Characterization of bacteria isolated from a platinum mine tailings dam

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DECLARATION

The experimental work conducted and discussed in this dissertation was carried out at the School of Biological Sciences, Microbiology, North West University, Potchefstroom Campus. This study was conducted from March 2006 to November 2008 under the supervision of Prof. C.C. Bezuidenhout and co-supervision of Prof. M.S. Maboeta.

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ABSTRACT

Contamination from various sources has a huge impact on soil health and microbial community composition. Metal contamination of soil in mining scenarios is of concern and is not adequately addressed, particularly with respect to the microbial community. The mining industry is one of the largest contributors to heavy metal contamination of soil in South Africa, especially since the country is one of the major mining countries in the world. Platinum mining is of special importance, since the largest percentage of the world's reserves of platinum group metals are found and mined in South Africa. Metals from mining activities become irreversibly immobilized in soil systems because they cannot be degraded and has a huge impact on soil systems. In this study, bacteria was isolated from soil samples collected from a platinum mine tailings dam outside Rustenburg. During the warm sampling season (March 2006) most isolates were found, especially in sites 3 and 4. During the colder and drier season (May 2006) there were less isolates. Most of the isolated cultures also displayed a wide temperature growth range, mostly between 24°C - 37°C. *Paenibacillus lautus* and *Bacillus subtilus* DN-10 had a growth range between 5°C - 40°C. Culturable metal tolerant bacteria were isolated, purified and identified using 16S rDNA sequences. Nine different species were found namely *Paenibacillus lautus* strain DS19, *Paenibacillus lautus*, *Paenibacillus* sp. C15, uncultured *Paenibacillaceae*, *Bacillus subtilis* strain DN-10, *Bacillus* sp. KDNB5, *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2. The ability of these strains to tolerate metal concentrations were explored by determining their minimum inhibitory concentrations for a selection of metals e.g. aluminum, barium, cobalt, chromium, cadmium, copper, iron, lead, manganese, nickel and mercury. Most isolates were able to tolerate >5mM of the Al\Ni alloy and cobalt. Transmission electron microscopy was used to determine the location of metals inside bacterial cells and electron dispersive X-ray analysis was used to determine the levels of metals inside microbial cells. *Bacillus subtilis* DN-10 (LDK0306) showed a high MIC (>5mM) for most metals used, except Hg. This strain also had a high percentage (10.26%) of Pb detected in its cells by EDX. This was the highest percentage detected. Plasmids were extracted from the identified strains and can help gain a better understanding of metal tolerance mechanisms used by these isolates.

Keywords: bacteria, metal tolerance, bacterial diversity, MIC, transmission electron microscopy, 16S rDNA

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CO dehydrogenase	Carbon monoxide dehydrogenase
DGGE	Denaturing Gradient Gel Electrophoresis
EDX	Energy dispersive X-ray analysis
FAD- containing proteins	Flavin adenine dinucleotide-containing
FISH	Fluorescence in situ hybridisation
MerP	Mercury-binding protein
MIC	Minimum inhibitory concentration
MFS	Major Facilitator Superfamily
NA	Nutrient Agar
PCR	Polymerase chain reaction
PYG-broth	Peptone Yeast Glucose Broth
rDNA	Recombinant DNA
RND family	Resistance-nodulation-cell division family
SEM	Scanning electron microscopy
TAE- buffer	Tris-acetate-EDTA buffer
TEM	Transmission electron microscopy
TGGE	Temperature Gradient Gel Electrophoresis

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CHAPTER 1

INTRODUCTION

1.1 GENERAL INTRODUCTION AND PROBLEM STATEMENT

Soil is an extremely complex environment that contains more microbial genera or species than any other habitat (Kang & Mills, 2006) and the number of species present in soil depends on the conditions available for their survival and growth (Stotzky, 1997). There are major interactions between the different organisms using it as a habitat and any environmental disturbances or changes that might prevail (Stotzky, 1997; Trevors & van Elsas, 1997; Robe *et al.*, 2003). The first organisms in the soil environment to be influenced and to adapt to these changes are usually microorganisms (Silver & Phung, 2005). Microorganisms are usually highly adaptable to environmental changes. Conditions such as temperature fluctuations, pH, salinity, carbon, energy sources and available water, may affect species composition and could either stimulate or inhibit microbial growth (Stotzky, 1997). Environmental changes make it necessary for organisms to adapt and develop tolerance to the various stressors in order to survive.

Metal contamination is one of the factors that have an impact on soil microbial community structures, because they cannot be degraded (Ahmed *et al.*, 2001; Pérez-de-Mora *et al*., 2006; Sauge-Merle *et al.*, 2012). The toxic effect and accumulation of heavy metals can have serious ecological health problems (Malik, 2004) due to their remarkable differences in mobility, biological availability and chemical behaviours (Wu *et al.*, 2006). Industrialization greatly contributes to metal contamination of the environment because metal containing sludge and wastewater is released into the environment by many industries such as mining, milling and surface finishing industries (Malik, 2004; Wang *et al.*, 2007). Mining produces chemical waste products that get dumped on tailings dams. This material normally contains high levels of metals and soil then acts like a sink for these contaminants that are released into the environment (Atlas, 1997; Yilmaz, 2003; Pereira *et al.*, 2005). A large amount of wastewater is generated through platinum mining and large amounts of tailings with high levels of metals (Cu, Ni and Cr) are disposed of (Maboeta *et al.*, 2006; Dobsen & Burgess, 2007; Wahl, 2007).

In South Africa metal contamination is widespread, since the country has one of the largest mining industries in the world. The country has 80% of the world's manganese reserves, 72% chromite reserves and a large proportion of many other minerals such as gold, platinum group metals, vanadium and nickel (Mbendi Information Services, 2005). It is also estimated that 87% of the world's platinum group metal reserves are in South Africa (Mbendi Information Services, 2005; Conradie, 2007). Figure 2.1 illustrates the large area in the Bushveld Complex of South Africa where a large concentration of platinum group metals can be found.

It is important for soil microorganisms to adapt to these scenarios. Soil diversity must be maintained since important microbial processes such as litter decomposition, carbon mineralization and nitrification can be negatively affected if diversity is depleted (Stotzky, 1997; Wuertz & Mergeay, 1997; Dubey *et al.*, 2006). Metal contamination can cause a loss in structural diversity (Wang *et al.*, 2007) and increased biomass respiration since the microorganisms will use more energy to regulate their biochemical functions (Pérez-de-Mora *et al.*, 2006; Giller *et al.*, 2009). This loss of energy in contaminated soils will cause microorganisms to lose some of their catabolic abilities thus affecting biogeochemical cycling (Atlas, 1997; Wenderoth & Reber, 1999). Microorganisms also experience various modifications in community structure and physiological activities as a result of metal contamination (Díaz-Raviña & Bååth, 1996; Hassen *et al.*, 1998). The response of the microbial community is dependent on the concentration, type and availability of the metals.

Microorganisms have developed resistance mechanisms which are either chromosomal or plasmid driven (Malik, 2004). Tolerance to metals is accomplished by two kinds of actions. The first possibility is through intrinsic properties that are related to the cell membrane structure such as extra-cellular polypeptides that bind to metals and cause precipitation (Wuertz & Mergeay, 1997; Vullo *et al.*, 2008). Another way for microorganisms to adapt is to develop specific mechanisms to deal with metal accumulation in cells such as efflux pumps and intercellular compartmentalization or sequestration (Wuertz & Mergeay, 1997). Some strains can cause the enzymatic transformation of metals and metalloids through oxidation. Metal precipitation is used to immobilize metals to a lower redox state, producing a less bioactive state which is often employed in wastewater treatment processes (Valls & de Lorenzo, 2002). Metal biosorption where metals are bound to cellular parts is also a very useful process, especially where metals are high in concentration such as in effluents from industrial areas (Yilmaz, 2003).

1.2 RESEARCH AIM AND OBJECTIVES

The aim of this study was to isolate and characterize metal resistant bacteria from a platinum mine tailings dam.

Specific objectives included the following:

- Enumerate metal tolerant bacteria isolated from a platinum mine tailings dam.
- Identify these isolates using phenotypic and sequencing data of 16S rDNA genes.
- Characterize these isolates by determining optimum growth temperatures and MICs for a selection of metals.
- Utilize transmission electron microscopy (TEM) and electron dispersive x-ray analysis (EDX) to determine the fate of selected metals on selected bacterial species.
- Investigate if plasmids may be responsible for the metal tolerance trait.

CHAPTER 2

LITERATURE REVIEW

2.1 SOIL AS AN ECOSYSTEM

Soil is an extremely complex ecosystem and there are various interactions and relationships between the different organisms using it as a habitat (Robe *et al.*, 2003; Dubey *et al.*, 2006). It serves as their source of nutrients and also as a sink for dead plant and animal materials (Stotzky, 1997; Wu *et al.*, 2006). Organisms are affected by root systems, organic and mineral particles present and the structure of the soil layer (Remenant *et al.*, 2008). There are many important functions of soil like plant anchorage, nutrient supply, water retention and conductivity, support of soil food webs and also environmental regulatory functions such as the cycling of nutrients and remediation of pollutants (van Bruggen & Semenov, 2000). These functions make soil health important for the functioning of terrestrial ecosystems (Remenant *et al.*, 2008). Soil health can be described as its continuous capacity to sustain biological productivity, maintaining the quality of its surrounding air and water environment and promoting the health of plants, animals and humans (Park *et al.*, 2011). Indicators of soil health include microbial biomass, soil basal respiration, enzyme activities and nutrient transformations (Niemeyer *et al.*, 2012). These factors can be a valuable tool for biological assessment of soil health.

Proper functioning and homeostasis of the eco-system is directly involved and influenced by soil microorganisms (van Bruggen & Semenov, 2000; Wakelin *et al.*, 2008). The three main factors that can influence soil health are physical, biological and chemical stresses. Physical stress includes factors like temperature, high pressure and osmotic potentials whereas biological stress includes nutrient deficiency or excess (van Bruggen & Semenov, 2000). Chemical stress is most common and includes pH fluctuations, antimicrobial substances and organic and inorganic substances in excess (van Bruggen & Semenov, 2000).

Environmental deterioration could destroy the soil ecosystem if there was not any resilience or buffering capacity in soils to stress and a high ability to regenerate (van Bruggen & Semenov, 2000). The soil ecosystem is maintained by microorganisms and their processes like geochemical cycling of carbon, nitrogen and phosphorus and litter decomposition (Wuertz & Mergeay, 1997; Remenant *et al.,* 2008; Stefanowics *et al.,* 2008). Soil fertility is dependent on microbial organic matter decomposition and nutrient cycles (Oliveira & Pampulha, 2006). This makes microbial activities of direct importance for agriculture and the sustainability of the environment (Wakelin *et al.*, 2008).

2.2 MINING IN SOUTH AFRICA

A variety and high quantity of minerals are produced in this country, including chrome, gold, vanadium and diamonds. The country has most of the world's manganese (±80%) and chromite (±72%) ore reserves (Mbendi Information Services, 2005). South Africa is also the world's largest producer of platinum group metals (87% of the world's reserves) (Conradie, 2007). In the Bushveld Complex of South Africa (Fig 2.1) the world's largest concentration of platinum group elements (PGE) can be found (Barnes *et al.*, 2004).

Pollution from mining activities should be especially important in South Africa, since it is one of the most important mining countries in the world and the country also has limited water resources. Mining can have a serious impact on water quality (Ochieng *et al*., 2010). Due to South Africa's water scarcity problem, sulfuric acid drainage and heavy metal pollution in streams due to mining activities is a serious concern (Ochieng *et al*., 2010).

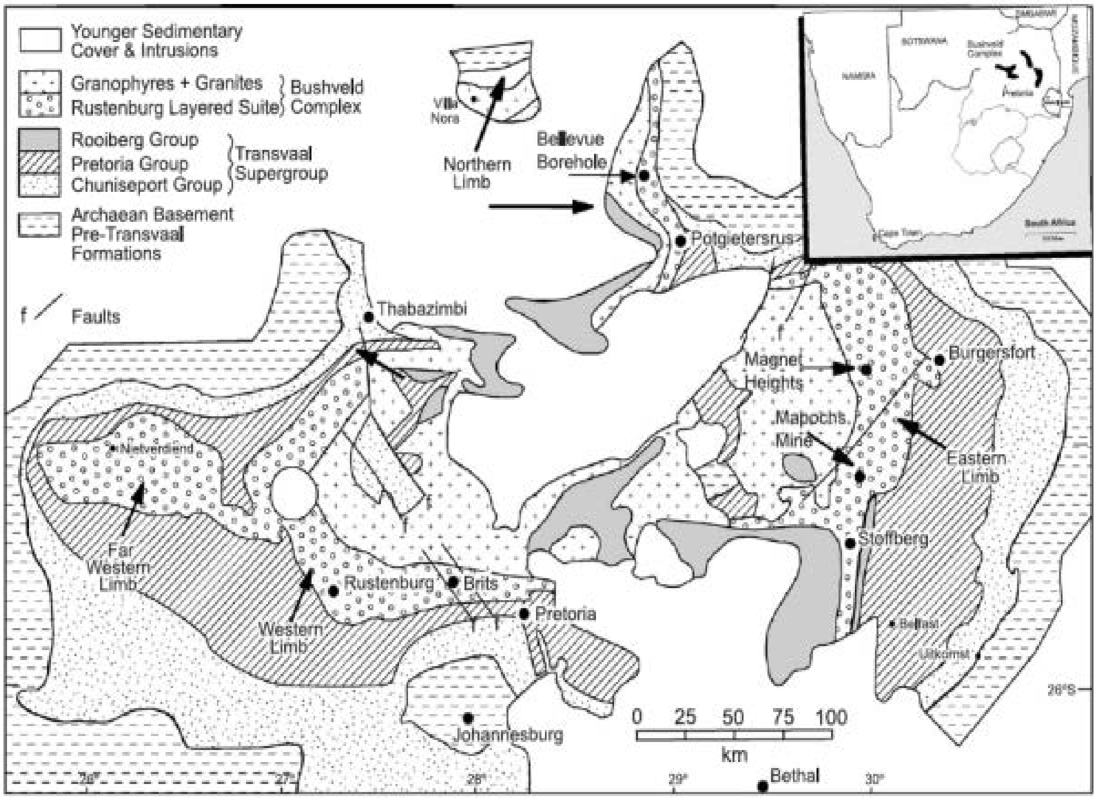


Figure 2.1 The geology and location of the Bushveld Complex in South Africa (Barnes *et al*., 2004).

2.3 THE EFFECTS OF MINING ON THE SOIL ECOSYSTEM

Metal pollution occurs because of the disposal and remediation of metal containing sludge from different industries, e.g. mining, processing and smelting industries (Tsezos *et al.*, 1997; Wang *et al.*, 2007; Perez *et al.*, 2008; Wang *et al.*, 2010). Several industries contribute to metal contamination of the environment. These include milling, surface-finishing and electroplating industries, but mining seems to be the biggest contributor to metal pollution in the environment (Malik, 2004; Colin *et al.*, 2012). Heavy metals can be extremely toxic to animals, plants and microorganisms and since they are not degradable, they tend to become irreversibly immobilized in the environment and can only be removed by extraction (Malik, 2004; Pereira *et al.*, 2005; Perez-de Mora *et al.*, 2006). Thus accumulation of heavy metals in the environment can cause serious ecological problems and health risks (Malik, 2004; Park *et al.*, 2011; Fonseca *et al.*, 2012*;* Sauge-Merle *et al.*, 2012).

Sometimes metals form complexes within cells that can be toxic and they have also been shown to contribute to the maintenance of antibiotic resistance genes due to the increase in selection pressure of the environment (Spain, 2003). There is also the possibility of metals becoming concentrated in higher trophic levels through biomagnification (Prescott *et al.*, 2005). It happens when metals in water and sediments cause a build-up in grains and vegetables grown in contaminated soils (Jézéquel *et al.*, 2005; Ying *et al.*, 2008; Colin *et al.*, 2012). This is harmful to human health and extremely toxic. Metals can cause damage to the nerves, liver and bones. Some are carcinogenic and cause birth defects or they can block the functional groups of vital enzymes (Malik, 2004; Wang *et al.*, 2012). An example of a metal that tends to accumulate in the environment and pose serious risks is cadmium (Cd). It enters soils via aerial deposition, through phosphate fertilizers and sewage sludge (Xu *et al.*, 2012).

Finely ground slurry from mining processes and the associated chemicals that reach tailings dam facilities contribute to metal pollution and to the stress put on microbiota (Maboeta *et al.*, 2006). This results in the inhibition of enzyme activity of soils and also has negative effects on microbial processes like geochemical cycling of carbon, nitrogen and phosphorus (Remenant *et al.*, 2008; Upchurch *et al.*, 2008). The presence of certain metals such as cobalt (Co), nickel (Ni) and zinc (Zn) not only limit soil functioning but also complicates rehabilitation efforts (Hattingh *et al.*, 2001). Mine tailings poses a potential hazard to surface and ground water pollution, offsite contamination by wind-transported materials and redistribution of contaminated soils (Ying *et al.*, 2008). A study done by Guo-li *et al.* (2008) involved investigating pollution at four different sites from different avenues including tailings pollution soil, mine drainage soil, dust in wind pollution soil and minerals transportation soil. Different forms of heavy metals was found in each zone and they found the area most contaminated with heavy metals to be the mine tailings. They concluded that in order to effectively address heavy metal contamination, much attention should be given to tailings pollution of soil.

2.4 METAL CONTAMINATION AND MICROORGANISMS

Microorganisms are some of the first organisms to be affected by metal contamination (Stotzky, 1997). Microbial community structure, taxonomic diversity, growth, function and replication are affected by metal pollution (Piotrowska-Seget *et al.*, 2005; Stefanowicz *et al.*, 2008; Ying *et al.*, 2008). Some metals such as zinc (Zn), nickel (Ni) and copper (Cu) are also essential elements and functions as cofactors that drive enzymatic reactions. Zinc, for instance, has an important role in the maintenance of the structure and functioning of several proteins, including metallothioneins and cells have a special mechanism for cellular Zn uptake (Sadineni & Schöneich, 2007). However, their cytoplasmic concentration has to be maintained at critical levels (Hassen *et al.*, 1998; Nies, 1999; Gleeson *et al.*, 2006). If metal concentration becomes too high in the cell it begins to form unspecific complex compounds on the cell wall that can have toxic effects (Nies, 1999).

In general, the effect of metals is inhibitory because they can block some essential functional groups of organisms, displace essential metal ions or even modify active conformations of biological molecules (Hassen *et al.*, 1998; Gleeson *et al.*, 2006; Colin *et al.*, 2012). An example of a toxic metal substituting an essential cation in an enzyme is Cd^{2+} that replaces Zn^{2+} and rendering the specific enzyme non-functional (Sandrin & Maier, 2003). Metals can inhibit microbial processes such as methane metabolism, litter decomposition, the conversion of nitrogen and sulfur and also dehalogenation and reductive processes (Sandrin & Maier, 2003; Oliveira & Pampulha, 2006; Qing *et al.*, 2007b). Usually metal toxicity will cause a decrease in soil microbial biomass because of a decrease in substrate utilization efficiency which is a result of the higher energy cost the microorganism will have when exposed to metal stress (Giller, 2009; Niemeyer *et al.*, 2012).

2.5 BACTERIAL TOLERANCE/RESISTANCE MECHANISMS TO METALS

Some microorganisms do adapt to metal contamination (Bontidean *et al.*, 2000; Pereira *et al.*, 2005; Wang *et al.*, 2012). Bacterial cells all have different ways of action when they are in a metal contaminated environment. The response of the organisms depend on the type of metal involved, the concentration and availability, the microbial species involved and also the nature of the medium (Benyehuda *et al.*, 2003; El Fantroussi & Agathos, 2005). Microorganisms become metal tolerant through the introduction of various mechanisms and resistance systems including the reduction of metals, enzymatic transformation, production of metal sulfides, precipitation, crystallization and efflux systems (Ahmed *et al.*, 2001; Bontidean *et al.*, 2000). Sometimes physiological or genetic adaptations or even morphological changes of the cells are necessary for adaptation to metal stress (Piotrowska-Seget *et al.*, 2005). Resistance to heavy metals is mostly conferred by products produced through genes that are present on plasmids and also in some instances through chromosomal genes (Silver *et al.*, 2001; Liu *et al.*, 2012). A selection pressure that is exerted in metal-abundant environments induces the mechanisms that produce tolerance. Figure 2.2 summarizes seven major mechanisms used by microorganisms for metal tolerance. The most common mechanisms used by microorganisms can be divided into three groups: efflux pumps, enzymes and metal binding proteins. These are all discussed below.

2.5.1 Efflux pumps

Metals or toxins are often removed from the cell by systems that are also involved in the transport of nutrients in the cell (Silver & Phung, 2005). This is a metabolism dependent bioaccumulation method. With these systems, metals are taken into the cells by nutrient uptake pumps only to be removed again by efflux pumps (Spain, 2003; Silver & Phung, 2005). Of the different mechanisms that occur the efflux pump mechanism seems to be more common and they exist either as ATPases or chemiosmotic ion/proton efflux systems (Figure 2.2).

ATPase membrane pumps have covalently phosphorylated intermediates from ATP. The ABC ATPases are commonly found. They have a phosphorylated intermediate and are usually multi-component primary active transporters (Pao *et al.*, 1998). A well-known example is the CopA/B efflux pump system that is expressed as four proteins and is essential for copper resistance (Figure 2.2). CopA is found in the periplasmic space and will catalyze the intake of copper while CopB is on the outside of the membrane and catalyzes the efflux of copper. The two remaining proteins are CopC in the periplasmic space and CopD on the inner membrane (Wei *et al.*, 2008).

P-type ATPases are ATP fuelled pumps that have a single catalytic subunit and a phosphorylated intermediate that has two conformations (Silver & Phung, 2005). The conformation changes with each phosphorylation and de-phosphorylation step. These pumps are involved in the transport of a variety of substrates including Cd^{2+}, Cu^{2+}, Na^{+}, H^{+}, and Mg^{2+} (Silver & Phung, 2005). They are divided into families based on ion specificity, number of transmembrane segments and their origin (bacterial and eukaryotic). *Bacillus subtilis*, *E. coli* and *Saccharomyces cerevisiae* are some examples of organisms with P-type ATPases (Palmgren & Axelsen, 1998).

There are three families of chemiosmotic ion exchanger pumps. The Major Facilitator Superfamily (MFS) are single membrane polypeptides (Figure 2.2) and they mainly transport small solutes in response to chemiosmotic ion gradients (Silver & Phung, 2005). The RND family is restricted to Gram negative bacteria (Rensing & Maier, 2003). They have three proteins that are distributed throughout the membrane to form a channel through the cytoplasm to the outside of the cell (Pao *et* *al.*, 1998). An example of the RND chemiosmotic ion exchange system is the CzcCBA system with 3 proteins CzcA, CzcB and CzcC (Pao *et al.*, 1998). The first protein (protein A) is on the inner membrane and protein C is found on the outer membrane. Protein B is found between the membranes, acts as the coupling protein and helps to form a continuous channel through the cell membrane (Pao *et al.*, 1998).

Another chemiosmotic ion exchanger family is the cation diffusion facilitator family. An example of this type of efflux system is the Zn transporter protein ZnT-n (Sadineni & Schöneich, 2007). This is also the only transport family that exclusively transports metals (Rensing & Maier, 2003).

2.5.2 Metal binding proteins

Some cells produce metal chelators called metallothioneins (Sandrin & Maier, 2003). They are small, low molecular weight proteins that are cysteine rich and they bind to metal ions (Blindauer *et al.*, 2002). Metallothioneins act as cytoplasmic metal cation-binding proteins (Silver & Phung, 2005). They bind both essential heavy metals such as Zn, Cu and Ni as well as more toxic heavy metals (Cd, Hg, Pb etc.) (Sauge-Merle *et al.*, 2012). A well-known metallothionein is BmtA that binds to zinc ions. These proteins were found in *Pseudomonas aeruginosa* and *Pseudomonas putida* (Blindauer *et al.*, 2002). Metallothioneins have an important role in the detoxification of heavy metals, protection of tissue against oxidative injury and also homeostatic regulation and transport of heavy metals (Haq *et al.*, 2003; Sauge-Merle *et al.*, 2012).

Phytochelatins are biosurfactants which are short, cysteine-rich peptides that have a higher metal binding capacity than metallothioneins (Vijayaraghavan & Yun, 2008). Peptides enhance the capacity of bacteria to accumulate Cd^{2+} and Hg^{2+} up to 20 times (Vijayaraghavan & Yun, 2008). Their general structure is $(\gamma\text{Glu-Cys})_n\text{Gly}$ (Vijayaraghavan & Yun, 2008). Biosurfactants have the possibility of being used in remediation of polluted areas. In order for this approach to be economical the biosurfactant must be recovered for reuse (Silver & Phung, 1996).

2.5.3 Enzymes in metal detoxification

Heavy metals are converted to less soluble forms through enzyme activity (Lloyd & Macaskie, 2000). Enzymatic transformation systems are less common than systems that use energy-dependent efflux pumps (Silver & Phung, 2005). A well-known reductase enzyme system is the enzyme involved in mercury resistance (Figure 2.2). The FAD-containing mercuric reductase is an intracellular, cytoplasmic enzyme that transforms Hg^{2+} to Hg^0, a less toxic form (Foster, 1983). Foster (1983) also indicated that in some species two enzymes are expressed to give broad spectrum metal resistance. They have an additional organomercurial lyase that cleaves C-Hg bonds to release the Hg-ion.

2.5.4 Other mechanisms

Metals are sometimes prevented from entering cells entirely. This may be because of low bioavailability or because of blockage at the cell wall and systems of membrane transportation (Hassen *et al.*, 1998). Metals can also become bound to specific binding proteins on the cell surface or inside the cell to form protein-metal associations (Hassen *et al.*, 1998; Bontidean *et al.*, 2000). Gram positive and Gram negative microorganisms contain similar metal binding functional groups that give the cell wall a negative charge making adsorption sites available for heavy metal cations (Johnson *et al.*, 2006; Vullo *et al.*, 2008; Ginn & Fein, 2008). Polysaccharides, proteins and lipids present on cell membranes have functional groups such as carboxylate, hydroxyl, phosphate, amine and sulphate groups that can bind metal ions (Vullo *et al.*, 2008). The binding of metal cations to negatively charged extra-cellular polysaccharides (EPS) is metabolism independent making bacteria that secretes these EPS a highly recommendable surface agent for the removal of heavy metals (Perez *et al.*, 2008).

Biosorption is a metabolism independent sorption method for heavy metals to living and dead biomass (Lloyd & Macaskie, 2000). In living organisms it can be influenced by metabolic activity because of differences in pH, nutrients and metabolites (Gadd *et al.*, 2001). Precipitation or binding of the metals can then occur within the cells and is a common mechanism that reduces the cell surface that the bacteria have available for nutrient uptake. When metals and bacteria react and bind, the metals precipitate on the cell surface and fine-grained minerals are produced (Southam, 2000). The process is influenced by the physical and chemical nature of the cell envelope (Gadd *et al.*, 2001).

Metals can thus be precipitated as carbonides and hydroxides through plasmid-coded mechanisms (Lloyd & Macaskie, 2000). Enzymatically generated ligands like sulfides and phosphates are used. The metal could also be transformed by certain enzymes to make it less toxic or less bio-available (Silver & Phung, 1996; Spain, 2003).

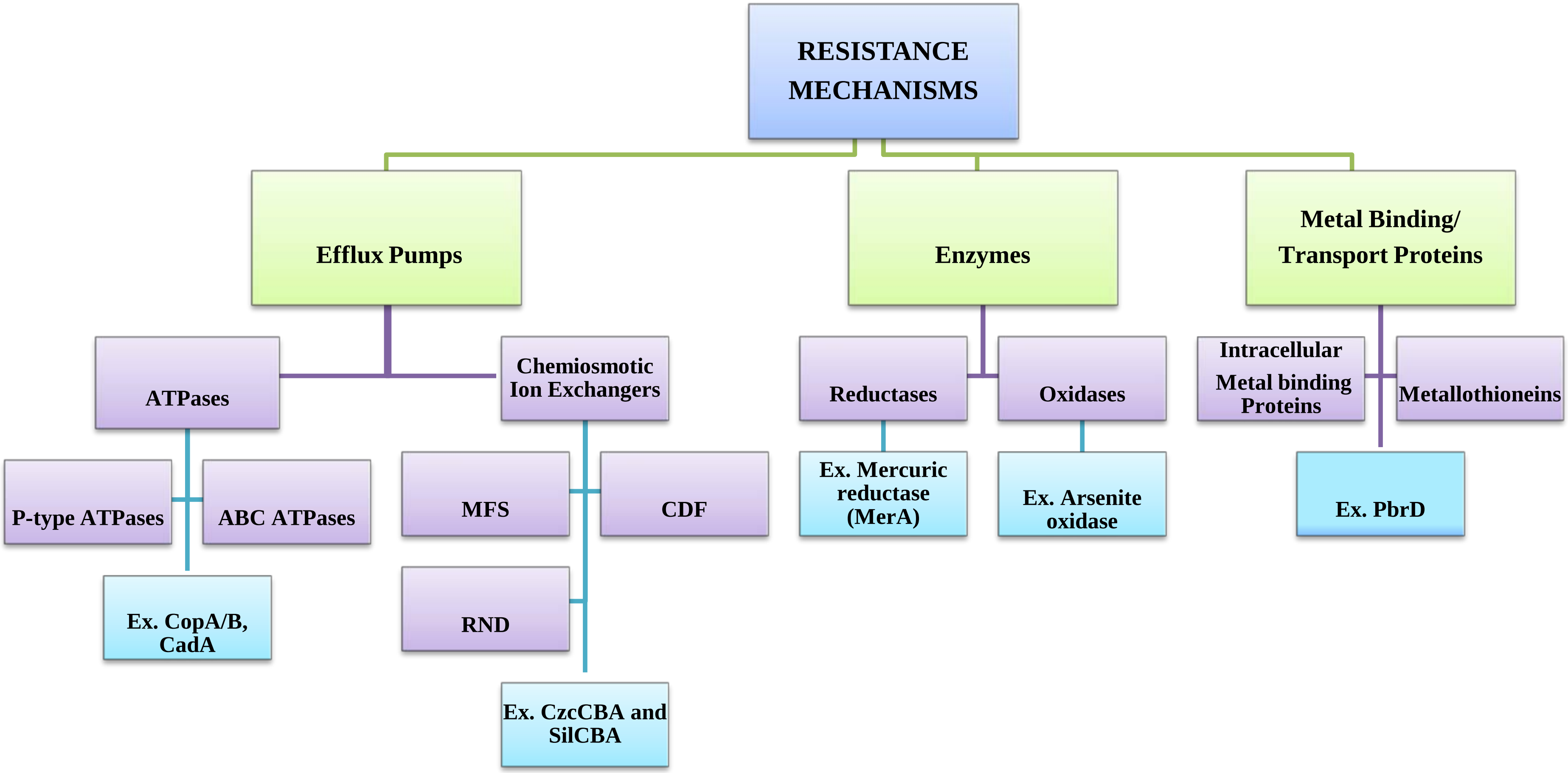


Figure 2.2 Resistance mechanisms found in microorganisms (Adapted from Silver & Phung, 2005).

2.6 CHARACTERISTICS OF SOME HEAVY METALS

Metals display different properties and some have biological functions in bacterial cells while others have no biological importance and could also be toxic to cells (Nies, 1999). Aluminum (Al) is a common element found in the environment and is widely used in the manufacturing industry. It is used in the manufacturing of kitchen utensils, medical and scientific equipment, wrapping and various containers. Aluminum salts are also used as food additives (Barabasz *et al.*, 2002). A rise of Al concentrations in the environment has been linked to changes in water pH to more acidic ranges, plant poisoning and drying forests (Barabasz *et al.*, 2002).

Barium (Ba) occurs naturally in surface waters and is released to the environment by industrial emissions (Choudhury & Cary, 2001). It is widely used in industry as industrial coatings, brake linings and in some adhesives. Some plants are known to bioaccumulate Ba from soil but no adverse effects have been reported (Choudhury & Cary, 2001).

Cadmium (Cd) is widespread, poisonous and one of the most toxic and highly mobile metals in the soil surface layer (Qing *et al*., 2007a). It is often found in fertilizers used in agriculture (Fonseca *et al*., 2012). It can cause a significant decrease in biological soil activities (Swalaha *et al.*, 2002; Qing *et al.*, 2007a; Vullo *et al.*, 2008). Accumulation of Cd by agriculturally important crops often happens and the metal is then consumed by animals and humans through their diet (Qing *et al.*, 2007b). Cadmium resistance is widely found and could be due to various efflux pump systems in both Gram positive and Gram negative microorganisms (Silver & Phung, 2005). Resistance to this metal has been observed in *Bacillus* strain H9 through the reduction of soluble Cd during growth and in *Bacillus subtilis* (Malik, 2004). A decrease in the activity of certain enzymes such as soil alkaline phosphatase, arylsulphatase and protease in the presence of Cd can be contributed to higher energy requirements for growth in Cd polluted environments or binding of Cd ions to sulphydryl groups (Lorenz *et al.*, 2006).

Chromium (Cr) is used as a component of stainless steel and some other alloys and dyes such as paints and pigments and is also an extremely toxic metal (Colin *et al.*, 2012). It has no biological function in cells and can be highly toxic to biological systems (Badar *et al.*, 2001). Chromium is less mobile in surface soil than some other metals such as nickel (Hattingh *et al.*, 2001). Resistance is associated with an interaction between chromate reduction and chromate efflux (Nies, 1999). A variety of bacteria with the ability to reduce chromate has been found. Remediation of chromate contamination in soils with the use of chromate-reducing bacteria will not result in permanent detoxification since any chromate left in the environment will be readily oxidized again (Nies, 1999).

Cobalt (Co) is one of the metals found in soil with high mobility and has been shown to limit soil functioning and complicate efforts for rehabilitation (Hattingh *et al.*, 2001). Cobalt and copper (Cu) are capable of inhibiting microorganisms if they occur in ionized forms and in high concentrations (Baker-Austin *et al.*, 2006). Resistance mechanisms to both Co and Cu usually involve oxidation or reduction active enzymes (Silver & Phung, 2005). Copper is a common industrial metal and is also an essential micronutrient that is involved in a variety of redox reactions (Colin *et al.*, 2012). It is required as a trace element and is a component in cytochrome c oxidase (Spain, 2003). Copper can affect the homeostasis of organisms if it becomes available in high amounts and can then have toxic effects on bacterial growth and metabolism (Badar *et al.*, 2001; Sokhn *et al.*, 2001). This could be due to the inactivation of certain enzymes. Copper also has the ability to interact easily with radicals and produce hydroperoxide radicals that contributes to its toxicity (Nies, 1999). It has also been found that Cu can bind strongly to organic matter found in soil and renders it unavailable to soil microbes (Fernández-Calviňo *et al.*, 2011).

Iron (Fe) is a very important heavy metal cation since it is the only metal macro-bioelement (Nies, 1999). Iron has a low mobility in soil ecosystems (Hatting *et al.*, 2001). It is not toxic to aerobic bacteria because of its low solubility (Nies, 1999). Anaerobic bacteria use Fe^{3+} as an electron acceptor (Nies, 1999). In soils Fe mostly exists as Fe^{3+} in aerobic geological systems and is a highly reactive form that can out-compete other metals in the area for metal binding sites. Iron could also be found in close spatial association with bacteria in soils in the form of Fe oxides and Fe silicates (Wightman & Fein, 2005).

Lead (Pb) is commonly used as a component of galvanizing materials and alloys and is an extremely toxic heavy metal. Effects of Pb include the denaturing of proteins, destabilization of membranes and retardation of electron transport chains and photosynthesis (van Hille *et al.*, 2003). In a study by Piotrowska-Seget *et al.* (2005), all the species and strains isolated from the soil of a former silver mine, displayed a very low tolerance to lead. Manganese (Mg) is an essential micronutrient with low toxicity and is normally part of enzymes and co-factors. It may also aid in the catalysis of certain reactions as well as the maintenance of protein structures. Manganese is also commonly used by bacteria in anaerobic respiration processes as an electron acceptor (Nies, 1999).

Mercury (Hg) and Hg compounds have a wide variety of uses. It is widely used in medicine as an antimicrobial or as preservatives of health care products (Hobman *et al.*, 2000). The metal is capable of forming stable carbon metal bonds. Inorganic and organic forms of Hg can be transformed by microorganisms to methylated forms. These then tend to accumulate in higher trophic levels

(Prescott *et al.*, 2005; Baker-Austin *et al.*, 2006). Mercuric ions are highly toxic but resistance to Hg exists widely for both Gram positive and negative microorganisms (Foster, 1983; Silver, 1996; Nies, 1999). Often the genes for resistance are found on plasmids and transposons. Resistance is only expressed when the organisms is exposed to sub-toxic levels of Hg (Foster, 1983; Silver & Phung, 2005).

Nickel (Ni) is a widely used metal particularly in electroplating processes. Nickel-containing effluents are common and can be very harmful to the environment because of their high mobility and high bio-availability (Hattingh *et al.*, 2001). Along with Co, Zn and Ni can have a limiting effect on soil functioning and rehabilitation (Hattingh *et al.*, 2001). At low concentration, Ni serves as a micro-nutrient for microorganisms (Nies, 1999). There are thus metal uptake pumps to keep sufficient intracellular levels of Ni and efflux pumps to remove excess amounts that might become toxic (Silver & Phung, 2005). Nickel availability influences urease production and function (Nies, 1999). Hydrogenase, urease and CO dehydrogenase are some Ni-containing enzymes in which nickel is bound to cysteine or histidine in the active sites (Nies, 1999). It does appear that uptake of the metal is linked to the metabolic state of cells (Malik, 2004).

2.7 THE ROLE OF PLASMIDS

2.7.1 Plasmid-borne resistance

Studies have determined that resistance to heavy metals such as Ag^+, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} are often coded for by plasmids (Silver, 1996; Silver & Phung, 1996; Ahmed *et al.*, 2001; Ugur & Ceylan, 2003). Large plasmids found in species of *Alcaligenes* carry numerous heavy metal resistance determinants. Resistance coded for on plasmids allows further manipulation and this can aid in enhancing the efficiency of microorganisms for remediation purposes (Malik, 2004). Furthermore, horizontal transfer of plasmids, transposons and genes is not uncommon (Abou-Shanab *et al*., 2007). A study by Alonso *et al.* (2000) on *Stenotrophomonas maltophilia* strains, showed that genes that carry the characteristic for metal and antibiotic tolerance were highly similar to that of the isoforms of the same set of genes found in *Staphylococcus aureus*. There was a high homology between genes coding for metal resistance from the two species (98.2% on DNA level). This indicates that gene transfer between Gram positives and Gram negatives is possible in the environment.

2.7.2 Association of metal tolerance to antibiotic resistance

There is some concern that metal pollution may have an effect on the antibiotic resistance of microbes because metal and antibiotic resistance are so closely associated (Hassen *et al.*, 1998). In a study done by Narancic *et al.* (2012), resistance to antibiotics such as ampicillin, nalidixic acid and erythromycin was noticed in microorganisms resistant to heavy metals. Resistance to metals and antibiotics is either acquired through a change in the genetics of the bacteria, exchange/transfer of resistance genes between bacteria or it occurs because of genetic mutations (Spain, 2003; Ahemad, 2012). Resistance to metals and antibiotics can be conferred through chromosomal genetic material eg. plasmids and transposons (Uger & Ceylan, 2003; Deredjian *et al*., 2011; Ahemad, 2012). Resistance can then be the result of mutations at target sites, the reduction of membrane permeability, inactivation of the drug or a rapid efflux (Foster, 1983; Baker-Austin *et al.*, 2006). Co-resistance occurs when genes for different resistances are located on genes of the same genetic element, which can be transposons, plasmid or an integron. The same genetic element may also be responsible where different antimicrobial agents attack the same target and initiate a common pathway to cell death. This phenomenon is called cross-resistance (Baker-Austin *et al.*, 2006). Metals in the environment lead to the maintenance of resistance genes for both metals and antibiotics (Spain, 2003). The genes for metal and antibiotic resistance may be located within close proximity on the same plasmid. This would make it more likely that the genes would be transferred together in the environment (Spain, 2003).

Some species such as *Stenotrophomonas maltophilia* shows intrinsic antibiotic resistance through various mechanisms. These mechanisms include efflux pumps, the presence of antibiotic-inactivating enzymes such as metallo-beta-lactamases and aminoglycoside-modifying enzymes and reduced permeability to metals and antibiotics (Alonso *et al.*, 2000). In a study by Calomiris *et al.* (1984), correlations were found between tolerance towards Cu^{2+}, Pb^{2+} and Zn^{2+} and resistance to multiple antibiotics. In this study it was found that microorganisms carrying resistance to both antibiotics and metals have been isolated from wound infections in humans treated with metal-based antimicrobial agents. *Pseudomonas aeruginosa* isolates generally have a broad resistance to both heavy metals and antibiotics. In this case it has been demonstrated that the mechanisms for tolerance are present on plasmids (Hassen *et al.*, 1998).

2.8 SELECTED TECHNIQUES AVAILABLE TO CHARACTERIZE METAL TOLERANT BACTERIA

2.8.1 Isolation and enumeration of bacteria

Studies aimed at characterizing bacterial species occurring in a specific environment can use culture dependent methods (Piotrowska-Seget *et al.*, 2005). Such an approach is normally easy to follow and cost effective although culture-dependent methods are widely criticized and may have some limitations. Plate count methods do not provide a true reflection of microbial community composition and structure since most soil species cannot be cultured (Malik *et al.*, 2008). It is estimated that only 1% of soil microorganisms is culturable and that culture based methods give a limited overview of soil diversity (Malik *et al.*, 2008). Piotrowska-Seget *et al.* (2005) used culture-dependent methods successfully to investigate the diversity of metal tolerant bacteria in heavily polluted soil. During this study it was also found that isolates were mainly Gram positive organisms. A similar approach was also followed by Oliveira & Pampulha (2006) to determine if heavy metal content had an effect on soil microbial characteristics in an area with well-known long-term pollution problems. In the latter study soil samples from the top layer of soil was used and dilutions were then prepared and plated on Tryptone Soy Agar (TSA). Various metal tolerant bacterial species could thus be isolated using this method.

2.8.2 Characterization and identification of bacteria

The use of morphology, colony data and various staining methods (capsule, spore and Gram staining) are helpful to group organisms into morphotypes. Various studies have made use of biochemical properties and morphological features of bacteria to group isolates (Hassen *et al.*, 1998; Vullo *et al.*, 2008). Qing *et al.* (2007a) used morphological, physiological and biochemical characteristics to group cadmium-resistant bacterial strains. In this study Biolog and 16S rDNA sequencing was also used to identify these strains (Qing *et al*., 2007a). Preliminary characterization was done based on cell growth, Gram staining reactions, microscopic observation and other standard biochemical tests. Identification could then be confirmed or achieved by using 16S rDNA sequences (Li *et al.*, 2006). These genes offer a useful, practical tool because of its presence in all bacteria and the method is also well established (Choudhary & Sar, 2009). Also, 16S rDNA genes contain conserved regions that are useful for primer design, as well as variable regions that can be used to distinguish sequences from each other (ChihChing *et al.*, 2008; Choudhary & Sar, 2009). Useful applications of 16S rDNA sequencing include the identification of new species, geographic diversity and applications in food microbiology. Pérez-de-Mora *et al.* (2006) also used amplified ribosomal

DNA restriction analysis (ARDRA) fingerprinting of the 16S rDNA fragment to determine microbial diversity in heavy metal contaminated soil. The use of molecular tools can help to give us insight into the changes undergone by microbial communities in response to changes in environmental conditions like heavy metal pollution (Pérez-de-Mora *et al.*, 2006).

2.8.3 Growth characteristics of identified strains

Minimum inhibitory concentrations (MIC's) of the various identified isolates to metals and growth temperature profiles could be used to further characterize the isolated strains (ChihChing *et al*, 2008). The lowest concentration of a metal at which growth is completely inhibited is accepted as the MIC (Hassen *et al.*, 1998). Such data could potentially be used as indications of the effect of metal contamination on soil bacteria and their activity. Minimum inhibitory concentrations data give an indication as to the toxicity of the individual heavy metals on the different bacterial strains. Testing the sensitivity of the strains to heavy metals in a liquid media can also give good insight into metal toxicity found in environments such as industrial effluents, incinerator residues, landfill municipal refuse and sewage sludge (Hassen *et al.*, 1998). Limitations of determining MIC values in a liquid medium include that metals often bind to components in the media and the determined MIC's may not be related to actual metal concentrations in the environment (Hassen *et al.*, 1998). Minimum inhibitory concentrations determination using a micro-dilution approach is still widely used (Piotrowaska-Seget *et al.*, 2005).

In a study done by Vullo *et al.* (2008) the MIC's of bacteria resistant or tolerant to Cu, Cd and Zn was determined by incubating them in a PYG-broth at different concentrations of these metals. Bacterial growth was checked every 24 hours by measuring absorbance at 600nm. Some also determine MIC's by visually checking for the lowest concentration of metals at which no growth occurred within a metal salt media (Piotrowaska-Seget *et al.*, 2005; Qing *et al.*, 2007a). However, absorbance determination is more reliable and trustworthy.

2.8.4 Electron Microscopy

Energy Dispersive X-Ray Microanalysis (EDX) can be used to study metal adsorption by bacterial cells. It can also be helpful to provide an estimation of the uptake capacity as well as the chemical nature of the metals after uptake (Malik, 2004). Transmission electron microscopy (TEM) could be used to determine if bacteria take metals up from their surrounding environment and what the location of metals in bacterial cells are (Mullen *et al.*, 1989; Tsezos *et al.*, 1997; Choudhury & Sar, 2009).

Choudhury & Sar (2009) used EDX to estimate the elemental content of bacterial biomass and the possible changes after metal uptake by the bacteria. There was a definite change in elemental content before and after metal uptake. This observation also strongly suggested that the metal binding to the bacterial cells took place due to the displacement of cellular potassium (ion exchange mechanisms). To further confirm metal adsorption to bacterial cells, unstained preparations of the test bacteria were viewed before and after metal uptake. The metals were visible as dark opaque rings seen inside the metal loaded cells.

Some studies determine metal adsorption by calculating the decrease in metal ion concentrations in the supernatant (Kao *et al.*, 2008). This only provides indirect evidence and can be misleading since metals might have disappeared due to other mechanisms like precipitation of adsorption to the surface of the vessel in which the experiment was conducted. Vullo *et al.* (2008) did a biosorption assay to determine Cu, Cd and Zn content in the supernatant. Another useful method to determine metal uptake is atomic absorption spectrophotometry. Choudhury & Sar (2009) used this approach by harvesting cells from the metal supplemented uptake by centrifugation and the supernatant is then used for metal estimation. This is a valuable method that is widely used (Morley & Gad, 1995; Zafar *et al.*, 2007).

2.9 SUMMARY AND CONCLUSION

The material discussed in this chapter provides an overview and insight into the aim and main objectives that was stipulated in Section 1.2. The chapter is divided into sections dealing with the different aspects around mining, metal pollution and microbial metal tolerance. Soil as an eco-system and factors influencing soil health was firstly discussed. The next two sections summarize mining in South Africa and the effects of mining on soil as an eco-system. The effect of metal pollution on microbial communities and different bacterial resistance or tolerance mechanisms is then discussed. Another section deals with the characteristics and toxic effect of the different metals used during this study. Then a discussion on plasmid-borne metal tolerance and its association with antibiotic resistance follows.

Literature was also used to demonstrate the principles of the various methods that are available to soil bacteria. These include culture based methods, molecular identification of the isolates by means of 16S rDNA sequencing, EDX and TEM to determine metal adsorption and where metals are present in bacterial cells.

CHAPTER 3

MATERIALS AND METHODS

3.1 SAMPLING REGIME AND AREA

Samples were collected from a platinum mine tailings dam (TD) near the towns of Rustenburg and Phokeng in the North West province of South Africa, on the western limb of the Bushveld Complex (Figures 2.1 and 3.1).

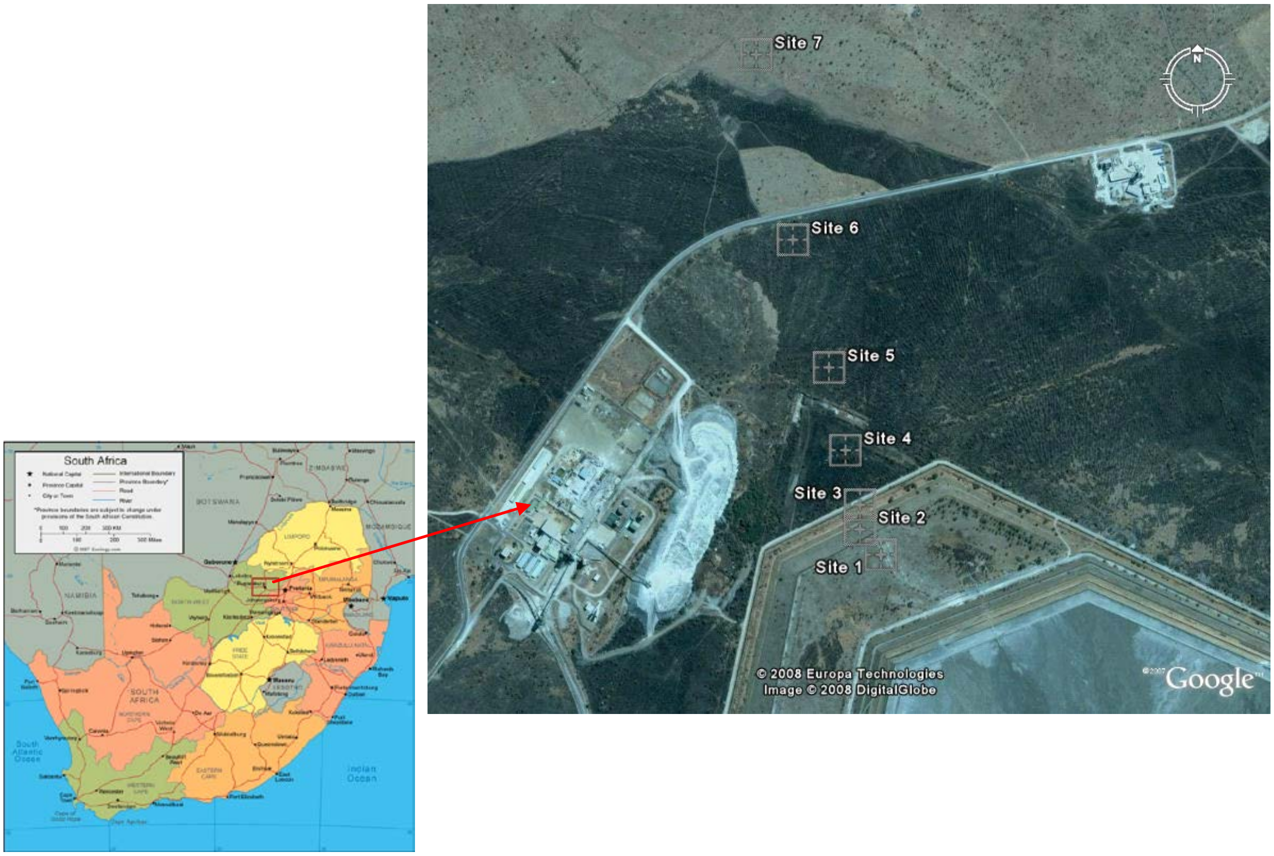


Figure 3.1 Map of South Africa and a satellite image of the tailings dam at the platinum mine near Rustenburg from where the samples were collected. The seven sites from where samples were collected are indicated.

The TD covers an area of 964ha. This region has an average rainfall of 450mm to 750mm per year and the ambient temperature can range between -6°C and 40°C in the area, with an average temperature of 19°C (Wahl, 2007). Seven sampling sites were used on and around the tailings dam and six different samples were collected from six random points, 5m apart, in each of the seven sites. The precise location of the seven different sites is indicated on Figure 3.1. Table 3.1 contains information about soil structure, pH and organic matter content (% C). Sites were situated linearly in a northern direction, downwind from the TD. Layout of the sampling areas gives an opportunity to determine if there is a gradient of pollution from the TD that affects microbiota growth and numbers in the area.

Table 3.1 Soil characteristics of the seven sampling sites (Adapted from Wahl, 2007).

		Particle size distribution				**Org. matter**	**pH values**	
Sites	**Distance**	**Sand %**	**Silt%**	**Clay%**	**>2mm**	**% C**	**Mar-06**	**May-06**
1	0m	68.30	19.00	12.70	0.0	0.14 ± 0.03^{A}	7.65 ± 0.26^{A}	7.09 ± 0.13^{A}
2	70m	76.30	13.80	9.90	0.0	0.13 ± 0.02^{A}	7.22 ± 0.09^{B}	7.05 ± 0.09^{A}
3	150m	45.90	28.00	26.10	2.3	1.01 ± 0.06^{B}	7.29 ± 0.12^{C}	7.06 ± 0.02^{A}
4	300m	28.10	27.10	44.80	11.5	1.05 ± 0.11^{B}	7.43 ± 0.08^{D}	7.04 ± 0.03^{A}
5	500m	26.40	25.40	48.20	6.1	1.19 ± 0.1^{B}	7.36 ± 0.06^{E}	6.9 ± 0.1^{B}
6	850m	32.90	16.00	51.10	5.6	1.11 ± 0.06^{B}	7.37 ± 0.12^{E}	6.84 ± 0.07^{B}
7	1350m	24.80	22.80	52.50	4.9	1.13 ± 0.07^{B}	7.47 ± 0.07^{F}	6.97 ± 0.19^{A}

A-F: Values sharing the same letter in superscript were not statistically different from each other.

Soil samples were taken from the top layers of soil (10cm) and then kept cool in Ziploc bags in a cooler box until they were analyzed. Samples were analyzed within 24h of collecting them. The samples were collected aseptically during two seasons, March 2006 for the rainy season and May

2006 for the dry season to determine if there is a seasonal variation in soil bacterial community structure.

3.2 ENUMERATION AND IDENTIFICATION OF BACTERIA

3.2.1 Isolation of metal resistant bacteria from soil

From each soil sample collected at the various sampling areas, 5g of soil was used for the isolation of microorganisms. It was diluted with 99ml phosphate buffer (pH 7.0, 0.5M phosphate, 0.8% w/v NaCl) and shaken manually for 1min. A dilution series (1:10) was then made for each of the soil samples using 1ml of the soil-buffer mixture in 9ml dH_2O. The dilutions were plated on 0.1% w/v Nutrient Agar (Biolab Diagnostics, Merck, Gauteng). Nutrient agar was enriched with aluminum and Al-Ni alloy (19.34mM final concentration). Plates were incubated at room temperature for 48h.

Standard plate count methods were used to determine bacterial levels ($cfu.g^{-1}$, colony forming units per gram) of soil tailing bacteria. Colony morphology with regards to colour, shape, size and texture was initially used to sub-divide colonies into morphotypes. The morphotypes were then named by using LD (Laurette Daniels) for all the isolates, and then a letter from the alphabet (A to V) followed by either 0306 or 0506 indicating the month and year when samples were collected. Single colonies were again sub-cultured on fresh 0.1% Nutrient Agar plates (containing the Al-Ni alloy) to obtain pure colonies. Further characteristics were determined using Gram, capsule and endospore staining on single, purified colonies (Harley & Prescott, 2002).

3.2.2 DNA extraction

A CTAB-PVP method was first attempted to obtain DNA for PCR amplification. This method is time consuming and inconsistent. A PCR template preparation kit (High Pure PCR template preparation kit from Roche Diagnostics, Mannheim, Germany) was then used. Isolates were grown overnight in 0.1% Nutrient Broth (Biolab Diagnostics, Merck, Gauteng) at 24°C in a dark incubator before extractions were performed. Instructions from the manufacturer (Roche Diagnostics, Mannheim, Germany) were followed (Appendix B).

3.2.3 Amplification of DNA

16S ribosomal DNA fragments were amplified using the eubacterial primers GM5F (5'-CCT ACG GGA GGC AGC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3') synthesized by Inqaba Biotech (South-Africa). Double concentrated PCR master mix (Fermentas, US) containing Taq DNA polymerase (0.05units/µl), dNTP (0.4mM) and Mg_2Cl (4mM) was used. PCR was performed using an I-Cycler (Bio-Rad, UK). The 25µl PCR reaction mixture contained 2 x PCR master mix, 50pmole primer mix, 100ng BSA and 100ng extracted DNA. Cycling conditions were as follows: denaturation at 95°C for 300sec; 30 cycles of 30sec of melting at 95°C, 30sec of annealing at 60°C, 60sec of extension at 72°C and final extension at 72°C for 300sec.

3.2.4 Confirmation of DNA amplification and sequence analysis

Electrophoresis was conducted using 5µl of PCR product. The agarose gels (2% w/v; Roche, Germany) contained 0.5µg/ml ethidium bromide (BioRad, UK). Each gel was also loaded with a DNA molecular weight standard (100 bp Molecular Weight Marker; Fermentas, US) to which the sizes and intensities of the template DNA bands could be compared. Electrophoresis was performed for 100min at 80V using 1X TAE buffer. Gel images were captured using a Gene Genius Bio Imaging System (Syngene, Synoptics, UK) and GeneSnap (version 6.00.22) software. Amplified

DNA fragments were sequenced by Inqaba Biotech, South Africa. BlastN searches (http://www.ncbi.nlm.nih.gov/BLAST) were used to identify the organisms using the DNA sequences.

3.3 GROWTH CHARACTERISTICS OF ISOLATES

3.3.1 Temperature growth ranges

Representatives of the identified species were inoculated on Al-Ni containing Nutrient Agar (NA) slants, in duplicate. Inoculated slants were incubated at 5°C, 24°C, 30°C, 37°C, 40°C and 45°C in various dark incubators for 48h and then visually checked for growth.

3.3.2 Minimum Inhibitory Concentration (MIC's)

The same representatives used above were used to determine the MIC's. The broth cultures of isolates were inoculated in a 96 well plate in a broth containing varying concentrations (0.75 to 5mM) of the selected metals (Table 3.2). The wells contained a final volume of 100µl each as shown in Table 3.2. The experiment was performed in duplicate over a period of 48h. Readings were taken every 12h using a Microwell 96 well plate reader. Absorbance was read at 520nm to determine the effect of the different metals at different concentrations on the various isolates. From the absorbance values growth for the different organisms in each of the metals was determined.

The absorbance values for each 96 well plate were read with the Microwell 96 well plate reader and then transferred to an Excel file (Microsoft Office, 2003). Growth rate (µ) was determined using the method described in (Lester & Birkett, 1999).

3.4. ELECTRON MICROSCOPY

3.4.1 Electron dispersive X-ray analysis (EDX)

Transmission electron microscopy and EDX analysis was used to determine the location of metal absorption activity within the cells. Cells from the different isolates were first grown at room temperature for 24h on 0.1% NA plates containing the different selected metals. Cells were then scraped from the media into 1.5ml centrifuge tubes and then washed five times with dH_2O. After washing the cells they were put onto carbon coated nickel studs (Tsezos *et al*., 1997; Thomson *et al.*, 2011). These studs were used for EDX analysis using the FEI Quanta 200 ESEM and the Oxford INCA X-sight 200 EDX System. Using electron detection beams the amount of metals inside cells was determined. Organisms were also grown on 0.1% NA without any metals added and these served as controls.

3.4.2 Transmission electron microscopy (TEM)

Cells from the different isolates were first grown at room temperature for 24h on 0.1% NA plates containing the different selected metals. Bacterial cells were inoculated into water agar, cut into small blocks and then fixed in Todd's fixative solution overnight. The agar blocks were then washed three times with 0.05M Cacodilate buffer for 15min each. After washing, the agar was dehydrated with acetone of increasing concentrations (70%, 90%, 100%). The agar was incubated at each concentration for 15min. Afterwards samples were put into resin and allowed to dry overnight. Samples were then embedded into fresh resin in a flat mould and cured in an oven at 65ºC overnight (Thomson *et al.*, 2011). The whole procedure was performed inside a fume cabinet. Samples were cut using a Reichert-Jung Ultracut E to prepare them for TEM.

3.5 PLASMID EXTRACTIONS

Plasmids were extracted from each species using a PeqGold Plasmid Miniprep Kit 1 (PeqLab – Biotechnology, Germany), following the instructions from the manufacturer (Appendix B). The plasmid samples were electrophoresed on a 0.8% w/v agarose gel using 1X TAE electrophoresis buffer. Fifteen microliters of the samples were mixed with 25 µl of loading dye and loaded into the wells. Electrophoresis was performed for 90 minutes in 1X TAE.

3.6 STATISTICAL ANALYSIS

Averages and standard deviations were calculated for the levels of metal tolerant microorganisms found and their MIC. Minimum inhibitory concentration values were represented in appropriate graphical presentations showing the differences in tolerance between the isolates found. Sigmastat 2.0 (Jandel Corporation) was used to run significance tests. Isolate numbers between the different sites were compared using One Way ANOVA on Ranks and the Kruskall-Wallis test (P=<0.05).

CHAPTER 4

RESULTS

4.1 ISOLATION AND ENUMERATION

Bacteria were cultured from the soil samples and grown in the media containing the Al-Ni alloy. Growth was observed within the first 24h of incubation at room temperature (±25°C). After grouping them into different morphotypes, the number of isolates from each of the six samples per site was determined using plate count methods. This was then used to calculate the $cfu.g^{-1}$ of soil in each sample and the average as well as total $cfu.g^{-1}$ was determined per site. Based on these calculations Figure 4.1 was used to graphically indicate which sites had higher growth. Statistical analysis showed that there is a significant difference ($p < 0.05$) between $cfu.g^{-1}$ of soil isolated in each site for the rainy season compared to the dry sampling season. There was also a significant difference ($p < 0.05$) in $cfu.g^{-1}$ of soil during the two seasons for the different sites. Bacterial numbers for the different sites decreased in the following order during the rainy season site 3> site 4> site 2> site 1> site 7> site 6 > site 5 and for the dry season site 1> site 5> site 3> site 2 = site 7> site 4> site 6>.

There were some differences in types and numbers of species isolated during the two different sampling periods (Table 4.1 and 4.3). During the warmer, rainy season (March 2006) the highest growth numbers were found in sites 3 and 4 while the lowest number was in site 5 (Figure 4.1). In contrast, the highest number of isolates was found in site 1 and the lowest number in site 6 during the dry season (May 2006).

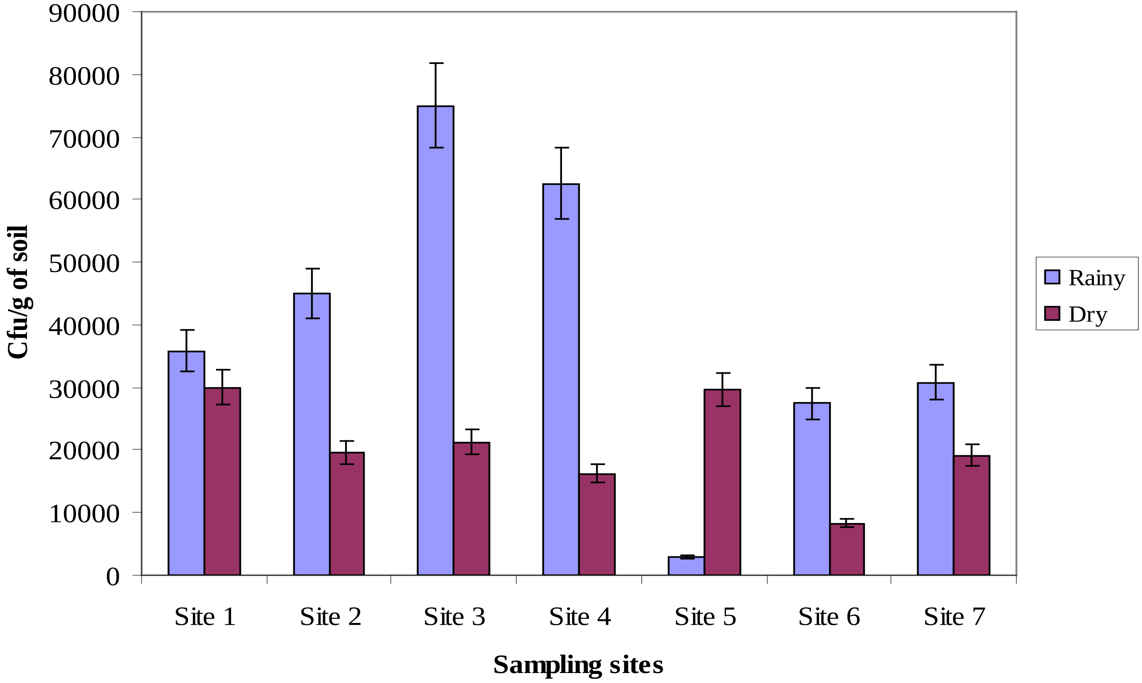


Figure 4.1 Different levels in colony numbers (cfu.g^{-1}) for the different sample sites during the two sampling periods.

Colony forming units for each of the organisms identified are indicated in Table 4.1. From Table 4.1, numbers for each of the identified strains found in the different sampling sites can be seen. In general more colonies were isolated from the soil during the warmer, rainy months in the first sampling period. As Figure 4.1 clearly demonstrates, the warm and rainy season had higher bacterial numbers except in site 5 where colony numbers were much higher during the dry season, but during the rainy season bacterial numbers in this site were extremely low. The number of metal tolerant species was higher in the area just next to the TD.

Table 4.1 Levels of species isolated, enumareated and identified in each of the sites.

	Morphotype	**Site 1**	**Site 2**	**Site 3**	**Site 4**	**Site 5**	**Site 6**	**Site 7**
Paenibacillus lautus	**LDB0306**	1666.67 ± 4082.48	12916.67 ± 11114.93	833.333 ± 7691.79	416.67 ± 1020.62	0 ± 0	3333.33 ± 3763.86	1250 ± 136931
Paenibacillus lautus **strain DS19**	**LDE0306**	416.67 ± 1020.62	4166.67 ± 4654.75	3333.33 ± 7011.90	47083.3 ± 56798.25	0 ± 0	13333.33 ± 10327.96	3750 ± 4937.10
Paenibacillus lautus **strain DS19**	**LDJ0306**	416.67 ± 1020.621	0 ± 0	416.67 ± 1020.621	0 ± 0	0 ± 0	416.67 ± 1020.621	0 ± 0
Paenibacillus lautus **strain DS19**	**LDN0306**	1250 ± 3061.86	7916.67 ± 17059.94	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Paenibacillus lautus **strain DS19**	**LDA0506**	17083.33 ± 24516.15	5000 ± 7905.67	14383.33 ± 22439.73	16250 ± 34049.6	25416.67 ± 45508.7	6250 ± 2622.02	6250 ± 6274.95
Paenibacillus lautus **strain DS19**	**LDD0506**	833.33 ± 1290.99	1666.67 ± 3027.65	0 ± 0	0 ± 0	1250 ± 3061.86	416.67 ± 1020.62	0 ± 0
Paenibacillus lautus **strain DS19**	**LDJ0506**	2083.33 ± 5103.10	1666.67 ± 3027.65	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Paenibacillus lautus **strain DS19**	**LDL0506**	416.67 ± 1020.62	3333.33 ± 5845.23	1250 ± 1369.31	0 ± 0	416.67 ± 1020.62	0 ± 0	7916.67 ± 13547.76
Paenibacillus lautus **strain DS19**	**LDN0506**	1250 ± 1369.31	0 ± 0	0 ± 0	0 ± 0	833.33 ± 2041.24	833.33 ± 1290.79	2916.67 ± 7144.35
Paenibacillus lautus **strain DS19**	**LDP0506**	2916.67 ± 7144.35	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Paenibacillus lautus **strain DS19**	**LDQ0506**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0	2083.33 ± 2457.98
Paenibacillus **sp. C15**	**LDF0306**	22916.67 ± 47917.03	4583.33 ± 5571.51	3333.33 ± 7011.9	3750 ± 7026.74	1250 ± 1369.31	2916.67 ± 3679.9	12083.33 ± 23686. 32
Uncultured *Paenibacileae*	**LDG0506**	416.67 ± 1020.62	2916.67 ± 7144.35	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
Uncultured *Paenibacileae*	**LDI0506**	4583.33 ± 5103.10	2916.67 ± 4587.12	5416.67 ± 8126.60	0 ± 0	1666.67 ± 4082.48	833.33 ± 2041.24	0 ± 0
Stenotrophomonas maltophilia	**LDC0306**	7916.67 ± 7317.22	2916.67 ± 4587.12	21666.67 ± 33862.47	0 ± 0	0 ± 0	0 ± 0	4583.33 ± 5571.51
Stenotrophomonas maltophilia	**LDS0306**	0 ± 0	0 ± 0	5000 ± 5000	5416.67 ± 5342.44	833.33 ± 1290.99	3750 ± 4677.07	6250 ± 8909.27
Bacillus **sp. KDNB5**	**LDH0306**	0 ± 0	0 ± 0	29583.33 ± 44705.05	4583.33 ± 4306.00	0 ± 0	2916.67 ± 7144.35	2083.33 ± 4005.21
Bacillus **sp. KDNB5**	**LDM0306**	0 ± 0	12500 ± 13693.00	2916.67 ± 2922.61	416.67 ± 1020.621	416.67 ± 1020.621	416.67 ± 1020.621	416.67 ± 1020.621
Bacillus subtilis **strain DN-10**	**LDK0306**	416.67 ± 1020.621	0 ± 0	416.67 ± 1020.621	0 ± 0	416.67 ± 1020.621	416.67 ± 1020.621	416.67 ± 1020.621
Bacillus cereus	**LDV0306**	833.33 ± 2041.24	0 ± 0	0 ± 0	833.33 ± 2041.24	0 ± 0	0 ± 0	0 ± 0
Bacillus cereus	**LDL0506**	416.67 ± 1020.62	3333.33 ± 5845.23	1250 ± 1369.31	0 ± 0	416.67 ± 1020.62	0 ± 0	7916.67 ± 13547.76
Alcaligenes **sp. DJWH 146-2**	**LDM0506**	0 ± 0	2083.33 ± 2457.98	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0

The isolated bacteria displayed a wide variety of morphological characteristics after a few days of incubation, including bright and significant colours, colony texture and shape. Colony morphology for a few of the isolated strains can be seen in Figure 4.2.

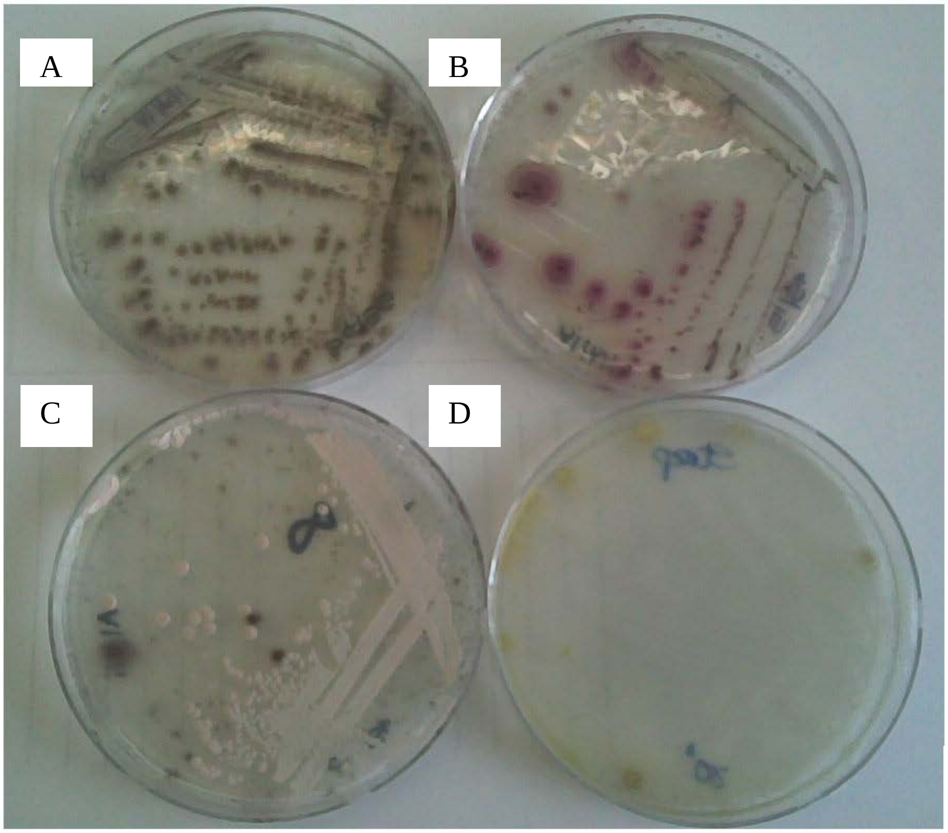


Figure 4.2 Some of the characteristics displayed by 4 different types of isolates.

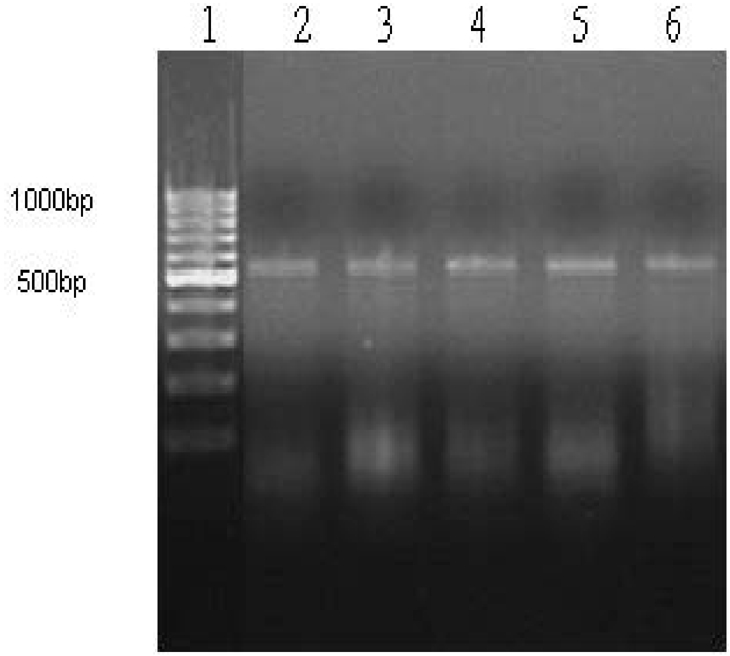
The isolates seen in Figure 4.2 were identified as A.) *Bacillus subtilis* (LDK0306) brown, irregular and woolly , B.) *Bacillus cereus* (LDO0506) deep purple, C.) *Paenibacillus lautus* (LDD0306) smooth, pink and D.) *Paenibacillus* sp. C15 (LDF0306) light yellow and smooth. These morphological characteristics (Figure 4.2) as well as the Gram stain (Table 4.3) made identification of different strains into morphotypes possible. However, 16S rDNA sequencing analysis provided a more accurate identification. Based on the morphological characteristics and staining results 22 isolates were initially identified during the rainy sampling season and 21 isolates for the dry sampling season.

4.2 IDENTIFICATION OF METAL TOLERANT BACTERIA

The DNA extraction done using the CTAB-PVP method was time-consuming and PCR amplification of DNA isolated using this method was inconsistent. Excessive shearing and the presence of primer dimers were found in the PCR when this method was used. A kit was then obtained and better results for further identification was obtained.

DNA isolation using the Fermentas High Pure DNA extraction kit yield DNA of high quality and was less time-consuming. The sequencing results demonstrated that nine different species were isolated from the area in and surrounding the mine tailings dam. Most isolates belonged to the genus *Paenibacillus* (Table 4.2 and Figure 4.5). Some isolates of the same species expressed different characteristics though. These included different temperature ranges and colony colour and texture, which may be because they belong to different strains of the same species. One option is to further characterize species by protein analysis. This can be done to investigate the expression of metal-binding peptides and proteins as was done by Sauge-Merle *et al.* (2012).

Amplification of the 16S rDNA was successfully done. In Figure 4.3 the molecular marker (O'GeneRulerTM 1000bp DNA Ladder, Fermentas Life Sciences, US) can be clearly seen in Lane 1 and fragments of 550bp (Lanes 2-6). There was no non-specific amplification. A few primer dimers can be seen in Figure 4.3 though.



1. Ladder
2. LDV0306 (*B. cereus*)
3. LDB0306 (*P. lautus*)
4. LDS0306 (*S. maltophilia*)
5. LDH0506 (*Bacillus* sp.)
6. LDE0306 (*P. lautus*)

Figure 4.3 A 1.5% ethidium bromide stained agarose gel (w/v) showing the amplified DNA sequences of 550bp.

As can be seen from Table 4.2 large parts of the nucleotide sequences were used for identification of isolates and that all identifications matched above 90%. The chromatographs in Appendix B show clear nucleotide peaks with little background noise. Sequencing results gave accurate identification but is supported by morphological characteristics and staining results. Variations in morphology between isolates of the same species may be due to differences in their metabolisms.

The sequencing results confirmed that most of the isolates were Gram positive except for *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2. In fact, all isolated strains were Gram positive bacteria with the exception of isolates LDC0306 and LDS0306 which belonged to the *Stenotrophomonas maltophilia* species and LDM0506 which were identified as *Alcaligenes* sp. DJWH146-2. *S. maltophilia* was only isolated during the rainy season and was then present in all seven sites. The colonies were light in colour (white or light blue). The *Alcaligenes* colonies were found only in the dry season from site 2 (Table 4.1) and capsules was visible with staining.

Relationships of bacterial isolates in this study is shown in Figure 4.4. It shows that all *Paenibacilli* formed a major cluster. *Bacillus* sp. formed another cluster and the *Stenotrophomonas* and *Alcaligenes* one.

Table 4.2 GenBank identification of Al-Ni alloy tolerant species found in this study in the area surrounding the tailings dam.

Species	**Nucleotides used**	**GenBank ID**	**Sequence ID**	**% Similarity**	**E value**
LDA0506 GMF5	402bp	*Paenibacillus lautus* strain DS19	363/402	90	2e-141
LDB0306 GMF5	170bp	*Paenibacillus lautus*	170/173	98	3e-80
LDC0306 GMF5	487bp	*Stenotrophomonas maltophilia* strain	486/487	99	0.0
LDE0306 GMF5	464bp	*Paenibacillus lautus* strain DS19	458/464	98	0.0
LDF0306 GMF5	463bp	*Paenibacillus* sp. C15	430/462	93	0.0
LDH0306 GMF5	483bp	*Bacillus* sp. KDNB5	483/483	100	0.0
LDJ0306 GMF5	502bp	*Paenibacillus lautus* strain DS19	497/502	99	0.0
LDK0306 GMF5	462bp	*Bacillus subtilis* strain DN-10	455/464	98	0.0
LDM0306 GMF5	479bp	*Bacillus* sp. KDNB5	479/479	100	0.0
LDN0306 GMF5	507bp	*Paenibacillus lautus* strain DS19	453/495	91	0.0
LDS0306 GMF5	500bp	*Stenotrophomonas maltophilia* strain	498/500	99	0.0
LDD0506 GMF5	443bp	*Paenibacillus lautus* strain DS19	435/438	99	0.0
LDG0506 GMF5	490bp	Uncultured *Paenibacillaceae* bacterium clone	489/490	99	0.0
LDI0506 GMF5	512bp	Uncultured *Paenibacillaceae* bacterium clone	501/503	99	0.0
LDJ0506 GMF5	484bp	*Paenibacillus lautus* strain DS19	482/482	100	0.0
LDL0506 GMF5	527bp	*Paenibacillus lautus* strain DS19	510/521	97	0.0
LDM0506 GMF5	502bp	*Alcaligenes* sp. DJWH 146-2	491/503	97	0.0
LDN0506 GMF5	498bp	*Paenibacillus lautus* strain DS19	497/498	99	0.0
LDO0506 GMF5	368bp	*Bacillus cereus* strain	73/89	82	1e-07
LDP0506 GMF5	481bp	*Paenibacillus lautus* strain DS19	478/481	99	0.0
LDQ0506 GMF5	500bp	*Paenibacillus lautus* strain DS19	496/498	99	0.0

The white colonies also had endospores and only one morphotype for this strain was identified. The most isolated strain from the area was *Paenibacillus lautus* DS19. They were isolated during both the sampling seasons. During the wet season no isolates were found at site 5 and the colonies where light in colour (grey to white). In the dry season *Paenibacillus lautus* DS19 was found at all of the sites. The colonies were also light in colour (white to grey) except for LDP0506 which was a deep purple colour. This morphotype was only found at site 1. The only morphotype found in site 4 was LDA0506. One morphotype of *Paenibacillus* sp. C15 was found at all the sites. They were light yellow colonies that were only isolated in the wet season. An uncultured *Paenibacillaceae* clone was only isolated during the dry sampling period and two representatives were found. None of these were found in site 4 and 7.

Bacillus sp. KDNB5 isolates were only found in the wet season and two morphotypes were identified. LDH0306 were light green colonies and LDM0306 were smooth, white colonies that produced endospores. These two were both found in all the sites except for site 1. *Bacillus cereus* was isolated during both the sampling periods. In the wet season morphotype LDV0306 was isolated from site 1 and site 4 and colonies were green/brown and woolly. Endospores were identified through staining. During the dry season LDO0506 was isolated. Colonies were smooth and brown in colour and was only found in site 4.

One of the two Gram negative species isolated in this study was *Stenotrophomonas maltophilia* and it was only found during the wet season. There were two morphotypes identified. LDC0306 were light blue colonies and the other isolate LDS0306 had white colonies. The other Gram negative isolate, *Alcaligenes* sp. DJWH 146-2, was milky white with a blue pigment. They were only found in the dry season and a capsule was present. The only site where isolates were found is site 2.

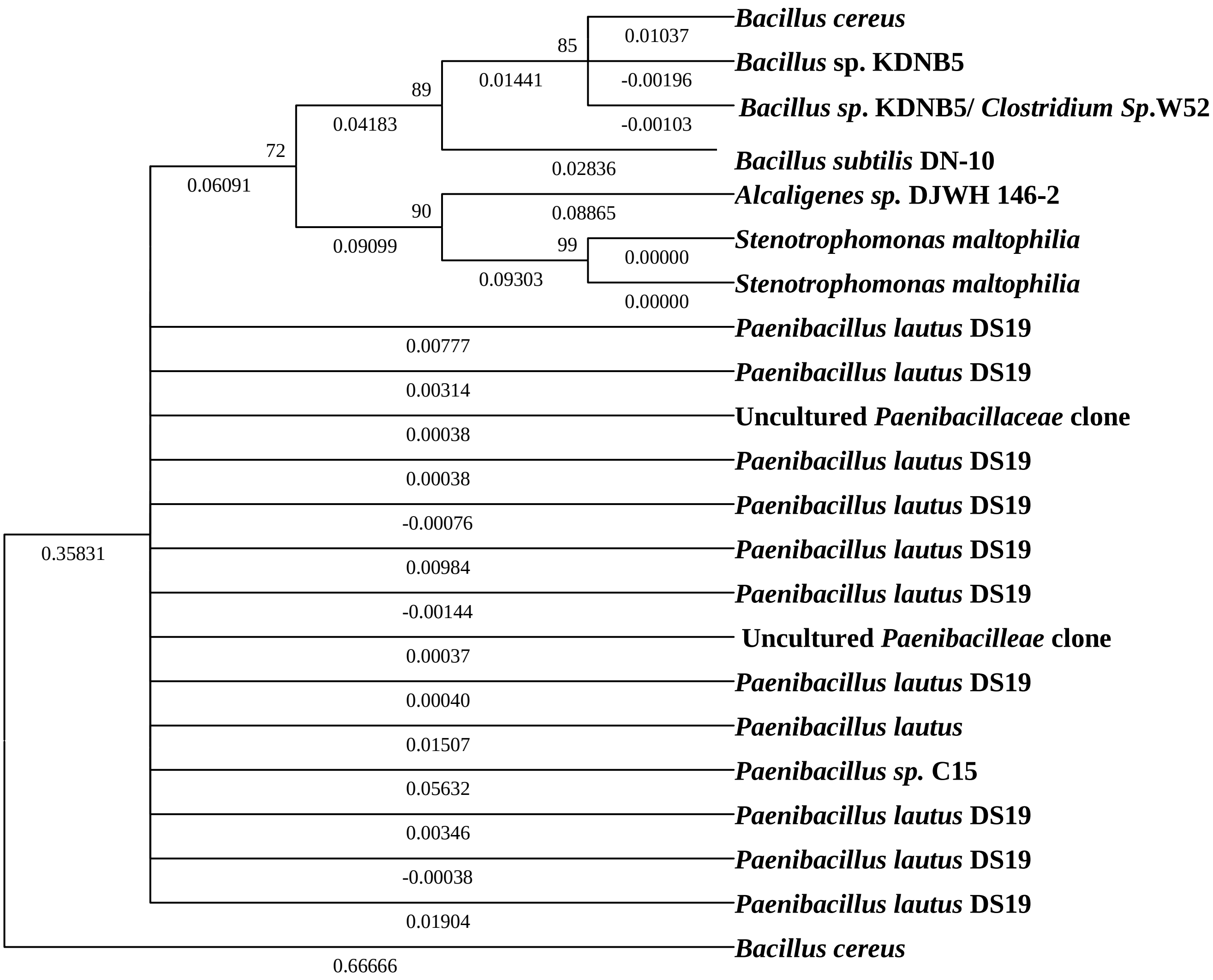


Figure 4.4 Neighbour-Joining phylogenetic tree for the metal resistant isolates from Table 4.2

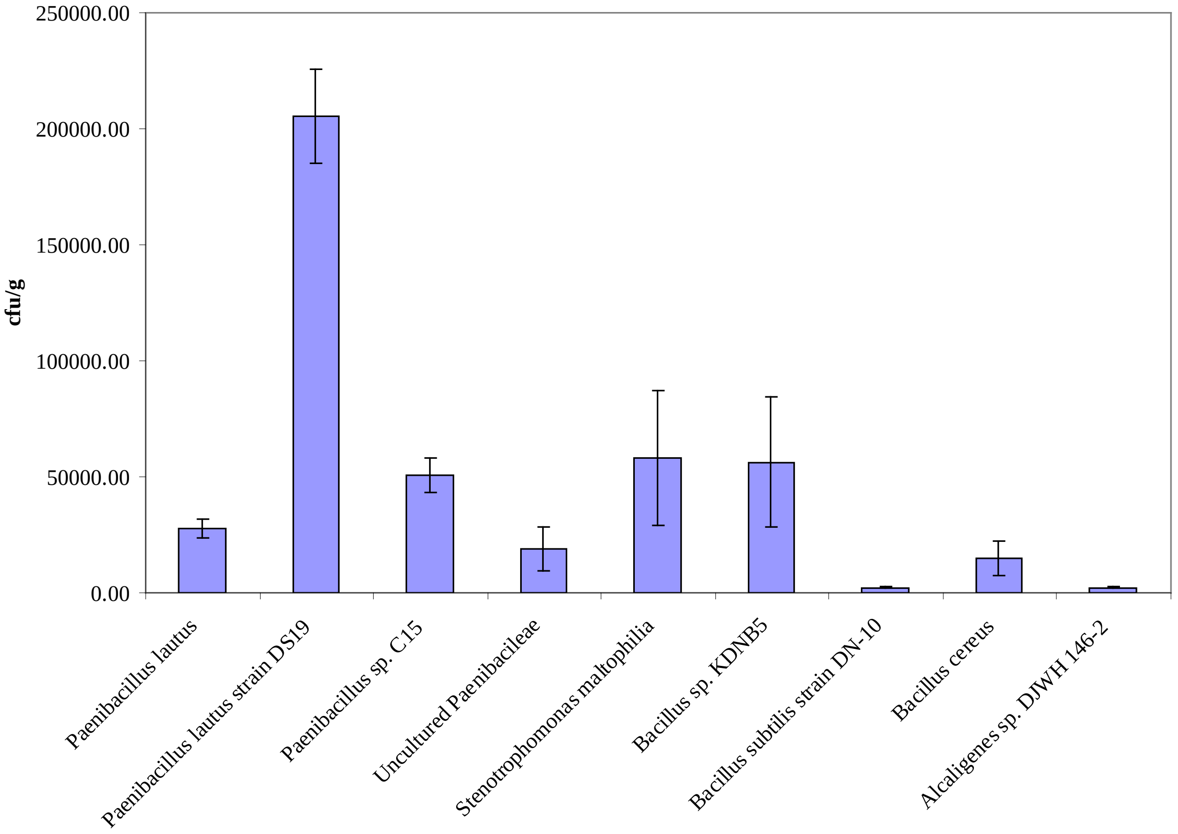


Figure 4.5 Colony forming units per gram of soil for each of the isolated strains.

Most of the isolates found during this study belonged to the group *Paenibacillus* and then *Bacillus* as Figure 4.5 shows. The most abundant isolate found was *Paenibacillus lautus* strain DS19 of which more than 200 000 cfu.g^{-1} of soil was isolated. This strain was found throughout all 7 sites during both the sampling periods. This was followed by *Stenotrophomonas maltophilia* and *Bacillus* sp. KDNB5. *Alcaligenes* sp. DJWH 146-2 was found in smaller numbers. It can be concluded then that Gram Positive isolates were more predominant than the Gram negative ones.

4.3 CHARACTERIZATION OF THE IDENTIFIED STRAINS

4.3.1 Temperature growth ranges for the isolates

Temperature growth at which selected morphotypes could grow is represented in Table 4.3. Most species were only able to grow between temperatures of 24°C and 37°C. *Paenibacillus lautus* (LDB0306), *Paenibacillus lautus* strain DS19 (LDJ0306, LDQ0506), *Bacillus subtilis* strain DN-10 (LDK0306) and *Bacillus cereus* (LDV0306) were able to tolerate low temperatures of 5°C. There were also two strains, *Paenibacillus lautus* strain DS19 (LDN0306) and *Stenotrophomonas maltophilia* (LDS0306) that were able to tolerate temperatures of up to 45°C. Both of these strains were also isolated during the warmer, rainy season. *Paenibacillus lautus* strain DS19 (LDJ0306) was able to grow at all the incubated temperatures except at 37°C.

No correlation between extremes in temperature (high or low) and endospore formation or capsules could be established. Most endospores were seen for isolates found during the rainy, warmer season though (Table 4.3). Six isolates from the rainy sampling season had endospores present, while only three of the isolates from the dry season had endospores. Capsules were found for *Alcaligenes* sp. DJWH 146-2 isolates isolated during the dry, colder month.

Table 4.3 The biochemical characteristics and temperature ranges of the different isolates found during the two sampling seasons.

						Temperatures (°C)					
Morphotype	**Isolate ID**	**Morphology**	**GS**	**S**	**C**	**5**	**24**	**30**	**37**	**40**	**45**
LDB0306	*Paenibacillus lautus*	White, round, woolly	+	Y	N/A	G	G	G	G	G	X
LDE0306	*Paenibacillus lautus* strain DS19	Grey, smooth	+	Y	N/A	X	G	G	G	X	X
LDJ0306	*Paenibacillus lautus* strain DS19	Grey edge, woolly middle	+	Y	N/A	G	G	G	X	G	G
LDN0306	*Paenibacillus lautus* strain DS19	Transparent smooth edge, grey pin	+	-	N/A	X	G	G	G	G	G
LDA05/06	*Paenibacillus lautus* strain DS19	Round, white, smooth	+	Y	N/A	X	G	G	G	X	X
LDD05/06	*Paenibacillus lautus* strain DS19	Pink, smooth	+	-	N/A	X	G	G	G	X	X
LDJ05/06	*Paenibacillus lautus* strain DS19	Pink, white edge	+	-	N/A	X	G	G	G	X	X
LDL05/06	*Paenibacillus lautus* strain DS19	White, smooth, round grey pin	+	-	N/A	X	G	G	G	X	X
LDN05/06	*Paenibacillus lautus* strain DS19	White, light, transparent smooth edge, grey pin	+	Y	N/A	X	G	G	G	X	X
LDP05/06	*Paenibacillus lautus* strain DS19	Deep purple	+	-	N/A	X	G	G	G	X	X
LDQ05/06	*Paenibacillus lautus* strain DS19	Woolly white edge, pink woolly middle	+	-	N/A	G	G	G	G	X	X
LDF0306	*Paenibacillus* sp. C15	Light yellow, smooth	+	-	N/A	X	G	G	G	G	X
LDG05/06	Uncultured *Paenibacillaceae*	Yellow, irregular	+	-	N/A	X	G	G	G	X	X
LDI05/06	Uncultured *Paenibacillaceae*	White, smooth, milky edge	+	Y	N/A	X	G	G	G	X	X
LDC0306	*Stenotrophomonas maltophilia*	Light blue, smooth	-	-	N/A	X	G	G	G	X	X
LDS0306	*Stenotrophomonas maltophilia*	White irregular	-	-	N/A	X	G	G	G	G	G
LDH0306	*Bacillus* sp KDNB5	Light green, smooth	+	-	N/A	X	G	G	G	G	X
LDM0306	*Bacillus* sp. KDNB5	White smooth, milky edge, grey pin	+	Y	N/A	X	G	G	G	G	X
LDK0306	*Bacillus subtilis* strain DN-10	Brown, irregular, woolly	+	Y	N/A	G	G	G	G	G	X
LDV0306	*Bacillus cereus*	Brown-green, woolly	+	Y	N/A	G	G	X	X	X	X
LDO05/06	*Bacillus cereus*	Smooth, milky edge, brown middle	+	-	N/A	X	G	G	G	X	X
LDM05/06	*Alcaligenes* sp. DJWH 146-2	White, smooth, milky edge, grey-blue pin	-	-	Y	X	G	G	G	X	X

GS: Gram stain; S: Endospore stain; C: Capsule stain; G: Growth visible; X: no growth

N/A: not available

4.3.2 Minimum Inhibitory Concentration

MIC analysis showed that some of the species were able to grow in most or all of the selected metals. Statistical results, however, showed that growth rate differences were insignificant (P= 0.05). Most of the strains used were able to tolerate high concentrations of most of the metals as illustrated in Table 4.4. In some cases metals seemed to stimulate growth of the bacteria at certain concentrations, others seemed to inhibit growth. This may be due to the fact that some metals like Cu, Co, Ni and Zn are essential to microorganisms at low concentration, whereas, others such as lead, cadmium and mercury are toxic even at low concentrations (Abou-Shanab *et al.*, 2007).

Table 4.4 Minimum Inhibitory Concentration (MIC) of the selected strains with the nine different metals used

Species	**Strain**	**Ba**	**Fe**	**Mn**	**Pb**	**Al/Ni**	**Cr**	**Cu**	**Co**	**Hg**
Bacillus sp. KDNB5	LDH0306	3	>5	>5	>5	>5	4.5	>5	>5	>5
Bacillus subtilis DN-10	LDK0306	>5	>5	>5	>5	>5	>5	>5	>5	4.5
Stenotrophomonas maltophilia	LDS0306	>5	>5	>5	3	>5	4	>5	>5	>5
Bacillus sp. KDNB5	LDM0306	>5	3.5	0	4.5	>5	>5	3.5	>5	4.5
Paenibacillus lautus	LDB0306	>5	>5	>5	>5	>5	>5	>5	>5	>5
Uncultured *Paenibacillaceae* clone	LDG0506	>5	>5	4.5	>5	>5	>5	>5	>5	>5
Alcaligenes sp. DJWH146-2	LDM0506	>5	>5	>5	>5	>5	>5	4.5	>5	>5
Bacillus cereus	LDO0506	3.5	>5	>5	>5	>5	>5	>5	>5	>5

Metal tolerance between the different strains was found in decreasing order as *Paenibacillus* lautus (LDB0306) > *Bacillus subtilis* DN-10 (LDK0306) = Uncultured *Paenibacillaceae* clone (LDG0506) = *Alcaligenes* sp. DJWH 146-2 (LDM0506) > *Bacillus cereus (LDO0506)* > *Bacillus* sp. KDNB5 (LDH0306) > *Stenotrophomonas maltophilia* (LDS0306) > *Bacillus* sp. KDNB5 (LDM0306).

Three *Bacillus* species were isolated during this study and demonstrated metal tolerance. Other studies have also shown that *Bacillus* strains display tolerance to certain metals (Piotrowska-Seget *et al.*, 2005; Colak *et al.*, 2011). In a study done by Piotrowska-Seget *et al.* (2005), *Bacillus cereus* isolates showed tolerance to Zn and Cu. Some strains of *Bacillus thuringiensis* with high tolerance to Cr and Cd have also been found in a study done by Hassen *et al.* (1998). The isolates were tolerant to Cu, Co and Zn, though Zn tolerance was a bit lower. All *Bacillus* sp. that were isolated in the present study were able to grow in high concentrations (>5mM) of Fe, Mn, Pb, Cr, Cu, Co and in two cases Hg.

Paenibacillus lautus (LDB0306) seems to be especially metal tolerant. It was able to tolerate all of the metals at concentration above 5mM. *Bacillus* sp. KDNB5 was not able to grow in the media containing Mn at all. This was the only metal that could completely inhibit growth of the strain. A high tolerance to Co was also found with all the strains.

4.4 ELECTRON MICROSCOPY

4.4.1 Electron dispersive x-ray analysis (EDX)

The EDX results clearly show that metals accumulated within the cells. In Table 4.5 the percentage metal (in weight) that was found in the different cells are indicated. Readings were taken for each of the eight isolates for all of the metals used. The levels of some of the metals adsorbed into or onto the cells were very low. Of the selected metals only Pb, Ba and Mn deposits could be found inside the cells. Nickel, Al, Cu, Co and Hg were present in very low percentages or were sometimes not detected at all. The microorganisms were able to grow in the presence of the metals although they are not detected inside the bacterial cell. This indicated growth in the presence of metals although there might not be specific mechanisms for metal adsorbance to the cell or uptake into the cell. Efflux mechanisms may thus be responsible for the metal tolerance traits observed.

Table 4.5 Concentration (%) of metals found in the cells (in weight).

Isolate	Strain	Metal	% metal
LDG0306	*Bacillus* sp.	Pb	10.26
LDG0506	*Paenibacillaceae* clone	Ba	7.62
LDJ0506	*Paenibacilluslautus*DS19	Ba	5.03
LDM0506	*Alcaligenes* sp. DJWH 146-2	Ba	8.63
LDS0306	*Stenotrophomonas maltophilia*	Ba	17.21
	Stenotrophomonas maltophilia	Mn	11.41
LDB0306	*Paenibacillus lautus*	Ba	1.56
LDO0506	*Bacillus cereus*	Mn	9.38
	Bacillus cereus	Fe	1.21
LDV0306	*Bacillus cereus*	Mn	13.89
LDK0306	*Bacillus subtilis*DN-10	Mn	8.83

4.4.2 Transmission electron microscopy (TEM)

The cells were not stained with uranyl or any other staining agents so that the metals would be clearly visible inside the cells as dark spots or areas. With the use of TEM, metals could be seen inside the cells, mostly near the cell surface. This probably indicates that metals enter the cell membrane and bound to proteins present in the cell membrane. From Figures 4.7- 4.9 manganese deposits are visible of the cell surface of various species. Metal uptake into cells are influenced by various aspects such as the sensitivity of cells to pH, metal/salt concentration etc.

All the strains selected for electron microscopy were also grown in media not containing any metals added, to serve as a control. By comparing the cells grown with and without metals, it was easy to identify which cells do contain metals inside their cells or on the cell after exposure to metal containing media. The cells were unstained so that when metals are retained by cells, they will be clearly visible. Figure 4.6 is an example of cells grown as a control. No metals were added to the media of these cells so none could be taken up by the cells.

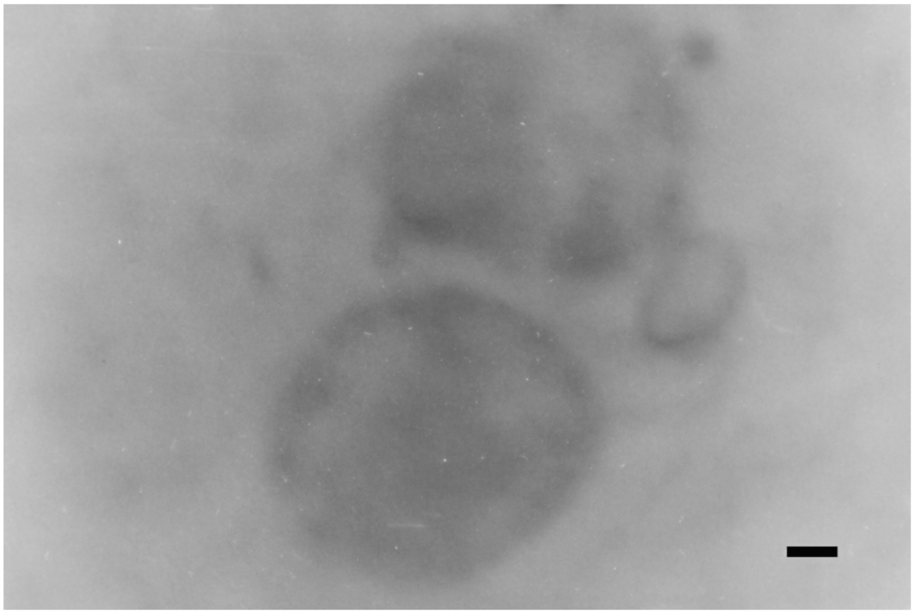


Figure 4.6 Electron micrograph of cells (*Bacillus* sp.) grown without metals in the media. (Scale of bar 0.1µm)

Metals occurring inside cells will thus be present as dark entities or spots as can be seen from the images (Figures 4.7 - 4.9). Metals were mostly seen on the cell membrane, looking almost like a crust around the cell (Figure 4.7 A). These images support the result from EDX analysis where metal content was measured and expressed as a percentage of the cell content.

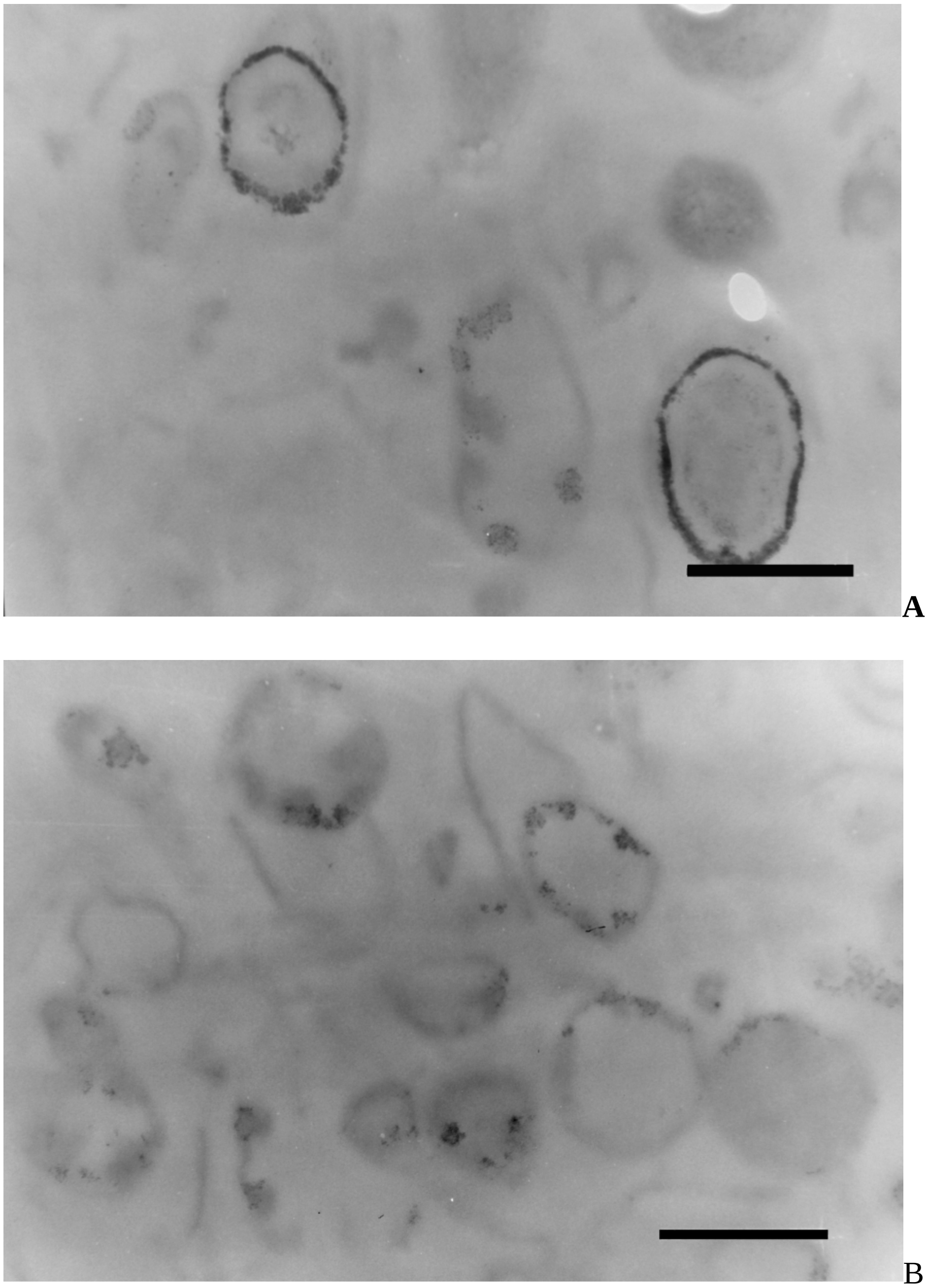


Figure 4.7 (A) and (B) Electron micrograph of *Bacillus cereus* clearly shows metal deposits on the cell membrane and some deposits inside the cells. The cells were grown on manganese enriched nutrient agar plates. (Scale of bars 0.5µm)

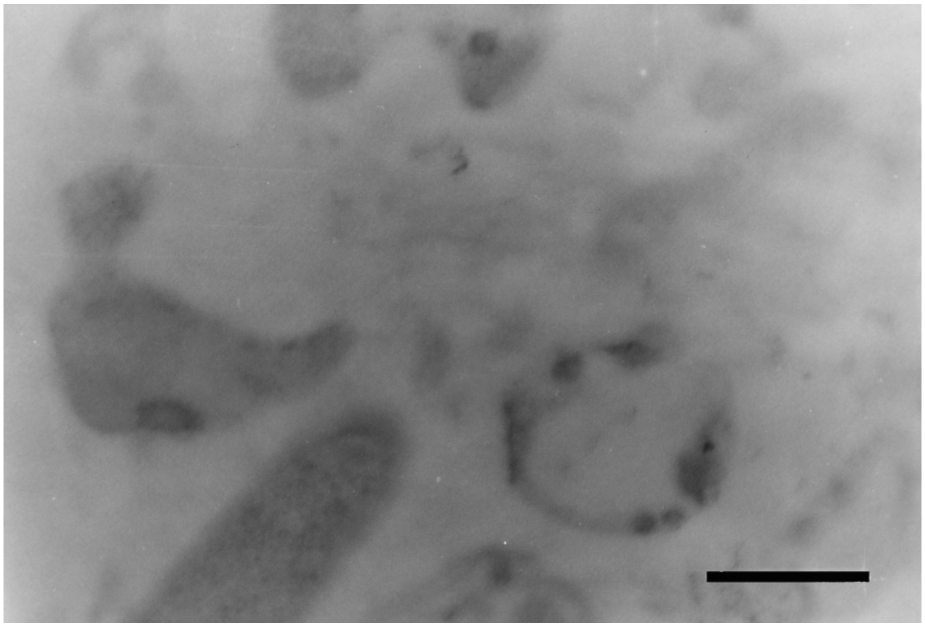


Figure 4.8 In the electron micrograph metal deposits are visible on the cell membrane of *B. subtilis*. (Scale of bar 0.5µm)

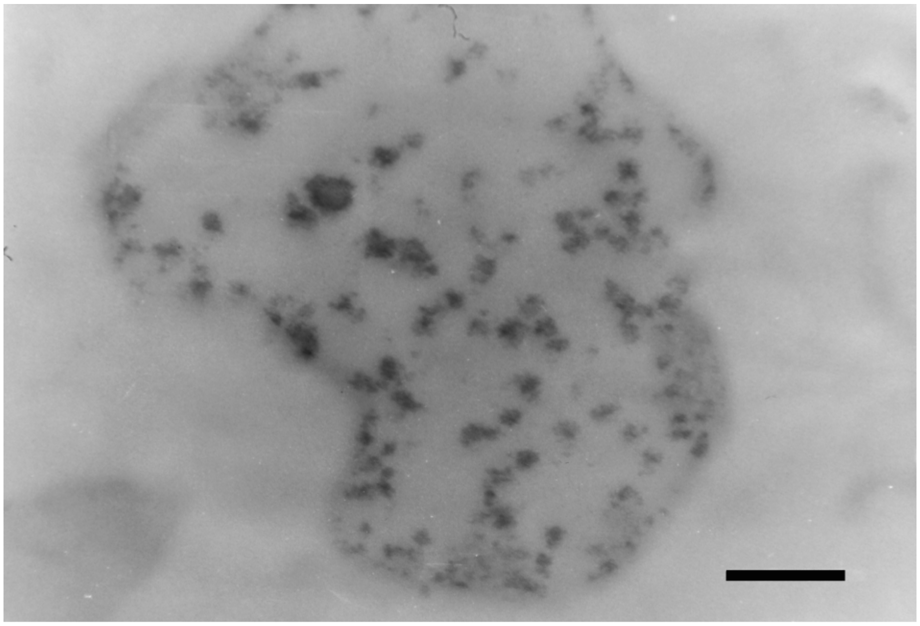


Figure 4.9 Metal deposits can be seen inside the cell of *B. cereus* as dark spots. (Scale of bar 0.5µm)

Hassen *et al*. (1998) suggested that the tolerance of certain strains to metals and their ability to adsorb them may be influenced by several factors such as isolation conditions, culturing techniques, selectivity and also the nature and specificity of the media. The possibility that other components in the media being used, react with the metals and influences data was also mentioned. These components can make the metals unavailable to the microorganisms.

4.5 PLASMID EXTRACTION

Figure 4.10 is an image of an ethidium bromide stained agarose gel (0.8w/v) that shows isolated plasmids. Plasmids extracted were around 10 000bp each in size. There was only one single band per strain, indicating that potentially only one plasmid was associated with metal resistance in all these species.

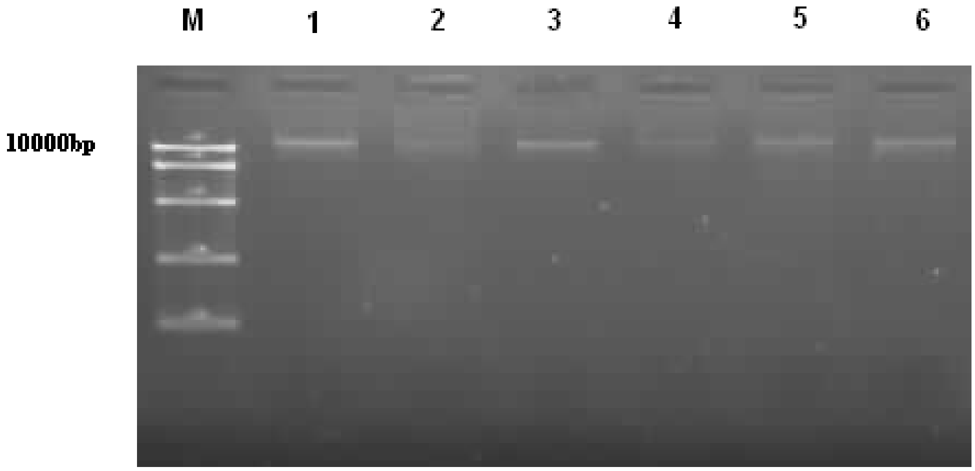


Figure 4.10 Agarose gels of plasmids that were extracted from a few selected strains. (1= 10 000bp high range genetic marker). Lane 2 contains *Paenibacillus lautus*, lane 3 *Paenibacillus* sp. *C15*, Lane 4 *Bacillus thuringiensis*, Lane 5 *Bacillus subtilis*, Lane 6 *Alcaligenes* sp. and lane 7 *Bacillus cereus.*

Plasmids from selected strains were then used to transform *E. coli* strain JM109. After transformation with the plasmids the *E. coli* expressed metal tolerance when grown on nickel-aluminum enriched NA plates.

In a recent study conducted, *E. coli* BL21(DE3)pLysSwas transformed with MerP genes from Gram positive and Gram negative strains (Kao *et al*., 2008). *Bacillus cereus* RC607 and *Pseudomonas* sp. K-62 were used in that specific study. The study showed that the host of mer operon genes was more effective biosorbents for the removal of heavy metals. Periplasmic protein merP and inner membrane protein merT expressed through this gene are cysteine-containing mercuric ion transport proteins. Although metal adsorption was detected with and without MerP protein expression, metal-biosorbent affinity was enhanced by expression of the gene. They also suggest that the origin of the MerP gene could have an effect on adsorption. In the study of Kao *et al*. (2008), the *E. coli* strains containing the gene from the Gram negative isolates showed a higher increase in metal affinity than the strains containing the MerP gene from the Gram positive isolate.

Other studies have also shown the effectiveness of other proteins in metal transport such as metallothioneins (MTs), phytochelatins (PCs) and other metal binding peptides (Malik, 2004). In a study done by Piotrowska-Seget (2005), it was found that it was mostly tolerance to Zn and Cd that was related to plasmids.

4.6 SUMMARY OF RESULTS

During this study metal tolerant bacteria were isolated from seven different sampling sites in an area surrounding a platinum tailings dam. Samples were collected during two sampling seasons, one rainy and one dry season. These isolates displayed various morphological and physiological characteristics. Based on these characteristics 22 different morphotypes were found during the rainy sampling season and 21 during the dry season. Using 16S rDNA sequences, selected isolates were identified and nine different species of bacteria were found. They were identified as *Paenibacillus lautus* strain DS19, *Paenibacillus lautus*, *Paenibacillus* sp. C15, uncultured *Paenibacillaceae*, *Bacillus subtilis* strain DN-10, *Bacillus* sp. KDNB5, *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2. Most isolates were Gram positive. Minimum inhibitory concentrations and growth temperatures were determined for these strains to characterize them further. Their MIC's for a selection of metals were determined including Al, Ba, Co, Cr, Mn, Pb, Hg, Ni and Fe. The isolates were mostly able to grow in the presence of all of these methods and the general trend seemed to be that the MIC is above 5mM. To help determine the fate of these metals inside bacterial cells, EDX analysis was used to measure the presence of metals present inside bacterial cells. Some of the cells did not have metals present inside their cells indicating that although they are able to tolerate metals and grow in their presence they do not necessarily interact with these metals or adsorb them. Transmission electron microscopy was used to determine if bacteria did however take up metals from the environment that was found in the cells by EDX.

CHAPTER 5

DISCUSSION

5.1 ENUMERATION AND IDENTIFICATION OF METAL TOLERANT STRAINS

5.1.1 Isolation and enumeration of soil bacteria

Different sampling sites were selected on and close to the tailings dam facility, up to 1350m away (see Table 3.1). These were selected to investigate if there were differences in the microbial community structures at the various sites and also to determine which metal tolerant organisms dominated at the various sites. The media that was used to isolate and purify the metal tolerant bacteria was 0.1% (w/v) nutrient agar with a high concentration (5mM) of Al-Ni alloy. Isolates were able to grow within 24h indicating that they are able to tolerate metal stress. Differences in community structure between the different sites were observed. This might have been influenced due to different soil compositions, fluctuations in soil temperature, pH and metal content. Isolates displayed various characteristics in morphology and growth temperatures.

In Figure 4.1 the bacterial levels (cfu.g^{-1}) of soil in each site is demonstrated for both the sampling periods. From this graph it is clear that higher bacterial levels were observed during the wet-warm sampling season. Each site had higher isolate numbers for this season, except for site 5. During the wet-warm season the highest bacterial levels (cfu.g^{-1}) was found at site 3 and during the dry season in site 1. Observations that indicated higher numbers of bacteria isolated during a wet-warm season could be expected, and the warmer ambient as well as the available soil water could be implicated. During dry seasons water availability and changes in metal toxicity is responsible for reduced levels of bacteria in metal contaminated soils. This aspect is demonstrated by Oliveira and Pampulha (2006) who also found that the bacteria levels were low in soils contaminated with Fe, Mn, Cu, Pb, Zn and Hg during the dry season.

Soil composition is also one of the major abiotic factors that can affect microbial community composition, because microorganisms attach to particulate matter in soils. The soil composition and particle surface nature will influence their ability to attach (Prosser, 1997). Transport of nutrients in soil depends on pore size because transport occurs mainly through diffusion (Prosser, 1997). Water plays a major role in nutrient transport. Thus, it makes sense that greater bacterial numbers and potentially greater diversity could be expected during rainy and warmer seasons. Wahl (2007) indicated that sites 1, 2 and 3 consist of high percentage sand (Table 3.1). Sites 1 and 2 were on the tailings dam and the larger particle sizes (>2mm) were because of the tailings material. The particle sizes at sites 4 – 7 were smaller (<2mm) indicating that it consisted predominantly of clay particles (Wahl, 2007). These soil characteristics may have had an impact on nutrient distribution and the ability of the bacteria to colonize and attach to particulate material in the soil. The numbers of different species isolated at sites 1-3 were greater than that at sites 4-7 and was observed during both seasons.

The pH of soil has a direct effect on the solubility of metals and affects their ability to form chelates in soil (Oliveira & Pampulha, 2006; Wu *et al.*, 2006; Wakelin *et al.*, 2008). Wu *et al.* (2006) found that a decrease in pH resulted in an increase in cation concentration in the soil solution. During their study, the solubility and mobility of metals such zinc were affected by the decrease in pH. The pH of soil can thus have an important influence on the availability of metals as a decrease in pH can increase the number of cations available to bind with bacteria and makes the metals more bioavailable to bacteria. During the June 2006 sampling period significant pH differences were observed (Table 3.1) between the sampling points 1-4 on the one hand and 5-7 on the other (Rauwane, 2009). These pH differences could thus also explain the differences in the bacterial levels that were observed.

5.1.2 Identification of isolates from the sampling sites

The different species found were identified as *Paenibacillus lautus* strain DS19, *Paenibacillus lautus*, *Paenibacillus* sp. C15, uncultured *Paenibacillaceae*, *Bacillus subtilis* strain DN-10, *Bacillus* sp. KDNB5, *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2. Details about their identification can be found in Table 4.2. Different morphotypes for most of these strains were found scattered across the tailings dam area. Most of these strains have also been identified by other studies as being metal tolerant (Springael *et al.*, 1993; Hassen *et al.*, 1998; Abou-Shanab *et al.*, 2007).

Different studies have indicated that Gram negative strains adapted better and grew under metal stress (Hassen *et al.*, 1998; Wenderoth & Reber, 1999). Benyehuda *et al*. (2003) claims that Gram negative organisms might be better able to adapt in metal contaminated soils because their cell walls might be a more efficient barrier to toxic metals. In the study done by Hassen *et al.* (1998), dominant species belonged to the genus *Pseudomonas* and *Proteus*. The opposite was found in this study, as most isolated strains were Gram positive and belonged to the genus *Paenibacillus* and *Bacillus*. This corresponds to the findings by Piotrowksa-Seget *et al.* (2005) where it was indicated that metal tolerance is more often associated with Gram positive organisms such as *Bacillus*, *Arthrobacter* and *Corynebacterium*. Piotrowksa-Seget *et al.* (2005) also mention that Gram negative organisms (eg. *Pseudomonas*, *Alcaligenes* and *Ralstonia*) can also display metal tolerance. Wenderoth & Reber (1999) also noticed a lower number of Gram negative strains were isolated, while Gram positive strains increased with higher metal concentration.

Six different strains of the two mentioned genera were identified, the most populous isolate being *Paenibacillus lautus* strain DS19 which constituted 42% of the total isolated strains. Two, Gram negative species, *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2, were isolated at low bacterial levels (cfu.g^{-1}). Isolate composition can be influenced by a variety of sampling conditions, culturing techniques and media composition (Hassen *et al.*, 1998). This may indicate that more Gram negative strains could have been found if another media was used or sampling techniques were adapted.

a) *Paenibacillus* sp.

In the present study *Paenibacillus* sp. was the mostly widely isolated genus. It was found in all 7 sites during both the sampling periods. The most commonly isolated strain was *Paenibacillus lautus* strain DS19.

Paenibacillus strains have been described as aerobic or facultative anaerobic endospore-forming bacteria that can degrade biopolymers and other polysaccharides (Shida *et al.*, 1997; Montes *et al.*, 2004). A few of the *Paenibacillus* isolates found in this study also produced endospores. This gives them a mechanism to cope with environmental stress and the ability to survive under harsh environmental conditions. Some *Bacillus* and *Paenibacillus* strains are closely related and many *Bacillus* species have general characteristics of *Paenibacillus* (Shida *et al.*, 1997).

b) *Bacillus* sp.

During the present study *Bacillus* strains were isolated during both sampling periods. Three morphotypes were found, *Bacillus* sp. KDNB5, *Bacillus subtilis* strain DN-10 and *Bacillus cereus*. Of the three, only *Bacillus cereus* was found during the dry sampling period. *Bacillus* sp. KDNB5 was not found at site 1, but high numbers were however found at the other sites, especially site 3. *Bacillus subtilis* strain DN-10 was found in site 1, 3 and 5-7 in low bacterial counts.

Bacillus species have been shown to display heavy metal tolerance in a number of studies (Hassen *et al.*, 1998; Yilmaz, 2003; Piotrowska-Seget *et al.*, 2005; Abou-Shanab *et al*., 2007). Selenska-Pobell *et al.* (1999) isolated *Bacillus* sp. from uranium waste piles that were able to tolerate a very wide variety of metals including Al, Ba, Cd, Co, Cr, Cs, Cu, Fe, Ga, Mn, Ni, Rb, Pb, Si, Sn, Ti and Zn. Abou-Shanab *et al.* (2007) also isolated four strains of *Bacillus* sp. from the rhizosphere of *Alyssum murale* and unplanted Ni-rich serpentine soil. In the study by Abou-Shanab *et al*. (2007) it was also found that these isolates displayed high MIC values to a combination of metals tested for. *Bacillus subtilis* and *Bacillus cereus* was shown to adsorb metals from solutions. In a study by Mullen *et al.* (1989) these strains adsorbed Cd and Cu from solutions. The same trend was seen by Vijayaraghavan & Yun (2008) and they described the potential of *Bacillus* species to be used as biosorbents of metals. Jezequel *et al.* (2005) investigated the bioaugmentation ability of *Bacillus* sp. for Cd and Malik (2004) also mentions their ability to take up Cd from the environment. *Bacillus* has also been described as a very efficient Cr and Ni reducer.

c) *Stenotrophomonas* sp.

During the present study, *Stenotrophomonas* sp. was isolated during the wet-warm season only. Isolates were distributed throughout sites 1-7. This was the second most isolated strain in this study.

The aerobic, non-fermentative, gram-negative *S. maltophilia* has been found widespread in the environment according to Rocco *et al.* (2009) and is a closely related relative of *Xanthomonas*. It has also been identified as a nosocomial pathogen associated with a wide variety of clinical syndromes like bacteremia and endocarditis and the ability to show resistance to a wide variety of antibiotics (Alonso *et al.*, 2000; Rocco *et al.*, 2009). Antibiotic resistance for this strain was not tested in the present study, but this might be interesting to follow up since high numbers of the strain was isolated from Al-Ni containing soil.

Genes similar to that of *Staphylococcus aureus* have been isolated from *S. maltophilia* for the regulation of Cd (Alonso *et al.*, 2000). Other studies have found that S. *maltophilia* is of high interest in bioremediation processes as it demonstrated the ability to degrade xenobiotic compounds and to detoxify high molecular weight polycyclic aromatic hydrocarbons (Pages *et al.*, 2008; Rocco *et al.*, 2009). These studies also found that this species had an intrinsic resistance to a wide variety of antibiotics. A cluster of genes for metal resistance have also been found in *S. maltophilia* D457 that seem to be isoforms of genes from *S. aureus* (Alonso *et al.*, 2000). Several other genes related to metal resistance have also been found in S. *maltophilia*. Rocco *et al.* (2009) identified several operons involved in storage, import and efflux of metals. These genes include (i) czc-genes (cobalt-zinc-cadmium resistance); (ii) genes involved in Cu metabolism and homeostasis (cop and cus operans); (iii) arsenic and Hg resistance genes. In a study by Yoon *et al*. (2006) *Stenotrophomonas*-like strain DS-16 was isolated from various environments in Korea, including soil. This strain grew at pH between 6.0 and 7.0 and at 30˚C. This was similar to trends present in this study.

d) *Alcaligenes* sp.

In the present study only one isolate was identified as *Alcaligenes* sp. and the isolate was only found in site 2 during the dry sampling period in very low numbers. Strains from the genus *Alcaligenes* have also been shown to display resistance or tolerance to a variety of metals and have been isolated from severely polluted sites (Springael *et al.,* 1993; Abou-Shanab *et al.*, 2007). Similarly the strain of *Alcaligenes* isolated in this study was from a severely polluted site and displayed resistance to all of the metals used in the MIC study. This strain had MIC values >5mM for all of the metals except for Cu for which it had a value of 4.5mM. Malik (2004) suggested that *Alcaligenes* carries large plasmids that have numerous heavy metal resistance determinants. According to Ahemad (2012) an *Alcaligenes xylosoxidans* strain has shown resistance to Ni, Cd, and Co and this was contributed to ncc systems.

5.2 CHARACTERIZATION OF IDENTIFIED MORPHOTYPES

5.2.1 Growth temperatures of isolates

Most of the bacterial strains isolated were able to grow at temperatures between 24°C and 37°C which are quite normal for soil microorganisms. A few of the strains were able to tolerate temperatures of 5°C**.** This was found with the Gram positive strains (*Paenibacillus lautus*, *Bacillus subtilis* and *Bacillus cereus*) and may indicate that they are facultative psychrophiles (Prescott, 2005). All of these strains were also the ones that produced endospores making them more resistant to harsh environmental conditions. Three of the strains were able to tolerate high temperatures (45°C). These belonged to the species *Paenibacillus lautus* strain DS19 and

Stenotrophomonas maltophilia. All of these strains were isolated during the warmer month of the rainy season.

5.2.2 Minimum inhibitory concentrations of the isolates to selected metals

The various strains displayed high MIC values for the different metals. This has also been seen in a study by Diaz-Raviña *et al.* (1994). In that study, they found that bacteria often were resistant to more than one metal and that contamination of a specific metal not only increased tolerance to that metal, but also increased tolerance to other metals occurring in the environment. Tolerance of the bacteria in this study could be expected because the soils they were isolated from contain Al, Fe, Mn, Cu, Ni, Ba, Cr, Pb, Se and Cd (Wahl, 2007). Some of these metals are also essential for bacterial growth (Cu, Co, Ni and Zn) as long as their concentrations are maintained at low levels inside cells (Hassen *et al.*, 1998; Abou-Shanab *et al.*, 2007). Many of these essential metals are taken into the cells by nutrient uptake pumps and if their concentration becomes too high they are removed again by efflux systems (Spain, 2003; Silver & Phung, 2005).

In the present study strains were also able to tolerate high concentration of Al-Ni alloy and Co. Resistance to Al could be expected since they were isolated from an area where there were high concentrations of Al (Wahl, 2007). A study by Wahl (2007) in the same area as the present showed that Al concentrations exceeded the benchmark values for microbes.

All the bacterial isolates used for MIC determinations were also able to tolerate Co concentrations above 5mM. According to Nies (1999) Co transport and detoxification in Gram negative microorganisms is often associated with RND-systems and in Gram positive microorganisms with CDF-system (both transport systems). A study done by Abdelatey *et al*.

(2011) also found that both Gram positive as well as Gram negative microorganisms showed Co resistance.

Mercury is said to be the most toxic metal to all bacteria (Spain, 2003), but most strains were also able to tolerate mercury at MIC >5mM except for *Bacillus subtilis* DN-10 and *Bacillus* sp. KDNB5 that had a MIC of 4.5mM. Ahemad (2012) describes Hg as a toxic metal that can cause protein denaturation, inhibition of various cell functions (eg. Cell division, enzyme activity and transcription), as well as cell membrane disruption. The present study did not explore the effects that the selected metals had on the different isolates, but this could be explored further in additional studies.

Paenibacillus lautus (LDB0306) had MIC values of >5mM for all of the metals. This strain was found at all sites in high numbers except site 5 where none was detected. In a study done by Abou-Shanab *et al.* (2007), strains of *Paenibacillus* also displayed tolerance to various metals. These authors also found a strain, *Paenibacillus lautus* AY509231, which displayed high MIC values for As, Cu, Ni, Pb and Zn. This is similar to the results for the *Paenibacillus lautus* strains isolated in this study.

Another strain with high MIC values is *Bacillus subtilis* DN-10 (LDK0306) which was present in samples from all the sites in the present study except sites 3 and 4. Both these strains (*Paenibacillus lautus* and *Bacillus subtilis*) also produce endospores. Various strains of *Bacillus* have been shown to have tolerance to heavy metals and exceptional heavy metal adsorption capacities. Abou-Shanab *et al.* (2007) found *Bacillus* sp. had high MIC values for Ni and Pb. These results are similar to results in this study.

In the present study *Alcaligenes* sp. DJWH 146-2 (LDM0506) and uncultured *Paenibacillaceae* clone (LDG0506) had high MIC values (>5mM). Various *Alcaligenes* strains, especially *Alcaligenes eutrophus*, have been found to be resistant to a variety of metals (Springael *et al.*, 1993; Van Roy *et al.*, 1997; Yilmaz, 2003). Abou-Shanab *et al*. (2007) isolated and tested *Alcaligenes* strains for MIC and found that these were resistant to high levels of Cd, Ni, Pb and Zn. This trend is similar to that observed in the present study. Van Roy *et al.* (1997) isolated a strain, *Alcaligenes eutrophus* var. *metallotolerans* from Zn-containing sediments that had two large plasmids (pMOL30 and pMOL28) carrying tolerance to different metals. Abou-Shanab *et al.* (2007) also tested MIC's for a *Stenotrophomonas* strain and found high tolerance to a wide variety of metals including Cd, Co, Cr, Cu, Ni, Pb, Zn and especially As (20mM). Tolerance to a high concentration of most metals was also found in this study for this species.

5.3 ELECTRON MICROSCOPY

5.3.1 Electron dispersive x-ray analysis (EDX)

With the EDX analysis it was seen that some isolates (Table 4.5) adsorbed high percentages of some of the metals, especially Pb, Ba and Mn. For most metals (Al, Ni, Cr, Cu, Co and Hg) measured uptake was lower than 1% or no metals were detected inside the cells at all. Metal uptake may have been influenced by factors such as pH of the media or media composition. Cell density, temperature and cell age can also have an influence on the cells ability to accumulate metals (Ledin, 2000). This could also indicate that although some strains were metal tolerant and were able to grow in media containing heavy metals, they did not necessarily interact with or take up any metals into their cells. Some bacterial species are able to tolerate metals present in their environment. Some bacteria even have certain strategies to prevent metals from entering cells (Hassen *et al.*, 1998).

In the present study *Stenotrophomonas maltophilia* adsorbed high concentrations of Ba (17.21%) and Mn (11.41%). Pages *et al.* (2008) also found *Stenotrophomonas maltophilia* to be tolerant to a wide range of metals and able to adsorb Cd. Accumulation was strongly associated with cell walls or incorporation into cells. Another strain with high metal accumulation was *Bacillus cereus* which was able to adsorb 13.89% Mn. Other *Bacillus* strains were also able to adsorb metals eg. *Bacillus subtilis* DN-10 adsorbed 8.83% Mn and *Bacillus* sp. adsorbed 10.26% Pb. *Bacillus cereus* was also able to adsorb Mn (9.83%) and Fe (1.21%). This study supports other findings that *Bacillus* sp. is considered to have good metal sorption capacity (Yilmaz, 2003).

When using these microorganisms for bioremediation several factors have to be considered. Strains need to be selected carefully and the nature of the media will also affect metal sorption capacity (Benyehuda *et al.*, 2003*).* Dead cell mass are not influenced by these factors but using dead cell mass requires cultivation, harvesting, drying, processing and storage prior to use. Using live mass, however avoids the need for all these processes and the system will be able to self-replenish. Based on EDX data, several of the isolates in the present study could be selected as candidates for bioremediation purposes.

5.3.2 Transmission electron microscopy (TEM)

Metals were observed on the membranes of some of the species that were analyzed by TEM. This may indicate that the metals were not actively taken into cells but that they are bound to cell structures such as proteins and become entrapped in the cell membrane. Metals may be attracted to the cell structures due to opposite charges of chemical functional groups. The ability to adsorb metals is valuable and could potentially be used to remediate metal polluted sites or water. These microorganisms have the possibility to be used in reactors as biosorbent materials.

Only two of the strains found in this study were able to adsorb metals from the media and take them into cells. Various factors could have influenced metal uptake as discussed previously (Benyehuda *et al.*, 2003*).* The fact that metals were not detected in the cells of all the species supports the data from EDX analysis and the conclusion that although they were able to grow and tolerate the metals and that the bacterial cells did not necessarily interact with them. Species where metals were detected on or inside cell walls include *Bacillus cereus* that had Mn deposits on the cell wall and *Bacillus subtilis*. In a study by Wu *et al.* (2006), Pb was found to bind to bacterial cells via adsorption and precipitation processes. In another study by Malik (2004), metal deposits of Ni were found in the cell wall as well as inside the cells of *Aspergillus niger*. Through X-ray and electron defraction analysis the conclusion could be made that Ni was accumulated as nickel oxalate dehydrate crystals. Cadmium uptake into the cells of *Bacillus* strain H9 and *Pseudomonas* strain H1 was also detected. The resistance of these two organisms was linked to plasmid-coded genes and the presence of methallothionein.

Bacillus strains isolated in this study showed the ability to tolerate metals like Pb, Mn and Fe. In a study done by Abou-Shanab *et al*. (2007) several *Bacillus* strains also displayed the ability to adsorb a variety of metals like As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn. Malik (2004) also detected Cd and Pb inside the cell of *Bacillus* sp.

5.4 PLASMIDS

Plasmids isolated may carry the genes that these organisms use for tolerance. In this study, plasmids isolated from different strains seemed all to be of the same size. In the study by Piotrowska-Seget *et al.* (2005), plasmids also seemed to be of the same size. In this study (Piotrowska-Seget *et al*., 2005) resistance to two of the metals used, Zn and Cd, was associated with plasmids. In some strains of *Alcaligenes* pMOL28 and pMOL30 plasmids have been isolated that carry multiple resistance determinants for a selection of metals (Taghavi *et al.*, 1997). Results from the present study show that plasmids were present in the various metal tolerant strains. The study has not conclusively demonstrated that the metal tolerant traits are associated with plasmids.

If tolerance mechanisms are expressed through plasmids, manipulation and enhancement of these strains will be useful. If their ability to absorb metals can be enhanced and their selectivity increased these would be suitable for use in biological remediation procedures. All isolates showed that they would be able to survive with fluctuations in temperature, nutrient levels, pH and salt concentrations. These characteristics would make control mechanisms less of a concern.

Similar studies have also shown that antibiotic resistance factors are often plasmid-borne and that these tend to be on the same plasmids as resistance factors for metals such as Hg on clinical isolates (Ugur & Ceylon, 2003). Ugur & Ceylon (2003) used strains of *Staphylococcus* sp., a nosocomial pathogen, to investigate the occurrence between plasmids and resistance to antibiotics (Penicillin G, amakacin, clindamycin, gentamycin and oxacallin) and metal tolerance ($K_2Cr_2O_7$ and $Pb(CH_3COO)_2$ and $AgNO_3$). Most of the strains showed resistance to these compounds, except for $AgNO_3$ to which most was sensitive. Future studies on bacterial isolates from platinum mines should consider establishing links between plasmids diversity and metal tolerance.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

This study was conducted to isolate and characterize metal tolerant bacterial strains from a platinum mine tailings disposal facility and the surrounding area. In order to do so, five objectives were undertaken. A brief discussion of the trends and conclusions for each objective is provided below.

6.1.1 Enumeration of metal tolerant bacteria

Metal tolerant bacteria were easily cultured even though the growth media used was low in nutrient content and contained high levels of Al-Ni alloy. The isolated strains also displayed metal tolerance to various heavy metals. Sampling was done during two seasons: a warm and wet season and a dry and cold season. More isolates were found during the rainy season (March 2006) than during the dry season (May 2006). This may indicate that conditions were harsher during the second sampling period due to lower temperatures and low soil moisture content. There were also some differences in colony numbers from the different sites. Sites 1 – 3 seemed to have higher colony numbers in general. At site 5 bacterial numbers were extremely low during the rainy sampling season, but during the dry season it had the second highest number of bacteria. Significant differences in bacterial growth during different seasons were also observed in a parallel study done by Rauwane (2009).

Twenty two different morphotypes were found in the different sites. Isolates displayed a variety of morphological characteristics and could grow under different temperatures. Gram positive as well as Gram negative species was isolated during this study. Some of the Gram positive isolates also produced endospores.

6.1.2 Identification using phenotypic and sequencing data of 16S RNA genes

With the use of 16S rDNA sequences nine different bacterial strains isolated from the soil samples, were identified. All identification matches were above 90% and chromatographs showed clear nucleotide peaks, indicating good quality DNA sequencing data. Species were identified as *Paenibacillus lautus* strain DS19, *Paenibacillus lautus*, *Paenibacillus* sp. C15, Uncultured *Paenibacillaceae*, *Bacillus subtilis* strain DN-10, *Bacillus* sp. KDNB5, *Bacillus cereus*, *Stenotrophomonas maltophilia* and *Alcaligenes* sp. DJWH 146-2. The most populous species found was *Paenibacillus lautus* strain DS 19. The species isolated in this study have also been shown in various other studies to display metal tolerance.

Although quite a large number of bacteria were isolated from the soil in this study, most species present in soil are not culturable, or the sampling and culturing techniques will not be sufficient to isolate all possible species. It has been estimated that only 1% of all soil organisms are culturable (Malik *et al.*, 2008; Upchurch *et al.*, 2008). Molecular assays, that are culture-independent, like profiling of soil DNA, rRNA and phospholipid fatty acids will give a better indication of bacterial community compositions and dynamics (Margesin *et al.*, 2011). Some methods that could be applied for this, is DGGE or TGGE and FISH (fluorescence in situ hybridization). These methods separate amplified rDNA fragments of the same length according to their base pair composition (Malik *et al.*, 2008). The number of bands that are produced will be proportional to the species composition, but the possibility also exists that community structure can be under-estimated (Malik *et al.*, 2008).

6.1.3 Characterization of isolates by determining optimum growth temperatures and MICs

Most strains were able to grow rapidly at temperatures from 24°C - 37°C. A few Gram negative strains were also able to grow at 5°C. Two of the isolates, *Paenibacillus lautus* strain DS19 and *Stenotrophomonas maltophilia,* were also able to grow at 45°C. Being able to grow over large temperature ranges that change over short temperature intervals is an important characteristic of soil bacteria. Ambient conditions in soil could change rapidly depending on environmental conditions.

Minimum inhibitory concentrations were determined for Al, Ba, Co, Cu, Cd, Cr, Fe, Mn, Hg and Ni. Most strains were able to tolerate high concentrations (>5) of most of these metals. *Paenibacillus lautus* displayed high MIC's (>5mM) for all the metals. All the isolates were able to grow with 5mM Al-Ni alloy and also at 5mM of Co.

6.1.4 Transmission electron microscopy (TEM) and electron dispersive x-ray analysis (EDX)

The results from EDX analysis showed that although the species isolated were able to grow in the presence of these metals, the metals were not taken up or adsorbed onto cells by all of them. Lead Ba, Mn and Fe could be detected inside cells when EDX analysis was used. *Stenotrophomonas maltophilia* had a high percentage of both Ba (17.21%) and Mn (11.41%) inside its cells.

TEM images of the cells confirmed the results from the EDX analysis. Only a few strains had metals present in or on their cell structures. Images comparing cells grown with and without metals were used to help determine whether metals are present inside cells. On most images metals are seen as dark deposits on cell walls. This confirms that metals are taken up into cell structures by some mechanism. Further investigations should be conducted to clarify how this process occurs.

6.1.5 Investigation if plasmids may be responsible for the metal tolerance trait

Plasmids from a number of the isolates were obtained but they were not classified. Further characterization of the isolated plasmids should be done to classify these. Plasmid characterization can be used to determine the specific genes responsible for tolerance and metal uptake mechanisms that are present. This information can also be used to genetically engineer these organisms to develop a higher uptake rate or higher tolerance, increase their selectivity and accumulating properties. Such genetically modified organisms will be useful in bioremediation studies. The plasmids can also be used to manipulate other organisms, as was done with *E. coli*, to become metal tolerant. This will help to understand the resistance mechanisms used by different species and can be used to enhance strains. Further electron microscopy could be conducted to determine exact location of the different metals inside the bacterial cells. This may also assist in a better understanding of the mechanisms involved in metal uptake.

6.2 RECOMMENDATIONS

1. Investigating bioremediation potential of the isolates

With the growing interest in biological treatment methods, the possibility to use these strains in reactor systems as biosorbent material could be useful. Biological treatment methods have various advantages. They are naturally occurring biocatalysts, with low hazardous waste emissions, “low-cost” and “low-tech” systems and limited secondary pollutants (Ahemad, 2012). These systems do, however, require control measurements to achieve optimal process concentrations, are susceptible to environmental changes, have limited tolerance to toxic compounds and require long time periods (Brandl and Faramarzi, 2006).

2. Further explore the relationship between antibiotic resistance and metal tolerance

Antibiotic resistance is a public health concern. Certain heavy metals (eg. $AgNO_3$, $CuSO_4$, $HgCl_2$ and $ZnSO_4$) have antimicrobial properties and are used as antiseptics and disinfectants (Ugur & Ceylan, 2003). Some studies have found that many antibiotic resistance genes are associated with genes related to metal tolerance and these are often plasmid-borne (Ugur & Ceylan, 2003; Qing *et al.*, 2007a). Ugur and Ceylan (2003) established that there is a relationship between metal tolerance and antibiotic resistance in *Staphylococcus* species that they isolated from clinical specimen.

3. The effect of the different metals used during this study have on bacterial cell activities can be investigated

Soil microbial communities can be affected by long-term exposure to heavy metals. Several studies have shown that metal exposure can have an effect on microbial activity, biomass, respiration and the structure of soil bacterial communities (Stefanowicz *et al.*, 2008; Upchurch *et al.*, 2008; Stefanowicz *et al.*, 2009; Wang *et al.*, 2010; Abdelatey *et al.*, 2011). This can in turn; affect ecosystem processes (eg. organic matter decomposition, nutrient turnover etc.) because of deterioration in the functions performed by soil bacterial communities (Wakelin *et al.*, 2008; Wang *et al.*, 2010). Thus, monitoring soil microbial communities can serve as an indicator of soil health and changes in soil conditions. Dehydrogenase activity and soil ATP content is two commonly used parameters to determine the effects of metal pollution (Oliveira & Pampulha, 2006).

4. Characterization of the isolated plasmids

Various studies have shown that horizontal gene transfer is largely responsible for adaptive traits in microbes (Ansari *et al.*, 2008). This can result in the spread of multiple antibiotic and metal resistance genes to native soil populations. Characterization of the plasmids found in this study can give a better understanding of tolerance mechanisms and transfer of these traits in the environment.

5. Determining metal tolerance mechanisms of the different isolates

Various metal tolerance mechanisms have been expressed in different bacterial strains (Silver and Phung, 1996; Abdelatey *et al.*, 2011; Ahemad, 2012). Understanding the mechanisms used by the strains used in a specific study can help optimize the use of these strains in bioremediation applications.

This study has demonstrated that bacterial strains from platinum mine tailings and the surrounding area had the ability to tolerate high levels of certain metals, that some could adsorb some of the metals and that plasmids may be involved in the metal tolerance trait. The main aim of the study was thus achieved by the successful execution of the five objectives.

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APPENDIX A

Table A.1 The volumes used for the different concentrations of metals

	B											**C**
Target [metal]	**0**	**0.75**	**1**	**1.5**	**2**	**2.5**	**3**	**3.5**	**4**	**4.5**	**5**	**0**
V metal	0	7.5	10	15	20	25	30	35	40	45	50	0
V diluent	100	82.5	80	75	70	65	60	55	50	45	40	90
V Bacteria	0	10	10	10	10	10	10	10	10	10	10	10
V Final	100	100	100	100	100	100	100	100	100	100	100	100

V: volume; B: Blank; C: Control

MINIMUM INHIBITORY CONCENTRATION LINE CHARTS

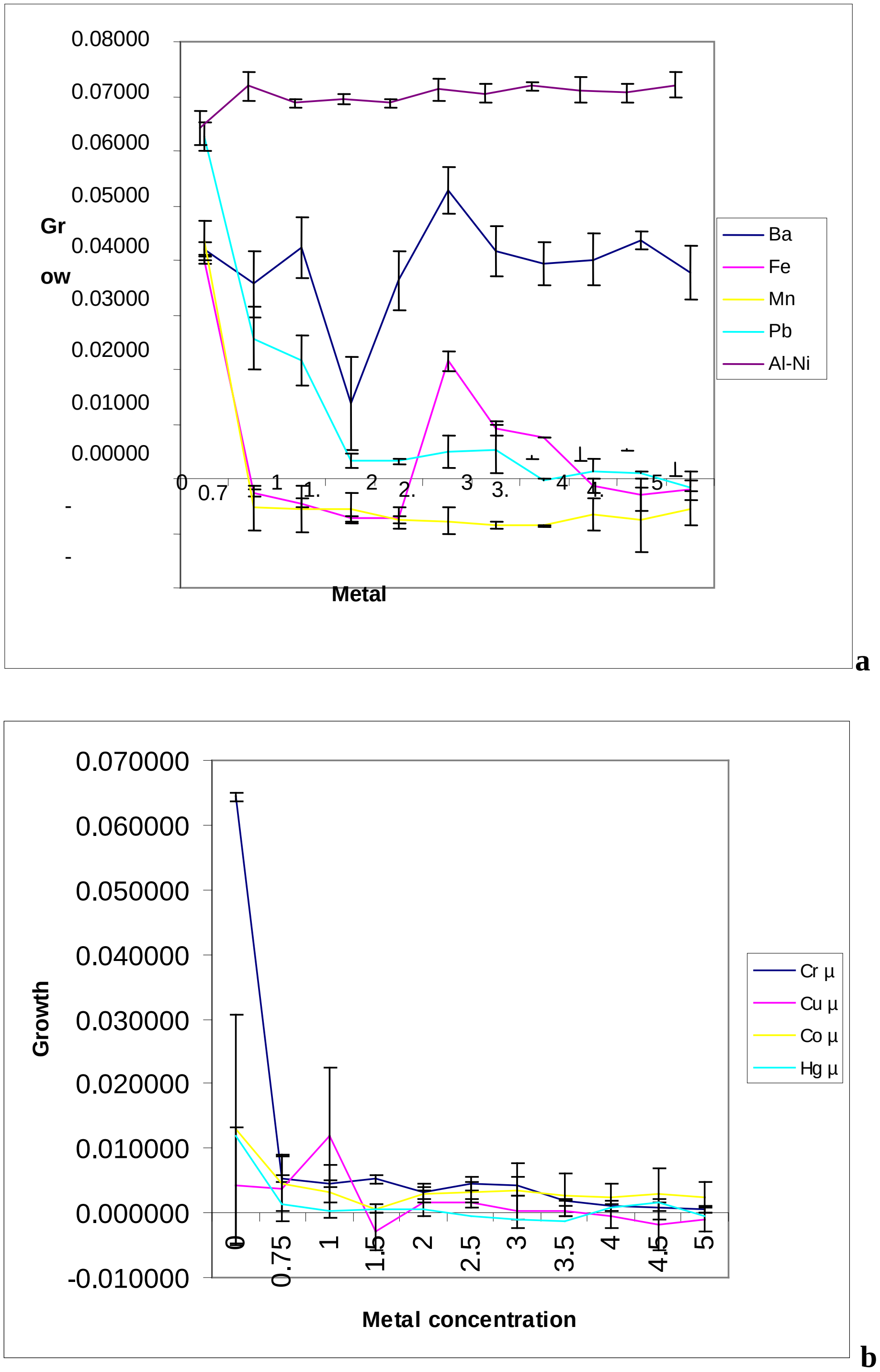


Figure A.1 Minimum inhibitory concentrations for *Bacillus* sp. KDNB-5 (LDM0306)

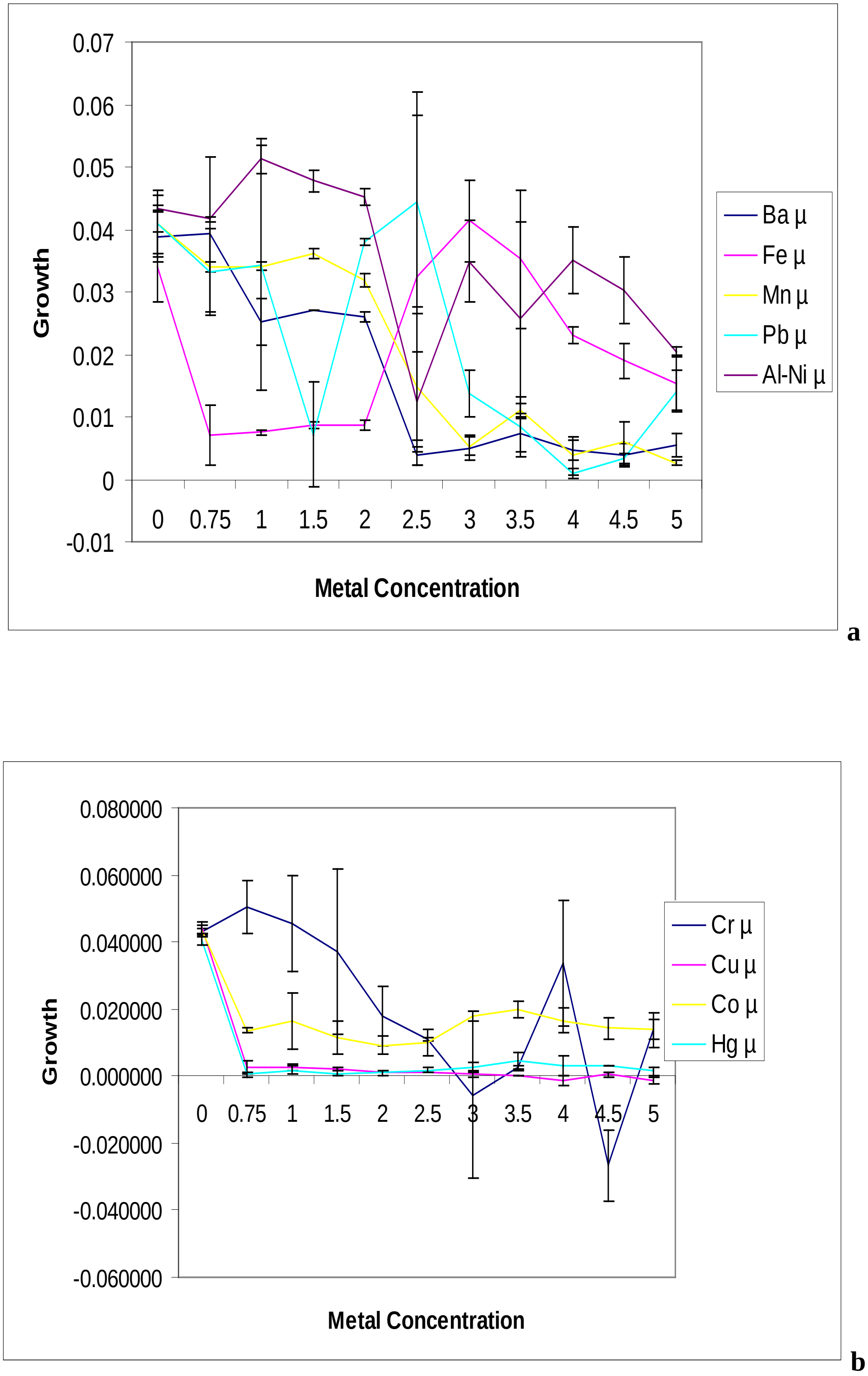
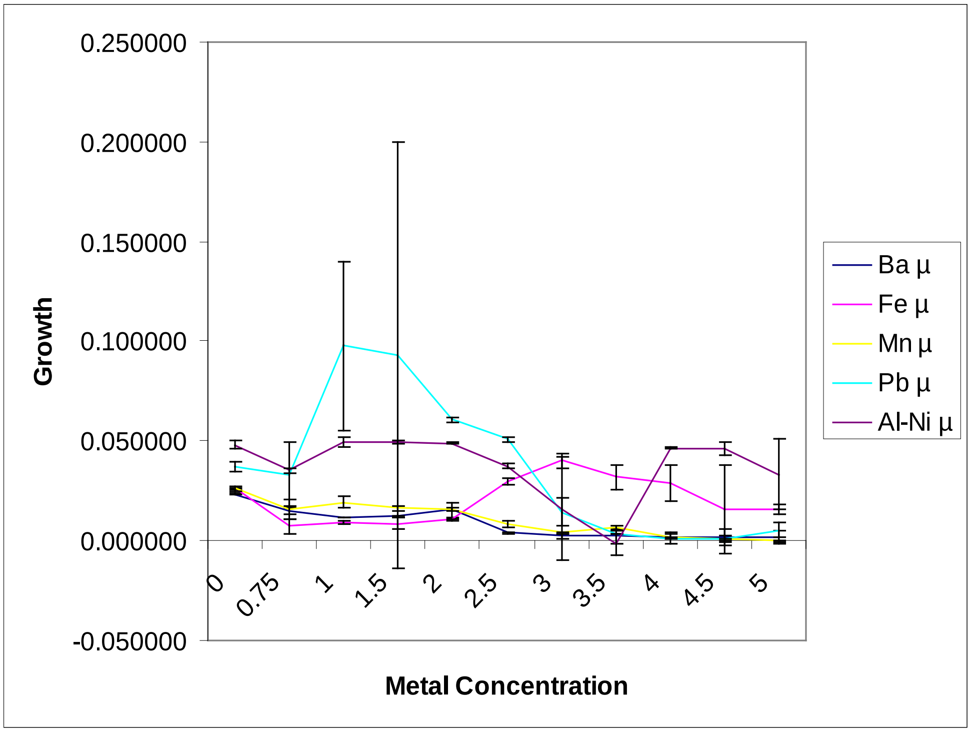
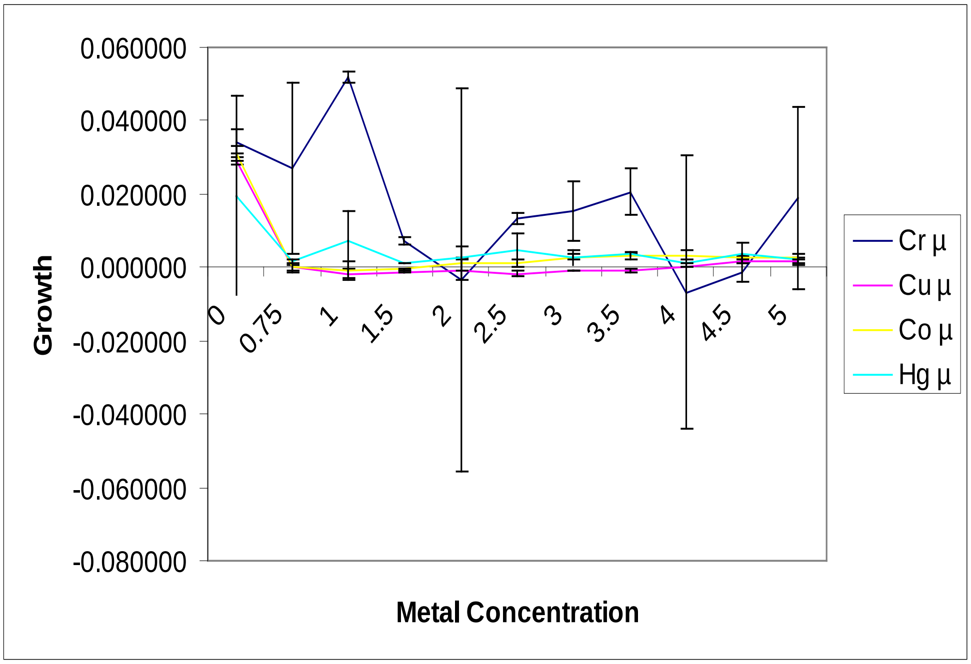


Figure A.2 Minimum inhibitory concentration for *Bacillus subtilis* strain DN-10 (LDK0306)



a



b

Figure A.3 Minimum inhibitory concentrations for Uncultured *Paenibacillaceae* (LDG0506)

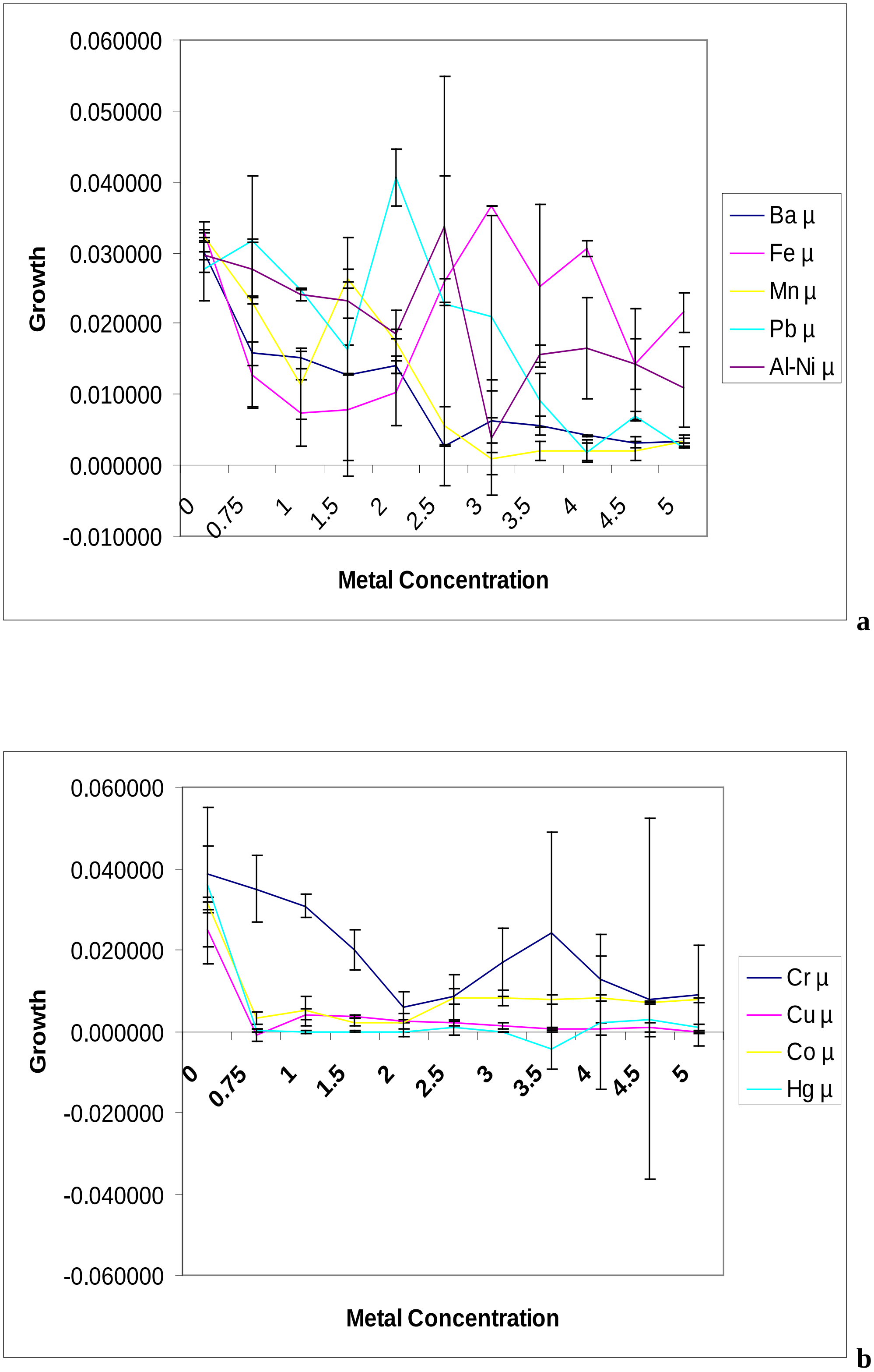
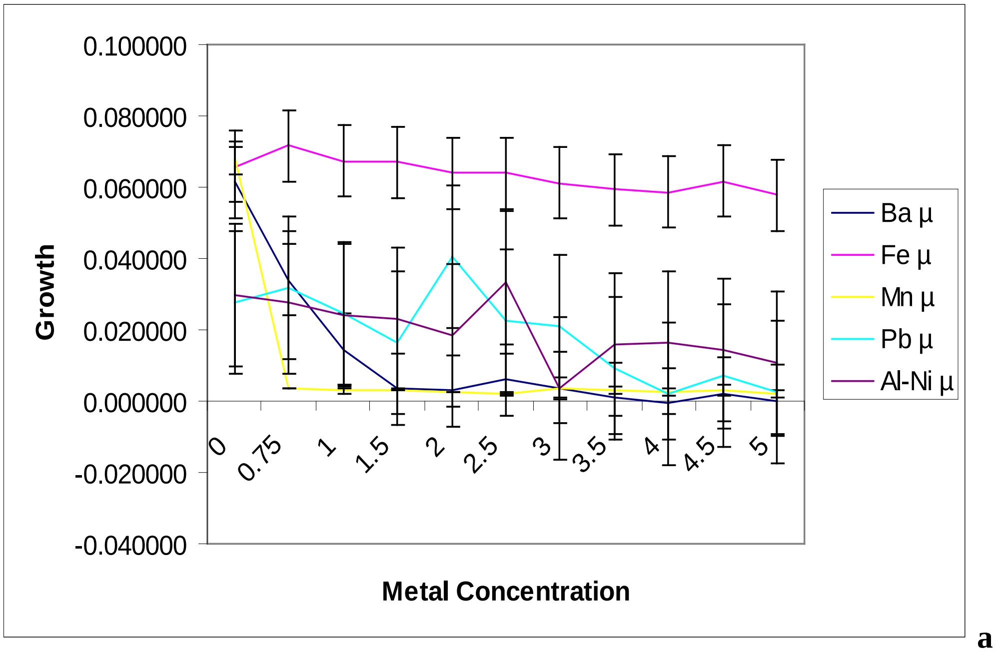


Figure A.4 Minimum inhibitory concentrations for *Alcaligenes* sp. DJWH 146-2 (LDM0506)



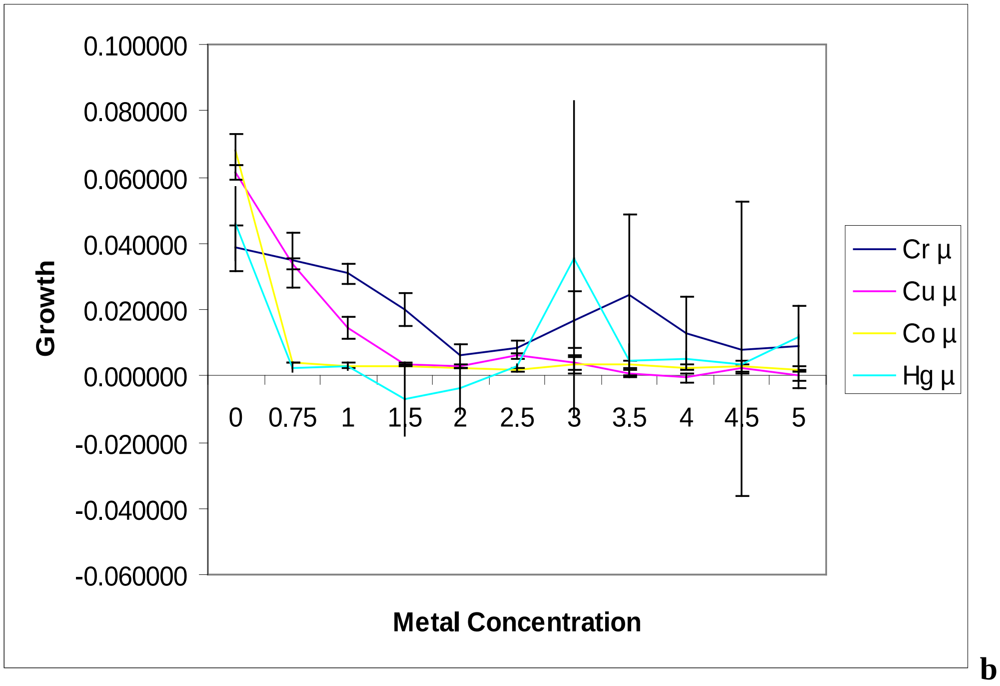


Figure A.5 Minimum inhibitory concentrations for *Bacillus cereus* (LDO0506)

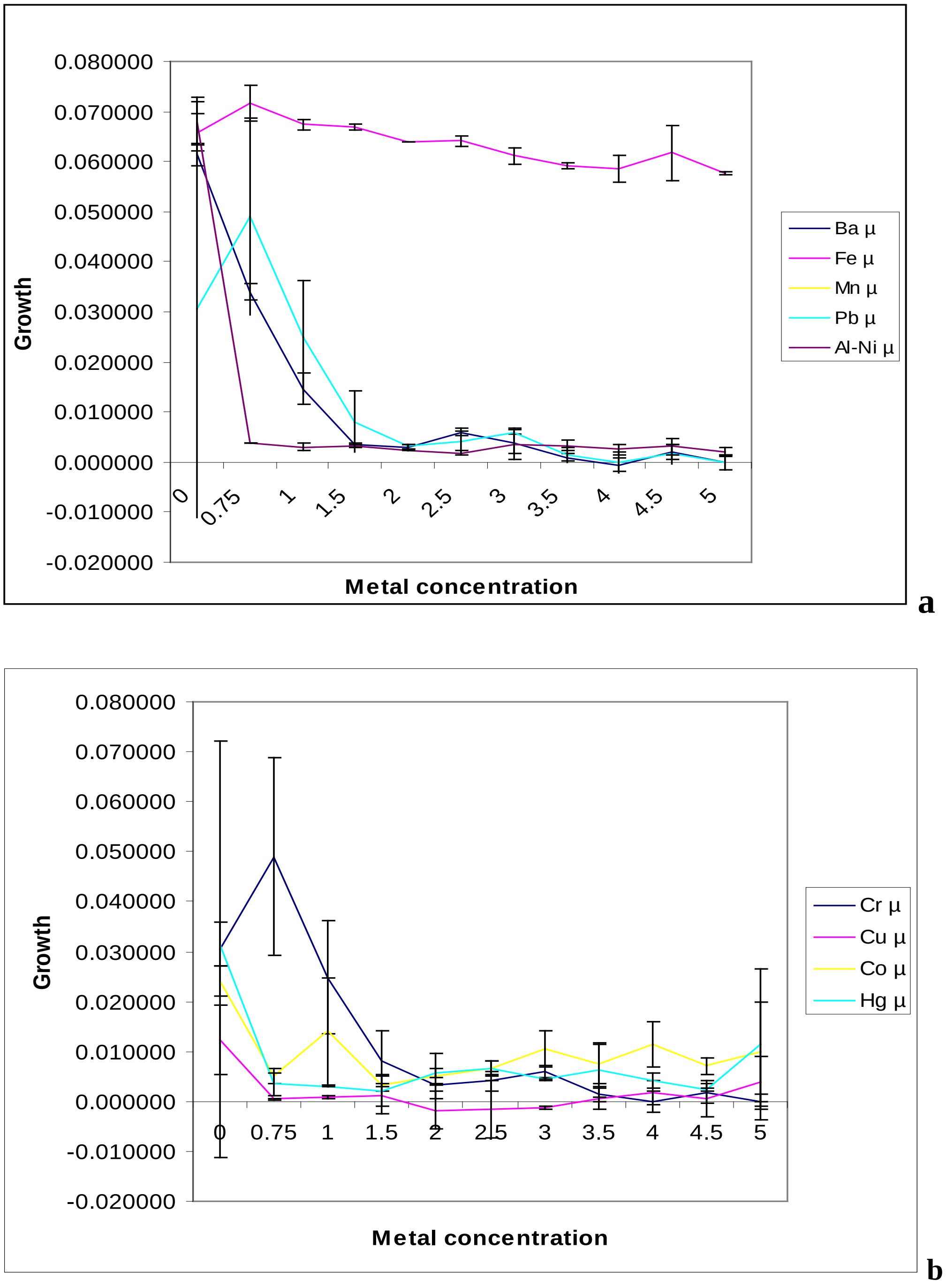
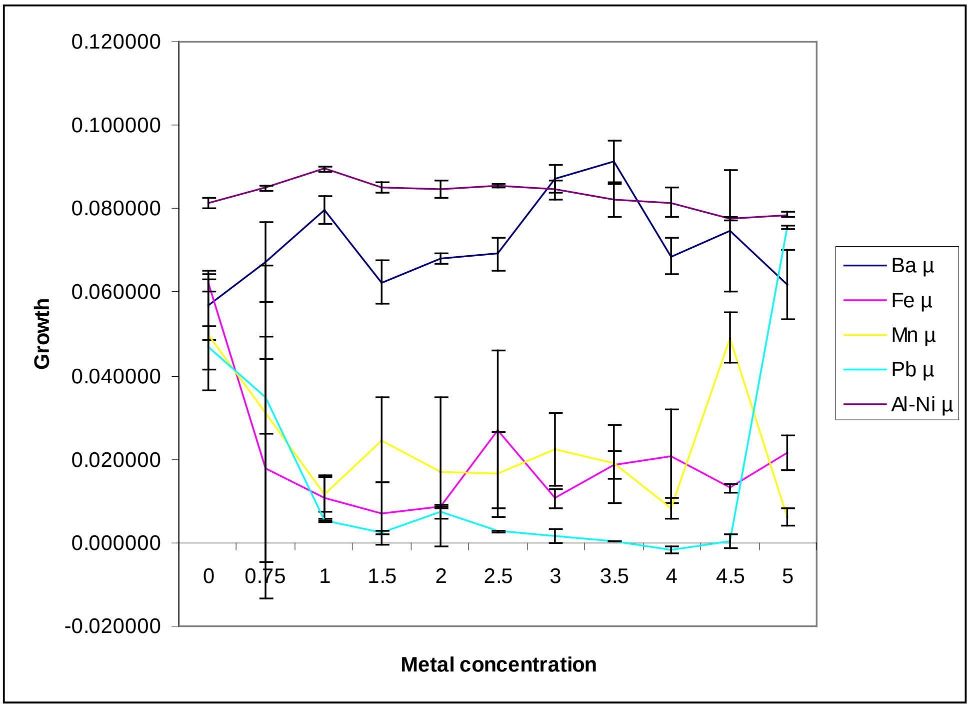
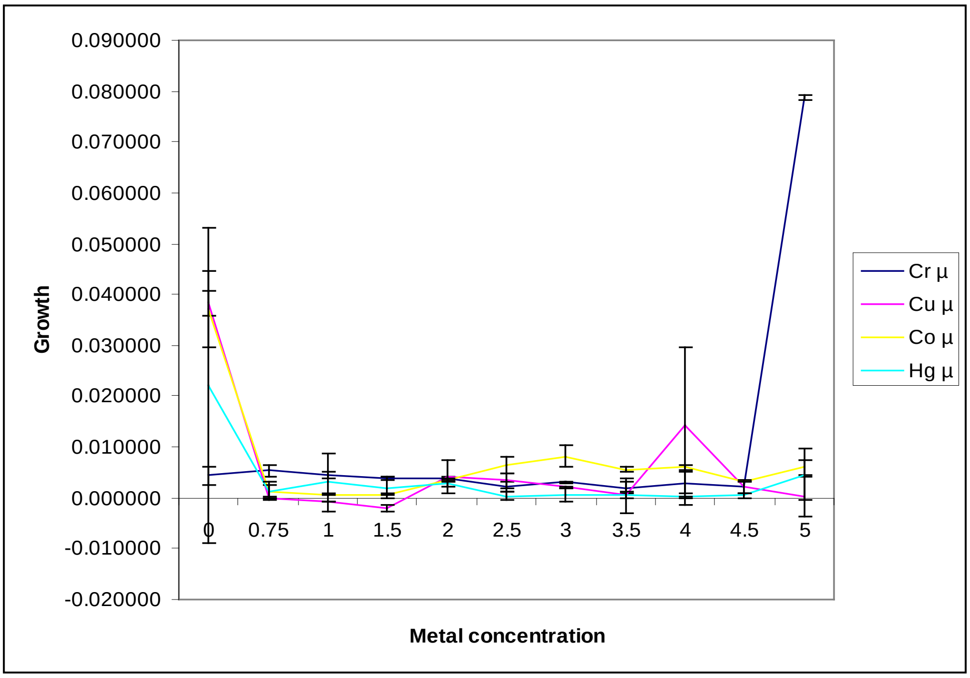


Figure A.6 Minimum inhibitory concentration of Stenotrophomonas maltophilia (LDS0306)



a



b

Figure A.7 Minimum inhibitory concentrations for Paenibacillus lautus (LDB0506)

APPENDIX B

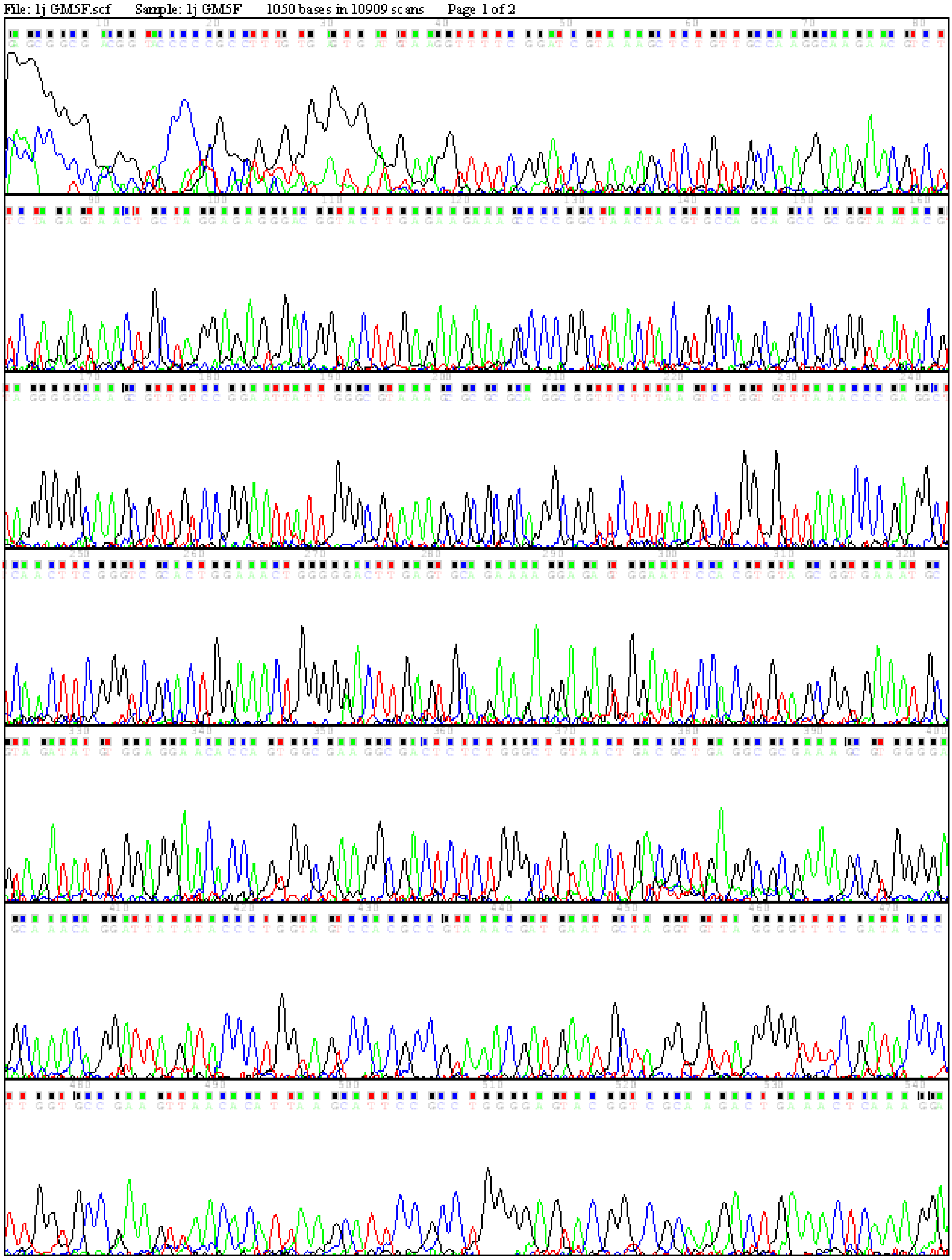
DNA ISOLATION AND SEQUENCING RESULTS

A. CTAB-PVP EXTRACTION

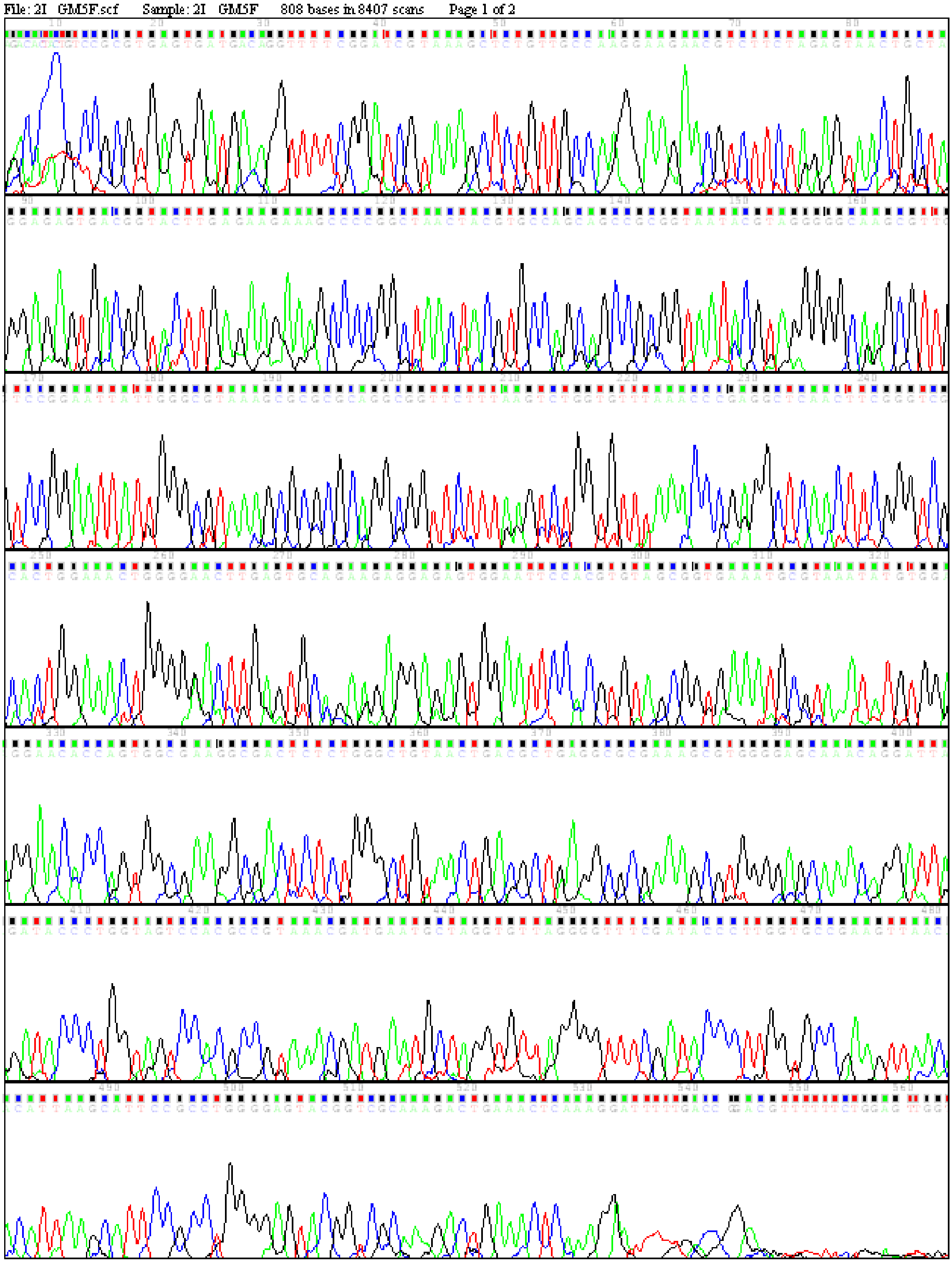
Purified isolates from the different sites were grown in 5ml nutrient broth for at 24°C for 12 hours. The cells were then collected in centrifuge tubes by centrifuging 2ml of the nutrient broth containing the cells at 13 400rpm for 5 min. The supernatant was discarded and then 25µl lysozyme was added to the pellet. The mixture was incubated for 30 – 60 min at 37°C. To this mixture 500µl hot CTAB, 100µl 5% PVP and 10µl proteinase K was added. This mixture was incubated for 90 min at 65°C and the tube was inverted every 5min. An equal volume of TE buffer Phenol:Chloroform:Isoamyl (25:23:1) was then added and the tube inverted at regular intervals for the next 10min. The mixture was then centrifuged for 5 min after which the aqueous phase was transferred to a new tube and an equal volume of TE buffered chloroform:isoamyl added. Tubes were again inverted for the next 10 min and then centrifuged for 5min. The aqueous phase was transferred to a new tube and 100µl of NaCl and 1000µl cold 95% ethanol added. The mixture was stored at -80°C for 24 hours and then centrifuged for 5min at 4°C. The supernatant was discarded and the pellet dried under vacuum. After re-suspending the pellet in 50µl DEPC and incubating it for 1 hour at 65°C the sample was ready for use and stored at 4°C until analyzed or used for PCR.

B. CHROMOTOGRAPHS

Paenibacillus lautus strain DS19 (LDJ0306)

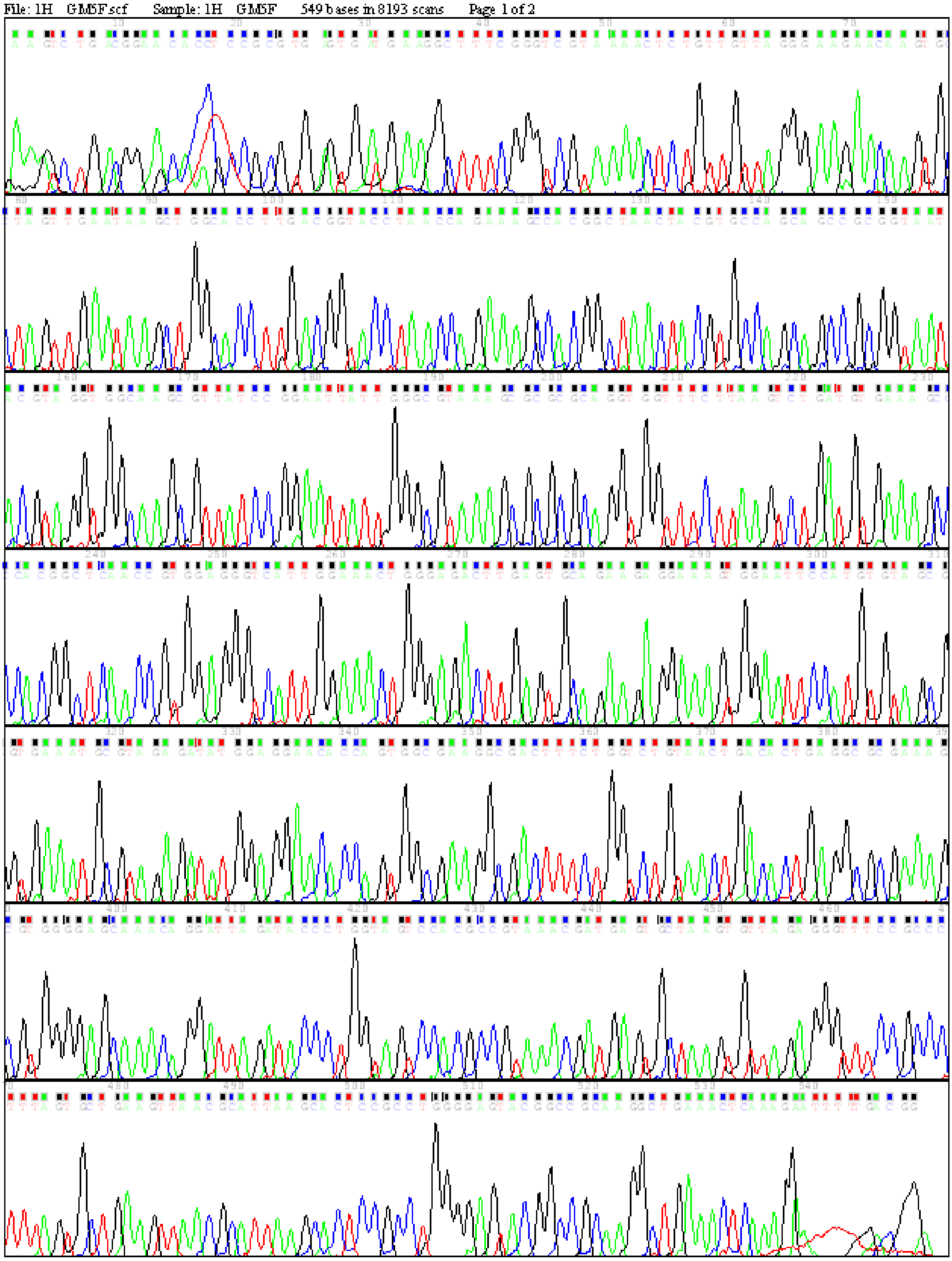


Uncultured *Paenibacillaceae* clone (LDI0506)

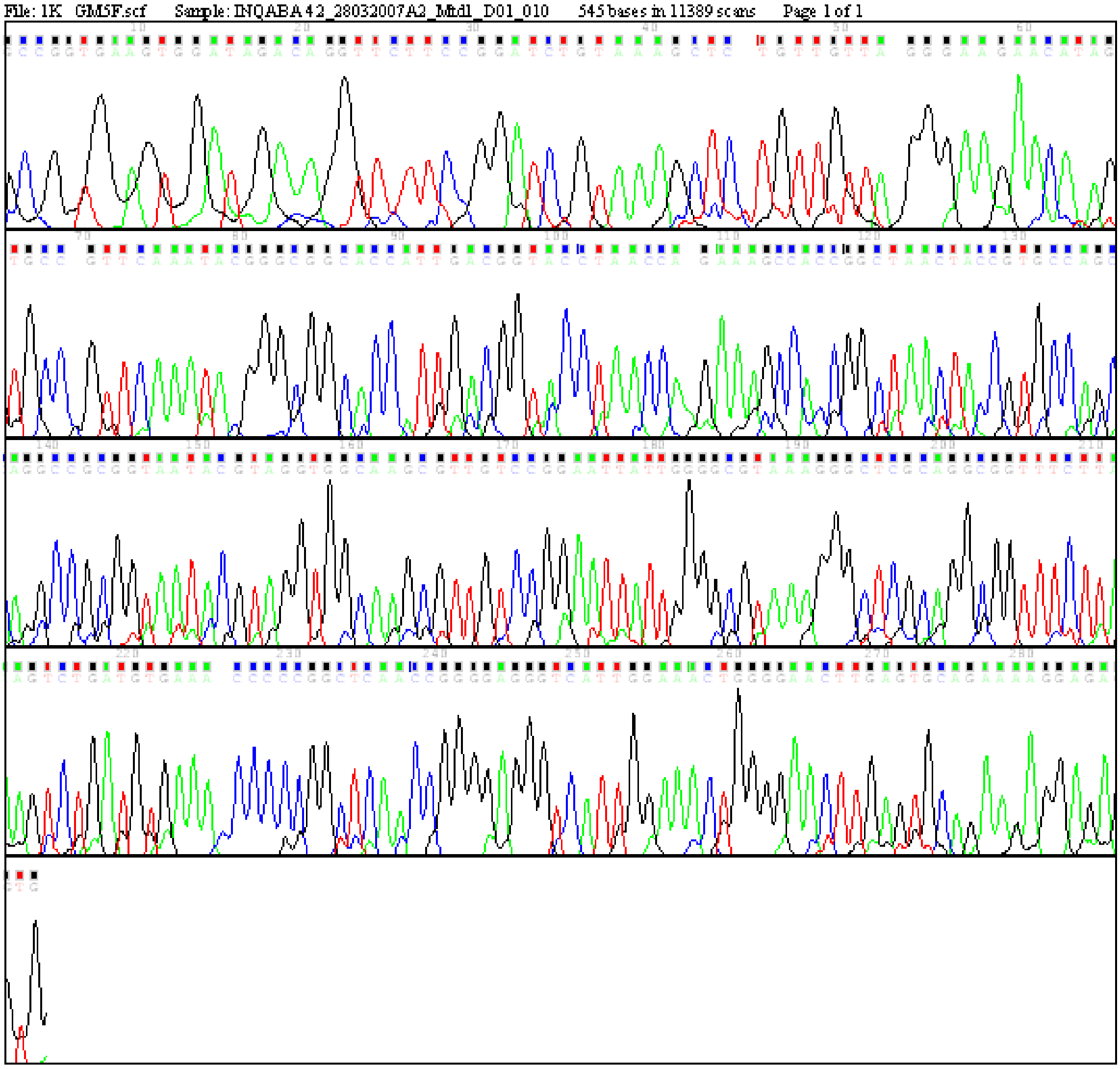


Bacillus sp. KDNB5 (LDH0306)

File: 1H GM5F.scf Sample: 1H GM5F 549 bases in 8193 scans Page 1 of 2

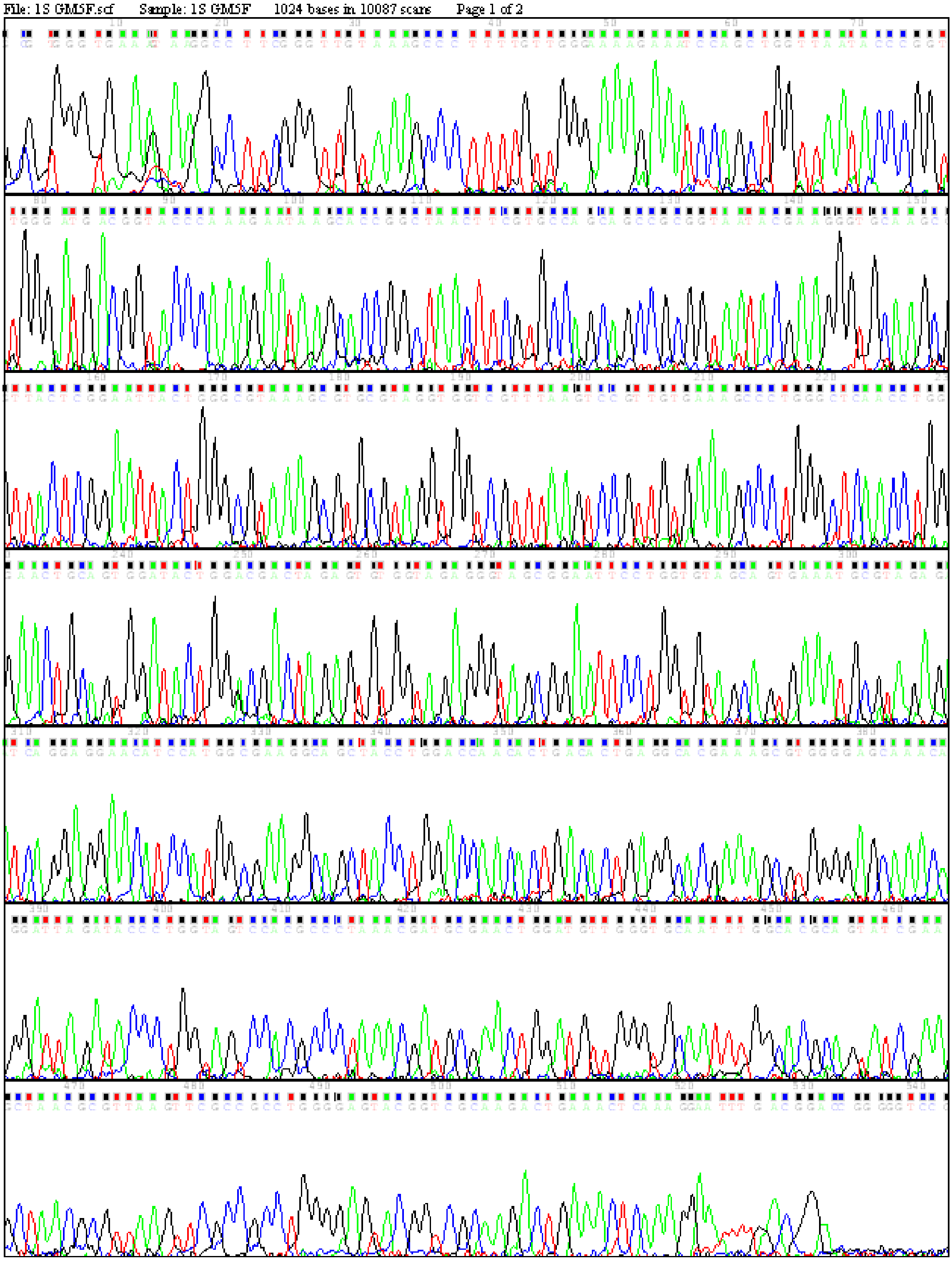


Bacillus subtilis strain DN-10



Stenotrophomonas maltophilia (LDS0306)

File: 1S GM5F.scf Sample: 1S GM5F 1024 bases in 10087 scans Page 1 of 2



Alcaligenes sp. DJWH 146-2 (LDM0506)