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Insights on metal-microbe interactions in *Bacillus* sp. and *Chromohalobacter* sp. from a solar saltern

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Abstract

Metal tolerant bacterial strains viz *Bacillus cereus* (RS-1), *Bacillus* sp. (RS-2) and *Chromohalobacter beijerinckii* (RS-3) were isolated from the surface sediments of a solar saltern in Ribandar Goa, situated in the vicinity of the Mandovi estuary influenced by mining activities. RS-1 that showed optimal growth at 20 psu salinity was tolerant to 10 mM Co^{2+} while hypersaline isolates RS-2 (100 psu) and RS-3 (200 psu) were tolerant to Ni^{2+} and Mn^{2+} at 1 mM and 10 mM respectively. Experimental studies revealed that growth was stimulated at low concentrations of metal amendments for all the isolates. Growth of RS-1 was stimulated by ~450% on addition of 100 µM Co^{2+} whereas for RS-2 and RS-3 it was at 100 µM Ni^{2+} (70%) and 5 mM Mn^{2+} (450%). The stimulation in growth was coupled to a dip in respiration rates for the isolates RS-1 and RS-3 when compared to metal unamended controls. The respiration rates for RS-1 and RS-3 during peak growth in the presence of metal were 17.0 and 27.5 compared to the controls which were 24.7 and 473.4 pg formazan $cell^{-1}$ day^{-1} respectively. Presence of Ni^{2+} stimulated the respiration rate (26%) in RS-2 when compared to the control (417.4 pg formazan $cell^{-1}$ day^{-1}). Co^{2+} and Mn^{2+} had a significant negative impact on the utilization of carbohydrates and carboxylic acids in RS-1 and RS-3 respectively. Ni^{2+} had a stimulatory effect on the utilization of BIOLOG GP2 substrates by RS-2. The phenotypic expressions observed above were correlated with the changes in whole cell protein profiles in the presence and absence of added metal. Addition of Co^{2+} to RS-1 resulted in a significant up-regulation of 57 kDa fraction while there was a conspicuous down-regulation of 29 kDa protein. The major protein fraction up-regulated in RS-2 in the presence of Ni^{2+} was a 59 kDa protein while most of the fractions were down-regulated. In RS-3, the addition of Mn^{2+} at 10, 100 and 1000 µM up-regulated a 50 kDa protein while the 53 kDa fraction was down-regulated. This study relates the metal induced regulation of proteins to phenotypic variations encountered in growth and substrate utilization.

Keywords: Metal Tolerance, Bacteria, Solar saltern, BIOLOG, Proteins

INTRODUCTION

Microorganisms have been exposed to varying concentrations of metals presumably since the beginning of life [1, 2] and have sustained by maintaining a homeostasis between the available metal concentration and microbial physiology [3, 4, 5]. However, in a contaminated environment the elevated concentration of metals is known to trigger adverse redox-reactions in the cell [6]. Metal induced stresses also exert a selective pressure on the microbial communities leading to the emergence of resistant strains [7, 8, 9]. Biomagnification of metals may cause residual effects on aquatic biota which are long lasting and highly deleterious due to their recalcitrant nature. Metals such as calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc are essential as they serve as micronutrients and are used for redox-processes; stabilize molecules through electrostatic interactions; serve as components of various enzymes; and for regulation of osmotic pressure.

Solar salterns are thalassohaline environments where seawater is evaporated to salt used for commercial purposes. During evaporation, the metals tend to concentrate and this is further amplified when these salterns are fed with metal contaminated water from the adjoining estuary. Heterotrophic bacteria play a pivotal role in regulating the cycling of metals in these salterns. Ventosa et al. [10] have reported that moderately halophilic or halotolerant bacteria have adapted to life at the lower range of salinities with the possibilities of rapid adjustment to changes in the extreme salt concentration. They employ various mechanisms to overcome metal stress such as precipitation of metals as phosphates, carbonates and/or sulfides; volatilization via methylation or ethylation. Other mechanisms such as intracellular sequestration with low molecular weight cysteine-rich proteins such as phytochelatins, metallothioneins and overexpression of metal-binding proteins, prevent entry of metals inside their cells. These microbiological processes can regulate the solubility of metals thereby governing bioavailability and potential toxicity. In salterns, continuous exposure of microbes to metals result in natural selection/evolution of highly resistant strains that adapt to the presence of metals.

Attri et al. [11] have reported extensive damage to the marine environment in Goa due to mining activities. The present study focuses on the influence of manganese, cobalt and nickel on

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halotolerant and halophilic bacteria. Cobalt, a central atom of vitamin B_{12} (cobalamine) is also involved in diverse enzymatic reactions in microorganisms. It forms an important component of several proteins like aminopeptidase, prolidase, nitrile hydratase, glucose isomerase etc [12]. Nickel on the other hand is required for enzymes such as urease, dehydrogenase and hydrogenase [13]. Similarly, manganese is an essential nutrient for all living organisms serving as a cofactor in a variety of enzymes including superoxide dismutase [14, 15, 16]. Manganese is also used by bacteria as an electron acceptor in anaerobic respiration [17].

Little is known about phenotypic changes in bacteria in response to metal stress and the primary effector molecules i.e protein expression especially in hypersaline environments. Therefore, this study aims to determine variations in phenotype and protein expression in response to metals and to delineate the possible role of these proteins in cellular adaptation to overcome metal stress. Hence, a comprehensive assessment of the various components of the metal microbe interactions would yield better insights on the capability of microbes in salterns to attenuate metals thereby preventing it from reaching toxic levels, a critical aspect of environmental monitoring.

MATERIALS AND METHODS

Bacterial isolates and growth conditions

Bacterial strains RS-1, RS-2 and RS-3 were isolated from the solar salterns of Goa (India) on 25% Nutrient Agar (100% corresponds to 13 g l^{-1} nutrient broth with 1.5% agar-agar) amended with metals and crude salt. The isolate RS-1 was isolated and maintained on the media amended with 400 ppm Co^{2+} and 2% crude salt while RS-2 and RS-3 were on 400 ppm Ni with 10% crude salt; and 400 ppm Mn^{2+} with 20% crude salt respectively. Metals were supplemented as chlorides facilitating biosorption of metal on the cell surface to its intracellular uptake [18].

Identification and characterization of the metal tolerant isolates

The three metal tolerant isolates were classified based on phylogenetic analysis of their 16S rRNA gene sequences. Bacterial DNA was isolated from single colonies on quarter strength nutrient agar plates using bacterial genomic DNA prep kit (Chromous Biotech RKT11/12). The 16S rDNA was amplified by PCR from single colonies [19] using oligodeoxynucleotide primers designed to anneal to conserved regions of the bacterial 16S rDNA. The 16S rRNA gene was amplified using the universal bacterial 16S forward primer 5'-CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG-3'and bacterial 16S reverse primer 5'-CCGGGATCCAAGCTTACGGC TACCTTGTTACGACTT-3'. The amplified product was purified on QIA quick PCR Purification Kit (QIAGEN) as recommended by the manufacturer. Cycle sequencing was performed on CFX 96, Real time system and the products were analyzed on a DNA sequencer. A segment of approximately 1500 bases of 16S rRNA gene (nearly the entire gene) was amplified and sequenced for each of the three isolates. Each sequence was compared with the sequence of strains belonging to the same phylogenetic group obtained from the GenBank database and from a BLAST search of the National Centre for Biotechnology Information (NCBI). Gram and spore staining was performed as per standard protocols using HiMedia Gram Stain; and Schaeffer and Fultons Spore Stain kit respectively [20].

Effect of metal on Carbon substrate utilization

The BIOLOG system was used to determine the effect of metal amendments on the ability of RS-1, RS-2 and RS-3 to utilize various substrates. A BIOLOG assay involves inoculating microbial cultures into microplates that contain 95 sole C-sources in addition to a "tetrazolium violet dye". The utilization of any C-source by the bacteria results in the respiration-dependent reduction of the dye and purple-color formation that can be quantified and monitored over time. Negative wells remain colorless, as does the reference well with no carbon source. For the BIOLOG assay a two day old culture of RS-1, RS-2 and RS-3 grown at 28 ± 2°C on 25% nutrient agar plates supplemented with 1 mM Co^{2+}, Ni^{2+} and Mn^{2+} at 2, 10 and 20% salinity respectively were harvested with sterile physiological saline (0.85%). The suspension was washed five to six times by centrifuging at 6000 rpm for 10 min at 10°C with subsequent vortexing each time. This was necessary to exclude any nutrients coming from the agar plates, which could otherwise result in a false-positive test. Almost the same turbidity (~0.133±0.014 at OD_{600}) was maintained for all the three isolates. The cell suspension was then divided in two sets, one amended with the respective metal at 1 mM concentration and the other unamended. Isolates RS-1 and RS-2 were inoculated in GP2 plates, while RS-3 was inoculated in GN2 with 150 µl of cell suspension. Separate plates were used for inoculation of sample with metal-amended and unamended suspensions. Incubation was done at 28 ± 2°C. Absorbance was measured at 0 day on a microplate reader (Synergy 2, BioTek) at 595 nm and thereafter at every 24-h intervals for a period of 5 days. Average well color development was computed and the values were normalized for each substrate [21]. Wells that showed the highest normalized value (compared to that of the previous day) were assumed to have the substrate that was most preferentially utilized during that particular period.

Effect of metal on growth and respiration

Tests were done at 10 µM, 100 µM, 1 mM and 5 mM amendments of Co^{2+}, Ni^{2+} and Mn^{2+} on RS-1, RS-2 and RS-3 respectively in order to determine bacterial growth and respiration in the presence of added metal. Inoculum was prepared by harvesting exponentially growing cells from metal supplemented 25% nutrient agar plates into physiological saline. The cell suspension was centrifuged at 6000 rpm for 15 minutes at 10°C and the pellet was washed twice with sterile saline and resuspended by vortexing. The optical density of the final cell suspension was measured at 600 nm using a microplate reader (Synergy 2, BioTek) and adjusted to a uniform cell density. The inoculum size was calculated by direct cell counts in a Neubauer cell-counting chamber at 400-X magnification with a bright field microscope (BX51 Olympus microscope). The experiment was carried out in 25% Nutrient broth containing filter sterilized triphenyl tetrazolium chloride (TTC) at a final concentration of 0.025%. Controls for each metal concentration were also included to correct for chemical oxidation and loss of metal due to adsorption. The experimental tubes were incubated at 28 ± 2°C in the dark. Samples (1 ml) were removed immediately after inoculation and at 2 day intervals for determining the total count and after 10 days of incubation for determining the respiring cell count. During the incubation period, respiring cells convert TTC to triphenyl formazan [22] that gives a deep pinkish appearance. The total count and the number of respiring cells (red) was determined after segregation of

cell aggregates by sonication at 15 Hz for 3s. The formazan in experimental tubes was determined and the values were corrected for the 0 day absorbance at the end of the experiment. The reduction of TTC is a result of electrons passing from the substrate (metal) through the enzymatic machinery of the cell and ultimately onto TTC. 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. The experimental readings were corrected for control to estimate the rate of respiration.

Protein Expression studies in the presence of metal

Inoculum was prepared by growing RS-1, RS-2 and RS-3 in quarter strength nutrient broth without metal. They were grown at 28 ± 2°C till mid-exponential phase. This culture was then used as inoculum for the following experiment. Four flasks of 100ml quarter strength nutrient broth were set up for each isolate as follows:

1. Media (unamended) + test organism
2. Media (amended with10 µM metal) + test organism
3. Media (amended with 100 µM metal) + test organism
4. Media (amended with 1000 µM metal) + test organism

The flasks were incubated for a total of six days. Total protein was extracted from 20 ml of media immediately after inoculation and also on each subsequent day till six days. The culture was centrifuged at 6000 rpm for 5 min at 4°C. Protein was extracted from the pellet with 0.1 ml protein extraction buffer (50 mM Tris-HCl [pH 8.0], 5 mM EDTA [pH 8.0], 0.1%Triton-X 100, 0.01% Lysozyme and 1 mM PMSF pH 8.0) by homogenization on ice for about 10 minutes using a sterile pestle, followed by sonication (Transsonic Digital S [Elma]) for 15 minutes at 25°C at ultrasound power of 100%.The reaction mixture was centrifuged at 6000 rpm for 15 min at 4°C. Supernatant was collected in sterile microfuge tubes and the protein quantitated by Bradford micro assay method [23] using a Microplate reader (Synergy 2, BioTek) for measuring absorbance. SDS-PAGE analysis was carried out as per Laemmli [24] on a 12% acrylamide gel. Equal amounts of protein were loaded into each well. Electrophoresis was performed at 50V for 15 min and then at 100V until completion. Gels were stained in 0.15% Coomassie Brilliant Blue. After destaining, gels were photographed in an Alliance 4.7 Gel doc system. Low Range-SDS-PAGE standards (BIORAD) were used to determine the molecular weights of proteins.

Protein quantity in each band was determined by taking serum albumin (66,200) as the reference band. Serum albumin was selected as the reference band based on the comparative closest quantification value obtained by analysis using UVI Band software (Version 12.14) with the value recommended by manufacturer. The quantification of the protein was dependent on the intensity of the reference band and the relative amount of each protein in other lanes calculated by the processing software. Up or down regulation of proteins (in percentage) was calculated by taking the corresponding control protein band as the baseline value.

RESULTS AND DISCUSSION

Identification and characterization of the metal tolerant isolates

Isolate RS-1 and RS-2 were Gram positive spore forming rods, whereas isolate RS-3 was a Gram negative non spore former. Distance Matrix was constructed with ~1500 bp of 16S rRNA sequences based on Nucleotide Sequence Homology (Using Kimura-2 Parameter and NCBI GenBank and RDP database). RS-1 was identified as *Bacillus cereus* (99%; GenBank Accession Number: JQ312120). Isolate RS-2 was detected to be *Bacillus* sp. (99%; GenBank Accession Number: JQ312119) and isolate RS-3 was detected to be *Chromohalobacter beijerinckii* (97%; GenBank Accession Number: JQ312118). The bacterial genera *Bacillus* represents the common soil bacteria and have been reported as contaminated soil inhabitants; however *Chromohalobacter beijerinckii* is a salt loving bacterium [25].

Effect of metal on Carbon substrate utilization

Color development in a well of the Biolog microtitre plate reflects the ability of the bacterial isolate to utilize that specific carbon source. Test for determining the metabolic capability of the isolates using Biolog GP2 and GN2 plate showed that they were capable of oxidizing a wide array of carbon substrates. Carbon source utilization patterns were obtained by determining the percentage of carbon sources utilized after a 5 day incubation period. The ability of a bacterium to utilize a compound is dependent on the presence of a transport system for that particular compound and the presence of favorable environmental conditions for the activation of the transport system [26]. Distinct transport systems for a few compounds like sugars, amides, amino acids etc. have been documented in various bacterial species [27, 28, 29]. These transport systems were found to be highly distinct with very little overlap between them [28]. Strain RS-1, RS-2 and RS-3 oxidized 63%, 87% and 95% carbon substrates respectively in the absence of metal amendment, out of the 95 tested. However, there was a significant change when amended with metals. Strains RS-1 and RS-3 showed a reduced ability to utilize substrates. Strain RS-1 could utilize only 14% substrates in the presence of Co^{2+}, whereas strain RS-3 could utilize 18% of the substrates in the presence of Mn^{2+}. Strain RS-2 showed an increase in substrate utilization when amended with Ni^{2+}, utilizing a total of 93% substrates in the presence of metal. Tables 1 & 2 give an account of the major carbon compounds (carbohydrates and amino acids) utilized by the three isolates. Considerable difference in the utilization of carbohydrates was observed by the isolates in the presence and absence of added metal. In unamended controls, D-Galactose was the most preferred sugar by isolate RS-1, followed by Xylitol, D-Trehalose and α-D-Glucose. In the presence of 1 mM Co^{2+}, α-methyl D-Mannoside was utilized with high levels of preference, followed by Mannan. It is important to note that Glucose was not used at all in the presence of Co^{2+}, while addition of Co^{2+} triggered the use of Mannan, L-Arabinose and D-Mannitol. Taqatose, β Methyl D-Galactoside, α Methyl D-Galactoside and L-Fucose were some of the other sugars that were not utilized in the presence of Co^{2+}. Chun et al. [30] have also reported that many carbon sources, such as NAcetyl-D-Glucosamine, L-Arabinose, and D-Galactose were preferred by *Bacillus megaterium* strain 91-51, one of the best biocontrol strains, and that Glucose, Maltose and Sucrose were utilized well by most of the *Bacillus* species. With strain RS-2 there was no major difference in carbohydrate utilization pattern with and without 1 mM Ni^{2+} amendments. In unamended controls, α-D-Glucose was utilized with high levels of preference on all 5 days, whereas in the presence of Ni^{2+}, α-D-Glucose was the most preferred sugar on the first day, with D- Mannose being preferentially

utilized for the rest of the period. Strain RS-3 displayed a pattern similar to RS-1. However, there was a lag phase of 24 hours before sugar utilization began. Gentobiose was the preferred sugar on the third and fourth day followed by α D-Glucose at the end of the incubation period. With 1mM Mn^{2+} amendments, D-Melibiose was used preferentially on the first and second day followed by Gentobiose for the next two days and N Acetyl D-Glucosamine on the last day. In general, Co^{2+} and Mn^{2+} had a significant negative impact on the utilization of carbohydrates and carboxylic acids in RS-1 and RS-3 respectively whereas Ni^{2+} had a stimulatory effect on the utilization of most of the substrates by RS-2.

Table 1. Most preferred carbohydrates by the isolates RS-1, RS-2 and RS-3 with 1mM Co^{2+}, Ni^{2+} and Mn^{2+} respectively (+) and without (-) metal amendments on different days of incubation.

Day	RS-1(- Co^{2+})	RS-1 (+Co^{2+})	RS-2 (-Ni^{2+})	RS-2(+ Ni^{2+})	RS-3 (-Mn^{2+})	RS-3 (+Mn^{2+})
1	D-Galactose	α Methyl D Mannoside	α-D-Glucose	α-D-Glucose	-	D-Melibiose
2	Xylitol	Mannan	α-D-Glucose	D-Mannose	D-Fructose	D-Melibiose
3	D- Trehalose	Mannan	α-D-Glucose	α-D-Glucose	Gentobiose	Gentobiose
4	D-Trehalose	Mannan	α-D-Glucose	D-Mannose	Gentobiose	Gentobiose
5	α-D-Glucose	L-Arabinose	α-D-Glucose	D-Mannose	α-D-Glucose	N Acetyl D-Glucosamine

Not utilised (-)

Table 2. Most preferred amino acids by the isolates RS-1, RS-2 and RS-3 with 1mM Co^{2+}, Ni^{2+} and Mn^{2+} respectively (+) and without (-) metal amendments on different days of incubation.

Day	RS-1 (-Co^{2+})	RS-1(+Co^{2+})	RS-2(-Ni^{2+})	RS-2 (+Ni^{2+})	RS-3 (-Mn^{2+})	RS-3 (+Mn^{2+})
1	L-Glutamic acid	-	L-Alanine	L-Alanine	-	L-Threonine
2	L-Asparagine	-	L-Alanine	L-Alanine	L-Leucine	Glutamic acid
3	L-Alanyl glycine	-	L-Alanine	L-Alanine	Hydroxy L-Proline	L-Leucine
4	L-Alanyl glycine	-	L-Alanine	L-Alanine	L-Proline	L-Leucine
5	L-Alanyl glycine	-	L-Alanine	L-Alanine	L-Proline	L-Leucine

Not utilised (-)

With respect to amino acid utilization in the absence of added Co^{2+}, L-Glutamic acid was the most preferred followed by L-Asparagine and L-Alanyl Glycine. However, it was interesting to note that in the presence of added Co^{2+} none of the amino acids could be utilized. With strain RS-2 there was no change in pattern of amino acid utilization in the presence and absence of Ni^{2+} amendment with L-Alanine being preferentially utilized both in the presence and absence of 1mM Ni^{2+}. The pattern of amino acid utilization by RS-1 and RS-2 showed similarity to that reported by Chun et al. [30] wherein the organism *B.megaterium* did not grow well on L-Histidine, L-Serine and L-Leucine. Absence of Mn^{2+} amendment resulted in amino acid not being utilized by strain RS-3 during the first 24 hours. Amino acid utilization began only on the second day with L-Leucine being the most preferred substrate followed by hydroxyl L-Proline on the third day and L-Proline till the end of the experiment. With Mn^{2+} amendment, amino acid utilization began immediately with L-Threonine being most preferred during the first 24 hours, followed by Glutamic acid on the second day and subsequently L-Leucine till the end of the experiment. Metal amendments significantly changed the preference for utilization of carbohydrates and amino acids especially in isolates RS-1 and RS-3.

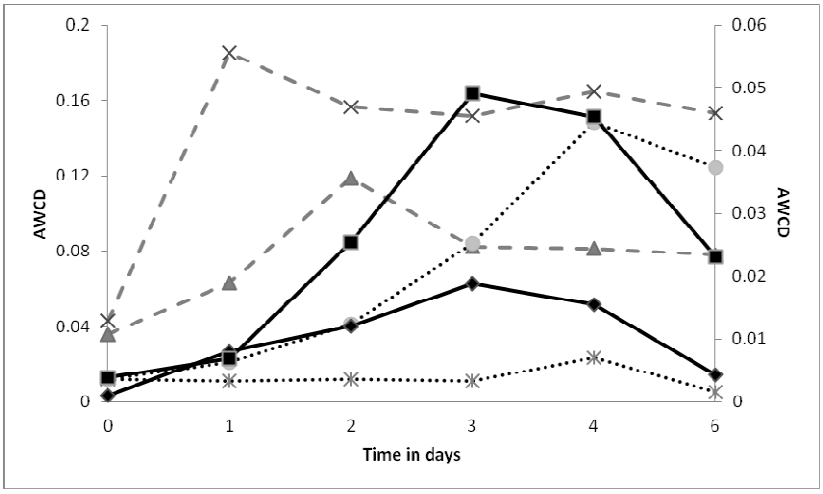

Fig 1. Average well colour development (AWCD) by isolate RS-1 with 1mM Co^{2+} (▪-✳-▪) and without (▪▪◆▪▪) Co^{2+} amendments (secondary Y axis), isolate RS-2 with 1mM Ni^{2+} (-▲-) and without (-x-) Ni^{2+} amendments (primary Y axis) and isolate RS-3 with 1mM Mn^{2+} (-♦-) and without (-■-) Mn^{2+} amendments (secondary Y axis).

Average well color development (AWCD) is an expression of the bacterial activity in a microtitre plate. AWCD analysis of the Biolog plates showed that isolates RS-1 and RS-3 developed highest AWCD during the same time period i.e. on the third day, both in the absence and presence of metal amendment (Fig. 1). However for isolate RS-2 maximum colour development occurred within 24 hours

in the absence of metal amendment, but required 48 hours in the presence of Ni^{2+}. AWCD values were lesser with metal amendment compared to unamended substrates, indicating decreased substrate utilization under metal stress. It could be inferred that Co^{2+}affected substrate utilization of RS-1 (83% decrease in AWCD) whereas the effect of Mn^{2+} on RS-3 was much less (61%). Ni^{2+} had the least effect on RS-2 with a minimal decrease of 46%. It may be possible that in RS-1 and RS-3 metal stress inactivates certain proteins important in cellular transport, or enzymes important in the metabolism of some substrates, thereby resulting in reduced substrate utilization. Shoeb et al. [31] have reported a unique mechanism of Ni tolerance in *Bacillus* wherein a 36 kDa protein identified as Flagellin by Peptide mass fingerprinting plays a major role. Flagellin is a component of flagellar filament which provides motility to the bacteria. Potential benefits of motility include increased efficiency of nutrient acquisition, avoidance of toxic substances and optimal access to colonization sites.

Effect of metal on growth and respiration

The growth profiles of RS-1, RS-2 and RS-3 in unamended media and with metal amendments of 10 µM, 100 µM, 1 mM and 5 mM are shown in Figs. 2, 3 & 4 respectively. The organism RS-1 and RS-2 showed maximum growth on the second day with 10 µM Co^{2+} amendments and on the fifth day with 100 µM Ni^{2+} amendments respectively. Though in RS-3 the growth peaked twice, the maximum growth was observed on the tenth day when amended with 5 mM Mn^{2+}.A decrease in growth was observed when metal concentrations increased beyond 5 mM. At the end of a 10-day incubation period, the respiring cell counts in control tubes of unamended media for strains RS-1, RS-2 and RS-3 were $6.7x10^6$, $5.2x10^6$ and $3.6x10^6$ cells ml^{-1} respectively. With metal amendment, the maximum total cell count was seen with isolate RS-1 at 10 µM Co^{2+} (2^{nd} day), RS-2 at 100 µM Ni^{2+} (5^{th} day) and for isolate RS-3 it was 5 mM Mn^{2+} (10^{th} day). However at the end of the ten day incubation period isolate RS-1 showed an increase in tolerance registering a maximum respiring cell count ($7.6x10^6$ cells ml^{-1}) at 100 µM Co^{2+} (Fig. 5a). Antony et al. [32] have observed that prolonged exposure of cells to Co^{2+} at 1 mM concentration resulted in morphological changes such as a reduction in cell surface to volume ratio. Decrease in cell surface area would decrease metal binding sites at the cell surface thereby enabling these isolates to tolerate high concentrations of Co^{2+} for longer duration. A similar adaptive strategy to counter heavy metal toxicity on exposure to heavy metals has been reported by Chakravarty et al. [33].

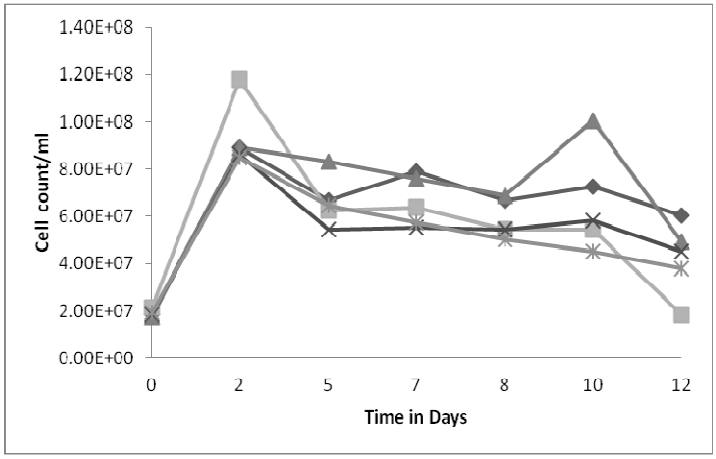

Fig 2. Growth of isolate RS-1 on 25% Nutrient broth with 2% crude salt and 0.01mM Co^{2+} (-■-), 0.1mM Co^{2+} (-▲ -), 1mM Co^{2+} (-x-), 5mM Co^{2+} (-□-) amendments along with unamended control (-♦-).

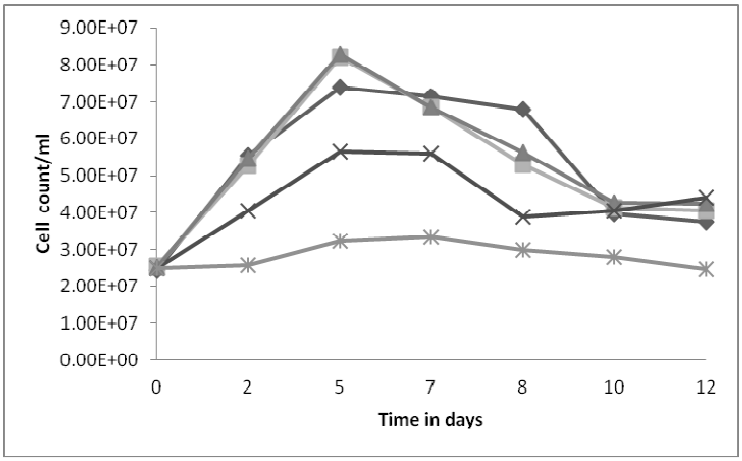

Fig 3. Growth of isolate RS-2 on 25% Nutrient broth with 10% crude salt and 0.01mM Ni^{2+} (-■-), 0.1mM Ni^{2+} (-▲ -), 1mM Ni^{2+} (-x-), 5mM Ni^{2+} (-□-) amendments along with unamended control (-♦-).

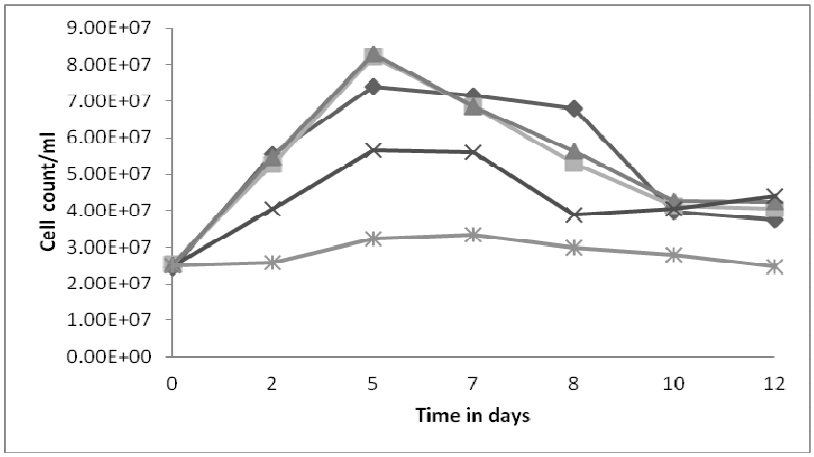

Fig 4. Growth of isolate RS-3 on 25% Nutrient broth with 20% crude salt and 0.01mM Mn^{2+} (-■-), 0.1mM Mn^{2+} (-▲ -), 1mM Mn^{2+} (-x-), 5mM Mn^{2+} (-□-) amendments along with unamended control (-♦-).

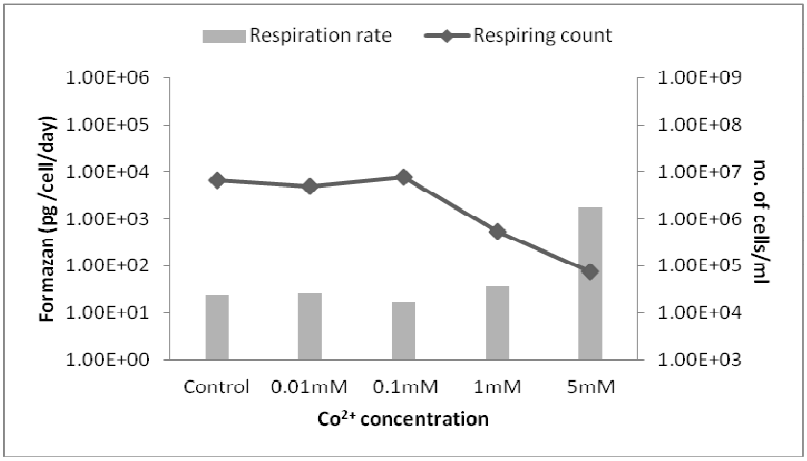

Fig 5a. Respiring count and respiration rate of RS-1 in unamended control and at different concentrations of Co^{2+}.

The trend was opposite for RS-2 which showed a drop in tolerance beyond 10 µM Ni^{2+} (maximum respiring cell counts were $7.5x10^6$ cells ml^{-1}) (Fig. 5b). Similar observations have also been documented by Cobet et al. [34] who reported toxicity of Ni at increasing concentrations in *Arthrobacter marinus* sp. Metals become toxic to the organism when their concentration is higher than the demand for metabolism, resulting in inhibition of metabolic pathways usually by strongly binding to enzymes, forming unwanted radicals or less stable reaction products [35].

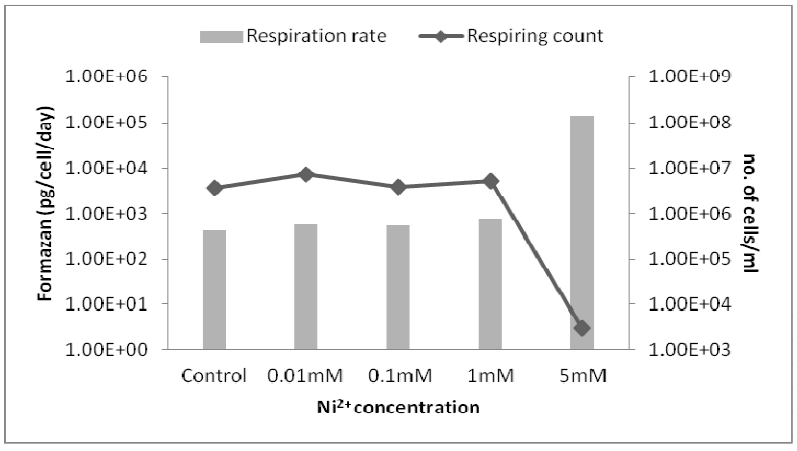

Fig 5b. Respiring count and respiration rate of RS-2 in unamended control and at different concentrations of Ni^{2+}.

Isolate RS-3 displayed a consistent respiring count till 1 mM Mn^{2+} and then a sharp increase at 5 mM Mn^{2+} ($7.3x10^7$ cells ml^{-1}) (Fig. 5c). The reduced Manganese toxicity in hypersaline isolate RS-3 might be due the formation of the less toxic forms of the metal and/or the change in the membrane of the cell in such a way that it confers higher levels of metal tolerance. Stimulation in total cell count of RS-1 was maximum at 100 µM Co^{2+} concentration (476%) after 10 days of incubation. A steady decline in cell counts was seen beyond 100 µM Co^{2+} amendments for isolate RS-1. With isolate RS-2 the total cell counts showed a normal growth curve till 1 mM, but showed a decline by one order at 5 mM Ni^{2+}. The percentage stimulation in total cell count of RS-2 was maximum at 100 µM Ni^{2+} concentration (69.7%) after 10 days of incubation. Similarly, the Mn tolerant isolate RS-3 showed a maximum increase in total cell counts at 5 mM (468%) after 10 days, indicating that isolate RS-3 had a high level of tolerance to Manganese.

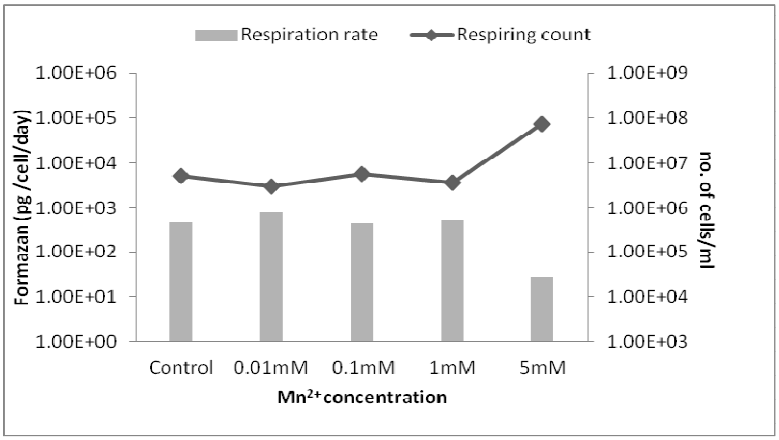

Fig 5c. Respiring count and respiration rate of RS-3 in unamended control and at different concentrations of Mn^{2+}.

Fig. 5a, b & c also show the effect of metal on the respiration rate. At the end of the incubation period, the percentage of respiring cells of RS-1 at 100 µM Co^{2+}, RS-2 at 10 µM Ni^{2+} and RS-3 at 5 mM Mn^{2+} accounted for 91%, 52% and 69% of the total count. Studies have shown that long-term heavy metal contamination of soils has harmful effects on soil microbial activity, especially microbial respiration [36]. Respiration rate for isolate RS-1 in unamended medium was 24.7 pg formazan $cell^{-1}$ day^{-1} and in media amended with 100 µM Co^{2+} it was 17.0 pg formazan $cell^{-1}$ day^{-1} (Fig. 5a). There was a steady decline in the respiration rate in the presence of Co^{2+} upto 100 µM; however the respiration rate rose sharply with increase in metal concentration. Comparatively, higher rates of respiration coupled with lower respiring/total counts observed could be considered as an index of stress. A decrease in respiration coupled with a decrease in cell count could indicate the presence of non-viable cells. With isolate RS-2, respiration rate was 528 pg formazan $cell^{-1}day^{-1}$ at 10 µM Ni^{2+} whereas in unamended media it was 417 pg formazan $cell^{-1}day^{-1}$ (Fig. 5b). Beyond 1 mM there was a drastic rise in respiration rate indicating the metal gets too toxic beyond that point. Small amounts of nickel are essential for the functioning of a number of nickel-containing enzymes including hydrogenase, urease, carbon monoxide dehydrogenase, and superoxide dismutase [37]. However, nickel is one of the most common metal contaminants in the environment and is often toxic to bacteria at high concentrations. This toxicity is generally a consequence of nickel binding to sulfhydryl groups of sensitive enzymes or displacing essential metal ions in a variety of biological processes. Cationic nickel (mostly Ni^{2+}) can cause significant oxidative stress in bacteria by facilitating production of oxidized bis-glutathione, which releases hydrogen peroxide [38]. The intracellular generation of superoxide by Ni and Co is reported to be toxic in *Escherichia coli* and superoxide dismutase is found to be involved in protection against this metal-induced oxidative stress [39]. Heavy-metal concentrations in cells are restricted through efflux mechanisms or by segregation as metal-thiols. Another important pathway is by altering the oxidation state of specific metal ions thereby reducing their toxicity. For many different metals, tolerance and homeostasis in bacteria involves a combination of these basic mechanisms [40]. In the case of Mn tolerant isolate RS-3, a different pattern was seen. The respiration rate peaked at 10 µM (813 pg formazan $cell^{-1}$ day^{-1}) and then decreased to 28 pg formazan $cell^{-1}$ day^{-1} at 5 mM, whereas in the unamended media it was 473 pg formazan $cell^{-1}$ day^{-1}(Fig. 5c). Ehrlich [41] and Emerson [42] have reported the tolerance of Gram-negative bacteria to elevated manganese concentrations appears to be due to enzymatic periplasmic oxidation of manganese. Redox cycling of Mn has a profound effect on the bioavailability and geochemical cycling of many essential or toxic elements [43].

Protein Expression studies in the presence of metal stress

Changes in protein profile of RS-1, RS-2 and RS-3 were investigated following exposure to 10, 100 and 1000 µM of Co^{2+}, Ni^{2+} and Mn^{2+} respectively. The response of each organism to different concentrations of metal was extremely diverse. Fig. 6a shows the SDS-PAGE analyses of proteins of isolate RS-1.The profile comprised thirty bands in the control representing protein concentrations between 9 to 138 ng. Two major protein bands, 29 kDa in the low molecular weight and 57 kDa in the medium molecular weight range showed considerable changes in concentration of protein fractions with cobalt amendments. 29 kDa protein was down- regulated (11%) as compared to the control, on exposure to 1000 µM of Co^{2+}; whereas the 57 kDa protein showed a 192% up- regulation at 1000 µM of Co^{2+} (Fig. 6b). No novel bands appeared on exposure to metal, however almost all the bands showed a measurable decrease in protein concentration compared

to the control. Similar observations were also made by Surosz et al. [44], Novo et al. [45] and Trehan et al. [46] wherein the synthesis of protein was down- regulated under heavy metal stress.

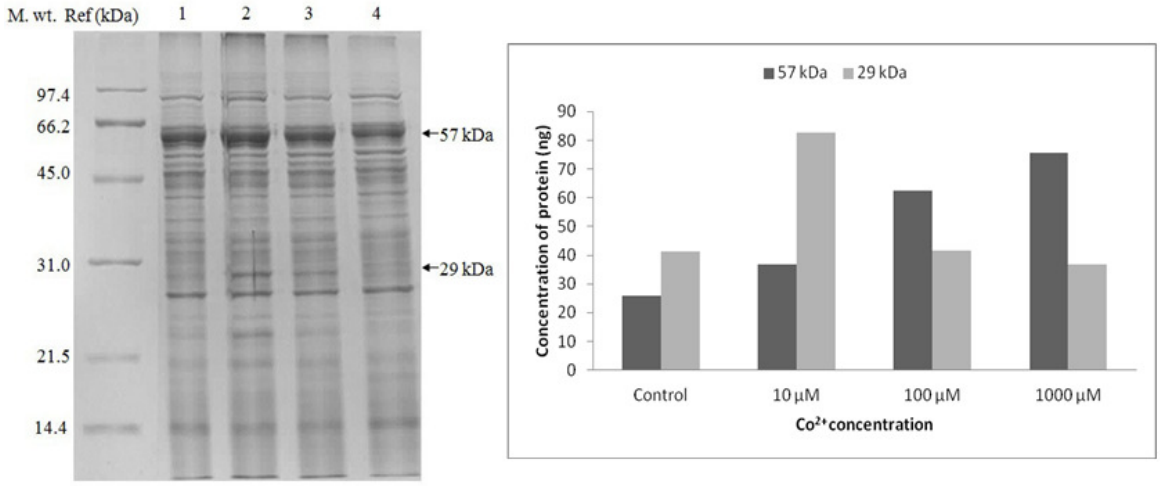

Fig 6. a) SDS-PAGE protein profile of isolate RS-1 after 24 hrs incubation. M - Marker, Lane 1 - Control, Lane 2 - with amendment of 10µM Co^{2+}, Lane 3 - with amendment of 100µM Co^{2+}, Lane 4 - with amendment of 1000µM Co^{2+} ; b) Concentration of major proteins expressed at varying Co^{2+} amendments.

Isolate RS-2 on the other hand seemed to be greatly affected by Ni^{2+} amendment (Fig. 7a). Out of 35 protein bands recorded in the control, only two proteins continued to be expressed on exposure to Ni^{2+}, with a percentage concentration ranging from 29 to 86 ng. These proteins were from the low and medium molecular weight range. 59 kDa appeared to be up-regulated to a considerable extent (94%) at a concentration of 100 µM whereas the 36 kDa protein was sustained even with the metal amendments and showed a marginal up-regulation at 1000 µM (14%) (Fig. 7b). Most of the other protein fractions were down-regulated in all the additions. Similar results were also obtained by Shoeb et al. [31], wherein an intense band of ~36 kDa molecular mass was found to be the only band of over expressed protein in presence of Ni^{2+}. All the other bands diminished as compared to control both in log phase culture and stationary phase culture. They have suggested that the ~36 kDa band protein is a constitutive protein of *Bacillus cereus* CMG2K4 which might play an active role in survival even in the presence of high concentration of Nickel. The over expressed ~36 kDa protein from Nickel tolerant strain of *B. cereus* was identified as Flagellin. Potential benefits of motility include increased efficiency of nutrient acquisition and avoidance of toxic substances. In bacterial cells, cation efflux-mediated Ni resistance is one of the best-known mechanisms of Ni detoxification [47, 48]. In addition, the up-regulation of genes encoding anti-oxidant enzymes is often the main response of many bacteria to various metals [49].

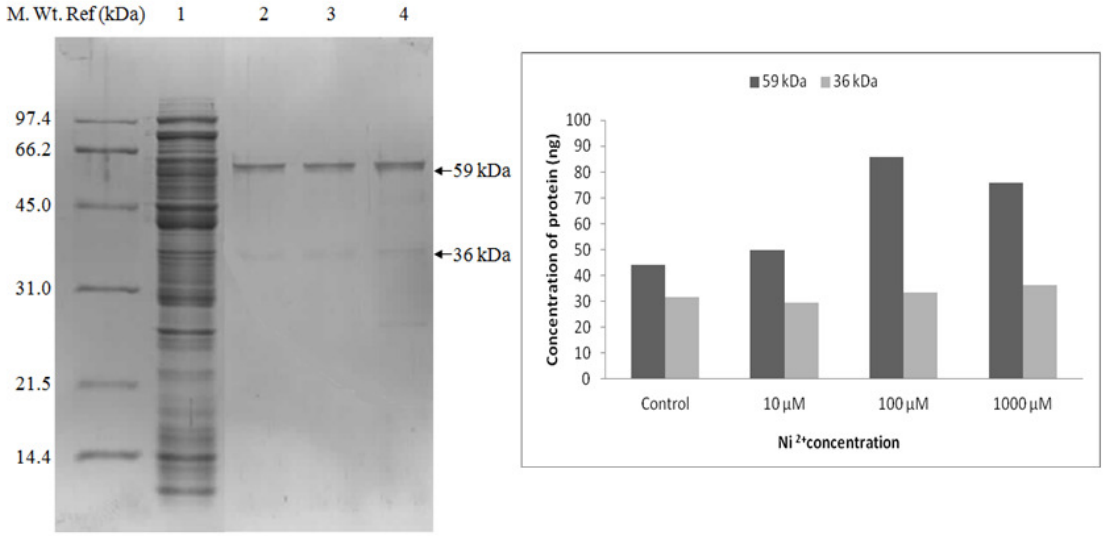

Fig. 7: a) SDS-PAGE protein profile of isolate RS-2 after 24 hrs incubation. M - Marker, Lane 1 - Control, Lane 2 - with amendment of 10µM Ni^{2+}, Lane 3 - with amendment of 100µM Ni^{2+}, Lane 4 - with amendment of 1000µM Ni^{2+}; b) Concentration of major proteins expressed at varying Ni^{2+} amendments.

Mn tolerant isolate RS-3 exhibited an altogether different profile, not only in terms of a variation in the banding pattern but also a significant variation in the magnitude of expression (Fig. 8a). A total of twenty one protein bands with a concentration ranging from 25 to 350 ng were expressed in the control. With Mn^{2+} amendments the proteins of significance were 50 kDa and 53 kDa. The 50 kDa band was up-regulated 23% at a concentration of 1000 µM whereas the 53 kDa protein disappeared with increase in concentration of

Mn^{2+} (Fig. 8 b). Complete elimination of some protein bands was also seen by Sinha et al. [50], Gianazza et al. [51] and Labra et al. [52] on cadmium treatment. In our study no new bands appeared on exposure to Mn metal. El-Gamal [53] has suggested that the decrease in protein band numbers in metal treated cells in comparison to control is indicative of cellular proteins being one of the main targets of heavy metal treatments. Trevors et al. [54] have suggested that different organisms show variations in response to metal treatments as well as to concentrations and have attributed it to the dissociation of some protein fractions, which moved to the lower molecular weight area.

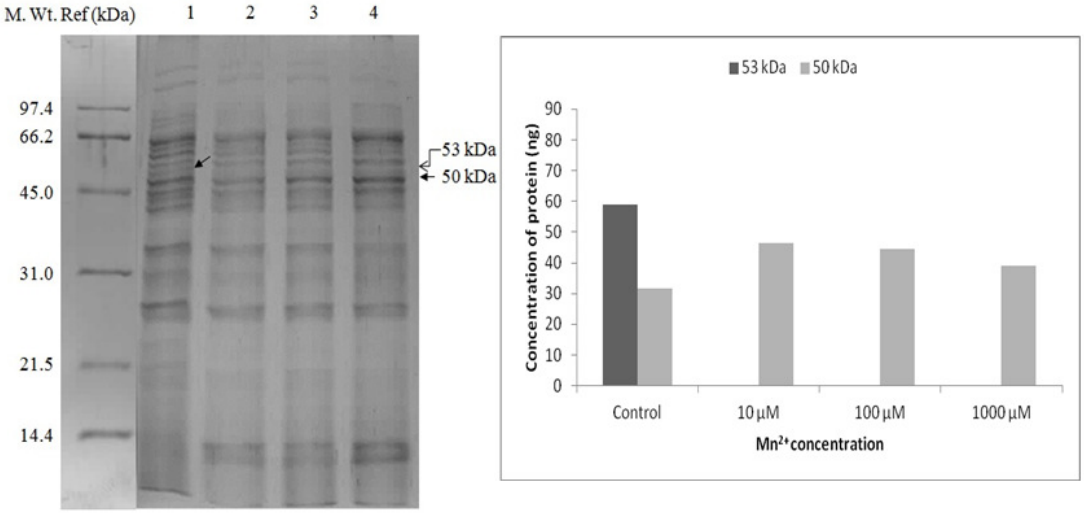

Fig 8: a) SDS-PAGE protein profile of isolate RS-3 after 24 hrs incubation. M - Marker, Lane 1- Control, Lane 2 - with amendment of 10µM Mn^{2+}, Lane 3 - with amendment of 100µM Mn^{2+}, Lane 4 - with amendment of 1000µM Mn^{2+} ; b) Concentration of major proteins expressed at varying Mn^{2+} amendments.

Imposition of any stress to bacteria results in adaptive responses that lead to changes in the regular metabolic process in the cells, which are then reflected in the alteration of the protein profiles [55]. Isolates RS-1, RS-2 and RS-3 showed variation in protein expression on exposure to metals. In the present study the variation observed in the expression of proteins could be an indication of cellular response or adaptation to counter metal stress.

CONCLUSION

The strains RS-1, RS-2 and RS-3 resistant to the Co^{2+}, Ni^{2+} and Mn^{2+} respectively were identified as *Bacillus cereus* (RS-1), *Bacillus* sp. (RS-2) and *Chromohalobacter beijerinckii* (RS-3). Co^{2+} and Ni^{2+} amendments stimulated growth of RS-1 and RS-2 at low concentrations. In case of RS-3, amendment with Mn^{2+} caused a conspicuous stimulation in growth coupled with a dip in respiration rate at 5 mM concentration and an increase in respiring counts. Ni^{2+} promoted the utilization of most of the substrates by RS-2, whereas Co^{2+} and Mn^{2+} had a significant negative impact on the utilization of carbohydrates and carboxylic acids in RS-1 and RS-3 respectively. The role of different proteins involved in metal stress in halotolerant and halophilic bacterial species has been documented, where the expression of 57 and 29 kDa (in RS-1), 59 (in RS-2), and 50 and 53 kDa (in RS-3) was specifically regulated with Co^{2+}, Ni^{2+} and Mn^{2+} amendments respectively. Hence the metal induced variations in phenotypic properties could be traced down to the changes in the synthesis of specific proteins and a high degree of functional diversity among the different bacteria.

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