THE IDENTIFICATION OF REGULATORY ELEMENTS IN *PSEUDOMONAS AERUGINOSA* AND *P. PUTIDA* RESPONSIVE TO SPECIFIC HEAVY METALS.

Nolan Lindsay Africander

A thesis submitted in partial fulfillment of the requirements for the degree

Master of Science

In the Department of Microbiology

University of the Western Cape

Supervisor: Professor Volker S. Brözel

UNIVERSITY of the
WESTERN CAPE

MARCH 1999

http://etd.uwc.ac.za/

DECLARATION

I, the undersigned, certify that the dissertation hereby submitted, and the work presented herein, to the University of the Western Cape for the degree of MSc., is my own original work and has not, to my knowledge, previously in its entirety or in part been submitted at any university for a degree.

Signature: ....

N. L. Africander (Student no. 9015639)

UNIVERSITY of the
WESTERN CAPE

Date: ....

http://etd.uwc.ac.za/

ACKNOWLEDGEMENTS

I would like to express my gratitude to the following people:

To my wife, Donita, for being my inspiration, for believing in me, and last but not least, for loving me.

My parents, for giving me the opportunity to obtain a tertiary education and for their constant love and support.

My in-laws for their continuous encouragement.

My promoter, Prof. V. S. Brözel for advice, enthusiasm and encouragement during the course of study; for continuous support and invaluable discussions in planning and finalising this manuscript.

My co-promoter, Prof. W. H. Van Zyl for essential advice during the course of study.

Pieter Gouws, for always being prepared to lend a helping hand and ear.

ESKOM and FRD for providing financial assistance.

Members of the Microbiology post-graduate lab for all the fun and support.

I would also like to thank my Creator.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

TABLE OF CONTENTS

	Page
DECLARATION	i
ACKNOWLEDGEMENTS	ii
LIST OF TABLES	xi
LIST OF FIGURES	xii
LIST OF ABBREVIATIONS	xiii
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	4
2.1 Heavy metals	4
2.1.1 General background	4
2.1.2 A history of global heavy metal pollution	5
2.2 Geophysical parameters that influence the toxicity of heavy metals to microorganisms in the environment	6
2.2.1 pH	7
2.2.2 Oxidation-reduction potential (Eh)	9
2.2.3 Anionic composition	9
2.2.4 Cationic composition	10
2.2.5 Clay minerals	10
2.2.6 Microorganisms	11
2.3 Effects of heavy metals on microorganisms and microbial mediated Processes	12
2.3.1 Abundance and Diversity	12
2.3.2 Methanogenesis	13
2.3.3 Respiration	15
2.4 Tolerance and resistance mechanisms of bacteria to heavy metals	15
2.4.1 Phosphate and exopolysaccharide as tolerance mechanisms to heavy metals	16
2.4.2 Plasmid-mediated resistance	17
2.4.3 Heavy metal resistance in biofilms	18
2.5 Bioindicators	20
2.5.1 Principles of bioindicators	20
2.5.2 Use of bacteria as indicators for the detection of heavy metals from the environment	21
2.5.3 Use of genetically engineered bioindicators	23

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

2.6 Bacteria under stress in unfavorable environments 24
2.6.1 Bacterial adaptation to stressful environments 24
2.6.2 Environmental stimuli that induce stress 25
2.6.3 Sensing of bacteria to environmental changes 28
2.6.4 Response to stress and subsequent repair 29

2.7 Translational control of gene expression in prokaryotes 31
2.7.1 Antisense RNA regulation in prokaryotes 32
2.7.2 Pseudoknots and *trans*-acting proteins 33
2.7.3 RNaseIII-mediated control 34
2.7.4 Attenuation of *ermC* mRNA translation 35

2.8 *Pseudomonas* in the environment 36

CHAPTER 3: Response of *Pseudomonas aeruginosa* and *P. putida* exposed to various heavy metals 38

3.1 Introduction 38

3.2 Materials and Methods 40
3.2.1 Microorganisms and growth conditions 40
3.2.2 Preparation of heavy metals 40
3.2.3 Minimal inhibitory concentrations determination of various heavy metals to *P. aeruginosa* and *P. putida* 40
3.2.4 Response of *P. putida* and *P. aeruginosa* to various heavy metals 41
3.2.5 Effect of time on the response of *Pseudomonas* sp. to heavy metals 42
3.2.6 Detection of stress proteins in *P. putida* and *P. aeruginosa* 42

3.3 Results and Discussion 43
3.3.1 Minimal inhibitory concentration of heavy metals to *P. putida* and *P. aeruginosa* 43
3.3.2 Response of *P. putida* to specific heavy metals at various concentrations 44
3.3.3 Exposure of *P. putida* and *P. aeruginosa* to 50mg/L of various heavy metals over time 50

CHAPTER 4: Reporter vector construction and screening of *Pseudomonas* gene libraries against heavy metals 58
4.1 Introduction 58

4.2 Materials and Methods 60

4.2.1 Construction of reporter plasmid pALACZSD 60
4.2.1.1 Amplification of *lacZ* gene using Polymerase Chain Reaction (PCR) 61
4.2.1.2 Modification of $pHBSK^{+/-}$ to serve as control vector 62
4.2.1.3 Cloning of the *lacZ* gene in $pMHBSK^{+/-}$ 62

UNIVERSITY of the WESTERN CAPE

4.2.1.4 Testing the functionality of the *lacZ* gene product 62
4.2.2 Revised cloning strategy for the construction of reporter plasmid pALACZSD 63
4.2.2.1 Digestion of yEP 62 63
4.2.2.2 Testing the functionality of the *lacZ* gene product 63
4.2.2.3 Hybridization of chemically synthesized oligonucleotides 63
4.2.2.4 Separation of hybridized products 64
4.2.2.5 Elution of hybridized oligonucleotides from the acrylamide gel 64
4.2.2.6 Digestion of hybridized oligonucleotides 65
4.2.2.7 Excision of the *lacZ* gene from pBL6.1 65
4.2.2.8 Cloning of the *lacZ* gene construct in pAL4000 66

4.2.3 Construction of *Pseudomonas* gene libraries 68
4.2.3.1 Isolation of *P. putida* and *P. aeruginosa* genomic DNA 68
4.2.3.2 Digestion of genomic DNA 68
4.2.3.3 Construction of *Pseudomonas* gene libraries 69

4.2.4 Transformation of recombinant DNA into the respective *Pseudomonas* hosts 69
4.2.4.1 Transformation of gene libraries in *P. aeruginosa* 69
4.2.4.1.1 Preparation of *P. aeruginosa* competent cells 69
4.2.4.1.2 Transformation of *P. aeruginosa* gene library into *P. aeruginosa* 70
4.2.4.2 Transformation of the *P. putida* gene library into *P. putida* 70
4.2.4.2.1 The $CaCl_2$ method 70
4.2.4.2.2 Electroporation 71
4.2.4.2.3 Triparental mating of *P. putida* gene library 72

4.2.5 Screening of the *Pseudomonas* gene libraries for heavy metal responsive promoters 73
4.2.5.1 The microtitre plate method 73
4.2.5.2 The agar plate method 73

4.3 Results and Discussion 75
4.3.1 Construction of reporter plasmid pALACZSD 75
4.3.2 Construction of *Pseudomonas* gene libraries 81
4.3.3 Transformation of gene libraries in *Pseudomonas* sp. 83
4.3.4 Screening of recombinant clones for heavy metal inducible promoters 85

CHAPTER 5: CONCLUSIONS 90

SUMMARY 91

REFERENCES 94

UNIVERSITY of the WESTERN CAPE

LIST OF TABLES

Page

Table 2.1: Oxidative defense genes in *E. coli.* 27

Table 3.1:The production of heavy metal responsive proteins in *P. putida* when exposed to various concentrations of heavy metals. 47

Table 3.1: (continued) The production of heavy metal responsive proteins in *P. aeruginosa* when exposed to various concentrations of heavy metals. 48

Table 3.2: Summary of the number of proteins induced and suppressed in *P. putida* when exposed to specific heavy metals at various concentrations. 49

Table 3.3: The production of metal responsive proteins in *P. putida* when exposed to 50 mg/ml of various heavy metals at specific time intervals. 52

Table 3.4: Summary of the number of proteins induced and suppressed in *P. putida* when exposed to various heavy metals at a concentration of 50 mg/L over time. 52

Table 3.5: The production of heavy metal responsive proteins in *P. aeruginosa* when exposed to 50 mg/ml of various heavy metals at specific time intervals. 55

Table 3.6: Summary of the number of proteins induced and suppressed in *P. aeruginosa* when exposed to various heavy metals at a concentration of 50 mg/L over time. 55

Table 4.1: Bacterial strains and plasmids used in this study 60

Table 4.2: Summary of clones screened for heavy metal inducible promoters 87

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

LIST OF FIGURES   Page

Fig. 2.1. The emission of heavy metals as compared with mine production. 6

Fig. 2.2. Mechanisms of heavy metal uptake in bacteria. 22

Fig. 3.1. MICs of *Pseudomonas* species to specific heavy metals. 43

Fig. 3.2A. Exposure of *P. putida* to specific heavy metals at various concentrations. 45

Fig. 3.2B. Exposure of *P. putida* to various heavy metal concentrations (continuation). 46

Fig. 3.3. Exposure of *P. putida* to specific heavy metals at a concentration of 50mg/L over time. 51

Fig. 3.4. Exposure of *P. aeruginosa* to specific heavy metals at a concentration of 50 mg/L over time. 54

Fig. 4.1. Chemically synthesized oligonucleotides containing RBS sequence 64

Fig. 4.2. Molecular features of pAL4000 66

Fig. 4.3. Schematic representation of the construction of plasmid pALACZSD 67

Fig. 4.4. Analysis of PCR product on a 0.8% agarose gel 76

Fig. 4.5. Digestion of pBL6.0 as shown on a 0.8% agarose gel 77

Fig. 4.6. Analysis of hybridized oligonucleotides on a 20% PAGE gel 78

Fig. 4.7. Restriction enzyme digest of hybridized oligonucleotides. 78

Fig. 4.8. Restriction enzyme digest of pAL4000 with *Bam* HI, *Nhe*I *and Bgl* II. 79

Fig. 4.9. Digestion of pALACZSD with *Sac*I to confirm *lacZ*SD insert. 80

Fig. 4.10. Partial digestion profile of *P. putida* genomic DNA cut with *Sau*3A. 81

Fig. 4.11. Digestion of *Pseudomonas* genomic DNA with *Sau*3A. 82

Fig. 4.12. Confirmation of insert in recombinant clones by digestion with *Sac*I. 83

Fig. 4.13. Agar-plate showing blue colonies for β-galactosidase activity for the agar-plate method. 89

UNIVERSITY of the WESTERN CAPE

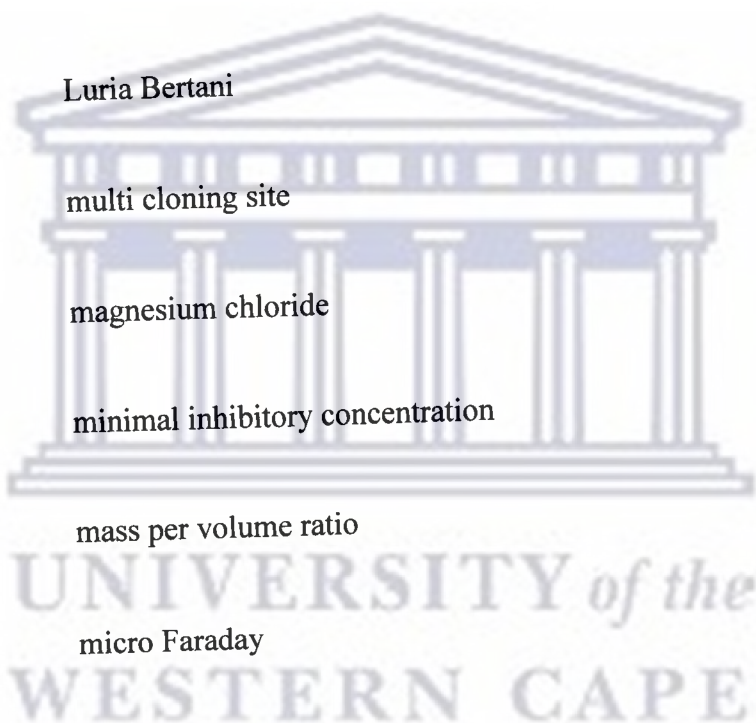
ABBREVIATIONS USED IN THE TEXT

Amp	Ampicillin
APS	ammonium persulfate
ATP	adenosine triphosphate
bp	base pair(s)
bisacrylamide	N, N'-methylene-bisacrylamide
$CaCl_2$	calcium chloride
Cd	cadmium
CFU	colony forming units
CH_3Cl	chloroform
Co	cobalt
CsCl	cesium chloride
CTAB/NaCl	hexadecyltrimethyl ammonium bromide/sodium chloride
Cu	copper
EDTA	ethylene diamine tetraacetic acid

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

EtBr	ethidium bromide
His	Histidine
Hg	mercury
IPTG	isopropyl-thio galactopyranoside
Kan	Kanamycin
kV	kilovolts
LB	Luria Bertani
MCS	multi cloning site
$MgCl_2$	magnesium chloride
MIC	minimal inhibitory concentration
m/v	mass per volume ratio
µF	micro Faraday
min	minute(s)
NaOH	Sodium hydroxide
NH_4OAc	ammonium acetate
Ni	nickel


UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

TEMED	N, N, N', N' tetramethylethylene diamine
Tet	Tetracycline
Tris	Tris-(hydroxymethyl) amino methane
UV	ultraviolet
V	Volts
VB	Vogel and Bonner
v/v	volume per volume ratio
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside
Zn	zinc

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

CHAPTER 1

INTRODUCTION

Heavy metals constitute a group of about sixty-five elements having a density greater than five (Gadd and Griffiths, 1978). Some metals, for example lead, copper, and nickel are extremely precious and profitable to man due to its widespread application in industry. As there has been an increase in heavy metal demand, necessary development of elegant and efficient methods for their isolation were also required. These methods, which led to an increase in industrial development, also resulted in an increase in the complexity and variety of heavy metals polluting the environment (Morby, 1996).

Some of the precious heavy metals such as copper, zinc and nickel, which are extremely pollutive at high concentrations, also serve as essential trace elements for microorganisms, for growth, reproduction, protective mechanisms and also at low concentrations as part of heme and other catalytic structures (Galvin, 1996). Other metals, which include cadmium, mercury and lead, have no known biological function and are therefore considered to be quite toxic and pollutive to the environment. A number of environmental factors force heavy metals into pollutant and toxic forms. These include pH, the type and concentration of ligands on which the metal could adsorb, the oxidation state of the mineral compounds and many others (Gadd and Griffiths, 1978, Babich and Stotzky, 1980). It is due to some of these factors that heavy metals trapped in sediments, silt and in soil, could become mobilized, concentrate in its environment and have adverse effects on its biota.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Microorganisms, which forms the basis of this study, play an essential part in the food web cycle and partake in very important key processes which can be disrupted by the presence of high heavy metal concentrations (Gadd and Griffiths, 1978; Babich and Stotzky, 1980). However, in such environments microorganisms have developed certain mechanisms to combat heavy metal toxicity. These mechanisms occur either at cellular level or at a molecular level (Nies, 1992; Gadd and White, 1993). The cellular mechanisms include exclusion, excretion, sequestration and transformation, and are primarily active as they are induced in the presence of heavy metals. In contrast, microorganisms also have passive tolerance mechanisms, which are not induced in the presence of heavy metals, but nonetheless improve cellular tolerance. At a genetic level, protein production takes place, and has previously been demonstrated in a number of laboratories (Blom *et al*., 1992; Lupi *et al*., 1995). The production of these additional proteins is sometimes referred to as stress proteins.

A typical response of microorganisms to unfavorable environmental conditions involves altered patterns of gene expression, and in some cases, synthesis of stress proteins. Stress factors that induce these responses include heat-shock, UV-light, irradiation, nutrient limitation, chemical stress and many others (Krueger and Walker, 1984; Grossman *et al*., 1985; Goff and Goldberg, 1985; Jenkins *et al*., 1988; Lupi *et al*., 1995; Dukan *et al*., 1996). The regulation of these stimulons appears to be central to the activity and survival of microorganisms. Previous studies performed to study the expression of stress proteins

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

in response to a wide range of stress conditions, were predominantly investigated using Gram negative *Escherichia coli* (Van Bogelen *et al.*, 1987).

Since our environment has become a sink for many industrial, agricultural and household pollutants, methods were developed to detect these toxic elements. Chemical methods developed to detect toxic elements are both expensive and difficult to use. In response to the need to detect heavy metal contamination numerous useful bioassays involving microorganisms have been developed. Tests using bacteria have included the measurement of growth inhibition, respirometry, viability of cells, ecological effects and bioluminescence (Bitton *et al.*, 1992).

Pseudomonas sp., which are natural inhabitants of water, soil and air, have been shown to be present in heavy metal polluted areas (Wong and So, 1993; Wong *et al.*, 1993; Wang *et al.*, 1997). The aim was therefore to subject *Pseudomonas aeruginosa* and *P. putida* to heavy metals and to evaluate the response of the bacteria to the metals. Based on these results, a possible reporter vector can be constructed based on fusions between regulatory elements of heavy metal response genes of *P. aeruginosa* or *P. putida* and a reporter gene. The plasmid can then be transformed into either *P. aeruginosa* or *P. putida*, which will subsequently serve as bioindicators for the specific detection of heavy metals in environmental samples.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

CHAPTER 2

LITERATURE REVIEW

2.1. Heavy metals

2.1.1 General background

Heavy metals are defined as elements having atomic weights between 63.546 and 200.590 and a specific gravity greater than five (Gadd and Griffiths, 1978; Kennish *et al.*, 1992). This definition is based on arbitrary physical parameters, and not on the chemical properties of the metals, such as physical state, color, density, and melting (Ebbing, 1987). A total of approximately 65 elements comply with the above-mentioned definition of heavy metals (Duxbury, 1985).

Heavy metals can bind various ligands to form different complexes, or to either oxidize or reduce them (Jernelöv and Martin, 1975). As a result of this they can become very toxic depending on the external geophysical parameters of the environment (Babich and Stotzky, 1980). Another contributing factor to heavy metal pollution is the high demand for heavy metals due to its widespread application in industry, with subsequent release thereof in the environment.

UNIVERSITY of the
WESTERN CAPE

The concern of heavy metal pollution is that it has adverse effects on human health (Barraquio and Knowles, 1989; Inbar and Ron, 1993; Galvin, 1996) and also lower life, especially microorganisms (Babich and Stotzky, 1980). The degree to which microbial processes can be affected by heavy metal pollution and how microorganisms have

http://etd.uwc.ac.za/

actually adapted to overcome heavy metal polluted environments will be reviewed in this study.

2.1.2 A history of global heavy metal pollution

The question as to when heavy metal pollution actually originated has been investigated. Records of atmospheric heavy metal pollution dating back to ancient times have been preserved in various types of natural deposits, especially polar ice caps, ombrogenic (nutrients derived exclusively from atmosphere) bogs, and aquatic sediments (Nriagu, 1996). Such deposits allowed researchers to establish the time and type of pollution that took place centuries ago (Nriagu and Pacyna, 1988).

It is speculated that heavy metal pollution began when trace amounts of metals were released into the environment as a result of wood being burned for fires (Nriagu, 1996), and according to Nriagu (1996) the emission of heavy metals may be compared with mine production as shown in Fig. 2.1. Emission may be compared with mine production of Cd, Cu, Pb, Ni, and Zn, where about 90% of the mine outputs were consumed in the twentieth century.

UNIVERSITY of the
WESTERN CAPE

Although regulation currently exists on the acceptable amounts of heavy metals allowed to be released in the environment, the ongoing expansion of industry and domestic activities continuously affects the levels of metals released to the environment (Gadd and White, 1993). The natural recycling of some metals that generally occur in biogeochemical cycles is therefore disrupted as a result of the large quantities of metals

http://etd.uwc.ac.za/

and pollutants that are currently entering the environment (Nriagu and Pacyna, 1988; Collins and Stotzky, 1992; Kern and Westrich, 1995; Nriagu, 1996).

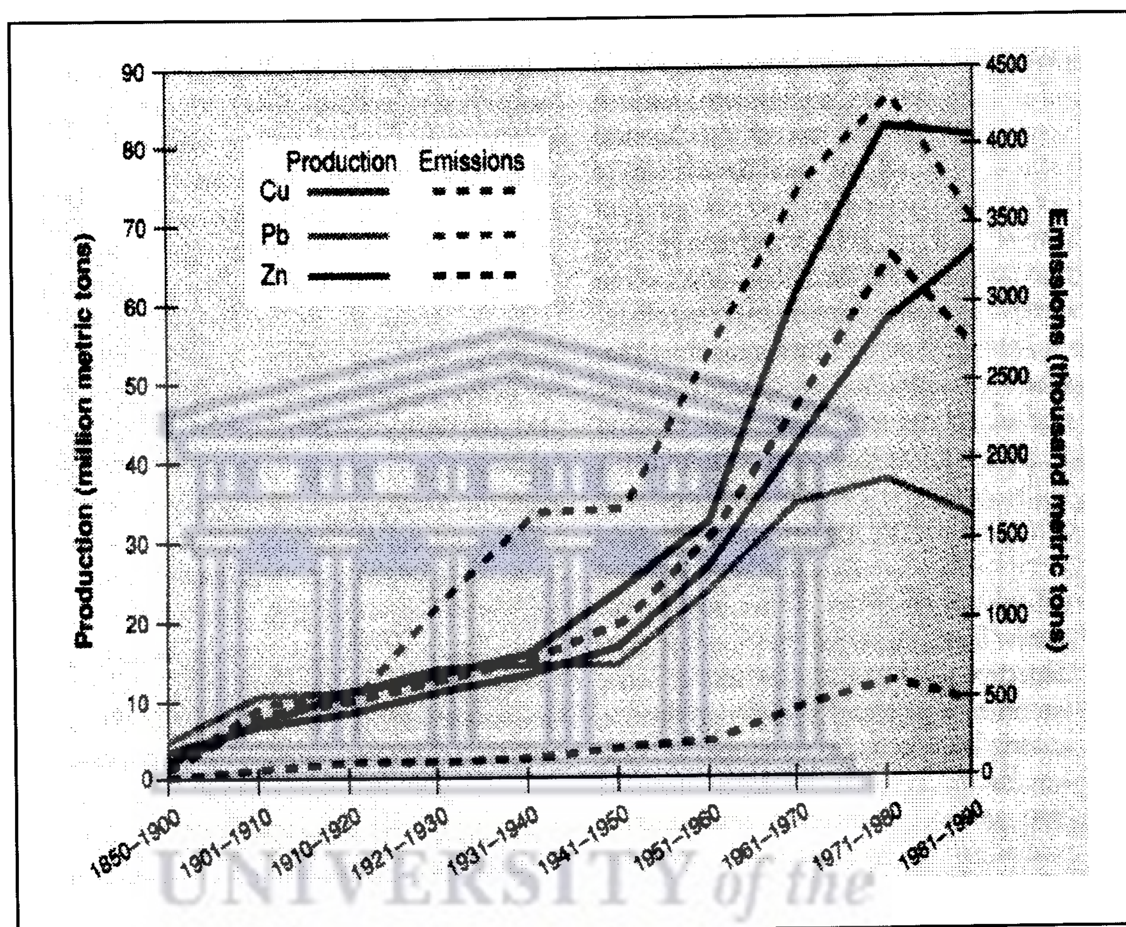


Fig. 2.1. The emission of heavy metals as compared with mine production (taken from Nriagu, 1996)

2.2 Geophysical parameters that influence the toxicity of heavy metals to microorganisms in the environment

Most studies of environmental toxicology have determined the concentrations of heavy metals that cause inhibitory and lethal responses in test organisms under controlled

UNIVERSITY of the
WESTERN CAPE

conditions. These studies are necessary to establish an initial cause and effect relationship between heavy metals and the biota. However, it also important to study the physicochemical characteristics of the environment into which the heavy metal is ultimately deposited (Babich and Stotzky, 1980).

These parameters influence the chemical form, mobility, and hence availability and toxicity of heavy metals to the biota. The determination of the level of toxicity of heavy metals to microbiota, or to the biota in general, is not a simple task. The reason for this is that not only the physicochemical characteristics of the environment but also the chemical form of the pollutant and the physiological state of the biota, affect toxicity. There are many geophysical parameters that influence the toxicity of heavy metals in the environment, but only parameters pertinent to this study will be mentioned. These include pH, (Eh), anionic and cationic composition, clay minerals and microorganisms.

2.2.1 pH

pH is a measure of the availability of H^+ ions in a system and can have a considerable effect on the availability and thus the toxicity of heavy metals in a given environment. In general at an acidic pH, metals exist as free ionic cations, while at an alkaline pH the cations precipitate as insoluble hydroxides or oxides. Most heavy metal hydroxides are insoluble (Gadd and Griffiths, 1978).

The pH at which precipitation occurs, varies for the different metals and the oxidation states of the same element. Some metals such as Cu have more than one valence state and

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

the oxidized state is favored by high pH. The hydroxides of these oxidized states are less soluble than those of reduced states, and precipitate at low pH values. Thus low pH generally increases the availability of metal ions to the biota, whereas high pH decreases the availability. Dean-Ross (1991) reported a detailed case study in which the response of attached bacteria to Zn was studied in outdoor mesocosms. The results indicated that water containing 0.1 mg/L Zn at pH 8.4, showed a higher microbial abundance and activity than at a higher concentration of Zn and lower pH (5mg/L Zn at pH 7.0 and pH 5.5). It is thus noteworthy that pH modulates the toxicity of a heavy metal to the bacterial communities. In a previous study by Ferris and co-workers (1989), it was indicated that biofilm counts increased rapidly to 10^4 CFU/ml in a neutral pH system, while counts decreased to 10^3 CFU/ml at acidic sites.

The toxicity of some heavy metals to microorganisms at various pH levels, can be attributed to the fact that the hydrolyzed speciation forms of these metals, which occur at high pH values, bind on the cell surface, thereby altering the net charge of the cell. Studies done by Collins and Stotzky (1992) show that the adsorption of heavy metals on the cell surface alter the charge of the cell and could affect the various physiological functions of the cell. It was thus deduced that biofilm metal uptake at neutral pH levels were enhanced by up to 12 orders of magnitude over acidic conditions.

UNIVERSITY of the
WESTERN CAPE

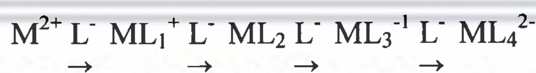
http://etd.uwc.ac.za/

2.2.2 Oxidation-reduction potential (Eh)

Whilst pH is a measure of the availability of H^+ ions in a system, the oxidation-reduction potential (Eh) is a measure of the availability of electrons. Positive Eh values indicate an oxidative environment and negative Eh values, a reducing environment (Babich and Stotzky, 1980). As the valency of many heavy metals is dependent on the Eh of the environment, differentially charged forms of the same element may exert different effects on the biota. For example, Cu precipitates from solution at any Eh when the pH is above 6. When the pH is less than 6 however, it precipitates at an Eh below +200 mV.

2.2.3 Anionic composition

Most heavy metals have a co-ordination number ranging from 1 to 4, sometimes 5 and 6, and can combine with different ligands to form a variety of complex co-ordination species. For example, a divalent metal (M^{2+}), forming a co-ordination complex with a monovalent negatively charged ligand (L^-) follows the sequence:



$$M^{2+} \xrightarrow{L^-} ML_1^{+} \xrightarrow{L^-} ML_2 \xrightarrow{L^-} ML_3^{-1} \xrightarrow{L^-} ML_4^{2-}$$

A further example shows that in the presence of increasing concentrations of Cl^- ions, divalent Hg^{2+} forms $HgCl^+$, $HgCl_2$, $HgCl_3^-$, and $HgCl_4^{2-}$. These complexes reach maximum concentrations at specific Cl^- concentrations: $HgCl^+$, $HgCl_2$, and $HgCl_3^-$ reach maximum concentrations at Cl^- levels of 10^{-7}M, 10^{-4}M, and 10^{-1}M, respectively while $HgCl_4^{2-}$ predominates at Cl^- levels above 10^{-1}M (Babich and Stotzky, 1980). Phosphate, thiosulphate, carbonate, and bicarbonate, like chloride, can form precipitates with heavy metals, depending on their concentrations and the pH of the solution. The addition of such anions to growth media often reduces heavy metal toxicity (Gadd and Griffiths, 1978; Howlett and Avery, 1997).

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

2.2.4 Cationic composition

The background concentrations and types of inorganic cations in an environment influence the biological uptake and, hence, the toxicity of heavy metal pollutants deposited into the environment. Cations such as Mg^{2+} and Ca^{2+} often reduce heavy metal inhibition. For example toxic effects of Ni, Co, Cd, Zn and Mn to *E. coli* were decreased in media with high magnesium content (Gadd and Griffiths, 1978; Bird *et al*., 1985).

2.2.5 Clay minerals

Clay minerals posses surfaces that are predominantly negatively charged and to which charge-compensating cations (e.g. H^+, K^+, NH_4^+, Na^+, Mg^{2+}, Ca^{2+}) are adsorbed. These cations are not permanent components of the clays and are constantly being exchanged by other cations in the environment (Babich and Stotzky, 1980; Collins and Stotzky, 1992). Heavy metals introduced into the environment may be exchanged for cations on the exchange complex of clay. The metal toxicants are thereby temporarily removed from solution and their uptake by the microbiota reduced.

UNIVERSITY of the
WESTERN CAPE

The adsorption of some heavy metals by clay minerals appears to be dependent on the pH of the environment. Collins and Stotzky (1992) reported that the uptake of cadmium and lead by the clay minerals koalinite, illite and montmorillonite, increased as the pH increased from 3 to 6. At higher pH values, the metals were precipitated sparingly as hydroxyl species. The increase was attributed to the adsorption of OH^- groups to the clays that acted as bridges to metal ions.

http://etd.uwc.ac.za/

2.2.6 Microorganisms

One potentially important geophysical parameter that has received little research attention is that of the microbial population. The surface of a bacterial cell plays an important role in the relationship between the cell and its environment. This is because the surface of the cell is in direct contact with the ambient environment of the cell, and both essential (non-toxic) and non-essential (toxic) metal ions are transported across the surface into the cell. For example in a soil environment, microorganisms are typically associated with the clay and organic fractions and would be expected to participate in the metal dynamics as previously described in Section 2.2.5.

Bacteria have a high surface area-to-volume ratio (Beveridge *et al*., 1988) and, as a strictly physical cellular interface, should have a high capacity for sorbing metals from solution. Evidence exists that bacterial cells are more efficient at metal removal than clay minerals on a dry weight basis (Walker *et al*., 1989). It was also previously shown that sorption of Cd by dead cells of *Paracoccus* sp. and *Serratia marscescens* was greater than that of montmorillonite when the solid to solution ratio was the same for both bacteria and clay (Kurek *et al*., 1982). Bacteria are therefore capable of reducing the amount of free heavy metals to the environment.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

2.3 Effect of heavy metals on microorganisms and microbial mediated processes

2.3.1 Abundance and Diversity

The effect of heavy metals on microbial communities varies, depending upon which groups of microorganisms are being considered, the metal involved, and on the particular environment. According to Babich and Stotzky (1980), it is important to note that some environments are highly susceptible to other pollutants, for example sulfur dioxide, other than heavy metals. When observing the effects of heavy metals on a specific environment, these other pollutants have to be accounted for. This factor creates difficulty in drawing any firm conclusion about the true significance of heavy metals on this type of environment and microbiota.

Heavy metals reduce the abundance of microorganisms. This is as they display a differential toxic action, one of the consequences of which will be the alteration of the qualitative composition of microbial communities (Duxbury, 1985). Heavy metal contamination of soil decreases microbial diversity and causes bacterial communities to lose part of their degradative abilities (Burkhardt, 1995). Comparisons of culturable counts revealed that heavy metal stress caused a decrease in the evenness of distribution of the 20 degradative capabilities of bacteria on 20 specific media containing an aromatic substrate as the sole source of carbon and energy. The presence of toxic inorganic ions in the environment usually inhibits exposed microorganisms but may also select variants able to tolerate high concentrations of these ions (Pacheco *et al.*, 1995). Bacteria isolated from soil samples, containing high exchangeable lead concentrations were mostly Gram-

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

positive (Pacheco *et al.*, 1995). This could be expected as the dry polluted environment probably favors spore-forming bacteria.

A survey of metal tolerance in moderately halophilic eubacteria showed that *Acinetobacter* strains were the most heavy metal tolerant and abundant, with the majority of them showing tolerance to Ag, As, Cd, Co, Cr, Cu, Hg, Ni, Pb and Zn. In contrast, *Flavobacterium* strains were the most metal sensitive and thus only a low number of isolates were detected (Nieto *et al.*, 1989). Direct molecular analysis of DNA has greatly enhanced the ability to assess the diversity of microorganisms growing in an ecosystem (Ward *et al.*, 1992). Furthermore, analysis of rRNA genes has confirmed the view that conventional identification methods requiring culturing do not detect many of the bacteria originally present in the system (Ward *et al.*, 1990; Schmidt *et al.*, 1991). Based on rRNA analysis (spacer regions between the 16 and 23rRNA genes), Pizzaro and co-workers (1996) found complex bacterial populations in a copper bioleaching process. The spacer region corresponding to *Thiobacillus ferroxidans* was the main product observed at high ferrous iron concentrations. At low ferrous iron concentrations, spacer regions of different lengths, corresponding to *Thiobacillus thiooxidans* and *Leptospirillium ferroxidans* were observed.

2.3.2 Methanogenesis

Methanogens are bacteria which occur in a reducing environment devoid of oxygen and are able to produce methane (marsh gas) from carbon dioxide and hydrogen (Prescot *et al.*, 1990). Ni, Co and Mo are essential elements for certain methanogens and

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

consequently the effects of these metals on methane production in natural environments have been investigated (Jones, 1982). No stimulation of methanogenesis in fresh water sediment slurries amended with 0.06 ppm Ni or Co or 0.096 ppm Mo could be found. However, slight stimulation was observed with some surface sediment samples. The presence of heavy metals is an important factor in anaerobic system toxicity, as it has been detected in significant concentrations in municipal sewages, as well as in industrial waters (Mosey and Hughes 1975; Hickey *et al.*, 1989). Anaerobic digesters receive 28-89% of the heavy metals entering secondary waste water treatment systems. Heavy metal toxicity is one of the major causes of digester upset or failure. For example, Bhattacharya and co-workers (1995) determined the effect of Cd on an anaerobic acetate enrichment system in both batch and continuous studies. Their results indicated that Free Cd, unlike total soluble or total added Cd, showed a good correlation to toxicity in batch systems. Based on these studies a free Cd level of 0.1 mg/L appeared to be a "safe limit" for acetate utilizing methanogenes.

While sulfide reduction is regarded as being much more important than methanogenesis in marine sediments, growing evidence exist that the reverse is true in fresh water systems (Jones *et al.*, 1982). In an investigation of the effects of various pollutants on methanogenesis in fresh water sediments, it was found that 1 and 10 ppm of $HgCl_2$ had no biological effects on the process. Capone and co-workers (1983) observed similar effects with 10 and 100 ppm $HgCl_2$. They also included several other heavy metals in their study and found that the effects were variable, depending not only on the metal itself but also on its form. CH_3HgCl inhibited methanogenesis at a concentration of 1000 ppm,

http://etd.uwc.ac.za/

whereas $HgCl_2$, $PbCl_2$ and $KCrO_7$ at similar concentrations caused an initial inhibition, followed by a period of stimulation. The chlorides of Ni, Cd and Cu, as well as $ZnSO_4$, PbS and HgS caused short-term inhibition, but no significant long-term effects were observed.

2.3.3 Respiration

Respiration is the enzymatic release of energy from organic compounds in the presence of oxygen (aerobic respiration) or in its absence (anaerobic respiration). Freedman and Hutchinson (1980a) investigated the respiration rates of soils polluted to varying degrees around a Ni-Cu smelter, and recorded lower rates of carbon dioxide efflux at more contaminated sites. Statistical analysis revealed that Cu had a greater influence on the efflux than similar amounts of Ni. Furthermore, the addition of Pb to soil samples also caused a decrease in respiration rates (Chang and Broadbent, 1981). Doelman and Haanstra (1979a) had previously shown that sandy soils exhibited about 15% decrease in respiration when amended with 375 ppm Pb, whereas clay soil required 1500 ppm Pb to experience the same degree of inhibition. Although impeded respiration is common in metal polluted soils, it is clear that respiration is highly dependent upon the prevailing soil conditions.

2.4 Tolerance and resistance mechanisms of bacteria to heavy metals

Microorganisms have evolved a number of heavy metal tolerance mechanisms namely: exclusion, excretion, sequestration and transformation. These mechanisms are primarily active, in that they are induced in the presence of heavy metals. Microorganisms also

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

have passive tolerance mechanisms that are not induced in the presence of heavy metals but nonetheless improve cellular tolerance to metals (Nies, 1992; Gadd and White, 1993). Various tolerance and resistance mechanisms utilized by microorganisms are discussed.

2.4.1 Phosphate and exopolysaccharides as tolerance mechanisms to heavy metals

Heavy metals have been found in the polyphosphate (polyP) granules of certain bacteria. It has been supported by research that bacterial cells use polyP to detoxify heavy metals (Pettersson *et al.*, 1990). Previous work also indicated that excess extracellular phosphate leads to the precipitation of metal-phosphate in the medium, and that the limitation of phosphate leads to phosphate starvation of the cell. Keasling and Hupf (1996) set out to circumvent this problem. They genetically manipulated the polyP level in *E. coli* and examined the effect the resulting polyP has on cell growth during exposure to heavy metals. The doubling time as well as phosphate amounts increased with increasing cadmium concentrations not toxic to the cells. In the presence of Cd resting cells of a *Citrobacter* sp., which have a surface-located acid-type phosphatase enzyme, released $HPO_4^{2-}$from a supplied substrate, for example glycerol 2-phosphate, and precipitated Cd^{2+} as $CdHPO_4$ at the cell surface (Macaskie, 1995).

UNIVERSITY of the
WESTERN CAPE

Copper resistant strains of *P. syringae* also showed an increase in exopolysaccharide (EPS) production in media containing $CuSO_4$ at 250 μg/ml. The production of EPS may enhance tolerance of *P. syringae* by binding to the heavy metal, thus making it unavailable. The EPS of *P. syringae* was found to be primarily alginate. Arsinate, cobalt,

http://etd.uwc.ac.za/

lithium, molybdenum, and mercury did not induce EPS production indicating that alginate is not induced in cells exposed to these heavy metals (Kidambi *et al.*, 1995).

2.4.2 Plasmid-mediated resistance

A *P. putida* strain was found to be resistant to Cd by producing metallothioneins that are able to chelate the heavy metal. Since previous work on these proteins are not conclusive certain questions about the metallothioneins remain unresolved (Silver *et al.*, 1990). Certain microorganisms have become resistant to heavy metals and other toxic pollutants by evolving circular strands of DNA, called plasmids. These plasmids carry resistant genetic traits.

The plasmid-determined cadmium resistance system in *Staphylococcus aureus* has been well-established (Silver *et al.,* 1989). The *CadA* system results in reduced net uptake of Cd^{2+} because of increased energy-dependent efflux of the toxic cation. Cloning and sequence analysis identified two genes called *cadC* and *cadA* (Silver *et al.*, 1989). While all experimental data has shown that the CadA ATPase protein is sufficient for resistance and decreased cadmium uptake, the role of *CadC* was unclear. Endo and Silver (1995) showed that the Cad C protein regulates transcription of the cadmium resistance system *in vitro*. Cad C proteins specifically associate with the *CadA* operator/promoter region DNA so that it is released from the DNA with the addition of Cd^{2+} or Pb^{2+}. *Listeria monocytogenes* also showed a similar mechanism of resistance to cadmium as *S aureus* and that these genes are present on Tn 5422 (Lebrun *et al.*, 1994).

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Alcaligenes eutrophus is also resistant to a variety of heavy metals via plasmid containing resistant genetic elements. One of these, the *czc* determinant of plasmid pMOL30, mediates inducible resistance to Co^{2+}, Zn^{2+} and Cd^{2+} in *A. eutrophus* (Nies *et al.,* 1987; Nies, 1992). The products of the genes *czcA, czcB* and *czcC* form a membrane-bound protein complex catalyzing an energy -dependent efflux of these three metal cations (Nies *et al.,* 1989b). The mechanism of action of *CzcCBA* is that of a proton/cation antiporter (Nies, 1995).

2.4.3 Heavy metal resistance in biofilms

A biofilm is a functional consortium of microorganisms organized within an extensive exopolymer matrix comprised mainly of hydrated polysaccharides. Biofilms are produced by a wide variety of environmentally and medically important microorganisms including *Staphylococcus*, *Pseudomonas*, *Desulfovibrio*, *Haloferax*, *Thermococcus*, and *Methanobacterium* (Costerton *et al*., 1995).

The benefits of growing in a biofilm environment include an increased ability to retain extracellular enzymes near the cell, resistance to desiccation, protection from toxic compounds, control of competition and predation, and an increased resistance to antibiotics (James *et al*., 1996). *Archaeoglobus fulgidus*, an anaerobic marine hyperthermophylic, forms a biofilm in response to environmental stresses. The biofilm is a heterogeneous, morphologically viable structure containing protein, polysaccharide, and metals. Production of the biofilm can be induced by non-physiological extremes of pH and temperatures, by high concentrations of metals, and by addition of antibiotics,

UNIVERSITY of the WESTERN CAPE

xenobiotics and oxygen. Cells within the biofilm show an increased tolerance to other toxic environmental conditions. Metals sequestered within the biofilm stimulate growth of *A. fulgidus* cells in metal depleted medium.

Because similar biofilms are formed by *A. profundus*, *Methanococcus jannaschii* and *Methanobacterium thermoautotrophicum*, biofilm formation might be a common stress response mechanism among the archaea (LaPaglia and Hartzell, 1997). The production of biofilm may enhance the survival of cells in dynamic environments by allowing the formation of colonies containing thousands of cells. Biofilm colonies might be able to sequester nutrients or limit contact with toxic materials to ameliorate environmental stress on a scale unattainable by single cells. One mechanism of protection appears to involve the sequestration of heavy metals. Cultures stressed by high concentrations of metals were able to incorporate metals into an insoluble matrix. Although the mechanism of sequestration is unknown, polypeptides found in biofilm may act as phytochelatins to trap metals. The stability of biofilm polymer in boiling 10M NaOH and the ability of *A. fulgidus* to incorporate metals into a polymer may have industrial or research applications (LaPaglia and Hartzell, 1997. For example, *A. fulgidus* cultures could be used to detoxify metal contaminated samples or to concentrate metals in an economically recoverable form. Studies have also shown that *Pseudomonas* strains appear to be copper tolerant (Cha and Cooksey, 1991) and can be isolated from microbial biofilms (Lin and Olson, 1995).

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

2.5 Bioindicators

2.5.1 Principles of bioindicators

Several bioassays have been developed for assessing the impacts of toxic chemicals such as heavy metals on natural and man-made ecosystems. These bioassays utilize fish, zooplankton, bacteria, fungi, and algae as test organisms (Bitton *et al.*, 1992). For example, bacteria can be used as specific and sensitive devices for sensing the bioavailability of a particular pollutant or pollutant stress. This is based on the ability of pollutants such as normal compounds or specific pollutants (those from activation of a degradative pathway) to induce responses in bacteria (Stichter *et al.*, 1997). Tests using bacteria have included the measurement of growth inhibition, respirometry, viability of cells, ecological effects and bioluminescence. Since heavy metals are not biodegradable, bacteria can alter the speciation of metal contaminants. In addition, recombinant DNA techniques have been very useful in the study of microbial ecology. The most common use of molecular genetic techniques has been the direct selection of strains or phenotypes that permits the direct selection of strains, or which confer unique and readily scored phenotypes (Lindlow, 1995).

In a heavy metal polluted environment, the signaling pathway activated in a bacterium will regulate the expression of one or more (sets of) genes. The extent of this gene expression serves as a measure of the available ('sensed') concentration of the compound (Stichter *et al.*, 1997). A rapid and sensitive way to measure such gene expression is to fuse relevant promoter sequences and promoterless reporter genes, such as those for

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

bacterial luciferase of *Vibrio* sp. (Engebrecht, 1985). Other biosensor strains were constructed to detect toxic compounds, generally by coupling the *lux* genes with a stress-inducible promoter (van Dyk *et al.*, 1994).

2.5.2 Use of bacteria as indicators for the detection of heavy metals from the environment

There are several advantages to using bacteria for adsorption of metals. The rapid growth of these organisms and their adaptability to an assortment of substrates and environments are some useful attributes. Following chemical treatment to desorb bound heavy metals microbial biomass can often be reused for metal reclamation. The various modes of uptake of heavy metals in bacteria are shown in Fig. 2.2.

Sludges have also been employed in the adsorption and subsequent removal of heavy metals from the environment (Bux *et al.*, 1995). Activated sludge is comprised of bacteria, fungi and yeasts, algae and protozoa. Ten sludges previously exposed to Zn^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, Cr^{3+} and Cr^{6+} exhibited biosorptive capabilities. An affinity series was found to be, in descending order, $Cu>Cd>Zn>Ni>Cr^{3+}>Cr^{6+}$. For the removal of the heavy metals from the sludges two acids, namely acetic- and hydrochloric acid, were used to efficiently remove the heavy metals from sludge surfaces. Desorption was found to be agent-dependent rather than sludge-dependent.

http://etd.uwc.ac.za/

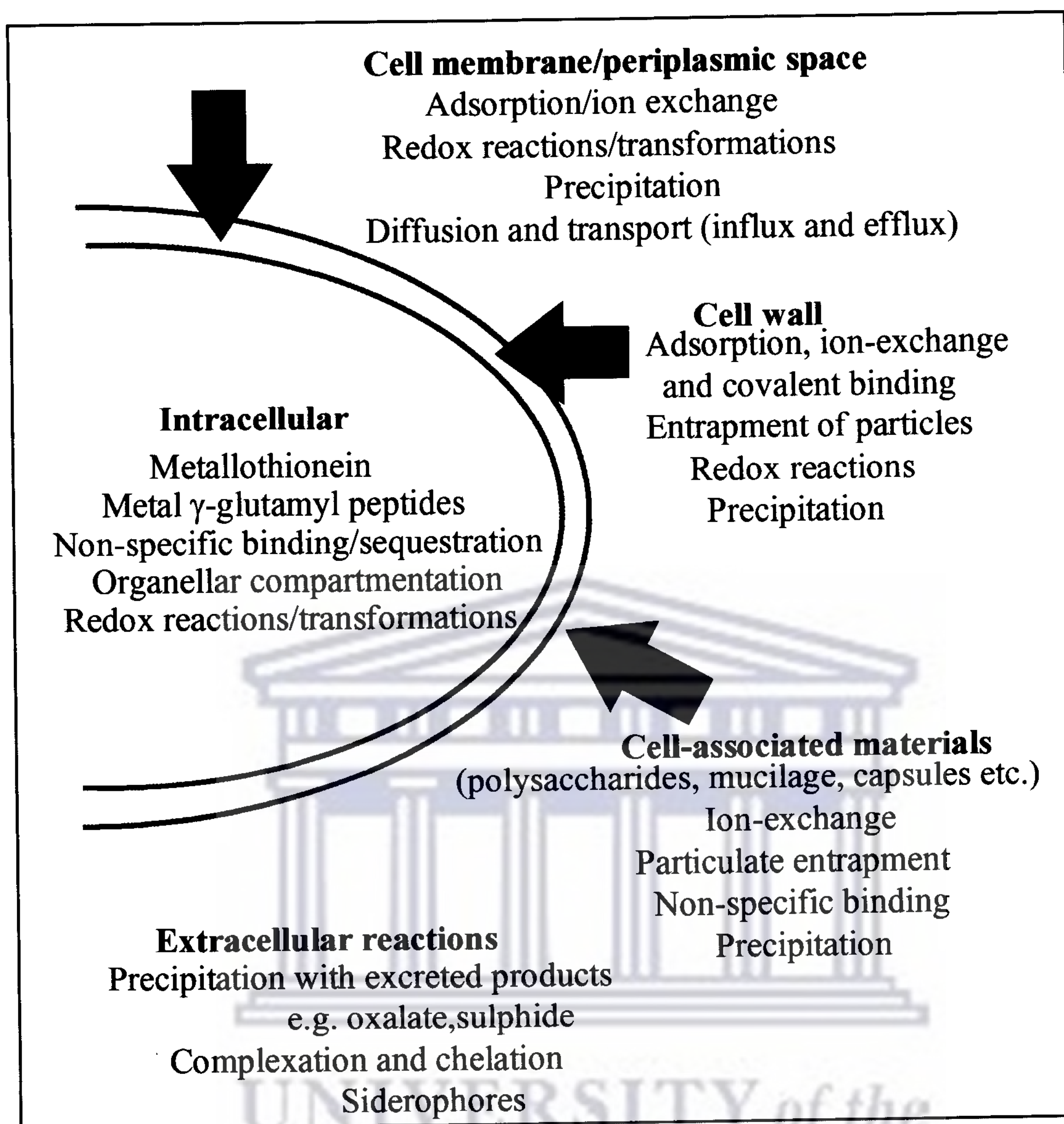


Fig. 2.2. Mechanisms of heavy metal uptake in bacteria (from Gadd and White, 1993).

UNIVERSITY of the
WESTERN CAPE

Wong *et al*., (1993) investigated Cu removal and recovery from industrial effluent using immobilized *Pseudomonas putida* II-11 strain isolated from a metal plating effluent. They eluted biomass after adsorption with 0.1M HCl and were able to recover 90% of adsorbed Cu. Elution with 0.1M EDTA yielded approximately 89% desorption and 94% desorption was found using HCl. It was postulated that the unrecoverable Cu might be

http://etd.uwc.ac.za/

trapped intracellularly. A fluorescent pseudomonad, *P. aeruginosa*, isolated from a deep-sea vent sample also tolerated Cd concentrations of up to 5mM. Following 140 hours of growth in the presence of the heavy metal, the organism was able to remove >99% of Cd from solution. Microanalysis further revealed that the Cd was removed by precipitation on the cell wall (Wang *et al*., 1997).

2.5.3 Use of genetically engineered bioindicators

E. coli strains have been genetically engineered to express a metallothionein and a Hg^{2+} transport system (Chen and Wilson, 1997). Though a number of metallothioneins have been expressed in *E. coli*, their stability has been a problem (Berka *et al*., 1988). A glutathione S-transferase system was subsequently used for the expression and stabilization of these metallothioneins. Overexpression of the fusion protein significantly increased the bioaccumulation of Hg^{2+} transported by Mer T and Mer P (Morby *et al*., 1995), and consequently protected the cells from the accumulated Hg^{2+}. Hg^{2+} remediation was generated by random mutagenesis of *P. putida* with a minitransposon containing *merTPAB*, the structural genes specifying organomercury resistance (Horn *et al*., 1994). The organism had the ability to cleave Hg from its organic moiety, thereby reducing the released Hg(II) to the less toxic form, Hg^0. Sousa *et al*., (1996) examined the metal-binding properties acquired by *E. coli* cells when one or two histidine (His) clusters, known to form stable co-ordination spheres around a distinct class of divalent cations (Hochuli *et al*., 1987), are expressed on their surface as hybrids to the Lam B protein. Expression of these metal-binding His clusters not only increased the ability of the recombinant bacteria to retain Cd^{2+}, but also caused cells to attach to Ni^{2+}-containing

UNIVERSITY of the WESTERN CAPE

solid matrices in a metal-dependent fashion. Furthermore, bioluminescence declined in modified *P. flourescens* as the metal concentration increased (Paton *et al.*, 1995). A developed bioassay, MetPAD^{TM}, also showed great potential for the rapid assessment of heavy metal toxicity in wastewater (Bitton *et al.*, 1992). The test involves *E. coli* cells that contain a reporter plasmid with the *lacZ* gene. ß-galactosidase activity is detected when the cells are exposed to various heavy metals. If this test is run in parallel with Microtox^{TM}, MetPAD^{TM} can be a suitable complementary assay for specific determination of heavy metal toxicity (Bitton *et al.*, 1992).

2.6 Bacteria under stress in unfavorable environments

2.6.1 Bacterial adaptation to stressful environments

The external environment in which organisms live is constantly changing. Microbial processes compelled to changes, require organisms to respond quickly to changing environments in order to compete successfully or survive. As a response to harmful environmental conditions, the cell may produce protective proteins, often referred to as stress proteins (Blom *et al.*, 1992). Different sets of genes and proteins are induced or suppressed by different types of stress (Jenkins *et al.*, 1988; Dorman, 1995). The adaptation involves resetting the transcriptional machinery of the cell. Stress proteins are thus expressed in response to a wide range of stress conditions. The typical response involves altered patterns of gene expression and in some cases, synthesis of stress specific proteins. Regulation of these stress stimulons is therefore central to the activity and survival of the microorganism (Lupi *et al.*, 1995).

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Information is available on the regulation of multiple operons involved in response to stressful environmental conditions (Yura *et al.*, 1993). The impressive ability of bacteria to adapt to environmental change is a cause of both wonder and concern. For example, some bacteria produce useful industrial products when undergoing adaptation to stressful growth conditions, while some bacteria frequently express virulence-enhancing factors in response to environmental cues (Dorman, 1996).

2.6.2 Environmental stimuli that induce stress

Well-characterized environmental stimuli that induce the production of stress proteins exist in bacteria. *E. coli* and *S. typhimurium* treated with low doses of oxidants such as hydrogen peroxide adapt to subsequent high doses of these oxidants by inducing the expression of numerous genes. Many of the proteins and the corresponding genes that are required for the bacterial defense against oxidative damage have been identified as shown in Table 2.2 (Storz *et al.*, 1990).

UNIVERSITY of the
WESTERN CAPE

Starvation proteins synthesized by *E. coli* at the onset of carbon starvation exhibited four temporal classes of synthesis in response to glucose or succinate starvation. This indicates the sequential expression of carbon starvation response (*cst*) genes. A *cst* mutant of *E. coli* showed greatly impaired carbon starvation survival. Thus, it appears that *E. coli* undergoes a significant molecular realignment in response to starvation, thereby increasing its resistance to this stress factor (Groat *et al.*, 1986). It has also been shown that several new polypeptides were formed when *P. aeruginosa* was under starvation. Glucose- or nitrogen-starved cultures of *E. coli* exhibited enhanced resistance

http://etd.uwc.ac.za/

to heat (57°C) or a H_2O_2 (15 mM) challenge, compared with their exponentially growing counterparts. The degree of resistance increases was directly proportional to the time for which the cells were starved prior to the challenge. The results indicated that 4 hours of starvation provided the maximal protection.

Table 2.1: Oxidative defense genes in *E. coli* (from Storz *et al.*, 1990)

Activity		Gene
Defense enzymes		
Superoxide dismutase	Manganese	*sodA*
	Iron	*sodB*
Catalase	HPI	*katG*
	HPII	*katE*
Alkyl hydroperoxide reductase	C22, F52	*ahpC, ahpF*
Glutathione reductase		*gorA*
DNA Repair enzymes		
Apurinic/apyrimidinic endonuclease	Exonuclease III	*xthA*
	Endonuclease IV	*nfo*
Endonuclease III		*nth*
DNA Polymerase		*polA*
Excision nuclease		*uvrA*
		uvrB
Exonuclease		*recB*
		recC
Metabolic enzymes		
NADH dehydrogenase		*ndh*
Glucose-6-phosphate		*zwf*
dehydrogenase		
Regulators		*soxR*
SoxR		*oxyR*
OxyR	rpoS	*katF*
KatF		*recA*
RecA		

Nickerson *et al.,* (1992) have also shown that *E. coli* W3110, grown in the presence of 5 % SDS, produced four unique protein spots and fifteen elevated (≥ threefold) protein spots on two-dimensional gels. Together these nineteen unique and elevated SDS-induced spots constituted 7.91 % of the total cell protein. Chemicals such as hypochlorous acid and 2-chlorophenol were shown to evoke or elicit stress responses in *E. coli* and *P. putida* respectively (Lupi *et al.*, 1995; Dukan *et al.*, 1996).

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Bacteria have developed several complex mechanisms, with a considerable degree of overlap, to allow them to cope with potential hazards. This means that proteins associated with one stimulon can be induced during exposure to other stress factors. For example, various heat shock proteins in *E. coli* are also synthesized when the cells are exposed to hydrogen peroxide, cadmium chloride, ethanol, UV light, puromycin as well as during amino acid deprivation (Kreuger and Walker, 1984; Grossman *et al*., 1985; Goff and Goldberg, 1985; Jenkins *et al*., 1988; Lupi *et al*., 1995; Dukan *et al*., 1996). Furthermore, Inbar and Ron (1993) have shown that pre-treatment of *E. coli* with sub-lethal concentrations of Cd induces tolerance to the metal. Cross-protection against Cd killing was also obtained by pre-incubation with heavy metal at elevated temperatures. However, in contrast to pre-treatment at elevated temperatures, exposure to sublethal cadmium concentrations did not induce thermotolerance (Inbar and Ron, 1993).

2.6.3 Sensing of bacteria to environmental changes

In some bacteria there is a division of labor in which signal reception and the biological response are partitioned between two proteins. For example, the histidine protein kinase and response regulator is composed of members which transmit environmental information between a sensing protein and a response regulating partner by phosphotransferase (Stock *et al.*, 1989). These proteins are referred to as the "two-component" signal transduction proteins. The operon structure of the *czc* determinant in *Alcaligenes eutrophus* CH34, and its regulation by Co^{2+}, Zn^{2+} and Cd^{2+} which are substrates of the CzcCBA efflux system, was investigated by van der Lelie and co-workers (1997). It appeared that that *czc* is regulated by a two-component regulatory

http://etd.uwc.ac.za/

system of a histidine kinase "sensor" and a response activator (Hoch and Silhavy, 1995), and perhaps three additional gene products. Reception of the environmental signal by the kinase results in autophosphorylation followed by phosphotransfer to the partner protein, which in turn elicits the required response within the cell. In the case of osmotic sensing in some members of the Enterobacteriaceae by the EnvZ/OmpR/response-regulator duo, genes involved in expressing proteins are differentially expressed as a result of OmpR phosphorylation by EnvZ (Stock *et al.*, 1989).

The nature of sensors and signals in the heat shock response is presently unknown. Recent proposals are that the free pool of DnaK or the free pool of DnaJ (major heat shock proteins) may serve as a thermometer, monitoring changes in cellular concentration of unfolded or denatured proteins. In addition, DnaK may act as a direct thermometer on the basis of the extremely sharp temperature dependency of its autophosphorylation, as well as its ATPase activities. In an entirely different model, ribosomes are assumed to serve as the sensors for both heat- and cold shock (Mager and De Kuijff, 1995).

UNIVERSITY of the
WESTERN CAPE

2.6.4 Response to stress and subsequent repair

The response and repair mechanisms by which bacteria recover from heavy metal stress are still unknown. However, research has shown that there appears to be considerable overlap in the type of stress proteins produced by bacteria in response to environmental stimuli. A discussion of the various systems (mechanisms) of response and repair will be briefly discussed. Mechanisms to be used as a model of response, include the SOS

response for DNA damage and the heat shock response repair mechanisms (Blom *et al.*, 1992).

The *proU* operon of *E. coli* and *Salmonella typhimurium* encodes a transport system for the osmoprotectant glycine-betaine. Its transcription is induced by about a hundred fold when the bacterial cell experiences an increase in osmotic pressure. These increases in osmolarity also correlates with a reduction in the linking number of the bacterial DNA. This means that DNA experiences an increase in negative supercoiling (Dorman, 1995). Furthermore, this change in linking number is accompanied by an increase in the free energy of the DNA, resulting in an enhanced ability to drive processes that depend on DNA strand separation (such as cruciform extrusion). It seems reasonable that these same changes in supercoiling could drive processes (such as formation of an open complex at the promoter) that would facilitate the transcription of operons such as *proU* (Dorman, 1995; Dorman, 1996).

The heat shock-induced response in *E. coli* transcription is carried out by RNA polymerase (RNAP) associated with the heat shock specific σ-factor, σ^{32}, the product of the *rpoH* gene. The specificity of the respective holoenzyme to bind to the promoters of the heat shock genes, is conferred by σ^{32}. At least 13 promoters are known to be transcribed by RNAPσ^{32}. The heat shock promoters differ from regular promoters with respect to the -35 region (consensus sequence TCTCNCCCTTGAA), the -10 region (consensus sequence CCCCATNTA), and the length of the spacer (13 to 17 nucleotides) separating these two regions. Promoters of the major heat shock genes such as *groES*,

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

groEL, *dnaK* and *dnaJ* are among the strongest found in *E. coli* (Mager and De Kuijff, 1995).

The level of heat shock gene transcription depends on the cellular concentrations of σ^{32}. Under heat shock conditions, σ^{32} levels increase by enhanced synthesis, elevated stability and increased activity of the factor. Among the proteins synthesized at high rate at high temperature are DnaK, DnaJ and GrpE, which play a central part in the stress response. This is achieved by mediating the refolding or degradation of heat-denatured polypeptides. Regulation of σ^{32}-mediated transcription activation occurs at various levels according to feedback mechanisms. Although transcription of the *rpoH* gene is rather complex, the main regulation of expression of the *rpoH* is at the translational level: DnaK and DnaJ are implicated in the attenuation of σ^{32} mRNA translation. In addition, heat shock proteins assist in the rapid degradation of σ^{32}, as well as in the inhibition of the factor. It is assumed that this is achieved by blocking its association with RNAP (reviewed by Mager and De Kuijff, 1995).

UNIVERSITY of the WESTERN CAPE

2.7 Translational control of gene expression in prokaryotes

In prokaryotes the level of gene expression is determined primarily by three elements: the rate of transcription, stability of the RNA transcript, and the efficiency of translation. In bacteria mRNA undergo rapid exponential decay, with the average mRNA having a half-life of 1.3 minutes at 37^{0}C (Arraiano, 1993). This mRNA instability best explains the rapid adaptation of microorganisms to a changing environment. The rate of gene expression in some bacterial species is altered in response to physiological signals, for

http://etd.uwc.ac.za/

example a change in growth rate (Nilsson *et al.*, 1984). Translational control therefore plays an integral part in the modulation of the efficiency of translation of mRNA as well as translation-coupled regulation of mRNA stability. There are numerous examples of translational control of prokaryotic gene expression (Gold, 1988). Regulatory mechanisms involve two major types: repression by *trans*-acting proteins that prevent the formation of competent initiation complexes, and modulation of mRNA secondary structure within the ribosome-binding site that occludes ribosome binding. Some examples will be mentioned.

2.7.1 Antisense RNA regulation in prokaryotes

The occurrence of antisense RNA regulation in bacteria has spurred the publication of a number of studies on this topic in both prokaryotes and eukaryotes. Antisense RNA regulation basically involves the synthesis of a short transcript that does not code for protein but has a high degree of complementarity with a second RNA enabling the two to hybridize, e.g. the hybridization to a mRNA which represses protein synthesis (Simons, 1988). Any process that includes single stranded RNA may be a target for this newly discovered mode of regulation. In many cases the antisense RNA gene is part of a complex regulatory system, and its natural role may not be the only or the main regulatory gene. Only during the examination of such regulatory circuits by overexpression of the antisense gene (as in the cases of Tn10 multicopy inhibition and *mic*F osmoregulation) is a strong effect on the target gene observed.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Antisense RNA generated from an unlinked locus has been implicated in the relative expression of two outer membrane proteins, OmpF and OmpC which function as passive diffusion pores for small hydrophilic molecules in *E. coli* (Mizuno *et al.*, 1984; Pines and Inouye, 1986). An increase in osmolarity causes a decrease in OmpF production and an increase in OmpC. The *mic*F gene, coding for 93 nucleotide *m*RNA-*i*nterfering *c*omplementary *micF* RNA (4.5S) and a rarer 174-nucleotide micF RNA form (6S), lies just upstream of *ompC* but is expressed in the opposite orientation. Both the *micF* and *ompC* genes are activated by high osmolarity. The *micF* RNA product forms a hybrid with *ompF* mRNA and inhibits its translation by blocking the Shine-Dalgarno (SD) sequence and the initiation codon; it may also result in the destabilization of the mRNA. Thus this method assures an equal amounts of OmpC and OmpF in the cell (Voorma, 1996).

2.7.2 Pseudoknots and *trans*-acting proteins

Regulation of translation may proceed through competition between a repressor protein and the ribosome for an overlapping binding site in the translation initiation region. Binding of the repressor protein prevents binding of the ribosome. However, an alternative repression mechanism involves the repressor protein trapping the ribosome on its initiation site and preventing further assembly of the 30S initiation complex (Portier and Grunberg-Manago, 1993).

An example of such translational regulation is found in the expression of ribosomal protein S15. This protein is able to control its own translation by binding to a region of

UNIVERSITY of the
WESTERN CAPE

the S15 mRNA that can fold into two mutually exclusive conformations, either a structure of two hairpins or one with a pseudoknot that is recognized and stabilized by S15. The bound S15 prevents the conversion of an inactive ribosome-mRNA complex to a productive 30S initiation complex by trapping the ribosome in an inactive transitory stage. Physicochemical studies with ribosomal protein S4 mRNA and the α operon mRNA suggest a mechanism similar to that of S15 regulation. Many pseudoknot structures have been detected in the past few years and their roles in processes such as translational autoregulation or ribosomal frameshifting have been studied in great detail (ten Dam *et al.*, 1992; Pleij, 1994).

2.7.3 RNaseIII-mediated control

RNaseIII often plays a role in the onset of mRNA degradation, but it has also been implicated in the translational regulation of several bacteriophage and *E. coli* genes. In the λPL transcript, several RNaseIII processing sites are present, one of which is found upstream of the CIII coding sequence. CIII mRNA is found in equilibrium between two alternative structures, A and B. In the A conformation, the SD sequence and the AUG are inaccessible to the 30S subunit, whereas in the B conformation, the AUG and the SD sequence are accessible and translation is efficient (Altuvia *et al.*, 1987). Furthermore, translation of CIII is greatly reduced in a host defective in RNaseIII and thus depends on its presence. Both alternative structures are recognized by RNaseIII, but the A structure is processed more efficiently. Nevertheless, processing per se seems not to be the mechanism for translational stimulation. It is proposed that RNaseIII catalyzes the

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

transition from the inactive A structure to the active B structure, rather than cleaving the CIII mRNA (Altuvia *et al.*, 1991).

2.7.4 Attenuation of *ermC* mRNA translation

The basis of erythromycin resistance is the posttranscriptional N^6, N^6-dimethylation of adenine nucleotides in 23S rRNA, which markedly reduces the affinity of ribosomes for erythromycin. The *ermC* mRNA encoding the N^6, N^6-dimethyladenine methylase contains a leader frame of 19 amino acids. This uORF is out of frame with the *ermC*-coding sequence. In addition, because of the presence of six complementary segments, several hairpin loop structures can be formed in the leader. In the most stable structure, the methylase AUG and part of the SD sequence are sequestered, preventing the translation of *ermC* mRNA. The induction model proposes that in the presence of erythromycin, ribosomes translating the leader peptide stall during synthesis of the hydrophilic carboxy-terminal portion, since erythromycin appears to block more severely the transpeptidation reaction involving hydrophilic amino acids (Dubnau, 1984). A consequence of ribosome stalling, the downstream *ermC* mRNA attains an open configuration that permits initiation at the methylase initiation codon. The apparent paradox is that erythromycin as an inhibitor of peptide bond formation acts as an inducer of protein synthesis. An explanation may be the minor fraction of ribosomes is methylated and provides a pool of resistant ribosomes for the translation of methylase.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

2.8 *Pseudomonas* in the environment

Bacteria belonging to the genus *Pseudomonas* are rod shaped, Gram-negative, non-sporulating and polarly flagellated. Based on the level of rRNA homology, the *Pseudomonas* genus is classified into five groups, which are designated rRNA homology group I to group V. Each group is then further subdivided into a number of species. The best known species of the genus are the fluorescent pseudomonads which belong to group I. *P. aeruginosa* is the best known of all pseudomonads, and also one of the most studied species of Gram- negative bacteria.

Pseudomonas strains are very common in natural habitats, particularly soil, water, spoiled foods and diseased plants (Ramos and Marqués, 1993). These bacteria have a capacity to grow in very simple media. Strains capable of degrading a vast variety of organic compounds, including recalcitrant xenobiotics (*P. cepacia* and *P. putida*), and strains stimulating plant growth (*P. flourescens*) have also been indicated (Silver *et al.*, 1990). These characteristics make this group of microbes interesting for biotechnological applications in industrial fermentation processes and control of environmental pollution.

UNIVERSITY of the
WESTERN CAPE

P. putida and *P. aeruginosa* have been indicated to be of importance in the process of minerilization of organic matter. In addition, an unusually high adaptation and catabolic potential have also been shown for these bacteria. *P. aeruginosa* and *P. putida* have been shown to be present in toxic environments, especially heavy metal polluted environments, and that they are able to survive these conditions (Mullen *et al.*, 1989; Iwasaki *et al.*, 1994; Wang *et al.*, 1997). These two *Pseudomonas* sp. were chosen for

http://etd.uwc.ac.za/

this study as bioindicators assuming that they posses heavy metal response regulatory elements.

http://etd.uwc.ac.za/

CHAPTER 3

RESPONSE OF *PSEUDOMONAS AERUGINOSA* AND *P. PUTIDA* EXPOSED TO VARIOUS HEAVY METALS

3.1 Introduction

Pollution of the environment by heavy metals arises as a result of many industrial activities, although sources such as agriculture and sewage disposal also contribute These pollutants are discharged or transported into the atmosphere, aquatic and terrestrial environments mainly as solutes or particulates and many reach high concentrations, especially near the site of entry. The effects of heavy metals on ecosystem function vary considerably and are of economic and public-health significance (Gadd and White,1993).

Once in the environment, heavy metals undergo transformation into various mobile forms and/or immobilization in the environment. This is contributed by ever-changing geophysical parameters in the environment such as pH, organic and inorganic ions, clays, minerals, oxidation-reduction potential and many others (Babich and Stotzky, 1985). Because of the adverse effects that heavy metal pollution has on human health, biota and in especially microorganisms, studies have shown that some bacteria are actually able to survive these harsh conditions. The ability of microorganisms to grow in the presence of high metal concentrations may result from specific mechanisms of resistance, for example, the rapid pumping out from the cell of toxic cations. Tolerance may result from the intrinsic properties of the microorganism, such as the possession of an impermeable cell wall, the production of extracellular polysaccharides or the lack of specific metal transport systems (Gadd and Griffiths, 1978; Babich and Stotzky, 1985).

As a response to harmful environmental conditions, the cell may produce additional proteins often referred to as stress proteins. Different sets of genes and proteins are induced by different stresses. Well-characterized prokaryotic examples include the heat shock response, the SOS response, oxidative stress, starvation response, and anaerobiosis.

http://etd.uwc.ac.za/

Chemicals like $CdCl_2$, H_2O_2, and ethanol also stimulate the synthesis of stress proteins in *Escherichia coli* and *Salmonella typhimurium*, some of which are unique. In their natural environments, bacteria are constantly exposed to conditions under which stress protein induction has been demonstrated in the laboratory. The regulation of these stress stimulons is therefore central to the activity and survival of the microorganism.

Since *Pseudomonas* sp. have been shown to exist in metal polluted environments, the aim was to determine what level of tolerance *P. aeruginosa* and *P. putida* exhibit when exposed to different heavy metals at various concentrations. Furthermore, a pollutive heavy metal concentration and the time taken to elicit stress protein production in the *Pseudomonas* sp. can also be established.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

3.2 Materials and Methods

3.2.1 Microorganisms and growth conditions

P. putida ATCC 12633 and *P. aeruginosa* PAOI (DSM) were routinely maintained on Nutrient Agar (Biolab) plates and incubated at 30^0C and 37^0C, respectively. For selective purposes *P. putida* and *P. aeruginosa* were periodically passed over on Pseudomonas Isolation Agar (PIA) (Difco).

3.2.2 Preparation of heavy metal solutions

Heavy metal salts were prepared according to Wong *et al.*,(1993). The metal salts (obtained from Saarchem) $Ni(NO_3)_2$, $CdCl_2$, $CoCl_2$, $ZnSO_4$, $PbNO_3$ and $CuSO_4$ were each prepared in terms of the metal ions Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+} at a final concentration of 1000mg/L. All solutions were sterilized by autoclaving.

3.2.3 Minimal Inhibitory Concentrations of various heavy metals against *P. aeruginosa* and *P. putida*

The Minimal Inhibitory Concentration (MIC) of each heavy metal was defined as the lowest concentration that inhibited confluent growth of bacterial cultures after 24h incubation. To obtain single colonies, *P. aeruginosa* and *P. putida* were streaked on Nutrient Agar plates and grown at 37^0C and 30^0C for 16-24h, respectively. Single colonies were picked and inoculated into Vogel and Bonner (VB) broth (Vogel and Bonner, 1956) which consists of $MgSO_4.7H_20$ 0.20g, Citric acid 2.00g, K_2HPO_4 10.0g, and $NaNH_4HPO_4.4H_2O$ 3.5g made up to 900ml. 5% Glucose was added as sole carbon source (Vogel and Bonner, 1956). Cultures were grown for 3-4h prior to spread plating

http://etd.uwc.ac.za/

onto VB agar plates supplemented with various concentrations of Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+}. Plates were incubated at 37^0C and 30^0C, respectively and results were interpreted after 24h growth.

3.2.4 Response of *P. putida* and *P. aeruginosa* to various heavy metals

P. putida was exposed to 1, 50, 100, 250, and 500mg/L Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+}, while *P. aeruginosa* was exposed only to 50mg/L of each heavy metal. Protein sampling and extraction was done according to Lupi *et al.,* (1995) with modifications. Cultures in early log phase (OD_{600}= 0.4) were supplemented with the various heavy metals at the concentrations stated above. Cultures grown in the absence of heavy metals were used as controls. The growing cultures were subsequently labeled with ^{35}S-methionine (ICN Biochemical Research Products, Ohio, USA) to a final concentration of 20µCi/ml. The labeled cultures were grown for a further hour at their respective growth temperatures. Following this incubation period, 1ml samples were removed and centrifuged for 3min at 15000x*g* in a microfuge (Beckman). The pellets were resuspended in 8µl lysis solution (10ml 0.5M Tris-HCl [pH 6.8], 17ml SDS [15%], 5ml glycine, 8ml β-Mercaptoethanol (solution made up to a 100ml with distilled water) and incubated at 100^0C for 4 min. The samples were incubated for 1h at 37^0C prior to the addition of lysis buffer (9.5M Urea, 2ml Nonidet P-40 [10%] (Boehringer Mannheim), 5ml β-Mercaptoethanol (solution made up to a 100ml with distilled water). Samples were either analyzed immediately or "flash-freezed" using ice-cold ethanol prior to storage at -70^0C.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

3.2.5 Effect of time on the response of *Pseudomonas* sp. to various heavy metals

P. putida and *P. aeruginosa* cultures were exposed to 50mg/L Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+} for 30min, 60min and 120min. Cultures not exposed to heavy metals at the above-mentioned time intervals were used as controls. Protein sampling and extraction were performed as described in Section 3.2.4.

3.2.6 Detection of stress proteins in *P. putida* and *P. aeruginosa*

The whole cell proteins of *P. putida* and *P. aeruginosa* were separated by discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (12.5% SDS-PAGE). [Separation gel (for SDS-PAGE): Distilled water 5.5ml, Separation buffer 1.5M Tris-HCl (pH 8.8) 5ml, Acrylamide (40%) 6ml, Bis-Acrylamide (2%) 3.2ml, SDS (10%) 150µl, APS (10%) 60µl, TEMED 10µl. Stacking gel (for SDS-PAGE): Distilled water 4.05ml, Stacking buffer 0.5M Tris-HCl (pH 6.8) 1.88ml, Acrylamide (40%) 0.96ml, Bis-Acrylamide (2%) 0.512ml, SDS (10%) 75µl, APS 37.5µl, TEMED 7.5µl], according to Laemmli (1970).

Gels were electrophoresed at 30mAmp, until the bromophenol blue dye front reached the bottom of the glass plates. For visualization of the radioactive proteins, gels were stained in Coomassie blue, vacuum dried and exposed to autoradiographic film (Kodak) for 24h at -70^{0}C. Films were developed and results interpreted on autoradiographs.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

3.3 Results and Discussion

3.3.1 Minimal inhibitory Concentration determination of heavy metal to *P. putida* and *P. aeruginosa*

The lowest concentration of heavy metal that completely prevented growth was termed the minimal inhibitory concentration (MIC). This was accomplished by employing a Vogel and Bonner minimal medium (Vogel and Bonner, 1956) as it contains less complexing agents and thus allow heavy metals to be more readily available in order to determine its toxicity to the microorganisms (Bird *et al.*, 1985). The tolerance levels of *P. putida* and *P. aeruginosa* to Cu^{2+}, Co^{2+}, Cd^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+}, expressed as MICs are shown in Fig. 3.1. On the basis of the MICs, the two *Pseudomonas* sp. showed similar susceptibilities to Pb^{2+} (1800mg/L), Ni^{2+} (500mg/L), Zn^{2+} (2400mg/L), except for Cu^{2+}, $Co^{2+}$and Cd^{2+} to which *P. aeruginosa* showed the highest tolerance levels than *P. putida* (Fig. 3.1).

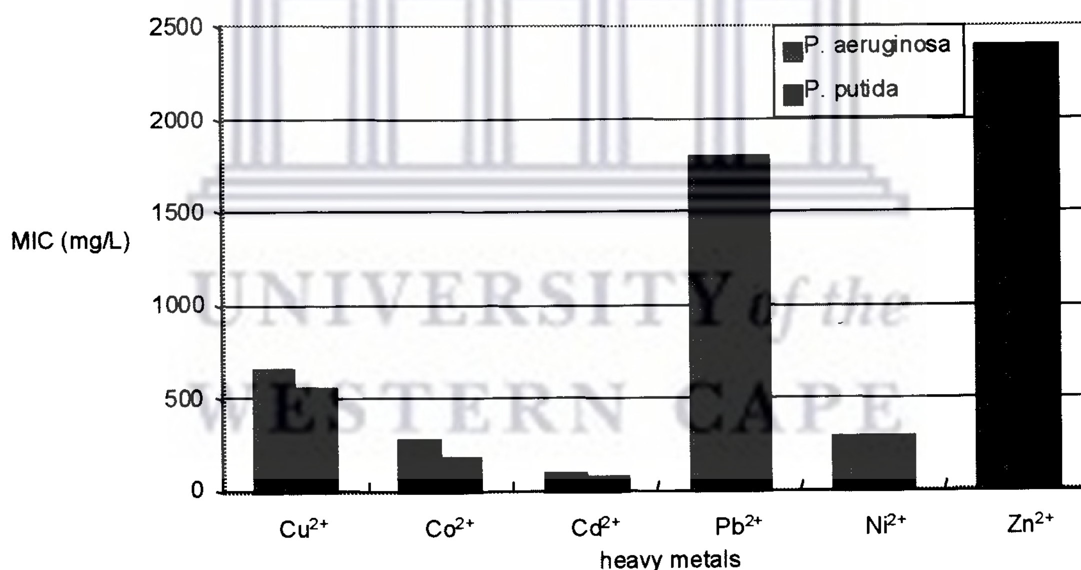


Fig. 3.1. MICs of *Pseudomonas* species to specific heavy metals (values are given as mg/L).

The genus *Pseudomonas* comprises a diverse group of bacteria and species such *as P. putida, P. aeruginosa, P. flourescens* and *P. syringae* have been shown to be present in heavy metal polluted environments (Wong *et al.*, 1993; Wang *et al.*, 1997). The tolerance of *P. putida* and *P. aeruginosa* to high heavy metal concentrations is an indication that

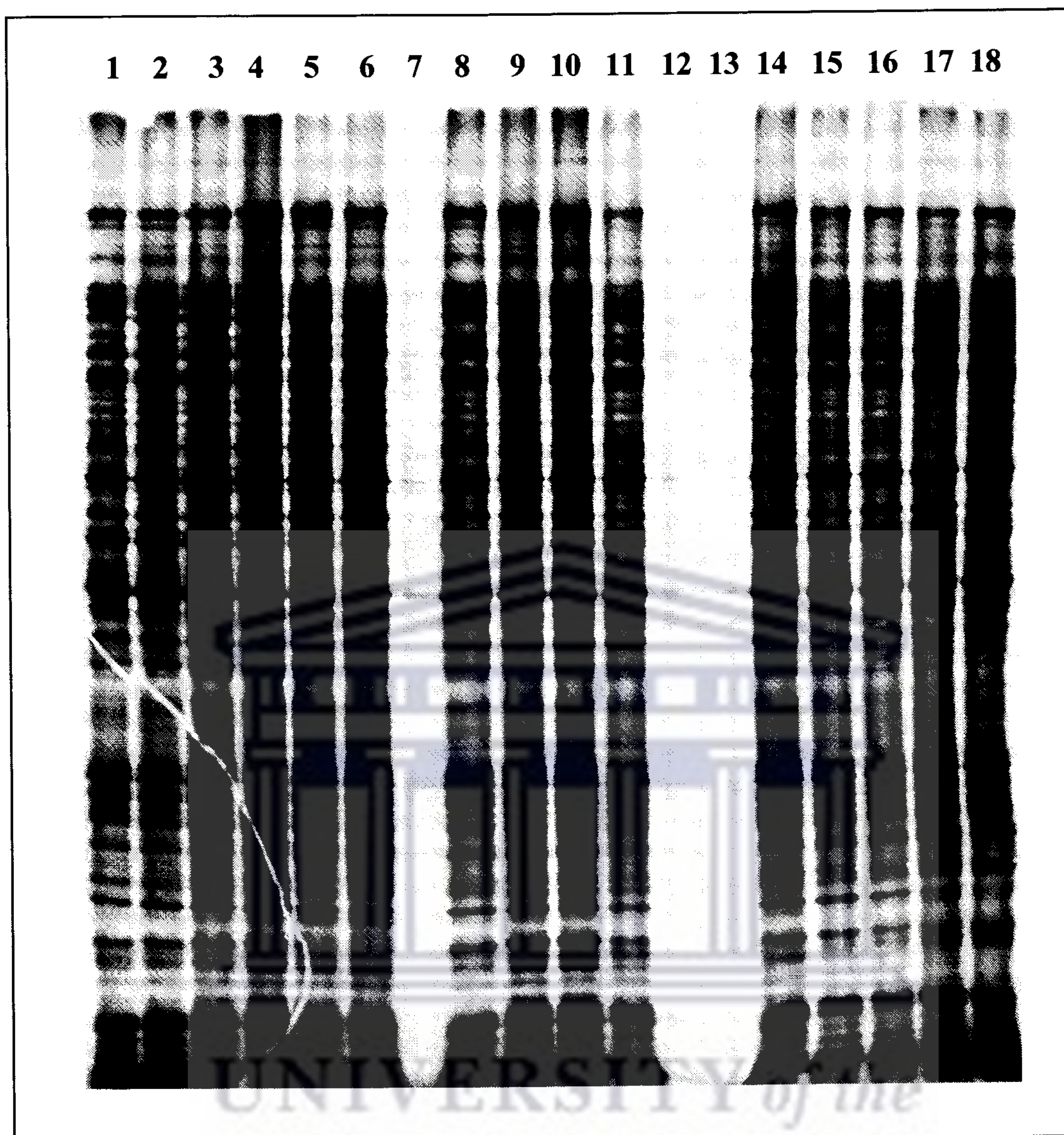
http://etd.uwc.ac.za/

both bacteria can be used as bioindicator organisms for heavy metals, even though *P. aeruginosa* showed a higher tolerance level to heavy metals than *P. putida.*

3.3.2 Response of *P. putida* to specific heavy metals at various concentrations

Since MICs are a mere indication of the heavy metal concentration that inhibits cell growth, it was also important to determine the effect heavy metals have on *de novo* protein synthesis. As shown in Fig. 3.2A, Fig. 3.2B and Table 3.1, exposure of *P. putida* to heavy metals at various concentrations induced and suppressed a number of proteins by comparing cells not exposed to heavy metals to those exposed.

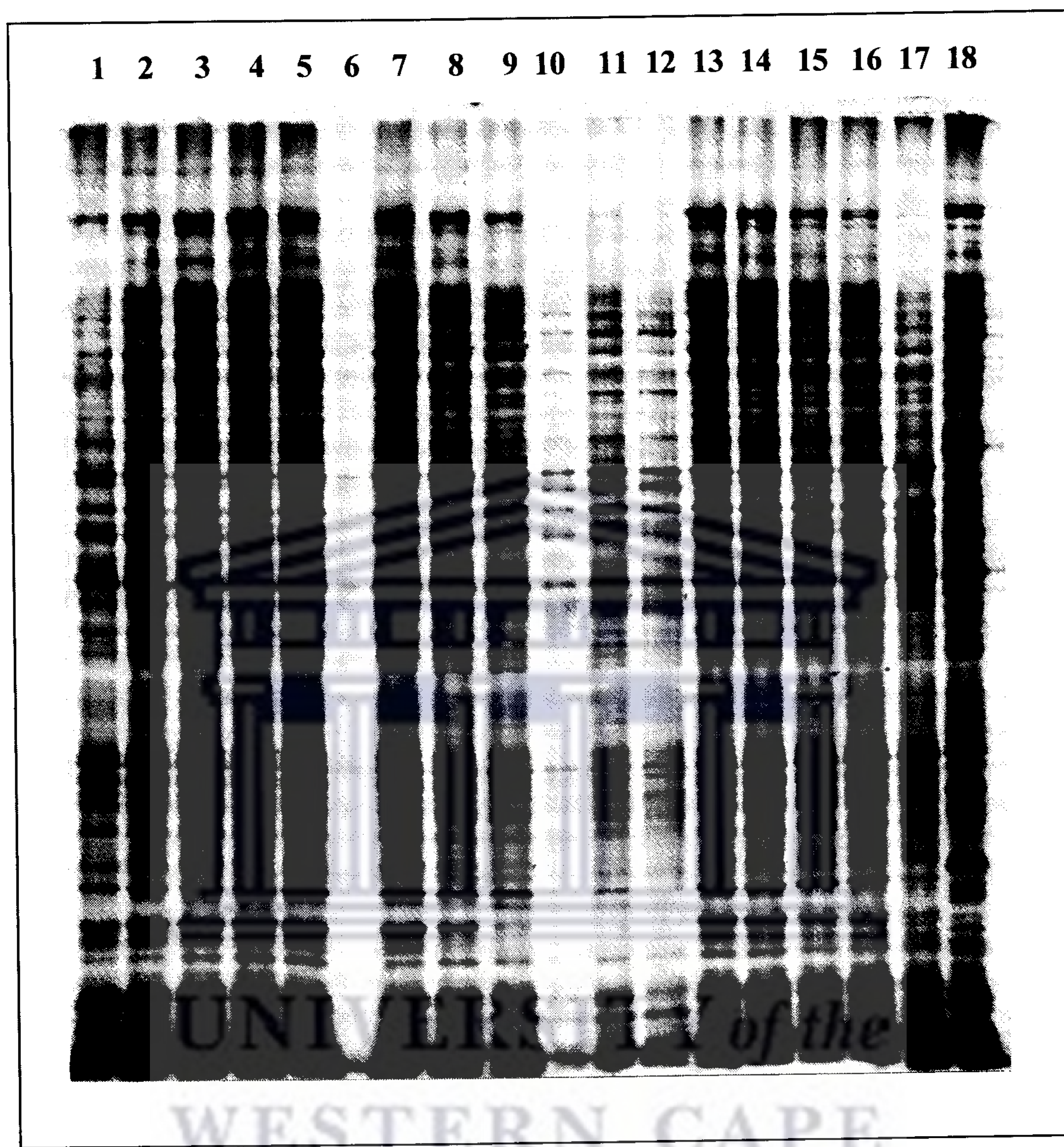
UNIVERSITY of the
WESTERN CAPE



UNIVERSITY of the
WESTERN CAPE

Fig. 3.2A. Exposure of *P. putida* to specific heavy metals at various concentrations. Lane 1: Control - *P. putida* without exposure to heavy metal. Lanes 2-6: *P. putida* exposed to 1, 50, 100, 250 and 500mg/L Ni^{2+}, respectively. Lanes 8-12: *P. putida* exposed to 1, 50, 100, 250 and 500 mg/L Cu^{2+}, respectively. Lanes 14-18: *P. putida* exposed to 1, 50, 100, 250, 500 mg/L Zn^{2+}, respectively. Lanes 7 and 13, no proteins loaded.

http://etd.uwc.ac.za/



UNIVERSITY of the
WESTERN CAPE

Fig. 3.2B. Exposure of *P. putida* to various heavy metal concentrations (continued). Lane 1: Control- *P. putida* without exposure to heavy metal. Lanes 2-7, except lane 6: *P. putida* exposed to 1, 50, 100, 250 and 500mg/L Pb^{2+}, respectively. Lanes 8-12: *P. putida* exposed to 1, 50, 100, 250 and 500 mg/L Cd^{2+}, respectively. Lanes 13-17: *P. putida* exposed to 1, 50, 100, 250, 500 mg/L Co^{2+}, respectively, Lane 18: *P. aeruginosa* not exposed to heavy metals.

http://etd.uwc.ac.za/

Table 3.1: The production of metal responsive proteins in *P. putida* when exposed to various concentrations of heavy metals.

No	Ni^{2+}					Cu^{2+}					Zn^{2+}				
	1	50	100	250	500	1	50	100	250	500	1	50	100	250	500
1	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
3	0	0	+	+	+	+	0	0	0	ND	0	0	0	0	0
4	+	+	+	+	+	0	0	0	0	ND	0	0	0	0	0
5	+	+	+	+	+	+	0	0	0	ND	+	+	0	0	0
6	0	0	0	+	+	0	0	0	0	ND	0	-	-	-	-
7	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
8	0	0	0	0	0	0	0	+	+	ND	0	0	0	0	0
9	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
10	0	0	0	0	0	+	+	+	+	ND	0	0	+	0	0
11	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
12	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
13	0	+	+	+	0	+	+	+	0	ND	+	+	+	0	0
14	0	0	0	0	0	0	0	0	0	ND	0	0	0	+	+
15	0	+	+	+	+	0	0	0	0	ND	0	0	0	0	0
16	0	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
17	0	0	0	0	0	-	-	-	-	ND	0	0	0	0	0
18	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	-
19	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
20	0	0	0	0	0	0	0	0	+	ND	0	0	0	0	0
21	0	-	+	+	+	+	+	+	+	ND	+	+	+	+	+
22	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
23	0	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
24	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
25	0	+	+	0	0	0	+	+	+	ND	+	+	0	0	0
26	0	0	0	0	0	0	0	0	0	ND	0	0	0	0	0
27	+	+	+	+	+	+	+	+	+	ND	+	+	+	+	+
28	0	0	+	+	0	+	0	0	0	ND	+	+	0	0	0
29	0	+	+	+	+	0	0	0	0	ND	0	0	0	0	0
30	0	+	+	+	+	+	+	+	0	ND	+	+	+	0	0
31	+	+	+	+	+	0	+	+	+	ND	0	+	+	+	+

Keynote: + - induced
- - suppressed
0 - no effect
ND - not determined

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Table 3.1 (continued): The production of metal responsive proteins in *P. putida* when exposed to various concentrations of heavy metals.

No	Pb^{2+}					Cd^{2+}					Co^{2+}				
	1	**50**	**100**	**250**	**500**	**1**	**50**	**100**	**250**	**500**	**1**	**50**	**100**	**250**	**500**
1	+	+	+	+	+	+	+	-	-	-	+	+	+	+	-
2	+	+	+	+	+	+	+	+	-	-	+	+	+	+	+
3	+	+	+	+	0	0	0	0	0	0	0	+	+	+	0
4	+	+	+	+	0	0	0	0	0	0	0	+	+	+	+
5	0	0	+	+	+	+	+	+	+	+	0	0	0	0	0
6	0	0	0	0	0	0	+	+	+	+	+	+	+	+	+
7	0	0	0	0	0	0	0	-	-	-	0	0	0	0	-
8	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9	0	0	0	0	0	0	0	-	-	-	0	0	0	0	-
10	0	0	0	0	0	-	-	-	-	-	+	-	-	-	-
11	0	0	0	0	0	0	+	0	0	0	0	0	0	0	0
12	0	0	0	0	0	+	+	+	+	+	0	0	0	0	0
13	0	0	0	0	+	+	+	0	0	0	0	0	0	+	+
14	+	+	+	+	+	0	0	0	0	0	+	0	0	0	-
15	+	+	+	+	+	0	0	+	+	+	0	0	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
18	0	0	0	-	-	0	0	0	0	0	0	0	0	0	0
19	0	0	0	0	0	0	0	0	-	-	0	0	0	0	-
20	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	0	0	0	0	0	0	0	0	0	0	0	0	0	0	-
23	+	+	+	+	+	+	+	+	+	0	+	+	+	0	0
24	0	0	0	0	0	0	0	0	0	-	0	0	0	0	0
25	0	0	+	+	+	+	+	+	+	+	0	0	0	0	0
26	0	0	0	0	0	0	0	-	-	-	0	0	0	0	0
27	+	+	+	+	+	+	+	+	0	0	+	+	+	+	0
28	0	0	0	0	0	+	+	0	0	0	+	+	0	0	0
29	+	+	+	+	+	0	+	0	0	0	0	0	0	0	0
30	+	+	+	+	+	0	0	0	0	0	+	+	+	0	0
31	+	+	0	0	0	0	0	0	0	0	+	+	+	+	+

Keynote: + - induced
- - suppressed
0 - no effect

In the study of stress effects it is equally important to consider not only the proteins which were induced as a result of a stress, but also those which were suppressed (Lupi *et al.*, 1995). This gives an overall indication of what the effect is of a stress on a given bacterium. The exposure of *P. putida* to a specific heavy metal at various concentrations

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

induced a high number of proteins and suppressed a few. Exposure to Cd^{2+} induced a considerable number of stress proteins at low concentrations, but as the concentrations were increased, the amount of stress proteins induced decreased with a subsequent increase in protein suppression. Since Cd^{2+} is considered to be a very toxic heavy metal, it is therefore expected that the cells will respond to the heavy metal at low concentrations by inducing stress protein production, which is later suppressed, as the concentration becomes too toxic.

Table 3.2: Summary of the number of proteins induced and suppressed in *P. putida* when exposed to specific heavy metals at various concentrations

Concentration (mg/L)	Cd^{2+}		Cu^{2+}		Pb^{2+}		Ni^{2+}		Zn^{2+}		Co^{2+}	
	I	S	I	S	I	S	I	S	I	S	I	S
1	11	1	12	1	13	0	6	0	11	0	12	0
50	13	1	11	1	13	0	13	1	12	1	12	1
100	10	5	12	1	14	0	16	0	10	1	12	1
250	8	7	11	1	14	1	16	0	8	1	11	1
500	7	8	ND	ND	13	1	14	0	8	2	7	7

Keynote: I - Induced
S - Suppressed
ND - not determined

For Cu^{2+}, Pb^{2+} and Zn^{2+} a constant number of proteins were induced and suppressed when exposed at different concentrations as shown in Table 3.2. The reason for not being able to determine the number of proteins being induced or suppressed at 500mg/L Cu^{2+} could be that global suppression of protein suppression took place or that the concentration of protein loaded onto the gel was not enough to observe. A small number

http://etd.uwc.ac.za/

of proteins were induced when exposed to 1mg/L Ni^{2+}, with a constant increase at higher concentrations. Protein production in *P. putida* was also found to be constant when it was exposed to Co^{2+} at concentrations of 1, 50, 100 and 250mg/L, with a decrease in protein induction and increase in protein suppression at 500mg/L. Blom *et al.*, (1992) have previously shown that $CdCl_2$ at a concentration of 80mg/L, induced a total number of 38 proteins in *E. coli* as determined by two-dimensional PAGE gels. It is therefore evident that various heavy metals such as Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+} and Co^{2+} are able to induce and suppress protein production in *P. putida* as resolved on a SDS-PAGE gel. Because the amounts and concentrations can be determined using two-dimensional PAGE gels, this method is not easy to perform and is only used in laboratories that does this work routinely (Groat *et al.*, 1986; Lupi *et al*, 1995). Most proteins were induced at a concentration of 50mg/L of the various heavy metals indicating that this concentration can be used as an index to study protein production.

3.3.3 Exposure of *P. putida* and *P. aeruginosa* to 50mg/L of various heavy metals over time

With the concentration determined for heavy metals to induce the most stress proteins in *P. putida*, the next step was to determine how long the synthesis of stress proteins would be maintained at this concentration. *P. putida* was exposed to 50mg/L of Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} over 30min, 60min and 120min. Analysis of whole-cell protein profiles as resolved on SDS-PAGE gels and autoradiography (Fig. 3.3), and also shown in Table 3.3 and Table 3.4, showed that differences in the number of proteins induced and suppressed over time occurred.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

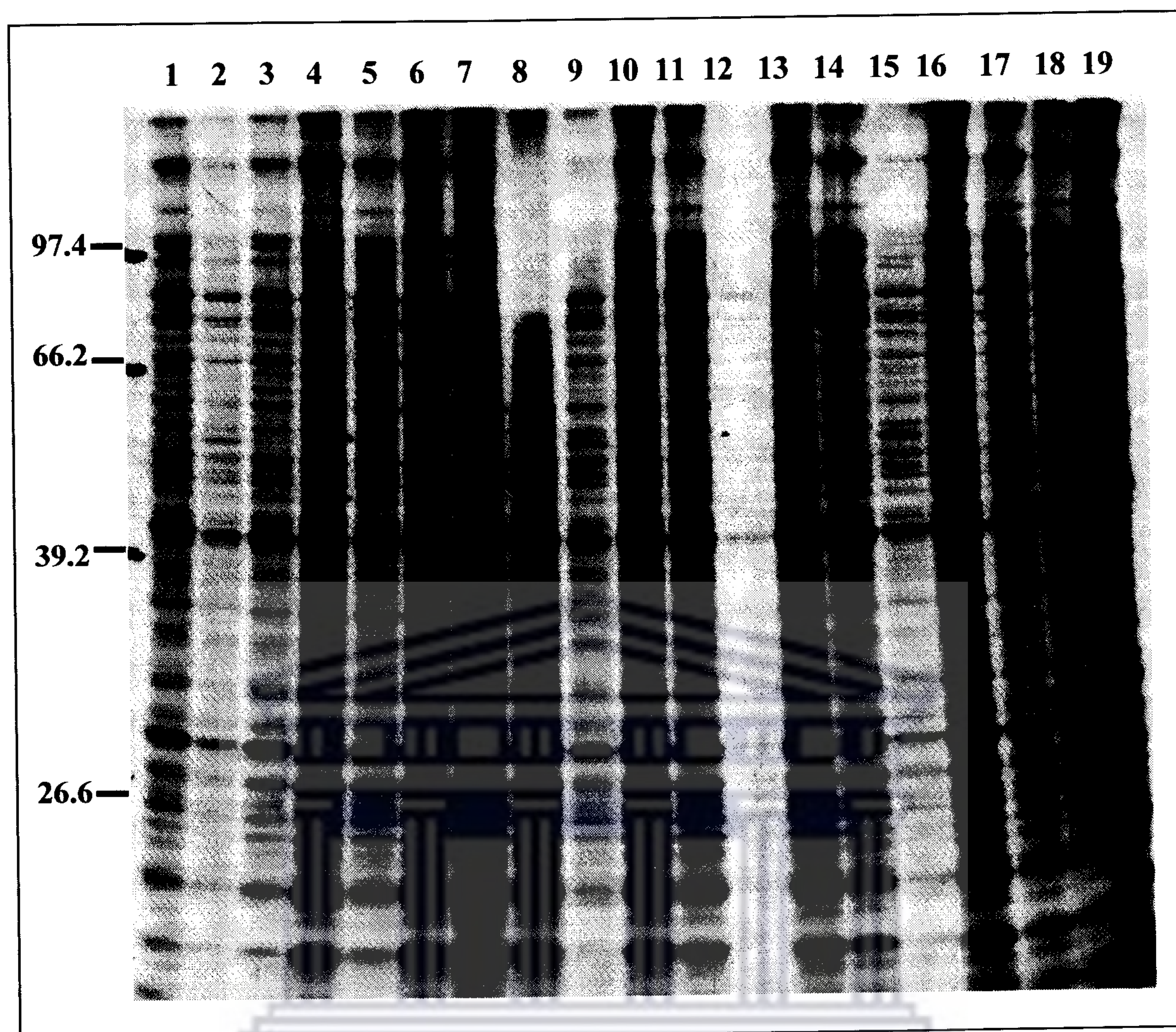


Fig. 3.3. Exposure of *P. putida* to specific heavy metals at a concentration of 50mg/L over time. Lane 1: Control - *P. putida* not exposed to heavy metals over 30min. Lanes 2-6: *P. putida* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 30min. Lane 7: repeat of lane 6. Lane 8: Control - *P. putida* not exposed to heavy metals over 60min. Lanes 9-13: *P. putida* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 60min. Lane 14: Control - *P. putida* not exposed to heavy metals over 120min. Lanes 15-19: *P. putida* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 120min.

http://etd.uwc.ac.za/

Table 3.3: The production of metal responsive proteins in *P. putida* when exposed to 50 mg/L of various heavy metals at specific time intervals.

No	30 min					60 min					120 min				
	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}
1	0	+	+	+	+	0	+	+	ND	+	0	+	+	+	+
2	0	+	+	+	+	0	+	+	ND	+	0	+	+	+	+
3	-	0	0	0	0	-	0	0	ND	0	-	0	0	0	0
4	0	+	+	+	+	0	0	0	ND	0	0	0	0	0	0
5	0	0	+	0	0	0	0	+	ND	0	0	0	+	0	0
6	+	0	0	0	0	+	0	0	ND	0	+	0	0	0	0
7	0	0	+	+	+	-	+	+	ND	+	-	+	+	+	+
8	-	+	+	+	+	-	0	0	ND	0	-	0	0	0	0
9	-	+	+	+	+	-	+	+	ND	+	-	+	+	+	+
10	-	0	+	0	+	-	0	0	ND	0	-	+	0	0	0
11	-	+	+	+	+	-	0	0	ND	0	-	0	0	0	0
12	-	0	0	0	0	-	0	0	ND	0	-	0	0	0	0
13	-	0	+	0	+	-	0	0	ND	0	-	+	0	0	0
14	-	0	+	0	+	-	0	0	ND	0	-	+	0	0	0
15	-	+	+	+	+	-	0	0	ND	0	-	+	0	0	0
16	-	0	+	+	+	-	0	0	ND	0	-	0	0	0	0
17	-	-	0	-	-	-	0	0	ND	0	-	0	0	0	0
18	0	0	+	+	+	0	0	0	ND	0	0	0	0	0	0
19	-	0	+	0	+	-	0	0	ND	0	-	+	0	0	0

Keynote: + - induced
- - suppressed
0 - no effect
ND - not determined

Table 3.4: Summary of the number of proteins induced and suppressed in *P. putida* when exposed to various heavy metals at a concentration of 50 mg/L over time.

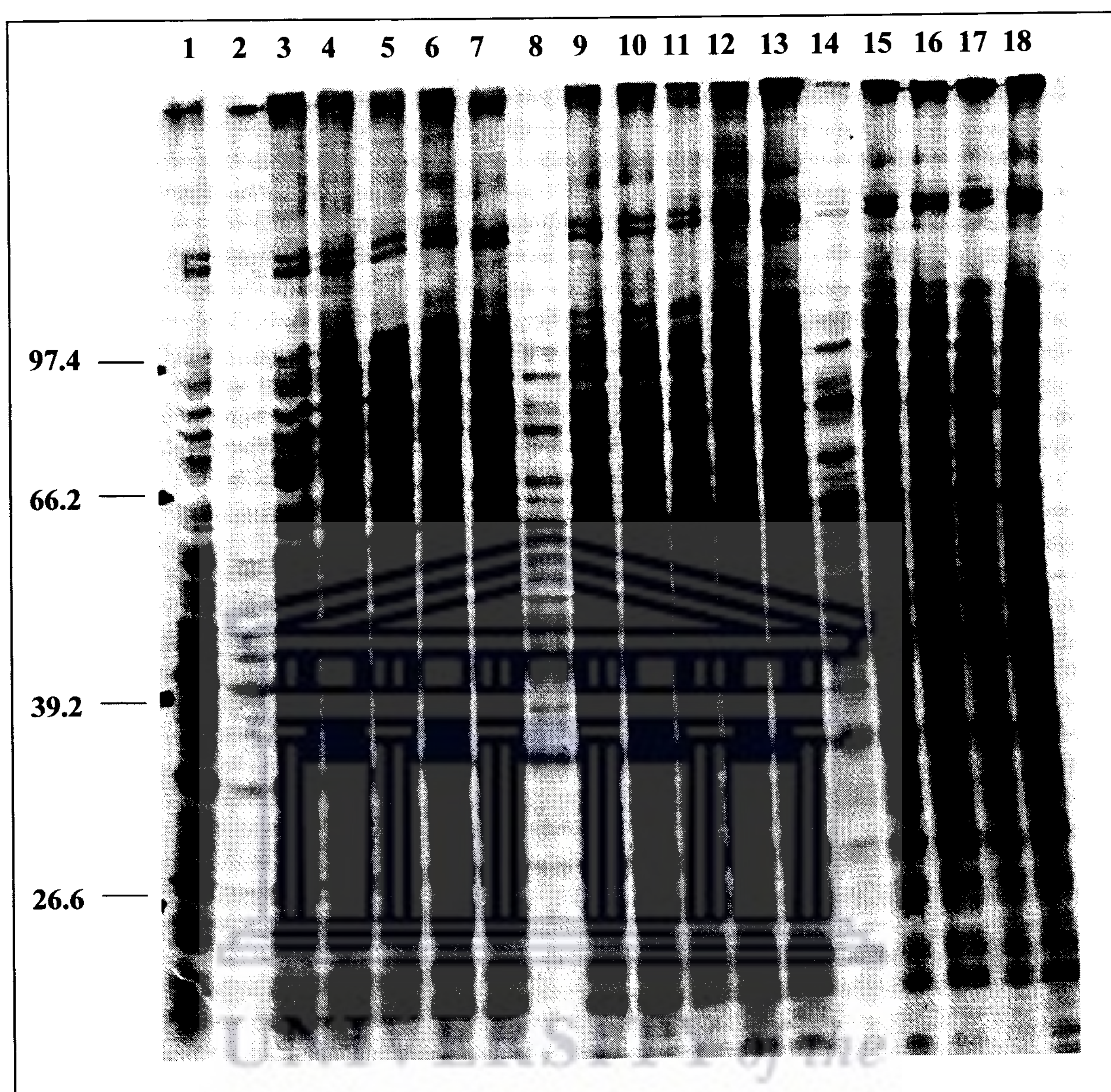
TIME (min)	Cd^{2+}		Cu^{2+}		Pb^{2+}		Ni^{2+}		Zn^{2+}	
	I	S	I	S	I	S	I	S	I	S
30	1	12	7	1	14	0	10	1	15	0
60	1	13	4	0	5	0	ND	ND	4	0
120	1	13	9	0	5	0	4	0	4	0

Keynote: I - Induced
S - Suppressed

UNIVERSITY of the WESTERN CAPE

When exposed to 50mg/L of the various heavy metals, as shown in Fig. 3.4 and also in Table 3.5 and Table 3.6, an almost similar response was observed in *P. aeruginosa* when compared to *P. putida*. Cd^{2+} suppressed most proteins in *P. aeruginosa*, whereas for Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} most of the stress proteins were induced within the first 30min of exposure to the heavy metals.

http://etd.uwc.ac.za/



WESTERN CAPE

Fig. 3.4. Exposure of *P. aeruginosa* to specific heavy metals at a concentration of 50 mg/L over time. Lane1: Control - *P. aeruginosa* not exposed to heavy metals over 30min. Lanes 2-6: *P. aeruginosa* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 30min. Lane 7: Control - *P. aeruginosa* not exposed to heavy metals over 60min. Lanes 8-12: *P. aeruginosa* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 60min. Lane 13: Control - *P. aeruginosa* not exposed to heavy metals over 120min.Lanes 14-18: *P. aeruginosa* exposed to 50mg/L Cd^{2+}, Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+} respectively, over 120min.

http://etd.uwc.ac.za/

Table 3.5: The production of metal responsive proteins in *P. aeruginosa* when exposed to 50 mg/L of various heavy metals at specific time intervals.

No	30 min					60 min					120 min				
	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}	Cd^{2+}	Cu^{2+}	Pb^{2+}	Ni^{2+}	Zn^{2+}
1	0	0	+	0	0	0	0	0	0	0	0	0	0	0	0
2	0	0	+	+	+	0	0	0	0	0	0	0	0	0	0
3	-	0	+	+	+	-	0	0	0	0	-	0	+	+	+
4	-	0	+	+	+	-	0	0	+	0	-	0	0	+	0
5	0	0	+	-	+	-	0	0	0	0	0	0	0	0	0
6	0	+	0	0	0	0	+	0	0	0	0	+	0	0	0
7	0	0	+	+	+	-	0	0	+	0	0	0	0	+	0
8	0	0	0	+	0	0	0	0	+	0	0	0	0	+	0
9	0	0	0	-	0	0	0	0	-	0	0	0	0	-	0
10	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0
11	0	0	0	+	0	0	0	0	+	+	0	0	0	+	0
12	0	+	+	+	+	0	0	0	0	0	0	0	0	0	0
13	-	+	+	+	+	-	0	0	0	+	-	0	0	0	+
14	-	0	0	0	0	-	0	0	0	0	-	0	0	0	0
15	0	0	+	+	+	0	0	0	-	+	0	0	0	0	+

Keynote: + - induced
- - suppressed
0 - no effect

Table 3.6: Summary of the number of proteins induced and suppressed in *P. aeruginosa* when exposed to various heavy metals at a concentration of 50 mg/L over time.

TIME (min)	Cd^{2+}		Cu^{2+}		Pb^{2+}		Ni^{2+}		Zn^{2+}	
	I	S	I	S	I	S	I	S	I	S
30	0	4	4	0	10	0	10	0	9	0
60	0	6	1	0	0	0	4	2	3	0
120	0	4	1	0	1	0	5	1	3	0

Keynote: I - Induced
S - Suppressed

By exposing *P. putida* and *P. aeruginosa* to 50mg/L of Ni^{2+}, Cu^{2+}, Zn^{2+}, Pb^{2+} and Cd^{2+} at various time intervals, it was possible to determine how long it takes the cell to respond

UNIVERSITY of the
WESTERN CAPE

to a stress. In other words, proteins, which were either suppressed or activated after exposure to heavy metals, reached the amount of proteins found in the control after extended exposure. During starvation stress, some new proteins are synthesized even after 4h (Jenkins *et al*., 1988), while a shift in temperature induces heat shock proteins within 1 to 2 min, returning to basal synthesis levels within 15 to 20 min (Niedhart *et al*., 1984). After 120 and 180 min of exposure to CPV, benzene, or TCE synthesis of new stress proteins was still found in *E. coli* (Blom *et al*., 1992).

In *P. putida* and *P. aeruginosa*, most stress proteins were produced during the first 30 min of exposure to Cu^{2+}, Pb^{2+}, Ni^{2+} and Zn^{2+}. Cd^{2+} seems to have suppressed most proteins. Therefore, the Cd^{2+} dosage might be detrimental to the cell or it might take the cell longer to adapt to the stressful conditions. Thus it may take the cell 20min to 3h or longer to adjust its cellular processes against the stress or ultimately face destruction. It is evident that the pattern of protein rearrangement as observed with heavy metal stress response is not unexpected and probably reflects the functions of the proteins and the need for an initial rapid adaptation to protect the cell against the damaging influence of the stress. This is followed by a period of functional metabolic enhancement during which the proteins expressed modulate metabolism under the altered physical or chemical conditions (Lupi *et al*., 1995).

It is also evident that stress protein synthesis is a more sensitive index of stress than growth rate, since pollutant concentrations at which little or no growth inhibition occurs evokes stress protein synthesis. These results show promising prospect for stress protein

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

analysis as an alternative and more sensitive method for measuring toxic effects in organisms at sublethal levels. Furthermore, the fact that individual chemicals induce unique proteins can conceivably provide a means of identifying pollutants in the environment (Blom *et al.*, 1992). It is evident that exposure of *P. aeruginosa* and *P. putida* to Cu^{2+}, Pb^{2+}, Ni^{2+}, Zn^{2+} and Cd^{2+} at 50mg/L over time does induce protein production and suppression.

http://etd.uwc.ac.za/

CHAPTER 4

REPORTER VECTOR CONSTRUCTION AND SCREENING OF *PSEUDOMONAS* GENE LIBRARIES AGAINST HEAVY METALS

4.1 Introduction

Microorganisms indigenous to metal-containing environments have evolved several distinct mechanisms of heavy metal tolerance. Some include common plasmid encoded resistance, sequestration by adsorption or by binding to detoxifying ligands, proteins or polymers. These and other heavy metal tolerance mechanisms revealed by microorganisms has led to their use as bioindicators of various toxic pollutants in the environment.

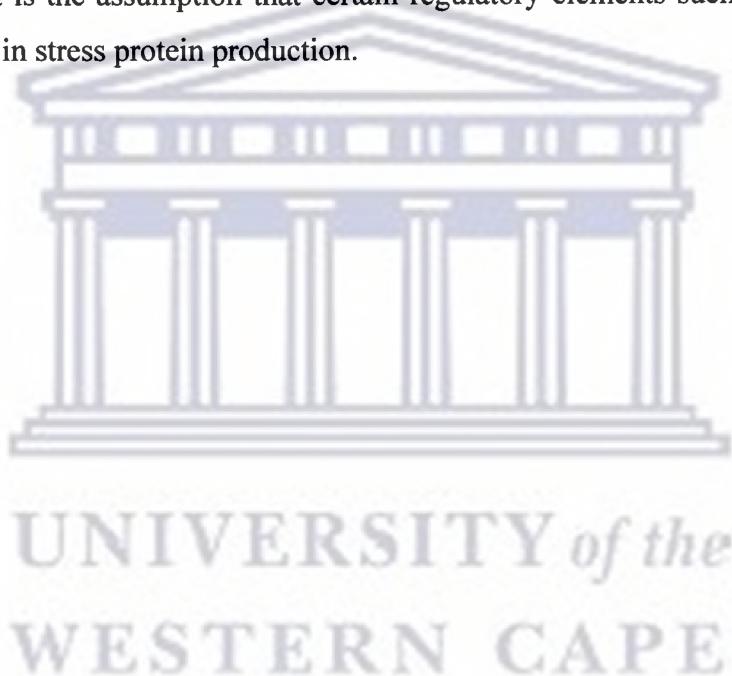
In response to the need to quantify toxicological contamination, numerous bioassays involving microorganisms have been developed (Paton *et al.*, 1995). Tests using bacteria have included the measurement of growth inhibition, respirometry, viability of cells, ecological effects and bioluminescence. Microorganisms can be used as specific and sensitive devices for sensing the bioavailability of a particular pollutant or pollutant class. This is based on the ability of pollutant (like the most normal compounds) to invoke non-specific (e.g., toxicity of stress) or specific (e.g., activation of a degradative pathway) responses in microorganisms. The signaling pathway thus activated will regulate the expression of one or more (sets of) genes. The extent of this gene expression serves as a measure of the available (sensed) concentration of the compound.

UNIVERSITY of the WESTERN CAPE

A rapid and sensitive way to measure such gene expression is to fuse relevant promoter sequences and promoterless reporter genes such as those for bacterial luciferases of *Vibrio* sp. The use of microbes to sense and report the presence of chemical compounds has recently provoked great interest. Whole-cell biosensors that can detect naphtalene and salicylate, toluene and mercury have been developed. A fusion of the lux genes and the regulatory elements of the isopropylbenzene catabolism operon were used to detect various hydrophobic pollutants, such as alkylbenzenes and several other aromatic and

http://etd.uwc.ac.za/

aliphatic hydrocarbons. Other biosensor strains were constructed to detect toxic compounds, generally by coupling the lux genes with a stress-inducible promoter. Intracellular-bioaccumalation processes with microorganisms optimized by genetic engineering could overcome the deficiencies of common metal cleanup processes and may be an alternative for removal and recovery of heavy metal such as Hg^{2+} from contaminated soil and water. The aim of this study was to construct a promoterless broad-host-range reporter vector system based on the molecular features of pAL4000 (Greener *et al*., 1992), by replacing the *luc* gene with the *lacZ* gene as its substrate is cheaper and easily assayable. Based on successful vector construction, gene libraries of *P. putida* and *P. aeruginosa* will be constructed, which will eventually be screened against various heavy metals as it is the assumption that certain regulatory elements such as promoters could be involved in stress protein production.


UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

4.2 Materials and Methods

4.2.1 Construction of reporter plasmid pALACZSD

All enzymes were obtained from Boehringer Mannheim. Genetic manipulations such as restriction enzyme digestion, ligations, dephosphorylation and transformations were performed according to standard protocols as described by Sambrook *et al.*, (1989).

Table 4.1 Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
Strains		
E. coli HB101	pro leu recA hsdR hsdM	D. R. Helinski
E. coli MC1061	Δ (*lac*) X74	W. H. van Zyl
Plasmids		
yEP62	Amp^r, *lacZ*	W. H. van Zyl
$pHBSK^{+/-}$	*Hinc*II site changed to *Bgl*II site, Amp^r	W. H. van Zyl
$pMHBSK^{+/-}$	*Nhe*I site, Amp^r	This study
pBL6.0	$pMHBSK^{+/-}$::*lacZ*, Amp^r	This study
pBL6.1	$pMHBSK^{+/-}$::*lacZ* with SD hybrid	This study
pAL4000	Tet^r, *luc*	D. R. Helinski
pALACZSD	Tet^r, pAL4000::*lacZ*SD	This study
pRK2013	Km^r, helper plasmid	D. R. Helinski
pALACZSD	Tet^r, *lacZ*	This study

UNIVERSITY of the WESTERN CAPE

4.2.1.1 Amplification of *lacZ* gene using Polymerase Chain Reaction (PCR)

Primers (PRIMER 1: 5'-GCTAGCTAGCTAGATGCAACGTCGTCACTGGGAAA-3' and PRIMER 2: 5'-GATCAGATCTACGCGAAATACGGGCAG-3') chemically synthesized by Genosys were used to amplify the *lacZ* gene hybrid flanked by *BglII-NheI* overhangs from the yeast episomal plasmid yEP62. The PCR reaction mixture contained the DNA template, 2.5mM $MgCl_2$, 175µM dNTP mix, 50pmol of each primer, 10X Taq polymerase buffer, 1U Taq polymerase and sterile distilled water to a final volume of 50µl. The PCR conditions were as follows: The reaction mixture was first denatured at 95^0C for 2min, followed by 35 cycles of dissociation at 94^0C for 1min; annealing at 65^0C for 30sec, extension at 70^0C for 2min, and a final elongation step of 72^0C for 5min.

The PCR product was electrophoresed on a 0.8% agarose gel containing (5µg/ml) ethidium bromide (EtBr). The *lacZ* gene (3kb) was excised from the gel and cleaned using the freeze-clean method of Benson (1984) with modifications as described below. The excised gel piece was passed through a 5ml syringe and collected in a 2ml-microfuge tube. One milliliter phenol was added and the sample vortexed for 1min, prior to an incubation period for 1h to overnight at -80^0C. Following the incubation the samples were centrifuged for 15min at 15000x*g*. The supernatant was transferred to a clean microfuge tube and the DNA extracted with an equal volume of Phenol/Chloroform/Isoamylalcohol (25:24:1) (PCI). The aqueous phase was precipitated with a one third volume of 7.5M NH_4OAc and 1 volume of isopropanol. DNA was stored at -20^0C for 1-2h and then centrifuged at 15000x*g* for 15min. The DNA pellet was

http://etd.uwc.ac.za/

washed with ice-cold 70% ethanol and dried at 65^0C for 10min. The dried pellet was suspended in sterile Millipore water for ligation purposes.

4.2.1.2 Modification of pHBSK$^{+/-}$ to serve as control vector

The pHBSK$^{+/-}$ used in this study had been modified by insertion of a *Bgl*II site in the *Hinc*II site in the laboratory of Prof. W. H. van Zyl, Department of Microbiology, University of Stellenbosch. Further modifications were made to the existing pHBSK$^{+/-}$, so that it could serve as a control vector in order to monitor β-galactosidase activity. This was performed by inserting a 12 monomer *Nhe*I linker (CTAGCTAGCTAG) into the *Eco*RV site of pHBSK$^{+/-}$, so that the *lacZ* gene with a *Nhe*I and *Bgl*II overhang could be cloned into the new vector referred to as pMHBSK$^{+/-}$.

4.2.1.3 Cloning the *lacZ* gene in pMHBSK$^{+/-}$

The amplified *lacZ* gene insert was subsequently ligated to the pMHBSK$^{+/-}$ vector containing *Bgl*II and *Nhe*I compatible termini, at room temperature overnight. Recombinant constructs were transformed into *E. coli* MC1061 using standard procedures (Sambrook *et al*., 1989). Upon plating on LB plates containing X-gal (20mg/ml in dimethylformamide), IPTG (250mg/ml in distilled water) and ampicillin (50μg/ml), no blue colonies were observed after incubation at 37^0C overnight.

4.2.1.4 Testing the functionality of the *lacZ* gene product

The synthesis of the complete *lacZ* gene was repeated by using a special thermostable DNA polymerase (Expand High Fidelity) with improved proofreading activity. Upon

http://etd.uwc.ac.za/

reinvestigation of the *lacZ* gene sequence from yEP62, it was discovered that the gene lacked the Shine-Dalgarno sequence for ribosome binding (also called ribosome binding site [RBS]). The cloning strategy was therefore revised.

4.2.2 Revised cloning strategy for construction of reporter plasmid pALACZSD

4.2.2.1 Digestion of yEP 62

yEP62 was digested with *Bam*HI and *Dra*I to yield the *lacZ* gene fragment with sticky and blunt ends, respectively. The *lacZ* gene fragment was subsequently electrophoresed on a 0.8% agarose gel, excised and freeze-cleaned (Benson, 1984).

4.2.2.2 Testing the functionality of the *lacZ* gene product

pHBSK$^{+/-}$, modified with a *Bgl*II site in the *Hinc*II site within the multicloning site, was digested with *Eco*RV and *Bam*HI. The complementary *lacZ* gene fragment was ligated to digested pHBSK$^{+/-}$ to produce a plasmid hereafter referred to as pBL6.0, about 6kb in size. Plasmid pBL6.0 was transformed into *E. coli* MC1061 cells prior to large-scale plasmid isolations being performed (Sambrook *et al*., 1989). The modified plasmid was digested with *Bam*HI, dephosphorylated using alkaline phosphatase and cleaned using the freeze clean method (Benson, 1984). Other cloning manipulations performed prior to testing the full functionality of the *lacZ* gene product are described below.

4.2.2.3 Hybridization of chemically synthesized oligonucleotides

Oligonucleotides, with a Shine-Dalgarno sequence, were chemically synthesized as the *lacZ* gene from yEP62 contained no RBS. The 46-monomer oligonucleotides are shown

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

in Fig. 4.1. The oligonucleotides were hybridized by initial denaturation at 95^{0}C for 5min, followed by annealing at 79.8^{0}C overnight.

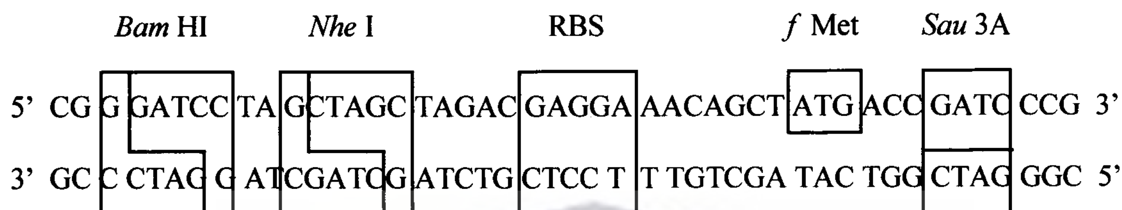


Fig. 4.1. Chemically synthesized oligonucleotides containing RBS sequence

4.2.2.4 Separation of hybridized products

The hybridized products were separated on a non-denaturing 20% polyacrylamide gel. The gel was electrophoresed at 200 V for 2.5h until the loading dye reached the end of the glass plates. The products were visualized by staining in EtBr (5µg/ml).

UNIVERSITY of the
WESTERN CAPE

4.2.2.5 Elution of hybridized products from the acrylamide gel

Following visualization with EtBr staining, hybridized products were excised from the gel and sliced into fine pieces. These pieces were transferred to a 2ml-microfuge tube and an elution solution (0.3M NaOAc, pH7.5) was added to cover the gel pieces (approximately twice the original volume of the gel slice). The DNA samples were incubated with shaking at 37^{0}C for 3-4h and centrifuged at 15000x*g* for 10min at room temperature. The supernatant containing the eluted DNA was removed, filtered through a

http://etd.uwc.ac.za/

blue tip with a glasswool plug and then briefly centrifuged at 15000x*g* for 20sec to remove any solid material. The supernatant was removed and precipitated with 2.5 volumes of ethanol for 1h at -20^{0}C. The DNA was pelleted by centrifugation and washed twice with 70% ice-cold ethanol. Following these steps the pellet was dried and resuspended in Tris-EDTA buffer for further manipulation.

4.2.2.6 Digestion of hybridized products

The hybridized products were digested with *Sau*3A and analyzed on a non-denaturing 20% PAGE gel. The digested fragments were excised from the gel and cleaned. The digested oligonucleotides were ligated to the *Bam*HI site of pBL6.0. Recombinants were transformed into *E. coli* MC1061, plated on LB plates with X-gal (20mg/ml), IPTG (250mg/ml) and ampicillin (50µg/ml), which was incubated at 37^{0}C overnight. The hybridized products were also digested with *Nhe*I to determine the presence of the restriction enzyme site.

UNIVERSITY of the
WESTERN CAPE

4.2.2.7 Excision of the *lacZ* gene from pBL6.1

pBL6.1 (pBL6.0::SD) was amplified by large-scale plasmid isolation (Sambrook *et al.*, 1989). Isolated plasmid DNA was digested with *Nhe*I and *Bgl*II to excise the *lacZ* gene containing the hybridized product with SD sequence.

http://etd.uwc.ac.za/

4.2.2.8 Cloning of the *lacZ* gene construct in pAL4000

The molecular features of the promoterless plasmid pAL4000 are shown in Fig. 4.2. The reporter gene of this plasmid, the firefly luciferase gene *luc* was replaced with a *lacZ* gene by digesting the plasmid with *Bgl*II and *Nhe*I to release the *luc* gene. The *lacZ* gene, with complementary ends, was ligated to pAL4000 yielding the plasmid hereafter referred to as pALACZSD (pAL4000:: *lacZ* SD). A schematic representation of construction of the reporter plasmid pALACZSD is shown in Fig. 4.3.

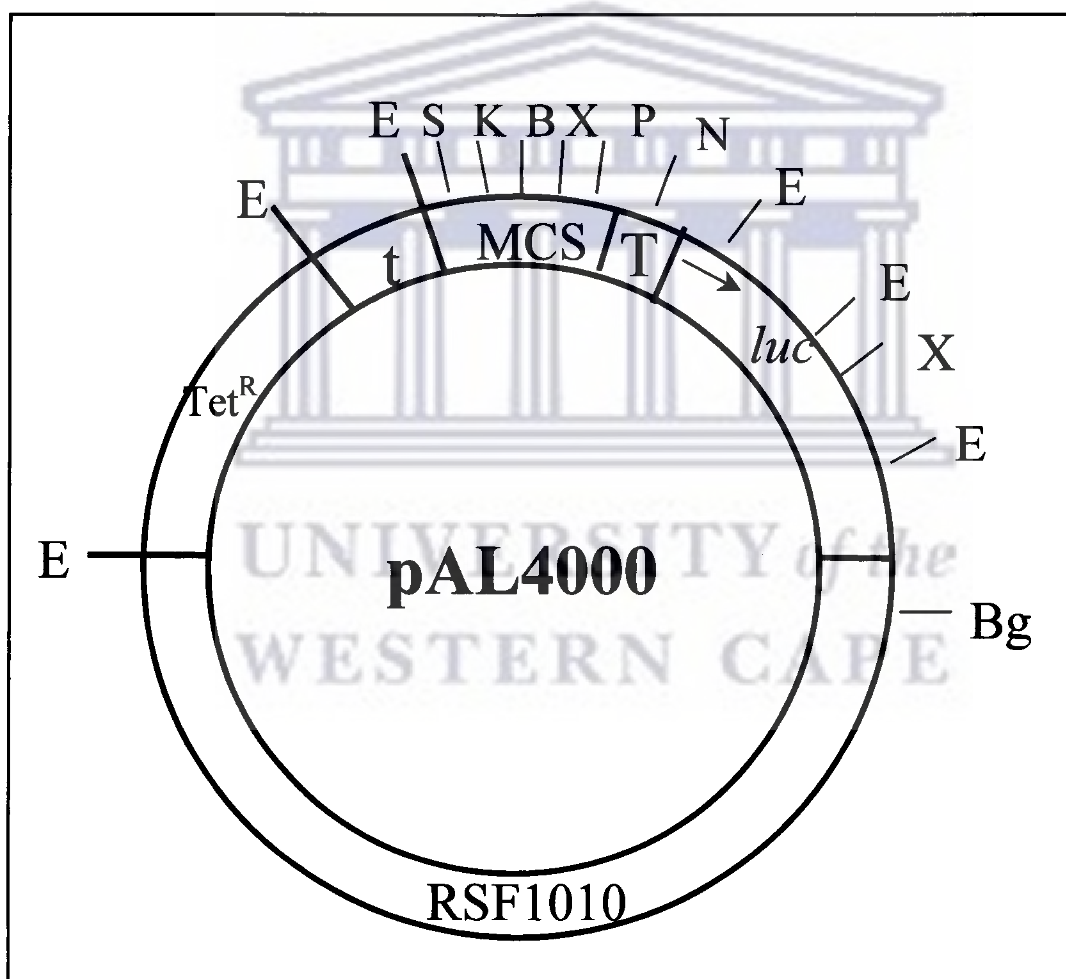


Fig. 4.2. Molecular features of pAL4000. K, P, E, B, Bg, S and X refer to sites for restriction enzymes *Kpn*I, *Pst*I, *Eco*RI, *Bam*HI, *Bgl*II, *Sac*I and *Xba*I, respectively.

http://etd.uwc.ac.za/

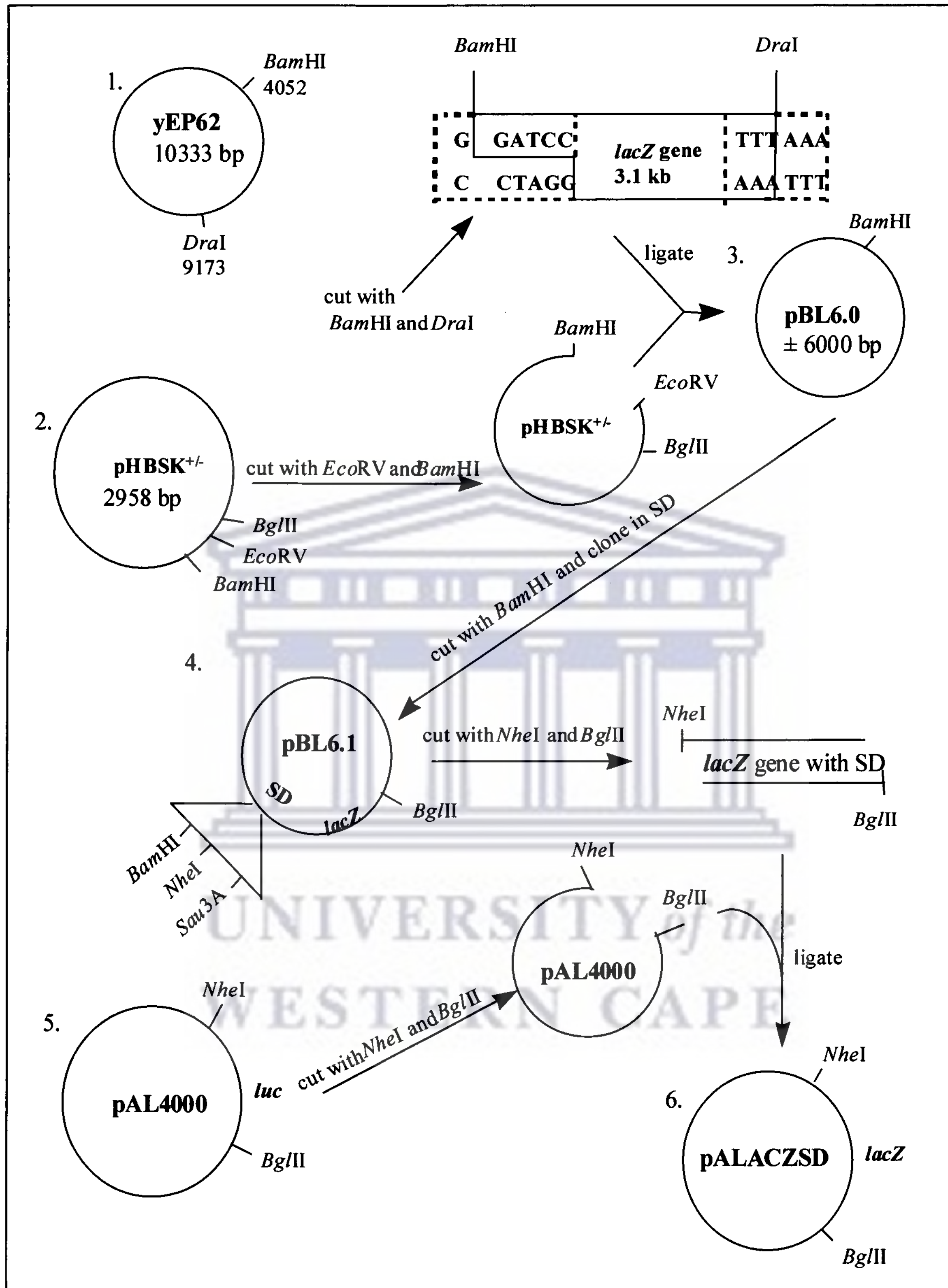


Fig.4.3. Schematic representation of the construction of reporter plasmid pALACZSD.

http://etd.uwc.ac.za/

4.2.3 Construction of *Pseudomonas* gene libraries

4.2.3.1 Isolation of *P. putida* and *P. aeruginosa* genomic DNA

P. putida and *P. aeruginosa* were grown in LB broth to saturation at 30^{0}C and 37^{0}C, respectively. Genomic DNA from the *Pseudomonas* sp. were isolated according to standard procedures (Ausubel *et al*., 1994), with a few modifications. The genomic DNA was isolated by lysis in 10% SDS and treated with 20mg/ml proteinase K, 5M NaCl and CTAB/NaCl (10% CTAB in 0.7M NaCl) solution. This was followed by a series of P:C:I extractions prior to alcohol precipitation of the nucleic acids. The genomic DNA was further purified using an ethidium bromide/CsCl gradient and ultracentrifuged overnight at 55000rpm in a Beckman VTi65 rotor. A single band visualized under UV light was removed and the EtBr was extracted using water-saturated isobutanol. The DNA was precipitated by adding 3 volumes of water and 2 volumes of 100% ethanol.

4.2.3.2 Digestion of genomic DNA

In order to create a partial digestion profile of the genomic DNA of the two *Pseudomonas* sp., DNA was digested with *Sau*3A at various time intervals (1-10min). After establishing the period required to obtain fragment sizes ranging from 100 to 1500 bp, the DNA was digested as follows:

UNIVERSITY of the WESTERN CAPE

The restriction enzyme digest mixture, without restriction enzyme, was incubated at 37^{0}C for 2min. An aliquot of *Sau*3A was added to the mixture, tapped lightly until dispersed and incubated at 37^{0}C for 4-5min. The reaction was stopped by the addition of 0.5M EDTA. Digested genomic DNA was separated on a 0.8% agarose gel prior to excision of

http://etd.uwc.ac.za/

DNA fragments in the size range of 100-1500 bp. The excised gel fragments were cleaned using the Gene Clean Kit (Biolabs) following the manufacturer's instructions.

4.2.3.3 Construction of *Pseudomonas* gene libraries

pALACZSD was digested with *Bam*HI to create compatible termini for ligation. After digestion, the enzyme was removed using the Gene Clean Kit (Biolabs), prior to the dephosphorylation of the plasmid at 37^0C for 1h. The samples were cleaned according to the Gene Clean Kit protocol. The dephosphorylated samples and the genomic DNA were separated on a 0.8% agarose gel and subsequently cleaned using the freeze-clean method (Benson, 1984). The phosphorylated vector and genomic DNA fragments were ligated at room temperature overnight. Recombinants were transformed into competent *E. coli* MC1061 cells, plated on LB plates containing tetracycline (40 µg/ml) and incubated at 37^0C overnight. Minipreps were performed on clones isolated from these plates (Sambrook *et al*., 1989). To confirm the presence of the insert in the recombinant clones, isolated plasmid DNA was digested with *Sac*I.

UNIVERSITY of the
WESTERN CAPE

4.2.4 Transformation of recombinant DNA into the respective *Pseudomonas* hosts

4.2.4.1 Transformation of gene libraries in *P. aeruginosa*

4.2.4.1.1 Preparation of *P. aeruginosa* competent cells

Bacteria were grown overnight on TN agar (Tryptone 5g, Dextrose 1g, Yeast extract 2.5g and Sodium nitrate 4g per 1000ml). A portion was inoculated into TN broth and incubated for 2-3h until it reached the mid-log phase (OD_{600} = 0.6). Cells were centrifuged at 4^0C at 8500xg for 10min. The pellets were resuspended in 0.5 volumes of

http://etd.uwc.ac.za/

cold, sterile 0.15M $MgCl_2$. Pellets were dispersed and incubated on ice for an additional 5min. The samples were centrifuged and the pellets were resuspended in 0.5 volumes of 0.15M $MgCl_2$, prior to a further incubation on ice for 20min. Following the incubation period, the samples were centrifuged and the pellets resuspended in 0.1 volumes of cold 0.15 M $MgCl_2$.

4.2.4.1.2 Transformation of *P. aeruginosa* gene library into *P. aeruginosa*

The plasmid DNA from 1000 clones in *E. coli* MC1061 was isolated by minipreps (Sambrook *et al*., 1989). The isolated DNA was transformed into *P. aeruginosa* using the method of Olsen *et al*., (1982), with a few modifications. Transforming DNA (10 µl) was placed in cold centrifuge tubes. Two hundred microliters of competent cells were added with mixing. The mixture was incubated on ice for 60min. Cells were heat pulsed for 3min in a water bath at 37^0C while gently swirling the tubes. The DNA-cell mixture was placed immediately on ice for 5min. After this, 0.5 ml of TN broth was added and the suspension was incubated at 37^0C for 2h. The cells were then plated on TN plates with 40µg/ml tetracycline and incubated at 37^0C for 24h.

UNIVERSITY of the
WESTERN CAPE

4.2.4.2 Transformation of *P. putida* gene library into *P. putida*

4.2.4.2.1 The $CaCl_2$ method

The transformation procedure used was essentially as described by Chakrabarty *et al.* (1975), with a few modifications. *P. putida* was grown overnight in LB broth at 30^0C in an environmental shaker at 180rpm. An aliquot (5ml) was inoculated in 100ml of fresh LB broth and grown for 3-4h until it reached an OD_{600}= 0.4. Forty milliliters of culture

http://etd.uwc.ac.za/

was added to cold centrifuge tubes and further chilled on ice for 10min. The cells were washed once with 20ml of 10mM NaCl by centrifugation at 7500xg for 10min. The cells were resuspended in half the original volume of 0.1M cold $CaCl_2$, kept on ice for 20min, harvested by centrifugation and resuspended in one tenth of the original volume of 0.1M $CaCl_2$. An aliquot (100µl) of these cells were mixed with 10µl of plasmid DNA and incubated on ice for 60min. The cell-DNA mixture was then subjected to a heat pulse at 42^0C for 2min, chilled and finally diluted in 900µl of fresh LB broth. The cells were allowed to grow at 30^0C in a shaker for 2h and 100µl aliquots were plated onto nutrient agar containing 20µg/ml tetracycline.

4.2.4.2.2 Electroporation

Electroporation was performed according to Iwasaki *et al.,* (1994), with a few modifications. An overnight culture of *P. putida* (1ml) was added to fresh LB broth and grown to its mid-log phase (OD_{600}=0.31) at 30^0C with shaking at 165 rpm. A modification in the recipe of LB was that 5g of NaCl were used per liter instead of 10g. Cultures were immediately placed on ice and cells were harvested by centrifugation at 8500x*g* for 10 min at 4^0C. Cells were washed in 20 ml of sterile 300mM sucrose at 8500x*g* for 10min at 4^0C. Cells were resuspended in 0.5 volumes of sterile 300mM sucrose, left on ice for 5-7min and pelleted by centrifugation at 7500xg for 10min at 4^0C. Cells were resuspended in 0.1 volumes of 300mM sucrose and left on ice for 30min. A 100µl volume of electrocompetent cells was aliquoted in prechilled sterile electroporation cuvettes (BioRad). Ligation mixtures and controls were added to cuvettes that were incubated on ice for 10min. DNA was transformed into all cells using an electroporation

UNIVERSITY of the
WESTERN CAPE

protocol. Electrical settings in the experiment were as follows: set voltage 2.5 kV(12.kV/cm); 25μF; pulse controller; parallel resistor, 200Ω. Immediately after discharge, 900μl of LB was added directly to each cuvette. The samples were transferred to test tubes and incubated at 30^{0}C for 2h with shaking at 140rpm. After incubation, 100μl of each transformation mix was spread plated onto LB plates containing X-gal and 20μg/ml tetracycline. Plates were incubated at 30^{0}C and checked for transformants after 24h.

4.2.4.2.3 Triparental mating of *P. putida* gene library

E. coli HB101 (pRK2013) with 40μg/ml kanamycin, *E. coli* MC1061 (recombinants) with 40μg/ml tetracycline and *P. putida* without antibiotic, were grown in LB broth overnight at 37^{0}C for *E. coli* strains and at 30^{0}C for *P. putida*. An aliquot (50μl) of each cell suspension was inoculated into 5ml LB broth, with their respective antibiotics, and incubated at 30^{0}C for 3-4h. A 100μl of each cell suspension was inoculated in 700μl LB broth and allowed to grow for 1h or overnight at room temperature. The cells were spreadplated onto selective plates and incubated at 30^{0}C for 48h.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

4.2.5 Screening of the *Pseudomonas* gene libraries for heavy metal responsive promoters

4.2.5.1 The microtitre plate method

Freshly transformed cultures of *P. aeruginosa* were grown in minimal broth containing tetracycline (20 μg/ml) for 2-3h. Cultures were subsequently exposed to 50 mg/ml of Cd^{2+}, Ni^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Pb^{2+} for 1h. Growth was then suppressed by placing the cultures on ice, prior to the determination of β-galactosidase activity. The assay was performed according to Miller *et al.,* (1988) with modifications. After a 20min incubation period on ice, 70μl of Z-Buffer (0.06M $Na_2HPO_4.2H_2O$, 0.04M $NaH_2PO_4.H_2O$, 0.01M KCl, 0.001M $MgSO_4.7H_2O$, 0.05M ß-Mercaptoethanol, pH 7.0 [do not autoclave]), 10μl 0.1% (vol/vol) SDS and 10μl of chloroform (CH_3Cl) was added to each well of the microtitre plates. Following another incubation of the microtitre plates at 28^0C for 10min, 25μl of 2-Nitrophenyl-β-galactopyranoside (ONPG) (dissolve 4g ONPG in 42.2ml of 1M $NaH_2PO_4H_2O$, 57.7ml of 1M $Na_2HPO_42H_2O$ and 900ml distilled water [do not autoclave]) was added. After a 30min incubation at room temperature absorbance readings were taken at 414nm on a Titertek Multiscan. About a 1000 clones were screened in this manner. The plasmid pALACZSD (*E. coli* MC1061) was used as a negative control, while pALACZSDIP (*E. coli* MC1061) served as a positive control in this assay.

4.2.5.2 The agar plate method

Clones from the *P. putida* gene library were screened using the agar plate method. Clones were grown to their mid-log phase in VB broth and then spread plated on VB minimal

http://etd.uwc.ac.za/

agar plates supplemented with 60mg/L of either Ni^{2+}, Cd^{2+}, Co^{2+}, Zn^{2+}, Pb^{2+} and Cu^{2+}. X-gal and tetracycline (20μg/ml) were added to plates afterwards. Again, plasmid pALACZSD (*E. coli* MC1061) was used as a negative control, while pALACZSDIP (*E. coli* MC1061) served as a positive control. Plates were incubated at 30^0C and blue color development was monitored.

4.3 Results and Discussion

4.3.1 Construction of reporter plasmid pALACZSD

The induction and suppression of proteins in *P. putida* and *P. aeruginosa* by heavy metals suggested that certain regulatory elements, which could include promoters, play an important role in gene expression. The construction of the promoterless broad-host-range reporter plasmid pALACZSD was based on the molecular features of pAL4000 (Greener *et al.*, 1992). pAL4000 is easy transformable in *Pseudomonas* and has a translational stop signal 5' from the translational initiation codons of the reporter gene, thereby ensuring that chimeric proteins are not produced. Chimeric proteins are generally regarded as less useful as reporters of transcriptional activity (Greener *et al.*, 1992). The aim was therefore to construct a promoterless broad host range reporter vector system that can be easily assayed.

As the substrate required for luciferase activity assays is a very expensive biochemical, it was not feasible to use such a reporter gene in the desired bioindicator system. It was therefore opted to use *lacZ* as reporter gene for this study. Fusions to *lacZ* are now most commonly used in gene regulation studies (Slauch and Silhary, 1991; Atlas *et al.*, 1992). β-galactosidase activity can be easily measured when X-gal is cleaved to produce an insoluble blue pigment, easily observable in most agar media (Atlas *et al.*, 1992). In addition, β-galactosidase cleavage of the substrate o-nitrophenol-β-D-galactoside (ONPG) cause the conversion of the colorless substrate into o-nitrophenol, a yellow compound easily quantified spectrophotometrically (Atlas *et al.*, 1992).

http://etd.uwc.ac.za/

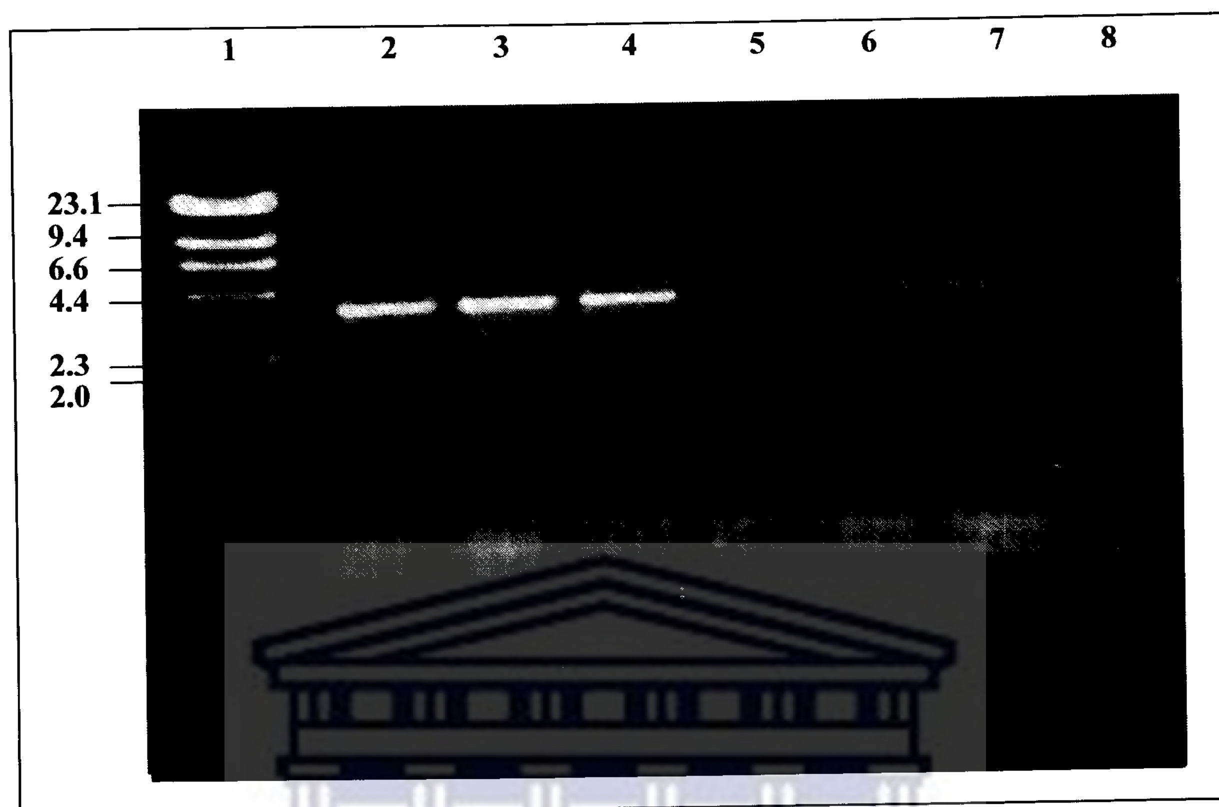


Fig. 4.4. Analysis of PCR product on a 0.8% agarose gel. Lane 1: MWM λ *Hind*III. Lanes 2-7: PCR product. Lane 8: Negative control (water blank).

UNIVERSITY of the
WESTERN CAPE

The *lacZ* gene, present in the yeast episomal plasmid yEP62, was amplified by PCR as shown in Fig. 4.4. The 3.1 kb fragment subsequently cloned in pHBSK$^{+/-}$ produced no B-galactosidase activity. It was discovered that there was no ribosome binding site or Shine Dalgarno sequence present on the *lacZ* gene. The problem was overcome by initially cloning the *lacZ* gene, obtained through restriction enzyme digestion from yEP62, in pHBSK$^{+/-}$ subsequently referred to as pBL6.0 (Fig. 4.5). The hybridized oligonucleotides, containing the Shine-Dalgarno sequence, as shown in Fig. 4.6 were digested with Sau3A (Fig. 4.7) to facilitate cloning in pBL6.0 digested with *Bam*HI. This plasmid referred to

http://etd.uwc.ac.za/

as pBL6.1 therefore contained pHBSK$^{+/-}$ as vector and the *lacZ* gene with Shine-Dalgarno as insert.

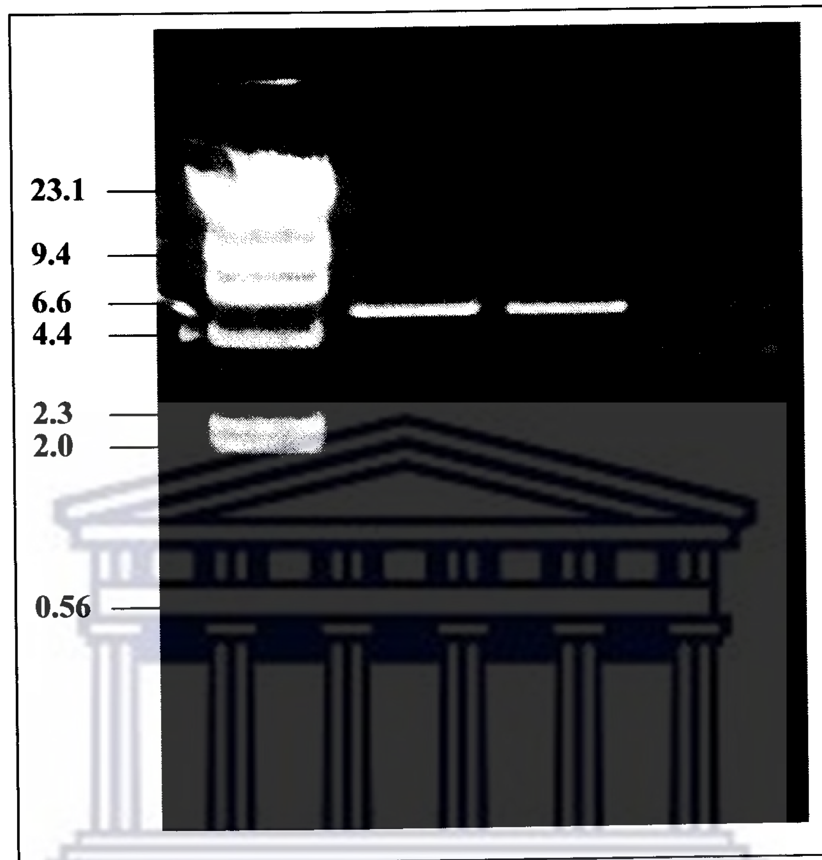


Fig. 4.5. Digestion of pBL6.0 as shown on a 0.8% agarose gel. Lane 1: MWM λ *Hind*III. Lane 2: pBL6.0 digested with *Bam*HI. Lane 3: pBL6.0 cut with *Bgl*II and Lane 4: pBL6.0 cut with *Sac*I.

UNIVERSITY of the
WESTERN CAPE

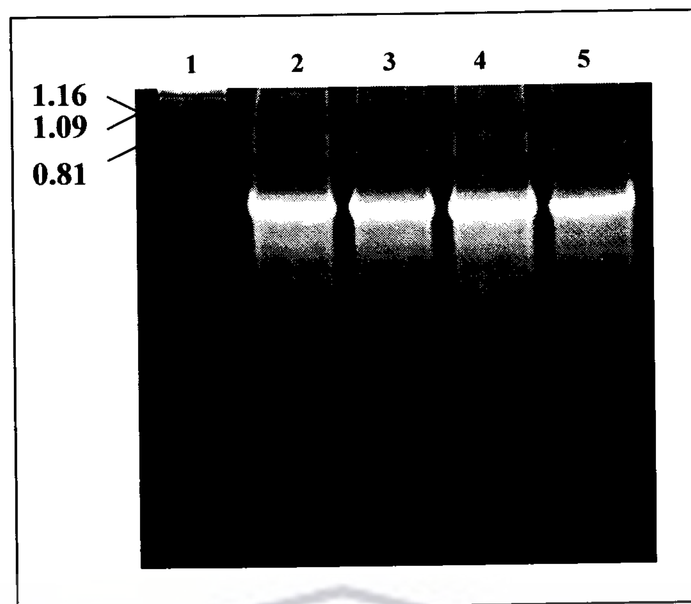


Fig. 4.6. Analysis of hybridized oligonucleotides as resolved on a 20% PAGE gel. Lane 1: MWM λ *Pst*I. Lanes 2-5: hybridized oligonucleotides.

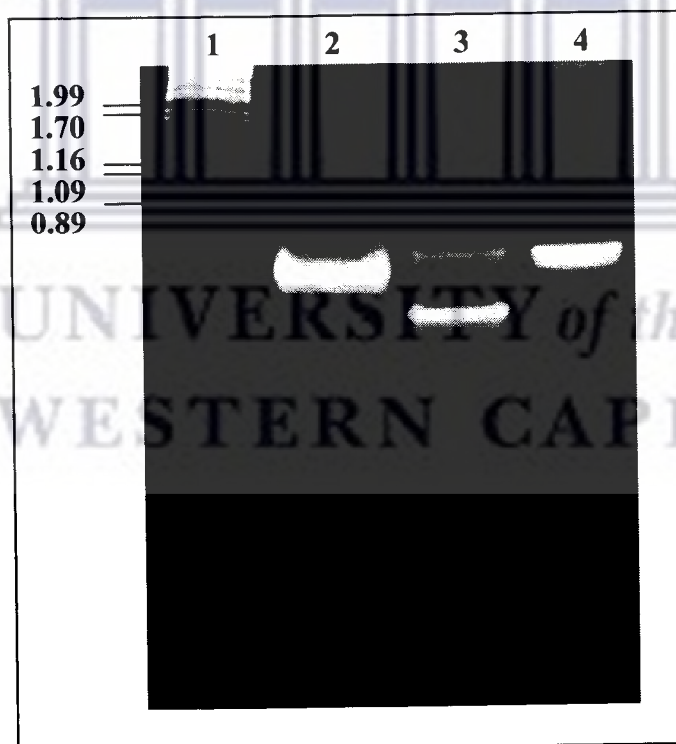


Fig. 4.7. Restriction enzyme digest of hybridized oligonucleotides as resolved on a 20% PAGE gel. Lane 1: MWM λ *Pst*I. Lane 2: uncut hybridized oligonucleotides. Lane 3: product cut with *Nhe*I and Lane 4: product cut with *Sau*3A.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

The promoterless broad-host-range plasmid pAL4000, digested with *BamHI*, was found to be a very large plasmid of about 12kb in size as shown in Fig. 4.8. For further size determinations the plasmid was double digested with *Nhe*I and *Bg*lII as well as to release the reporter gene *luc* which is 1.8 kb in size as shown in Fig. 4.8 The *lacZ* gene containing the Shine-Dalgarno sequence could therefore be easily cloned into pAL4000 containing *Nhe*I and *Bgl*II overhangs.

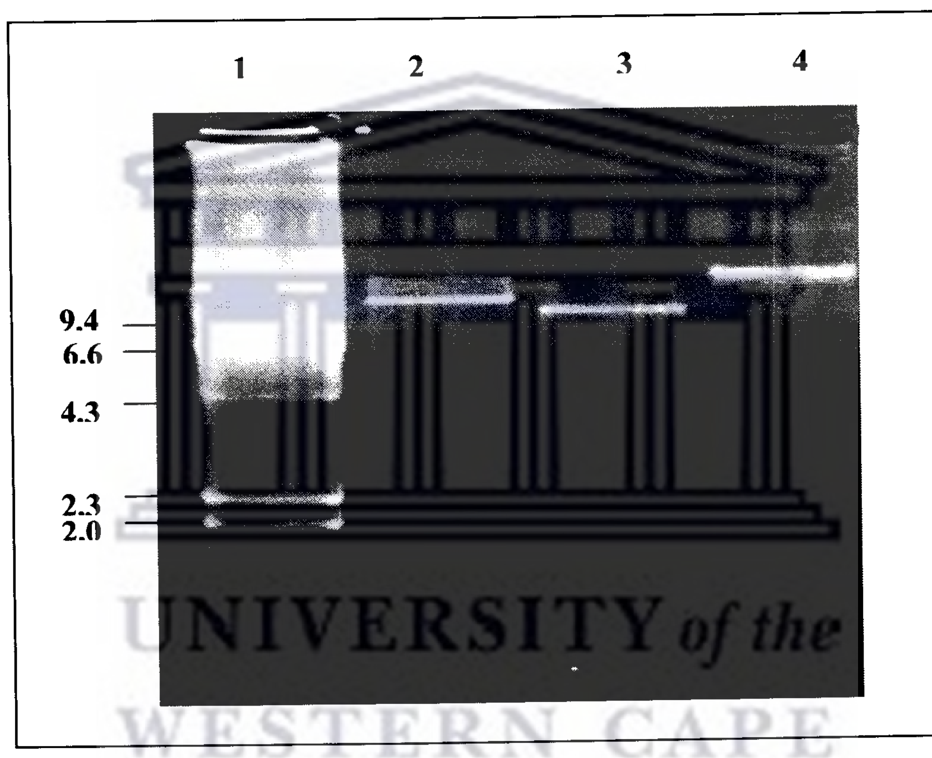


Fig.4.8. Restriction enzyme digest of pAL4000 as shown on a 0.8% agarose gel. Lane 1: MWM λ *Hind*III. Lane 2: digestion with *Bam*HI. Lane 3: digestion with *Nhe*I *and Bgl*II. Lane 4: pAL4000 uncut.

To confirm the insertion of the *lacZ* gene into pAL4000, the recombinant plasmid pALACZSD was digested with *Sac*I. Since one *Sac*I site is present on the *lacZ* gene and one on the vector, two DNA fragments were expected as shown in Fig. 4.9.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

By cloning the strong *lac*1promoter in the *Bam*HI site of pALACZSD the functionality of the reporter gene product could be tested. Cells of *E. coli* MC1061, *P. putida* and *P. aeruginosa* produced β-galactosidase when transformed with this pALACZSD promoter fusion in the presence of IPTG (inducer). No β-galactosidase activity was detected in the absence of inducer, or in cells transformed with either pAL4000 or pALACZSD. PALACZSD only produces an inherent basal level of β-galactosidase activity. It was therefore concluded that the reporter gene vector constructed (pALACZSD) was sensitive and reliable, only encoding for expression of β-galactosidase when an active promoter was found within the multi cloning site (MCS).

Fig. 4.9. Digestion of pALACZSD to confirm *lacZ*SD insert as shown on a 0.8 % agarose gel. Lane 1: MWM λ *Hind*III. Lane 2: uncut pALACZSD. Lane 3: pALACZSD cut with *Sac*I.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

4.3.2 Construction of *Pseudomonas* gene libraries

Promoters are segments of DNA to which RNA polymerase attach in order for transcription to take place (Glick and Pasternak, 1994). They are normally small in sequence size and contain a consensus –10 region and –35 region. To find heavy metal responsive promoters in *P. putida* and *P. aeruginosa*, a gene library of each organism was constructed using pALACZSD as vector. Isolated genomic DNA of *P. putida* and *P. aeruginosa* were both partially digested with *Sau3A* for 1-10min to determine what time interval gives fragments of size 100-1500 bp as shown in Fig. 4.11. After determining the exact time to obtain genomic DNA fragments in size range of 100-1500 bp, a high concentration of genomic DNA was digested with *Sau3A* for 5min as shown in Fig. 4.12.

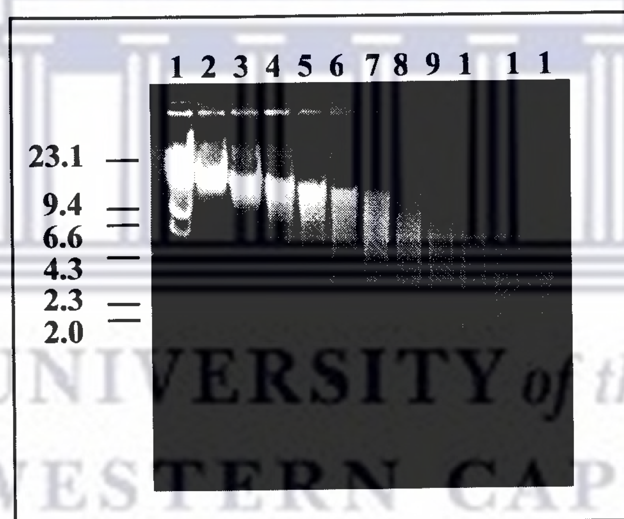


Fig. 4.10. Partial digestion profile of *P. putida* genomic DNA cut with *Sau*3A as shown on a 0.8 % agarose gel. Lane 1: λ *Hind*III MWM. Lane 2: Uncut *P. putida* genomic DNA. Lanes 3-12: *P. putida* genomic DNA digested with *Sau*3A for 1-10min, respectively.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

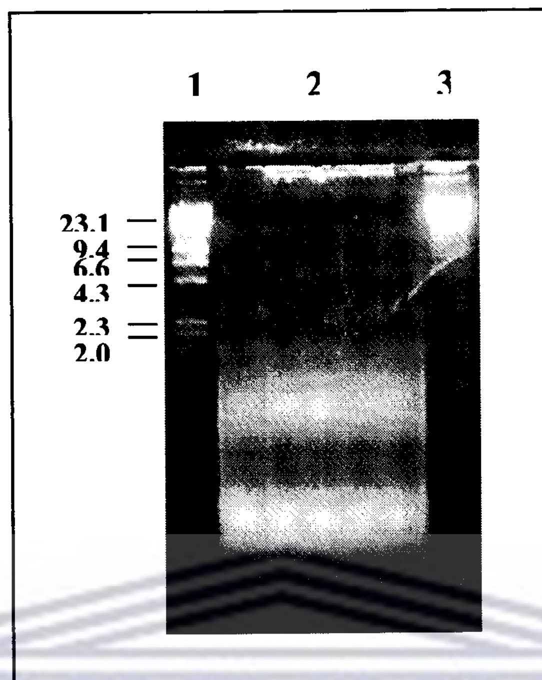


Fig. 4.11. Digestion of *P. putida* genomic DNA with *Sau*3A. Lane 1: λ *Hind*III MWM. Lane 2: *P. putida* genomic DNA digested with *Sau*3A for 5min. Lane 3: Uncut *P. putida* genomic DNA.

The genomic DNA was cloned into pALACZSD to obtain recombinant clones. To confirm the insertion of DNA fragments into the vector, twenty randomly selected clones were digested with *Sac*I as shown in Fig. 4.12. The digestion of the control plasmid pALACZSD with no insert, revealed two fragments of approximately 12kb and 1.8 kb. Digestion of the recombinants with *Sau*3A showed that the resulting small fragments were larger compared to the small (1.8 kb) fragment of pALACZSD. This thus confirmed the insertion of the genomic DNA fragments as shown in Fig. 4.12. The gene libraries of *P. putida* and *P. aeruginosa* were therefore successfully constructed using pALACZSD as vector system.

http://etd.uwc.ac.za/

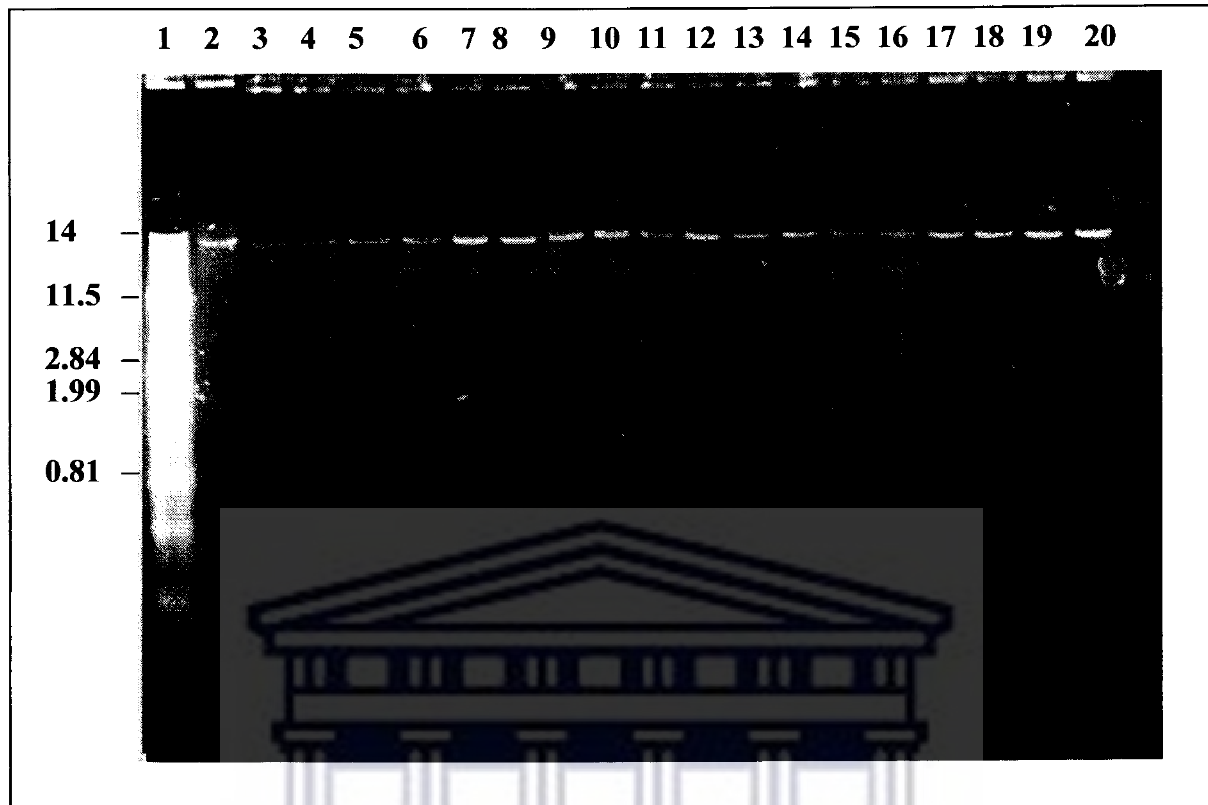


Fig. 4.12. Confirmation of insert in recombinant clones by digestion with *Sac*I as shown on a 0.8% agarose gel. Lane 1: λ *Pst*I MWM. Lane 2: pALACZSD digested with *Sac*I. Lanes 3-22: randomly selected recombinant clones digested with *Sac*I.

4.3.3 Transformation of gene libraries in *Pseudomonas* sp.

Transformation of bacteria with DNA is an important method in genetic analysis studies and many different types of transformation methods exist. For example, *E. coli* cells have a high transformation efficiency using the $CaCl_2$ method, whereas for *Bacillus subtilis*, the polyethylene glycol (PEG) method is preferred. In *P. aeruginosa* the $MgCl_2$ method was found to give efficient results (Iwasaki *et al.*, 1994).

Another successful method in transforming DNA from one host to another is triparental mating (Sokol, 1987; Greener *et al.*, 1992). This method relies on the presence of a helper

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

plasmid. In addition, electroporation has recently been used to transform various bacterial species. This method uses an electric current to generate membrane distortions, thereby allowing the uptake of DNA into the cell. Electroporation is a simple and easy method compared with chemically induced competence (e.g. the $CaCl_2$ method), and allows Gram-negative bacteria to be electrotransformed at high frequencies and efficiencies (Iwasaki *et al.*, 1994).

The gene libraries of *P. putida* and *P. aeruginosa* were transformed into their respective hosts by employing a number of the above-mentioned transformation methods. Firstly, gene libraries were transformed into *E. coli* MC1061 to allow genetic recombination i.e. the strain is $recA^+$ (Watson *et al.*, 1987). The sole reason for this is that the *Pseudomonas* sp. used in this study does not allow genetic recombination. $RecA^+$ is a protein found in most bacteria that is essential for DNA repair and DNA recombination (Glick and Pasternak, 1994). A high copy number of plasmids will be made in *E. coli* whereas in *P. putida* and *P. aeruginosa* the number will be low.

When employing the $MgCl_2$ method for transformation in *P aeruginosa*, isolated recombinant clones from *E. coli* were easily transformed into competent *P aeruginosa*. Although a thousand clones were transformed in this manner, the method only appeared applicable in small scale studies and not for use when screening genomic libraries. As the mechanisms of electrotransformation are not fully understood, in most cases of optimization of the procedure, for electrotransformation with bacteria is empirical. The method of electroporation was followed as set out by Iwasaki *et al.* (1994). *P. putida* competent cells in their mid-log phase were used as these cells are capable of taking up the recombinant plasmid pALACZSD when the bacterium is replicating. However a

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

number of problems were encountered when using this electroporation method as described by Iwasaki and co-workers (1994).

For example, recombinant DNA at low concentrations was not easily transformed. Furthermore, the large size of the plasmids (±13kb) could also have caused the low transformation efficiency. Montario and co-workers (1992) noted that the number of electrotransformants decreased exponentially with an increase in plasmid sizes (10.2-25kb). It is speculated that ligation products (gene libraries) could also not be transformed using electroporation because of the large recombinant clones. Therefore, electroporation as well as the $MgCl_2$ method, is not feasible for use in our gene library studies. Triparental mating proved to be the method of choice because high transformation efficiencies were obtained. Recombinant clones were transformed from *E. coli* with the aid of the helper plasmid, pRK2013, into *P. putida*. It is a single and easy method for transformation to study gene libraries in *Pseudomonas* species.

4.3.4 Screening of recombinant clones for heavy metal inducible promoters

When screening these recombinant clones for heavy metal inducible promoter activity using the microtitre plate method against Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, negligible β-galactosidase activity was determined for the isolated clones. The positive control (pALACZSDIP) showed high β-galactosidase activity due to the *lacI* promoter insert. PALACZSD used as the negative control showed a basal level of β-galactosidase activity as expected. Untransformed *Pseudomonas* cells were also included as controls to determine the effect the heavy metals have on them. The screening method was found to be very reliable due to various observations. The heavy metal concentration (50mg/L)

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

was found to optimal, i.e. not too toxic or too low, allowing normal cell synthesis to take place when exposed to the various heavy metals. A full spectrum of proteins as well as heavy metal responsive proteins were produced when exposed to heavy metals. The β-galactosidase activity was also found to be stable in the bacterial cell and could be easily determined.

According to Holloway (1996), the genome size of *Pseudomonas* is approximately 5.9×10^6 bp in size. The size of the genome relative to a single cloned fragment (n) would therefore be:

n = genome/fragment, therefore n= $5.9\times10^6/1000 = 5900$.

In order to determine the number of clones needed to be screened to cover the entire genome of a *Pseudomonas* species, the following formula can be used (Old and Primrose, 1994):

N = ln (1-P)/ln (1-1/n), where N = number of clones required and P = probability. At a 90 % confidence level, N = (ln (1-0.9)/ln (1-1/5900)

= -2.9957323/-0.0001695

=13 585 clones to cover the genome of the two *Pseudomonas* sp.

On average, 18 heavy metal responsive proteins were detected (includes induced and suppressed proteins), when *P. putida* was exposed to Cd^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} (Table 3.1). As it was assumed that 18 heavy metal regulatory elements should be responsible for the induction and suppression of these proteins, then at least one heavy

UNIVERSITY of the WESTERN CAPE

metal regulatory element should have been detected with the assay, when screening 755 clones. For *P. aeruginosa*, the same scenario was considered. As 14 heavy metal responsive proteins were produced, once again, at least one heavy metal regulatory element should have been detected when screening 970 clones. In total 1012 clones of *P. aeruginosa* were screened against the various heavy metals.

Table 4.2: Summary of clones of *P. aeruginosa* screened for heavy metal inducible promoters

Promoters	**Heavy metals**				
	Zn^{2+}	**Pb^{2+}**	**Cu^{2+}**	**Ni^{2+}**	**Cd^{2+}**
Constitutive					
weak	784	782	815	487	796
strong	130	132	99	67	83
Heavy metal inducible	0	0	0	0	0
no of clones	914	914	914	554	879

As shown in Table 4.2, the only promoters detected with the assay were those being weakly constitutive and those strongly constitutive. Heavy metals did not affect the activity of these promoters. No heavy metal inducible promoters were detected when the recombinant clones were exposed to heavy metals.

A possible reason for not detecting any heavy metal inducible promoter activity when exposing cells to heavy metals could be that the regulatory elements were erroneously overlooked, or that possible non-functional regulatory elements were initially cloned into

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

the vectors. In addition, it could also be that regulation occurs at the translational, and not transcriptional level, when cells are exposed to heavy metals. In translational control the most common type of regulation involves repression by a protein. The inhibitory effect could be attributable to direct blocking of the ribosomal binding site, or to the stabilization of an inhibitory mRNA secondary structure (MacCarthy and Gualerzi, 1990). Cases of translational activation have also been reported, but are in general less well understood. The most likely mode of action of positive effectors would be to stabilize inhibitory structures in the mRNA. RNase III seems to be able to activate this process via cleavage, but also via binding alone (Altuvia, 1987). Since no heavy metal inducible promoters could be detected, it seems possible that the presence of heavy metals could somehow eleviate the secondary structure of mRNA thereby allowing ribosome binding and the subsequent translation of stress proteins or metal responsive proteins.

The agar plate method did not prove feasible as a fast method to determine β-galactosidase activity, because the cells took 4 days to grow on media containing heavy metals. Colonies turning blue had to be closely monitored for interpreting the results because a high number of background colonies also turning blue developed afterwards. Fig. 4.13 shows color development of positive control pALACZSDIP on an agar plate supplemented with Cu^{2+}. The heavy metal had no effect on β-galactosidase activity.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

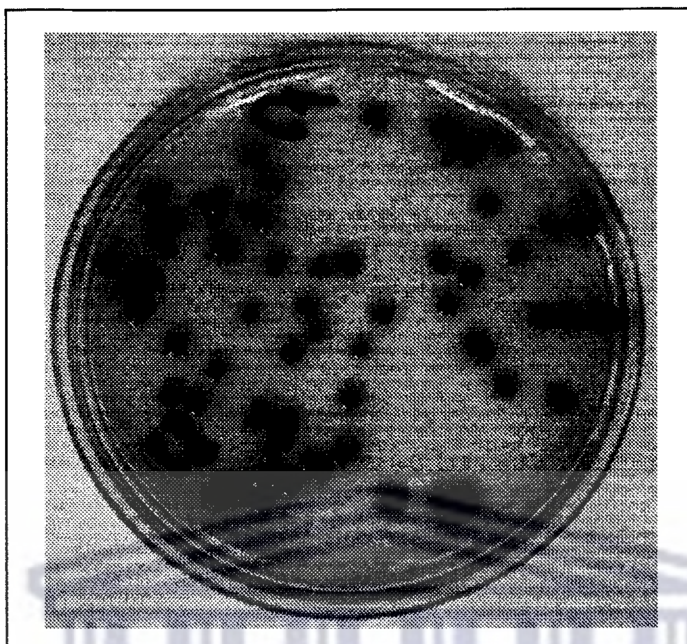


Fig. 4.13. Agar-plate of *P. putida* (pALACZSDIP) showing blue colonies for β-galactosidase activity.

UNIVERSITY of the
WESTERN CAPE

CHAPTER 5

CONCLUSIONS

Bacteria are constantly exposed to an ever-changing environment, where they either have to adapt to survive or to compete successfully. Exposure of the two *Pseudomonas* sp., *P. putida* and *P. aeruginosa,* to heavy metals showed that these organisms are capable of tolerating stressful conditions.

The data presented in this thesis, provides evidence that *P. putida* and *P. aeruginosa* can be used as test organisms to study the effect of heavy metals at the *de novo* synthesis level, as well as at the DNA molecular level. The production of heavy metal responsive proteins by *P. putida* and *P. aeruginosa* in response to heavy metals, is an indication of a possible tolerance or resistance mechanism used by these species to cope with heavy metal stress. In addition, these responses may be part of a global regulatory system that deals with toxic pollutants and other stresses. The gene library construction of the two *Pseudomonas* sp. and the subsequent transformation thereof into their respective wild type hosts, were successful. However, no heavy metal inducible promoters were detected when these gene libraries were screened against Cd^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}. The conclusion is therefore, that heavy metal inducible promoters might have been erroneously omitted during screening, or that the production of heavy metal responsive proteins is at the translational level.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

The identification of regulatory elements in *Pseudomonas aeruginosa* and *P. putida* responsive to specific heavy metals.

by

Nolan Africander

Promoter : Prof. V. S. Brözel

Co-promoter : Prof. W. H. van Zyl

Department : Microbiology

Degree : M. Sc. (Microbiology)

SUMMARY

Bacteria are constantly exposed to changes in the environment, whether it is climatic conditions or pollution. In order to survive or compete successfully they have to develop certain tolerance or resistance mechanisms. The aim of this study was therefore to evaluate the effect that heavy metals may have on *P. aeruginosa* and *P. putida,* as they have been shown to be present in heavy metal polluted environments. A further aim was to screen gene libraries of the two organisms, using a developed screening assay, to search for heavy metal inducible elements.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Initially, the Minimal Inhibitory Concentration (MIC) of heavy metals to *P. aeruginosa* and *P. putida*, were determined. The next step was to expose *P. putida* to various concentrations (1, 50, 100, 250 and 500mg/L) of Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}. Heavy metal responsive proteins were visualized on a 12.5% SDS-PAGE gel. The results indicated that most heavy metal responsive proteins were produced at a concentration of 50mg/L. Based on these results, *P. putida* and *P. aeruginosa* were further exposed to 50mg/L Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} over time intervals of 30 min, 60min and 120 min in order to determine at what time most of the proteins were produced.

A promoterless broad-host-range vector pALACZSD, which was based on the molecular features of pAL4000, was constructed using various DNA molecular manipulations. This vector was subsequently used in the construction of gene libraries of *P. aeruginosa* and *P. putida*. The libraries were initially transformed into *E. coli* MC1061, and then into their respective wild type hosts. These gene libraries were subsequently screened for heavy metal inducible promoters, using a developed screening assay, by exposing them to 50mg/L of Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}.

UNIVERSITY of the
WESTERN CAPE

The MIC values for *P. aeruginosa* and *P. putida* when exposed to these heavy metals were high, indicating that *P. aeruginosa* and *P. putida* were capable of tolerating high concentrations of heavy metals. Exposure of *P. putida* to various heavy metals concentrations produced on average about 20 heavy metal responsive proteins. Difficulties were experienced when trying to verify SDS-PAGE results with two-dimensional PAGE gels. The reason for not using two-dimensional PAGE gels is that

http://etd.uwc.ac.za/

they are difficult to perform. At a concentration of 50mg/L, *P. putida* and *P. aeruginosa* produced approximately 18 and 14 heavy metal responsive proteins respectively. Construction of vector pALACZSD, the subsequent use thereof in gene library construction of *P. putida* and *P. aeruginosa*, transformation in *E. coli* MC1061 and final transformation into their respective hosts were all successful. Screening of the two *Pseudomonas* gene libraries against 50mg/L of Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+}, yielded no heavy metal inducible promoters. However, weak and some constitutive promoters were detected.

It was therefore concluded that heavy metals such as Cd^{2+}, Cu^{2+}, Co^{2+}, Ni^{2+}, Pb^{2+} and Zn^{2+} are able to induce heavy metal responsive proteins in *P. putida* and *P. aeruginosa.* Furthermore, no heavy metal inducible promoters were evident when screening at a molecular level. It is speculated that certain heavy metal responsive elements driving heavy metal responsive protein production, might be controlled at a translational level.

http://etd.uwc.ac.za/

REFERENCES

Aldea, M., T. Garrido, and A. Tormo. 1993. Gearbox gene expression and growth rate. World Journal of Microbiology and Biotechnology. **9:** 414-420.

Altuvia, S., H. Locker-Giladi, S. Koby, O. Ben-Nun, and A. B. Oppenheim. 1987. RNase III stimulates the translation of the *cIII* gene of bacteriophage λ. Proc. Natl. Acad. Sci. USA. **84:** 6511-6515.

Arraiano, C. M. 1993. Post-transcriptional control of gene expression: bacterial mRNA degradation. World J. Microbiol. Biotech. **9:** 421-432.

Atlas, R. M., G. Sayler, R. S. Burlage, and A. K. Bej. 1992. Molecular approaches for environmental monitoring of microorganisms. Biotechniques. **12:** 706-716.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. 1994. Current protocols in molecular biology, John Wiley and Sons, Inc., USA.

Babich, H., and G. Stotzky. 1980. Environmental factors that influence the toxicity of heavy metal and gaseous pollutants to microorganisms. Crit. Rev. Microbiol. **8:** 99-145.

http://etd.uwc.ac.za/

Barraquio, W. L., and R. Knowles. 1989. Beneficial effects of nickel on *Pseudomonas saccharophila* under nitrogen-limited chemolithotrophic conditions. Appl. Environ. Microbiol. **55:** 3197-3201.

Benson, S. A. 1984. A rapid procedure of isolation of DNA fragments from agarose gels. Bio-Techniques. **2:** 66-67.

Berka, T., A. Shartzman, J. Zimmerman, J. Strickler, and M. Rosenburg. 1988. Efficient expression of the yeast metallothionein gene in *E. coli*. J. Bacteriol. **170:** 21-26.

Beveridge, T. 1988. The bacterial surface: general considerations towards design and function. Can. J. Microbiol. **34:** 363-372.

Bhattacharya, S. K., R. L. Madura, V. Uberoi and M. R. Hoghighi-Poden. 1995. Toxic effects of cadmium on methanogenic system. Water Res. **29:** 2339-2345.

Bird, N. P., J. G. Chambers,R. W. Leech, and D. Cummins. 1985. A note on the use of metal species in microbiological tests involving growth media. J. Appl. Bacteriol. **59:** 353-355.

Bitton, G., B. Koopman, and O. Agami. 1992. MetPAD^{TM}: a bioassay for rapid assessment of heavy metal toxicity in wastewater. Water Environ. Res. **64:** 834-836.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Blom, A., W. Harder, and A. Matin. 1992. Unique and overlapping pollutant stress proteins of *E.coli*. Appl. Environ. Microbiol. **58:** 331-334.

Brawn, M. K., and I. Fridovich. 1985. Increased superoxide radical production evokes inducible DNA repair in *E. coli*. J. Bacteriol. **260:** 922-925.

Brown, T. A. 1990. Gene Cloning: An introduction. 2nd ed. Chapman and Hall, London.

Bux, F., F. M. Swalaha and H. C. Kasan. 1995. Assessment of acids as desorbents of metal ions bound to sludge surfaces. Water SA. **21:** 319-324.

Burkhardt, C., H. Insam, T. C. Hutchinson and H. H. Reber. 1993. Impact of heavy metals on the degradative abilities of soil bacterial communities. Biol. Fertil. Soils. **16:** 154-156.

Capone, D. G., D. D. Reeses, and R. P. Kiene. 1983. Effects of metals on methanogenesis, sulfate reduction, carbon dioxide evolution, and microbial biomass in anionic saltmarsh sediments. Appl. Environ. Microbiol. **45:** 1586-1591.

Cha, J. S., and D. A. Cooksey. 1991. Copper resistance in *Pseudomonas syringae* mediated by periplasmic and outer membrane proteins. Proc. Natl. Acad. Sci USA. **88:** 8915-8919.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Chakrabarty, A. M., J. R. Myloni, D. A. Friello, and J. G. Vacca. 1975. Transformation of *Pseudomonas putida* and *Escherichia coli* with plasmid-linked drug resistance factor DNA. Proc. Natl. Acad. Sci. USA. **72:** 3647-3651.

Chang, F. H., and F. E. Broadbent. 1981. Influence of trace metals on carbon dioxide evolution from a Yolo soil. Soil. Sci. **132:** 1316-1323.

Chang, J. S., and J. Hong. 1994. Biosorptin of mercury by the inactivated cells of *Pseudomonas aeruginosa* PU21 (Rip64). Biotechnol. Bioeng. **44:** 999-1006.

Chen, S., and D. B. Wilson. 1997. Construction and characterization of *E. coli* genetically engineered for bioremediation of Hg^{2+}-contaminated environments. Appl. Environ. Microbiol. **63:** 2442-2445.

Collins, Y. E., and G. Stotzky. 1992. Heavy metals alter the electrokinetic properties of bacteria, yeasts, and clay minerals. Appl. Environ. Microbiol. **58:** 1592-1600.

Costerton, J. W., Z. Lewandowski, D. E. Caldwell, D. R. Korber, and H. M. Lappin-Scott. 1995. Microbial biofilms. Annu. Rev. Microbiol. **49:** 711-745.

Dean-Ross, D. 1991. Effects of combined treatments of zinc and pH on attached bacterial abundance and activity. Can. J. Microbiol. **37:** 491-496.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Doelman, P., and L. Haanstra. 1979a. Effect of lead on respiration and dehydrogenase activity. Soil. Biol. Biochem. **11:** 475-479.

Dorman, C. J. 1995. DNA topology and the global control of bacterial gene expression: implications for the regulation of virulence gene expression. Microbiol. **141:** 1271-1280.

Dorman, C. J. 1996. Flexible response: DNA supercoiling, transcription and bacterial adaptation to environmental stress. Trends in Microbiol. **4:** 214-216.

Dubnau, D. 1984. Translational attenuation: The regulation of bacterial resistance to the macrolide-lincosamide-streptogramin B antibiotics. CRC Crit. Rev. Biochem. **16:** 103-132.

Dukan, S., S. Dadon, D. R. Smulski, and S. Belkin. 1996. Hypochlorous acid activates the heat shock and soxRS systems of *E. coli*. Appl. Environ. Microbiol. **62:** 4003-4008.

Duxbury, T. 1985. Ecological aspects of heavy metal responses in microorganisms. Adv. Microbiol. Ecol. **8:** 185-235.

Ebbing, D. D. 1987. General Chemistry. 2nd edition. Houghton Mifflin Company, Boston.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Endo, G., and S. Silver. 1995. CadC, the transcriptional regulatory protein of the cadmium resistance system of *Staphylococcus aureus* plasmid pI258. J. Bacteriol. **177:** 4437-4441.

Engebrecht, B. D., M. Simon, and M. Silverman. 1985. Measuring gene expression with light. Science. **227:** 1345-1347.

Ferris, F. G., S. Schultze, T. C. Wilten, W. S. Fyfe and T. J. Beveridge. 1989. Metal interactions with Microbial Biofilms in Acidic and Neutral pH Environments. Appl. Environ. Microbiol. **55:** 1249-1257.

Freedman, B., and T. C. Hutchinson. 1980a. Effects of smelter pollution on forest leak litter decomposition near a nickel-copper smelter at Sadbury, Ontario. Can. J. Biol. **58:** 1722-1736.

Gadd, G. M., and A. J. Griffiths. 1978. Microorganisms and Heavy Metal Toxicity. Microbial Ecology. **4:** 303-317.

Gadd, G. M., and C. White. 1993. Microbial treatment of metal pollution in a working biotechnology? Trend. Biotechnol. **11:** 353-359.

Galvin, M. R. 1996. Occurrence of metals in waters: An overview. Water SA. **22:** 7-18.

UNIVERSITY of the
WESTERN CAPE

Gawel, J. E., B. A. Ahner, A. J. Fiedland, and F. M. M. Morel. 1996. Role for heavy metals in forest decline indicated by phytochelatin measurements. Nature **381:** 64-65.

Glick, B. R., and J. J. Pasternak. 1994. Molecular Biotechnology: Principles and applications of recombinant DNA. American Society for Microbiology Press, Washington DC.

Goff, S. A., and A. L. Goldberg. 1985. Production of abnormal proteins in *E. coli* stimulates transcription of *lon* and other heat shock genes. Cell. **41:** 587-595.

Gold, L. 1988. Posttranscriptional regulatory mechanisms in *Escherichia coli*. Annu. Rev. Biochem. **57:** 199-233.

Greener, A. S., M. Lehman, and D. R. Helinski. 1992. Promoters of the broad host range plasmid RK2: Analysis of transcription (initiation) in five species of Gram-negative bacteria. Genetics **130:** 27-36.

Groat, R. G., J. E. Schultz, E. Zychlinsky, A. Bockman, and A. Matin. 1986. Starvation proteins in *E. coli* : Kinetics of synthesis and role in starvation survival. J. Bacteriol. **168:** 486-493.

UNIVERSITY of the
WESTERN CAPE

Grossman, A. D., W. E. Taylor, Z. F. Burton, R. R. Burgess, and C. A. Gross. 1985. Stringent response in *Escherichia coli* induces expression of heat shock proteins. J. Mol. Biol. **186:** 357-365.

Hamer, D. H. 1986. Metallothionines. Annu. Rev. Biochem. **55:** 913-951.

Hickey, R. F., L. Vanderwielen, and M. S. Switzenbaum. 1989. The effects of heavy metals on methane production and hydrogen and carbon monoxide levels during batch anaerobic sludge digestion. Wat. Res. **23:** 207

Hoch, J. A., and T. J. Silhavy. 1995. Two-component signal transduction. Washington, DC. American Society for Microbiology Press.

Hochuli, E., H. Dobeli, and A. Schacher. 1987. New metal chelate adsorbents selective for proteins and peptide containing neighboring histidine residues. J. Chromatography **411:** 177-184.

Holloway, B. W. 1996. *Pseudomonas* Genetics and Taxonomy *In* Nakazawa, T., Furukawa, K., Haas, D and S. Silver (ed) Molecular Biology of *Pseudomonas*. American Society for Microbiology, Washington, DC.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Horn, J. M., M. Brunke, W. D. Deckwer, and K. N. Timmis. 1994. *Pseudomonas putida* strains which constitutively overexpress mercury resistance for biodetoxification of organomercurial pollutants. Appl. Environ. Microbiol. **60:** 357-362.

Howlett, N. G. and S. V. Avery. 1997. Induction of Lipid Peroxidation during Heavy Metal stress in *Saccaromyces cerevisiae* and influence of plasma membrane fatty acid unsaturation. Appl. Environ. Microbiol. **63:** 2971-2976.

Inbar, O., and E. Z. Ron. 1993. Induction of cadmium tolerance in *E. coli* K12. FEMS Microbiology letters **113:** 197-200.

Iwasaki, K., H. Uchiyana, O. Yagi, T. Kurabayaski, K. Ishizuka, and Y. Takamura. 1994. Transformation of *Pseudomonas putida* by electroporation. Biosci. Biotech. Biochem. **58:** 851-854.

James, S. G., C. Holmström, and S. Kjelleberg. 1996. Purification and characterization of a novel antibacterial protein from the marine bacterium D2. Appl. Environ. Microbiol. **62:** 2783-2788.

Jernelöv, A., and A. Martin. 1975. Ecological implications of metal metabolism by microorganisms. Annu. Rev. Microbiol. **29:** 61-77.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Jenkins, D. E., J. E. Schultz, and A. Matin. 1988. Starvation-induced cross protection against heat or H_2O_2 challenge in *E. coli*. Appl. Environ. Microbiol. **170:** 3910-3914.

Jones, J. G. B. M. Simon, and S. Gardner. 1982. Factors affecting methanogenesis and associated anaerobic processes in the sediments of a stratified eutrophic lake. J. Gen. Microbiol. **128:** 1-11.

Karamushka, V. I., T. G. Bruzina, and Z. R. Ul'berg. 1995. Accumulation of Gold (III) by the cells of Cyanobacterium *Spirulina platensis*. Microbiol. **60:** 157-160.

Keasling, J. D., and G. A. Hupf. 1996. Genetic manipulation of polyphosphate metabolism affects cadmium tolerance in *E. coli*. Appl. Environ. Microbiol. **62:** 743-746.

Kennish, M. J. 1992. Ecology of estuaries: Anthropogenic effects. CRC Press Inc., Boca Raton. FL. (USA).

Kern, U., and B. Westrich. 1995. Sediment contamination by heavy metal n a Lock-regulated section of the river Necktar. Mar. Freshwater Res. **46:** 101-106.

Kidambi, S. P., G. W. Sundin, D. A. Palmer, A. M. Chakrabarty, and C. L. Bender. 1995. Copper as a signal for Alginate synthesis in Pseudomonas syringae pv. Syringae. Appl. Environ. Microbiol. **61:** 2171-2170.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Koncz, C., W. H. R. Langridge, O. Olsson, J. Schell, and A. A. Szalay. 1990. Bacterial and firefly luciferase genes in transgenic plants: Advantages and disadvantages of a reporter gene. Develop. Genet. **11:** 224-232.

Kreuger, J. H., and G. C. Walker. 1984. *groEL* and *dnaK* genes of *Escherichia coli* are induced by UV irradiation and nalidixic acid in an htpR$^+$-dependent fashion. Proc. Natl. Acad. Sci. USA **81:** 1499-1503.

Kurek, E., J. Czaban, and J. M. Bollag. 1982. Sorption of cadmium by microorganisms in competition with other soil constituents. Appl. Environ. Microbiol. **43:** 749-752.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:** 680-685.

LaPaglia, C., and P. L. Hartzell. 1997. Stress-induced Production of Biofilm in the hyperthermophile *Archaeglobus fulgidus*. Appl. Environ. Microbiol. **63:** 3158-3163.

Lebrun, M., A. Audurier, and P. Cossart. 1994. Plasmid-borne cadmium resistance genes in *Listeria monocytogenes* are present on Tn5422, a novel transposon closely related to Tn917. J. Bacteriol. **176:** 3049-3061.

Lin, C., and B. Olsen. 1995. Occurrence of *cop*-like copper resistance genes among bacteria isolated from a water distribution system. Can. J. Microbiol. **41:** 642-646.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Lindlow, S. E. 1995. The use of reporter genes in the study of microbial ecology. Mol. Ecol. **4:** 555-566.

Lovely, D. R., and J. D. Coates. 1997. Bioremediation of metal contamination. Curr. Opin. Biotechnol. **8:** 285-289.

Lupi, C. G., T. Colangelo, and C. A. Mason. 1995. Two-dimensional gel electrophoresis analysis of the response of *Pseudomonas putida* KT 2442 to 2-chlorophenol. Appl. Environ. Microbiol. **61:** 2863-2872.

Macaskie, L. E. 1995. Copper tolerance, phosphate activity and copper uptake by a heavy metal accumulating *Citrobacter* species. Microbios. **84:** 137-153.

Mager, W. H., and A. J. J. de Kuijff. 1995. Stress-induced transcriptional activation. Microbiol Rev. **59:** 506-531.

McCarthy, J. E. G., and C. Gualerzi. 1990. Transcriptional control of prokaryotic gene expression. Trend. Genetic. **6:** 78-85.

Miller, J. H. 1972. Experiments in Molecular Biology, Cold Spring Harbor Press, Cold Spring Harbor, New York.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Niedhardt, F. C., R. A. VanBogelen, and V. Vaughn. 1984. The genetics and regulation of heat-shock proteins. Annu. Rev. Genet. **18:** 295-329.

Nies, D. H. 1992. CzcR and CzcD gene products affecting regulation of cobalt, zinc and cadmium resistance (*czc*) in *Alcaligenes eutrophus*. J. Bacteriol. **174:** 8102-8110.

Nies, D. H. 1995. The cobalt, zinc and cadmium efflux system czcABC from *Alcaligenes eutrophus* functions as a cation-proton antiporter in *E. coli*. J. Bacteriol. **177:** 2707-2712.

Nies, D. H., M. Mergeay, B. Friedrich, and H. G. Schlegel. 1987. Cloning of plasmid genes encoding resistance to chromate and cobalt in *Alcaligenes eutrophus* CH34. J. Bacteriol. **169:** 4865-4868.

Nies, D. H., A. Nies, L. Cha, and S. Silver. 1989b. Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA. **86:** 7351-7355.

Nies, D. H., and S. Silver. 1989. Plasmid determined inducible efflux is responsible for resistance to cadmium, zinc and cobalt in *Alcaligenes eutrophus*. J. Bacteriol. **171:** 896-900.

UNIVERSITY of the
WESTERN CAPE

Nieto, J. J., R. Fernandez-Castillo, M. C. Marquez, A. Ventosa, E. Quesada, and F. Ruiz-Berraquero. 1989. Survey of metal tolerance in moderately halophilic eubacteria. Appl. Environ. Microbiol. **55:** 2385-2390.

Nilsson, G., J. G. Belasco, S. . Cohen, and A. Von Gabain. 1984. Growth-rate dependent regulation of mRNA stability in *Escherichia coli*. Nature **312:** 75-77.

Nriagu, J. O., and J. M. Pacyna. 1988. Quantitative assessment of worldwide contamination of air, water and soils by trace metals. Nature **333:** 134-139.

Nriagu, J. O. 1996. A history of global heavy metal pollution. Science **272:** 223-224.

Old, R. W., and S. B. Primrose. 1994. Principles of Gene Manipulation. Fifth edition. Blackwell Scientific Publications. Oxford.

Olsen, R. H., de Busscher, G., and W. R. McCombie. 1982. Development of broad host-range vectors and gene banks: self cloning of the *Pseudomonas aeruginosa* POA chromosome. J. Bacteriol. **150:** 60-69.

Paton, G. I., C. D. Campbell, L. A. Glover, and K. Killham. 1995. Assessment of bioavailability of heavy metals using *lux* modified constructs of *Pseudomonas flourescens*. Let. Appl. Microbiol. **20:** 52-56.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Pacheco, S. V., R. Miranda, and C. Cervantes. 1995. Inorganic-iron resistance by bacteria isolated from a Mexico City freeway. Antonie van Leeuwenhoek **67:** 333-337.

Pines, O., and M. Inouye. 1986. Antisense RNA regulation in prokaryotes. Trends Genet. **2:** 284-287.

Pizarro, J., E. Jedlicki, O. Orellana, J. Ramero, and R. T. Espejo. 1996. Bacterial populations in samples of bioleached copper ore as revealed by analysis of DNA obtained before and after cultivation. Appl. Environ. Microbiol. **62:** 1323-1328.

Pettersson, A., L. Kunst, B. Bergman, and G. M. Roomans. 1985. Accumulation of aluminum by Anabaena cylindrical into polyphosphate granules and cell walls: an X-ray energy dispersive microanalysis study. J. Gen. Microbiol. **131:** 2544-2548.

Pleij, C. W. A. 1994. RNA pseudoknots. Curr. Opin. Struct. Biol. **4:** 337-344.

Podlesek, Z., B. Herzog, and J. Ambrozic. 1993. Inducible resistance zinc ions in *Bacillus subtilis* 168. FEMS Microbiol. Lett.**113:** 201-204.

Portier, C., and M. Grunberg-Manago. 1993. Regulation of ribosomal mRNA translation in bacteria-The case of S15. *In* Translational regulation of gene expression (ed. J. Ilan), **2:** 23-47. Plenum Press, New York.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Prescot, L. M., J. P. Harley, and D. A. Klein. 1990. *In* Microbiology. Wm. C. Brown Publishers. USA. pages 126-127.

Ramos, J. L., and S. Marqués. 1993. Gene expression in *Pseudomonas.* World J. Microbiol. Biotech. **9:** 433-443.

Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. Molecular Cloning: A Laboratory Manual. 2nd ed. Cold Spring Harbor Laboratory Press. Cold Spring Harbor, New York.

Schippers, B., A. W. Bakker, and P. A. H. M. Bakker. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. **25:** 339-358.

Schmidt, T. M., E. F. DeLong, and N. R. Pace. 1991. Analysis of a marine picoplankton community by 16S rRNA gene cloning and sequencing. J. Bacteriol. **173:** 4371-4378.

UNIVERSITY of the WESTERN CAPE

Silver, S., G. Nucifora, L. Chu, and T. K. Misra. 1989. Bacterial resistance ATPases: primary pumps for exporting toxic cations and anions. Trends Biochem. Sci. **14:** 76-80.

Silver, S., A. M. Chakrabarty, B. Iglewski, and S. Kaplan. (eds.) 1990. *Pseudomonas:* Biotransformation, Pathogenesis, and Evolving Biotechnology. Washington DC: American Society for Microbiology.

http://etd.uwc.ac.za/

Simons, R. W. 1988. Naturally occurring antisense RNA control-A brief review. Gene **72:** 35-44.

Slauch, J. M., and T. J. Silhavy. 1991. Genetic fusions as experimental tools. Meth. Enzymol. **204:** 213-248.

Sokol, P. M. 1987. Tn5 insertion mutants of *Pseudomonas aeruginosa* deficient in surface expression of ferripyochelin-binding protein. J. Bacteriol. **169:** 3365-3368.

Sousa, C., A. Cebolla, and V. de Lorenzo. 1996. Enhanced metallo adsorption of bacterial cells displaying poly-His peptides. Nature Biotech. **14:** 1017-1020.

Stichter, P., M. C. M. Jaspers, K. Stemmler, H. Harms, A. J. B. Zehnder, and J. R. van der Meer. 1997. Development and characterization of a whole-cell bioluminescent sensor for bioavailable middlechain alkanes in contaminated groundwater samples. Appl. Environ. Microbiol. **63:** 4053-4060.

Stock, J. B., A. J. Ninfa, and A. M. Stock. 1989. Protein phosphorylation and regulation of adaptive responses in bacteria. Microbiol. Rev. **53:** 450-490.

Storz, G., L. A. Tarkaglia, S. B. Farr, and B. N. Arnes. 1990. Bacterial Defenses against oxidative stress. Trend. Genet. **6:** 363-368.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

ten Dam, E., K. Pleij, and D. Draper. 1992. Structural and functional aspects of RNA pseudoknots. Biochem. **31:** 11665-11676.

Unz, R. F., and K. L. Shuttleworth. 1996. Microbial mobilization and immobilization of heavy metals. Curr. Opin. Biotech. **7:** 307-310.

VanBogelen, R. A., P. M. Kelly, and F. C. Niedhardt. 1987. Differential induction of heat shock, SOS, and oxidative stress regulons and accumulation of nucleotides in *Escherichia coli*. J. Bacteriol. **169:** 26-32.

van der Lelie, D., T. Schwuchow, U. Schwidetzky, S. Wuertz, W. Baeyens, M. Mergeay, and D. H. Nies. 1997. Two-component regulatory system involved in transcriptional control of heavy metal homeostasis in *Alcaligenes eutrophus*. Mol. Microbiol. **23:** 493-503.

van Dyk, T. K., W. R. Majarian, K. B. Konstantinov, R. M. Young, P. S. Dhurjati, and R. A. LaRossa. 1994. Rapid and sensitive pollutant detection by induction of heat shock gene-bioluminescence gene fusions. Appl. Environ. Microbiol. **60:** 1414-1420.

Volesky, B. 1987. Biosorbents for metal recovery. Trend. Biotechnol. **5:** 96-101.

UNIVERSITY of the
WESTERN CAPE

http://etd.uwc.ac.za/

Voloudakis, A. E., C. L. Bender, and D. A. Cooksey. 1993. Similarity of between Copper resistance genes from *Xanthomonas campestris* and *Pseudomonas syringae*. Appl. Environ. Microbiol. **59:** 1627-1634.

Vogel, H. J., and D. M. Bonner. 1956. Acetyl-ornithase of *Escherichia coli* – partial purification and some properties. J. Biol. Chem. **218:** 97-106.

Voorma, H. O. 1996. Control of translation in prokaryotes. *In* Hershey, J. W. B., M. B. Mathews, and N. Sonenberg. 1996. Translational control. Cold Spring Harbor Laboratory Press. Cold Spring Harbor. N. Y.

Walker, S. G., C. A. Flemming, F. G. Ferris, T. J. Beveridge, and G. W. Bailey. 1989. Physicochemical interaction of *Escherichia coli cell* envelopes and *Bacillus subtilis* cell walls with two clays and the ability of the composite to immobilize heavy metals from solution. Appl. Environ. Microbiol. **55:** 2976-2984.

UNIVERSITY of the
WESTERN CAPE

Wang, C. L., P. C. Michels, S. C. Dawson, S. Kitisakkul, J. A. Barros, J. D. Keasling, and D. S. Clarke. 1997. Cadmium removal by a new strain of *Pseudomonas aeruginosa* in aerobic culture. Appl. Environ. Microbiol. **63:** 4075-4078.

Ward, D. M., M. M. Bateson, R. Weller, and A. L. Ruff-Roberts. 1992. Ribosomal RNA analysis of microorganisms as they occur in nature. Adv. Microb. Ecol. **12:** 219-286.

http://etd.uwc.ac.za/

Ward, D. M. , R. Weller, and M. M. Bateson. 1990. 16S rRNA sequences reveal numerous uncultured microorganisms in a natural community. Nature (London) **345:** 63-65.

Watson, J. D., N. H. Hopkins, J. W. Roberts, J. A. Steitz, and A. M. Weiner. 1987. Molecular biology of the gene. Vol 1. 4th ed. The Benjamin/Cummings Publishing Company, California.

Weissenhorn, I., C. Leyval, and J. Berthelin. 1995. Bioavailability of heavy metals and abundance of abuscular mycorrhiza in a soil polluted by atmospheric deposition from a smelter. Biol. Fertil. Soils. **19:** 22-28.

Wong, P. K., and C. M. So. 1993. Copper accumulation by a strain of *Pseudomonas putida*. Microbios. **73:** 113-121.

Wong, P. K., K. C. Lam, and C. M. So. 1993. Removal and recovery of Cu (II) from industrial effluent by immobilized cells of *Pseudomonas putida*II-11. Appl. Microbiol. Biotechnol. **39:** 127-131.

Wood, J. M. 1974. Biological cycles for toxic elements in the environment. Science **183:** 1049-1052.

UNIVERSITY of the WESTERN CAPE

http://etd.uwc.ac.za/

Yura, T., H. Nagai, and H. Mori. 1993. Regulation of the heat-shock response in bacteria. Annu. Rev. Microbiol. **47:** 321-350.

http://etd.uwc.ac.za/