General introduction

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Metals play an integral role in the life processes of microorganisms. Some metals, such as cobalt, chromium, copper, iron, zinc, manganese, nickel etc. are essential micronutrients for growth. The others such as, silver, aluminium, cadmium, gold, lead and mercury etc. since have no biological role, can be considered as nonessential metals. Essential metals function as catalysts for biochemical reactions, stabilize protein structures and bacterial cell walls, and help in maintaining osmotic balance (Hughes and Poole, 1989; Poole and Gadd, 1989; Ji and Silver, 1995). Essential transition metals like iron, copper and nickel are involved in redox processes, whereas magnesium and zinc stabilize various enzymes and DNA through electrostatic forces. Iron, magnesium, nickel and cobalt are part of complex molecules with a wide array of functions; and potassium and sodium are required for regulation of intracellular osmotic pressure (Nies, 1992).

While characterizing two-strains of hydrogen-oxidizing bacteria, Bartha and Ordal (1965), for the first time, demonstrated the importance of nickel as a trace element in the microbial growth. The knowledge regarding the utilization of nickel by microorganisms was augmented manifold when nine nickel dependent enzymes, such as urease, NiFe-dehydrogenases, CO-dehydrogenase, acetyl-CoA-synthase, methyl Co-enzyme-M reductase, aci-reductone dioxygenase, methylenediurease, certain superoxide dismutases and some glyoxylases were discovered (Friedrich *et al.*, 1982; Mulrooney and Hausinger, 2003). Among these nine nickel-dependent enzymes, discovered so far, seven enzymes were structurally characterized by crystallography and nuclear magnetic resonance (NMR) (Mulrooney and Hausinger, 2003). Nickel specific sensing and transport systems, involved in the uptake of Ni^{2+} ion from extracellular environment into the cell, have also been characterized in detail (Eitinger and Friedrich, 1991). In *Escherichia coli* the best characterized nickel transport operon is *nik*; where *nikR* is associated with regulation of the operon and five other *nikABCDE* encoding components of a typical ATP-dependent transport system (Navarro *et al.*, 1993). In addition to the 'nik-transporter', another single gene product HoxN was also found to play a role in nickel-transport and mutation at the *hoxN*-locus reduced the activity of two nickel-enzymes, hydrogenes and urease in *E. coli* (Eberz *et al.*, 1989; Eitinger and Friedrich, 1991; Wolfram *et al.*, 1995). In *E. coli* nickel is also transported into the cell by a constitutive Mg^{2+} transport system (Park *et al.*, 1976).

Although essential as a micronutrient, but at high concentrations metal ions are toxic to microorganisms. Toxicity occurs through the displacement of essential metals from their native binding sites or through ligand interactions. Nonessential metals bind with greater affinity to thiol-containing groups and oxygen sites than do essential metals (Hughes and Poole, 1989; Poole and Gadd, 1989). Toxicity results from alterations in the conformational structure of nucleic acids and proteins and interference with oxidative phosphorylation and osmotic balance (Poole and Gadd, 1989). Bacteria have adapted themselves to metal contaminated environment(s) through a variety of chromosomal and plasmid-mediated resistance systems.

Microbial metal resistance mechanisms:

Selection pressure from a metal-containing environment has led to the development of resistance systems to virtually all toxic metals (Rouch *et al.*, 1995). These systems are mostly plasmid-mediated and very specific, and were found in virtually all eubacterial groups studied (Silver and Misra, 1984; Ji and Silver, 1995). Researches carried out since the early 1970s, enabled to identify several microorganisms being resistant towards certain metals through one (or more) of the mechanisms like; (i) metal exclusion, (ii) active transport of the metal from the cell or organism, (iii) intracellular sequestration of the metal by protein binding, (iv) extra-cellular sequestration, (v) enzymatic detoxification of the metal to its less toxic form, and (vi) reduction in metal sensitivity of cellular

targets. These reports included mostly aerobic microorganisms, with prominent examples being *Staphylococcus* sp., *Escherichia coli*, *Pseudomomonas aeruginosa*, and *Bacillus* sp. (Nakahara *et al.*, 1977; Marques *et al.*, 1979; Harnett and Gyles, 1984; Schwarz and Hobel, 1989; Belliveau *et al.*, 1991; Wang and Shen, 1995). Resistance has been reported for mercury and organomercurials in obligate anaerobes like *Bacteriodes* and *Clostridium* species too. With the help of cell surface electron-transport-systems, changing the valency of a metal through oxido-reduction allows bacteria to detoxify and regulate the movement of the metal ion across the membrane; e.g., reduction of chromate (Cr^{+6}) (Wang and Shen, 1995) and arsenate (As^{+5}) (Cervantes *et al.*, 1994).

In cyanobacteria *Synechococcus* and in *Pseudomonas* a new metal binding protein named Metallothionein has been discussed in some detail. The protein is approximately 60-amino acid in length, one third of which is cysteine residue, and associated with intercellular metal-ion-chelating activity (Turner and Robinson, 1995).

The presence of metal ions can up-regulate genes to initiate metal resistance by enzymatic detoxification (Misra, 1992; Silver and Walderhaug, 1992). The most prominent example is Hg^{2+} resistance coded by *mer* operon. Mercury is highly toxic because of its high affinity towards thiol groups and thereby results into inactivation of enzymes and other essential cellular proteins (Kozak and Forsverg, 1979; Rudrik *et al.*, 1985). Bacteria have adapted to the presence of Hg^{2+} by evolving a set of genes that form a resistance by operon-encoded reductase enzyme. The gene products of this operon not only detoxify Hg^{2+} by evaporation but also involve themselves in transport and self-regulation (Weiss *et al.*, 1977; Ni'bhriain *et al.*, 1983; Liverelli *et al.*, 1993).

In contrast to that of mercury, chromium or arsenic, chemical reduction of Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} into the metallic form is not possible, mainly because NAD(P)H-dependent reduction of the divalent metal cations is energetically not favored. Therefore, bacteria reduce the accumulation of these metal ions by active efflux mechanism(s). Unlike mercury, other metals do not evaporate out of the cell, but remain inside and could be re-oxidized. Hence, possible mechanisms of resistance towards Cd^{2+}, Zn^{2+}, Co^{2+} and Ni^{2+} include only complexation and active efflux. Methylation and other covalent modification of these metals might not prove to be plausible mechanisms for resistance, because the resultant organometallic compounds would be unstable, mutagenic, and more toxic than the divalent cations (Nies, 1992). In eukaryotes, two different metal-ion-binding factors are known: (i) a small, cysteine rich protein called metallothioneins, that are synthesized under heavy metal-stress condition and are found in wide variety of animals, lower eukaryotes (Kojima and Kagi, 1978; Hamer, 1986), plants (De Miranda *et al.*, 1990) and cyanobacteria (Turner and Robinson, 1995); (ii) In addition to metallothionein, an efficient Cd^{2+} binder in plants have been discovered and named as phytochelatin (Rauser, 1990), Activity of phytochelatin synthase is Cd^{2+} dependent and therefore autoregulated; whose product binds with cadmium ion in cytoplasm (Grill *et al.*, 1989). In Gram-positive multiple metal resistant bacterium *Staphyloccus aureus*, Cd^{2+} and Zn^{2+} efflux is catalyzed by a membrane-bound CadA protein, a P-type-ATPase (Tynecka *et al.*, 1981). The *czc* determinant from the Gram-negative multiple-metal-resistant bacterium *Alcaligenes eutrophus* CH34 (currently known as *Ralstonia metallidurans* CH34) encodes proteins required for Co^{2+}, Zn^{2+} and Cd^{2+} efflux (CzcA, CzcB and CzcC) and regulation (CzcD) of the operon (Nies *et al.*, 1987; Nies *et al.*, 1989). In early 1990's the hypothetical model of *czc* operon mediated efflux system showed that CzcA works as a cation-proton antiporter, CzcB as a cation-binding subunit, and CzcC as a modifier protein required for changing the substrate specificity of the system from Zn^{2+} solely to Co^{2+}, Zn^{2+}, and Cd^{2+} (Nies, 1992).

Nickel resistance in Bacteria: to high Nickel Nickel resistance in bacteria: onickel ion:

Plasmid encoded resistance to Ni^{2+} has been characterized most extensively in *Ralstonia metallidurans* CH34 (formerly known as *Alcaligenes eutrophus* CH34) (Mergeay *et al.*, 1985). The strain was reported to harbor the conjugative plasmid

pMOL28 (MW 163 kb), which confers resistance against a range of metals, including nickel. The resistance was mediated by an inducible energy dependent specific efflux system (Siddiqui and Schlegel, 1987; Senfuss and Schlegel, 1988; Liesegang *et al.*, 1993). Extensive molecular characterization of the nickel efflux system was achieved by cloning the resistance gene (*cnr*) from pMOL28 (Siddiqui *et al.*, 1989).

Cloning and sequencing of *cnr* determinants from 8.5 kb *Eco*RI-*Pst*I fragment of plasmid pMOL28 of *A. eutrophus* strain CH34 showed that the operon is made up of three structural genes, *cnr*C, *cnr*B and *cnr*A, and three regulatory genes *cnr*Y, *cnr*R and *cnr*H. Transformation of recombinant plasmid containing *cnr* operon (pVDZ'2) in two plasmid-cured *A. eutrophus* host, strains AE 106 and H16 conferred inducible nickel and cobalt resistance to the strains (Liesegang *et al.*, 1993). The amino acid sequences derived from *cnr* and *czc* structural genes share significant homology, and the genes are arranged in the same order too. The gene products CnrC (418 amino acids) and CzcC (346 amino acids) share 30% identity at the amino acid level, and both are hydrophilic proteins. CnrB (395 amino acids) and CzcB (521 amino acids) share 28.5% identity, and CnrA (1,076 amino acids) and CzcA (1,064 amino acids) share 45.8% identity. The hydropathy patterns of the deduced amino acid sequences revealed two strongly hydrophobic regions and two hydrophilic regions in both the proteins and a striking similarity of the hydrophobic and hydrophilic patterns in these regions (Liesegang *et al.*, 1993). Secondary structure analysis of CnrA predicted six hydrophobic alpha helices. These are probably transmembrane helices, as predicted for CzcA (Nies, 1992). These findings clearly indicate that CnrA represents the internal membrane protein of *cnr* resistance system and the resistance conferred follows the same mechanism as predicted for *czc* system. The efflux of Ni^{2+} by pMOL28 was found to be highly sensitive to protonophores but not to inhibitors of F_0 channel of ATPase (Nies, 2003). The result suggested that the pMOL28 encoded nickel efflux is dependent on chemiosmotic potential rather than on ATP. In 1994, another nickel resistant bacteria, *Alcaligenes xylosoxidans* strain 31A (now *Achromobacter xylosoxidans* strain 31A) was isolated from a copper galvanization tank, which was found to tolerate nickel (40 mM), cobalt (20 mM), zinc (10 mM) and cadmium (1mM) (Schmidt and Schlegel, 1994). The strain was recorded to bear two conjugative mega-plasmids of 200 kb and 340 kb, named pTOM9 and pTOM8 respectively. In plasmid-less metal sensitive strain of *A. eutrophus* AE104, both these above-mentioned plasmids when transformed conferred resistance to nickel, cobalt, and cadmium (Schmidt and Schlegel, 1994). The 14.5 kb *Bam*HI fragment of the plasmid pTOM9 was found to possesses two nickel resistance systems. (i)The *ncc* system (8kb), made up of seven genes, *nccYXHCBAN*, conferring high-level (30-40 mM) nickel resistance as well as cobalt and cadmium resistance on *A. eutrophus* AE104 but could not be expressed in *E. coli*, and (ii) The *nre* system (1.8 kb) of *A. xylosoxidans* conferring low-level (3-5 mM) nickel resistance was capable of expressing in *E. coli*. The predicted amino acid sequences of *ncc*Y, *ncc*X and *ncc*H shared significant homology at amino acid level with Cnr-proteins expressed from *cnr* operon (NccY and CnrY, 59%; NccX and CnrX, 76%; and NccH and CnrH, 67%). Additionally, NccH showed significant similarities to a new subfamily of sigma factors, the ECF subfamily. The 437-amino acid long NccC revealed 75% identity with CnrC and 29% identity with CzcC. The ORF of *ncc*B shared 75% identity with CnrB and 31% identity with CzcB. The largest ORF predicted from *ncc* determinants, *ncc*A composed of 1,076 amino acids has shown 89% identity with CnrA and 49% identity with CzcA. About 70 bp downstream from *ncc*A, the last ORF (NccN) (213 amino acids) shared 66% identity with CzcN.

The mechanism of resistance conferred by the *ncc* genes has not been characterized in detail. Previous studies on *czc* and *cnr*, and their close homology with *ncc* gene-products enabled Stoppel and Schlegel (1995) to conclude that the resistance towards high concentration (40 mM) of nickel in a strain *A. xylosoxidans* 31A bearing *ncc* locus is an energy dependent efflux system. The largest of the Ncc proteins, NccA (116 kDa), contains a double set of six extended hydrophobic region that possibly spans the membrane. NccA is thought to form a

membrane tunnel, which allows ion transport across the membrane. NccB and NccC contain small hydrophobic regions of about 15 amino acids in their amino-terminal parts. Both proteins show significant similarities to CzcB or CzcC, which are assumed to be involved in metal cation binding or substrate (metal cation) specificity, respectively. NccH function as a sigma factor and belongs to the ECF subfamily of σ^{70} factors. NccY and NccX may have association with the regulation.

In addition to the *ncc* locus, *A. xylosoxidans* 31A was also found to possess another low-level (3-5 mM) nickel resistance genetic system, accommodated in a DNA stretch of 4.2 KB, named as *nre*-system. Detailed study on *nre* determinants revealed the existence of two ORFs, NreA and NreB. Unlike *cnr* and *ncc*, *nre* determinants were found to express in *E. coli* (Grass et al., 2001; Schmidt and Schlegel, 1994). It was shown by Grass *et al.* (2001) that, NreB being a nickel transporter was responsible for Ni^{2+} efflux and resistance in the host. In *E. coli* and A. *eutrophus* AE104, NreB showed reduced uptake of nickel compared to that of wild-type cells and the histidine-rich C-terminus of NreB was found necessary for maximum nickel resistance.

Nucleotide sequence analysis of a 4.8 kb *Sal* I-*Eco* RI fragment generated from a 70 kb plasmid, pEJH501, of *Hafnia alvei* 5-5 has led to the understanding of an another novel high-level nickel resistance system in bacteria. The *ncr* operon of *H. alvei* is made up of five genes, *ncrABCYX*. The *ncr*A and *ncr*B gene product share 70-78 % identity with *nre*B and *nre*A. Although 152 amino acid long ORF resulting from *ncr*C did not show any similarity with any of the known heavy metal resistance protein, but absence of any promoter-like sequence upstream of *ncr*C suggested that *ncrABC*, conferring nickel resistance, should be read as a single transcript (Park *et al.*, 2004). The NcrY and NcrX were expected to perform regulatory function, and NcrA and NcrB were predicted as main transmembrane protein associated with efflux of Ni^{2+}, Co^{2+} and Zn^{2+}. (Park *et al.*, 2003). Both NcrB and NcrC are necessary for nickel resistance and transport.

In *Klebsiella oxytoca* CCUG 15788, genes conferring resistance to nickel (10 mM $NiCl_2$) was found to be located in a 4.3 kb *Hin* dIII fragment of chromosomal DNA (Stoppel and Schlegel, 1995). The nucleotide sequence of this novel nickel-resistance determinant was deposited under accession no. AY492000 (by Park and Lee, 2003) as *nir*-locus. The *nir* gene cluster was found to consist of four genes *nirABCD*. Nucleotide sequence of *nir* deteminants showed 97% identity with *ncr* locus. Two major ORFs of *nir*, NirA and NirC, showed 88% and 91% identity with NcrA and NcrB, respectively. Domain search analysis of the NirA showed 34.2% similarity with a putative transmembrane protein (DUF 894) of unknown function, under protein family pfam05977. On the other hand, the amino acid sequence of *nirC* showed highest similarity with a high-affinity-nickel transport protein NicO (44.5%) under protein family pfam03824. All the members of this protein family are nickel transporters, involved in the incorporation of nickel into H_2-uptake hydrogenase and urease enzymes and are essential for the expression of catalytically active hydrogenase and urease enzymes. The NirC has also shown 40.6% similarity with the ABC-type uncharacterized transport system, associated with ion efflux mechanism. Although there were no published documents about the functioning of *nir*-system, but its similarities with the transmembrane cation efflux proteins indicate its functional resemblance with the other known cation efflux system.

The bacteria harboring the nickel resistance determinants, discussed so far, were isolated from the metal contaminated ecosystems, for exmple, the strain *R. metallidurans* CH34 (containing *cnr* system) was isolated from a zinc decantation tank in Liege, Belgium (Mergeay *et al.*, 1978); *A. xylosoxidans* 31A (containing *nre* and *ncc* locus) was isolated from the metal working industry in Holzminden, Germany (Schmidt and Schlegel, 1989); *Hafnia alvei* 5-5 (containing *ncr* locus) was isolated from the rhizophere of nickel hyper-accumulating tree *Sebertia acuminata* (Stoppel and Schlegel, 1995); and *Klebsiella oxytoca* CCUG 15788 (containing *nir* locus) was isolated from a

mineral oil emulsion of a metal working industry in Goetborg, Sweden (Stoppel *et al.*, 1995). Abundance of metal resistant bacteria in the metal contaminated environment urges one to think that these resistances arose as a result of human pollution in recent centuries. The other view regarding the origin of metal resistance genetic system is that they arose soon after life began, in a world already polluted by volcanic activities and other geological sources. Like antibiotic resistance determinants, toxic heavy metal resistance determinants were also preexistent to recent human activities that created polluted environments (Misra, 1992). Therefore, one is tempted to explore the existence of metal resistant bacteria in a so called 'metal unpolluted' environments. The ease of isolation of metal resistant bacteria from metal contaminated environment and presumably the dearth of suitable enrichment methods to isolate resistant bacteria from a mixed microbial population of a so called metal-uncontaminated ecosystem, might have led the earlier authors to conclude that the metal resistant bacteria could not be isolated from the places not contaminated with heavy metals (Stoppel and Schlegel 1995).

The present study was undertaken with a view to explore the possibilities of isolate metal resistant bacteria from a free flowing snow-fed river Torsa of northern West Bengal, India, a metal unpolluted ecosystem. The physiological and molecular genetic characterization of the nickel resistant isolates of the said river was therefore obvious.

The major objectives of the study were:

1. *Construction of a database on the water quality of Torsa River.*

2. *Isolation and characterization of nickel resistant bacteria from the river water.*

3. *Search for novel genetic system(s) conferring nickel-resistance in suitable gram-negative bacterial isolates of the river.*

4. *Taxonomic characterization of gram-negative nickel resistant bacterial taxa.*