Characterization of heavy metal tolerant bacterial plasmids isolated from a platinum mine tailings dam

by

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DECLARATION

I declare that, this dissertation for the degree of Master of Environmental Science at the North-West University, Potchefstroom Campus hereby submitted, has not been submitted by me for a degree at this or another University, that it is my own work in design and execution, and that all material contained herein has been duly acknowledged.

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Tladi Abram Mahlatsi Date

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ABSTRACT

The development of metal-tolerance and antibiotic resistance in bacteria may be caused by metals polluting a particular environment. During mining and mineral processing activities, large quantities of metals are deposited into the soil. These high concentrations of metals are evolutionary pressures selecting for microorganisms tolerant to these metals. Metal-tolerance maybe conferred to these organisms by mobile genetic elements such as plasmids. This study describes the characteristics of plasmids isolated from various bacteria that displayed an ability to withstand high metal concentrations. The isolated plasmids were individually transformed into *Escherichia coli* JM109. Transformants were then evaluated for metal-tolerant capabilities using a microdilution approach. Plasmids were then isolated from the transformants and the concentration of the plasmid DNA ranged between 11.75 ï 118.06 ng/µl. These plasmids were of the same size as the original ones. This demonstrated that successful transformations with plasmid DNA were conducted. In order to determine the compatibility group, plasmids were subjected to PCR amplification using IncQ, IncP-9 and IncW specific primers. Only the IncW provided positive results. To demonstrate that the plasmids were free of genomic DNA, a 16S rDNA PCR test was included. The plasmids that were positive for IncW PCRs were all negative for the rDNA PCRs. Plasmids were stably inherited and at least three, isolated from three different Gram positive species, belonged to the Inc W group of plasmids. These were originally isolated from *Paenibacillus ginsingari, Paenibacillus lautus* and *Bacillus cereus*. Minimum inhibition concentrations (MICs) were carried out to determine the ability of transformed *E. coli* JM109 to tolerate metals at varying concentrations. Results indicated that transformed *E. coli* JM109 developed ability to grow in the presence of several heavy metals. Some strains were resistant to high concentrations (+10 mM) of Ni^{2+}/Al^{3+}, Pb^{2+} and Ba^{2+}. The order of metal resistance was Ni/Al=Pb>Ba>Mn>Cr>Cu>Co=Hg. All the

transformants were sensitive to 1 mM of Co^{2+} and Hg^{2+}. Moreover, protein profiling was used to determine the impact of plasmids on *E. coli* JM109. Proteins were extracted from both transformed and un-transformed *E. coli* JM109 using acetone-SDS protocol and subjected to one-dimensional (1D) and two-dimensional (2D) Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS- PAGE). Transformed *E. coli* JM109 were grown under the metal stress. One dimension SDS-PAGE illustrated general similarity of the profiles except for two banding positions in the 30 to 35 kDa region where bands were present in the transformants that were grown in the Ni/Al alloy containing media. Two-dimensional electrophoresis PAGE analysis showed that some of the proteins were up-regulated while others were down-regulated. The largest numbers of proteins were from 15 ï 75 kDa. The majority of these proteins had isoelectric points (p*I*) between 5 and 6. It was concluded that plasmids isolated from various heavy metal-tolerant bacterial species were successfully transformed into *E. coli* JM109 rendering various new metal-tolerant *E. coli* JM109 strains. Furthermore, the study showed that metal resistance was due to the presence of the plasmids. Two-dimensional SDS-PAGE resolved more differences in the protein expression profiles. Since the plasmids rendered the *E. coli* JM109 tolerant to metals tested, it also can be concluded that the change in the protein profiles was due to the effects of the plasmids. Furthermore, plasmids were also re-isolated from the transformants and these plasmids were of the same size as the original ones.. All the plasmids in this study were also stably inherited, a feature associated with IncW plasmids. More detailed genetic characterization of these plasmids is required. Plasmids isolated and characterized in this study may hold biotechnology potential. Such features should be exploited in follow-up experiments.

Keywords: *Escherichia coli* JM109, Plasmids, Transformation, Metal, Ni^{2+}/Al^{3+} alloy, 1-D SDS-PAGE, 2D-PAGE.

CHAPTER 1

LITERATURE REVIEW

1.1 PLATINUM MINING AND TAILINGS DISPOSAL FACILITIES

The African continent is the producer of a variety of the worldôs most important minerals and metals. These are gold, platinum group metals (PGMs), diamonds, uranium, manganese, chromium, nickel, bauxite and cobalt (Mbendi Information Services, 2012). South Africa is the worldôs largest producer and supplier of PGMs, supplying 56.7% of the world production (DME, 2007; GDACE, 2008). Platinum group metals include platinum (Pt), palladium (Pd), rhodium (Rh), ruthenium (Ru), osmium (Os) and iridium (Ir). These metals are used in various industries e.g. the manufacturing of jewellery and industrial applications such as the electrical, chemical and petroleum refining industries (DME, 2007; Lofersky, 2007). The mining industry in South Africa contributes over US$7 billion annually to the gross domestic product (GDP) of the country. This comes at significant environmental costs. These include water, from the runoff of metals into the water systems; air, dust from the tailings facilities and soil pollution due to the generation of hazardous wastes.

Tailings disposal facilities (TDFs) are structures built to store mill and waste tailings from mines and contain hazardous waste such as metals and other toxic compounds (Rico *et al*., 2008). Mining companies employ rehabilitation strategies to try and mitigate the effects of the hazardous tailings material. These include covering the material with soil, adding compost to the material etc, (Maboeta *et al*., 2006; Wahl, 2007; Rauwane, 2008). Rehabilitated TDFs are subjected to all environmental and meteorological conditions prevailing at the particular locality (Zandarín *et al*., 2009). Wet conditions may result in

the mobilization of contaminants such as metals into the surrounding soil environment. This might lead to soil and groundwater pollution and may result in decreased biological diversity (Ledin and Pedersen, 1996; Liu *et al*., 2006; Liu *et al*., 2008).

It is particularly the presence of metals and the fact that they may become bioavailable that is of environmental concern. These metals include chromium (Cr), nickel (Ni), copper (Cu), zinc (Zn), arsenic (As), selenium (Se), strontium (Sr), molybdenum (Mo), technetium (Tc), cadmium (Cd), mercury (Hg), and lead (Pb). Those that are known to be persistent in platinum tailings materials are Cr, Ni, Cu, Al, Zn, Pb, Mn and Fe (Wahl, 2007; Kargar *et al*., 2012). Some of these metals are critical for bacterial enzyme activity but are required in extremely low concentrations. When a certain threshold is exceeded these metals lead to toxic effects after entering the cell (Nies, 1999). It is not always that metals are toxic, but some metal-cations have an important role in biochemical reactions. Though at high concentrations, metal ions tend to form unspecific complex compounds in the bacterial cell thus leading to toxic effects. Nies and Silver (2005) and Nies (1999) described mechanisms in which the cells deal with toxic levels of metals. Cells use systems such as the ATP-binding cassette (ABC), P-Type ATPases and metal inorganic transport (MIT) to accumulate metals ions into the cytoplasm. Metals cannot be degraded. Athigh concentrations the metal ions are pumped out of the cell by efflux mechanisms. It could also bind to thiol-containing molecules. Lastly metals ions can also be reduced to a less toxic state.

Tailings disposal facilities are not sterile environments (Ellis *et al*., 2003; Frey *et al*., 2006) although microbial activities and biomass may be reduced when microbes are exposed to metals in the tailings materials and soil (Ranjard *et al*., 2000; Wang *et al*., 2000; Sandaa *et al*., 2001; Gremion *et al*., 2004; Rajapaksha *et al*., 2004). Wahl (2007) found mites from

the orders Prostigmata, Oribatiba formerly Cryptostigmata, Mesostigmata and insects from the order Collembola common in sampling sites on and close to a platinum mine TDF in North West Province, South Africa. Collembola species were represented by the families Sminthuridae, Entomobryidae and Isotomidae. Wahl (2007) it was concluded that the mite taxa that dominated in the TDF may be indicative of the fact that this is a polluted ecosystem. Their predominance is due to their ability to reproduce on the mine tailing and their tolerance to metal concentrations. Furthermore, Rauwane (2008) showed that various fungi and bacteria species were present at sites on and close to a platinum TDF. This was the same TDF where Wahl (2007) worked. The presence of metals, even at low concentrations has the ability to inhibit enzymatic activity with their stress impact to the microorganisms in the soil (Ashman and Puri, 2002; Maboeta *et al*., 2006). In a study by Kandeler *et al*. (1996), it was demonstrated that an elevation in the concentration of metals in the soil exerted a negative influence on microbial activity.

1.2 METALS AND MICROBES

The non-biodegradability of metals is responsible for their persistence in the environment and subsequent bioaccumulation in the food chain. Metals are soluble in water at low pH (Verma *et al*., 2001). In polluted soils, microorganisms are the first to be subjected to effects of pollution and have to overcome the adverse conditions (Bersch *et al*., 2008). Bacterial cells are directly exposed to the metals and the higher the concentration thereof in the environment, the greater the toxic effect may be when the metal becomes bio-available. Once inside the cell, metals inhibit various essential enzymes causing decreased fitness. Metals also increase DNA damage by the production of reactive oxygen species (Schmidt *et al*., 2009). Metal ions also form unspecific complex compounds in the cell, leading to toxic effects (Nies, 1999). They may cause conformational change in protein structures (Bar *et al*., 2007), nucleic acids and phospholipids and ultimately arrest cellular

proliferation (Jerke *et al*., 2008; Bong *et al*., 2010). Hydroxide radicals are formed from adverse redox-reaction in the cell and they cause deleterious reactions such as peroxidation of lipids, causing membrane disruption and oxidation of proteins, causing their inactivation (Sharma *et al*., 2006).

Once present in certain matrices, metals may become more difficult to remove compared to the other pollutant types (Mejáre and Bülow, 2001). This means that the detrimental environmental effects are prolonged. However, although soil microbes are the first to be affected by elevated concentrations of metals, their genomes are also subjected to extremely fast evolutionary processes and this allows them to survive (Kuo *et al*., 2009). Besides evolutionary processes, various processes are used by microorganisms to mitigate the effects of high concentrations of metals e.g, biosorption (Deng *et al*., 2003). Biosorption includes binding of metals by metallothioneins, extracellular polymers, to the cell wall, compartmentalization inside cell and formation of insoluble metal sulphides. Metal detoxification processes may also include decreased uptake, active efflux and volatilization (Giller *et al*., 1998; Valls *et al*., 2000; Majáre and Bülow, 2001). In addition to this, superoxide dismutases (SOD) that are involved in the mechanism of defence against oxidative stress posed by metals such as cobalt, cadmium and nickel may also play an important role in reducing the toxicity of metals (Geslin *et al*., 2001).

Metals are thus classified as stressors and their presence in the environment may have adverse effects on the composition of microbial communities occurring in the contamination areas leading towards a lower bacterial diversity and selection of only the metal tolerant strains (Srinath *et al.,* 2002). Some of the bacterial populations have evolved in such a way that they are not present in soils with low to moderate concentrations of metals, but only found in highly polluted environments. This aspect, however, could have

beneficial remediation applications. In one example, Azabou *et al*. (2007) demonstrated that bacterial populations in metal polluted environments adapted to the conditions and would be suitable for remediation purposes. They obtained a mixed sulphate-reducing bacterial (SRB) population from sewage sludge in which they used phosphogypsum as sulphate source. The authors demonstrated that, when sulphate and zinc chloride was used, the SRB population could tolerate up to 150 mg/l of zinc. Under these conditions up to 95% zinc removal was achieved. It was concluded that concentrations of metals decreased because they reacted with sulfide produced by the SRB population (Azabou *et al*., 2007).

Microorganisms also have both beneficial and harmful roles in the mining and mineral processing of metals (Brierley & Brierley, 2002). Firstly, microorganisms break down certain toxic constituents used in mineral processing as well as concentrate and immobilize soluble metals released during mining and mineral processing activities. Secondly, certain bacteria are responsible for one of the most persistent and destructive environmental problems namely acid rock drainage (ARD). This is responsible for the pollution of both surface and groundwater resources from mining industries. Yet, the very same bacteria responsible for ARD are also commercially exploited for cost-effective, efficient and environmentally sound extraction of base metals such as copper, cobalt and uranium. Bacterial species are also used for the pre-treatment of ores and mineral concentrates in which precious metals such as gold and silver are embedded in sulphide minerals (Brierley & Brierley, 2002).

Metal exposure leads to establishment of tolerant Gram positive (*Bacillus* spp., *Arthrobacter* spp., *Firmicutes* spp and *Corynecaterium* spp.) as well as Gram negative (*Pseudomonas* spp., *Alcaligenes* spp., *Ralstonia* spp. and *Burkholderia* spp.) bacterial communities (Wuertz & Mergeay, 1997; Ellis *et al*., 2003; Kozdrfjj and van Elsas, 2001;

Piotrowska-Seget *et al*., 2005). Hu *et al*. (2007) reported *Bacillus cereus* resistance to metals in Beijing, China. Rauwane (2008) also showed that various Gram positive species, in particular *Paenibacillus* spp. as well as *Bacillus* spp., were prevalent in platinum mine TDF in South Africa. It was demonstrated that *Arthrobacter* spp. was widespread in chromium-stressed soils (Viti and Giovannetti, 2005). Strains of *Bacillus mycoides* and *Micrococcus luteus* that are tolerant to silver have been reported (Piotrowska-Seget *et al*., 2005). The Gram negative *Stenotrophomonas maltophilia* have been observed from sediments of the metal polluted Iskenderun Bay in Turkey (Martyar *et al*., 2008). Wuertz & Mergeay (1997) reported the presence of *Pseudomonas aeruginosa* in factories from a zinc-decertified area in Belgium. Metal tolerance is thus wide spread in nature and from the literature it is evident that it has to do with adaptive mechanisms of organisms.

1.3 PLASMIDS AND METAL TOLERANCE

Initially it was thought that the adaptation and tolerance of bacteria to different soil or environmental conditions was due to spore formation. However, it was demonstrated that mobile genetic elements may also play a role (Szpirer *et al*., 1999; Gadd, 2010). Plasmids are non-chromosomal, double stranded, self-replicating DNA found in bacteria and various yeast species. They consist of variable assortment of genes involved in maintenance, transfer and certain phenotypic characters. The only traits shared by all plasmids are the genes involved in replication and its control (Szpirer *et al*., 1999; Jerke *et al*., 2008). However, several other traits are also carried on plasmids such as genes giving bacterial hosts the capability of degrading and detoxifying a wide variety of hazardous compounds as well as rendering organisms tolerant to antimicrobial substances (Kulkarni & Chaudhari, 2006; Zhang *et al*., 2007; Wei *et al*., 2008).

Plasmids play important roles in evolutionary processes among different microorganisms and within microbial communities by giving members access to useful genes (Burian *et al.*, 1997; Arber, 2000; Sobecky, 2002). Their existence is based on promiscuity and they give their host the ability to adapt within constantly changing environments. (Kües & Stahl, 1989). Broad-Host-Range (BHR) plasmids can transfer their genes across distant phylogenetic groups (Gerdes *et al*., 2000). Thus, the ability to transfer to new hosts enhances plasmids survival and reduces the chance of plasmid extinction (Bergstrom *et al*., 2000). Moreover, it also provides the host with the ability to adapt to adverse conditions.

Piotrowska-Seget *et al.* (2005) showed that bacterial species isolated from metal polluted soils were able to survive due to the presence of plasmids. These authors isolated the plasmid, transformed these into *E. coli* DH5Ŭand then demonstrated that the transformants had similar metal tolerant characteristics that the original species. Plasmids electrophoresis profiles of the original host species and the transformants were the same (Piotrowska-Seget *et al.,* 2005).

Abou-Shanab *et al*. (2007) and Piotrowska-Seget *et al*. (2005) provided evidence and concluded that some of the bacterial strains possess several genes encoding metal tolerance. These genes are located within bacterial chromosomes, plasmids or on transposons. Some metal resistance determinants can move from plasmids to chromosomes or from the chromosome to the plasmid, thus making the plasmid the source of resistance genes. The presence of these genes in bacteria provides them with an evolutionary advantage and leads to their increase in numbers in the environment. They also spread these metal tolerance genes to adjacent bacteria by horizontal transfer. Resistant genes shared this way, rather than by R-factor, are shared within as well as across both Gram-positive and Gram-negative bacterial communities and populations. In addition to this the

R plasmids in *Escherichia coli* can help the host develop resistance to several metallic ions such as mercury, cobalt and nickel (Nakahara *et al*., 1977).

Cross resistance of microorganisms to antibiotics and metals has been reported (Nakahara *et al*., 1977; Karbasizaed *et al*., 2003). Such metal and antibiotic resistant populations will adapt faster by distribution of resistant-factor (R-factor) under metal stress conditions than by mutation and natural selection, leading to an increase in their numbers (Verma *et al*., 2001; Gosh *et al*., 2000).

Metal resistance studies provide useful information on the mechanisms of metal and antibiotic resistance, plasmid genetics and physiology of the microbes in polluted environments. Hassen *et al*. (1998) demonstrated that the broad resistance of different strains to antibiotics might indicate that the latter is rich in plasmids that carry simultaneous resistance to antibiotics and metals.

Plasmid genes may code for proteins involved in metal reduction and for specific transport systems e.g. efflux pumps (Silver, 1992). Thus, the resistance may be due to plasmid mediated proteins which are responsible for reduction and binding and removal of organic ligands (Gosh *et al*., 2000). The use of engineered microorganisms displaying heterologous proteins or peptides for biosorption of heavy metals has been explored (Krishnaswamy and Wilson, 2000). Genetically engineered *E. coli* JM 109 has the ability to accumulate and tolerate a number of metals compared to the original host strain (Deng *et al*., 2007). Studies of Deng and Wilson (2001) as well as Deng *et al*. (2003) have shown that genetically engineered bacteria with bioaccumulation capacity and affinity for both mercury and nickel from wastewaters and industrial effluent could be generated. In these cases sensitive strain were transformed with plasmids. This confirms that cells containing plasmids with metal tolerance genes have high ability to accumulate metal ions from multi-

component solutions under various environmental conditions. Deng *et al*. (2007) showed that genetically engineered *E. coli* JM109 have the ability to take-up/bind cadmium at greater capacity than the original host strains harboring the cadmium transport gene (*cdtB*) that were isolated from wastewater. When *Escherichia coli* DH5Ŭ was transformed with plasmids from Zn and Cd tolerant bacterial strains from soil, the transformants showed similar tolerance for Zn and Cd (Piotrowska-Seget *et al*., 2005). These studies demonstrated that tolerance to metals was associated with plasmids.

1.4 INCOMPATIBILITY PLASMIDS

The diversity of plasmids is enormous and this is linked to the genes they may contain **(**Gillings *et al*., 2008). Jerke *et al.* (2008) noted that plasmids from different and often geographically separate taxa as well as those existing in the same cell may share similar core genes, but that these genes are different enough to allow plasmids to be incompatible. Plasmids are promiscuous and facilitate the transfer of resistance genes to be shared amongst related and unrelated species (KŤes and Stahl, 1989; Davison, 1999; Sobecky, 2002; Kelly *et al*., 2009; Suzuki *et al*., 2010). Mobility of these genetic elements has been associated with transformation (uptake of free DNA fragments) and conjugation (exchange of DNA by direct contact) processes. In aquatic environments, such processes are influenced by several factors, including environmental (temperature, salt levels etc,) as well as inherent plasmid construction factors (Suzuki *et al*., 2010). The latter factors are also used to classify plasmids into various groups incompatibility groups (Gilmour *et al*., 2004). Plasmids from specific incompatibility groups have originally been associated with enteric bacteria (Suzuki *et al*., 2010). Since the 1990ôs they have also been associated with polluted soils, manure and other environments (Götz *et al*., 1996; Krasowiak *et al*., 2002).

Before nucleotide sequence analysis of plasmid replicons and the use of hybridization methods to identify plasmid relatedness, the main methods of demonstrating relatedness of plasmids was by incompatibility classification methods (Datta, 1979; Stanisich, 1988). Plasmid incompatibility (Inc) is the failure of two genetically distinguishable plasmids to co-exist. These plasmids cannot be stability inherited in a host in the absence of an external selection pressure (Datta, 1979; Novick, 1987). Van Der Lelie *et al.* (1988) showed that plasmids isolated from one organism may be poorly transformed into an unrelated species and that incompatibility factors may be critical in mediating the transformation process. Miller and Cohen (1993) confirmed this deduction by demonstrating that repeat sequences found near the plasmid origin of replication are essential for incompatibility mediation and key elements in plasmid replication controlling plasmid copy number. More than 20 incompatibility groups exist. However, it was Götz *et al.* (1996) that first developed PCR based detection of IncP, IncN, Inc W and IncQ plasmids of environmental bacteria. Host ranges of incompatibility plasmids have been shown. IncP has a broad-host range, while IncN has intermediate host range of transfer and replication. IncW due to its lack of hostsô signatures are not reduced to any host because of their promiscuity (Suzuki *et al*., 2010). The study of soil microcosm (Pukall *et al*., 1996) confirms the limited host range of IncN plasmid found mostly in *Enterobacteriales.* IncW plasmids are found in a variety of bacteria including *Alpha-, Beta-, Gamma-, Deltaproteobacteria*, and *Bacteriodes* due their broad-host range (Fernandez-Lopez *et al*., 2006; Caballero-Flores *et al*., 2012). Another incompatibility group plasmids is IncQ plasmid, which is one of the few non-transmissible plasmids and can replicate in Gram-positive bacteria and is highly promiscuous (Rawlings and Tietze, 2001; Suzuki *et al*., 2010).

When characterising plasmids, determining the incompatibility group is thus important. It allows for the ability to trace and follow the evolution of antimicrobial resistance,

including metal resistance, as well as mechanisms associated with such resistance. It helps in the reconstruction of plasmid transfer network among microorganisms and the ability to track the pathway of gene dissemination (Suzuki *et al*., 2010).

1.5 METALLOREGULATORY PROTEINS

Though bacteria are affected by high concentrations of metals, some have the ability to thrive under these conditions. This can only happen in an organism that possesses an operon for a protein-based detoxification system (Silver, 1996). Metalloregulatory proteins are one of such protein classes that are essential in metal tolerance in organisms. They specifically recognize one or more types of metal ions (Chen and He, 2008). At elevated concentrations of metal ions these particular proteins recognize and detoxify the metals allowing the bacteria to survive (Chen and He, 2008). Membrane proteins also have the capacity to protect microorganisms against metal toxicity (Felício *et al*., 2003). In response to environmental stress conditions (elevated metals concentrations, oxidizing agents, starvation, extreme pH and osmotic conditions), stress proteins such as, heat shock proteins, starvation proteins and molecular chaperons are readily induced in microorganisms (Kiliç *et al*., 2010). In addition to metalloregulatory proteins, several other stress related protein groups are induced during unfavorable conditions.

It has been shown that proteins related to antioxidative defence mechanisms are differentially regulated in response to metal toxicity (Requejo and Tena, 2005; Le Lay *et al*., 2006; Lee *et al*., 2010; Costa *et al*., 2010). Redox-active metals can catalyze the formation of hydroxyl radicals to produce ROS (reactive oxygen species), and thereby causing oxidative stress in cells. Shanmuganathan *et al.* (2004) suggested that metal-induced oxidative stress in cells can be partially responsible for the toxic effects of heavy metals. Moreover, proteins involved in glutathione (GSH) biosynthesis are differentially

regulated under metal stresses (Ahsan *et al*., 2009). Fulladosa *et al.* (2006) described that exposure of living beings (e.g bacteria and animals species or humans) to sub-lethal levels of environmental pollution tends to trigger a number of defence mechanisms at the cellular and molecular levels, such as methallothioneins (MTs) proteins as they protect the cells against excessive metal uptake (Bauman *et al*., 1993). Other groups of proteins, such as heat-shock proteins (HSPs) are a class of functionally-related proteins whose expression is increased when cells are exposed to high temperatures and other stressors, with several being up-regulated after heavy metal exposure (Zhang *et al*., 2005; Chen *et al*., 2009; Visioli *et al*., 2010). Heat shock proteins can also act as the first defence against heavy-metal-induced stress.

Bacteria under various environmental stress forms respond by utilizing sensors to monitor their surroundings. They incorporate responses from many sensors to change gene transcription and adapting protein synthesis. These will inevitably include metal-binding proteins, when elevated metal ions are present in the environment. This increased response to metal stress leads to greater protein production (Binet *et al*., 2003; Gordon *et al*., 2008).

When studying the behavior of microorganisms under metal stress conditions, it is important to use techniques based on genomic as well as proteomics approaches. The proteome is highly dynamic as it continually changes in response to external and internal events, whilst the genome is a rather constant entity except when the roles of mobile elements such as plasmids are concerned. According to Wilkins *et al*. (1996) proteomes differ from cell to cell or cell to tissue and is constantly changing through its biochemical interactions with the genome and the environment. Proteomics are defined as the comprehensive analysis of the entire protein complement expressed in a cell or any biological sample at a given time and under specific conditions (Wasinger *et al*., 1995;

Wilkins *et al*., 1996; Graham *et al*., 2007). However, to understand the proteome, it is not only necessary to identify all of its protein constituents but also to better understand the characteristics of these proteins (Wilkins *et al*., 2006). In understanding of proteomes, several techniques have been developed.

1.6 MOLECULAR TECHNIQUES

1.6.1 PRINCIPLE OF PLASMID ISOLATION

Plasmids are useful to bacterial cells because they may carry genes which can allow bacteria to grow in non-ideal environments (Ghosh *et al*., 2000). DNA, whether plasmid or chromosomal does not exist as a free molecule in a cell, but rather as a complex association with RNA and proteins. Therefore, it is essential to purify plasmid DNA from chromosomal DNA, RNA and proteins to have the pure workable plasmid DNA. There are three basic steps in the purification of DNA in order of getting the pure workable DNA. Most of the techniques currently employed for the isolation of plasmids DNA are the alkaline lysis method (Sambrook *et al*., 1989). Many of the commercially available plasmid extraction kits are based on this method. Bacterial cells are cultivated overnight to provide sufficient biomass. These cells are then concentrated into a pellet. The cells are resuspended in a buffer containing RNAse and SDS/alkaline lysis buffer (Bigot and Charbit, 2009). The next buffer neutralizes the resulting lysate and creates appropriate condition for binding of plasmids DNA to a silica column. Precipitated proteins, genomic DNA and cell debris are pelleted by centrifugation. The plasmid DNA/supernatant is loaded onto a column and centrifuged. Contaminations are removed by washing the DNA on the matrix with ethanolic buffer. Pure plasmid DNA is eluted under low ionic strength conditions with an appropriate buffer (Macherey-Nagel, 2009; BN Products and Services, 2009). Should a silica column not be available then the plasmid DNA solution (supernatant) could be transferred to a fresh microfuge tube and then be precipitated using

a high salt alcohol procedure Sambrook *et al.* (1989). Purified plasmid DNA is used for further analysis including transformation into bacterial cells.

1.6.2 PRINCIPLE OF TRANSFORMATION

Bacterial transformation involves the transfer of genetic information on a plasmid by the direct uptake of this exogenous, or foreign DNA into the bacteria cell of interest, which results in the acquisition of a new genetic trait that is stable and heritable (van Dyk *et al*., 2007). This process is essential to the field of molecular biology in that it allows for the propagation, genetic expression and isolation of recombinant DNA molecules (van Dyk *et al*., 2007).

Transformation was first described in *Streptococcus pneumonia* by Griffith (1928). As much as transformation depends on DNA concentration, it also depends on bacterial cells competency. According to Streips (1991) few bacterial species undergo the transformation process naturally. Thus for most bacteria to become competent they need to be manipulated. Competency is a normal physiological state, which changes the structure and permeability of the cell membrane for easier cellular entrance of plasmid DNA (Streips, 1991). *Escherichia coli* do not develop competence naturally. However, Mandel and Higa (1970) and Cohen *et al*. (1972) found that the treatment of *E. coli* cells with $CaCl_2$ at low temperature allowed cells to be the recipient of plasmid DNA. It has been found that *E. coli* cells and plasmid DNA productively interact in a suitable environment of low temperatures and calcium ions (Primrose *et al*., 2001). Following successful transformation, the efficiency of the process is determined, as the number of transformed cells generated by 1 ε g of supercoiled plasmid DNA (Tu, 2008). However, recent studies by Li *et al*. (2010) and Sha *et al*. (2011) showed that the $CaCl_2$ method of transformation with increased concentration of $CaCl_2$ and an improved heat-shock method could

effectively improve transformation efficiency and frequency. Electrophoresis of isolated plasmid after transformation allowed the separation and confirmation that competent *E. coli* cells had acquired the plasmid of interest.

1.6.3 AGAROSE GEL ELECTROPHORESIS

Electrophoresis is a technique that separates and purifies macromolecules, especially proteins and nucleic acids that differ in size, charge or conformation (Wilson and Walker, 2000). When these charged molecules are placed in an electric field, they migrate toward either the cathode or anode, depending on their charge. Nucleic acids have a consistent net negative charge imparted by their phosphate backbone and when the pH of the electrophoresis system is more than 8. They migrate toward the anode. The gel can either be agarose or polyacrylamide (Sambrook and Russel, 2001).

Agarose is a mixture of polysaccharides isolated from seaweeds. It is typically used at concentrations of between 0.5 to 3%. The higher the agarose concentration the stiffer the gel and the smaller the pores and greater the sieving capacity becomes. Agarose gels have a large range of separation, but relatively low resolving power. By varying the concentration of agarose, fragments of DNA from about 200 to 50 000 base pairs (bp) can be separated using standard electrophoretic techniques (Wilson and Walker, 2000). However, a dye such as ethidium bromide (EtBr) is added to the gel, where the dye molecules bind (intercalates) to the DNA. Exposure of the bound DNA to UV light causes the dye to fluoresce and the DNA can be seen (Wilson and Walker, 2000). Aleem et al (2003) have shown that by using agarose gel electrophoresis, there is a possibility of differentiating the sizes of multiple metal resistant plasmids DNA.

Agarose DNA electrophoresis is used for analysis of the DNA structure, success of DNA isolation, analytical techniques such as restriction enzyme mapping, confirmation of the

size of plasmids, whether insertions are successfully achieved, whether the polymerase chain reaction (PCR) were successful and whether the amplicons are of the correct size. Piotrowska-Seget (2005) used gel electrophoresis for the confirmation of successful transformation and re-extraction of plasmids from heavy metal tolerant bacteria. It has also been used to test for the presence of plasmids from various environments such as industrial wastewater and for the comparison whether the strains have lost the plasmids containing the specific resistance gene (Zolgharnein *et al*., 2007; El-Deeb, 2009). Anjum *et al*. (2011) used agarose electrophoresis to characterised plasmids. These authors used specific primer systems for the detection of conjugative plasmids and incompatibility groups in metal and antibiotics resistant bacterial isolates. Agarose electrophoresis is also used during preparative techniques such as the separation of fragments in the recovery and cloning and the quantitation of individual DNA fragments in a mixture (Brody & Kern, 2004).

1.6.4 SDS-PAGE

Polyacrylamide is used to separate proteins on the basis of their shape/size, which relates the proteins to their relative molecular masses. Co-polymerization of monomeric acrylamide and the cross-linker bisacrylamide forms a lattice of cross linked, linear polyacrylamide strands. The pore of a polyacrylamide gel is determined by the concentration of acrylamide and the ratio of acrylamide to bisacrylamide (Wilson and Walker, 2000).

Prior to electrophoresis protein samples are treated with a buffer that contains the anionic detergent sodium dodecyl sulfate (SDS) and reducing agent (ß-mercaptoethanol). The ß-mercaptoethanol reduces any disulphide bridges that are holding the protein tertiary structure together (Wilson and Walker, 2000). SDS binds to the proteins and dissociates most multi-chain proteins. Each SDS-coated protein chain has a similar charge-to-mass

ratio. During electrophoresis, the separation of the SDS-protein chains is based primarily on size, and the effect of conformation is eliminated. Thomson-Carter and Pennington (1989) have demonstrated that SDS-PAGE can be used as a tool in differentiation studies. While, Archer *et al*. (1984) showed reproducible plasmid differentiation pattern obtained by SDS-PAGE.

Sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (SDS-PAGE) with 10% polyacrylamide gel resolves proteins that range from 20 to 200 kilodaltons (kDa). SDS-PAGE is the most widely used method for analysing protein mixtures qualitatively. This technique has been applied in verifying microbial strain authenticity, rapid classification and identification, determining sample purity, protein composition monitoring, ecological and epidemiological studies, blotting applications and establishing protein sizes (Plikaytis *et al*., 1986; Schägger & Von Jagon, 1987; Pot *et al*., 1994; Coenye *et al*., 2001). Barreau *et al.* (1993) showed and concluded that by using cluster analysis based on SDS-PAGE profiles differences between species were effectively demonstrated. They could verify the results with complementary techniques. Gómez-Zavaglia *et al.* (1999) showed that SDS-PAGE is a useful tool in the analysis of the whole-cell protein profiles for resolving taxonomic status and distinguishing strains and subspecies.

1.6.5 TWO-DIMENSIONAL ELECTROPHORESIS

Two-Dimensional gel electrophoresis is one of the proteomic techniques which separates proteins according to their isoelectric point (p*I*) in the first dimension and followed by the second dimension that separates proteins according to their molecular weight (MW). The molecular weight is measured in daltons (Da) (Figeys, 2005). Classical 2-D electrophoresis with pH gradients is performed with the utilization of carrier ampholytes (CA) which were limited in terms of their resolution, reproducibility and protein-loading capacity (Görg,

2000). However, the use of commercial Immobilized pH Gradient (IPG) strips were rejected at first, but after the suitable strips were found they have been used ever since (Bjellqvist *et al*., 1982). Their pH gradients range between 4-7, 5-8, 3-10 and 3-12. IPG-strips are rehydrated so that they can absorb the protein sample and that the protein could be distributed evenly across the whole strip. This limits the precipitation of excess proteins in uneven areas of the gel. After rehydration of the IPG-strip, the isoelectric focusing is the following step that applies an electric field. Figure 1 is an illustration of the IPG focussing process.

The second dimension is by SDS-PAGE. Proteins migrate into the second dimension (a SDS-PAGE gel) and are separated according to the molecular weight (MW) (O'Farrell, 1975; Figeys, 2005). This process makes it possible for the simultaneous analysis of mixture of proteins or hundreds of thousands of gene products and a high possibility of identification of specific proteins.

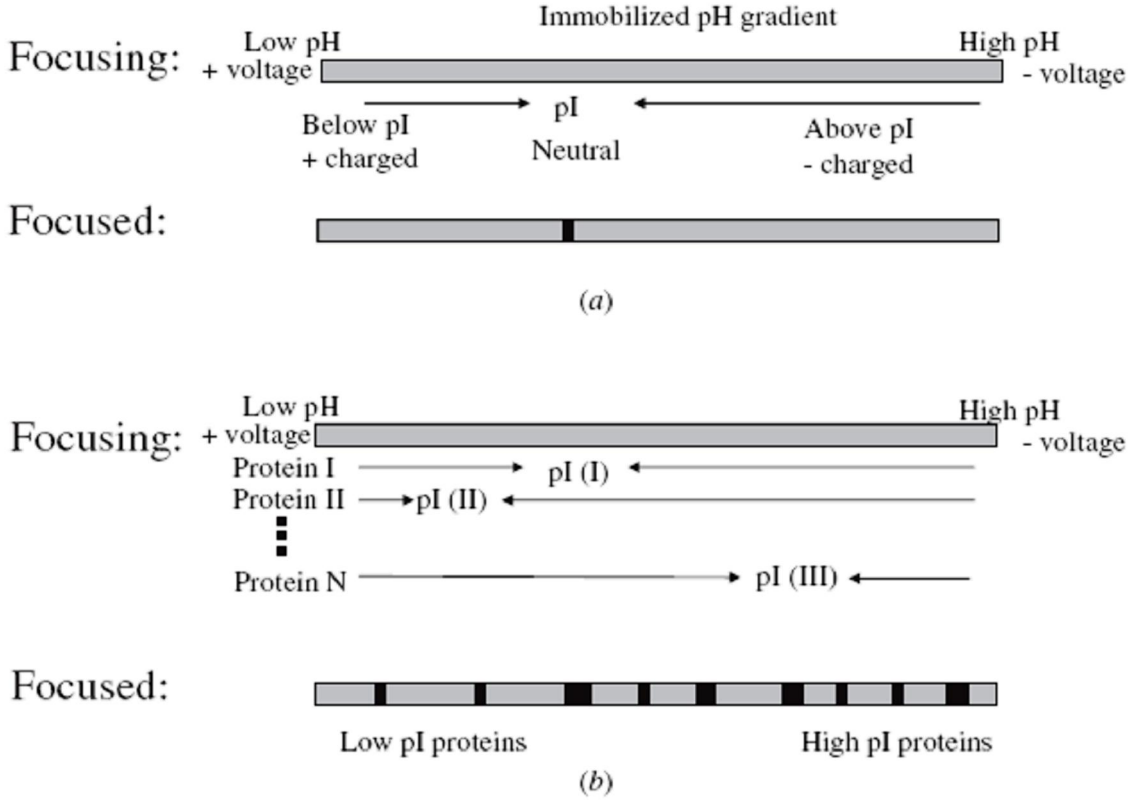

Figure 1.1: Isoelectric focusing on an immobilized pH gradient (IPG) strip. (*a*) Shows the focusing of a single protein, whilst (*b*) focuses on a mixture of multiple proteins (Görg, 2000).

In clinical research, proteomics are used as a tool for biomarker discovery. In the field of medicine for example, blood proteins from patients with various disease states are compared to those of healthy individuals. Using this approach, researchers were able to use the protein of interest for biomarker identity (Colantonio and Chan, 2005). Proteomics is also applied to scenarios where proteins involved in the carcinogen mitigation for screening, diagnosis, prognosis, monitoring response to treatment and when detection of recurrent diseases are studied (Cho, 2007). Furthermore, proteomics methods may be useful for pathogen discovery.

Plant proteomics contributes in biomedicine fields, by identifying and characterizing the allergens in agronomy, transgenic crops, genotyping and studies of food quality and traceability (Jorrin-Novo *et al.*, 2009). In addition it has the potential to make characterization of tissue specific expression products in animal and plants possible. In plant biology, proteomics can be utilised to map translated genes and loci controlling their

expression in order to be used for the identification of proteins accountable for the variation of complex phenotypic traits (Müllner, 2003).

Proteomics applicability is not only based on clinical application in pharmacological related research. It is also important in the optimization of bacterial or fungal strains used in fermentation processes. This makes it possible to identify key metabolic enzymes and regulatory proteins. Thus, with application of proteomic techniques, different protein patterns from a specific organism exposed to different environments can be compared (Melin, 2004).

1.7 AIM

The aim of the study was to characterize metal tolerant bacterial plasmids isolated from a platinum mine tailings dam using molecular (genotypic and proteomic) profiling techniques.

1.8 OBJECTIVES

The objectives of the study were to determine the:

- ability to transform *Escherichia coli* strain JM 109 with plasmids that were isolated from various heavy metal tolerant bacteria
- capabilities conferred to transformed *Escherichia coli strain* JM 109 by the plasmids with respect to metals and antibiotics tolerance;
- impact of the isolated plasmids on protein expression profiles (1D and 2D-PAGE) of *Escherichia coli* JM109.
- difference between aluminium-nickel alloy up-regulated and non-regulated protein profiles of the transformants.

CHAPTER 2

MATERIALS AND METHODS

2.1 PARENTAL STRAINS AS SOURCE OF PLASMIDS

Metal tolerant species were obtained from an unpublished study done by Daniels (2008). These were the parental strains of plasmids used in the current study. The isolates were Gram positive bacilli and a Gram negative *A. faecalis*. They were identified as *Paenibacillus lautus*, *validus* and *ginsingagri*; *Bacillus cereus*, *Bacillus subtilis*, *Alcaligenes faecalis* and *Stenotrophomonas maltophilia* (Daniels, 2008).

2.2 PLASMIDS DNA EXTRACTIONS

Five millilitres of LB-Broth (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl per litre (Merck)) containing 0.38 M Aluminium-Nickel alloy was inoculated with a colony of an overnight culture of the original parental species or transformants and incubated at 37°C with constant agitation at 250 rpm overnight. Growth was observed by turbidity of the medium. Then 5 ml overnight cultures were used to inoculate fifty millilitres of the LB-Broth in a 250 ml conical flask. These were then incubated at 37°C for eighteen hours with constant agitation at 250 rpm. In both cases LB-broth contained 0.38 M Aluminium-Nickel alloy. Cells were harvested by centrifugation (4000 x g at 4°C).

Plasmids DNA were extracted from the overnight cultures using a peqGOLD plasmid miniprep kit (PEQLAB Biotechnology, Germany). Three different buffers were used in order to separate chromosomes and the cell debris from plasmids during extraction. The extracted plasmids were captured by the column and eluted using the elution buffer. This was done following instructions from the manufacturer. Minor modifications were necessary and included the two step growth regime mentioned above. Briefly, cells were

resuspended in lysis buffer/solution I containing RNase A. This was done by vortex-mixing. Solution II was added and the solution gently mixed. After the mixture was incubated in room temperature for 2 minutes, solution III was added for lysate neutralisation and gently mixed until a white precipitate formed. The mixture was centrifuged at 4000 x g for 10 minutes at room temperature. Clear supernatant was transferred to PerfectBind DNA column that was placed in a 2 ml collection tube and centrifuged at 10 000 x g. The PerfectBind DNA column was washed with PW plasmid buffer for removal of protein contamination. It was washed twice with DNA wash buffer. After the wash steps the PerfectBind DNA column containing plasmids was dried by centrifugation (2 minutes at 10 000 x g at room temperature). The plasmid DNA was eluted using elution buffer and was stored at -20°C until further analysis.

2.3 SPECTROSCOPIC ANALYSIS AND ELECTROPHORESIS

DNA concentration (ng/µl) and purity of DNA was assessed by NanaDrop, ND-1000 (Nanodrop Technologies, US) spectrophotometer. This was done immediately after collection. The success of the plasmid isolation was also confirmed by 1% (w/v) agarose gel electrophoresis. Ten microliter (µl) of the isolated plasmid DNA was mixed with 10 µl of loading dye (6X Orange Loading Dye, Fermentas, US) and loaded into the wells of a 1% (w/v) agarose gel. The gel contained ethidium bromide (1 µg/ml) (Bio-Rad, UK). The electrophoresis buffer was 1X TAE (40 mM Tris, 20 mM Acetic acid, and 100 mM EDTA pH 8.0). Five microliter (µl) DNA molecular weight marker (1 kb, O'GeneRuler, Fermentas, US) was used for the comparison and confirmation of the molecular and weight of the plasmids. Gel electrophoresis was performed at 70 volts for 40-60 min in a Mini Sub-cell GT and a Power-Pac (Bio-Rad, US). The images of the gels were captured using a Gene-Genius Bio Imaging System (SynGene, Synoptics, UK) and GeneSnap software version 6.08 (SynGene, UK).

2.4 POLYMERASE CHAIN REACTION (PCR)

PCR amplification was carried out using C1000 Thermal Cycler (BioRad, US). The 25 µl reaction mixture included 2X PCR master mix (0.05 U/µl *Taq* DNA polymerase, 4 mM $MgCl_2$ and 0.4 mM dNTPs (Fermentas, US), specific primers ((100 pmole/µl) Table 2.1) and nuclease-free water (Fermentas, US). Primers were synthesized by Inqaba Biotech (SA). Reagents were mixed by brief centrifugation (10 000 x g for 30 seconds Minispin, Eppendorf, Germany). The following conditions were used with different annealing temperatures. For each Inc primer set the initial denaturation was at 94°C for 300 seconds. The next step consisted of denaturation at 94°C for 60 seconds. Annealing temperatures varied depending on the primer set in Table 2.1 as stipulated and was always for 60 seconds and the extension at 72°C for 60 seconds. These conditions were for 35 cycles. This final extension was 72°C for 600 seconds. The 16S rDNA conditions were as follow initial denaturation at 95°C for 300 seconds, the next 35 cycles consisted of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds, extension at 72°C for 60 seconds. The final extension was at 72°C for 600 seconds. All reactions were conducted using 100 ng of plasmid DNA.

The polymerase chain reaction was used to test whether the plasmids could be classified into one of three incompatibility (Inc) groups (IncP, IncQ and IncW; Götz *et al*., 1996). Primers used in this study amplify the origin of replication of these incompatibility plasmids (Table 2.1).

2.5 TRANSFORMATION AND PLASMID RE-EXTRACTION

The calcium chloride (Saarchem, SA) heat-shock method (Cohen *et al*., 1973) was used for the transformation. Competent *E. coli* JM109 (Promega, Madison, WI, USA) cells were prepared and transformed with plasmids, listed in Table 3.1. The transformants were selected and spread on Luria-Bertani agar (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, 15

g Agar, per litre from Merck, Germany), supplemented with 0.38 M Aluminium-Nickel alloy. This aluminium-nickel alloy was used since both nickel (Ni) and aluminium (Al) are metal pollutants associated with platinum mining (Maboeta *et al*., 2006). Plasmid DNA was extracted using the peqGOLD plasmid miniprep kit (Section 2.2; PEQLAB Biotechnology, Germany). Extracted plasmid DNA was stored at -80°C until further analysis.

Table 2.1: Oligonucleotide primers for PCR amplification of IncP-9, IncQ, IncW and 16S rDNA. F, forward primer and R, reverse primer.

PRIMER NAME	PRIMER SEQUENCES (5'-3')	SIZE (bp)	ANNEALING TEMPERATURE	REFERENCE
IncP-9				
Ori 3Fd	5ô- CCA CCG ACA CTG ATG GTC TG -3ô	800	54	Krasowiak *et al*., 2002
Rep 3Rc	5ô- ACC GTG ATG CGT ATT CGT G -3ô			
IncQ				
oriV 1	5ô- CTC CCG TAC TAA CTG TCA CG -3ô	436	57	Götz *et al*., 1996
oriV 2	5ô- ATC GAC CGA GAC AGG CCC TGC -3ô			
IncW				
oriV 1	5ô- GAC CCG GAA AAC CAA AAA TA -3ô	1 140	58	Götz *et al*., 1996
oriV 2	5ô- GTG AGG GTG AGG GTG CTA TC -3ô			
16s rDNA				
341F	5ô- CCT ACG GGA GGC AGC AG -3ô	500	56	Muyzer *et al*., 1993
907R	5ô- CCG TCA ATT CCT TTG AGT TT -3ô			

2.6 MINIMUM INHIBITORY CONCENTRATION (MIC) OF METALS

MIC is determined as the lowest concentration of a metal that completely inhibits bacterial growth after an incubation of 24ï 36 hours at 37°C (Piotrowska-Seget *et al.,* 2005). This was achieved by adapting the methods of Piotrowska-Seget *et al*. (2005) and Bar *et al*. (2007). The individual metals were added as $HgCl_2$ (Sigma), $CuCl_2$ (Merck, Germany), Al/Ni alloy (Merck, Germany), $Pb(NO_3)_2$, $MnCl_2$ (Merck, Germany), $BaCl_2$ (Saarchem, SA), $CoCl_2$ (Saarchem, SA) and $CrK(SO_4)_2$ (Saarchem, SA) to LB-Broth (media) (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, Merck, Germany). Metal concentrations were 1, 2.5, 5 and 10 mM for each metal (Piotrowska-Seget *et al*., 2005). The tubes containing transformed *E. coli* JM109 were incubated for 18 hours at 37°C with constant agitation at 250 rpm.

Strains were considered resistant if there was growth or turbidity in the tubes. There are no acceptable standard that can be used to distinguish metal-resistant from metal-sensitive bacteria. LB-Broth without metals and *E. coli* JM 109 untransformed cells were used as controls. The MICs of the corresponding environmental strains were also determined. In this case incubation was at 25°C for 1 week.

The agar plate method was also used as it has been used in previous studies (Akinbowale *et al*., 2007; Abou-Shanab *et al*., 2007). This was done to confirm the microdilution approach. Liquid overnight cultures were prepared in LB-Broth and were spotted onto metal containing LB-agar plates. Cultures were incubated for 24 to 48 hours. LB-agar plates without metals were used as controls.

2.7 ANTIBIOTIC RESISTANCE DETERMINATION

Mueller-Hinton agar and antibiotic discs were used to determine antibiotic resistance/susceptibility of *E. coli* JM109 transformed with the various plasmids from the heavy metal tolerant bacteria (Bauer *et al*., 1966). Antibiotics included Kanamycin (30 µg), Streptomycin (300 µg), Chloramphenicol (30 µg), Trimethoprim (2.5 µg), Neomycin (30 µg), Ciprofloxacin (5 µg), Oxy-Tetracycline (30 µg), Ampicillin (30 µg), Amoxylin (100 µg), Cephalothin (30 µg), and Erythromycin (15 µg) (Mast Diagnostics, UK). The cultures were spread plated onto LB agar. These plates were allowed to dry before antibiotic discs were placed onto the surface. The plates were then incubated at 37°C for 16ï 18 hours. Inhibition zones were then measured (in mm) using a ruler and compared to NCCLS (1999) standards to indicate whether the transformant was susceptible, resistant or intermediate resistant.

2.8 PROTEIN EXTRACTIONS

Bacterial proteins were extracted from 10 ml culture extract, suspension of both transformed and non-transformed overnight *E. coli* JM109 cultures by an acetone-SDS extraction procedure (Bhaduri & Demchick, 1983). Minor modifications were made. Cultures were grown in Luria-Bertani agar (10 g Tryptone, 5 g Yeast Extract, 10 g NaCl, 15 g Agar per litre, from Merck, Germany), induced with Aluminium-Nickel alloy and incubated at 37ºC for 24 hours with constant agitation at 120-150 rpm. Thereafter, 2.0 ml of an overnight culture was pipetted into a 2.0 ml sterile microfuge tube and centrifuged at 13 400 rpm for 5 minutes in a centrifuge (Minispin, Eppendorf, Germany). The supernatant was discarded and the steps repeated until all of the 10 ml culture was centrifuged. The pellet was washed twice with phosphate buffered saline (0.1 mM K_2HPO_4, 0.1 mM KH_2PO_4, 0.85% (w/v) NaCl) solution without Mg^{2+} and Ca^{2+} and centrifuged at 13 400 rpm for 5 minutes. Supernatant was discarded and the pellet suspended in 10 ml ice-cold

acetone (Merck, Germany) and allowed to stand on ice for 5 minutes. The cells were collected by centrifugation at 13 400 rpm for 5 minutes. The supernatant was then discarded. The remaining acetone was removed by leaving the microfuge tubes on bench tops at room temperature. Proteins were extracted from the pellet by resuspension in 200 µl of extraction buffer (0.125 mM Tris pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 10% (v/v) ß-mercaptoethanol) with addition of 2 µl of protease inhibitor cocktail I (Melford, UK). This was incubated at 100ºC for 10 minutes. Glass beads were added in small quantities to each sample. The samples were vortexed for 2 minutes and centrifuged at 13 400 rpm for 90 seconds. Supernatant was then transferred into a sterile microfuge tube. Proteins were purified using the ReadyPrep™ 2-D Cleanup kit (Bio-Rad, US) according to the instruction manual. Protein concentrations were determined by the Bradford (1976) assay using a Bovine Serum Albumin Standard Set (Fermentas, US).

2.9 GEL-BASED SEPARATION OF PROTEINS

2.9.1 SDS-PAGE

Protein sample concentrations ranging between 4ï 8 µg/µl were prepared and mixed with loading in a loading buffer (0.125 mM Tris pH 6.8, 4% (w/v) sodium dodecyl sulphate, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, 0.002% (w/v) bromophenol blue). The mixtures were then incubated at 100ºC for 10 minutes, immediately transferred to ice and loaded on a gels consisting of 12% (w/v) acrylamide (resolving) and 6% (w/v) acrylamide (stacking). The pH of the stacking gel was 6.8 and that of the resolving gel 8.8. Five microliters of 14.4 to 116.0 kDa unstained protein molecular weight marker (Fermentas, US) was also loaded on each gel. The electrophoresis buffer was a Tris-Glycine buffer (0.25 M Tris base, 1.92 M glycine, 1% (w/v) SDS). Electrophoresis was done for two hours at 110 volts using a Mini-PROTEAN® 3 cell and PowerPac UniversalÊ (Bio-Rad,

US). After two hours of running, the gel was stained with 0.13% (w/v) Coomassie Brilliant blue R-250 (Saarchem, SA) in 50% (v/v) methanol, 10% (v/v) acetic acid glacial and 40% of ultra-pure water (ddH_2O) for 1 hour. The gel was distained overnight with 40% (v/v) methanol, 10% (v/v) acetic acid glacial and 50% of ultra-pure water (ddH_2O). The gel images were captured using a Gene-Genius Bioimaging system (Syngene, UK) and GeneSnap software version 6.08 (SynGene, UK).

2.9.2 TWO-DIMENSIONAL ELECTROPHORESIS AND PROTEIN STAINING

For the first dimension, rehydration of protein samples were carried out by passive rehydration in which 100-200 µg of protein extracted were mixed with 125 µl of rehydration/sample buffer (8 M urea, 2% (w/v) CHAPS, 50 mM DDT, 0.2% (v/w) 100X Bio-Lyte 3/10 (or 4/7) ampholyte, 0.002% bromphenol blue) (Bio-Rad, US). Samples were pipetted into adjacent channels of rehydration/equilibration trays using 7 cm IPG strips (Bio-Rad, US) with nonlinear pH gradients (pH 4ï 7), according to manufacturerôs instructions. The strips were placed gel down in order to absorb the sample and were overlaid with 2ï 3 millilitre of mineral oil and allow to incubate for sixteen hours at room temperature.

After rehydration, proteins were separated by isoelectric focusing (IEF) on an IEF Protean Cell (Bio-Rad, US). Parameters were according to the protocol of the manufacture. First-dimension linear separation was carried from 0 to 250 V for 20 minutes. The second step was at 4000 V for 2 hours with linear increase, followed by 4000 V for 10 000 Vh with rapid increase. The last step served as a holding step where samples were held at 500 V with a time limit of 25 000 Vh.

Once isoelectric focusing was completed the 7 cm IPG strips were equilibrated with DTT equilibration buffer for 15 minutes, followed with equilibration in iodoacetamide buffer for 15 minutes at room temperature as described by the manufacturer. Second-dimension was carried out in a Laemmli system (Laemmli, 1970) described in 2.9.1, were the 7 cm IPG strips were loaded on the SDS-PAGE gels. Electrophoresis was at constant 180 volts, using Mini-PROTEAN® 3 cell and Power-PacÊ (Bio-Rad, US) until the dye front reached the bottom of the gel. An unstained protein molecular marker (Fermentas, US) was loaded to each gel. The gels were stained with Coomassie and distained and images captured as described in section 2.9.1.

2.10 QUALITATIVE ANALYSIS OF 2D-PAGE PROTEINS

The comparative spot pattern analysis across multiple gels was accomplished by using PDQuest image analysis software version 7.4 (Bio-Rad, US). Histograms comparing spot quantity were generated with this software.

CHAPTER 3

RESULTS

A total of 13 plasmids were transformed into *E. coli* JM109. These were isolated from various Gram positive metal tolerant species that were originally isolated from platinum mine tailings material. The plasmids and the transformants were characterised and the results are presented in this chapter.

3.1 TRANSFORMATION EFFICIENCY

Transformation efficiency is defined as the number of cells (cfu) produced by 1 εg of plasmid DNA in a transformation reaction (Tu *et al*., 2008). In the present study, the transformation efficiency ranged between $2.5x10^4$ and $6.0x10^4$ per microgram (εg).

3.2 PLASMIDS EXTRACTION AND RE-EXTRACTION

According to the NanoDrop spectrophotometric data, plasmid DNA concentrations ranged between 11.75 and 118.06 ng/εl and the purity (A_{260nm}/A_{280nm} ratios) between 1.62 and 1.86. Furthermore, Figure 3.1 shows plasmids DNA bands on a 1% (w/v) agarose gel. These electrophoresis results support the spectrophotometric data indicating relatively good quality and quantity of plasmid DNA. The quantities varied but sufficient to produce clear bands greater than 10 kb in size (Figure 3.1).

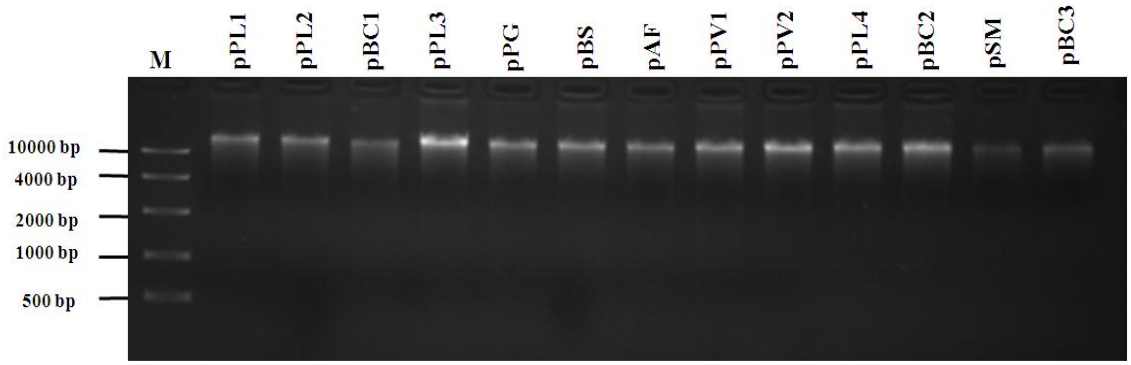

Figure 3.1: Ethidium bromide stained agarose gel electrophoresis showing plasmids isolated from transformed *E. coli* JM109 (p is plasmid and AF, BC, BS, PG, PL, PV and SM represents the parental species from which the plasmids were originally isolated. Also see Table 3.1). A 10kbp molecular weight marker is shown in lane M.

3.3 PLASMIDS STABILITY

To evaluate plasmid-stability, the metal-tolerant *E. coli* JM109 strains were sub-cultured over a 14 day period on LB agar plates that did not contain any heavy metals. After this sub-culturing process all strains were streaked on, and were able to grow on LB agar supplemented with 0.38 M nickel-aluminium alloy. The results of this study are similar to those of Hägg *et al*. (2004) who showed that transformed *E. coli* JM109 has the ability to retain plasmid DNA irrespective of whether the strain was grown in rich LB medium or in minimal medium. These cultures obtained after the plasmid stability tests were grown in LB medium containing 0.38 M of nickel-aluminium alloy. All the various strains were thereafter subjected to plasmid isolation as described above. The isolated plasmids were then subjected to agarose gel electrophoresis. Figure 3.2 shows that plasmids DNA of similar sizes were isolated.

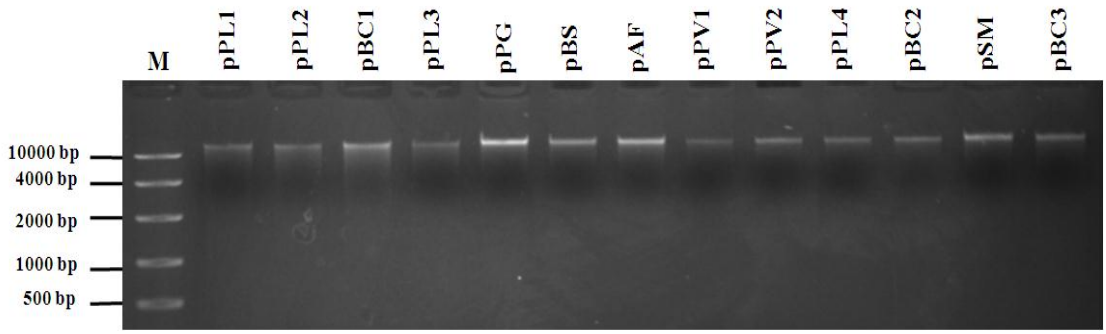

Figure 3.2: Ethidium bromide stained agarose gel electrophoresis showing plasmid profile of transformed *E. coli* JM109 after the stability test. A molecular weight marker of 10 kbp is shown in lane M.

3.4 MINIMUM INHIBITORY CONCENTRATION (MIC) OF HEAVY METALS

E. coli JM109 was successfully transformed with various plasmids that were originally isolated from different bacterial species (Table 3.1). Several strains of *E. coli* JM109 (Table 3.1), that could potentially tolerate heavy metals at relatively high concentrations were thus produced. To test whether these strains exhibited metal tolerance, they were subjected to growth trials in LB containing 0.38 M nickel-aluminium alloy. MICs for the various *E. coli* JM109 strains to Al^{3+}/Ni^{2+}, Pb^{2+}, Ba^{2+}, Mn^{2+}, Cr^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+} were determined using the approach described above. Table 3.1 shows that all strains could grow in media containing 10 mM Ni^{2+}/Al^{3+}, Pb^{2+} and in most cases also Ba^{2+}. However, all transformants were sensitive to 1 mM of Co^{2+} and Hg^{2+}. The overall trend of tolerance to heavy metals was in the order: Ni/Al=Pb>Ba>Mn>Cr>Cu>Co=Hg.

Table 3.1: Minimum Inhibitory Concentration (MIC) of the transformed *E. coli* JM109 against heavy metals. (NG = no growth).

			MIC (mM)							
Plasmids	**Strain**	**Species of Origin**	**Al/Ni**	**Pb**	**Ba**	**Mn**	**Cr**	**Cu**	**Co**	**Hg**
pPL1	***E. coli* JM109**/pPL1	*Paenibacillus lautus*	>10	>10	>10	Ć2.5	È1	1>	1>	1>
pPL2	***E. coli* JM109**/pPL2	*Paenibacillus lautus*	>10	>10	>10	>10	Ć2.5	Ć2.5	1>	1>
pBC1	***E. coli* JM109**/pBC1	*Bacillus cereus*	>10	>10	>10	Ć2.5	È1	1>	1>	1>
pPL3	***E. coli* JM109**/pPL3	*Paenibacillus lautus*	>10	>10	>10	Ć5	È1	È1	1>	1>
pPG	***E. coli* JM109**/pPG	*Paenibacillus ginsingagri*	>10	>10	>10	>10	È1	È1	1>	1>
pBS	***E. coli* JM109**/pBS	*Bacillus subtilis*	>10	>10	>10	Ć2.5	È1	È1	1>	1>
pAF	***E. coli* JM109**/pAF	*Alcaligenes faecalis*	>10	>10	È5	Ć5	Ć2.5	Ć2.5	1>	1>
pPV1	***E. coli* JM109**/pPV1	*Paenibacillus validus*	>10	>10	È5	Ć5	È1	Ć2.5	1>	1>
pPV2	***E. coli* JM109**/pPV2	*Paenibacillus validus*	>10	>10	>10	>10	È1	È1	1>	1>
pPL4	***E. coli* JM109**/pPL4	*Paenibacillus lautus*	>10	>10	È5	Ć5	È1	Ć2.5	1>	1>
pBC2	***E. coli* JM109**/pBC2	*Bacillus cereus*	>10	>10	>10	Ć5	>10	È1	1>	1>
pSM	***E. coli* JM109**/pSM	*Stenotrophomonas maltophilia*	>10	>10	>10	Ć5	>10	Ć2.5	1>	1>
pBC3	***E. coli* JM109**/pBC3	*Bacillus cereus*	>10	>10	>10	>10	>10	È1	1>	1>
Untransformed *E. coli*JM109			NG	NG	NG	NG	NG	NG	NG	NG

3.5 POLYMERASE CHAIN REACTION (PCR)

Amongst all the plasmids tested only three (pPL1, pBC1, pPG) tested positive for IncW group (Figure 3.3). In Figure 3.3 a band of the expected size is shown in all three lanes, although the bands in lanes 1 and 2 were fainter than the one in lane 3. The PCRs for IncP and IncQ were all negative. The IncW plasmids in this study were originally isolated from metal-tolerant *Paenibacillus lautus* (pPL1), *Bacillus cereus* (pBC1) and *Paenibacillus ginsingari* (pPG). None of these three plasmid DNA samples tested positive for 16S rDNA.

The three IncW plasmids (pPL1, pBC1, pPG) conferred resistance to Ni^{2+}/Al^{3+}, Pb^{2} and Ba^{2+} at levels of 10 mM if not greater. In addition to this, pPG also conferred resistance to Mn^{2+} at concentrations of 10 mM or higher. Another plasmid that did not belong to any of the three compatibility groups tested for, pAF also conferred low-level tolerance to Cr^{2+} and Cu^{2+} (2.5 mM).

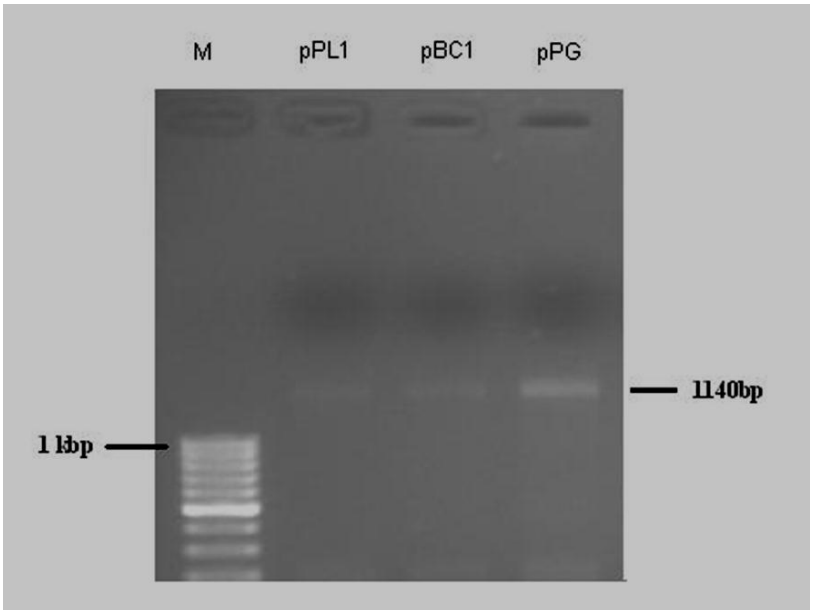

Figure 3.3: A gel of PCR amplified fragments of IncW origin of replication. These were amplified from plasmids isolated from transformed *E. coli* JM109. A 100 bp molecular weight marker (O'GeneRuler, Fermentas Life Sciences, US) is shown in lane M.

3.6 ANTIBIOTIC RESISTANCE

The majority of metal resistant isolates were also resistant to one or more antibiotics. Antibiotic resistance profiles for each of the environmental (parental) isolates are summarized in Table 3.2. This illustrates the antibiotic susceptibility pattern of the parental species. All the isolates were resistant to Trimethoprim and most were also resistant to oxy-tetracycline. All of the isolates were sensitive to Neomycin, Streptomycin and Cephalothin. Antibiotic resistance patterns of *E. coli* JM109 strains (Table 3.3) were not the same as the parental strains (Table 3.2).

Table 3.2: Antibiotic Resistance Profile of the Original Cultures

Antibiotic	Concentration	Growth Inhibition Zone											
		A	B	C	D	E	F	G	H	I	J	K	L
Amoxylin	10 ε g	I	R	R	R	R	R	R	S	S	R	R	S
Ampicillin	30 ε g	R	R	R	R	R	R	R	S	S	R	R	S
Cephalothin	30 ε g	R	R	R	R	R	R	R	R	R	R	R	R
Chloramphenicol	30 ε g	S	S	R	I	I	I	S	R	R	I	S	R
Ciprofloxacin	5 ε g	S	I	S	S	S	S	I	S	I	S	I	S
Erythromycin	15 ε g	I	S	S	I	S	I	S	S	R	S	S	I
Kanamycin	30 ε g	R	I	I	R	R	I	R	I	I	R	R	R
Neomycin	30 ε g	S	S	S	S	S	S	S	S	S	S	S	S
Oxy-Tetracycline	30 ε g	R	R	R	R	R	R	R	R	I	R	R	S
Streptomycin	300 ε g	S	S	S	S	I	I	S	S	S	S	S	S
Trimethoprim	2.5 ε g	R	R	R	R	R	R	R	R	I	R	R	R

Note: R ï resistant, IR ï intermediate resistant, S ï susceptible, *Paenibacillus lautus* (A, B, C, D, E), *Bacillus cereus* (F), *Paenibacillus validus* (G), *Paenibacillus ginsingagri* (H, I), *Bacillus thuringiensis* (J), *Alcaligenes faecalis* (K), *Stenotrophomonas maltophilia* (L).

Table 3.3: Antibiotic Resistance Profile of Transformed *Escherichia coli* JM109

Antibiotic	Concentration	Growth Inhibition Zone												
		A	B	C	D	E	F	G	H	I	J	K	L	M
Amoxylin	10 ε g	S	S	S	S	S	S	S	S	R	S	S	S	S
Ampicillin	30 ε g	S	S	S	S	S	S	S	S	R	S	S	S	S
Cephalothin	30 ε g	S	S	S	S	S	S	S	S	R	S	S	S	S
Chloramphenicol	30 ε g	S	I	S	S	S	S	S	S	S	S	S	S	R
Ciprofloxacin	5 ε g	S	S	S	S	S	S	S	S	S	S	S	S	S
Erythromycin	15 ε g	R	R	R	R	R	I	I	R	S	I	R	I	R
Kanamycin	30 ε g	S	S	S	S	S	S	S	S	S	S	S	S	S
Neomycin	30 ε g	S	S	S	S	S	S	S	S	S	S	S	S	S
Oxy-Tetracycline	30 ε g	I	R	I	R	S	I	R	R	R	I	I	I	I
Streptomycin	300 ε g	S	S	S	S	S	S	S	S	S	S	S	S	I
Trimethoprim	2.5 ε g	S	S	S	S	I	S	S	S	R	S	S	S	S

Note: R ï resistant, IR ï intermediate resistant, S ï susceptible, transformed *E. coli* JM 109 per plasmid pPL1, pPL2, pPL3, pPL4 (A, B, C, D) respectively, pBC1, pBC2, pBC3 (E, F, G), pPV1, pPV2 (H, I), pPG (J), pBS (K), pAF (L), pSM (M).

.

3.7 PROTEOMIC ANALYSIS

3.7.1 SDS-PAGE ANALYSIS

SDS-PAGE was performed to analyse protein-banding patterns from different metal tolerant bacterial species isolated from a platinum mine tailings dam. The protein profiles are illustrated in Figure 3.4, showing the banding patterns of the different environmental isolates. The banding patterns were quite different even between isolates of the same species e.g, lanes B, C, G that represents *Paenibacillus ginsengagri* and lanes A, E, F, J that represents *Paenibacillus lautus*. There were, however, some bands that distinguish the various bacterial strains. The plasmids from these species were transformed into *E. coli* JM109.

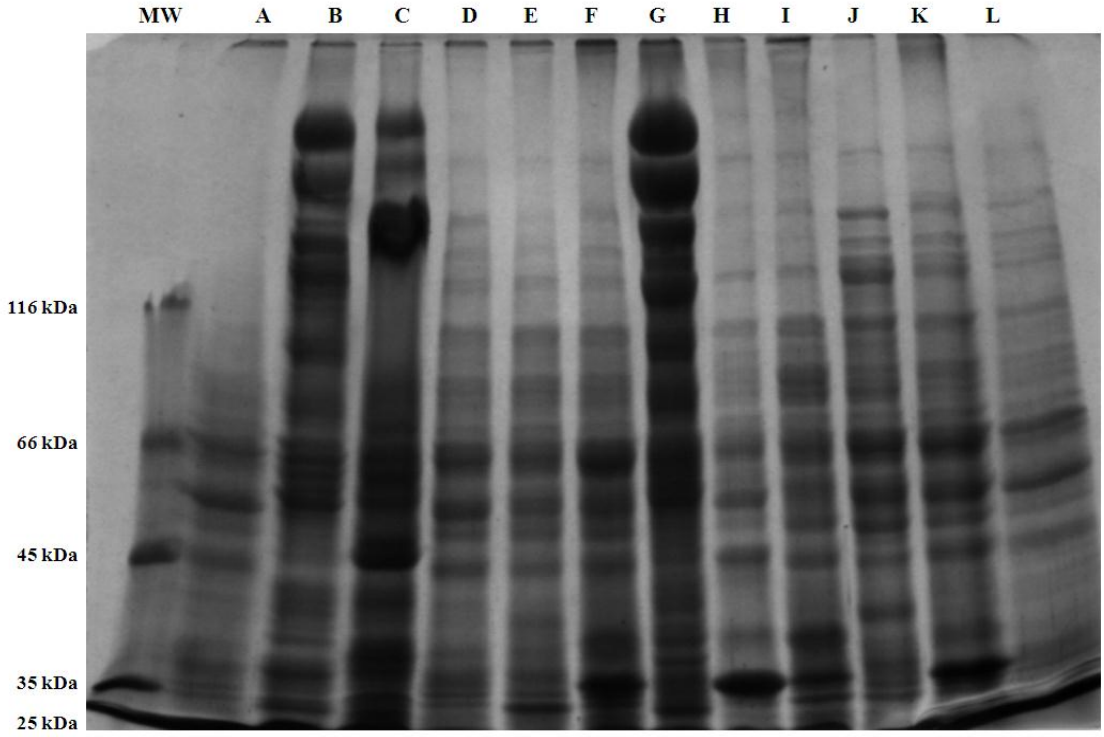

Figure 3.4: SDS-PAGE protein expression patterns of various heavy metal tolerant bacteria. MW = molecular weight marker (kDa). Protein patterns: Lanes A, E, F, J represent *Paenibacillus lautus*, B, C, G, *Paenibacillus ginsengagri*, D *Bacillus thuringiensis*, H *Alcaligenes faecalis*; I *Paenibacillus validus*, K *Stenotrophomonas maltophilia* and L *Bacillus cereus* protein profiles.

Figure 3.5 shows the SDS-PAGE proteins profile of transformed and un-transformed *E. coli* JM109 strains. The transformed *E. coli* JM109 strains were grown in LB broth containing 0.38 M of Al/Ni alloy. These profiles were generally very similar. This could be expected since all were *E. coli* JM109, although they were transformed with various plasmids. However, there were bands in the 30 to 35 kDa region present in the transformants that were grown in the Ni/Al containing media. These bands are indicated by arrows. The larger bands were present in strains *E. coli* JM109 transformed with pPL1, pPL2, pBC, pPL3, pPG and pBS but absent in the profiles pAF, pPV1, pPV2 and the untransformed *E. coli* JM109. The smaller band was present in all the transformants except pPV2.

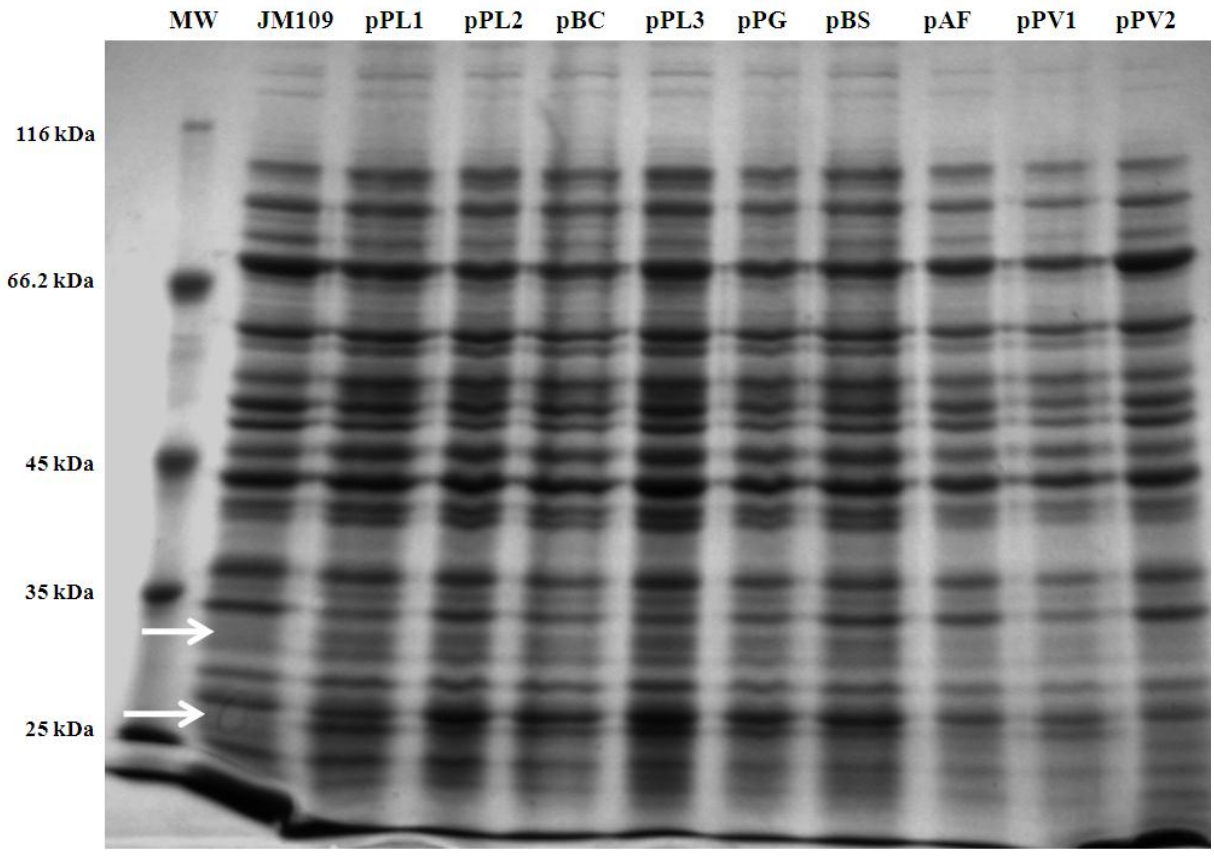

Figure 3.5: SDS-PAGE of untransformed and transformed *E. coli* JM109. Lane MW = molecular weight marker (kDA). Lane JM109 was untransformed followed by transformed *E. coli* JM109 strains. Instead of labeling the lanes numerically, the labels used were of the plasmids that the *E. coli* JM109 was transformed with. The arrows indicate major differences between the protein samples.

These differences in the SDS-PAGE profiles could thus be justified by this explanation. Resolution of this protein methodology is in such a way that very little differential protein expression could be observed in Figure 3.5. In Figure 3.5 it can also be seen that the differentially expressed bands (30 to 35 kDa) were present in the IncW plasmid transformed *E. coli* JM109 strains (pPL1, pBC and pPG). Due to low bacterial protein alteration level by one-dimensional SDS-PAGE, further analyses were not considered.

3.7.2 TWO-DIMENSIONAL ELECTROPHORESIS (2D-PAGE)

Two-dimensional SDS-PAGE offers greater protein expression differentiation (O'Farrell, 1975; Figeys, 2005). This is also demonstrated in Figures 3.6 to 3.9. From these images it is evident that some of the proteins were up-regulated while others were down-regulated. Protein expression differences across the gels were observed. The largest numbers of proteins were from 15ï 75 kDa. Since a pH 4-7 IPG strip was used for the isoelectric focusing and the majority of the proteins had p*I* values ranging from 5-6. From the comparative analyses with PDQuest™ software, close to 150 proteins were two-to-three fold up-regulated in the induced *E. coli* JM109 strains and hundreds were down-regulated. Fifteen proteins were selected to demonstrate this aspect. The various gels in Figures 3.6 to 3.9 were analysed by PDQuest™ 2D-PAGE analysis version 7.4 software (BioRad, US). Examples of the output bar charts are provided in each of the figures. The data from the output files are summarized in Table 3.4.

In Figure 3.6 one of *E. coli* JM109 strains transformed with an IncW plasmid (pPG) that was grown in Ni/Al containing LB media is compared to *E. coli* JM109 grown in metal free LB media (control). From these gels it is evident that several protein expression levels were either up ï or down-regulated. A similar pattern is observed when the same control is compared to the protein expression of the *E. coli* JM109 strain that was transformed with another incW plasmid (pPL1) and grown under similar conditions as the *E. coli* JM

109/pPG strain (Figure 3.7). According to the PDQuest^{TM} results for both these strains protein number 1, 2, 13 and 14 were down-regulated in the strain grown in media containing the metals. The rest of the proteins were up regulated. However, in the case of strain pPL1 protein 12 was also down regulated.

Figure 3.8 shows the comparison between 2D-PAGE gels of the strain *E. coli* JM109/pPL1 that was grown in Ni/Al alloy (Figure 3.8 A) and one that was grown in the absence of the alloy (Figure 3.8 B). In this case proteins 1, 2, 4 to 8 and 13 were up-regulated and the rest down-regulated. In Figure 3.9, when the 2D-PAGE gel profiles of *E. coli* JM109/pPL1strain (A) and control *E. coli* JM109 (B) both grown in the absence of Ni/Al alloy was compared, it showed that proteins 3, 10 and 11 were up-regulated and most down-regulated.

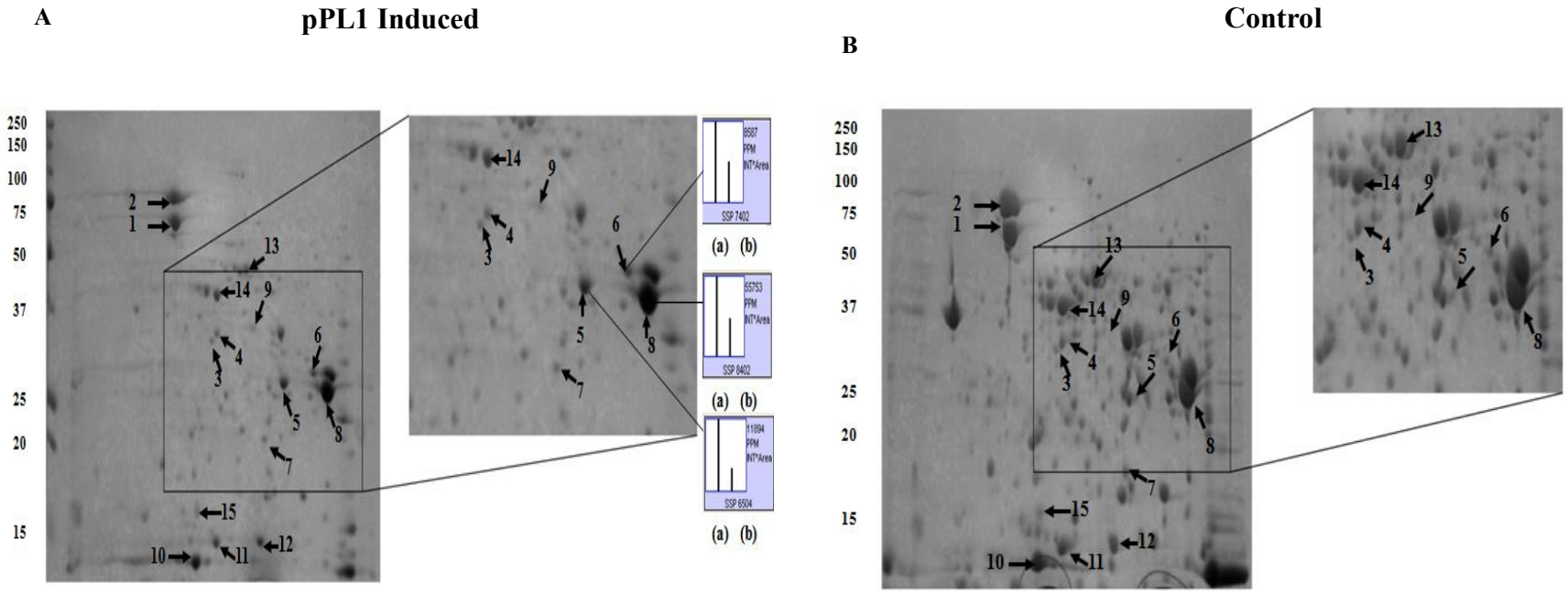

Figure 3.6: 2D-PAGE profiles of (A) transformed *E. coli* JM109/pPL1 grown in LB broth containing Ni/Al and (B) control (*E. coli* JM109) untransformed and not exposed to any metals. Different numbers shows the relative position of the differentially expressed proteins. The bar charts are the representatives of comparison between the same numbered spot (proteins) on the gels.

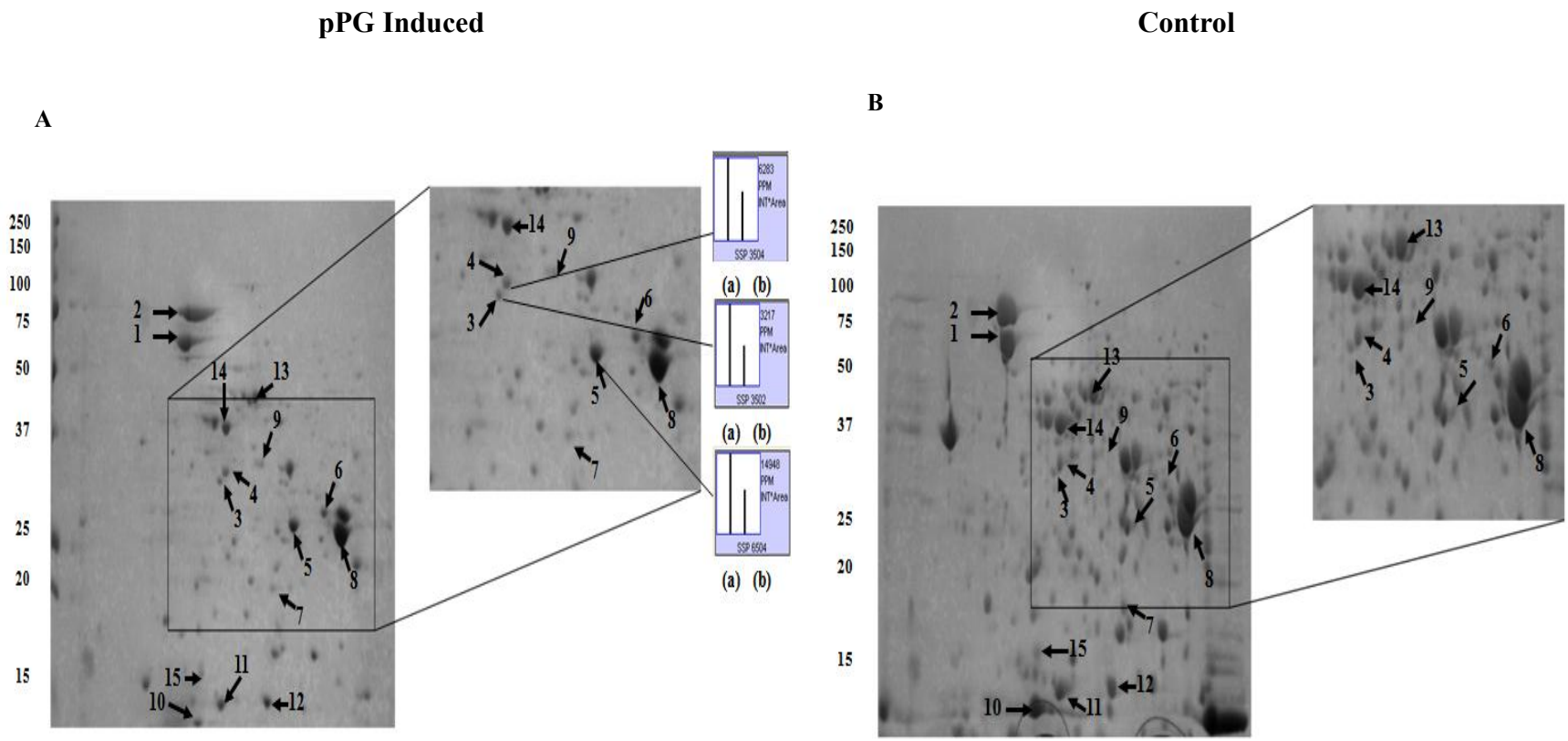

Figure 3.7: 2D-PAGE profiles of transformed *E. coli* JM109/pPG (A) grown in LB broth containing of Ni/Al alloy. Control is represented by (B) untransformed *E. coli* JM109 un-exposed to any of metals. Different numbers showing relative position of the differentially expressed proteins. The bar charts are the representatives of comparison between the same numbered spot (proteins) on the gels.

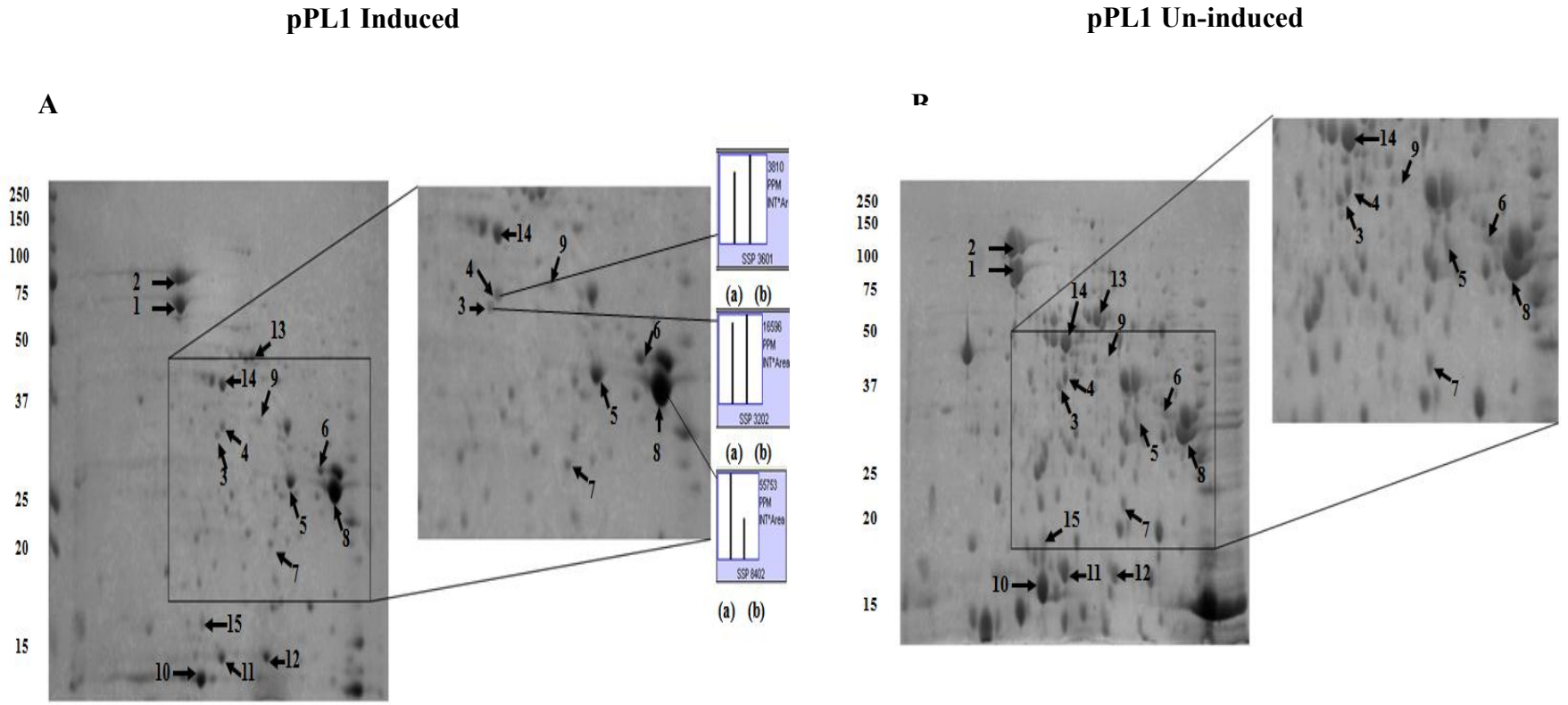

Figure 3.8: A comparison of protein expression from transformed *E. coli* JM109. Image show 2D-PAGE profile of (A) transformed *E. coli* JM109 (pPL1) grown in media containing Ni/Al alloy, (B) is transformed *E. coli* JM109 (pPL1) not grown in media Ni/Al alloy. The bar chart shows the difference of protein regulation.

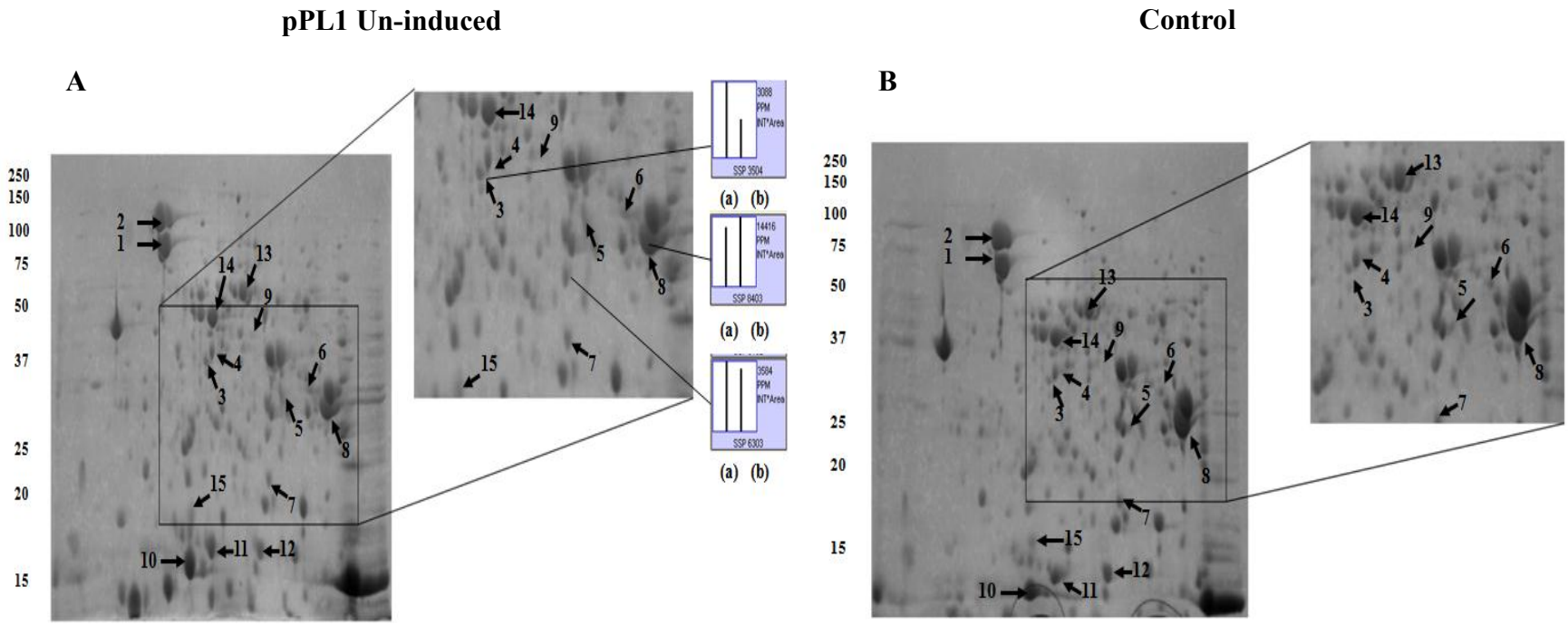

Figure 3.9: 2D-PAGE gels of transformed *E. coli* JM109/pPL1 (A) and (B) as un-transformed *E. coli* JM109 (control) both grown in the absence of Ni/Al alloy.

Table 3.4: Regulation of protein expression of transformed *E. coli* JM109 (pPL1 and pPG) induced and un-induced with Ni/Al alloy compared to the control (untransformed and un-induced *E. coli* JM109) showing up-regulation (u) and down-regulation (d).

	Regulation compared to control (*E. coli* JM109)		**Regulation compared to Un-induced transformant**	**Regulation compared to un-induced control (*E. coli* JM109)**
Protein no:	**pPG**	**pPL1**	**pPL1 Induced**	**pPL1 Uninduced**
1	d	d	u	d
2	d	d	u	d
3	u	u	d	u
4	u	u	u	d
5	u	u	u	d
6	u	u	u	d
7	u	u	u	d
8	u	u	u	d
9	u	u	d	d
10	u	u	d	u
11	u	u	d	u
12	u	d	d	d
13	d	d	u	d
14	d	d	d	d
15	u	u	d	d

3.8 SUMMARY OF RESULTS

The transformation of *E. coli* JM109 with plasmids from metal tolerant bacteria was successful. Plasmids were stably maintained within *E. coli* JM109 when grown on metal free media. After the stability tests plasmids of similar sizes were isolated. Minimum inhibitory concentration (MIC) revealed that some transformants were tolerant to high concentrations (>10 mM) of some metals (Ni/Al, Pb, Ba) but sensitive in low concentrations of Co and Hg. PCR was used to test whether the plasmids could be classified into one of three incompatibility (Inc) groups IncP, IncQ and IncW. These were originally isolated from *P. lautus* strain 1, *B. cereus* and *P. ginsingagri*. The originally isolated metal tolerant bacteria and as well as the transformants were also subjected to antibiotic susceptibility testing using disk diffusion method. The results indicated that most of the parental strains were resistant to several antibiotics but that the transformants were not. The only similarity between the parental strains and transformants was their susceptibility to Neomycin.

Protein expression of *E. coli* JM109 un-transformed as well as strains that were transformed with various plasmids was studied. One dimension SDS-PAGE illustrated general similarity of the profiles except for two banding positions. However, 2D-PAGE resolved more differences in the protein expression profiles. Several proteins were up- and/or down-regulated in the presence of metals. Results suggest that these proteins expression levels are impacted on by the plasmids as they exhibit greater metal resistance. Furthermore, it was showed that most proteins were up-regulated in the presence of the nickel-aluminium alloy.

CHAPTER 4

GENERAL DISCUSSION

Heavy metals are extensively released into the environment by mining operations including platinum mining. They have major impacts due to their toxicity and their inability to be degraded. However, microorganisms adapt to conditions where the concentrations of metals are high by the uptake of various mobile genetic elements including plasmids. These mobile elements sometimes present the hosts with new traits including antibiotic resistance, metal resistance, xenobiotic degradation, etc (Smets and Barkay, 2005; Pal *et al*., 2005; De Boever *et al*., 2007; De Gelsder *et al*., 2008). According to Silver (1996) and Shakya *et al.* (2011) soil microorganisms can resist metal toxicity by transforming metals into less toxic forms. This is achieved by immobilising metals on the cell surface or by precipitation or biomethylation.

4.1 PLASMID PREVALENCE

In the present study several unknown plasmids isolated from different bacterial species that could tolerate high concentrations (>10 mM) of metals, were transformed into *E. coli* JM109. The new genetically modified strains were thus all resistant to high concentrations of various metals. Several authors have previously reported that genetically engineered *E. coli* JM109 with known plasmids of different traits and genes could be resistant to high levels of metals (Krishnaswamy and Wilson, 2000; Deng and Wilson, 2001; Deng *et al.*, 2003; Deng *et al*., 2007). Furthermore, plasmids might be the source of genes that provided *E. coli* JM109 in the current study with metal resistant mechanisms or activated resistant mechanisms that were present on the chromosome (Silver and Misra, 1988; Lakzian *et al*.,

2002). Results of the present study showed that metal resistance was due to the presence of the plasmids and not other mobile genetic elements.

Plasmids isolated and analysed in this study were of the same size, implying that most of them were potentially transported/exchanged between bacterial species in order to develop metal tolerant traits. Schmidt *et al*. (2009) suggested that plasmids might be involved in metal efflux transporters that could lead to increased levels of resistance to metals and other antimicrobials. Ni^{2+} resistance is based on efflux mechanism that is driven by resistance, nodulation and cell division (RND) transporters, whereas Pb^{2+} is transported by CadA P-type ATPase and also by metal ion efflux mechanisms (Nies, 1999). It may thus be possible that the resistance to metals in the present study is caused by similar efflux mechanisms.

4.2 PLASMID STABILITY TEST

The study also demonstrated that the plasmids were inherited in a stable manner. After several generations of sub-culturing onto LB media not having the selective pressure (nickel aluminium alloy) to maintain the plasmids, the plasmids could still be isolated from the *E. coli* strains. These strains also maintained the metal tolerance profiles as originally indicated. Häag *et al.* (2004) showed that plasmids do not always depend on the presence of antibiotics or metals nor minimal or rich media for stability. Stability of plasmids in *E. coli* is not uncommon and had been demonstrated previously (Liu *et al*., 2001; Phillip *et al.,* 2009). The broad spectrum IncW plasmids are known to be stably inherited in *E. coli* K12 even in the absence of antibiotic selection pressures (Sarovich and Pembeton, 2007). Phillips *et al*. (2009) determined the complete sequence of an IncW derived plasmid because of the stability with which this plasmid was inherited in *E. coli* K12 as well as the suitability of the plasmid for the expression of genes from streptomyces. Previous studies

have shown that most bacteria are likely to lose their plasmids after a few cycles of sub-culturing. Thus, this study demonstrated that the various genetically modified *E. coli* strains generated have the ability to stably maintain the plasmids for long periods. In this regard, it is expected that the strains would be capable of surviving in metal polluted environments.

4.3 MINIMUM INHIBITORY CONCENTRATION (MIC) OF HEAVY METALS AND ANTIBIOTIC RESISTANCE

Untransformed *E. coli* JM109 cells were used as controls and were sensitive to all the metal concentrations tested. The transformed *E. coli* JM109 were resistant to high concentrations (10 mM) of Ni^{2+}/Al^{3+}, Pb^{2+} and Ba^{2+}. However, all the transformants were susceptible to Co^{2+} and Hg^{2+}. The order of metal tolerance was Ni/Al=Pb>Ba>Mn>Cr>Cu>Co=Hg. These results are similar to Hassen *et al.* (1998) who also observed that tolerance by *Providencia rettgeri* and *Pseudomonas aeruginosa* was greater to Cu while *Pseudomonas aeruginosa*, *Pseudomonas paucimobilis* and *Klebsiella rhinoscleromatis* was greater to Cr than to both Co and Hg. The lower MICs of the *E. coli* JM109 for Cu^{2+} as observed in the present study is consistent with the results of Giller *et al*. (1998) using *Rhizobium leguminosarum* bv. *trifolii* and *Klebsiella* sp.. The later Gram negative species were also sensitive to copper.

A large body of evidence indicates that metal tolerance and antibiotic resistance are often linked in clinical isolates (Calomiris *et al*., 1984; Grewal and Tiwari, 1990; Sabry *et al*., 1997). In the present study it was however, unlikely that metal tolerance and antibiotic resistance were linked. The parental species had different antibiotic resistance patterns to the transformants. According to Thomas (2000) conjugative gene transfer mediated by plasmids with Broad-Host-Range (BHR) is generally believed to be a common and

widespread mechanism for the transfer of genes across a broad phylogenetic range of bacteria. The findings of the present study are similar to those reported by Grewal and Tawari (1990) and Alonso *et al*. (2002). These authors reported that plasmids conferred to bacterial species, tolerance to metals and other antimicrobial agents. They showed that even though the plasmids were promiscuous, they did not render the hosts with antibiotic resistance characteristics. However, they presented them with greater heavy metal tolerance. Malik *et al*. (2008) also showed that no correlation between the antibiotics and metal resistance patterns. A recent study on sewage pollution, Garcia-Armisen *et al*. (2011) has also shown the lack of association between bacterial resistance to both antibiotics and metals. The examples listed presents similar observation to the results of the current study. These findings suggest that, in the present study, the antibiotic and heavy metal resistance may also not be genetically linked.

4.4 PLASMID INCOMPATIBILITY CLASSIFICATION

Using a PCR based approach it was shown that 3 of the 13 plasmids belonged to the IncW incompatibility group. The stability and the broad host range potential of the plasmids in this study could, to some degree, be explained by this positive result (Sarovich and Pembeton, 2007). The other plasmids tested negative for IncP-9, IncQ and IncW PCRs. Although there are a large number of incompatibility plasmid groups, PCRs could have been designed to test whether these plasmids belonged to any of those groups. The IncP-9, IncQ and IncW groups were initially selected because they were found to occur in bacteria isolated from soil, manure, fish farm sediment, sewage, compost and wastewater samples (Götz *et al*., 1996). Plasmids belonging to these groups are of environmental significance and carry both degradation and antimicrobial resistance markers (Krasowiak *et al*., 2002). It was thus not unexpected that the plasmids in our study, originally isolated from metal-tolerant *Paenibacillus lautus* (pPL1), *Bacillus cereus* (pBC1) and *Paenibacillus ginsingari*

(pPG), were able to be transformed into *E. coli* JM109 and to display stability over several generations.

The transfer of plasmids to new hosts is often accompanied by genetic rearrangements which may facilitate gene expression or plasmid stability in the new host (Davison, 1999). Previous studies have found that genetically engineered *E. coli* has the potential to remove and recover metals from wastewaters and soils at a faster rate than chemical precipitation and ion exchange (Deng and Wilson, 2001; Deng *et al.*, 2003; Deng *et al*., 2007). Results presented in this study suggest that the plasmids isolated may be suitable for conferring heavy-metal resistance to a bacterial consortium and may hold promise for bioremediation of toxic metals.

4.5 PROTEIN EXPRESSION

The proteome is the sum of all the proteins present in a cell at a specific period or under specific physiological conditions (Washburn and Yates III, 2000). Electrophoresis methods to study diversity of proteins produced by cells provide a limited overview of the soluble proteins. The main methods are one dimension SDS-PAGE or 2D-PAGE. In the present study both approaches were used. However, 2D-PAGE gave better resolution of the proteins of *E. coli* JM109 strains. This was particularly so when the protein expression profiles of induced and un-induced transformed *E. coli* JM109 were compared to untransformed *E. coli* JM109. It was demonstrated that transformed *E. coli* JM109 grown under the metal stress either up or down regulated proteins compared to control conditions (non-transformed *E. coli* JM109 grown in LB broth).

Ahsan *et al.* (2009) suggested that there is a need to use proteomics to evaluate the effects of metals on living bacteria. In these studies the focus should be on demonstrating the global protein expression as a consequence of metal exposure. Pereira *et al.* (2006) used

one dimension SDS-PAGE to demonstrate protein expression profiles in *Rhizobium* spp. in relations to high levels of heavy metal contamination in soil. These authors suggested that protein alterations could be an indicator to estimate the level of stress imposed on *Rhizobium* communities, when these are exposed to metal stress. In the present study such an alteration was not feasible when the *E. coli* JM109 transformants were considered. The alteration level was too low and no further analysis was considered for the one dimension SDS-PAGE.

Two dimension polyacrylamide gel electrophoresis (2D-PAGE) can be used for more complex protein mixtures (Nowacka *et al*., 2012). In this analysis the most laborious and time-consuming is the sample preparation step. This is, however, essential for high quality/resolution and reproducibility of the 2D-PAGE. However, using 2D-PAGE individual proteins can be isolated, purified and sequenced. Birko *et al*. (2007 and 2009) used 2D-PAGE to demonstrate that differentially expressed proteins namely Factor C and Factor A are necessary to induced expression of proteins that play a role in cellular communication in stressed *Streptomyces griseus*.

A study of *Pseudomonas fluorescens* isolated from metal polluted water and soil demonstrated that 69 cellular protein spots were differentially expressed, when the isolates were grown in the presence of metals (Sharma *et al*., 2006). The relative molecular weight (MW) of these proteins ranged between 21.5ï 90 kDa with *pI* from 4.6 to 6.2 kDa. According to Sharma *et al.* (2006) the exposure of specific organism to metals does not affect the cells viability directly but rather the expression of proteins for survival and their response to stress. Furthermore, Bar *et al.* (2007) used 2D-PAGE and mass spectrometry to study and identify proteins involved in metal resistance in *Klebsiella* spp. These authors found differential expression of more than 11 proteins but selected 2 proteins for

identification. These two proteins were DNA gyrase and L-isoaspartate carboxylmethyl transferase type II. The molecular weight (and *pI*) of these proteins were between 21.64 kDa (5.0) and 17 kDa (7.4), respectively. The proteins differentially expressed in the present study were, to some extent, similar to the ones from Sharma *et al.* (2006) and Bar *et al.* (2007).

Sano *et al*. (2006) showed production of heavy metal binding protein (HMBP) by *E. coli* BL21. The MW of the HMBP was in the order of 30 kDa. In the present study differentially expressed protein profiles were also observed in the same MW range when the protein expression profiles of metal stressed transformed *E. coli* were considered. Bauman *et al*. (1993) and Luque-Garcia *et al*. (2011) demonstrated that low-molecular-weight proteins have the capacity to bind both physiological required as well as toxic heavy metals. All of these studies support the findings of the present study, i.e. that low MW proteins involved in metal binding were differentially impacted on by plasmids that were originally isolated from various metal bacterial species. These results demonstrate that the plasmids affect the protein expression profiles of the *E. coli* JM109. Results also demonstrated that proteins 4 to 8 are mostly up-regulated when the *E. coli* JM109 transformed strains are grown in Ni/Al containing media.

CHAPTER 5

CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

The aim of the study was to characterize heavy metal tolerant bacterial plasmids isolated from metal tolerant bacteria that were isolated from a platinum mine tailings dam. The objectives were (i) to determine the ability to transform *Escherichia coli* JM109 with plasmids that were isolated from various heavy metal tolerant bacteria (ii) to determine whether the metal tolerance capabilities were also conferred to the transformed *Escherichia coli* JM109 by the plasmids (iii) to determine whether the plasmids belonged to one of 4 incompatibility groups (iv) to determine the impact of the plasmids on the protein expression profiles (1D-PAGE and 2D-PAGE) of the transformed *Escherichia coli* JM109 under metal stress.

(i) TRANSFORMATION OF *Escherichia coli* JM109

Plasmids isolated from various heavy metal-tolerant bacterial species were successfully transformed into *E. coli* JM109 rendering various new metal-tolerant *E. coli* JM109 strains. The results also demonstrated that the plasmids were stability maintained in these new strains over several day by sub-culturing on media that did not contain any metals. Furthermore, the same size plasmids were then isolated from these *E. coli* JM109 transformants that were subjected to the plasmid stability test.

(ii) METAL TOLERANCE OF TRANSFORMED *Escherichia coli* JM109

The various plasmids conferred similar metal tolerant capabilities to *Escherichia coli* JM109 as the parental species from which the plasmids were originally isolated. Plasmids, however, did not convey the same antibiotic resistance patterns of the parental species to

the transformants. This is a finding that cannot be explained but it could be speculated that the antibiotic resistance capabilities in the parental species were not due to genes occurring on plasmids.

(iii) INCOMPATIBILITY GROUP CLASSIFICATION

PCR based methods was used to classify plasmids into one of four incompatibility groups. Three of plasmids tested positive for the IncW *oriT* gene. These plasmids were originally isolated from metal tolerant *Paenibacillus* spp. and *Bacillus cereus* isolates. IncW plasmids are promiscuous and would relatively easily transfer between Gram positive and Gram negative bacteria. These plasmids are known to be stably maintained in bacterial cells, even in the absence of selective pressures. These latter two aspects were demonstrated in the present study.

(iv) PROTEIN PROFILES OF TRANSFORMED *Escherichia coli* JM109

The study further demonstrated that proteins expression of transformed *E. coli* JM109 after the exposure of metals resulted in changes in the expression profiles of a number of proteins. The one dimension SDS-PAGE was less informative than the 2D-PAGE. Several proteins were up-regulated and a number down-regulated when the transformed *E. coli* JM109 were exposed to the Ni/Al metal alloy. Since the plasmids rendered the *E. coli* JM109 tolerant to metals and using support from literature, it can also be concluded that the change in the protein profiles were due to the effects of the plasmids.

5.2 PROSPECTS AND RECOMMENDATIONS

Results of this study provided information regarding role of plasmids in heavy metal-tolerance. The ability to transform *Escherichia coli* JM109 with these plasmids and the fact that the plasmids could be directly linked to the metal tolerant trait opens up possibilities of

using biotechnological approaches, specifically genetic engineering, for toxic metal detoxification. However, several aspects need to be considered before such biotechnological applications could be put in place.

- It is of critical importance to sequence and annotate the entire plasmids to determine which genes are responsible the metal tolerance traits. Next generation sequencing technology should be explored to sequence the plasmids. The technology has become cost effective.
- Transformation of the plasmids into different bacterial (other Gram negative and positive) species should be investigated. It should then be determined if the plasmids give the same traits to the new transformants.
- The remaining plasmids should be tested, by PCR, whether they belong to any of the other Inc groups.
- The proteomics study should be expanded to include profiles where other metals are used to stress the transformants.
- Protein spots from the 2D-PAGE should be excised and sequenced using MALDI-TOF technology. The plasmids and protein sequencing will provide more insight into the mechanisms of metal tolerance.
- Transformants should be investigated for biosorption of metals.

However, with the advanced genetic engineering and molecular biology it should be possible to mimic the proteins that can bind specific metals from engineered bacteria. Finally, it can be concluded that the experiments in this study were successfully completed and useful results were obtained that could be further considered in biotechnological applications by the successful execution of the objectives.

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