Chapter 4

Taxonomy and growth-physiology of some Gram-negative nickel resistant Torsa isolates possessing inducible nickel resistance mechanism & Application of molecular biology and bioinformatics tools in understanding the nature of nickel resistance genetic system(s) in them.

4. Taxonomy and growth-physiology of some Gram-negative nickel resistant Torsa isolates possessing inducible nickel resistance mechanism & Application of molecular biology and bioinformatics tools in understanding the nature of nickel resistance genetic system(s) in them.

It is apparent from the PCR amplification study (discussed in the section 3.3 of Chapter 3) that most of the metal resistant Torsa isolates contain unexplored/ diverse nickel–resistance determinant(s), other than *cnr*, *ncc*, *nre*, *nir* or *ncr* (Liesegang *et al.*, 1993; Schmidt and Schlegel, 1994; Grass *et al.*, 2001; Stoppel *et al.*, 1995; Park *et al.*, 2004) to help them survive in an environment containing high concentration of nickel. The nickel resistant Torsa isolates showed a varied range of MTC (Maximum Tolerable Concentration) towards $NiCl_2$. Depending on MTC of nickel, the isolates (listed in Table 2.4 of Chapter 2) were categorized into three-classes:

I. *'Low level' nickel resistant isolates:* The members of this group demonstrated MTC in the range of 3– 5 mM $NiCl_2$. A total of 52 isolates fell in this group and 11 isolates were presumed to contain well-explored genetic systems (*czc*, *cnr* and *nre*) (discussed in section 3.4).

II. *'Moderate level' nickel resistant isolates:* The isolates showing MTC of 5.5– 7.5 mM $NiCl_2$ were grouped into this category. There were six isolates in this group, viz., 6NiCo 43, NiVa 61, 5CoNi 34, BB1A, BB37 and NiVa 51. Members of this group either did not produce any amplicon or did produce one or more amplicon(s) of the sizes other than the expected ones; which clearly indicated that either well-characterized nickel-resistance determinants were absent or they were present in a sequence–divergent group form.

III. *'High level' nickel resistant isolates:* There were only two representatives in this group. Both of the isolates, NiVas 113 and NiVas 114, showed MTC of 12 mM $NiCl_2$ and generated amplicons of varied sizes with bbrc1-bbrc2 and nre1-nre2 primer pairs (Figure 3.2 and 3.8).

The 'moderate' and 'high' –level nickel resistant isolates of Torsa River were subjected to several physiological and biochemical tests for their probable identification up to the genus level. Growth physiology was studied following methods described by earlier authors (Stoppel *et al.*, 1995; Stoppel and Schlegel, 1995, Grass *et al.*, 2001; Park *et al.*, 2003), to understand the phenomenon of induction of nickel resistance in these isolates. Southern hybridization of restricted genomic DNA of some isolates using *czc*, *cnr*, *ncc*, *nre* and *nir* specific probes yielded results that threw some light on the genome sequences in the context of probable metal resistance genetic systems.

4.1. Biochemical properties of some selected nickel resistant Torsa isolates:

Most of the nickel resistant strains, isolated from anthropogenically nickel polluted sites of Belgium, Germany and Zaire belonged to the genus *Alcaligenes* (Stoppel and Schlegel, 1995), whereas amongst the 200 nickel resistant bacteria isolated from the rhizosphere of nickel-hyperaccumulating plant *Sebertia acuminata*, the predominant species was identified as *Burkholderia* (Stoppel and Schlegel, 1995). *Pseudomonas pseudoalkaligenes* and *Klebsiella oxytoca* were found to be the predominant taxa among the nickel resistant bacterial population isolated from mineral oil emulsion (Stoppel *et al.*, 1995). The diversity of nickel resistant bacteria isolated from various ecosystems encouraged taxonomic investigation of the nickel resistant isolates of Torsa. Physiological and biochemical tests of eight Torsa isolates (6NiCo 43, NiVa 61, NiVa 51, BB1A, BB 37, 5CoNi 43, NiVas 113 and NiVas 114), showing resistance to more than 5 mM $NiCl_2$, were performed following techniques recommended in '*The Prokaryotes*' (Grimont and Grimont, 1992).

4.1.1. Materials and Methods:

4.1.1.1. Chemicals and reagents:

All chemicals used were of analytical grade and were purchased from Qualigens Fine Chemicals, India (unless otherwise mentioned). De-ionized double distilled water was used to prepare all chemicals and media. A 'digital pH-meter' (SYSTRONICS, India) was used for measuring the pH, and a digital balance (SARTORIUS) was used for weighing purpose.

4.1.1.2. Media and culture condition:

The nickel resistant bacterial strains were maintained in Nutrient Agar (NA) media (HIMEDIA, India Ltd.) supplemented with 1mM $NiCl_2$. For biochemical tests, cultures were grown overnight in a nutrient agar/ nutrient broth at 35 ^{0}C and one loop-full culture/ 1% inoculum (v/v) were added to the respective media. Culture media used for the experiments were supplied by HIMEDIA, India Ltd. (unless otherwise mentioned).

4.1.1.3. Biochemical tests:

The following biochemical tests were conducted for the identification and characterization of the strains:

I. Gram Reaction: Gram reaction was performed following protocols stated by Cappuccino and Sherman (1996). A smear of the culture was made on a glass slide, air-dried and was heat-fixed. The smear was flooded with crystal violet for one minute, washed with tap water and flooded with gram's iodine for one minute followed by washing and decolorization with 95% ethanol. After washing it was counter stained with safranin for 45 seconds, washed with tap water, air dried and finally examined under compound light microscope with oil immersion objective lens. Gram-negativity of the isolates was also tested on McConkey Agar plate.

II. Indole test: Cells were grown in 5ml tryptophan broth (g/l; NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4; L-tryptophan, 10.0, pH 7) and were incubated at 35 ^{0}C for 24h. 0.2 ml of the test reagent (p-dimethylaminobenzaldehyde, 0.5gm; Isoamyl alcohol, 0.5 ml; Conc. HCl, 2.5 ml) was then carefully added into the full-grown culture and the tubes were allowed to stand for 10min after a gentle shaking. A dark red colored ring on the surface was regarded as positive test.

III. Methyl Red test: The organisms were grown in 10 ml of glucose peptone broth for 2-3 days at 30 ^{0}C. The medium contained (g/l) - peptone, 5.0; KH_2PO_4, 5.0; glucose 10.0; and the pH of the medium was adjusted to 7.4. Few drops of methyl red reagent (5 mg dissolved in 30 ml of 95% ethanol and the final volume was made up to 50 ml with water) were added to the growing culture. Methyl red positive reaction was indicated by bright red coloration and negative reaction was indicated by yellow color of the culture medium.

IV. Voges-Proskauer test: One loop full of overnight grown test culture was inoculated in 5 ml of the sterilized buffered glucose broth (g/l, Protease peptone, 7.0; Glucose, 5.0; K_2HPO_4, 5.0; pH 7.0) and was incubated at 35 ^{0}C for 48h. 1 ml aliquot of the full-grown culture was taken in a separate test tube and 0.6 ml of α-naptha solution (α- napthol, 5.0gm dissolved in 100ml ethanol) and 0.2 ml 7(N) KOH was sequentially added and mixed well by shaking. Development of pink to crimson color at the surface within 5min considered as positive test.

V. Catalase test: The organisms were grown on Tryptic Soya Agar (TSA) plates for 24- 28 h at 35 ^{0}C to get the isolated colonies. A drop of H_2O_2 was put over a colony. As a negative control H_2O_2 was always dropped on any part of the plate devoid of any bacterial growth. Catalase positive character was indicated by effervescence of gas bubbles from the colony surface, immediately after the addition of H_2O_2.

VI. Oxidase test: The test was performed by adding bacterial culture on a strip of filter paper which was initially impregnated with 1% (w/v) aqueous solution of N-N-dimethyl-p-phenylenediamine. Development of pink color within 30 seconds indicated the oxidase positive tests.

VII. Citrate utilization test: The Simmons Citrate medium was used for this test. The composition of the Simmons Citrate medium was as follows (g/l); NaCl, 5.0; $MgSO_4.7H_2O$, 0.2; $NH_4H_2PO_4$, 1.0; K_2HPO_4, 1.0; Na-Citrate, 5.0; bromothymol blue, 0.02; Yeast Extract, 0.05; agar powder, 20.0; pH 6.8. Blue coloration of the media after

24- 48 h of inoculation was recorded as positive reaction.

VIII. Urease test: Urease activity was tested with a medium having following composition (g/l): part A: peptone, 1.0; glucose, 1.0; NaCl, 5.0; KH_2PO_4, 2.0; phenol red, 0.12; agar, 20.0; pH 8.0; and part B (g/l): urea 40.0. Part A was sterilized at 15psi for 15 min and Part B was filter sterilized. After cooling it to 40^0-50^0 C they were mixed together and were poured into the plates. In each plate, a loop-full of overnight-grown culture was streaked and incubated at 30 ^{0}C for 1– 2 days. Development of deep red color at the periphery of the bacterial colonies at the plates was indicative of positive test.

IX. Lysine Decarboxylase test: Moeller Decarboxylase broth [(g/l) Peptic digest of animal tissue, 5.0; beef extract, 5.0; Dextrose, 0.5; bromo cresol purple, 0.01; pyridoxal, 0.005; cresol red, 0.005; pH 6.0] supplemented with 1% L-lysine was used for this experiment. 50 µl of overnight grown culture(s) were added in 5 ml broth and incubated at 35 ^{0}C for 18 –20 h. Change of color from yellow to red was recorded as positive test.

X. Ornithine Decarboxylase test: Moeller Decarboxylase broth [(g/l) Peptic digest of animal tissue, 5.0; beef extract, 5.0; Dextrose, 0.5; bromo cresol purple, 0.01; pyridoxal, 0.005; cresol red, 0.005; pH 6.0] supplemented with 1% L-Ornithine was used for this experiment. 50 µl of overnight grown culture(s) were added in 5 ml broth and incubated at 35 ^{0}C for 18–20 h. Change of color from yellow to red was recorded as positive test.

XI. Nitrate reduction test: The enzyme nitrate reductase is a molybdenum-containing membrane bound enzyme, which reduces nitrate to nitrite in presence of an electron donor. Nitrate reduction and denitrification (as a function of dissimilatory reduction of nitrate denoting the presence of the enzyme nitrate reductase) was examined in the medium having following composition (g/l); KNO_3, 1.0; peptone, 5.0; pH 7.5. 5 ml volume of medium was dispensed in each tube and sterilized at 15 psi for 15 min. Each culture tube was inoculated with a single colony and incubated at 30 ^{0}C for 24 h. Formation of nitrite in the culture medium was tested by adding 1.0 ml test reagent (0.8g of sulfanilic acid dissolved in 100 ml of 5(N) acetic acid and 0.5 g of α – napthalamine dissolved in 100 ml acetic acid, mixed well before use). Development of red color indicates positive test for nitrate reductase. To monitor the production of gas by nitrate respiration, inverted Durham tubes were placed in culture medium.

XII. Lipase test: One loop full of overnight grown culture was streaked on Tributyrin agar plate (1.0 ml Tributyrin added in 100 ml sterile Tributyrin agar base) and was incubated overnight at 35 ^{0}C. Isolates showed opaque zone around the growth area was considered as lipase positive.

XIII. Gelatin hydrolysis test: Gelatin hydrolysis was examined in a medium composed of (g/l) gelatin, 20.0 & nutrient broth, 10. After melting, 6ml of the medium was distributed in each of the tubes, plugged with cotton and sterilized at 15 psi for 15 min. each tube was inoculated with freshly grown culture by stabbing, and incubated for 2 –3 days at 35 ^{0}C. The culture tubes, after growth of the organisms, were kept at 4 ^{0}C along with an un-inoculated (control) tube. The tubes that did not solidify even at 4 ^{0}C indicated the liquefaction of gelatin and the isolates were recorded as gelatinase positive.

XIV. Starch Hydrolysis: For this test organisms were streaked on Starch agar plates [(g/l); Nutrient Agar, 23.0; soluble starch, 5.0; pH 7.2] and incubated at 35 ^{0}C for 1 –2 days. After the growth of the culture the petri-plates were flooded with Lugol's iodine solution. Appearance of colorless zone surrounding the bacterial growth indicated the hydrolysis of starch leaving the other part dark blue in color due to the reaction of starch with iodine.

XV. Casein hydrolysis: Skim Milk Agar plates were streaked with overnight grown loop-full culture(s) and caseinase producing bacteria showed a clear zone at the periphery of the growth.

XVI. Fermentation of sugars: Fermentation of sugars was tested using 'Phelol Red Broth base' medium [(g/l); protease peptone 10.0; beef

extract, 1.0; Sodium chloride, 5.0; phenol red 0.018; pH 7.4] containing different sugars at a concentration of 1% (w/v). An aliquot of 50 µl overnight grown culture was inoculated in 5 ml of test media and incubated at 35 °C for 24 –48 h. Change in the color of the media from red to yellow was recorded as positive test.

XVII. Utilization of organic acids: To assay the utilization of organic acids as a carbon source, inorganic M70 media (Veron, 1975) was used. To prepare 1L of M70 medium, 10 ml of 'Metal-70' solution [(mg/l): H_3PO4, 1960; $FeSO_4$. $7H_2O$, 55.6; $ZnSO_4$. 7H2O, 28.7; $MnSO_4$. $4H_2O$, 22.3; $CuSO_4$. 5H2O, 2.5; $Co(NO_3)$. $6H_2O$; H_3BO_4, pH 6.2] was added in to 490 ml of 'P-Ca-Mg solution' [(mg/l); $CaCl_2$. $2H_2O$, 14.7; $MgSO_4$. $7H_2O$, 123; KH_2PO_4, 680; K_2HPO_4, 2610, pH 7.2], the resultant mixture was filter-sterilized and added in 500 ml sterile 'Nitrogen Base' solution [(g/l); NaCl, 10; NH_4SO_4, 1.0]. The substrate was filter-sterilized and added to the sterile M70 media at a concentration of 10 % (w/v). To the 10 ml of media 100 µl of growing cultures were added separately and growth was observed after 2 –6 days after incubation at 35 °C.

4.1.2. Results and Discussion:

All the eight nickel-resistant Torsa isolates, tested for number of biochemical and physiological properties, showed poor growth in defined basal media [(g/l); $(NH_4)_2SO_4$, 2.0; $MgSO_4$, 0.5; K_2HPO_4, 0.5; KCl, 0.1; pH 7.2] supplemented with glucose/ sucrose/ fructose/galactose as a sole carbon source. But all of them showed copious growth in nutrient rich heterotrophic media (viz, nutrient broth/ agar, luria broth/ agar, trypticase soy broth/ agar etc.); which indicated the copiotrophic nature of the isolates. All the isolates tested were gram-negative, catalase positive, motile, and formed slightly concave, smooth edged, white colonies on NA plates. The detailed physiological and biochemical properties of the isolates are listed in Table 4.1. Depending on the physiological and biochemical properties of the isolates, 5CoNi 34, NiVas 113 and NiVas 114 were identified as *Enterobacter* sp.; isolate 6NiCo 43 and NiVa 51 as *Serratia* sp.; and isolates NiVa 61, BB 1A and BB 37 were identified as member of the genus *Pseudomonas*, *Acinetobacter* and *Moraxella* respectively. The results of physiological and biochemical characteristics of the isolates (Table 4.1) were confirmed by MTCC (Microbial Type Culture Collection), Chandigarh, India.

4.1.3. Conclusion:

The Torsa isolates showing MTC for Ni^{2+} more than 5mM (i.e., within the range 5.5 –12.0 mM) predominantly belonged to the family of *Enterobacteriaceae*. Along with the members of *Enterobacteriaceae*, the members of the family *Moraxellaceae* and *Pseudomonadaceae* were also found. Among three *Enterobacter* sp., isolate NiVas 113 and NiVas 114 exhibited indistinguishable responses during biochemical tests (see Table 4.1), produced amplicons of comparable size with bbrc1-bbrc2 primers (Figure 3.2) and demonstrated similar MTC towards Ni^{2+}, Co^{2+}, $Cd^{2+}$and Zn^{2+} (see Table 2.4). Therefore it was assumed that these two isolates were taxonomically similar and were the representatives of same genus and species.

Among two strains of *Serratia* sp. (NiVa 51 and 6NiCo 43), NiVa 51 was found to tolerate comparatively higher (7.5 mM) concentration of nickel ion, and differences in their biochemical properties implied that they were probably the members of different *Serratia* species.

Isolate BB1A and BB 37 generated a ~500 bp amplicon with bbrc1-bbrc2 primer pair (Figure 3.2) and the restriction digestion analysis of the amplicon(s) (see figure 3.4) showed similar RFLP. Moreover, the results of numerical taxonomy identified them as *Acinetobacter* sp. strain BB1A and *Moraxella* sp. strain BB 37 respectively. Although it is very difficult to distinguish between *Acinetobacter* and *Moraxella* by biochemical tests, the former gave negative oxidase test in contrast to the later (Holt *et al.* 1994). Two different organisms have generated similar amplicon with the same pair of primers revealed identical sequence. Therefore the exploration of the nature and function of the gene predicted from the sequence of ~500 bp amplicon with bbrc1-bbrc2 primer pair may help in

Table 4.1. Biochemical and physiological characteristics of some nickel resistant copiotrophic bacteria isolated from Torsa River. All taxa are gram-negative, motile, catalase positive, indole negative and do not produce endospore.

Characteristics	BB1A	BB37	NiVa61	5CoNi43	NiVa51	NiVas113	NiVas114	6NiCo43
Pigment	-	-	+	-	-	-	-	+
Methyl Red	-	-	-	-	+	+	+	+
Voges-Proskauer	-	-	-	+	+	+	+	-
Oxidase	-	+	+	-	-	-	-	-
Urease	-	-	-	-	+	+	+	-
Lysine Decarboxylase	+	-	-	+	-	-	-	+
Ornithine Decarboxylase	-	-	-	+	+	+	+	-
Nitrate reductase	-	-	+	+	+	+	+	+
Lipase (Tween80)	-	-	nd	-	-	-	-	-
Starch	-	-	+	-	-	-	-	-
Gelatin	-	-	+	-	-	-	-	-
Casein	-	-	+	-	+	-	-	-
Acid production from carbohydrates:								
Glucose	-	-	-	+	+	+	+	+
Sucrose	+	+	-	+	+	+	+	+
Fructose	+	-	+	+	-	+	+	-
Arabinose	-	-	+	+	-	+	+	+
Mannitol	-	-	+	+	+	+	+	-
Mellibiose	-	-	+	+	+	+	+	+
Xylose	-	-	+	+	+	+	+	+
Adonotol	-	-	+	+	+	-	-	-
Cellibiose	-	-	-	+	+	+	+	-
Trehalose	-	-	+	+	+	+	+	+
Sorbitol	-	-	-	+	+	+	+	+
Lactose	-	-	+	+	+	+	+	-
Rhamnose	-	-	-	+	-	+	+	-
Raffinose	-	-	+	w	-	+	+	-
Utilization of organic acid salts as carbon source;								
Malonate	-	-	+	-	-	-	-	-
Acetate	+	+	+	+	+	+	+	+
Lactate	-	-	+	nd	+	-	-	nd
Oxalate	+	+	w	-	-	-	w	w
Tartarate	-	-	w	-	w	-	-	w
Citrate	-	-	+	+	+	+	+	+
Succinate	-	-	+	+	+	+	+	-

+, strain showed positive reaction; -, strains showed negative reaction; nd, not done; w, weak positive.

providing a new insight on the evolution and origin of these two genera from a common ancestor.

4.2. Study of the induction of nickel resistance:

The nickel-resistant bacteria isolated from the metal contaminated environments, such as, zinc decantation tank in Liège, Belgium (Mergeay *et al.*, 1985), metal working industry at Holzminden, Germany (Schmidt and Schlegel, 1994), from soil-litter mixture underneath the canopy of the nickel-hyper-accumulating tree *Sebertia accuminata* in New Cladonia (Stoppel and Schlegel, 1995) and mineral oil emulsion tank in Gotebörg, Sweden (Stoppel *et al.*, 1995), have shown considerable diversity in the regulation of expression of genes involved in nickel resistance.

In presence of sub-millimolar concentration of nickel and cobalt, the transcriptional response of the regulatory locus of inducible *cnr* system was studied in detail by a constructed transcriptional fusion of *cnrYXH* and *luxCBAD* (pMOL1550) and *cnrYXHC'* and *luxCBAD* (pMOL1551), luciferase reporter gene (Tibazarwa *et al.*, 2000). The study showed that in the strain *Ralstonia eutropha* 104 harboring pMOL1550/ pMOL1551, transcription from *cnrYXH* was maintained at a basal level in absence of nickel and cobalt, and transcription starts when cells are exposed to 0.02 mM Ni^{2+}, or 0.1 mM Co^{2+} and the rate of transcription reached its maximum when exposed to 0.3 mM Ni^{2+} or 2 mM Co^{2+}. Between 0-0.3 mM, transcription was positively induced with increasing nickel concentration (Tibazarwa *et al.*, 2000). As shown with Tn5-*lac* Z fusion, *cnr* is the best induced by 128 µM Ni^{2+}while other metal

served as less efficient inducers (Grass *et al.*, 2000).

The regulation of expression of nickel resistance of *Klebsiella oxytoca* CCUG15788 was studied with whole cells, where beginning of the exponential growth phase in liquid media containing 5 mM Ni^{2+} was found to be dependent on the condition of pre-growth condition (Stoppel *et al.*, 1995). Cells pre-grown in 5 mM Ni^{2+} have shown lag phase of 2-3 h whereas cells pre-grown in nickel free media showed lag phase of 12h. From these observations, earlier authors have concluded that nickel resistance in *K. oxytoca* CCUG15788 is an inducible property (Stoppel *et al.*, 1995).

In *Achromobacter xylosoxidans* 31A, *nreB* was specifically induced by nickel but not by cobalt and zinc (Grass *et al.*, 2001). Cells of *A. xylosoxidans* AX1 containing *nre*B-*lac*Z operon fusion on plasmid pTOM9 were induced by 0.3 mM Ni^{2+}.

The cells of *Hafnia alvei* 5-5, containing *ncr* determinants for nickel resistance, when exposed to 5 mM $NiCl_2$, showed lag phase of 15 h before growing exponentially. But when cells were pre-exposed to 0.5 mM Ni^{2+}, the lag phase was reduced to 8 h (Park *et al.*, 2003), clearly indicating the fact that the nickel resistance genetic system in *H. alvei* 5-5 is an inducible genetic system.

Pickup *et al.* (1997) isolated number of nickel-resistant bacteria, from a sewage-contaminated place of Cambria, UK, and all of them were found to be the members of family *Enterobacteriaceae*. The strains were found to possess a conjugative plasmid which responded to the inducible nickel resistance. The authors demonstrated that when cells were pre-exposed overnight to 0.5 mM $NiCl_2$ and then inoculated in nutrient broth (NB) containing 2.5 mM Ni^{2+}, the lag phase of the cells were remarkably reduced to 3h compared to the cells incubated overnight without any vestige of Ni^{2+} ion in the medium. From this observation the authors concluded that the nickel resistance property in those isolates was inducible.

Following the modus operandi stated by Stoppel *et al.* (1995), Pickup *et al.* (1997) and Park *et al.* (2003), similar growth experiments were performed to understand the nature of genetic system(s) conferring nickel resistance in Torsa isolates, 6NiCo 43, NiVa 61, NiVa 51, BB1A, BB 37, 5CoNi 43 and NiVas 114, having MTC for nickel ranging between 5.5 -12.0 mM. The isolates showed poor growth in the defined basal salt media supplemented with glucose; hence the nutrient broth was used as growth medium. As isolate NiVas 113 and NiVas 114 responded identically during biochemical characterization and were suspected to be the member of the same genus and species, growth curve of only NiVas 114 was constructed for analysis.

4.2.1. Materials and Methods:

4.2.1.1. Reagents and Chemicals:

All metal salts ($NiCl_2$, $6H_2O$; $CoCl_2$, $6H_2O$; $ZnSO_4$, $6H_2O$; $CuSO_4$, $7H_2O$, and CH_3COOPb, $2H_2O$) used in the experiment were of analytical grade and were supplied by Qualigens fine chemicals, India. Deionized double distilled water was used in the preparation of stock solutions and media. Required amounts of metal salts were dissolved to prepare 1M or 0.1M stock solutions, which were filter-sterilized and added in a sterile nutrient broth media before inoculation.

4.2.1.2. Pre-incubation of cultures:

One loop full of overnight grown culture of the isolates, 6NiCo 43, NiVa 61, NiVa 51, BB1A, BB 37, 5CoNi 43 and NiVas 114, were separately inoculated in 10 ml of NB and were grown at 35 ^{0}C for 15 –20 h. 1ml of such cultures were centrifuged at 5000 rpm for 5 min at 4 ^{0}C and the pellet was washed twice with sterile 0.87 (M) NaCl. The washed cells were suspended in 0.5 ml 'defined basal salt medium' [(g/l); $(NH_4)_2SO_4$, 2.0; $MgSO_4$, 0.5; KH_2PO_4, 0.5; KCl, 0.1; pH 7.2] containing 1% glucose(w/v) and 0.1% nutrient broth (w/v), and was incubated overnight either in presence or in absence of various concentrations (0.005 –0.100 mM) of added Ni^{2+}/ Co^{2+}/ Cu^{2+}/ Pb^{2+}/ Zn^{2+} ion(s). Cultures grown in presence of added metal ion(s) were considered as "induced pre-culture(s)" and in absence of the same were considered as "un-induced pre-culture(s)".

4.2.1.3. Growth in liquid media:

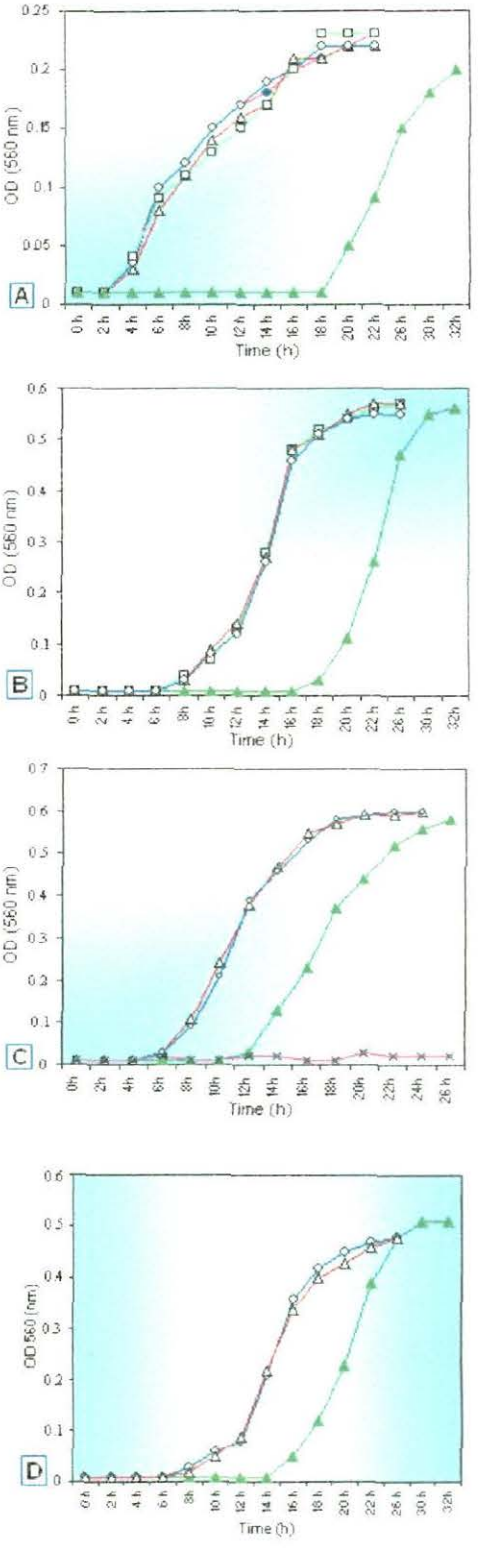
An aliquot of 0.1 ml of both induced and un-induced cultures were separately inoculated into two separate 100 ml Erlenmeyer flasks containing 10 ml nutrient broth supplemented with 3mM $NiCl_2$. The flasks were incubated at 35 °C with shaking (100 rpm) and optical density of the cultures were then measured at regular intervals at 560 nm in a spectrophotometer (Shimadzu, Japan), using sterile un-inoculated NB as a blank. Two nickel–sensitive (MTC <0.4 mM) Torsa-isolates, NiVa 101 and BB1C (tentatively identified as an *Acinetobacter* sp.), were similarly inoculated in nutrient broth containing 3 mM $NiCl_2$ which served as the negative control.

The normal growth curves of four nickel-resistant strains, NiVa 51, NiVas 114, NiVa 61, and BB1A, in heterotrophic medium were generated to understand their copiotrophic nature. For this, the strains were cultured overnight in nutrient broth (NB) at 35 °C and each culture was diluted using the same medium to an optical density of 0.6 at 560 nm. After adjustment, 1ml of each culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml NB. The flasks were incubated at 35 °C with shaking and optical density was measured at every 15 min interval for a total of 3 –5 hours after inoculation.

4.2.2. Results and Discussion:

Growth experiments in liquid media revealed that nickel resistance in these isolates (BB1A, BB37, NiVa 61, 5CoNi 34, NiVa 51, NiVas 114 and 6NiCo 43) was an inducible property (Figure 4.1). When un-induced cultures i.e. cells incubated overnight in basal salt medium containing 1% (w/v) glucose and (w/v) 0.1% NB without any added metal ion, were inoculated in NB containing 3 mM $NiCl_2$, the growth initiated exponentially after a lag phase of 9-19 h (Figure 4.1), but when cells were pre-grown in the presence of micro-molar concentration of Ni^{2+}/ Co^{2+}/ Cu^{2+}/ Zn^{2+}/ Pb^{2+} (induced pre-culture) and inoculated in NB containing 3 mM $NiCl_2$, they started growing exponentially only after a lag phase of 2-8 h (Figure 4.1).

When un-induced cells of the isolate BB1A were inoculated into NB containing a challenge concentration of 3 mM $NiCl_2$, they initiated the exponential growth after a lag phase of 19 h. Whereas, cells pre-grown in presence of 50 µM $NiCl_2$ (induced pre-culture) grew exponentially after a lag phase of only 2-3 h when exposed to the same medium (Figure 4.2-A). It has also been observed that when cells of BB1A were pre-grown



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in presence of 20-30 µM of lead acetate/ copper sulfate/ zinc sulfate and inoculated in NB containing 3 mM $NiCl_2$, the lag phase was reduced to the same extent (2- 3 h). The results suggested that micro-molar concentrations of nickel, lead, copper and zinc were sufficient for induction of nickel resistance in BB1A.

Under laboratory conditions, un-induced cells of NiVa 61, NiVas 114 and 6NiCo 43 showed a lag phase of 13 h in NB containing 3 mM $NiCl_2$ (Figure 4.1-C, F & G). In all the cases the duration of the lag phase was shortened to 3-6 h, provided the cells were pre-incubated in micro-molar (5-100 µM) concentrations of $ZnSO_4$ and $NiCl_2$. In case of isolates NiVa 61 and 6NiCo 43, only 5 and 8 µM of $ZnSO_4$ was found sufficient to induce nickel resistance respectively, whereas a minimum of 100 µM $ZnSO_4$ was required to induce nickel resistance in NiVas 114 (Figure 4.1-C, G & F). In addition to nickel and zinc, pre-exposure of cells in 50 µM $CoCl_2$ was also found to induce nickel resistance in isolate 6NiCo 43 (Figure 4.1- G). For all these three isolates, NiVa 61, NiVas 114 and 6NiCo 43, a minimum of 20 µM, 50 µM and 100 µM $NiCl_2$ respectively, were found essential for the reduction of lag phase in the same 3 mM nickel challenged medium (Figure 4.1 C, G & F).

In case of the isolates 5CoNi 34 and BB 37, the un-induced cells showed the lag phase of 15 and 17 h respectively in the NB medium containing 3mM $NiCl_2$, but pre-incubation in 8-10 µM zinc sulfate or 50 µM nickel chloride could effectively reduce the duration of lag phase by 8 -10h (Figure 4.1-D & B). Out of these two isolates, BB37 showed a similar reduction of lag phase in 3 mM nickel challenged media, when cells were pre-exposed to 50 µM Cu^{2+} (Figure 4.1-B).

The un-induced pre-culture of the isolate NiVa 51, when inoculated in NB containing 3mM $NiCl_2$, the cells entered into the log phase after a lag phase of 9 h, but the same culture when induced by pre-exposing the cells in Ni^{2+} or Zn^{2+} or Co^{2+} containing media, the lag phase was remarkably shortened to 2 h. It was also recorded from the study that a minimum of 100 µM of $ZnSO_4$/ 50 µM of $NiCl_2$/ 100

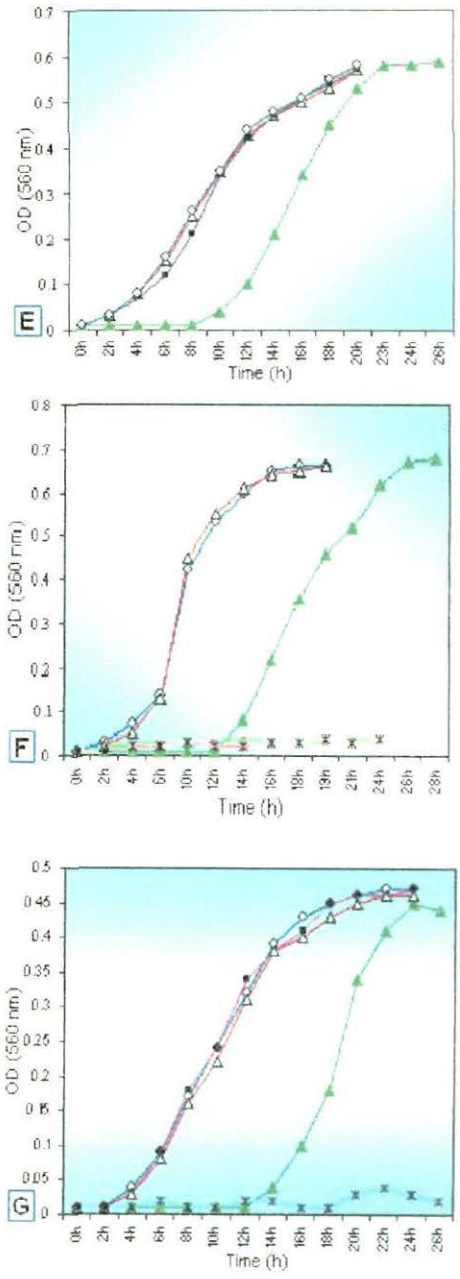

Figure 4.1. Induction of nickel resistance in some Torsa River isolates which tolerated more than 5 mM $NiCl_2$. Cells were grown in nutrient broth media containing 3 mM $NiCl_2$. Symbol definitions; ▲, growth curves of un-induced cells; Δ, growth curves of Zn^{2+} induced cells; ○, growth curves of Ni^{2+} induced cells; •, growth curves of Pb^{2+} induced cells; □, growth curves of Cu^{2+} induced cells; ■, growth curves of Co^{2+} induced cells; *, Growth curves of nickel sensitive isolate BB1C (negative control); and x, growth curves of nickel sensitive isolate NiVa 101 (negative control).
[A], Growth curves of the isolate BB1A; [B], Growth curves of the isolate BB37; [C], Growth curves of the isolate NiVa 61; [D], Growth curves of the isolate 5CoNi 34; [E], Growth curves of the isolate NiVa 51; [F], Growth curves of the isolate NiVas 114; [G], Growth curves of the isolate 6NiCo 43.

μM $CoCl_2$ was essential in order to induce nickel resistance in the isolate.

Similar growth experiments, demonstrating inducible property of nickel resistance in *Ralstonia metallidurans* CH34, *Burkholderia* strain 32W-2, *Klebsiella oxytoca* CCUG 15788, and *Hafnia alvei* M622 has been described by earlier authors (Stoppel & Schlegel 1995; Stoppel *et al.* 1995; Park *et al.* 2003). A considerable diversity in the minimum inducing concentration of Ni^{2+} could be noted from these studies. The nickel resistance system in *K. oxytoca* reached full induction only after pre-growth at the challenge nickel concentration of 5 mM, while *R. metallidurans* CH34 were induced by 0.5 mM as against the challenge nickel concentration of 3 mM. The nickel resistance system in both *Burkholderia* strain 32W-2 and *H. alvei* M622 could be induced by 1 mM Ni^{2+}. In contrast to all the earlier whole cell induction studies of nickel resistance system, the Torsa isolates showed full induction at a much lower inducing concentration (0.02 mM –0.1 mM) of nickel chloride. Except nickel, other metals (cobalt and zinc) proved to be less efficient inducers of *nre* (Grass *et al.*, 2001); and as shown with Tn5-*lac* Z fusion, *cnr* was best induced by 128 μM Ni^{2+} but not by other metal(s) (Grass *et al.*, 2000). In addition to nickel, the Torsa isolates showed full induction of nickel resistance determinant when pre-exposed to Co, Cu, Zn and Pb. Therefore, nickel resistant determinant of the Torsa-isolates having MTC of nickel >5 mM, showed considerable diversity in the nature of induction.

Similar growth experiments in a defined basal salt medium containing 1% (w/v) glucose/ fructose/ arabinose/ lactose/ sucrose were performed, but could not be acquiescent to turbidometric analysis due to initiation of poor growth of almost all the isolates even after a lag phase of 38-48 h.

4.2.3. Conclusion:

The importance of heavy metal resistance in ecological studies was recognized recently (Mergeay, 1991). Till date, no information is virtually available about the incidence and abundance of metal resistant bacteria in an environment that was not being considered as contaminated, if not said polluted, by heavy metals. In the present study, we have provided information for the first time about the incidence of nickel resistance in fractions of the recoverable copiotrophs isolated from River Torsa of India [Table 2.4], the metal content analysis of the river water, monitored round-the-year, indicated safe limit(s) for the metal(s) (tested) [Table 2.2] which people use for bathing and other purposes.

The copious growth of the nickel and/ or zinc resistant strains (NiVa 51, NiVa 61, NiVas 114 and BB1A) in nutrient broth exhibiting a mean generation time (g) of 13-25min, in contrast to insignificant growth by the same isolates in basal salt medium containing 1% glucose (w/v) and 0.1% nutrient broth (v/v), explained the copiotrophic nature of the strains used in this study. However, the 'g' values of the same strains were increased by 5 to 8 times in nutrient broth containing 3mM nickel chloride.

The Ni-resistant Torsa isolates, showing MTC of more than 5mM towards Ni, were examined for the induction of Ni-resistance by pre-culturing the isolates in NB containing non-lethal concentration of various metal ions (5-100 μM) and then rowing them in NB supplemented with 3mM $NiCl_2$. Control strains, BB1C and NiVa 101, showed no growth in 3mM $NiCl_2$ containing NB medium even after incubation of 26 h (Figure 4.1- C, F & G).

The growth profile of the isolates indicated that the nickel resistance properties of these strains was of inducible nature, since the pre-incubation of the cells in micro-molar concentration of metal cation had a significant effect on the reduction of lag time compared to the un-induced cells challenged with 3mM $NiCl_2$. We have attempted to explain the reason behind the difference in proportion of nickel resistant copiotrophic colonies recovered from water samples in different sampling months round the year (Table 2.3). Growth studies conducted with isolate 6NiCo 43, NiVa 61, BB 37 and 5CoNi 34, revealed that pre-exposure of the cells to 5 –10 μM zinc sulfate (= 325 –650 ppb Zn^{2+}) was found effective in inducing the nickel resistance of the isolates. This phenomenon indicates the fact that

the fraction of river water copiotrophs that possess such inducible nickel resistant genetic system (requiring ppb level of zinc for induction) would get the selective growth advantage over metal-sensitive copiotrophs on 3mM nickel containing plates. The recovery of nickel resistant copiotrophs on metal-containing nutrient agar plates was poor in sampling-months when zinc content of the river was found below the inducible concentration (>200 µM), while in sampling months that recorded high Zn^{2+}content (555.46-691.32 ppb) the recovery was high (see Table 2.3). Data obtained from the similar induction studies enabled to conclude that nickel resistance in several Torsa isolate was induced by pre-exposing the cells in 4 µM Zn^{2+} (260 ppb) where the lag phase was found to be reduced by 6-8 h in all the cases. Although a high correlation (0.8) value between lead content and nickel resistant bacterial population was scored, but, except BB1A, the nickel resistance in other bacterial isolates could not be induced by pre-exposing the cells in Pb^{2+} containing medium. The lead ion content of the river therefore may not have any biological significance in inducing nickel resistance. The high correlation score (0.87) between lead and zinc ion content of the river may be explained by the dissolution of these two metals from the same geological source(s).

Undoubtedly, the nickel resistant strains, e.g., 6NiCo 43, NiVa 61, BB 37 and 5CoNi 34, bear the potential genetic material for enriching the genomic of nickel resistance in prokaryotes. Furthermore, the property of induction of metal resistance by ppb level of bio-available metal ions in the environment could be genetically manipulated in constructing microbe-based biosensors.

4.3. Molecular genetic characterization of nickel resistant determinant(s) of three Torsa isolates that tolerate more than 6 mM nickel chloride:

Considerable diversity in nickel resistance phenotype along with the diversity in resistance genes have been observed among the bacterial strains isolated from metal contaminated/ enriched environments. Among the metal resistance determinants of which nickel resistance has been one of component, discovered so far, *cnr* and *ncc* operon shared high degree of nucleotide sequence homology as well as in protein sequence of the transcribed genes (Schmidt and Schlegel, 1994), and could not be expressed in *E. coli* (Lisegang *et al.*, 1993; Schmidt and Schlegel, 1994). On the other hand, *nre* operon, which co-reside with *ncc* operon in plasmid pTOM9 of *Achromobacter xylosoxidans* 31A, exhibited only limited sequence identity with *cnr* and *ncc*, and was found to express in *E. coli* (Schmidt and Schlegel, 1994; Grass *et al.*, 2001). The *nirB* gene of *nir* operon, discovered from *Klebsiella oxytoca* CCUG 15788, shared certain degree of sequence homology with *nreB*, and high degree (95%) of sequence identity with *ncrB* gene of *Hafnia alvei* 5-5. Both, *nir* and *ncr* operon, were found capable of expressing in *some* members of *Enterobacteriaceae* including *E. coli* (Stoppel *et al.*, 1995; Park *et al.*, 2003).

Stoppel and Schlegel (1995) isolated large number of nickel resistant bacteria from the rhizosphere of nickel hyper-accumulation tree *Sebertia accuminata* and also from anthropogenically nickel polluted sites. The restricted total-genomic-DNA of the nickel resistant strains (a total of 50 isolates) were subjected to DNA-DNA hybridization with the probes prepared from the inserts of the recombinant plasmids containing *cnr*, *ncc*, *nre* and *nir* operon (Stoppel and Schlegel, 1995). On the basis of hybridization signals, the nickel resistant determinants of the strains were grouped into four classes: (a) '*cnr/ncc*' type determinants, generated strong hybridization signal with *cnr* and *ncc* probes; (b) '*cnr/ncc/nre*' type determinants, yielded hybridization signal with *cnr*, *ncc* and *nre* probes; (c) *K. oxytoca* type determinants gave strong hybridization signal with *nir* probe; and (d) the others had no hybridization reaction with any of the DNA probe used. The majority of the strains, showed hybridization signal with *cnr*, *ncc* and *nre* probes. Only two strains, out of 50, yielded strong hybridization signal with *nir* probe, and subsequently it was one strain among them, *H alvei* 5-5, which was found to harbor a novel high-level nickel resistant determinant gene named *ncr* (Park *et al.*, 2003 and 2004).

(Park et al., 2003 and 2004).

It was described earlier that nickel resistant bacteria were isolated from the waters of Torsa River of India. PCR analysis of the isolates (section 3.2) clearly indicated that most of the isolates failed to produce amplicons with primers designed from well characterized metal resistance operon that conferred resistance to metals including nickel or exclusively nickel. Some of the isolates, having MTC of nickel between 5.5 –12 mM, showed an inducible nickel resistance phenomenon (discussed in section 4.2). In order to explore the genetic constituent that rendered nickel resistance in isolates of Torsa, the restricted genomic DNA of three isolates BB1A, NiVa 51 and NiVas 114 were subjected to DNA-DNA hybridization using *cnr*, *ncc*, *nre* and *nir* probes. The rationale behind the selection of these three isolates rested on the choice of higher MTC of nickel (6.5 -12 mM) shown by the isolates in the pool of nickel resistant isolates (Table 2.4 of Chapter 2).

4.3.1.1. Materials and Methods:

All the reagents required for the following methods were prepared according to the protocols described by Davis *et al.* (1986), (unless otherwise mentioned). The molecular biology grade chemicals used for the preparation of reagents were purchased from Sisco Research Laboratory Ltd. (SRL, India Ltd.).

4.3.1.1.1. Preparation of total genomic DNA:

The methods employed for the isolation and quantification of total genomic DNA are discussed in section 3.2.2 of Chapter 3.

4.3.1.1.2. Restriction Digestion of the genomic DNA:

An aliquot of 50 µl of genomic DNA preparation (that contain ~ 5 µg DNA) was taken in a 0.5ml microcentrifuge tube and was suspended in 81 µl of sterile double distilled water. To the suspended DNA solution 15 µl of 10X restriction buffer and 4 µl of *Eco*RI restriction enzyme (GENEI, India) was added, the ingredients were mixed by vortexing and centrifugation, and incubated overnight at 37 ^{0}C. On the next day the DNA was extracted by consecutive phenol-chloroform (Tris-equilibrated phenol and chloroform, 1:1) and chloroform-iso amylalcohol (24:1) treatment, and precipitated by dehydrated alcohol. The pellet was dried under vacuum and suspended in 25 µl TE (Tris-acetate 10.0 mM: EDTA 1.0 mM), checked on 1% agarose gel (with ethidium bromide) and the DNA was estimated in an UV-vis spectrophotometer (Shimadzu, Japan).

4.3.1.1.3. Southern Hybridization:

'DIG High prime DNA labeling and detection kit', purchased from Roche Diagnostics GmbH, Penzberg, Germany, was used for the experiments following manufacturer's instructions.

I. Preparation of probes:

a. PCR amplification: For the preparation of probes, the primers (listed in Table 3.2) were used to amplify specific region of *cnr*, *ncc*, *nre* and *nir* determinants (see Chapter 3). PCR using bbrc1-bbrc2 primers yielded ~700 bp amplicon from the positive controls *R. metallidurans* CH34 and *A. xylosoxidans* 31A, representing partial *cnrA* and *nccA* gene respectively. Using genomic DNA of *K. oxytoca* CCUG 15788 as template, a 950 bp amplicon, representing partial *nirA* gene, was generated by nir1-nir2 primer pair. As the nucleotide sequence of the 950 bp amplicon of *K. oxytoca* has 98% sequence homology with *ncrA* gene, no further probe for *ncr* locus was used in the experiment. An amplicon (200 bp) generated from *nreA* gene by using nre1-nre2 primer pair from the genomic DNA of *A. xylosoxidans* 31A was used as a probe for detection of *nre* locus. The PCR conditions used for the amplification are described in section 3.2.4 of Chapter 3.

b. Elution of the PCR products from low melting point agarose and labeling of the amplicons:

Setting and running of the gel was performed at 4 ^{0}C in 1X TAE following techniques stated in 'Basic Methods in Molecular Biology' (Davis *et al.*, 1986). The required band (s) were sliced out by sterile scalpel and taken in a 2.0 ml microcentrifuge tube, and were melted at 65 ^{0}C for 5 min. To the molten agarose equal volume of TAE buffer was added and agarose particles were removed by repeated phenol extraction. Finally DNA was precipitated and

suspended in TE (10mM Tris-Cl: 0.1 mM EDTA, pH 7.5), quantified and used in labeling reaction.

The random primed DNA labeling (non-radioactive) DIG- (Digoxigenin-dUTP) DNA labeling kit (Roche diagnostics, Germany) was used for the labeling reaction following manufacturer's instructions. Approximately 1.0 µg of template DNA was taken in 16 µl of sterile double-distilled water; denatured in a boiling water bath and immediately transferred in an ice bath. To the denatured DNA 4 µl of 'DIG-High Prime' was added, thoroughly mixed and incubated overnight at 37 °C. On the next day 2 µl of 0.2 M EDTA was added to the mixture to stop the reaction.

II. Transfer of DNA to the nylon membrane:
In an agarose gel (1%) the *Eco*RI restricted genomic DNA of the isolates (BB1A, NiVa 51 and NiVas 114), positive control (selected on the basis of probe used) and negative controls (listed in Table 3.1 of chapter 3) were loaded in separate lanes. Into the same agarose gel the PCR products generated by the isolates with specific primers (see Figure 3.2, 3.8 and 3.9 of chapter 3) and the unlabeled probe DNA were also loaded in separate lanes. The gel was electrophoresed at 50 mV for at least 5 h. The electrophoresed DNA and PCR products were then transferred to a positively charged nylon membrane by capillary transfer method (Sambrook *et al.*, 1989) after depurination (0.25 N HCl, for 15 min); denaturation (1.5 M Nacl, 0.5 mM NaOH, for 20 min); and neutralization (1.0 M Tris-Cl, 2.0 M NaCl, pH 5.0, for 25 min). After transfer, the DNA on the membrane was fixed using a UV-crosslinker (Stratagene, USA).

III. Pre-hybridization:
The membrane was placed in a plastic hybridization bag and was allowed to wet in 10 ml of preheated (40 °C) DIG-Easy Hyb solution. The hybridization bag was properly sealed with care so that no air bubbles remained inside. The sealed bag was agitated in a water bath at 40 °C for 30 min.

IV. Hybridization:
The pre-hybridization solution was forced out from the bag and 10 ml of fresh DIG-Easy Hyb solution was introduced again. The DIG-labeled DNA probe was denatured in boiling water bath for 5 min and was immediately placed in ice bath. The denatured probe was then introduced in to the hybridization bag and was mixed well avoiding foaming. The bag was immediately placed into a water bath at 50 °C and agitated for 15-16 h.

V. Post hybridization wash:
After hybridization the membrane was placed in a plastic container and washed twice for 5 min in 50 ml Solution-A (2X SSC, 0.1% SDS) at 25 °C under constant agitation. Stringent washes were also done twice for 15 min (2 X 15 min) in solution-B (0.5 X SSC, 0.1 % SDS) at 50 °C.

VI. Blocking of membranes:
After hybridization and stringent washes, the membrane was rinsed in 'washing buffer' (0.1 M Maleic acid; 0.15 M NaCl; pH 7.5; 0.3% Tween 20). Then the membrane was incubated for 30 min in 100 ml 1X 'Blocking solution' (prepared by diluting 10X Blocking reagent with wash buffer). The membrane was dipped into 20 ml 'Antibody solution' (10 µl of Anti-Digoxigenin-AP was added in 50 ml Blocking solution) for 30 min and then washed twice in 10 ml wash buffer for 15 min to remove the unbound antibody.

VII. Detection of hybridization by color development:
After blocking, the membrane was transferred into a glass tray and equilibrated with 20 ml detection buffer (0.1 M Tris-Cl, 0.1 M NaCl, pH 9.5) for 5 min. Then the membrane was placed in a fresh glass tray and 10 ml of freshly prepared 'color-substrate solution' (200 µl NBT/BCIP stock solution to 10 ml detection buffer) was poured on it. The membrane was preserved in dark and the reaction was stopped after 16 h by transferring the membrane in water.

4.3.1.2. Results and Discussion:

Bacteria resistant to nickel have so far been isolated from ecosystems polluted by heavy metals, such as waste waters, mine deposits, industrial composts and cooling waters of the metal-processing industries. Metal resistant bacteria isolated and characterized in detail from such ecosystems were

R. metallidurans CH34 (Brim *et al.*, 1999), *Alcaligenes denitrificans* 4a-2 (Kaur *et al.*, 1990), *A. eutrophus* KT02 (Schmidt *et al.*, 1991), *Klebsiella oxytoca* (Stoppel *et al.*, 1995), *Hafnia alvei* 5-5 (Park *et al.*, 2003) etc. During this study, we have isolated number of nickel resistant bacteria from Torsa River water free of toxic level of heavy metal contamination. Incidence of nickel resistant bacteria in such ecosystem may provide evidence supporting the hypothesis that *bacteria evolved with the heavy metal resistant gene(s) in a pre-historic earth already contaminated with heavy metal elements due to volcanic activities* (Misra, 1992).

With an objective to identify nickel resistance determinants of Torsa isolates, having MTC of nickel ≥ 3mM, we have first conducted the PCR amplification with the primers designed from the well known metal resistance determinants responsible for imparting resistance to heavy metals including nickel or exclusively nickel. The results of the PCR amplification indicated towards the probabilities of existence of novel or diverse genetic loci for nickel resistance.

Three Torsa isolates, representing different taxa, tentatively identified as *Acinetobacter* sp. strain BB1A, *Serratia* sp. strain NiVa 51 and *Enterobacter* sp. strain NiVas 114, demonstrated moderately high MTC of Ni^{2+} (6.5 mM, 7.5 mM and 12.0 mM respectively) than the other Torsa-isolates (see Table 2.4 of Chapter 2). Growth-physiology of these isolates in liquid medium (see section 4.2) confirmed the inducible nickel resistance phenotype. The results of the PCR amplification could not be interpreted to describe the nature of genetic determinants involved in nickel resistance of these isolates. All three isolates generated one or more than one amplicon(s) with different primer pair(s) designed to amplify well known nickel resistant determinants (see Table 4.2). Although the results of the PCR amplification, restriction analysis and sequencing of the PCR product, indicated the absence of *czcA*, *cnrA*, *nccA*, *nreA* and *nirA* homolog sequences in the isolates, but probability of existence of DNA region having weak homology with any of these well known nickel resistant determinants could not be ruled out.

With an aim to identify DNA regions having at least partial homology, if any, with *czc, cnr, ncc, nre,* and *nir* genes, in the genome of the isolates, Southern hybridization was performed with operon-specific probes. The result of the hybridization for each strain is discussed under the following headings:

Table 4.2. The number and size of the amplicon(s) generated by three Torsa isolates with specific primers.

Name of the Isolates	Primer used for amplification	Number of amplicon(s)	Approximate Size of the amplicon(s)
Acinetobacter sp. strain BB1A	bbrc1-bbrc2	1	0.5 kb
	nre1-nre2	2	0.2 kb & 0.5 kb
Serratia sp. strain NiVa 51	nre1-nre2	1	0.2 kb
	nir1-nir2	1	0.9 kb
Enterobacter sp. strain NiVas 114	bbrc1-bbrc2	4	2 .0 kb, 1.5 kb, 0.8 kb & 0.6 kb
	nre1-nre2	2	0.2 kb & 0.7 kb

I. *Acinetobacter* sp. strain BB1A: The 500 bp amplicon generated by bbrc1-bbrc2 primer pair was sequenced (see Figure 3.10-B of Chapter 3) and analyzed. It did not show any homology with any gene sequences of *cnr* or *ncc* operons. With the nre1-nre2 primer pair, the isolate has also generated two amplicons of 200 and 500 bp, but none of the PCR product(s) of the strain yielded detectable hybridization signal with *cnr*, *ncc*, *nre* or *nir* specific probe(s). But the Eco RI restricted genomic DNA of the isolate generated a weak hybridization signal with *nirA* probe only (Figure 4.2).

II. *Serratia* sp. strain NiVa 51: Although isolate NiVa 51 generated 200 bp and 900 bp amplicons (equal to the size of positive control, see Figure 3.8 and 3.9) with nre1-nre2 and

nir1-nir2 primer pair respectively, but none of them hybridized with the *nre* and *nir* specific probes. Therefore, the amplifications can be regarded as spurious amplification or product of non-specific priming. The *Eco* RI restricted genomic DNA of the isolate generated a weak hybridization signal only with *nir* probe, but not with any other probes used in the study (Figure 4.3).

III. *Enterobacter* sp. strain NiVas 114: Isolate NiVas 114 produced four amplicons with bbrc1-bbrc2 primer pair (see Figure 3.2 of chapter 3) and two amplicons with nre1-nre2 primer pair (see Figure 3.8 of Chapter 3). The *cnr* specific probe generated weak hybridization signal with 0.8 kb and 1.5 kb amplicons of the isolate generated by bbrc1/2 primers (Figure 4.4), but *nre* probe did not hybridize with any amplicon generated by nre1/2 primers. The *Eco* RI restricted genomic DNA of the isolate yielded a weak hybridization signal with *cnr* specific probe only (Figure 4.4).

From the result of the Southern hybridization experiment it could be assumed that the nickel resistant determinant of isolates, BB1A, NiVa 51 and NiVas 114, were diverse in nature. The weak hybridization signal observed in all the cases with *nir* or *cnr* specific probe indicated towards the probability of existence of DNA regions, having a degree of sequence identity with sequence of amplified DNA region of *nirA* or *cnrA* gene. The PCR products of the isolate NiVas 114, showed weak hybridization signal with *cnr* specific probe which may enable to predict certain gene function related to metal resistance after analysis of the cloned sequence.

4.3.2. Cloning and sequencing of amplicons generated hybridization signal with *cnr* probe:

Isolate NiVas 114 (tentatively identified as *Enterobacter* sp.) yielded four amplicons of 2.0 kb, 1.5 kb, 1.0 kb and 0.6 kb, with bbrc1-bbrc2 primer pair. In Southern hybridization experiments, the 1.5 kb and 1.0 kb amplicons generated weak hybridiza-

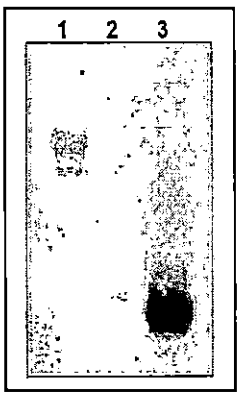

Figure 4.2. Results of Southern hybridization of the restricted total genomic DNA using *nir* probe. Lanes 1 & 2, *Eco* RI restricted genomic DNA of BB1A and *Escherichia coli* XL1 Blue (negative control); and lane 3, Probe DNA positive control (amplicon bracketing a 0.95 kb region of *nir*A gene).

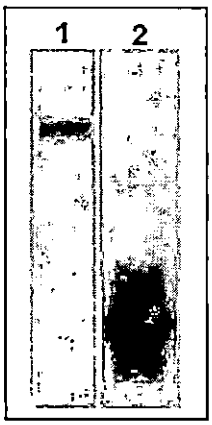

Figure 4.3. Results of Southern hybridization of the restricted total genomic DNA of isolate NiVa 51 using *nir* probe. Lanes 1, *Eco* RI restricted genomic DNA of NiVa 51; and lane 2, Probe DNA positive control (amplicon bracketing a 0.95 kb region of *nir*A gene).

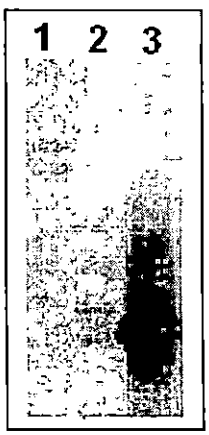

Figure 4.4. Results of southern hybridization experiment using *cnr* specific probe. Lane definitions, lane 1, *Eco* RI digested genomic DNA of isolate NiVas 114; lane 2, PCR products of the isolate NiVas 114^T generated by using bbrc primers; and lane 3, the unlabeled probe DNA positive control (PCR amplicon generated from *Ralstonia metallidurans* CH34 using bbrc1/2 primers).

-ation with *cnr*-specific probe. Both these amplicons were cloned, sequenced and analyzed to score their percent identity with the probe (partial *cnrA* gene) and to predict their probable role in the host.

4.3.2.1. Materials and Methods:

All the chemicals were used in the experiment were purchased from SRL Fine Chemicals (SRL India Ltd.) and deionized double distilled water was used for the preparation of reagents. The reagents were either filter-sterilized or autoclaved before use, and were preserved in glass containers.

4.3.2.1.1. Elution of the PCR products from low melting point agarose:

Approximately 100 µl of PCR product of isolate NiVas 114 (generated by bbrc1-bbrc2 primers) were directly precipitated by dehydrated ethanol and were suspended in 25 µl TE (10mM Tris-Cl: 1 mM EDTA, pH 7.5). 20 µl of the suspended DNA was loaded in a 'Low Melting Point Agarose' gel and were subjected to electrophoresis at 40 –45 mV for at least 8 –10 h. Setting and running of the gel was performed at 4 °C in 1X TAE following techniques stated in 'Basic Methods in Molecular Biology' (Davis *et al.*, 1986). The required band (s) were sliced and taken in an 2 ml microcentrifuge tube, and were melted at 65 °C for 5 min. to the melten agarose equal volume of TAE buffer was added and agarose particles were removed by repeated phenol extraction. Finally DNA was precipitated and suspended in TE (10mM Tris-Cl: 0.1 mM EDTA, pH 7.5), quantified and used in cloning reaction.

4.3.2.1.2. Cloning of the PCR product:

The pGEM®–T Easy Vector System II, purchased form Promega Corporation, Madison, USA, was used for the cloning of PCR products following manufacturers instructions.

I. *Ligation:*

The vector (25 ng) and the insert DNA were taken in the molar ratio of 1:2 and were suspended in 4µl sterile double distilled water. 5 µl of 2X rapid ligation buffer and 1 µl of T4 DNA ligase were added and thoroughly mixed by vortexing and centrifugation. The mixture was kept at 4 °C for at least 16 h, heated at 60 °C for 10 min and 5 –7 µl was used to transform competent *E. coli* cells.

II. *Transformation:*

E. coli XL1–Blue was inoculated in 50 ml Luria–Bertaini (LB) broth (Miller, 1972) containing 50 µg/ml Ampicillin. After incubation at 37 °C for 4-5 h with shaking, when the optical density at 660 nm reached 0.5 to 0.6, cells were harvested by centrifugation and suspended in approximately half the volume of the original culture with chilled $CaCl_2$. This was incubated in ice for 30 min and was centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 1/10th volume of 100 mM chilled $CaCl_2$ and incubated at 4 °C for overnight.

III. *Selection of recombinants:*

The DNA to be transformed was incubated with the competent cells (100 µl) in a microcentrifuge tube and was kept in ice for 30 min. Then a heat shock at 42 °C for 2 min was given followed by incubation at 37 °C shaker with 1 ml LB for 1 h. Recombinants were selected by blue-white screening technique using Luria–Bertaini Agar (LA) plate containing 100 µg/ml Ampicillin, X-gal and IPTG. Recombinant-plasmids were isolated and screened by alkaline lysis method (Brinboim and Doly, 1979). Purified clones were checked by *Eco* RI restriction digestion followed by agarose gel electrophoresis.

4.3.2.1.3. Sequencing of the inserts of the recombinants:

The recombinant plasmids were directly used for sequencing of the inserts using primers for T7 and SP6 promoters. Nucleotide sequencing was performed with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kits (Parkin-Elmer) using specific primer and the reaction was analyzed in an 'AB1 PRISM 377 DNA Sequencer'.

4.3.2.1.4. Analysis of the sequence:

Analysis of the sequence was done with the help of several bioinformatics tools.

I. *Restriction analysis*: For determination of restriction map of the sequence, the software package NEB CUTTER v. 2.0 of New England Biolabs Inc. was used from the website http://tools.neb.com/NEBcutter2.

II. *Determination of open reading frame*: To determine the largest possible protein-coding region the software package 'Fast PCR v 3.3.67' of the

Institute of Biotechnology, University of Helsinki, Finland, was used.

III. *Similarity Search*: For similarity search studies of nucleic acid and amino acid sequences the BLAST N and BLAST P programs were used (Altschul *et al.*, 1997) from the website http://www.ncbi.nlm.nih.

IV. *Conserved domain search* (CDD): The Domain analysis of the amino acid sequence of the inserts was done using the program of Marchler-Bauer and Bryant (2004) of the website http://www.ncbi.nlm.nih.

V. *Structure and function analysis*: For determination of probable secondary and three-dimensional structure of the peptides, the software packages, 'PredictProtein' from the website http://www.embl-heigelberg.de and 'SWISS-MODEL' (Schwede *et al.*, 2003; Guex and Peitsch, 1997) was used. The function of the peptides was analyzed using the ProtFun 2.1 software of the website http://www.cbs.dtu.dk.

4.3.2.2. Results and Discussion:

The PCR products of NiVas 114, that yielded positive hybridization signal with *cnr*–specific probe were purified from agarose gel and were subsequently cloned into pGEM®T–easy vector and were transformed into *E. coli* XL1Blue strain (Figure 4.5 A & B). Plasmid extracted from the resulting clones were digested with *Eco* RI and were electrophoresed on 1% agarose gel (Figure 4.5 C) to ensure that the plasmids contained the appropriate inserts. Pair-wise alignment conducted with the sequence of the probe DNA (partial *cnrA*) and the nucleotide sequence of the inserts, showed 40–46 % identity.

I. *Sequence analysis of the 1 kb insert DNA of the clone pBC 1.0*:

The nucleotide sequence of the insert (1062 bp) of pBC1.0 was deposited to EMBL nucleotide sequence database under accession no. AM039520. The restriction map of the sequence has been represented in Figure 4.6. Similarity search studies with BLASTN program showed high similarities with Serine acetyltransferase gene (83-85%) and glycerol-3-phosphate dehydrogenase gene (90-91%) of *Samlonella typhimurium* and *Escherichia coli*. Two ORFs, ORF1 (75 amino acids) and ORF2 (269 amino acids), were retrieved from the sequence and were shown in Figure 4.7.The BLASTP analysis, of the ORF1 showed 62-75% similarities with the amino acid sequence of the inner-membrane enzyme glycerol-3-phosphate dehydrogenese of *E. coli*, *S. typhimurium*, *S. enteritica*, *Vibrio cholerae* and *Yersinia pestis*, and the 269-amino-acid long ORF2 showed high (90-95%) similarities with the amino acid sequences of the Serine Acetyltransferase of *E. coli*, *S. typhimurium* and *Yersinia pestis*.

The domain analysis of ORF1 and ORF2 showed that they are the representatives of protein family pfam01210 and pfam06426 respectively. The amino acid region 1-71 of ORF1 showed an alignment score of 21.6 % with the N-terminal end of GpsA protein (Glycerol-3-phosphate dehydrogenase) of the pfam01210 (Figure 4.8). The 117 amino acids at the N-terminal end of ORF2 yielded percent alignment value of 60.8 with the CysE protein (Serine acetyltransferase) of pfam06426 associated with amino acid transport and metabolism function (Figure 4.9). Analysis of both the ORFs indicated that the insert of the clone pBC1.0 could be hypothesized to form inner membrane proteins, like that of glycerol-3-phosphate dehydrogenase. From the result of the BLASTP and domain search analysis it was difficult to predict the role of the peptides as a part of a metal-cation transporter, although the probability of (75-amino acid long) ORF1 as a metal sensor protein cannot be ruled out.

II. *Sequence analysis of the 1.5 kb insert DNA of the clone pBC 1.5:*

The nucleotide sequence of the insert of the clone pBC1.5 was deposited in EMBL nucleotide database under accession no. AM003901. The restriction map of the sequence has been given in the Figure 4.10. The nucleotide sequence of the clone showed 82–85% identity with nucleotide sequence of putative integral membrane resistance protein of *Salmonella typhimurium* and *Escherichia coli*. The sequence has been predicted to code for two ORFs made up of 164 (ORF1) and 139 (ORF2)

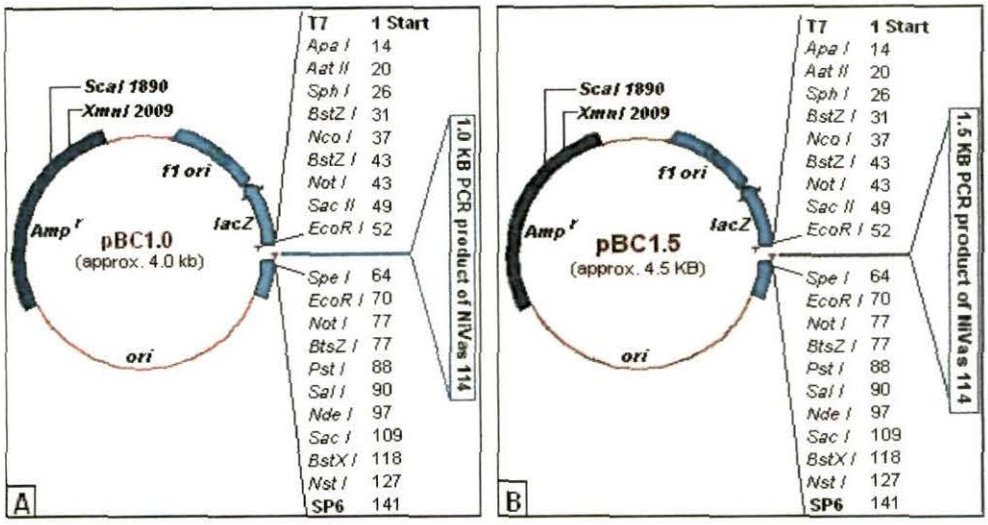

Figure 4.5. Construction of pBC1.0 (A) and pBC1.5 (B) recombinant plasmids (not in scale) harboring 1.0 kb and 1.5 kb PCR products of NiVas 114 respectively. (C), Agarose gel electrophoresis of the PCR products of isolate NiVas 114 generated by bbrc1-bbrc2 primer pair (lane 4), and the *Eco* RI restricted products of plasmids pBC1.0 (lane 3) and pBC1.5 (lane 2). Lane 1, *Eco* RI restricted vector DNA; M, Molecular size marker; arrows indicating the amplicons cloned in the vector.

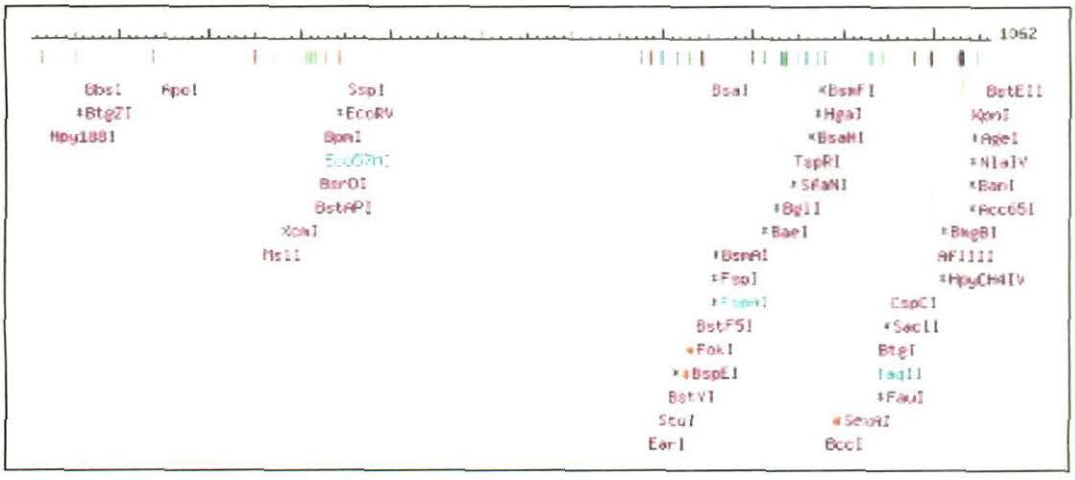

Figure 4.6. *Restriction map of 1062 bp insert of pBC1.0. #, Cleavage affected by CpG methylation; and *, Cleavage* affected by other methylations.

```
                          ORF1
                5'    ttggcatgatgctcggacagggcagcgat
                            M  M  L  G  Q  G  S  D
   30 gtaaaaagcgcgcaggagaagactggtcaggtggttgaaggctat
      V  K  S  A  Q  E  K  T  G  Q  V  V  E  G  Y
   75 cgcaataccaaagaagttcgcgaactggcgcaccgtttcggtgtt
      R  N  T  K  E  V  R  E  L  A  H  R  F  G  V
  120 gaaatgccaataaccgaggaaatttatcaggtattgtattgcgga
      E  M  P  I  T  E  E  I  Y  Q  V  L  Y  C  G
  165 aaaaatgcgcgcgaggcagcattgaccttattaggtcgtgcgcgc
      K  N  A  R  E  A  A  L  T  L  L  G  R  A  R
  210 aaggacgagcgcagcagcaattagccgggaagtatcgtcacctga
      K  D  E  R  S  S  N  *
      ORF2
  255 atgacccagccagcgcagaactggctggtcattaactatcgtctg
      M  T  Q  P  A  Q  N  W  L  V  I  N  Y  R  L
  300 gagcaagcaatgccgtgtgaagaactggatatcgtctggaataat
      E  Q  A  M  P  C  E  E  L  D  I  V  W  N  N
  345 attaaagccgaagcccgagcgttggccgactgtgagcccatgctg
      I  K  A  E  A  R  A  L  A  D  C  E  P  M  L
  390 gccagtttctatcacgcggcgctacttaagcacgaaaatctcggc
      A  S  F  Y  H  A  A  L  L  K  H  E  N  L  G
  435 agcgccctgagctatatgctcgccaataaactggcttcccctatc
      S  A  L  S  Y  M  L  A  N  K  L  A  S  P  I
  480 atgcctgctattgcgattcgcgaggtggtggaagatattaaagcc
      M  P  A  I  A  I  R  E  V  V  E  D  I  K  A
  525 gaagcccgagcgttggccgactgtgagcccatgctggccagtttc
      E  A  R  A  L  A  D  C  E  P  M  L  A  S  F
  570 tatcacgcggcgctacttaagcacgaaaatctcggcagcgccctg
      Y  H  A  A  L  L  K  H  E  N  L  G  S  A  L
  615 agctatatgctcgccaataaactggcttcccctatcatgcctgct
      S  Y  M  L  A  N  K  L  A  S  P  I  M  P  A
  660 attgcgattcgcgaggtggtggaagaggcctacgccgcagatccg
      I  A  I  R  E  V  V  E  E  A  Y  A  A  D  P
  705 gagatgattgcttctgccgcctgcgacatccaggctgtgcgcacg
      E  M  I  A  S  A  A  C  D  I  Q  A  V  R  T
  750 cgagacccggcggtggataaatactccacgccgctgctgtatctc
      R  D  P  A  V  D  K  Y  S  T  P  L  L  Y  L
  795 aaaggcttccacgccttacaggcgtaccgcatcggccactggtta
      K  G  F  H  A  L  Q  A  Y  R  I  G  H  W  L
  840 tggaatgagggacgccgcgcgctggccatcttcctgcaaaaccag
      W  N  E  G  R  R  A  L  A  I  F  L  Q  N  Q
  885 gtgtccgtgaccttccaggtcgatattcacccagcggcgaaaatt
      V  S  V  T  F  Q  V  D  I  H  P  A  A  K  I
  930 ggccgcgggattatgctcgatcacgccaccggcattgttgttggt
      G  R  G  I  M  L  D  H  A  T  G  I  V  V  G
  975 gaaacggcggtgatcgaagatgacgtgtcgatcctgcaatccgtt
      E  T  A  V  I  E  D  D  V  S  I  L  Q  S  V
 1020 acgctgggcggtaccggtaaaaccagcggcggtcaccatcgaa 3'
      T  L  G  G  T  G  K  T  S  G  G  H  H  R
```

Figure 4.7. The nucleotide sequence (EMBL Acc. No. AM003901) of 1062 bp insert in the recombinant plasmid pBC 1.0, and ORFs (ORF1 and ORF2) from the sequence.

```
ORF1:   1    MNLGQGSDVKSAQERTGQVVEGYRNTKEVRELAHKFGVEMPITEEIYQVLYCGKNAREAA  60
GpsA:   259  LLLGQGLSIDEALERIGQVVEGVRTAKAVYELAKKLGIEMPITEAVYRVLYEGLDPKEAI  318

ORF1:   61   LTLLGRARKDE  71
GpsA:   319  EELMGRDLKPE  329
```

Figure 4.8. Pair-wise alignment of the ORF1 protein sequence (of pBC1.0) with the amino acid sequence of 329 amino acid long GpsA (Glycerol-3-phosphate dehydrogenase) inner membrane protein.

```
ORF2:   151   EMIASAACDIQAVRTRDPAVDKYSTPLLYLKGFHALQAYRIGHWLWNEGRRALAIFLQNQ  210
CysE:   2     EMLGSAREDIRAVRERDPAARSYLEVLLYYPGFHALWAHRIAHWLWNRGRKLLARLLSSL  61

ORF2:   211   VSVTFQVDIHPAAKIGRGIMLDHATGIVVGETAVIEDDVSILQSVTLGGTGKTSGGHH  268
CysE:   62    SRFLFGIEIHPGAKIGRGLFIDHGTGVVIGETAVIGDDVTIYHGVTLGGTGKESGKRH  119
```

Figure 4.9. Pair-wise alignment of the predicted ORF2 protein (of pBC1.0) with the amino acid sequence of 194 residue long Serine acetyltransferase (CysE) enzyme of pfam06426. The enzyme performs Amino acid transport and metabolism *function.*

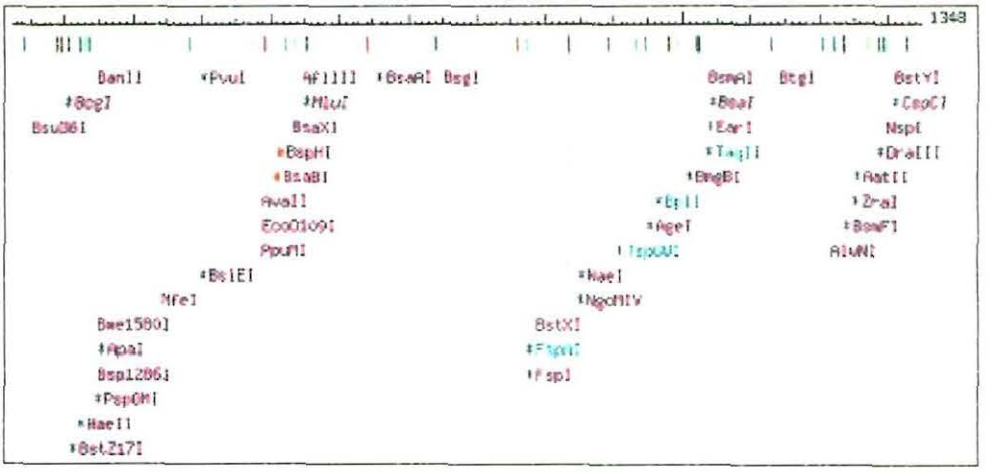

Figure 4.10. Restriction map of 1348 bp insert of pBC1.5. #, Cleavage affected by CpG methylation; and *, Cleavage *affected by other methylations.*

amino acids (Figure 4.11). The BLASTP analysis of the ORF1 showed a highest of 93% identity with hypothetical integral membrane protein of *S. typhimurium* and *E. coli*. Similarity search studies of ORF2 showed 95-96% identities with Ham1 protein of *E. coli, S. enteritica* and *Shigella flexneri*.

The domain analysis of the ORF1 generated an alignment score of 97.6% with the YGGT protein family (pfam02325) (Figure 4.12). The members of this protein family consists of a repeated amino acid sequence found in conserved hypothetical integral membrane proteins and the function of this region and the proteins, which possess it, is unknown. Amino acid sequence of ORF2 yielded 69.2% identity with Ham1P protein of HAM1 protein-family (pfam01725) (Figure 4.13). The proteins of this family controls 6-N-hydroxylaminopurine (HAP) sensitivity and mutagenesis in *Saccharomyces cerevisiae*. The Ham1 protein protects the cell from H-aminopurine, either during synthesis of deoxyribonucleotide triphosphate or during replication of DNA level by an unknown mechanism.

Structure function analysis of the ORF1 revealed four transmembrane helices spanning the amino acid region 4–27, 64–83, 88–112 and 143–162 (Figure 4.14), and therefore was identified as a transmembrane protein predicted to perform transport and binding function in the membrane. Both, BlastP and domain analysis, of the ORF1 and ORF2 failed to draw any similarities with any of the existing cation-transport protein. Pair-wise alignment of the ORF1 and ORF2 generated insignificant (15-18%) similarities with the amino acid sequence of the probe DNA. Therefore, the role of the ORFs as a part of a cation efflux system could not be established. But, the secondary structure of the ORF1, an integral membrane protein, further raises the hope to explore it as a novel cation-transporter in the isolate.

4.4. Conclusion:

Among the nickel resistant bacterial isolates of Torsa River, only eight strains were found to tolerate more than 5 mM $NiCl_2$. Biochemical tests were conducted to identify the taxonomic status of these isolates. The results of the biochemical tests were conducted to identify the taxonomic status of these isolates. The results of the biochemical tests indicated that all of them are the members of the class gamma-proteobacteria and they belonged to

```
                                                5'  gcaatttc
   9 ggtgcgcgcgattacccccaataataaggaaacctgaggaacgcc
     ORF1
  54 atgaagacgttgactttcctgctttcaacggtcattgaactgtat
     M  K  T  L  T  F  L  L  S  T  V  I  E  L  Y
  99 acgatggcgctgctgttgcgcgtctggatgcagtgggcccgttgt
     T  M  A  L  L  L  R  V  W  M  Q  W  A  R  C
 144 gatttttacaatccattctcacaatttatcgtgaaaatcacgcag
     D  F  Y  N  P  F  S  Q  F  I  V  K  I  T  Q
 189 cccgttgtggggccgcttcgccgcgtcattccggcaatggggcca
     P  V  V  G  P  L  R  R  V  I  P  A  M  G  P
 234 attgacagttcatctctgctgatggcgtttattctgagcgttatc
     I  D  S  S  S  L  L  M  A  F  I  L  S  V  I
 279 aaagcgatcgtgctgtttatggtcatcactttccagccgattatc
     K  A  I  V  L  F  M  V  I  T  F  Q  P  I  I
 324 tggatttcagccgttctgatcctggttaaaaccgtcggctcgctg
     W  I  S  A  V  L  I  L  V  K  T  V  G  S  L
 369 atcttctgggtcctgctggtgatggcgatcatgagctgggtaagc
     I  F  W  V  L  L  V  M  A  I  M  S  W  V  S
 414 cggggtcgtagcccggtggagtacgcgttgattcagctgactgaa
     R  G  R  S  P  V  E  Y  A  L  I  Q  L  T  E
 459 ccgttgctgcgtccgattcgtagcctgctgcctgcaatgggcgga
     P  L  L  R  P  I  R  S  L  L  P  A  M  G  G
 504 atcgacttctccccgatgcttctcgtcttctgctgtacgtgctga
     I  D  F  S  P  M  L  L  V  F  C  C  T  C  *
 549 atatgggtatcgcggaactgttacaggcgacgggtaatatgctgc
 594 tgccggggctgtggatggcgttatgagtgcagttagccctgcgcc
 639 gacggctggttttacgctgtcattcagccgaaagccagccgcgac
 684 agtattgttgggctgcatggcgacgagctaaaaagtcgccatcac
 729 ttgccccgccggttgacggccaggcgaatgcgcattctgaccaaa
 774 tatctggctaaacagttcgcgtcgctaaaagccaggtcatcattg
 819 agaaaggtgaattggccggcataaacaggtaaaaatccttaaccc
 864 gcattctatcccgacggaagtcgcggctctgaaagaacaggacta
         ORF2
 909 aaccatgcagaaagttgttctcgccaccggtaacgccggtaaagt
         M  Q  K  V  V  L  A  T  G  N  A  G  K  V
 954 gcgcgagctggcctcgctattaaatgattttgggctggacgtggt
      R  E  L  A  S  L  L  N  D  F  G  L  D  V  V
 999 ggcccagaccgagctgggcgtggactccgccgaagagaccggcct
      A  Q  T  E  L  G  V  D  S  A  E  E  T  G  L
1044 gacgtttatcgaaaacgccattctgaaagcgcgccacgccgcgca
      T  F  I  E  N  A  I  L  K  A  R  H  A  A  Q
1089 gatcaccggactgcccgcgatagccgatgactccggtctggccgt
      I  T  G  L  P  A  I  A  D  D  S  G  L  A  V
1134 ggattttctgggcggtgcgccggggatttactccgcccgctattc
      D  F  L  G  G  A  P  G  I  Y  S  A  R  Y  S
1179 cggggtggacgccaccgaccagcagaatctggaaaagctgcttat
      G  V  D  A  T  D  Q  Q  N  L  E  K  L  L  M
1224 ggccctgaaagacgtccctgacgaacagcgtaccgcgcagttcca
      A  L  K  D  V  P  D  E  Q  R  T  A  Q  F  H
1269 ctgcgtgctggtctacatgcgtcacgcggaagatcccacgccgat
      C  V  L  V  Y  M  R  H  A  E  D  P  T  P  I
1304 tgtctgtcacggcagctgaccgggcgtgtcacccg
      V  C  H  G  S  *
```

Figure 4.11. Nucleotide sequence (EMBL Acc. No. AM039520) of the 1348 kb insert of pBC1.5 and its two ORFs (ORF1 and ORF2) retrieved from the sequence.

```
ORF1: 1   MKTLTFLLSTVIELYTMALLLRVWMQWARCDFYNPFSQFIVKITQPVVGPLRRVIPAMGP  60
Yggt: 1   MNLLLSLLSSLLDIYSFLLLIRALLSWVPNIWYNPPGRFLVKLTDPYLNPFRRIIPPIGG  60

ORF1: 61  IDSSSLLMAFILSVIKAIVL  80
Yggt: 61  IDFSPIVAIIILQFLQFILL  80
```

Figure 4.12. Pair wise alignment of the amino acid sequence (of ORF1 of pBC1.5) with Yggt protein of pfam02325.

```
ORF2:  4    VVLATGNAGKVRELASLLNDFGLDVVAQTELGVDSAEETGLTFIENAILKARHAAQITGL  63
Ham1P: 1    LVFATGNPGKLKEVKEILSDFGLIEIVQLDLE-DYPEETGSTFEENALLKARAAAEAVGK  59

ORF2:  64   PAIADDSGLAVDFLGGAPGIYSARYSGVDATDQQNLEKLLMALKDVPDEQRTAQFHCVLV  123
Ham1P: 60   PVIAEDSGLFVEALNGFPGVYSARYVGETIGD---IEKILKLLEGV--ENRKAYFVSVIA  114

ORF2:  124  YMRHAEDPTPIVCHG  138
Ham1P: 115  FADDGE---PEVFEG  126
```

Figure 4.13. Pair wise alignment of the amino acid sequence of ORF2 (of pBC1.5) with Ham1P protein (pfam01725) of HAM1 protein family.

```
MKTLTFLLST VIELYMALLL RVWMQWARCD FYNPFSQFIV KITQPVVGPL 50
oooHHHHHHH HHHHHHHHHH HHHHHHHiii iiiiiiiiii iiiiiiiiii

RRVIPAMGPI DSSSLLMAFI LSVIKAIVLF MVITFQPIIW ISAVLILVKT 100
iiiiiiiiii iiiHHHHHHH HHHHHHHHHH HHHoooooHHH HHHHHHHHHH

VGSLIFWVLL VMAIMSWVSR GRSPVEYALI QLTEPLLRPI RSLLPAMGGI 150
HHHHHHHHHH HHiiiiiiii iiiiiiiiii iiiiiiiiii iiHHHHHHHH

DFSPMLLVFC CTC 163
HHHHHHHHHH HHo
```

Figure 4.14. *Distribution of predicted transmembrane helices (4-27, 64-83, 88-112 & 143-162) of ORF1 protein derived from nucleotide sequence of the insert of pBC1.5 [H, helix; I, Loop; o, Others].*

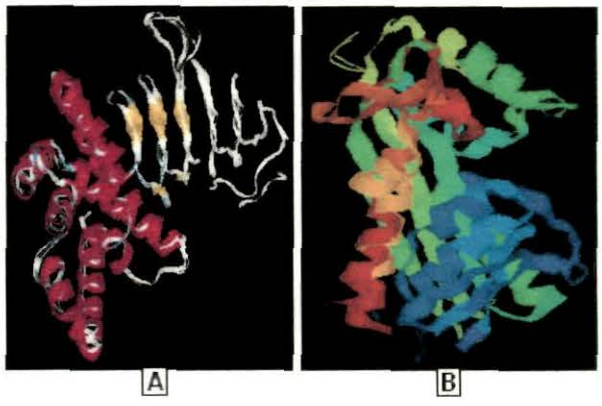

Figure 4.15. Probable 3D structures of the proteins derived from amino acid sequences predicted from each ORF2 of 1062 kb (A) *and* 1348 kb (B) *PCR products*

the genus *Acinetobacter, Pseudomonas, Moraxella, Serratia* and *Enterobacter*. Growth studies in liquid medium containing 3mM $NiCl_2$ implied that the nickel resistance genetic system(s) of these isolates wereinducible in nature. Cells pre-incubated in a medium containing 20-100 µM $NiCl_2$ (induced pre-cultures), showed considerable reduction in lag phase compared to un-induced pre-cultures. In addition to nickel, pre-incubation of cells in other metal ions (Co^{2+}/ Zn^{2+}/Cu^{2+}/Pb^{2+}) were also found to induce the nickel resistance in these isolates. Interestingly, in case of isolates 6NiCo 43, NiVa 61, BB 37 and 5CoNi 34, pre-exposure of the cells to 5.0 –10 µM zinc sulfate (equivalent to 325 –650 ppb of Zn^{2+}) was found to induce the nickel resistance. Compared to other sampling months, the high nickel-resistant CBC (Copiotrophic Bacterial Count) on 3 mM $NiCl_2$ containing plates in three sampling months, January, February and March (see Table 2.3 of Chapter 2), can therefore be corroborated with the higher Zn^{2+} ion content of the river water. The isolates having nickel resistant genetic determinants induced by ppb level of Zn^{2+} ion would therefore will get a selective advantage to form colonies on 3 mM $NiCl_2$ containing plates after 14-16 h of incubation out-competing others.

Compared to other nickel resistant strains isolated from the waters of Torsa River, three isolates, identified as *Acinetobacter* sp. BB1A, *Serratia* sp. NiVa 51 and *Enterobacter* sp. NiVas114, showed relatively higher MTC of Ni^{2+}. The results obtained from the PCR analysis using *cnr-ncc*, *nir-ncr* and *nre* specific primers (see section 3.3 of Chapter 3), did not indicate any thing specific about the nature of genetic determinants, but it served as a precursor to think and explore more vertically. In order to explore the degree of homology with the existing nickel resistance genetic determinants, the restricted genomic DNA of the isolates as well as their PCR products were subjected to Southern hybridization using *cnr, ncc, nre* and *nir* specific probes. Although the three isolates generated amplicon(s) with nre1-nre2 primer pair, the restricted genomic DNA, or the PCR products derived from them, failed to produce any detectable hybridization signals with *nre*-specific probe DNA. Rather, the restricted genomic DNA of both BB1A and NiVa 51 generated only a weak hybridization signal with *nir*-specific probe. On the other hand, restricted genomic DNA as well as the PCR products of the isolate NiVas 114 (generated by *cnr-ncc* specific primers) showed a weak hybridization signal with *cnr* specific probe.

The PCR products of NiVas 114 (approximate size 1.0 kb and 1.5 kb), that hybridized weakly with *cnr* probe, were cloned in pGEM T-easy vector. The recombinant plasmids harboring approximately 1kb and 1.5 kb amplicons were named as pBC1.0 and pBC1.5 respectively. The sequence of the clones showed only 41-46% identity with the sequence of the probe DNA, explaining the reason of getting a weak hybridization signal at low stringency. Four ORFs were predicted from the clones (Figure 4.7 and 4.11) and the analysis of the amino acid sequence of the ORFs failed to yield significant identity with the amino acid sequence of any of the known cation efflux protein.

The conserved domain search studies indicated that the amino acid sequences of the ORFs showed identities with Glycerol-3-phosphate-dehydrogenase, Serine acetyltransferase, hypothetical integral membrane protein and Ham1 protein of the protein familie pfam01210, pfam06426, pfam02325 and pfam01725 respectively. Whereas the amino acid sequence derived from the sequence of probe DNA (partial CnrA protein), showed identities with putative silver efflux pump and AcrB cation/ multidrug efflux pumps of protein family pfam00873 and COG3696 respectively. The probable three-dimensional structures predicted from two ORFs using SWISS-MODEL software, failed to show existence of any cation-transport channel (Figure 4.15) within them. Thus, from the present state-of-art, it is very difficult to hypothesize any function to the predicted ORFs

to be a part of a cation transporter; but further structure function analysis of predicted protein from ORF1 may yield some important information(s). The results obtained from the induction studies, DNA-DNA hybridization, and sequence analysis of the cloned PCR products indicated the fact that all these strains are worthy to the scientific community in terms of exploring novelty and diversity of nickel resistance genetic system. The nickel resistance system of strain BB1A is of principal importance, because nickel-resistance of the strain could be induced by various other metal ions (Pb, Zn, Cu) in addition to nickel. Vertical exploration leading to targeting and sequencing of the nickel resistant gene of these isolates will definitely enrich the knowledge of metal resitant genomics of bacteria. To facilate the process of gene transfer and targeting it was necessary to know the phylogeny and systematic position of these isolates.

4.5. **Summary of chapter 4:**
Several nickel resistant strains were isolated from Torsa River of India. Eight isolates among them showed maximum tolerable concentration (MTC) of 5.5 mM $NiCl_2$ and above. Numerical taxonomy enabled to identify them as the members of the class gamma-proteobacteria, and assign them to the following genus: *Acinetobacter*, *Moraxella*, *Pseudomonas*, *Serratia* and *Enterobacter*. The nickel resistance phenotype and the nature of inducibility exhibited by the Torsa isolates were studied in some detail. Cells pre-grown in a medium containing micromolar concentration of Ni^{2+}/ Co^{2+}/ Cu^{2+}/ Pb^{2+}/ Zn^{2+} (Induced pre-cultures) could selectively reduce the duration of lag phase compared to the uninduced pre-cultures in nickel challenged medium. Southern hybridization results have shown that the *nir*-specific probe hybridized weakly with the restricted genomic DNA of NiVa 51 and BB1A, while *cnr* specific probe generated faint hybridization signal with both PCR products and restricted genomic DNA of the isolate NiVas 114. The PCR products of NiVas 114 which produced hybridization signal, were cloned in pGEM T-easy vector and were sequenced. The pair wise alignment of the nucleotide sequence of the clones showed only 41-46% similarity with the sequence of the probe DNA (partial *cnrA*). The analysis of the amino acid sequence predicted from ORFs of the sequenced DNA regions did not show significant homology with any existing cation-transport protein. The ORF1 protein of the 1.5 kb amplicon was predicted to form an integral membrane protein which could be a part of a novel cation-transporter.