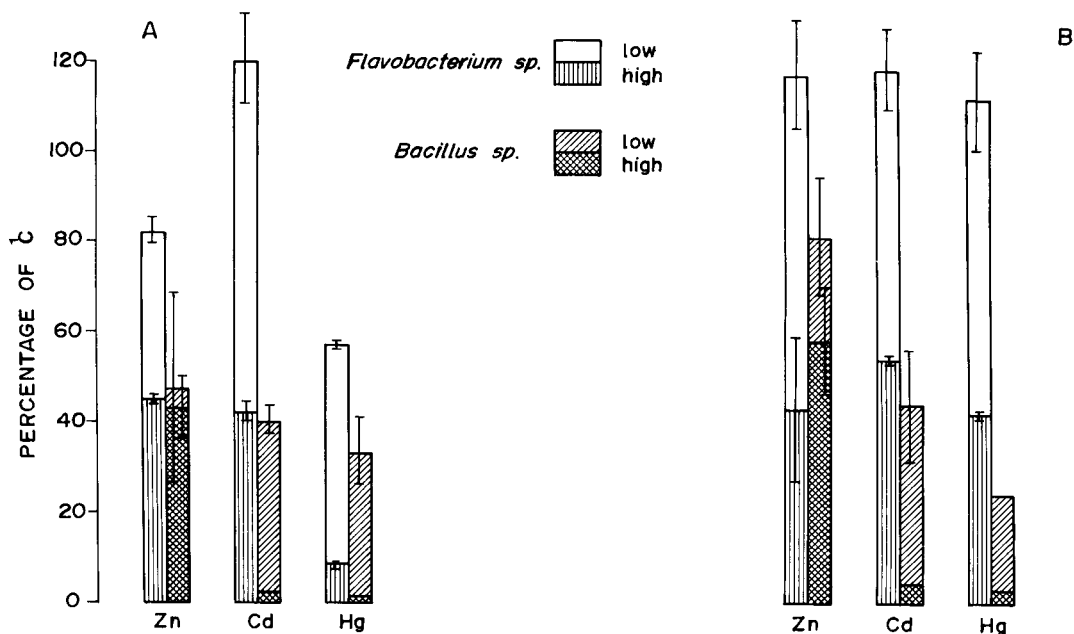


Fig. 2. Effect of glucose concentration in the presence of heavy metals on (a) respiration and (b) incorporation of [¹⁴C]glucose (% with reference to control). Bars represent \pm standard deviation of the mean ($n=2$), which are omitted when smaller than the symbol.

number of workers to the importance of maintaining low nutrient concentrations for such studies. (Nystrom and Kjellberg, 1987; Zelibor *et al.*, 1987). All experiments reported here have been carried out at low concentrations of organic content to minimize any error induced by complexing of metals with high levels of substrate. This would also minimize the effect of the medium composition on the bioavailability of the test salts of the metals, and would to a certain extent simulate the natural conditions like a nearshore or coastal ecosystem. McNicol (1980) has reported that if bacteria were allowed first to grow on permissive media and then replica plated onto metal amended agar, the physiological stress on the isolates would be reduced. In the present work, physiological stress was reduced by growing the cultures first on low nutrient medium and then on the same medium to which metal concentrations were added. Due caution was also taken to reduce the cell density of the inoculum to 10^3 ml^{-1} to minimize 'gratuitous' resistance (Duxbury and McIntyre, 1989).

Growth studies using increase in turbidity and heterotrophic potential measurements (¹⁴C uptake and respiration) were used to assay the effect of metals on cultures *in vitro*. Zn was the least inhibitory of the three metals to both the isolates as determined by the above methods. Increasing the concentration of Zn tenfold had little effect on the growth. With prolonged incubation there was a shift in the peak of the growth curve for both the cultures; both the cultures adapted themselves and growth exceeded the control in the case of *Bacillus sp.* (Fig. 1). This adaptive mechanism has been reported in *Escherichia coli* and *Pseudomonas putida*. These organisms synthesize Cd binding protein which sequesters the Cd^{2+} present in the cells and allows the cells to resume

normal metabolic growth (McEntee *et al.*, 1986; Nystrom and Kjellberg, 1987). The survival of a marine *Pseudomonas* sp. with increasing Cd concentration has been attributed to detoxification by precipitation as sulfide. (Flatau *et al.*, 1991). Prolonged incubation in the case of *Flavobacterium* sp. did not help this strain to overcome initial lag, and at no time did growth exceed control values. Stimulatory effects that are sometimes encountered with lower concentrations (Loka Bharathi *et al.*, 1990) were not noticed in the case of Hg. The detoxifying mechanisms differ in higher organisms where exposure to metals involves an adsorption of the metal to specific or nonspecific site. If adsorption occurs, the metal is translocated through the limiting surface membrane for transportation to the sequestration site (Phillips, 1980) and this is probably fairly specific, as on the chorion of the developing eggs of the herring *Clupea herringus* (Rosenthal and Spirling, 1974). This perhaps explains why the relative toxicities of these metals are so different from their toxicities to higher organisms.

The heterotrophic potential (^{14}C glucose utilization) studies showed clear cut metal toxicity. Zn was found to be the least inhibitory for respiration. Zn is a micronutrient and essential for the bacteria as a constituent of certain enzymes (Stanier *et al.*, 1980), and a specific uptake system is expected to be operative (Brynhildsen *et al.*, 1988). Cadmium is a relatively rare element with no biological function (Babich and Stotzky, 1978) but, like other pollutants (Hg, Cr, etc.), is more abundant in the circumscribed geographic area due to industrial use.

Our experiments showed increased toxicity of Cd with increased glucose concentration. While increasing glucose did not have much effect on respiration in *Bacillus* sp., it did in the presence of Zn, in *Flavobacterium* sp. It was greatly reduced in the presence of metal. One plausible reason is that Cd is taken up by an energy driven system specific for another essential element like magnesium (Silver, 1978). Respiration of *Bacillus* sp. in the presence of Hg is inhibited over 60% at $15\ \mu\text{g}$ glucose per l and nearly 99% at $45\ \mu\text{g}$ glucose per l. Cd is also known to affect microbial respiration (Babich and Stotzky, 1978). In the case of *Acinetobacter*, the effects of Cd were presumably strengthened by glucose limitation in chemostat cultures (Tan, 1983).

Different mechanisms have been adopted by different organisms to detoxify metal. Komura and Izaki (1971), Schottel (1978), and Silver (1981) have all shown that Hg resistance in *Bacillus* sp. is plasmid mediated. With the present *Bacillus* isolate, the inhibition increased with increasing concentration of glucose. However, this is not in agreement with the findings for *Klebsiella* sp. by Brynhildsen *et al.* (1988), who found Hg less inhibitory at higher concentration of glucose. Perhaps, like Cd, Hg is taken by an energy driven system specific for another essential element (Silver, 1978).

Lower concentrations of metals are shown otherwise to be stimulatory (Doyle *et al.*, 1975; Loka Bharathi *et al.*, 1990). In the present study, there was a stimulatory influence on ^{14}C incorporation and respiration in the case of *Flavobacterium* sp. in the presence of Cd. Perhaps a lower concentration of glucose permits a lower availability of the metal within the cells, which tends to stimulate rather than inhibit. Nonetheless, in certain cases the same concentration may be inhibitory to sensitive organisms (Goulder *et al.*, 1979) like *Bacillus* sp. in our study. Yet, a low Cd concentration in the range $1.1\text{--}6.2\ \mu\text{g}\ \text{l}^{-1}$ was reported to inhibit DNA mediated transformation without decreasing the viability of the bacterial population (Groves *et al.*, 1974). Mayfield *et al.* (1980) found that one of their isolates could not grow with $5\ \text{mg}\ \text{l}^{-1}$ of Hg ion in continuous culture, but the same isolate tolerated an Hg concentration of $10\ \text{mg}\ \text{l}^{-1}$ in a mixed continuous

culture. Hence, a metal concentration to which one particular group of organisms are sensitive may fall within the normal physiological range of another. Thus, comparing these two organisms under *in vitro* conditions, *Flavobacterium* sp. was better adapted to metal. It has also been pointed out by Duxbury (1986) that Gram negative bacteria have greater tolerance to heavy metals than Gram positive counterparts and perhaps this could be the reason why the marine environment is dominated by Gram negative bacteria. It is probably the adaptability to different metals under continued exposure, as shown by the present isolate, *Bacillus* sp., that has made some of the Gram positive organisms successful inhabitants not only of near shore environments (Nair and Loka Bharathi, 1982) but also of ferromanganese nodules of deep sea sediments (Chandramohan *et al.*, 1988). Further experiments on metal-microbe interactions with these organisms would give insight into their mode of tolerance, adaptation and possibly even their ability to contribute to metal accretion.

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