Chapter 1

Introduction

1.1. Background

Heavy metal(s) are widespread pollutants of great concern as they are non-degradable and thus persistent. These metals are used in various industries from which effluents are consequently discharged into the environment. Introduction of metals in various forms into the environment can produce numerous modifications of microbial communities and affect their activities (Doelman *et al*., 1994; Hiroki, 1994; Staezecka and Bednatz, 1993). Common sources of heavy metal pollution include discharge from industries such as electroplating, plastics manufacturing, fertilizer producing plants and wastes left after mining and metallurgical processes (Zouboulis *et al*., 2004).

Although some heavy metals are essential trace elements, most can be, at high concentrations, toxic to all forms of life, including microbes, humans and animals. Heavy metals generally exert an inhibitory action on microorganisms by blocking essential functional groups, displacing essential metal ions or modifying the active conformations of biological molecules (Doelman *et al.*, 1994; Gadd and Griffiths, 1978; Wood and Wang, 1983). However, at relatively low concentrations, some heavy metal ions (e.g. Co^{2+}, Cu^{2+}, Zn^{2+} and Ni^{2+}) are essential for microorganisms since they provide vital cofactors for metallo-proteins and enzymes (Eiland, 1981; Doelman *et al*., 1994).

In order to survive in heavy-metal polluted environments, many microorganisms have developed means of resistance to toxic metal ions (Nies and Silver, 1995; Nies, 1999). These mechanisms include: metal exclusion by permeability barriers, active transport of the metal away from the cell organism, intracellular sequestration of the metal by protein binding,

extracellular sequestration, enzymatic detoxification of the metal to a less toxic form and reduction in metal sensitivity of cellular targets (Bruins *et al*., 2000; Nies and Silver, 1995; Silver, 1996). The detoxification mechanisms may be directed against one metal or a group of chemically related metals. Furthermore, the detoxification mechanisms may vary depending on the type of microorganism (Nies and Silver, 1995). Most microorganisms are known to have specific genes for resistance to toxic ions of heavy metals. Mostly, the resistance genes are found on plasmids or on chromosomes (Nies, 1999). Plasmid-encoded metal resistance determinants have been reported to be inducible (Silver *et al*., 1981; Rosen, 2002).

The intake and subsequent efflux of heavy metal ions by microbes normally includes a redox reaction involving the metal. As such, some bacteria even use them for energy and growth (Nies, 1999). Bacteria that are resistant to heavy metals also play an important role in biogeochemical cycling of those metal ions (Spain, 2003). Also, since the oxidation state of a metal ion may determine its solubility, many scientists have been attempting to use microbes that are able to oxidize or reduce metals in order to remediate metal-contaminated sites (Spain, 2003). Thus, it is worthwhile to note that although some heavy metals are important and essential trace elements and others are toxic at high concentrations to microbes, some microbes have adapted to tolerate the presence of metals or even to use them to grow. Furthermore, a number of interactions between microbes and metals have important environmental and health implications.

1.2. Heavy metal ions and toxicity mechanisms

Microbes encounter metals and metalloids of various types in their surrounding environments and therefore it is not surprising that they interact with them, sometimes to their benefit, at other times to their detriment (Ehrlich, 1997). Of particular interest are the base metals including cobalt,

copper, nickel, zinc, cadmium and the oxyanions (antimonate, arsenate and arsenite).

These metals play an integral role in life processes of microorganisms. Some metals such as calcium, cobalt, chromium, copper, iron, potassium, magnesium, manganese, sodium, nickel and zinc, are essential, serve as micronutrients and are used for redox-processes, to stabilize molecules through electrostatic interactions, as components of various enzymes, and for regulation of osmotic pressure (Bruins *et al*., 2000). Many other heavy metals have no known biological role, (e.g. silver, aluminium, cadmium, gold, lead, antimony, arsenic and mercury), and are nonessential (Bruins *et al.*, 2000) and highly toxic to microorganisms. At high concentrations, however, heavy metal ions form unspecific complex compounds, which lead to toxic effects. Even very important trace elements like zinc or nickel and especially copper are toxic at high concentrations. Thus, the intracellular concentration of heavy metal ions has to be tightly controlled, and heavy metal resistance is just a specific case of the general demand of every living cell for some heavy metal homeostasis systems (Nies, 1999).

Most metal ions have to enter the bacterial cell in order to have physiological or toxic effect (Nies, 1999). Many divalent metal cations (e.g. Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+}) are structurally very similar. Also, the structure of oxyanions such as chromate resembles that of sulfate, and the same is true for arsenate and phosphate. Thus to be able to differentiate between structurally very similar metal ions, the microbial uptake systems have to be tightly controlled. Microorganisms use fast and unspecific uptake systems driven by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. These uptake systems are constitutively expressed (Nies, 1999) and thus, they lead to the accumulation of heavy metal ions within the microbial cell.

Since high concentrations of heavy metal ions within the microbial cells are very toxic, microorganisms have been forced to develop metal-ion homeostasis factors or metal-resistance determinants (Nies and Silver, 1995; Bruins *et al*., 2000). These resistance determinants encode proteins, which play a role in detoxification mechanisms for the survival of microorganisms in heavy-metal contaminated environments. Another type of uptake system has high substrate specificity, is slower, and often uses ATP hydrolysis as the energy source (Nies and Silver, 1995). As opposed to constitutively expressed unspecific uptake systems, ATP-dependent uptake systems are inducible.

Inside the cell, the toxicity of heavy metal ions may occur through the displacement of essential metals from their native binding sites or through ligand interactions (Nies, 1999; Bruins *et al.*, 2000). Heavy metal cations especially those with high atomic numbers, e.g. Hg^{2+}, Cd^{2+} and Ag^{+}, tend to bind SH groups (Nies, 1999). The minimal inhibitory concentration (MIC) of these heavy metal ions is a function of the complex dissociation constants of the respective sulfides (Nies, 1999). By binding to SH groups, the heavy metal ions may inhibit the activity or the functioning of sensitive enzymes. The MIC is defined as the minimum concentration of a heavy metal at which microbial growth is completely inhibited by toxicity of heavy metal ion (Yilmaz, 2003).

Some bacterial species are capable of exhibiting higher MICs than *Escherichia coli*, which have been tested and show resistance to heavy metal ions. For example, studies of Yilmaz (2003) indicated that a newly characterized *Bacillus circulans* strain EB1 exhibited high minimal inhibitory concentration values for heavy metal ions (Table 1.1). Hence, the bacterial species exhibiting high MIC could be of great significance since they could possibly be applied for bioremediation in heavy metal contaminated environments and other industrial processes such as biomining. Biomining

refers to the use of living organisms (e. g. bacteria, plants) to accumulate in their cells precious metals like gold, silver, platinum, from mine tailings (Acevedo, 2000). Then, these organisms can be collected and the metals be recovered.

Table 1.1. Minimal inhibitory concentrations (MICs) in mM for *E. coli* and *Bacillus circulans* EB1 (Adapted from Nies, 1999; Yilmaz, 2003)

Heavy metal ion	*E. coli*	*B. circulans*
Cu^{2+}	1.0	2.5
Co^{2+}	1.0	2.0
Cd^{2+}	0.5	2.0
Ni^{2+}	1.0	10.0
Zn^{2+}	1.0	22.0

Other heavy metal ions may interact with the physiological ions, thereby inhibiting the function of the respective physiological cations (Nies, 1999). In Gram-negative bacteria, metal cations can bind to glutathione. This results in the formation of bisglutathionato complexes, which interact with molecular oxygen to form bisglutathione (GS-SG), the metal cation, and hydrogen peroxide (Kachur *et al*., 1998). As the reduction of bisglutathione back to glutathione requires NADPH, heavy-metal cations can cause considerable oxidative stress. In addition, at high levels, both essential and non-essential heavy metal ions can damage cell membranes, alter enzyme specificity, disrupt cellular functions, and damage the structure of DNA (Bruins *et al*., 2000).

1.3. Microbial resistance mechanisms to heavy metal ions

Despite the diverse types of toxic metal ions and the varied methods by which they interact with microorganisms, many bacteria have developed means of resistance to the toxic metal ions and this is one factor that makes microbial cells special organisms. The bacterial cells acquire a gene, or genes which normally code for proteins and enzymes that perform specific functions either to protect the bacterial cell, or block or alter the incoming toxic metal, or both (Cavicchioli and Thomas, 2002).

According to Nies and Silver (1995), since heavy metal ions cannot be degraded or modified like toxic organic compounds, there are only three possible mechanisms for heavy metal ion resistance. Firstly, the accumulation of the respective ion can be diminished, not by interference with the uptake (that would be tolerance) but by efflux, an active extrusion of the heavy metal ion from the cell. Cations can as well be segregated into complex compounds by thiol-containing molecules while on the other hand some heavy metal ions may be reduced to less toxic oxidation states (Nies, 1999). Some studies reveal that there are other possible mechanisms for metal resistance: intra- and extra-cellular sequestration, enzymatic reduction and the reduction in the sensitivity of cellular targets to metal ions (Rai *et al*., 1981; Macaskie and Dean, 1989; Huang *et al*., 1990; Avery and Tobin, 1993; Brady *et al*., 1994; Veglio *et al.*, 1997; Wagner-Döbler *et al*., 2000; Liu *et al*., 2004; Malik, 2004). These resistance mechanisms explain why some microorganisms are able to survive in metal contaminated environments.

1.3.1. Microbial resistance to oxyanions

Detoxification of heavy metal ions by reduction has been extensively studied and As(V) reduction to As(III) is well-documented (Ji and Silver, 1992; Wu and Rosen, 1993). A metal compound that can be reduced should be able to

diffuse out of the cell or it might reoxidise itself. However, most reduction products are quite insoluble (e.g. Cr^{3+}) or even more toxic (e.g. As^{3+}) than the educts (Nies, 1999). Therefore, if the cell chooses to detoxify such a compound by reduction, an efflux system should be present to export the reduced products (Nies, 1999). If the heavy metal cannot be reduced by cellular means or reduction is undesirable, the only choice is between complexation and efflux or both. However, the cost of complexation is huge compared to efflux if a fast growing cell is considered (Nies, 1999).

Resistance mechanisms can be chromosomal or plasmid-mediated. Efflux systems are the major currently-known group of plasmid resistance systems. They are either ATPases (e.g. Cd^{2+} ATPase of Gram-positive and arsenite ATPase of Gram-negative bacteria) or chemiosmotic (as divalent cation efflux system of soil *Alcaligenes* and the arsenite efflux system of the chromosome of Gram-negative bacteria and plasmids of Gram-positive bacteria). Microorganisms use efflux systems to export toxic metals from their cytoplasm. Non-essential metals normally enter the cell through normal nutrient transport systems but are readily exported (Silver *et al.*, 1989, Nies and Silver, 1995).

The oxyanions of arsenic enter the bacterial cells via transporters of other compounds. In *E coli,* arsenate uptake is always mediated by phosphate transporters, Pit and the Pst pumps (Bennet and Malamy, 1970; Rosenberg *et al*., 1997; Nies, 1999; Rosen, 2002) (Fig. 1.1). Under abundant phosphate conditions, the less specific Pit system fulfills the phosphate needs of the cell and leads to arsenate accumulation (Elvin *et al.*, 1987). Under conditions of phosphate starvation, a highly specific Pst-system (for phosphate uptake) is induced (Surin *et al.*, 1987) and it uses the PstS phosphate-binding protein and the PstABC ATPase complex for inner membrane uptake. But the Pit system appears to be the predominant system for arsenate uptake (Willsky, 1980; Rosen, 2002). Similarly, in the eukaryote, *Saccharomyces cerevisiae*,

several phosphate transporters participate in arsenate uptake (Bun-ya *et al*., 1996; Yompakdee *et al*., 1996; Nies, 1999; Rosen, 2002).

Since the chemical properties of arsenic and antimony oxyanions are similar, it is likely that they are transported by the same pathway (Simon, 1996). One route of entry for Sb(III) is via the glycerol facilitator, G1pF polyol transporters (Rensing *et al*., 1999; Sanders *et al*., 1997; Rosen, 2002). It is also detoxified by all systems giving resistance to arsenite, by efflux (Rosenstein *et al*., 1992; Nies and Silver, 1995; Sanders *et al.*, 1997).

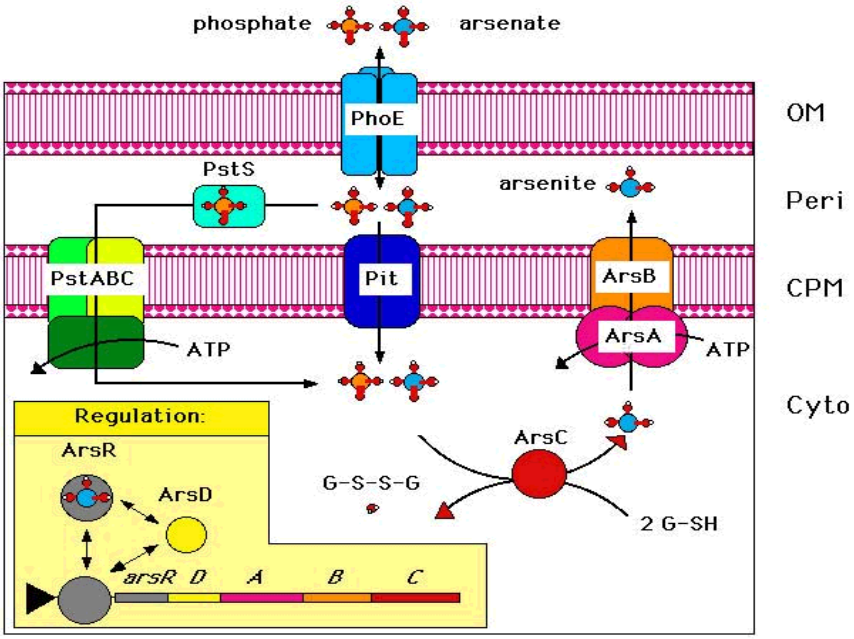

Fig. 1.1. Transport and resistance to arsenate *in E. coli*. OM, outer membrane; Peri, periplasmic space; CPM, cytoplasmic membrane; Cyto, cytoplasmic space; G-SH, reduced glutathione; G-S-S-G, oxidized glutathione (Nies and Silver, 1995).

Resistance to toxic oxyanions of arsenic and antimony is conferred by the *ars* operon of plasmid R773 in *Escherichia coli* (Rosen, 1996; Bhattacharjee *et al.*, 2000). Both oxyanions are transported in the cytoplasm by the Pit or the Pst system. When the arsenate is taken up, it is reduced to arsenite prior to extrusion or sequestration (Ji and Silver, 1995; Nies and Silver 1995; Rensing *et al.*, 1999; Rosen, 2002). In both *E. coli* and *S. aureus*, arsenate is reduced

to arsenite by the ArsC enzyme encoded by the *ArsC* gene from the *ars* operon, and effluxed through the arsenite-specific ArsB protein (Nies and Silver, 1995); glutathione and thioredoxin serve as sources of reducing potential in both *Escherichia coli* and *Staphylococcus aureus* respectively (Nies and Silver, 1995). The ArsR repressor protein and the ArsD co-regulator protein regulate the *arsRDABC operon* (Fig 1.1). The *ars* determinants also govern resistance to antimonite (Novick and Roth, 1968), which appears to be an alternative substrate for the ArsB-efflux system (Nies and Silver, 1995).

1.3.2. Resistance to divalent metal ions

There is no general mechanism of resistance to the different metals and the resistance determinants differ from one species to another. Resistance mechanisms to the different divalent metals have been described by Nies (1999). Most divalent heavy metal ions are accumulated within the cells by the fast and unspecific CorA (metal transport system, MIT) Mg^{2+} transport system (Nies, 1999). Highly specific divalent heavy metal ion transport systems are also expressed but they are inducible since they are expressed by the cell in time of need or starvation (Nies, 1999).

1.3.2.1. Cadmium

In gram-positive bacteria, accumulation of Cd^{2+} leads to the expression of the *CadA* resistance system (Fig 1.2), which is located on plasmid p1258 and related plasmids (Novick and Roth, 1968; Nies and Silver, 1995). *CadA* mediates resistance by active ion efflux (Tynecka *et al*., 1981; Nies and Silver, 1995). The *CadA* resistance determinant is inducible (Yoon *et al*., 1991; Nies and Silver, 1995) and the *CadC* gene product (Yoon *et al*., 1991, Nies and Silver, 1995)) is known to be trans-acting DNA-binding regulatory protein of this system (Fig. 1.2). Cation efflux is catalyzed by the CadA protein

(Tsai *et al.*, 1992; Oden *et al*., 1994; Nies and Silver, 1995), which is a P-type ATPase (Silver *et al*., 1989; Tsai and Linet, 1993; Nies and Silver, 1995). ATP serves as a source of energy for CadA-catalyzed cadmium transport (Tsai *et al*., 1992; Nies and Silver, 1995).

In gram-negative bacteria, cadmium seems to be detoxified by RND-driven (resistance, nodulation, cell division) systems like Czc, which is mainly a zinc exporter (Nies and Silver 1995; Nies, 1999) and Ncc, which is mainly a nickel exporter (Schmidt and Schlegel, 1994). Cyanobacteria, however, contain metallothionein-like proteins (smt) which have been characterized (Olafson *et al*., 1979; Nies, 1999) (Fig 1.3 D). It has also been found that amplification of *smt* metallothionein locus increases cadmium resistance and deletion of *smt* decreases resistance (Gupta *et al*., 1993; Turner *et al.,* 1993, Turner *et al*., 1995; Nies, 1999).

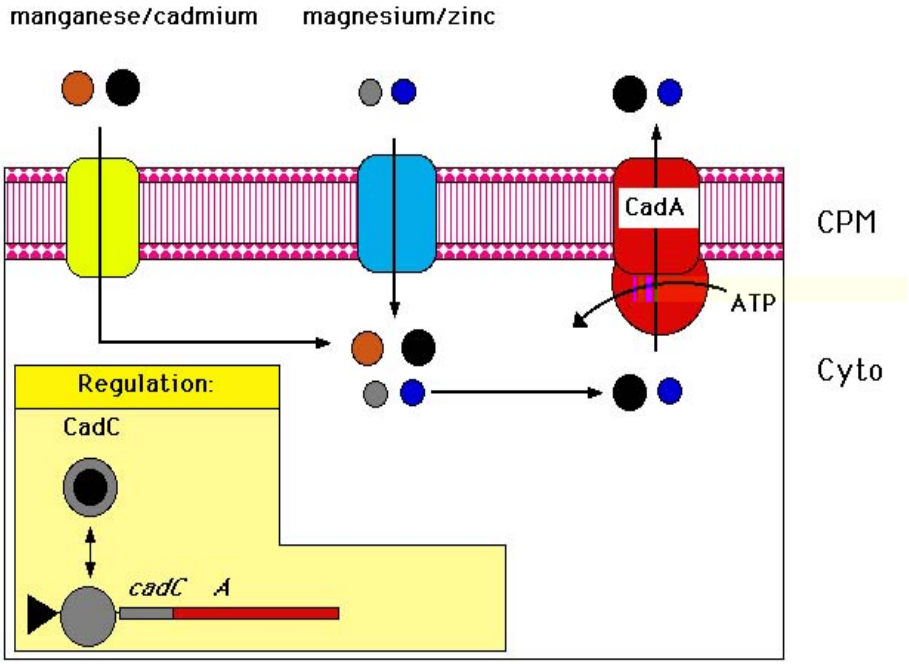

Fig. 1.2. Transport of and resistance to cadmium and zinc in *Staphylococcus aureus* (Nies and Silver, 1995).

Metallothioneins are thiol-containing, cystein-rich heavy metal-binding proteins that sequester metals thus preventing accumulation of potentially toxic-free metal ions within the cell (Zhou and Goldborough, 1994; Ybarra and Webb, 1998; Andrews, 2000). The heavy metal ion binding occurs through the interactions of the ions with the thiol groups of cysteine residues. The metallothionein genes are arranged as an operon called the *smt* locus, containing both *smtA* (coding the metallothionein protein) and *smtB* (coding the repressor, regulatory protein) genes (Ybarra and Webb, 1998). Metallothionein expression, from the gene to the functional protein, is induced by heavy metal ions and the regulation of transcription to mRNA is dependent upon the interaction between these heavy metal ions and the repressor protein regulating transcription, again via the interaction with the thiol groups present on the repressor protein (Erbe *et al*., 1995; Ybarra and Webb, 1998).

1.3.2.2. Zinc

Zinc is one of the essential trace elements and it is not biologically redox reactive and is thus not used in respiration (Spain, 2003). Zinc may be complexed by various cellular components (Daniels *et al.*, 1998; Palmiter, 1998, Nies, 1999). It is a component in a variety of enzymes and deoxyribose nucleic acid- (DNA) binding proteins like zinc-finger proteins (Chou *et al.*, 1998; Nies, 1999). Unspecific and fast uptake of Zn^{2+} is mediated by CorA (MIT) Mg^{2+} transport systems in some bacterial species and by the fast and unspecific MgtE system in others (Fig 1.3C). There are two systems for zinc detoxification in bacteria, P-type ATPases (which transport only zinc across the cytoplasmic membrane) and RND-driven transporters (transporting zinc across the complete cell wall of Gram-negative bacteria (Fig. 1.3C).

In *E. coli*, the ZntA P-type ATPases may be responsible for the efflux of zinc (Beard *et al*., 1997; Rensing *et al*., 1997; Nies, 1999) and the related ZiaA transporter in the cyanobacterium *Synechosystis* (Thelwell *et al*., 1998; Nies,

1999). Moreover, P-type ATPases mediating cadmium resistance, efflux zinc as well in most cases (Nies, 1999). Slow efflux of zinc in *Staphylococcus aureus* is catalyzed by cation-diffusion facilitator (CDF) transporters, which also mediate the resistance to cobalt (Xiong and Jayaswal, 1998).

1.3.2.3. Cobalt

Co^{2+} is rapidly accumulated by the CorA system in most bacterial cells (Smith *et al.*, 1993). Resistance to cobalt in Gram-negative bacteria is based on the transenvelope efflux system driven by a resistance, nodulation, cell division (RND) transporter (Nies, 1999). Members of CDF bacteria have also been found to detoxify Co^{2+} (Conklin *et al.*, 1994; Nies, 1999).

1.3.2.4. Nickel

Nickel is accumulated by the fast and unspecific CorA (metal transport system, MIT) Mg^{2+} transport system in bacteria and *Saccharomyces cerevisiae* (Hmiel *et al.*, 1989). Highly specific nickel transporters are either HoxN chemiosmotic transporters or ATP-binding cassette (ABC) uptake transporters, which use periplasmic nickel-binding protein (Nies, 1999). It is detoxified by sequestration and/or transport (Nies, 1999). The best-known nickel resistance in bacteria, *Ralstonia* sp. CH34 and related bacteria is based on nickel efflux driven by RND transporter (Fig. 1.3A). Two systems have been described, a nickel/cobalt resistance (Cnr) (Liesegang *et al*., 1993; Nies, 1999), and a nickel/cobalt/cadmium resistance (Ncc) (Schmidt and Schlegel, 1994; Nies, 1999). Both are closely related to the cobalt/zinc/cadmium resistance system (Czc) from *Ralstonia* strain CH34 (Nies, 1999).

1.3.2.5. Copper

Copper ions are required as co-factors by many enzymes, such as oxidases and hydroxylases, but are highly toxic when present in excess (Lu *et al*., 1999). They are possibly accumulated by the CorA-Mg^{2+} transporter and additionally by P-type ATPases under copper starvation (Fig 1.3B).

Mechanisms for copper metabolism occur naturally in all living organisms, and they are generally chromosomally encoded (Liu *et al*., 2002). In contrast, mechanisms that specify resistance to copper in bacteria are often plasmid-encoded (Cooksey, 1993). The plasmid and chromosomal systems may interact with each other to maintain copper homeostasis in bacteria (Rogers *et al*., 1991; Brown *et al*., 1995; Fong *et al*., 1995; Gupta *et al*., 1995). Inside the cells, copper may be bound by various compounds to form copper complexes (Nies, 1999). P-type ATPases seem to detoxify copper via efflux in some species, however, the copper resistance systems of the *Pseudomonas* type usually encode four proteins (circles with A, B, C or D) (Fig 1.3B), which bind copper in the periplasm or close to the outer membrane (Nies, 1999).

1.3.2.6. Mercury

Mercury is one of the most toxic elements tested in *Escherichia coli*. Affinity of the mercury for thiol groups is stronger than the affinity of cadmium for sulfide (Nies, 1999). It binds to sulfhydryl groups of enzymes, thereby inactivating vital cellular functions (Wagner-Döbbler *et al*., 2000). As a response to toxic mercuric compounds globally distributed by geological and anthropogenic activities, microorganisms have developed an array of resistance determinants to overcome the poisonous environments. Three types of resistance to mercury have been reported: reduced uptake of mercuric ions (in *Enterobacter aerogenes*), methylation and reduction.

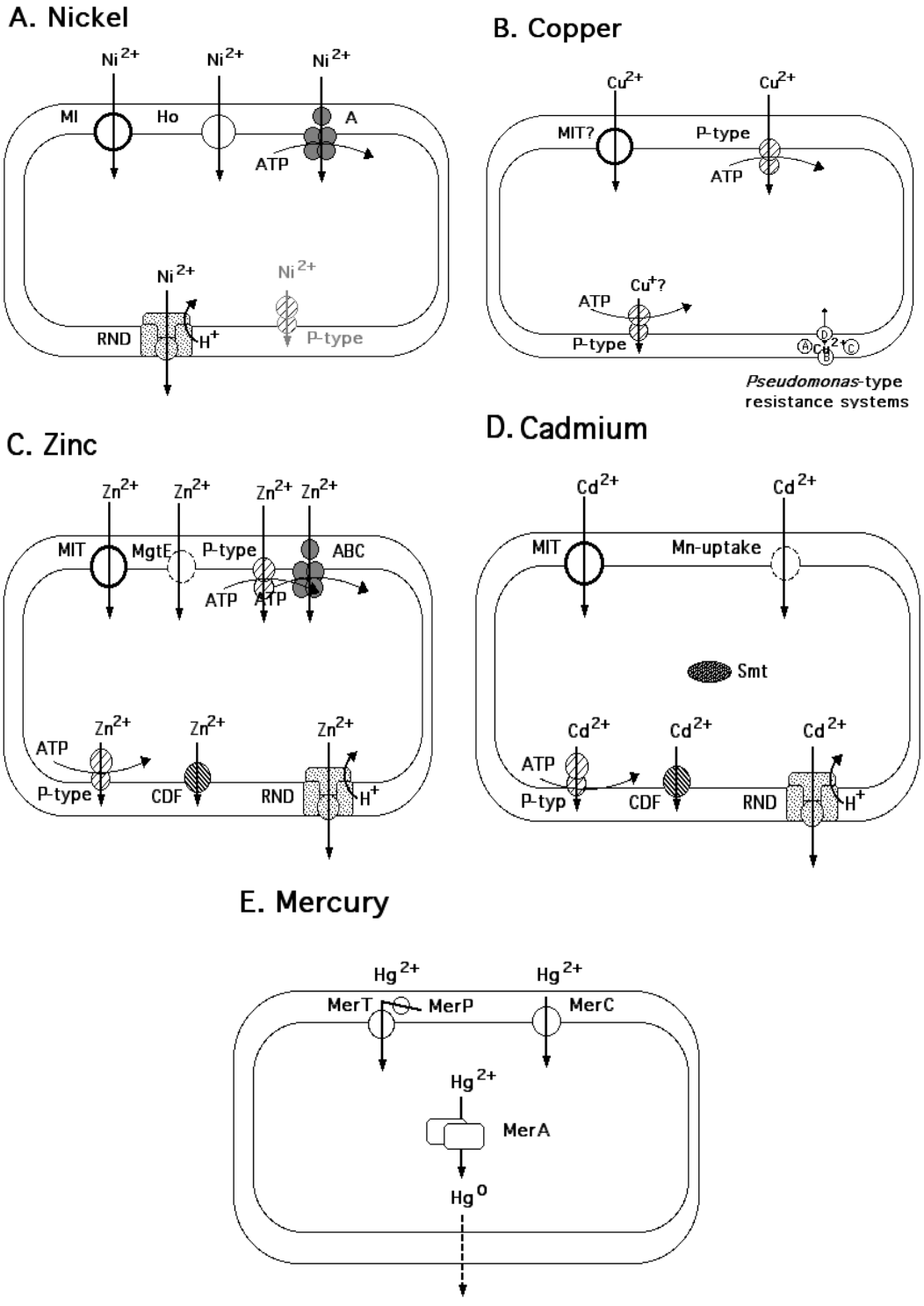

Fig. 1.3. Protein families involved in transport and detoxification of divalent metal ions (Adapted from Nies, 1999).

The resistance determinants encode the proteins involved in the transport inside the cell (Nies, 1999). An extensively studied resistance system is based on clustered genes in *mer* operon, which allows bacteria to detoxify Hg^{2+} into volatile metallic mercury by enzymatic reduction (Komura and Izaki, 1971; Summers, 1986; Misra, 1992; Silver, 1996; Silver and Phung, 1996; Nies, 1999; Wagner-Döbbler *et al*., 2000).

Mercury-resistance determinants have been found in a wide range of Gram-negative and Gram-positive bacteria isolated from different environments. These resistance determinants vary in the number and identity of genes involved and are encoded by the *me*r operon located on plasmids (Summers and Silver 1972; Griffin *et al.*, 1987; Radstrom *et al.*, 1994).

1.4. Heavy metal pollution: world-wide problem

Heavy metals are naturally occurring elements (Nies, 1999; Mighall *et al.*, 2002) and can be found deep in the earth crust (Malle, 1992). Due to natural erosion processes like weathering and abrasion of rocks, soils and sediments by wind and water, a small but significant fraction of natural metals are continuously being mobilized and transported in the environment. Volcanic eruptions, forest fires and aerosol formation above seas also contribute to the natural transport of metals. These processes cause cycling of metals in the environment, resulting in natural background levels in the air, surface waters and soil (Mighal *et al.*, 2002; Raab and Fieldman, 2003).

There are three main sources of environmental heavy metal pollution; industry, agricultural use and sewage sludge. The increase in industrial activities has intensified environmental pollution problems and the deterioration of several ecosystems with the accumulation of toxic metals. Other common sources of heavy metals include electroplating, plastics manufacturing, fertilizer producing plants, pigments and wastes left after

mining and metallurgical processes (Zouboulis *et al*., 2004). Tailings from metal-mining operations are also a significant source of contamination, and can lead to contamination of the surrounding topsoils, and, because of leaching, sometimes the groundwater too. It has been estimated that several billion tons of tailings waste exist in the USA alone (Wewerka *et al*., 1978). These metals are also used in various industries from which effluents are consequently discharged into the environment, resulting in high metal concentrations. The levels of metals in sewage sludge reflect the extent of industrialization of the area served by the local sewage system. Significant quantities may be added by metal-contaminated wastewater runoff derived from sources including atmospherically deposited metals, residues from pesticide usage, phosphate detergents and industrial effluent, particularly from the metal-processing industry (Zhu & Tabatabai, 1995). Growing attention is being given to health hazards presented by the existence of heavy metals in the environment; their accumulation in living tissues throughout food chain, poses a serious health problem (Zouboulis *et al*., 2004).

1.4.1. Atmospheric heavy metal pollution

1.4.1.1. Metalloids air pollution: arsenic

Arsenic is primarily emitted into the atmosphere by high-temperature processes such as coal-fired power generation, smelting, burning vegetation and volcanism. Natural low-temperature biomethylation and microbial reduction also release arsenic into the atmosphere; microorganisms can form volatile methylated derivatives of arsenic under both aerobic and anaerobic conditions, and can reduce arsenic compounds to release arsine gas (Cheng & Focht, 1979; Tamaki & Frankenberger, 1992).

Arsenic is released into the atmosphere primarily as As_2O_3 or, less frequently, as one of several volatile organic compounds and it exists in the air in the

form of particulate matter (Coles *et al*., 1979). These particles are dispersed by the wind to a varying extent, depending on their size, and the particles are returned to the earth by wet or dry deposition. Arsines that are released from microbial sources in soils or sediments undergo oxidation in the air, reconverting the arsenic to less volatile forms that settle back to the ground (Wood, 1974; Parris & Brinckman, 1976). Rabano *et al*., (1989) collected size-fractionated aerosol samples at an urban site during 1987. A greater proportion (75%) of the arsenic was observed in the fine particles (< 2.5 µm). Similarly, Waldman *et al*., (1991) reported that 65 % of the arsenic in aerosol samples collected at an urban site (China) was associated with fine particles (< 2.5 µm). Kelley *et al*., (1995) monitored arsenic in aerosol collected from the Kola Peninsula (Russia). They found 68 % of arsenic associated with fine particles (< 1 µm), 26 % with coarse particles (1-10 µm) and 7 % with large particles (> 10 µm). The atmospheric residence time of particulate-bound arsenic depends on particle size and meteorological conditions, but a typical value is about 9 days (US EPA, 1982).

1.4.1.2. Air pollution with divalent metals: mercury as an example

Environmental contamination with mercury compounds can have devastating effects as mercury toxicity is cumulative. Mercury is more volatile than any other sulfhydryl-reactive metals and therefore it is more highly absorbed in the elemental (HgO) form. An excellent review of literature pertaining to the toxicity of HgO from dental amalgams has been presented by Lorschieider *et al*., (1995). HgO vapor is efficiently absorbed through the lungs and quickly passes the blood-brain barrier and once absorbed, it has a low excretion rate and thus accumulating in the kidneys, neurological tissue, liver and other body parts (Nylander *et al*., 1987). Mercury is also genotoxic: inorganic Hg(II) is capable of strong reversible interactions with the nitrogens in purines and pyrimidines, and organic mercury compounds, (e.g. methyl-mercury), also produce irreversible damage to nucleic acids (Betti *et al*., 1992).

1.4.2. Heavy metal pollution on land

Similar to mercury, cadmium plays no known biological role in metabolism, as no enzyme has been identified which specifically requires cadmium or mercury as a cofactor. These two metals, however, are extremely hazardous to life and have been involved in historic poisoning episodes of human populations and wildlife, resulting from ingestion of contaminated foods and prey (Bonhomme *et al*., 1996; Orloff *et al*., 1997; Fiedler *et al*., 1999).

Majority of Cd ingested is in the form of Cd-MT (cadmium bound to metallothionein protein) from food previously exposed to Cd. Upon absorption, Cd-MT accumulates in the body parts like kidneys. Cd-MT is said to be more nephrotoxic form of Cd because once the threshold is exceeded (approximately 200 µg/g), Cd may distribute to other cellular proteins, and compromise the function of renal tubules. Other toxic effects resulting from Cd pollution in animals include inflammation and cancer when inhaled, or enteropathy and nutrient mal-absorption syndromes when ingested (Valberg *et al*., 1977). Experimental poisonings with Cd have also been shown to have cardiovascular effects such as increased blood pressure, anaemia, and cardiomyopathy, effects on the reproductive system in both sexes, and skeletal effects (Frustaci *et al.*, 2000).

In prokaryotes, there is a considerable amount of evidence documenting a decrease in microbial biomass as a result of long-term exposure to heavy metal contamination (McGrath *et al.,* 1995). Analysis of a polluted environment with heavy metals from other sources such as Cu and Zn in animal manures (Christie and Beattie, 1989), run-off from timber treatment plants (Bardgett *et al.*, 1994), past applications of Cu-containing fungicides (Zelles *et al*., 1994) and analysis of soils in the vicinity of metal-contaminated

army disposal sites (Kuperman and Carreiro, 1997) confirm that a decrease in the microbial biomass occurs at a relatively modest, and sometimes even at a low (Dehlin *et al*., 1997) metal loading (Ghorbani *et al.*, 2002).

1.4.3. Water pollution with heavy metals

Accumulation of metals in an aquatic environment has direct consequences on man and ecosystem. Cd, Hg and Zn are common environmental pollutants, which are widely distributed in the aquatic environment (Merian, 1991). Their sources are mainly from weathering of minerals and soils (Merian, 1991); atmospheric deposition from non-ferrous metal mines, smelters, refineries, urban storm-water runoff (Field and Lager, 1975) and domestic effluents (Preuss and Kollman, 1974). An extensive literature on the aquatic toxicity of Zn and especially its toxicity to fish has been reviewed by Alabaster and Lloyd (1980) and by Spear (1981). Cd has also been found to be toxic to fish and other aquatic organisms (Rao and Saxena, 1981).

Marine organisms naturally accumulate considerable quantities of organic arsenic compounds. In marine animals the bulk of this arsenic is present as arsenobetaine, whereas marine algae contain most of the arsenic as dimethylarsinoylribosides. Humans are therefore exposed to these arsenic compounds through any diet that includes seafood (WHO, 2001).

1.5. Bioremediation

Generally, significant amounts of contaminants are released into the environment from various sources including industries, agricultural applications and other human activities. The problems associated with these contaminants have long-term and short-term effects on the overall ecosystem depending upon the type of contaminants, their chemical species, spatial distribution and their concentration. Organic contaminants may be degraded

biologically and carbon-dioxide and water are the final products (Bosecker, 2001), which are not hazardous and may be recycled. In contrast to toxic organic and inorganic substances, the metallic species released into the environment cannot be degraded either biologically, chemically or even physically (Bosecker, 2001; Valls and Lorenzo, 2002; Singh and Cameotra, 2004). Metal pollutants such as arsenic, antimony, cadmium, mercury, copper, chromium, nickel, cobalt, and zinc cannot be broken down to non-toxic forms. Since the presence of heavy metals in the environment is a worldwide problem, effective removal of heavy metals from wastewater and industrial wastes has engaged the urgent attention of many researchers.

Conventional methods such as precipitation, oxidation/reduction, ion exchange, filtration, membranes or reverse osmosis and evaporation have been used for the treatment of metal pollution (Nuhoglu and Oguz, 2003), however, they all exhibit several disadvantages such as high cost, low selectivity and high energy consumption and they are not eco-friendly (Gupta *et al*., 2000; Barros *et al*., 2003; Liu *et al*., 2004; Zouboulis *et al*., 2004). Another disadvantage of conventional treatment techniques is the production of toxic sludge as a result of the chemicals used (Gupta *et al*., 2000), which is often difficult to dewater and also requires extreme caution in its disposal (Kapoor *et al*., 1999). Unpredictable metal ion removal and high reagent requirements are also disadvantages (Barros, *et al*., 2003).

Amongst the chemical adsorbents, only ion exchange resins are considered as the option for remediation with least ecological problems (Gupta *et al*., 2000). The treated water/effluent is often sufficiently pure that it can be recycled and reused, and the sorbed metals can be recovered and purified in well regeneration processes (Mckay, 1995). However, the chemical resins are expensive and the increasing demand for eco-friendly technologies has led to the search for low-cost alternatives (Gupta *et al*., 2000). Environmentally friendly processes need to be developed to clean up the contaminated

environment without creating harmful waste products and to reduce metal content in wastewater or discharge to acceptable levels at affordable cost.

The search for alternative and innovative treatment techniques has focused attention on the use of biological materials such as algae, fungi, yeast and bacteria for the removal and recovery technologies and has gained importance during recent years because of the better performance and low-cost of these biological materials (Veglio *et al*., 1997; Kratochvil and Volesky, 1998; Volesky, 2001; Iqbal and Edyvean, 2004). Besides flexibility to handle the range of physico-chemical parameters in effluents, selectivity to remove only the desired metals and the cost-effectiveness are some of the added advantages of biological clean-up techniques (Malik, 2004). Many microorganisms have been intensively examined for their abilities to be applied in bioremediation of heavy metals.

Bioremediation is the use of biological systems, almost invariably microorganisms to clean up a contaminated site (Bains, 1993). Microbes can remove heavy metals from contaminated solutions either by bioaccumulation, precipitation or biosorption (Lovley and Coates, 1997; Terry and Stone, 2001; Akhtar *et al*., 2004; Liu *et al.*, 2004; Malik, 2004; Öztürk *et al*., 2004). Bioaccumulation of heavy metals is the retention and concentration of heavy metals by microbes. The heavy metal ions are transported across the microbial cell membrane from the outside to the inside of the cell where they are concentrated. In biosorption, however, heavy metal ions, especially the positively charged metal ions, are sequestered through the adsorption of metals to the negative ionic groups of cell surfaces (Lovley and Coates, 1997; Malik, 2004).

Bioremediation leads to changes in the solubility, sorption characteristics, transport properties and toxicity metals (Malik, 2004). In bioremediation, solubilization of heavy metal contaminants provides a means of removal from

the solid substance. Some microbial species subject heavy metal ions to enzymatic reduction, the reduced form of heavy metal ions are quite insoluble and they precipitate out of solution (Nies, 1999). Other microbes can mobilize heavy metals through autotrophic and heterotrophic leaching which can result in volatilization and such processes lead to dissolution of insoluble metal compounds (Malik, 2004).

1.5.1. Heavy metal ion removal during biosorption

Microbial biomass can passively bind large amounts of metals during biosorption, thus providing a cost-effective solution for wastewater management. Biosorption does not consume cellular energy; positively charged metal ions are sequestered, primarily through the adsorption of metals to the negative ionic groups on cell surfaces, also, on the polysaccharide coating found on most of bacterial cell-walls, or other extracellular structures such as capsules (Lovley and Coates, 1997; Malik, 2004). However, on prolonged contact with the metal-bearing solution, the living biomass may also be able to sequester the metal intracellularly by an active process known as bioaccumulation (Gupta *et al*., 2000). In biosorption, heavy metal ions can be sorbed and/or complexed to either living or dead biomass (Lovley and Coates, 1997). Several studies on the application of growing microbial cells for metal removal have shown that they are better than non-viable cells due to the microbe's ability of self-replenishment, continuous metabolic uptake of metals after physical adsorption, and the potential for optimization through development of resistant species and cell surface modification (Malik, 2004).

Although the use of living microbes may allow development of a single-stage process for removal of most pollutants from the industrial effluents, there are significant practical limitations to uptake by living cell systems. These include sensitivity of the systems to extreme pH, high metal/salt concentration and

the requirement of external metabolic energy (Poole, 1995; Dömnez and Aksu, 2001). Some studies reveal that the solution pH is the most important parameter affecting the biosorption process (Vasudevan *et al*., 2002; Nuhoglu and Oguz, 2003; Malik, 2004; Öztürk *et al*., 2004). Other factors affecting biosorption process include initial concentration of the metal salt, temperature, contact time, agitation speed as well as the concentration of the biosorbent (microbes applied for bioremediation) in solution (Nuhoglu and Oguz, 2003; Akhtar *et al.*, 2004; Iqbal and Edyvean, 2004).

Adsorption isotherms have been commonly used to describe experimental results for the sorption of metal ions by microorganisms. The most commonly used adsorption isotherms are the Langmuir and Fruendlich equilibrium models (Iqbal and Edyvean, 2004; Liu *et al*., 2004; Rangsayatorn *et al*., 2004). The initial rapid uptake is believed to be due to binding of metal ions to the cell wall. Many studies have also shown that at low metal ion concentrations, the amount of metal ion accumulated per unit of a cell mass is directly proportional to the concentration of the metal ion in a solution (Omar, 2002). Kinetic studies of sorption are significant since the data can be used:

- For determining the time required to reach equilibrium
- To evaluate the maximum adsorption capacity (Singh *et al*., 2001; Rangsayatorn *et al*., 2004).

The metal uptake (q) for the construction of sorption isotherms, is determined as follows:

$$q = V(C_i - C_{eq}) / M$$

where C_i and C_{eq} are the initial and residual concentration of metal ion concentrations, respectively; V is the volume of sample solution (*l*); and M is the dry weight of added biomass (*g*).

Some microbes can bind and collect a wide range of heavy metals with no specific preference, whereas others are specific for certain types of metals (Hosea *et al*., 1986; Volesky and Kuyucak, 1988; Vieira and Volesky, 2000). The origin of microbial species plays a significant role in explaining the behavior of microbes during biosorption process. Recent studies show that microbial species isolated from contaminated sites possess excellent capability of metal removal (Malik, 2004).

1.5.2. Bioaccumulation of heavy metal ion from contaminated solutions

Bioaccumulation is the accumulation of materials which are not critical components of an organism by that organism. Usually, it refers to the accumulation of metals (Bains, 1993). Heavy metal ions accumulate in microbial cells through membrane transport proteins by active transport (Nies, 1999). Various microbial species including bacteria, fungi, algae and actinomycetes have been shown to be efficient in bioaccumulation of heavy metal ions from polluted effluents (Yakubo and Dudeney, 1986; Premuzic *et al*., 1991; Wong *et al*., 1993). In comparison with biosorption, bioaccumulation is a growth dependent process (Gupta *et al*., 2000)

When microbial biomass accumulates metals from the solution, the amount of metal ions in a contaminated solution becomes reduced. Transport of the metal across cell membrane yields intracellular accumulation, which is dependent on the cell's metabolism. This means that this process may take place only with viable cells. It is often associated with an active defense system of the microorganism, which reacts in the presence of toxic metal. The transport process may be mediated by the same metabolism used to convey metabolically important ions such as potassium, magnesium and sodium. The metal transport systems may be confused by the presence of heavy metal ions of the same charge and ionic radius associated with essential ions.

Basically, the metal ions which are adsorbed on the cell surface by metabolism-independent process, biosorption, may be transported by the second, metabolism-dependent intracellular intake inside the cell (Huang *et al*., 1990).

1.6. Biotechnological uses of heavy metal resistant bacteria

A major impetus driving research on heavy metal resistant microbes is the biotechnological potential associated with them and their cellular products. Generally, there are many beneficial uses of microbes in the environment, including fixing atmospheric nitrogen and absorbing phosphates for uptake by roots, managing populations of pests' species, protecting animals or plants against infections and pests attack (Cavicchioli and Thomas, 2002). However, heavy metal resistant microbes play significant role in some biotechnological processes where some groups of microbial species are not applicable.

From a biotechnological point of view, there are at least three areas of using heavy metal resistant microbes according to Nies (1999):

- Adding metal resistance to a microorganism to facilitate a biotechnological process which may not be linked to heavy metals,
- Heavy metal resistant bacteria may be used for any kind of bio-mining of expensive metals, directly on ores or by recovering metals from effluents of industrial processes,
- Heavy metal resistant bacteria may be used for bioremediation of metal-contaminated environments.

Microbial processes applied to mining operations have gained increasing interest in the past years (Acevedo, 2000). Other potential and current applications include the mining of gold, copper and other heavy metals, desulphurization of coal and oil and tertiary recovery of oil (Karavaiko, 1985;

Kelley and Tuovinen, 1988; Lawrence and Poulin, 1995; Rawlings, 1997; Brierley and Brierley, 1999).

In addition, enzymes may be needed to break down specific contaminants and such enzymes are encoded by the genes in the microbes. Hence, considerable research is under way to identify and characterize these genes (Cavicchioli and Thomas, 2002). Such genes may be inserted into other microbes and cloned for use in other biotechnological processes (Cavicchioli and Thomas, 2002). Some microbes, in addition to having enzymes involved in detoxification, may have enzymes that may tolerate harsh conditions (temperature, pH, high organic contaminants, etc.) and can be used in other industrial processes.

Heavy metal-resistant bacteria do not only supply enzymes that are active under harsh conditions, but are themselves tools for the evaluation and remediation of heavy metal contaminated environments (Nies, 2000). For example, *Ralstonia* sp. CH34 is a Gram-negative bacterium with a remarkable set of resistance determinants, enabling it to live in environments that are heavily contaminated with toxic metal ions (Nies, 2000). Metal resistance determinants in this bacterium are inducible, hence, their regulatory systems can be used to develop biosensors that measure the biologically important concentrations of heavy metals in an environment (Nies, 2000). Resistance based on metal ion efflux detoxifies only the cytoplasm of the respective cell. Therefore, this resistance mechanism cannot be used directly to develop biotechnological procedures. However, metal ion efflux can protect a cell in a metal-contaminated environment. Thus, the cell can be enabled to mediate biochemical reactions such as precipitation of heavy metals (Nies, 2000).

Finally, the industrial use of metals has led to the pollution in the environment. Consequently, heavy metal removal is a challenge for environmental

management. Since the conventional processes (e.g. ion-exchange, chemical precipitation, etc.) to remove metals from contaminated environments have been found to be sensitive to environmental conditions, heavy-metal resistant microbes can thus play a significant role in cost-effective heavy metal bioremediation.

1.7. Aim and objectives

1.7.1. Aim of the research

Recent studies show that microbes isolated from heavy metal contaminated environments and electroplating effluent-contaminated sludge (Malik, 2004) are excellent candidates for bioremediation. For the purpose of this study, bacterial strains have recently been isolated from various locations in the Consolidated Murchison Antimony Mine, Gravellote, in Limpopo Province. This mine might have in addition to high levels of antimony some arsenic metalloids. Also, there may be other heavy metals present in the earth crust in the Antimony Mine including Cu, Zn, Ni, Cd, and Co, which often form the major component of heavy metal pollution in the environment.

Studying resistance of these bacterial strains to heavy metals as well as testing for their potentials in bioremediation could be of great significance since the results could provide some information on the possible use of these bacterial strains for bioremediation of heavy metals in metal contaminated environments and other industrial purposes.

Hence the aims of this study are to investigate the resistance to some divalent heavy metal ions and the oxyanions of arsenic and antimony by some bacterial strains isolated from an antimony mine and to explore their bioremediation capacities.

1.7.2. Objectives

The specific objectives are:

- To determine the effects of heavy metals on the growth of the bacterial isolates in shake flask cultures.

- To study resistance of bacterial strains to some divalent heavy metal ions (Cu^{2+}, Ni^{2+}, Zn^{2+}, Cd^{2+}, Co^{2+}) and the oxyanions of arsenic and antimony.

- To determine the minimal inhibitory concentrations (MIC) of bacterial strains to heavy metal ions.

- To investigate the bioremediation potentials of the bacteria in heavy metal polluted medium.

Chapter 2

Materials and methods

2.1. Chemicals

Table 2.1. Chemicals used for the experiments in the laboratory

Reagent	**Company**
Agar powder	MERCK, Darmstadt, Germany
Ammonium chloride	SAARCHEM Pty Ltd, Johannesburg, RSA
Cadmium chloride hemi-pentahydrate	SIGMA Chemical Co. St. Louis, MO, USA
Calcium chloride dehydrate	SAARCHEM Pty Ltd, Johannesburg, RSA
Cobaltous chloride	SAARCHEM Pty Ltd, Johannesburg, RSA
Crystal violet	Protea Pharmaceuticals, Johannesburg, RSA
Cupric chloride	SIGMA Chemical Co. St. Louis, MO, USA
D(+) Glucose monohydrate	SAARCHEM Pty Ltd, Johannesburg, RSA
di-sodium hydrogen orthophospate anhydrous	SAARCHEM Pty Ltd, Johannesburg, RSA
Hydrochloric acid	Riedel-deHaën AG, Micor, Johannesburg
Iodine solution	Protea Pharmaceuticals, Johannesburg, RSA
Magnesium chloride	SAARCHEM Pty Ltd, Johannesburg, RSA
Mueller-Hinton agar and Mueller-Hinton broth	OXOID LTD, Basingstoke, Hampshire, England
Nickel chloride	SIGMA Chemical Co. St. Louis, MO, USA
Potassium antimony tartrate	Riedel-deHaën AG, Micor, Johannesburg
Potassium chloride	NT laboratory supplies, Excom, Johannesburg
Safranin O	Protea Pharmaceuticals, Johannesburg, RSA
Sodium arsenate	SIGMA Chemical Co. St. Louis, MO, USA
Sodium arsenite	SIGMA Chemical Co. St. Louis, MO, USA
Sodium chloride	Riedel-deHaën AG, Micor, Johannesburg
Sodium gluconate	SIGMA Chemical Co. St. Louis, MO, USA
Sodium hydroxide granules	Riedel-deHaën AG, Micor, Johannesburg
Sodium sulphate anhydrous	SAARCHEM Pty Ltd, Johannesburg, RSA
TRIS	ICN Medicals, Inc, Aurora, Ohio
Tryptone	SIGMA Chemical Co. St. Louis, MO, USA
Yeast extract	SIGMA Chemical Co. St. Louis, MO, USA
Zinc chloride	SIGMA Chemical Co. St. Louis, MO, USA

2.2. Equipment

The following equipment was used in carrying out experimental work

- Environmental rotary shaker incubator (New Brunswick, New Jersey)
- Spectronic Genesys 5 spectrophotometer (Beckman)
- SpetrAA 110 series (Varian) atomic absorption spectrophotometer
- Cadmium hollow cathode lamp (Varian)
- Nickel hollow cathode lamp (Varian)
- Copper hollow cathode lamp (Varian)
- Biohazard biological safety cabinet
- - 2o^{o}C and - 80^{o}C refrigerators
- Light microscope (Zeiss, Micron), 40X magnification

2.3. Bacterial strains

Six bacterial strains were obtained from Professor Litthauer's laboratory, Department of Biochemistry, University of Free State (UFS). These bacterial strains were isolated from samples collected from different locations in the Murchison Antimony Mine, Gravellote, Limpopo Province (Table 2.2).

2.4. Sample description

Table 2.2. Bacterial cultures isolated from samples collected from various sites in Consolidated Murchison Antimony Mine.

Sample ID No.	Sample collection site
GM 10(1) and GM 10(2)	Silt from North wall in dam 2
GM 14	Biofilm from North in dam 2
GM 15	Water from dam 3 at Danger sign
GM 16	Water and silt from draining hole No. 5 in dam 3
GM 17	Water and sludge from dam 3

2.5. Maintenance of bacterial cultures

Stock bacterial cultures were maintained on tryptone-yeast extract-glucose (TYG) agar plates containing 5 g/l tryptone, 3 g/l yeast extract, 1 g/l glucose and 16 g/l agar, at pH 6.5. Agar medium was supplemented with 100 µM of arsenite. TYG agar medium was sterilized by autoclaving at 121^{o}C for 30 minutes and 20 ml was transferred into the Petri dishes to form nutrient agar plates. A loopful of bacterial cultures from the stock samples was streaked on nutrient agar plates and incubated in an environmental incubator (New Brunswick, New Jersey) at 37^{o}C for 24 h. The cultures were stored refrigerated at - 80^{o}C in glycerol and sub-cultured after every 8 weeks.

2.6. Determination of bacterial morphology by Gram-staining

Bacterial smears were prepared by placing a drop of distilled water in the middle of the microscopic slide and mixed with a loopful of bacterial culture. The bacterial culture was fixed by passing the microscopic slide through a flame. Bacterial smears were then stained with crystal violet, washed off with distilled water after 1 min and flooded with Lugol's iodine for 60 sec. Thereafter the slides were washed off with 70% ethanol to remove crystal violet stain and counter-stained with Safranin O for 30 sec. After drying, the slides were observed under the light microscope. Gram-positive cells incorporated little or no counter-stain and remained blue-violet in appearance and the Gram-negative bacteria took on a pink color.

2.7. Effects of metals on cell growth and resistance

The effects of metals on growth and resistance were determined by growing the bacteria in TYG broth containing 5 g/l tryptone, 3 g/l yeast extract and 1 g/l glucose, at pH 6.5. The cultures were grown in the absence and presence

of different concentrations of divalent metal ions or oxyanions of arsenic and antimony. Analytical grade salts of $Na_2HAsO_4.7H_2O$, $NaAsO_2$, $C_4H_4KO_2Sb.½H_2O$, $ZnCl_2$, $CuCl_2.2H_2O$, $NiCl_2.6H_2O$, $CdCl_2.H_2O$ and $CoCl_2.6H_2O$ were used to prepare 1 M stock solutions to be used in the experiments. Metal solutions were sterilized by 0.2 µm pore-size Millipore sterile filters.

Liquid cultures of the isolates were pre-incubated in a 100 ml metal-deficient TYG broth at 37^oC until they reached mid-exponential growth phase. One milliliter sample of each bacterial culture was transferred into the 100 ml TYG broth supplemented with a range of concentrations (0 – 10 mM) of divalent heavy metal ions (Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+}) and arsenate, arsenite and antimonate oxyanions (0 – 100 mM) in 250 ml Erlenmeyer shake flasks. Inoculated metal-deficient TYG broth was used as a control. The cultures were then incubated on a rotary shaker (New Brunswick, New Jersey) at 37^oC at 200 rpm for 48 h. One milliliter samples were collected at 6 h intervals and growth was monitored by reading the absorbance at 560 nm using Spectronic Genesys 5 spectrophotometer. Similar experiments were performed using Mueller-Hinton (MH) broth.

Growth profiles were determined by using graphs of absorbance versus time. The effects of different concentrations of heavy metal ions on growth were also evaluated. Percentage resistance was determined by expressing the absorbance reading of the cells treated with heavy metal ions as a percentage of the untreated ones at the time the cells were harvested for spectrophotometric measurements.

2.8. Determination of minimal inhibitory concentrations (MICs)

2.8.1. Toxicity testing on TRIS-buffered mineral salt agar medium

Heavy metal ion resistance testing was done by determining the minimal inhibitory concentrations (MICs) of the divalent metals ions (Zn^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+} and Cd^{2+}), initially on TRIS-buffered mineral salt agar medium containing (per liter of deionized water) 2 g sodium gluconate, 6.06 g TRIS-HCl (pH 7.0), 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2. g $MgCl_2.6H_2O$, 0.003 g $CaCl_2.2H_2O$, 0.23 g $Na_2HPO_4.12H_2O$ and 20 g agar (Mergeay, 1985). Analytical grade salts of $ZnCl_2$, $CuCl_2.2H_2O$, $NiCl_2.6H_2O$, $CdCl_2.H_2O$ and $CoCl_2.6H_2O$ were dissolved in deionized water to form 1 M stock solutions, filter sterilized using 0.2 µm pore-size Millipore filters and used to prepare series of different concentrations (0-25 mM) in TRIS-buffered mineral salt agar medium. The medium was adjusted to pH 7.0. Mid-log phase cultures were streaked on agar plates and incubated at 37^{o}C for 72 h. Positive controls consisted of metal-deficient agar medium inoculated with bacterial cultures and negative controls consisted of metal-supplemented agar medium without bacterial cultures. The lowest concentration of heavy metal ion that completely inhibited growth after 72 h was termed as the minimal inhibitory concentration (MIC).

2.8.2. Toxicity testing on Mueller-Hinton (MH) agar medium

Minimal inhibitory concentrations were also determined on Mueller-Hinton (MH) agar medium. MH agar medium was prepared by dissolving 30 g/l of the MH powder in deionized water (Yilmaz, 2003). MH medium was sterilized separately by autoclaving at 121^{o}C for 15 minutes. Analytical grade salts of $ZnCl_2$, $CuCl_2.2H_2O$, $NiCl_2.6H_2O$, $CdCl_2.H_2O$ and $CoCl_2.6H_2O$ were dissolved in deionized water to form 1 M stock solutions and filter-sterilized. MH agar medium was cooled down to 50^{o}C and sterile 1 M stock solutions were used

to prepare different concentrations (0-25 mM) in MH agar medium at pH 7.0. After mixing, the medium was poured in to round flat-bottom plastic Petri dishes to a uniform depth of 4 mm. Bacterial cultures were inoculated on agar plates and incubated at 37^{o}C for 72 h. Positive controls consisted of metal-deficient agar medium inoculated with bacterial cultures and negative ones consisted of metal-supplemented agar medium without bacterial cultures. The lowest concentration of heavy metal ion that completely inhibited growth (MIC) was determined.

2.8.3. Toxicity in Mueller-Hinton (MH) broth

MH broth was prepared by dissolving 21 g/ l of MH powder in deionized water. The medium was sterilized separately by autoclaving for 15 min at 121^{o}C and spiked with different concentrations (0-25 mM) of filter-sterilized divalent metal ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}). Bacterial cultures were pre-incubated in 100 ml metal-deficient MH broth to a mid-log phase and 1 ml sample was transferred into 10 ml MH broth containing metals in test tubes. Positive controls consisted of metal-deficient medium inoculated with bacterial cultures and negative ones consisted of metal-supplemented medium without bacterial cultures. The tubes were incubated on an environmental rotary shake incubator (New Brunswick, New Jersey) at 37^{o}C for 72 h. The lowest concentration of heavy metal ion that completely inhibited growth (MIC) was determined by checking the level of turbidity in the medium.

2.9. Heavy metal accumulation by bacterial cultures

Liquid cultures were pre-incubated in 100 ml of 21 g/l metal-deficient MH broth until they reached mid-log phase and 1 ml bacterial sample was transferred in to 50 ml MH broth supplemented with heavy metal ions (Cu^{2+}, Co^{2+} and Ni^{2+}) at final concentrations of 50 or 100 mg/l in 250 ml Erlenmeyer shake flasks. Cultures were incubated at 37^{o}C on an environmental rotary

shaker incubator (New Brunswick, New Jersey) at 200 rpm. Five ml samples of each culture were collected at 6 h intervals (including the time zero, t_o) for 48 h. The samples were centrifuged for 10 min at 6000 x g using GS-15R centrifuge (Beckman) and the supernatant was used for residual metal analysis by using a SpetrAA 110 series (Varian) atomic absorption spectrophotometer (AAS). The amount of the metal ion removed by the bacteria was determined by the difference between the initial and residual concentrations and the values were expressed as percentages of the controls at the time of the cell harvest. All the experiments were performed in duplicates and the average values were determined.

Chapter 3

Results

3.1. Bacterial growth and resistance in heavy metal-containing media

In this study, six bacterial strains were Gram-stained and the results are shown in table 3.1. Two of the isolates [GM 10(1) and GM 10(2)] were Gram-positive but the others (GM 14, GM 15, GM 16 and GM 17) were found to be Gram-negative. All the isolates except GM 17 were bacterial rods. Preliminary studies on resistance showed that isolates GM 10(1), GM 10(2) and GM 14 were not resistant to the heavy metals (data not shown); hence, subsequent experiments were done using three bacterial strains (GM 15, GM 16 and GM 17). It was observed that at high metal concentrations, microbial growth and resistance decreased in a concentration dependent manner and growth occurred rapidly in the first 12 hours of incubation at lower concentrations.

Table 3.1. Gram-Staining results of GM isolates and their morphology

Sample ID number	Gram-Stain	Morphology
GM 10(1) and GM 10(2)	+	Rod
GM 14	-	Rod
GM 15	-	Rod
GM 16	-	Rod
GM 17	-	Cocci

3.1.1. Effects of AsO_2^-, AsO_4^{3-} and SbO_4^{3-} on bacterial growth in TYG broth

To determine growth of bacteria in the absence and presence of the oxyanions (AsO_2^-, AsO_4^{3-} and SbO_4^{3-}), the bacterial cultures were grown in TYG shake flask cultures at 37^oC for 48 h and growth was determined by reading the absorbance at 560 nm. Growth profiles were determined by using graphs of absorbance versus time.

Fig. 3.1 shows the growth profiles of GM 15 in the absence and presence of different concentrations (0-100 mM) of AsO_2^-, AsO_4^{3-} and SbO_4^{3-}, where the 0 mM served as a control. Growth of GM 15 in the presence of all oxyanions (AsO_2^-, AsO_4^{3-} and SbO_4^{3-}) increased rapidly with time of incubation at low concentrations (0-5 mM), however, at high concentrations (20-100 mM), it was slow. There were longer lag periods of growth, even at low concentrations of SbO_4^{3-}, than AsO_4^{3-}. AsO_2^- was more toxic than AsO_4^{3-}; growth was completely inhibited by AsO_2^- at concentrations above 5 mM (Fig. 3.1c).

Fig. 3.2 and 3.3. show the growth curves of GM 16 and GM 17 respectively in the absence and presence of different concentrations (0-100 mM) of AsO_2^-, AsO_4^{3-} and SbO_4^{3-}. The growth patterns of GM 16 and GM 17 were similar. The isolates grew rapidly at low concentrations of AsO_4^{3-} and SbO_4^{3-} in the early hours (0-3 h) of incubation and the maximum growth was reached after 12 hours of incubation. However, at 20 to 100 mM of AsO_4^{3-} , longer lag phases of growth were observed. Both isolates showed concentration dependent inhibition of growth in the presence of AsO_4^{3-} and SbO_4^{3-}. The results also showed that AsO_2^- was more toxic to both isolates than AsO_4^{3-} since growth at concentrations above 5 mM was completely inhibited (Fig. 3.2c and Fig. 3.3c).

3.1.2. Bacterial resistances to AsO_2^-, AsO_4^{3-} and SbO_4^{3-} in TYG broth

To determine the resistance to AsO_2^-, AsO_4^{3-} and SbO_4^{3-}, GM isolates were cultured in TYG media in Erlenmeyer shake flasks at 37^{o}C and pH 6.5 in the presence of different concentrations (0-100 mM) of AsO_2^-, AsO_4^{3-} and SbO_4^{3-}. Percentage resistance was determined by expressing the absorbance reading of the cells treated with heavy metal ions as a percentage of the untreated ones at the time the cells were harvested, and graphs of % resistance versus concentrations of metals were plotted. Bacterial isolates were highly resistant at low concentrations and less resistant when the concentrations of metals were increased.

Fig. 3.4 shows the % resistances of the GM isolates in TYG broth containing AsO_2^-, AsO_4^{3-} and SbO_4^{3-} at 37^{o}C for 48 h. The bacterial strains studied were more resistant to high concentrations of AsO_4^{3-} than AsO_2^- and SbO_4^{3-}. The isolates were resistant up to 100 mM of AsO_4^{3-} and 20 mM of SbO_4^{3-}. The results showed that the most resistant isolate to AsO_4^{3-} was GM 16 and the least resistant was GM 15. For example, GM 16, GM 17 and GM 15 showed 90 %, 60 % and 58 % resistance respectively to 80 mM of AsO_4^{3-} after 48 h (Fig. 3.4a).

The results also showed that AsO_2^- was more toxic than AsO_4^{3-} and SbO_4^{3-} to the bacterial cells. Generally, the results have shown that all the isolates were less resistant to high concentrations of the oxyanions. The order of resistance ranged as $AsO_4^{3-} > SbO_4^{3-} > AsO_2^-$.

3.1.3. Effects of divalent metal ions on bacterial growth in TYG broth

Growth of bacterial strains in the absence and presence of different concentrations (0-10 mM) of divalent metals ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}) was initially determined in TYG shake flask cultures at 37^{o}C for 48 h. Growth was monitored by reading the absorbance at 560 nm. Generally, the growth profiles of GM 16 and GM 17 were similar in TYG medium but GM 15 showed a different growth pattern. The two isolates (GM 16 and GM 17), grew well at all metal concentrations up to 1 mM but GM 15 could only tolerate very low concentrations (up to 0.5 mM).

When GM 15 was grown in TYG medium containing different concentrations of Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}, its growth was slow during the first 3 h of incubation at all concentrations (0-10 mM) of Zn^{2+}, Ni^{2+}, Co^{2+} and Cd^{2+} (Fig. 3.5a, b, c and e), however, growth increased with an increase in time of incubation at low concentrations (0-1 mM) of Ni^{2+}, Co^{2+} and Cu^{2+}. At high concentrations (5-10 mM) of Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}, growth was inhibited. Cd^{2+} was the most toxic metal to GM 15 since growth was completely inhibited at concentrations above 0.1 mM. (Fig. 3.5e). In the case of GM 16 and GM 17, both isolates grew rapidly within the first 12 h of incubation at low concentrations (0-1 mM) of Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+} (Figs. 3.6 and 3.7). However, at high concentrations (5-10 mM) of Zn^{2+}, they grew slowly within the first 12 h of incubation but the growth rate increased thereafter. High concentrations (5-10 mM) of Ni^{2+}, Co^{2+} and Cu^{2+} inhibited growth of GM 16 and GM 17. The toxic level of Cd^{2+} in GM 16 and GM 17 was lower than what was observed in GM 15 cultures. The inhibitory concentration in GM 15 was 0.5 mM compared to 1.0 mM in GM 16 and GM 17 (Figs. 3.6e and 3.7e).

3.1.4. Bacterial resistances to divalent metal ions in TYG broth

To determine the resistance of the isolates to divalent metals, the bacterial cells were cultured in TYG media in Erlenmeyer shake flasks at 37^{o}C and pH 6.5 in the presence of different concentrations (0-10 mM) of Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}. Percentage resistance was determined by expressing the absorbance reading of the cells treated with heavy metal ions as a percentage of the untreated ones at the time the cells were harvested, and graphs of % resistance versus concentrations of metals were plotted. Fig. 3.8 shows resistance patterns of the bacterial isolates (GM 15, GM 16 and GM 17) in the TYG broth. The isolates showed different degrees of resistance to the heavy metals. All the isolates showed over 80 % resistance to Zn^{2+}, Ni^{2+} and Cu^{2+} at low concentrations (0-1 mM) when grown for 48 h. In the presence of Co^{2+}, only GM 16 showed over 80 % resistance at concentrations up to 1 mM; GM 15 and GM 17 reached the same levels of resistance at 0.5 mM Co^{2+} (Fig. 3.8c). Compared with the other metal ion, Cd^{2+} was the most toxic metal to all the isolates. Above 0.5 mM Cd^{2+}, GM 15 and GM 16 were not resistant (Fig. 3.8e) and GM 15 could only tolerate up to 0.1 mM Cd^{2+} when only 40 % resistance was observed.

The results suggest that in TYG medium all the isolates were most resistant to Zn^{2+}, Ni^{2+} and Cu^{2+}, moderately resistant to Co^{2+} and least resistant to Cd^{2+}. It was interesting to observe that the resistance patterns of the isolates in the presence of Zn^{2+}, Ni^{2+} and Cu^{2+} were similar (Fig.3.8a, b and d). Although Cd^{2+} was the most toxic metal to the isolates, GM 16 and GM 17 showed over 70 % resistance at 0.5 mM above which growth was completely inhibited.

3.2. Bacterial growth and resistance to heavy metal ions in MH broth

3.2.1. Effects of divalent metal ions on bacterial growth in MH broth

Since most MIC determinations are done in MH agar or broth, (Yilmaz, 2003), it was necessary to determine the growth profiles of the isolates using this medium. The bacterial cultures were therefore, grown in MH broth supplemented with different concentrations (0-10 mM) of divalent metal ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}). Growth profiles of GM isolates are shown in figures 3.9-3.11. Zn^{2+} appeared to be the least toxic of the metals to GM isolates since growth was observed at all concentrations up to 10 mM. All of the GM isolates followed similar growth pattern in the presence of Zn^{2+}, however, 10 mM inhibited growth of GM 15 after 42 h (Figs. 3.9a, 3.10a and 3.11a). Growth increased rapidly at all concentrations (0-10 mM) of Zn^{2+} within 12 h, reached the maximum level at 12 h and remained stationary thereafter.

The isolates grew in the media containing 5 mM of Ni^{2+} and Cu^{2+} while in the presence of Co^{2+} and Cd^{2+}, they grew at concentrations up to 1 mM (Figs. 3.9-3.11). This indicates that Co^{2+} and Cd^{2+} were toxic to the GM isolates. For GM 15 and GM 17, similar growth patterns were observed in Cu^{2+} and Cd^{2+}-containing MH media, however, growth was inhibited at concentrations above 1 mM in the presence of Cd^{2+} (Fig. 3.9d; e and 3.11d; e). GM 16 also showed similar growth patterns in Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}-containing MH media, but Co^{2+} inhibited growth at concentrations above 1 mM (Fig. 3.10).

3.2.2. Bacterial resistances to divalent metal ions in MH broth

Resistances of the GM isolates to divalent metals were also evaluated in MH broth (pH, 7.0) at 37^{o}C for 48 h. Percentage resistance was determined by expressing the absorbance reading of the cells treated with heavy metal ions as a percentage of the untreated ones at the time the cells were harvested, and graphs of % resistance versus concentrations of metals were plotted. All the isolates showed higher resistances in MH broth than in TYG medium. Generally, the isolates were resistant up to 5 mM of the divalent metals in MH broth (Fig. 3.12) but in TYG broth the highest concentration showing maximum resistance was 1 mM after 48 h. At a high concentration (10 mM) of Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}, the isolates were not resistant (Fig. 3.12b, c, d and e).

GM 16 and GM 17 were found to be the most resistant organisms to Zn^{2+} and Cu^{2+}-containing media after 48 h (Fig. 3.12a; d). They showed about 80 % resistance and GM 15 showed about 65 % resistance to 5 mM Zn^{2+} when grown for 48 h. In the presence of Cu^{2+}, GM 17 showed 38 % resistance, GM 16 over 80 % and the least resistant organism was GM 15 showing less than 10 % resistance at 5 mM. However, in Ni^{2+} and Cd^{2+}-containing media, GM 15 was the most resistant bacteria with over 50 % resistance at 5 mM after 48 h.

Co^{2+} was found to be the most toxic element to the GM isolates in the MH broth. At 5 mM, GM 15 and GM 16 were not resistant and only about 30 % resistance was observed for GM 17 after 48 h (Fig. 3.12c).

3.3. Heavy metal tolerance in GM isolates

3.3.1. Toxicity of divalent metal ions in solid media

Toxicity effects of the divalent metals were done by determining the minimal inhibitory concentrations (MICs) of the divalent metal ions during growth of the GM isolates cultured on TRIS-buffered mineral salt agar (TMA) medium (Mergeay, 1985) and Mueller-Hinton agar (MHA) (Yilmaz, 2003). Bacterial cultures were grown at a series of increasing concentrations (0-30 mM) of divalent heavy metal ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}). The lowest concentration that completely inhibited the growth of bacterial isolates was termed the minimal inhibitory concentration (MIC). MIC values were determined after the incubation of the cultures at 37^{o}C for 72 h. MICs of different metals in agar media are presented in Table 3.2.

Generally, the MICs in MHA were much higher than in TMA for all the isolates (Table 3.2). Zn^{2+} was the most tolerated of all the metals by the GM isolates in TMA medium. Of all the isolates, GM 17 was the most tolerant isolate to Zn^{2+} in both TMA and MHA (Table 3.2). In TMA, Cu^{2+} and Cd^{2+} were the most toxic elements to all the GM isolates. MICs of 0.02 mM (Cu^{2+} and Cd^{2+}) were observed for GM 15, whereas GM 16 and GM 17 showed the MIC of 0.5 mM Cu^{2+}. Ni^{2+} and Co^{2+} showed the intermediate results in TMA for all the GM isolates. Ni^{2+} was the most tolerated metal by GM 15 and GM 16 in MHA (Table 3.2).

GM 15 was the least tolerant of all the GM isolates in TMA; however, there was little difference between the levels of tolerance by the isolates to Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+} in MHA.

Table 3.2. Minimal inhibitory concentrations (MICs) in mM of the divalent metal ions during growth of GM isolates cultured on TRIS-buffered mineral salt agar medium (TMA) and Mueller-Hinton agar (MHA).

Metal ion	GM 15		GM 16		GM 17	
	TMA	MHA	TMA	MHA	TMA	MHA
Zn^{2+}	1.0	10	10.0	8	12.0	20
Ni^{2+}	0.2	12	5.0	12	4.0	8
Co^{2+}	0.1	5	1.8	5	1.8	5
Cu^{2+}	0.02	8	0.5	5	0.5	8
Cd^{2+}	0.02	8	1.2	8	1.2	8

3.3.2. MICs of heavy metal ions in Mueller-Hinton broth (MHB) and Mueller-Hinton agar (MHA)

Toxicity testing in liquid medium was conducted to determine the precise concentrations of metals at which the bacterial cultures could grow. The toxicity results assisted in designing the biosorption experiments, where the concentration of the metal that allowed growth of bacterial cultures was used for the biosorption experiments. Table 3.3 shows the MICs of the divalent metals in mM during growth of bacterial isolates in MHA and MHB. Generally, the MIC values were found to be higher in MHA than in MHB where the conditions of diffusion, complexation and availability of metals were different from those in solid medium. Co^{2+} and Cd^{2+} were found to be the most toxic elements to GM 15 in MHB.

Zinc toxicity

Zn^{2+} appeared to be tolerated by all the GM isolates and the MICs were in the range of 8-20 mM. High MICs were observed in MHA for both GM 15 and GM 17; however, similar MICs were observed in MHA and MHB for GM 16. GM 17 was the most tolerant of all the organisms with MIC of 20 mM in MHA.

Nickel toxicity

All the GM isolates tolerated Ni^{2+} with the MIC values ranging between 5-12 mM (Table 3.3). GM 15 and GM 16 showed the same level of tolerance to Ni^{2+} in MHA with the MIC of 12 mM. GM 16 showed the same MICs in both MHA and MHB and the same was true for GM 17. GM 15 was the least tolerant in MHB with MIC of 5 mM.

Cobalt toxicity

Co^{2+} was the least tolerated metal by both GM 16 and GM 17 in MHA, however, GM 15 was the least tolerant to Co^{2+} in MHB. High MICs were observed in MHB than MHA for both GM 16 and GM 17 (Table 3.3). All the isolates showed the same level (5 mM) of tolerance to Co^{2+} in MHA.

Copper toxicity

Cu^{2+} was well tolerated by all the isolates with GM 16 being the least tolerant in MHA (Table 3.3). No major differences were observed between the MICs in MHA and MHB for both GM 16 and GM 17, however, a higher MIC in MHA than in MHB was observed for GM 15.

Cadmium toxicity

Cd^{2+} was highly toxic to GM 15 in MHB with the MIC of 1.0 mM (Table 3.3). The same level (8 mM) of tolerance was observed in MHA for all the isolates. MICs in MHA were higher than in MHB.

Table 3.3. Minimal inhibitory concentrations (MICs) in mM of the divalent metal ions during growth of GM isolates cultured in Mueller-Hinton agar (MHA) medium and MH broth (MHB).

Metal ion	GM 15		GM 16		GM 17	
	MHA	MHB	MHA	MHB	MHA	MHB
Zn^{2+}	10	8	8	8	20	8
Ni^{2+}	12	5	12	12	8	8
Co^{2+}	5	2	5	10	5	8
Cu^{2+}	8	6	5	6	8	8
Cd^{2+}	8	1.0	8	6	8	6

3.3.3. Order of toxicity of divalent metal ions in TMA, MHA and MHB

The order of toxicity of the divalent metal ions to the GM isolates is shown in Table 3.4. The results suggest that toxicity of the heavy metal ions to the bacterial isolates depended on the type of medium used. Zn^{2+} was the least toxic of all the metals and Cu^{2+} was found to be the most toxic to the GM isolates in TMA. The order of toxicity was similar for both GM 16 and GM 17 in TMA. When MHA was used, the order of toxicity was different in MHA for all the isolates; Ni^{2+} was the least toxic to both GM 15 and GM 16 whereas Zn^{2+} was the least toxic to GM 17 in MHA. Co^{2+} was the most toxic metal to the GM isolates.

The toxicity study of the metal ions to the GM isolates in MHB showed that Zn^{2+} was the least toxic to GM 15, whereas Ni^{2+} was the least toxic to GM 16. The toxic levels of the different divalent metals were similar in MHB in case of GM 17.

Table 3.4. The increasing order of toxicity of the divalent metal ions to the GM isolates in TRIS-buffered mineral salt agar medium (TMA), Mueller-Hinton agar (MHA and Mueller-Hinton broth (MHB).

GM isolates	TMA	MHA	MHB
GM 15	$Zn^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+} = Cu^{2+}$	$Ni^{2+} > Zn^{2+} > Cd^{2+} = Cu^{2+} > Co^{2+}$	$Zn^{2+} > Cu^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+}$
GM 16	$Zn^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+} > Cu^{2+}$	$Ni^{2+} > Zn^{2+} = Cd^{2+} > Cu^{2+} = Co^{2+}$	$Ni^{2+} > Co^{2+} > Zn^{2+} > Cu^{2+} = Cd^{2+}$
GM 17	$Zn^{2+} > Ni^{2+} > Co^{2+} > Cd^{2+} > Cu^{2+}$	$Zn^{2+} > Ni^{2+} = Cu^{2+} = Cd^{2+} > Co^{2+}$	$Zn^{2+} = Ni^{2+} = Cu^{2+} = Co^{2+} = Cd^{2+}$

3.4. Bioremediation capacities of the GM isolates

The biosorption capacities of the GM isolates were investigated during the course of growth of the bacterial cells in MH broth at 37^{o}C in the presence of 50 mg/l or 100 mg/l of the heavy metal ions (Ni^{2+}, Cu^{2+} and Cd^{2+}). At various time intervals, the cells were harvested by centrifugation and the residual metal concentration in the supernatant was determined by atomic absorption spectroscopy (AAS).

Generally, the biosorption results suggested that the capacities of the isolates to remove the metals from the media depended on the initial metal concentration. Fig. 3.13 shows growth and biosorption profiles of GM 15 in the presence of divalent metals. This isolate was incapable of removing Cd^{2+} since its growth was completely inhibited when the initial metal concentration in the medium was either 50 mg/l or 100 mg/l (Fig. 3.13a; b). Although GM 15 cells grew well in the presence of 50 mg/l or 100 mg/l Cu^{2+}, the biosorptive capacity was low. Only 21 % of Cu^{2+} was removed after 48 h when the initial

concentration was 100 mg/l (Fig. 3.13c), but at an initial concentration of 50 mg/l, the amount of Cu^{2+} removed was negligible. However, the GM 15 cells were able to grow well in the presence of Ni^{2+} and could remove 44 % of Ni^{2+} after 48 h when the initial concentration was 50 mg/l whereas only 20 % of Ni^{2+} was removed after 30 h when the initial concentration was 100 mg/l (Fig. 3.13e; f).

When GM 16 was grown in the presence of the metal ions, the cells did not have the capacity to remove Ni^{2+} from the media although they grew very well either in the presence of 50 mg/l or 100 mg/l of Ni^{2+} (Fig. 3.14e; f). However, in the presence of Cu^{2+}, GM 16 cells grew well and were capable of removing 65 % after 36 h when the initial concentration was 100 mg/l. In fact, 30 % of Cu^{2+} was removed within the first 6 h (Fig. 3.14c). Surprisingly, at an initial concentration of 50 mg/l, Cu^{2+} removal by the GM 16 cells was negligible, although there was sufficient cell growth.

The results in Fig. 3.14a and b also showed that the GM 16 cells were moderately efficient in removing Cd^{2+} from the culture media. After 48 h, 48 % and 19 % of Cd^{2+} were removed by the cells when the initial concentrations were 100 mg/l and 50 mg/l respectively.

Fig. 3.15 shows the growth profiles and the biosorption capacities of GM 17 isolate in the presence of Cd^{2+}, Cu^{2+} and Ni^{2+}. Similar to the biosorption capacity of GM 16, Ni^{2+} removal by GM 17 cells was negligible although there was good growth of the cells (Fig. 3.15e; f). However, GM 17 cells were able to remove 25 % of the initial 100 mg/l after 36 h whereas only 18 % was removed after 48 h when the initial concentration was 50 mg/l (Fig. 3.15a; b). Cd^{2+} removal occurred mostly during the exponential growth phase and 27 % was removed after 12 h when the initial concentration was 100 mg/l (Fig. 3.15a), but only 18 % was removed when the initial concentration was 50 mg/l. Compared to GM 16, the removal of Cu^{2+} by GM 17 was less in the

medium containing initial concentration of 100 mg/l. GM 17 cells removed 25 % (Fig. 3.15c) compared with 65 % by GM 16 cells (Fig. 3.14c). At initial concentration of 50 mg/l, the amount of Cu^{2+} removed was negligible (Fig.3.15d).

A summary of the maximum biosorption capacities of the three GM isolates in the presence of the metal ions (Cu^{2+}, Cd^{2+} and Ni^{2+}) are presented in Table 3.5 and Table 3.6. The results showed that GM 16 had the high capacity for removal of Cu^{2+} and Cd^{2+} (65 % and 48 % respectively) at initial concentration of 100 mg/l, and only GM 15 had the capacity to remove 44 % of Ni^{2+} at initial concentration of 50 mg/l.

Table 3.5. Maximum biosorption capacities of the GM isolates in MH broth when the initial concentration of the metal ion was 100 mg/l.

GM isolates	% Cu^{2+} biosorbed	Time (h)	% Cd^{2+} biosorbed	Time (h)	% Ni^{2+} biosorbed	Time (h)
GM 15	21.4	48	1.5	30	20.0	30
GM 16	65.0	36	48.0	48	ND	NA
GM 17	25.0	36	27.3	12	ND	NA

ND – Not detected

NA – Not applicable

Table 3.6. Maximum biosorption capacities of GM isolates in MH broth when the initial concentration of the metal ion was 50 mg/l.

GM isolates	% Cu^{2+} biosorbed	Time (h)	% Cd^{2+} biosorbed	Time (h)	% Ni^{2+} biosorbed	Time (h)
GM 15	7.3	30	13.0	48	44.3	48
GM 16	2.6	12	19.1	48	ND	NA
GM 17	6.7	18	17.6	18	ND	NA

ND – Not detected

NA – Not applicable

Chapter 4

Discussion

4.1. Bacterial resistances to As and Sb oxyanions

The three bacterial isolates (GM 15, GM 16 and GM 17) used in the present study, were isolated from samples collected from an antimony mine and were aerobic and identified as Gram-negative microbes. The isolates were investigated for their resistance to antimony and arsenic oxyanions and some divalent heavy metal ions (Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+}). They were also tested for their ability to biosorb/accumulate metals from aqueous solutions.

Growth of all the GM isolates decreased in a concentration dependent manner in TYG media containing AsO_4^{3-} and SbO_4^{3-} (Fig. 3.1a; b). The bacteria grew rapidly at low concentrations (up to 10 mM), but growth was inhibited at high concentrations (above 10 mM). Arsenate was less toxic to all the isolates than arsenite. GM 16 was the most resistant of all the isolates in AsO_4^{3-} containing media (Fig. 3.4a). It showed 90 % resistance to 80 mM of AsO_4^{3-} after 48 h, followed by GM 17 which showed 60 % resistance after 48 h. GM 15 was the least resistant isolates showing 58 % resistance to 80 mM AsO_4^{3-} (Fig. 3.4a). All the GM isolates were resistant up to 20 mM of SbO_4^{3-} and the most resistant isolate to SbO_4^{3-} was GM 15 showing 90 % resistance while GM 16 was 80 % resistant to 20 mM of SbO_4^{3-} after 48 h. Isolate GM17 was the least resistant of all the GM isolates to SbO_4^{3-}. It showed only 45 % resistance to 20 mM of SbO_4^{3-} (Fig. 3.4b).

The results also demonstrated that AsO_2^- was more toxic than AsO_4^{3-} and SbO_4^{3-} to the bacterial cells. All the isolates showed over 80 % resistance to 5 mM of AsO_2^- whereas at 20 mM, the resistance was less than 30 % after 48 h. Generally, the results have shown that all the isolates were less resistant to

high concentrations (10 - 100 mM) of the oxyanions. The order of tolerance ranged as AsO_2^- > SbO_4^{3-} > AsO_4^{3-}.

In general, toxicity of arsenic and antimony is dependent on their oxidation states: trivalent arsenic and antimony forms are known to be more toxic than the pentavalent derivatives (Cervantes, 1994). Arsenate acts as a phosphate analog because of the similarity in their structures, and arsenate competes with phosphate in the formation of ATP (Coddington 1986). Arsenate can be enzymatically added to an ADP to form an ADP-arsenate molecule. Unlike ATP, this ADP-arsenate complex is spontaneously hydrolyzed in the absence of enzymes, and the energy released will not be captured by a hydrolyzing enzyme (Coddington 1986). Arsenate is, therefore, an uncoupler because it results in a futile cycle whereby the cell continues to exert energy in synthesizing this ADP-arsenate complex but cannot store the energy for future use.

Hallberg *et al*., (1996) reported that arsenite was at least 10 times more toxic than arsenate to *Thiobacillus caldus* and it decreased its growth and viability. The toxicity of arsenite lies in its ability to bind to the sulfur groups of essential cysteines in proteins (Nies, 1999). Sulphur groups can be important in maintaining the three dimensional structure of proteins which affect the function of proteins and enzyme-substrate interactions. Because arsenite binds to two sulfur groups, it can cross-link proteins, distorting their overall shape and impeding their function (Coddington, 1986). Furthermore, it has been reported that, early microorganisms have not evolved detoxification mechanisms to cope with arsenite, but cope with arsenate (Rosen, 2002). Also, there are no arsenate-specific efflux systems; hence, cytosolic arsenite accumulates as a result of arsenate reduction (Nies, 1999; Rensing 1999) and from uptake by aquaglyceroporin (Rosen 2002). Thus the mechanisms to cope with arsenate evolved to use existing arsenite extrusion systems (Rosen, 2002).

Based on the resistance results, the GM isolates showed high resistance to arsenate even at high concentrations which indicated that all the isolates could survive in arsenate contaminated environments; hence, they could further be investigated for the possibility of being used in bioremediation of arsenate. Surprisingly the resistances of the isolates to arsenate were higher even at 80 mM than in antimonate containing media, in which less than 30 % resistance was observed at 80 mM. This suggests that the ability of the GM isolates to survive and remove antimonate from a contaminated site could be less than that of arsenate. Since arsenite was more toxic to the isolates than the other oxyanions, the chances of their survival in arsenite contaminated environment could be less. Therefore it is difficult to predict their potentials for the bioremoval of arsenite.

4.2. Resistance of the GM isolates to the divalent metal ions

In order to asses the bioremediation potentials of the isolates, it was also necessary to evaluate their resistance profiles and the minimal inhibitory concentrations (MICs). Resistance of the isolates was determined in both TYG and MH media. The resistance patterns shown by the three isolates to the heavy metal ions and the degree of resistance differed among them. However, the resistances were generally higher in MH than TYG media. Furthermore, the MICs were higher in agar media than the broth. For example, whereas GM 15 isolates could tolerate up to 5 mM of the divalent metal ions in MH media, they could only survive at concentrations below 1 mM in TYG media. Similar trends of resistance were shown by GM 16 and GM 17. The resistance results suggest that all the GM isolates could be considered for the bioremediation of Zn^{2+} contaminated environments.

The bacterial isolates were also found to be less tolerant to Cu^{2+} in TMA. Copper toxicity is based on the production of hydroperoxide radicals

(Rodriguez Montelongo *et al*., 1993) and its radical character makes Cu^{2+} very toxic (Nies, 1999), and many organisms are reported to be sensitive to copper (Gordon *et al*., 1994). Also, the nature of growth media, pH and temperature of incubation could affect the metal tolerance (Malik, 2004).

Generally, all the GM isolates were found to be resistant to high concentrations of Zn^{2+} (up to 10 mM) which indicated that these bacterial isolates could survive in zinc polluted environments. Zn plays a vital role in cells as a component of a variety of enzymes and DNA-binding proteins, such as zinc-finger protein which also exists in bacteria (Chou *et al*., 1998). Zn^{2+} could be readily removed from the medium by bacteria for use inside the cells. However, when the metal ion reaches toxic levels, bacterial cells use detoxification mechanisms to maintain the homeostatic levels of the metal ion (Nies, 1999).

The levels of tolerance of the GM isolates to Zn^{2+}, Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+} were higher than those reported for *E. coli* in TRIS-buffered mineral salt medium. The MICs of the GM isolates vary among the metals whereas the values reported for *E. coli* by Nies (1999) showed similar MICs for Zn^{2+}, Ni^{2+}, Co^{2+} and Cu^{2+}. These observations suggest that the toxicity of the metal ions to the bacterial isolates were dependent on the bacterial strains. Also, the bacterial isolates were more tolerant to the metal ions in MHA than TMA which suggests that the bacterial tolerance to the metal ions was dependent on the type of medium used. Hassen *et al*., (1998) also reported that some media support better growth of microbes than the others.

The minimal inhibitory concentrations of heavy metal ions were also determined in liquid media for the bacterial strains in order to establish baseline levels of resistance against which to compare values determined on both the agar and in broth media, and with the other bacterial species. Generally, the MIC values were found to be higher in solid medium (MHA)

than in liquid medium (MHB) (Table 3.3). Toxicity testing in liquid medium allows a good evaluation of metal toxicity in polluted environments, such as industrial effluents and sewage sludge leachates (Hassen *et al*., 1998). Liquid medium toxicity testing is different from toxicity testing on solid medium, where the conditions of diffusion, complexation and availability of metals are different from those in solid medium. Hassen *et al*., (1998) also tested the levels of tolerance of environmental bacteria to the different divalent metal ions including Cu^{2+}, Co^{2+}, Cd^{2+} and Zn^{2+} in nutrient broth and reported that the test in liquid media was sensitive at concentrations 10 to 1000 times lower than those obtained in solid media.

4.3. Biosorption of Cd^{2+}, Cu^{2+} and Ni^{2+} by GM isolates

The biosorption capacities of the bacterial isolates were assessed in MH broth containing 50 mg/l and 100 mg/l of Ni^{2+}, Cu^{2+} and Cd^{2+}. The biosorption of Zn^{2+} and Co^{2+} could not be investigated due to the unavailability of the hollow cathode lamps for these metal ions. The inability of GM 15 to remove Cd^{2+} from the medium could be due to the toxicity of Cd^{2+} to this isolate. Its growth was completely inhibited in MH broth when the initial concentrations of Cd^{2+} were 50 mg/l or 100 mg/l. GM 16 and GM 17 grew very well in Cd^{2+}-containing media, where GM 16 was capable of removing 48 % and 19 % Cd^{2+} when the initial metal concentrations were 100 mg/l and 50 mg/l respectively. However, GM 17 only removed 25 % of the initial 100 mg/l after 36 h whereas only 18 % was removed after 48 h when the initial concentration was 50 mg/l. These observations suggest that GM 16 was able to remove Cd^{2+} better than GM 15 or GM 17.

The greater portion of Cd^{2+} was removed even when the cells seemed to be in the stationary phase and when the initial concentrations were 50 mg/l or 100 mg/l indicating that Cd^{2+} biosorption was independent of the growth cycle of the cells. However, most of Cd^{2+} biosorption by GM 17 occurred in the

active growth phase. Malik (2004), reported that metabolically active cells from exponential growth phase probably contain highly active enzymes, some of which may be involved in complexing and binding metal ions. Metal ion uptake in yeasts, for example, is also known to involve an initial rapid biosorption of metal ions to negatively charged sites on the cell wall (Brady *et al*., 1994; Krauter *et al*., 1996), followed by a slower, energy-dependent entry in to the cell (Malik, 2004). Hence, GM 16 and GM 17 might have different mechanisms for Cd^{2+} removal from the contaminated medium.

The biosorptive capacities of all the GM isolates were higher when the initial Cu^{2+} concentration in the medium was 100 mg/l than when initial concentration was 50 mg/l. The amounts of Cu^{2+} biosorbed by GM 15, GM 16 and GM 17 were negligible in medium containing 50 mg/l initial concentration. There were also considerable differences in Cu^{2+} uptake capacities of the three isolates when the initial concentration was 100 mg/l. GM 16 could remove 65 % Cu^{2+}, but only 25 % and 21 % Cu^{2+} were removed by GM 17 and GM 15 respectively. These observations showed that GM 16 was the most effective isolate for the removal of Cu^{2+} ions from the aqueous solution. Several other bacteria have been tested for bioaccumulation of Cu^{2+} under growing conditions. Öztürk *et al*., (2004) observed that the initial metal ion concentration remarkably influenced the equilibrium metal uptake and adsorption yield of Cu^{2+} by *Streptomyces coelicolor* A3(2). The higher the initial concentration of Cu^{2+}, the larger was the amount of the metal ion adsorbed.

There are conflicting reports on whether cell metabolism is essential for Ni uptake (Malik, 2004). The amount of Ni^{2+} ion biosorbed by GM 15 increased slowly from the first hour of incubation to 12 h. The metal uptake increased further after 24 h to 48 h which indicated a biphasic mode of Ni^{2+} accumulation. The isolate was able to accumulate a maximum of 48 % Ni^{2+} after 48 h when the initial concentration of Ni^{2+} was 50 mg/l in a medium, but

the accumulation was negligible when the initial concentration was 100 mg/l. Several studies have indicated that Ni^{2+} biosorption is complex. For example, strains of *Pseudomonas* spp. (*P. fluorescens 4F39* and *P. stutzeri*) have often been used for biosorption of Ni^{2+} (Lopez *et al*., 2000; Ramteke, 2000; Malik, 2004), where Ni^{2+} was biosorbed rapidly on cell surfaces. Öztürk *et al*., (2004) found that the uptake of Ni^{2+} was a very rapid metabolism-independent passive binding process. The Ni^{2+} uptake by *Streptomyces coelicolor* A3(2) took place within 5 min of incubation (Öztürk *et al*., 2004). Akhtar *et al*., (2004) also observed that the biosorption of Ni^{2+} by immobilized and free cells of *Chlorella sorokiniana* was extremely rapid reaching equilibrium in 15 and 20 min respectively. Probably, in the present study, Ni^{2+} biosorption might have occurred rapidly in the initial period of incubation, but thereafter was extruded into the medium by an unknown mechanism. Ni^{2+} biosorption therefore, needs further investigation to find the actual mechanism involved by this GM 15 isolate.

Isolates GM 16 and GM 17 were highly tolerant to Ni^{2+} (Figs. 3.14e; f and 3.15e; f), however, they were totally incapable of removing nickel from the aqueous solutions, which might indicate that GM 16 and GM 17 have low binding capacity for Ni^{2+} and the mechanism of resistance to Ni^{2+} is unlikely to involve simple adsorption or uptake of the metal. Many metal tolerant microbes have been reported to have relatively low Ni^{2+} binding capacities (Chan *et al*., 1991; Holan and Volesky, 1994; Basnakova and Mackaskie, 1997; Williams *et al*., 1998; Deng *et al*., 2003; Malik, 2004). Tsezos *et al*., (1996) found that the biosorption of Ni^{2+} by different strains of microorganisms was less than that of the other metals. Similarly, Yilmaz (2003) found that *Bacillus circulans* strain EB1 which was capable of removing some metal ions, did not efficiently uptake Ni. This is probably due to the intrinsic chemical properties of nickel ions leading to the steric hindrance of biosorption (Tsezos *et al*., 1995; Deng *et al*., 2003). Furthermore, the biosorption process is sensitive to ambient conditions, such as pH, ionic

strength and the presence of other organic and inorganic components (Chang and Hong, 1994; Dömnez, *et al*., 1999; Deng *et al*., 2003; Akhtar *et al*., 2004; Öztürk *et al*., 2004). Possibly these isolates might have developed mechanisms other than biosorption/bioaccumulation in order to survive in the presence of high concentration of Ni^{2+}.

4.4. Summary and conclusions

The three bacterial isolates (GM 15, GM 16 and GM 17) showed varying degrees of resistance to the different metalloids (arsenic and antimony). Isolate GM 16 was the most resistant to AsO_4^{3-}, whereas isolates GM 15 and GM 17 showed the same level of resistance after 48 h of incubation. Isolates GM 16 and GM 17 showed the same level of resistance whereas GM 15 was the least resistant to SbO_4^{3-}. AsO_2^- was the most toxic oxyanions to the GM isolates, however, isolate GM 15 was the most resistant of all the isolates to AsO_2^-. The toxicity of the oxyanions was concentration dependent.

All the isolates were resistant to high concentrations (10 mM) of Zn^{2+}, but they could only resist up to 1 mM of Ni^{2+}, Co^{2+}, Cu^{2+} and Cd^{2+} in TYG medium. The levels of resistance of the isolates to the different metal ions were higher in MH medium than TYG medim. From the toxicity studies, the bacterial tolerance was dependent on the type of medium used. It was observed that the MIC values were higher in MH medium than in TRIS-buffered mineral salt medium. Also, the MICs were found to be higher on a solid medium than in liquid medium. Zn^{2+} was found to be the most tolerated metal ion whereas Cu^{2+} appeared to be highly toxic to the GM isolates in TMA. However in MH medium, Co^{2+} was toxic to the isolates.

The results on biosorption studies demonstrated that GM 16 was most effective in the removal of Cu^{2+} and Cd^{2+} from the contaminated medium while GM 15 was found to be most effective in the biosorption of Ni^{2+} from the

aqueous solutions. These observations indicate that GM 15 could be used for bioremediation of Ni^{2+} and GM 16 could be used for bioremediation and Cu^{2+} and Cd^{2+} from the contaminated aqueous environment. Compared with GM 15 and GM 16, GM 17 had less of an ability to remove Cu^{2+} and Cd^{2+}, since it could only remove 20 % of the metal ions.

4.5. Further investigations

In the last few decades, with the advent of sophisticated techniques (e.g. Analytical electron microscope, AEM; Transmission electron microscope, TEM; X-ray and electron diffraction analysis, XRD; Scanning electron microscope, SEM) facilitating deeper insight into the cell structure, many studies have focused on the localization of accumulated metals inside the cell (Maier *et al*., 1990; Matsunaga *et al*., 1999). Eventually, one can also predict through these results, whether the uptake is metabolism-dependent or an adsorption phenomenon. Hence the use of these techniques can clarify all predictions made from the metal biosorption profiles of the microbes.

The biosorption process is critically linked to a number of factors that affect the biosorption capacity:

- pH
- Biomass concentration
- Initial metal ion concentration
- Temperature

Hence, it would be necessary to determine the kinetic properties of biosorption, which are significant for determining the time required to reach equilibrium and to evaluate the maximum adsorption capacity.

Chapter 5

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Appendix

Reagents preparation

Preparation of biological media

Three different types of media, TYG (broth and agar), Mineral salt agar medium, MH (broth and agar), were used in this research.

TYG broth: It was prepared by dissolving 5.0 g of tryptone, 3.0 g of yeast extract, 1.0 g of glucose in a liter of deionized water. Medium was adjusted to pH 6.5 with 1 M hydrochloric acid and 1 M sodium hydroxide and sterilized by autoclaving at 121^{o}C for 30 minutes.

TYG agar (for culture maintenance): It was prepared by dissolving in a liter of deionized water 5.0 g of tryptone, 3.0 g of yeast extract, 1.0 g glucose, 16 g of agar powder, and 1 M of $NaAsO_2$ was added to a final concentration of 100 µM. Medium was adjusted to pH 6.5 with 1 M hydrochloric acid and 1 M sodium hydroxide and sterilized by autoclaving at 121^{o}C for 30 minutes.

Mineral salts agar medium: It contained (per liter of deionized water) 2 g sodium gluconate, 6.06 g TRIS-HCl (pH 7.0), 4.68 g NaCl, 1.49 g KCl, 1.07 g NH_4Cl, 0.43 g Na_2SO_4, 0.2. g $MgCl_2.6H_2O$, 0.003 g $CaCl_2.2H_2O$, 0.23 g $Na_2HPO_4.12H_2O$ and 20 g agar (Mergeay, 1985). Medium was sterilized by autoclaving at 121^{o}C for 30 minutes.

MH broth: It was prepared by dissolving 21 g of MH broth in a liter of deionized water and sterilized by autoclaving at 121^{o}C for 30 minutes.

MH agar: It was prepared by dissolving 30 g of MH agar in a liter of deionized water and sterilized by autoclaving at 121^{o}C for 30 minutes.

Preparation of stock solutions

$CdCl_2.H_2O$ (Mr = 228.34 g/mol): It was prepared by dissolving 11.47 g in a 50 ml of deionized water to form 1 M sock solution and filter sterilized using 0.2 µm pore-size Millipore filter paper.

$CoCl_2.6H_2O$ (Mr = 237.93 g/mol): It was prepared by dissolving 11.8965 g in a 50 ml of deionized water to form 1 M sock solution and filter sterilized using 0.2 µm pore-size Millipore filter paper.

$CuCl_2.2H_2O$ (Mr = 170.5 g/mol): It was prepared by dissolving 8.525 g in a 50 ml of deionized water to form 1 M stock solution and filter-sterilized using 0.2 µm pore-size Millipore filter paper.

$Na_2HAsO_4.7H_2O$ (Mr = 312.0 g/mol) : It was prepared by dissolving 15.6 in 50 ml l of deionized water to form 1 M stock solution. The solution filter sterilized using 0.2 µm pore-size Millipore filter paper.

$NiCl_2.6H_2O$ (Mr = 237.7 g/mol) stock solution: It was prepared by dissolving 11.885 g in 50 ml of deionized water to make 1 M stock solution. The solution was filter sterilized using 0.2 µm pore-size Millipore filter paper.

$ZnCl_2$ (Mr = 136.3 g/mol) stock solution: It was prepared by dissolving 6.815 g in 50 ml of deionized water to make 1 M stock solution. The solution was filter sterilized using 0.2 µm pore-size Millipore filter paper.

$NaAsO_2$ (Mr = 129.9 g/mol) : It was prepared by dissolving 6.495 g in 50 ml of deionized water to form 1 M stock solution and filter sterilized using 0.2 µm pore-size Millipore filter paper.

$C_4H_4KO_2Sb.½H_2O$ (Mr = 333.93 g/mol): It was prepared by dissolving 16.6965 g in 50 ml of deionized water to form 1 M stock solution and filter sterilized using .2 µm pore-size Millipore filter paper.

Table 5.1. The concentrations of the metal ions expressed in mg/l and in mM concentrations.

Metal ion	Molecular weight (g/mol)	50 mg/l	100 mg/l
		Concentration in mM	
Ni^{2+}	237.7	0.210	0.421
Cu^{2+}	170.5	0.293	0.586
Cd^{2+}	228.34	0.218	0.438