

CHAPTER 1

INTRODUCTION

1. Introduction

1.1. Background

Although heavy metals are naturally present in the soil, geologic and anthropogenic activities have resulted in the increase in the concentrations of these elements to the amounts that are harmful to both plants and animals [1]. Some of the heavy metals are trace elements and are essential for many physiological processes in the biological systems, but at higher concentrations it might have deleterious effect leading to toxicity [2]. In recent few decades, due to increase in industrialization and population there has been a significant increase in the utilization and release of chemicals including heavy metals into the environment [3]. Industries such as metallurgical, electroplating, metal finishing, tanneries, chemical manufacturing, mine drainage and battery manufacturing are main sources of metal discharge in the environment [4]. The exposure of heavy metals causes intoxication particularly neurotoxicity [5], nephrotoxicity [6], genotoxicity [7] or carcinogenicity [8] through the generation of reactive oxygen and nitrogen species. Many studies have reported the toxic and carcinogenic effects of metals in human and animals [9]. They interact with the cellular components including DNA and proteins and cause oxidative deterioration of biological macromolecules leading to malfunctioning of cell and eventually toxicity [9]. Studies in the recent past have shown that metals such as cadmium, chromium, nickel, mercury, lead and arsenic possess the ability to generate free radicals resulting in depletion of enzyme activities, damages lipid bilayers and DNA [7]. These metals also have the ability to form complex unspecific compounds due to coordinating abilities and presence of incompletely filled *d* orbitals causing detrimental effects on the biological system [10].

Recent studies have suggested that certain metals such as cadmium and nickel can function as endocrine disruptors by mimicking the actions of estrogens and might contribute to the development of breast cancer [11]. Epidemiological

studies have linked cadmium exposure with pulmonary cancer [12] and few studies indicated its link with bladder and pancreatic cancer [13]. A recent report suggests nephrotoxic effects of cadmium due to their accumulation in the epithelial cells of proximal tubules of the kidney [14, 15]. The exact mechanism of cadmium mediated toxicity is poorly understood.

Chromium exposure is also reported to be linked with respiratory conditions including nasal ulceration and lung cancers. Even though chromium is found in various oxidative states including +6, +3 and 0, Cr (VI) is prevalent and epidemiologically linked to the development of respiratory cancers [16]. The mechanism of chromium induced toxicity is well documented and leads to oxidative stress, DNA strand breaks, DNA-protein cross links and formation of DNA adducts [17, 18]. Nickel is also considered as a toxic and carcinogenic metal and its exposure is suggested to cause multiple respiratory system complications from irritation and inflammation to pulmonary edema and cancer [19]. Due to poor mutagenic ability it might exerts toxic effects through non-genotoxic mechanisms [20].

The chronic exposure to the heavy metals such cadmium, chromium, cobalt, lead and nickel are well reported to cause obstructive airway diseases, emphysema, end-stage renal failure, diabetic and renal complications, deregulated blood pressure, bone disorders, immune suppression and various cancers [21], so it is important to understand the molecular mechanism of detoxification adopted by the biological system. Importantly heavy metals are non-degradable in nature, and their accumulation in the food chain of the ecosystem is a serious health and environmental concern which needs immediate focus.

1.2. Heavy metals and Detoxification Mechanisms

Microorganisms such as bacteria, algae, fungi and yeast etc. have evolved to adapt the changing environmental conditions and have developed various molecular and biochemical processes to overcome adverse environmental

conditions. Some bacteria have developed the tolerance to environmental stress such as heavy metals and toxic chemicals [22]. Reports suggest the role of several functional groups present on the bacterial cell wall which can play important role in the metal ions sorption from the environment [22]. The various other mechanisms involves energy dependent efflux of metal [3], precipitation of the metal as insoluble salts [23], alteration in the permeability of membrane [24, 25], production of chelating agents [26] and biochemical transformation of metal ions [24]. Even though the bacteria have developed resistance to the environmental pollutants particularly heavy metals, there is a lack of understanding on the molecular mechanism of metal detoxification.

Bacteria such as *Bacillus subtilis* [27], *Staphylococcus aureus* [28] and *Lactobacillus plantarum* [29] have evolved active efflux mechanism consisting of two plasmid encoded genes *cadA* and *cadC*. CadA, a CPx-type ATPase catalyzes the efflux of cadmium and CadC which is transcriptional repressor binds to cad operator DNA and is released in presence of metal ions such as cadmium, lead or bismuth. Chromium resistant bacteria have also evolved similar membrane transports that catalyze the efflux of chromium from the cytoplasm. ChrA protein is the best example studied in *Pseudomonas aeruginosa* that function as chemiosmotic pump and extrudes chromium from the cytoplasm. In both Gram negative and positive bacteria, nickel is taken up by dedicated transporters such as NikABCD import pump or high affinity nickel/cobalt permeases [24]. In *Mycobacterium tuberculosis*, the transcriptional factor NmtR inhibits expression of the gene for NmtA, an ATP dependent transporter responsible for nickel and cobalt [30]. Another protein KmtA, represses the expression of a putative cation diffusion facilitator (CDF) metal exporter and this is reported to detect the basal levels of cytosolic nickel or cobalt and exports them out of the cell [31]. Besides the transporter proteins, reports have also shown that heavy metals induces oxidative stress in the bacterial system and antioxidant enzymes seems to play an important role in combating metal induced oxidative stress [32]. Heavy metal affects lipid peroxidation and plasma

membrane permeabilization, leading to an increase in the formation of lipid peroxides [33]. Lipid peroxides are highly unstable and decomposes to form a complex series of compounds including reactive carbonyl compounds attacking biomolecules such as proteins and DNA leading to damage [34]. The importance of antioxidant enzymes is generally emphasized in preventing oxidative stresses by scavenging ROS or reduction of oxidized glutathione to reduced form [35]. Superoxide radicals generated is converted to H_2O_2 by the superoxide dismutase (SOD). The accumulation of H_2O_2 is prevented in the cell by catalase (CAT), peroxidases (POX) or by the ascorbate glutathione cycle where ascorbate peroxidase (APOX) reduces it to H_2O [36]. Heavy metals are known to modulate these enzymes in the bacterial system. SOD is considered as first line of defense against generation of oxygen species. The increase in the activity was observed in *Streptococcus bovis* in response to mercury stress [36]. The increased levels of SOD activity was also observed in Cd resistant *Ochrobactrum* sp. (CdSp9), lead resistant *Bacillus* sp. (PbS6) and arsenate resistant *Bacillus* sp. (AsSP9) in response to Cd, Pb and As respectively [37]. In case of chromium SOD activity did not change in the *Escherichia coli*. Similar observation was found in *Xanthomonas campestris* exposed to cadmium [38]. The catalase activity showed variable response in Cd resistant *Ochrobactrum* sp. (CdSp9), lead resistant *Bacillus* sp. (PbS6) and arsenate resistant *Bacillus* sp. (AsSP9). In the gram negative CdSP9, the catalase activity was very high as compared with the other two strains [39].

Increased antioxidative activity has been recorded in variety of stress conditions such as drought and toxic levels of metal (Al, Cu, Cd & Zn) [40]. The role of thiol compounds such as glutathione is very crucial in oxidative stress [41]. Heavy metals are known to deplete free thiols [42] and the enzymes glutathione reductase (GR) and glutathione peroxidase (GSHPx) maintains the optimal levels of glutathione in the cell. The enzyme GSHPx removes peroxides formed during the normal metabolic activity and oxidizes glutathione reduced (GSH) to glutathione oxidized (GSSG) form. The increased levels of GSHPx have been

observed in the bacteria in response to metal stress [42]. The induction of GSHPx and GR has been demonstrated in *Pseudomonas putida* in response to arsenate [42]. In rumen bacteria *S. bovis* and *S. ruminantium* a relatively high levels of GR activity was observed [35, 43, 44].

Even though the role of the transporter proteins and antioxidant enzymes in heavy metal detoxification is well reported in the literature, integrated understanding of the molecular mechanism in conferring metal resistance to the bacteria is still lacking. Also the metal resistant bacteria have gained much attention in the recent past for their application in the remediation of metals from the aqueous solution due to their ubiquity and smaller size which leads to high surface area and uptake (biosorption) of metals at faster rates [45, 46]. The molecular understanding of the mechanism of metal detoxification in the metal resistant bacteria might find application in the area of bioremediation.

1.3. Heavy metals and Bioremediation

The remediation of heavy metal contaminated soil and water have been a major challenge for a long time [47]. The traditional physiochemical methods such as electrochemical treatment, ion-exchange, precipitation, reverse osmosis, evaporation and oxidation/reduction for heavy metal removal from waste streams are expensive, not ecofriendly and inefficient for metal removal [48]. In recent past biosorption has emerged as an alternative to these traditional methods with several advantages such as technically easy, potential for regeneration and sludge free operations [3]. In addition, it is an ecofriendly and low cost method for remediating heavy metals [49, 50]. At higher concentration heavy metals are potent toxicants that interfere with the normal biochemical reactions of the human body. The release of metals into the environment also causes environmental pollution problems as they are non-biodegradable and accumulate in the living system [51]. Exploiting taxonomically diverse microbes particularly bacteria in remediating heavy metals and restoration of polluted environment have been a major focus of research in recent times.

The studies in the early 1980s have shown how *Bacillus subtilis* was able to grow in the range of toxic metals including copper, iron, magnesium, gold and lead [52]. Fein *et al.* [53] showed through the potentiometric titration of *B. subtilis* that changing the pH of the environment, and altering the cell surface charge, affects the ability of the bacterial species to bind metals in solution, however, this is not true for all bacterial species [52]. In the environments such as chemical drainage or mining sites, there are bacterial species that not only survive in the extreme pH conditions but also have evolved the mechanisms to cope with high concentration of metals that might be toxic to human and other species and such bacterial species might contribute in the bioremediation of heavy metal contaminated water or soil [52]. Halttunen *et al.* [54] showed that *Lactobacillus* and *Bifidobacterium* species can bind to lead and cadmium in solution and importantly the metal remained sequestered and did not dissociate even after 48 h. Ibrahim *et al.* [55] also compared the abilities of *Lactobacillus rhamnos* LC-705 and *Propionibacterium freudenreichii* to bind and absorb to lead and cadmium and reported the maximal binding takes place within 1 h of exposure and was influenced by pH as found in *B. subtilis* and *E. coli* [56].

Choudhary *et al.* [57] reported heavy metals such as Co, Cd, Ni etc. sequestration by *Pseudomonas* J007 strain which was tolerant to a high concentration (100 μ M). Naik *et al.* [58] showed *Bacillus* strain IST105 detoxifies the hexavalent chromium from the electroplating effluents. Yilmaz [59] reported the metal tolerance and biosorption capacity of *Bacillus circularis* strain EB1. Nithya *et al.* [60] carried out assessment and characterization of heavy metals in Palk Bay sediment bacteria. Although the identification and characterization for heavy metal resistant bacteria are well reported in the literatures, limited information is available on the exploitation and applicability of the bacterial strains in the area of heavy metal remediation.

With the above background and considering the possible importance of metal resistant bacteria from the bioremediation point of view, the present research is

focused on characterization of metal resistant bacterial strains and to study the mechanism both at biochemical and molecular levels.

1.4. Aims and objectives of the present study

Recent reports have highlighted the significant role of microbes in metal detoxification, but lack of information available on the molecular mechanism of metal detoxification has limited their application in the field of bioremediation. So the present study is focused on the characterization of metal resistant bacterial strains from the soil samples collected from the nearby/adjoining areas of mining sites of East Singhbhum district Jharkhand (India) and the mechanistic study of metal detoxification using molecular and proteomic approaches. Main objective of the present study are as follows:

1. Screening, isolation and characterization of metal resistant bacterial strains from the soil samples of nearby/adjoining areas of mining sites.
2. Optimization of culture conditions for the metal resistant bacterial strains and the study of their growth responses in the presence of metals.
3. Investigation of the enzymatic and non-enzymatic antioxidant system and their role in metal detoxification.
4. Biophysical analysis of metal resistant bacterial strains to study metal-microbe interactions.
5. Proteomic approach to identify the differentially expressed proteins and their functions in metal detoxification.
6. Cloning and characterization of *lipA* gene followed by the *In silico* prediction of its protein structure using bioinformatics tools.