

**IDENTIFICATION AND CHARACTERISATION OF
BACTERIAL GENES ASSOCIATED WITH
RESISTANCE TO AND/OR DEGRADATION OF
ENVIRONMENTAL POLLUTANTS**

A thesis submitted for the degree of

DOCTOR OF PHILOSOPHY

By

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DECLARATION

“I, Belinda Davis, declare that the PhD thesis entitled Identification and Characterisation of Bacterial Genes Associated with Resistance to and/or Degradation of Environmental Pollutants is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work”.

Signature

Date

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PUBLICATIONS

Ng SP, Davis B, Palombo E and Bhave M (2009) A Tn5051-like *mer*-containing transposon identified in a heavy metal tolerant strain *Achromobacter* sp. AO22 isolated from an industrial site in Australia. BMC Research Notes. 2:38.

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ABBREVIATIONS

<	Less than
>	Greater than
≥	Greater than or equal to
I	One
II	Two
+	Plus
-	Minus
#	Number
α	Alpha
β	Beta
λ	Lambda
σ	Sigma
°C	Degrees Celsius
®	Registered trademark
μg	Microgram
μg/kg	Micrograms per kilogram
μg/L	Micrograms per liter
μg/mL	Micrograms per milliliter
μg/μL	Micrograms per microliter
μL	Microliter
μM	Micromole
%	Per cent
A	Adenine
A	Absorbance
Ag ⁺	Silver
Ala	Alanine
Amp	Ampicillin
ANGIS	Australian National Genomic Information Service
Asn	Asparagine
AsO ₂ ⁻	Arsenite
AsO ₄ ³⁻	Arsenate
Asp	Aspartic acid
ATPase	Adenosine triphosphatase
ATSDR	Agency for Toxic Substances and Disease Registry
bp	Base pairs
BSM	Basal salt medium
C	Cytosine
Cd(II)	Cadmium
CdCl ₂	Cadmium chloride
cDNA	Complementary DNA
C-Hg	Carbon-Mercury
Co(II)	Cobalt
Co(NO ₃) ₂	Cobalt nitrate
CO ₂	Carbon dioxide
CrO ₄ ²⁻	Chromium oxide
C-terminal	Carboxy-terminal

Cu(II)	Copper
Cys	Cysteine
dCTP	Deoxycytosine triphosphate
dH ₂ O	Distilled water
dNTP	Deoxynucleotide triphosphate
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EPS	Extracellular polymeric substance
G	Guanine
g/L	Grams per liter
GI	Gastrointestinal
Glu	Glutamic acid
Gly	Glycine
GSH	Glutathione
Hg(0)	Mercury
Hg(II)	Mercury
HgCl ₂	Mercuric chloride
HgX ₂	Mercury compound
HMW	High molecular weight
i.e.	For example
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobase
kDa	Kilo daltons
L	Liter
LB	Luria broth
LMW	Low molecular weight
M	Molar
Met	Methionine
MgCl ₂	Magnesium chloride
mg/L	Milligrams per liter
mg/mL	Milligrams per milliliter
MIC	Minimum Inhibitory Concentration
mL	Milliliter
mM	Millimole
MMC	Methylmercury chloride
NA	Not applicable
NAT	Nutrient agar plus 0.05 mM Tris pH 7.5
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NaOH	Sodium hydroxide
NBT	Nutrient broth plus 0.05 mM Tris pH 7.5
NCBI	National Center for Biotechnology Information
ng	Nanogram
Ni(II)	Nickel
nm	Nanometers
nM	Nanomole
NMR	Nuclear magnetic resonance
N-terminal	Amino terminal

OD	Optical density
OP	Operator/Promoter
P	Phosphorous
PAH	Polycyclic Aromatic Hydrocarbon
Pb(II)	Lead
Pb(NO ₃) ₂	Lead nitrate
PCR	Polymerase chain reaction
Phe	Phenylalanine
PMA	Phenylmercury acetate
pmol	Picomole
ppm	Parts per million
Pro	Proline
PYR	Pyrene
RNA	Ribonucleic Acid
rpm	Revolutions per minute
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
Sb(III)	Antimony
SDS	Sodium dodecyl sulfate
Ser	Serine
sp.	Species
SSC	Sodium citrate buffer
T	Thymidine
TAE	Tris-Acetate-EDTA
TE	Tris-EDTA
TeO ₃ ²⁻	Tellurite
Thr	Threonine
TM	Trademark
Tris	Tris hydroxymethylaminoethane
Tyr	Tyrosine
URF	Untranslated reading frame
U/μL	Units per microliter
U	Units
UV	Ultra violet
V	Volts
VU	Victoria University
W	Watts
X	Times
X-gal	5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside
Zn(II)	Zinc
Zn(NO ₃) ₂	Zinc nitrate
ZnS	Zinc sulfide

SUMMARY

Bacteria were previously isolated from two separate sites, one contaminated with lead and the other with Polycyclic Aromatic Hydrocarbons (PAHs). *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 were identified from the lead contaminated site, while *Stenotrophomonas maltophilia* was identified from the PAH contaminated site. Minimum Inhibitory Concentration assays (MICs) were previously performed on *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 and they were found to be resistant to varying levels of heavy metals and polymerase chain reactions suggesting the presence of mercury (*mer*), copper (*pco*) and cadmium, zinc and cobalt (*czc*) resistance genes. *S. maltophilia* VUN 10010, was previously investigated for its ability to degrade pyrene and other HMW PAHs as a sole carbon and energy source. The purpose of the current project was to further characterise the MICs of these isolates, along with biofilm capabilities. The genetic basis of their heavy metal resistance was also investigated.

Alcaligenes sp. AO22 was further characterised as *Achromobacter* sp. AO22 by analysis of the gene encoding 16S RNA, while *Arthrobacter* sp. E9 was further characterised as *A. woluwensis*. It was also determined that *S. maltophilia* VUN 10010 was not a pure isolate, that a *Mycobacterium* species was also present. Attempts were made to isolate the *S. maltophilia* from the *Mycobacterium*, however this was not achievable. For further experiments, it was decided to work with VUN 10010 as a mixed culture.

This thesis confirmed the heavy metal MICs for *Achromobacter* sp. AO22 and *A. woluwensis* E9 and determined the heavy metal MICs for Consortium VUN 10010. Using a variety of methods, it was demonstrated that both *Achromobacter* sp. AO22 and *A. woluwensis* E9 isolates retained similar levels of heavy metal resistance when compared to the original study. Consortium VUN 10010 was determined to be resistant to heavy metals at levels comparable to *Achromobacter* sp. AO22 and *A. woluwensis* E9. All isolates were tested for their MIC in the presence of the organomercurial compounds methylmercury chloride and phenylmercury acetate, with only VUN 10010 showing a slight resistance to these compounds.

All isolates were investigated for the presence of genes relating to mercury resistance. Results indicated that all three isolates possessed the *merR* gene and it was positioned

divergently from the remainder of the operon. Each isolate also possessed the transport genes, *merT* and *merP*, while partial *merA* genes were amplified. Attempts were made to amplify the 3' region of *merA* and beyond (i.e. *merD*, *merE*), however no amplification was achieved. Internal sequences of the *merB* gene, encoding organomercurial lyase, were achieved for all three isolates, while attempts were not successful at amplifying the *merC* gene, encoding an additional transport protein.

Further analysis was performed to investigate the expression of the *mer* genes. In the presence of HgCl₂, *Achromobacter* sp. AO22 was found to rapidly adapt and grow at a rate comparable to control cells, while it was found that *A. woluwensis* E9 was unable to adapt as rapidly or match the tolerance levels of *Achromobacter* sp. AO22. RNA was extracted from induced and control (no HgCl₂) *Achromobacter* sp. AO22 cells and Reverse Transcriptase PCR (RT-PCR) was performed. It was determined that *merR* transcripts were induced in cells exposed to HgCl₂, while *merT* and *merP* transcripts were upregulated. Very little expression of *merA* was determined.

An investigation was performed to determine the abilities of all three isolates to form biofilms in the presence of heavy metals. *Pseudomonas aeruginosa*, which is capable of forming biofilms in a range of industrial and environmental settings, was used as a comparative control in all biofilm experiments. It was determined that all three isolates were capable of forming biofilms in the presence of mercuric chloride, lead nitrate, zinc nitrate, cadmium chloride and cobalt nitrate.

MIC analysis of the three isolates also indicated the ability to grow in the presence of lead nitrate and cadmium chloride. Primers were designed based on the *pbr* operon of *C. metallidurans* CH34 and the *cad* operon of *S. aureus* pI258. No data was obtained to suggest sequences were present relating to these lead and cadmium resistance mechanisms. To investigate the presence of PAH degradation genes in Consortium VUN 10010, primers were designed based on six of reported genes found in PAH degradation pathways. The presence of PAH-related genes could not be found in this isolate.

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BACKGROUND

Bacteria had previously been isolated from two sites, one contaminated with heavy metals and the other site Polycyclic Aromatic Hydrocarbons (PAHs), and depending on their origin was determined to grow in the presence of heavy metals and/or degrade PAHs. This project focused on the genetic basis of these characteristics, searching for the presence of genes relating to heavy metal resistances and PAH degradation. Such information on the structure and function of these genes will facilitate decisions on the suitability of these indigenous isolates for their use in a number of applications such as the development of biosensors and biosorption systems and their use as biofilms. This literature review will provide an overview on the chemistry, uses and toxicity of heavy metals and PAHs and will discuss a number of genetic systems that encode resistances to and/or degradation of these contaminants. Such systems will also be discussed with respect to their role in the construction of biosensor and biosorption systems and how bacteria capable of forming biofilms demonstrate higher resistances than planktonic forms in the presence of these contaminants. The bacteria used in this project were isolated by previous researchers. Background information on their studies will also be provided in this chapter.

1.1 Chemistry, uses and toxicity of heavy metals

The term 'heavy metals' refers to metallic elements that have high atomic weights (>100) and a relative density >5. Some heavy metals, such as cobalt, copper and zinc, are essential micronutrients for biological systems, although they may be toxic in larger amounts. Other metals, such as mercury, cadmium and lead, are biologically non-essential and toxic in any quantity (Gadd, 1992). According to the Agency for Toxic Substances and Disease Registry (ATSDR(a)), arsenic, lead and mercury comprise the top 3 in the list of top 20 hazardous substances, while cadmium is ranked 7th. Major sources of heavy metal contamination in the environment are the combustion of fossil fuels, the operation of smelters and other industrial activities such as mineral mining and processing, brewery and distillery wastes and the generation and use of agricultural chemicals (Gadd, 1992). Mercury occurs in three chemical forms;

- i. unreactive elemental mercury, which is a shiny, silver-white odorless liquid.
- ii. inorganic mercurial salts and minerals (i.e. mercuric chloride, mercuric iodide, mercuric oxide, mercuric sulphide, mercurous chloride).
- iii. organic mercurials (i.e. methylmercury chloride, phenylmercury acetate, ethyl mercury and merbromin) (Graeme and Pollack Jr, 1998).

Lead is a bluish-gray metal found in small amounts in the earth's crust, although most of the lead present in the environment is due to human activities (ATSDR(b)). Cadmium is a natural element in the earth's crust and is usually found as a mineral, combined with other elements such as oxygen (cadmium oxide) or sulphur (cadmium sulphate and cadmium sulphide) (ATSDR(c)).

Zinc, a bluish-white shiny metal, is one of the most common elements in the earth's crust and is found in air, soil, and water and is present in all foods (ATSDR(d)). It is an essential co-factor for enzymes that control cell processes such as DNA synthesis, growth, brain development, behavioral responses, reproduction, fetal development, membrane stability, bone formation and wound healing. Zinc deficiencies may result in growth retardation, anorexia, delayed sexual maturation, iron-deficiency anemia and alterations in taste (Barceloux, 1999a). Cobalt is a naturally occurring element that may be found in rocks, soil and water (ATSDR(e)). It is a relatively rare metal of gray colour and is ductile, brittle, and magnetic. Cobalt is a necessary element in the formation of vitamin B₁₂ (hydroxocobalamin), which catalyses reactions such as the synthesis of methionine, the metabolism of purines and folates and the formation of methylmalonic acid in succinic acid (Barceloux, 1999b).

Heavy metals and their compounds are used widely in a number of industrial applications, which often leads to environmental contamination, including of the air, water and soil. Such contaminations may result in human exposure, which can often lead to detrimental health problems. Figure 1.1 shows examples of heavy metal structures, while the uses, sources of contamination and the target organs of the above mentioned heavy metals are summarised in Table 1.1.

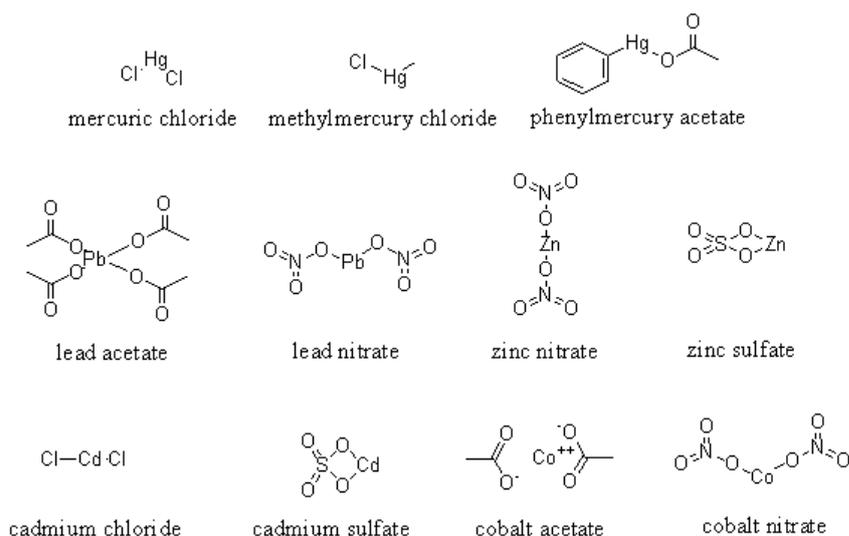


Figure 1.1 Examples of the chemical structures of some heavy metal compounds (<http://chemeth.chemexper.com>).

1.2 Chemistry, uses and toxicity of Polycyclic Aromatic Hydrocarbons (PAHs)

Polycyclic aromatic hydrocarbons (PAHs) comprise two or more fused benzene rings (Figure 1.2), which may be in linear, angular or cluster arrangements. Generally PAHs consist only of carbon and hydrogen atoms, although nitrogen, sulphur and oxygen atoms may be substituted into the benzene rings to form heterocyclic aromatic hydrocarbons. The stability of PAHs depends on the arrangement of the benzene rings, angular PAHs such as pyrene, benzo[*a*]pyrene and coronene are the most stable, while linear PAHs such as naphthalene and anthracene are the least stable. PAHs are hydrophobic compounds, which makes them more persistent in the environment with PAH solubility decreasing as the number of fused benzene rings increases (Cerniglia, 1992). PAHs have been reported to occur in both water and land environments.

Table 1.1 Uses, contamination and toxicity of heavy metals

Heavy metal	Uses	Contaminating sources	Target organs
Mercury	Production of chlorine gas and caustic soda. Used in dental fillings, batteries, skin lightening creams and antiseptic creams.	Contaminated fish and shellfish. Inhalation of vapors from spills, incinerators and the burning of mercury-containing fuels. Release of mercury from dental work and medical treatments. The practice of rituals that involve mercury.	Nervous system, brain, kidneys, developing fetus, lungs, GI tract, heart, skin and eyes.
Lead	Production of batteries, ammunition, solder and pipes and X-ray shields.	Contaminated food and water. Exposure to lead-based paints. Working in industries that use lead. Use of health care products and folk remedies.	Nervous system, kidneys, reproductive system and blood.
Zinc	Production of paint, rubber, wood preservatives, ointments and alloys such as brass and bronze. Used in coatings to prevent rust.	Breathing in zinc particles in the air of manufacturing sites. Contaminated water near manufacturing or waste sites. Contaminated water or beverage that has been stored in containers or flows through pipes that have been coated with zinc to prevent rust.	Stomach, blood, pancreas and lungs.
Cadmium	Production of batteries, pigments, metal coatings and plastics.	Breathing contaminated workplace air, and air near the burning of fossil fuels or municipal waste. Contaminated water and foods (i.e. shellfish, liver, kidney).	Lungs, stomach and kidneys.
Cobalt	Production of alloys. Used in aircraft engines, magnets grinding/cutting tools, artificial joints, medical sterilisation and research.	Working in industries that use cutting and grinding tools and those involving mining, smelting, refining or processing of cobalt or ores. Working at nuclear, irradiation or nuclear waste facilities.	Lungs, heart, skin and stomach.

ATSDR(a-f) provides summaries from which this information was sourced.

They are formed naturally during thermal geologic reactions associated with fossil fuels and mineral production, during the burning of vegetation in forest and bush fires and by some bacterial and plant reactions (Cerniglia, 1992). The uses, sources of contamination and the target organs of PAHs are summarised in Table 1.2.

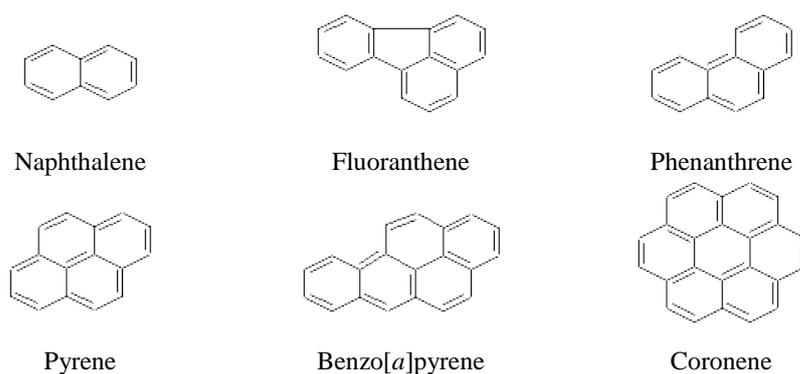


Figure 1.2 Examples of the chemical structures of some PAHs (<http://chemeth.chemexper.com>).

Table 1.2 Uses, contamination and toxicity of PAHs

Uses	Contaminating sources	Target organs
Production of coal tar, crude oil, creosote and roofing tar.	Breathing air, containing PAHs in the workplace of coking, coal-tar and asphalt production plants, smokehouses and municipal waste facilities.	Carcinogenic -lungs -stomach Skin
Used in dyes, plastics and pesticides.	Breathing air, containing PAHs from cigarette smoke, wood smoke, vehicle exhausts. Eating contaminated food and charred meats. Drinking contaminated water or cow's milk.	In animals -skin -immune system -reproductive system

Reference: ATSDR(g)

1.3 Microbial resistance mechanisms to environmental contaminants

Bacterial cells resistant to the above environmental contaminants have previously been isolated (Ji and Silver, 1995; Nies, 1999; Bruins *et al.*, 2000). Analysis of their genetic and physiological systems has revealed that they may possess one or more of the four main types of mechanisms to protect against such contaminants. The four main types of mechanisms are as follows:

Efflux systems: These types of mechanisms export toxic metal ions to the outside of the cell (Bruins *et al.*, 2000). These may be non-ATPase or ATPase-linked and are generally highly specific for a particular heavy metal ion (Nies and Silver, 1995). P-type ATPases are common in heavy metal resistance mechanisms. These may be described as a family of membrane proteins that perform active ion transport across biological membranes (Apell, 2003).

Accumulation and complexation: These types of mechanisms serve to prevent the exposure of essential cellular components to the contaminant and may be a result of the presence of metallothioneins or cysteine-rich proteins (Bruins *et al.*, 2000).

Reduction: The contaminating ions are enzymatically reduced upon entry to the cell and the less toxic, reduced ions are exported from the cell into the environment (Nies, 1999).

Alteration of cellular components: Some microorganisms may adapt to the presence of heavy metals by altering the sensitivity of cellular components, which may be achieved through mutations that decrease the sensitivity but do not alter basic function or by increasing the production of the sensitive cellular component (Rouch *et al.*, 1995).

A number of genes, located on bacterial plasmids and chromosomes, have been identified that encode specific resistance to a number of heavy metal ions, including Ag^+ , AsO_2^- , AsO_4^{3-} , Cd(II) , Co(II) , CrO_4^{2-} , Cu(II) , Hg(II) , Ni(II) , Sb(III) , TeO_3^{2-} and Zn^{2+} (Ji and Silver, 1995). The following sections will outline bacterial genetic systems for resistances to mercury (*mer*), lead (*pbr*) and cadmium, zinc and cobalt (*czc*, *cad*) and for the degradation of polycyclic aromatic hydrocarbons (*nah*, *pah* and *phn* for example).

1.4 Mercury resistance

One of the most widely studied bacterial heavy metal resistance operons is the *mer* operon, encoding mercury resistance, in a number of Gram-positive bacteria and Gram-negative bacteria. Two of the most widely studied *mer* operons are Tn501 and Tn21. The Tn501 *mer* operon was originally isolated from plasmid pVS1 from a *Pseudomonas aeruginosa* strain isolated in Australia (Misra *et al.*, 1984; Brown *et al.*, 1986; Barrineau *et al.*, 1984). This archetypal *mer* operon contains five genes, *merR*, *merD*, *merT*, *merP* and *merA*. Tn21 also carries an archetypal *mer* operon, with an additional transport gene (*merC*) and was originally isolated on plasmid NR1 from *Shigella flexneri* in Japan (Nakaya *et al.*, 1960). Plasmid pDU1358 of *Serratia marcescens* differs from the above two operons in that it carries an additional lyase gene (*merB*) (Griffin *et al.*, 1987). Figure 1.3 provides an overview of the functions of the various genes/proteins of the *mer* operons, which will be discussed in the following sections.

1.4.1 Regulation of the *mer* operon

1.4.1.1 MerR

Extensive work has been carried out on the regulation of the *mer* operon by the *merR* gene, which encodes a negative regulator of the remainder of the *mer* operon. In Gram-negative bacteria, the *merR* gene is generally transcribed separately and in the opposite direction compared to the other genes of the operon (Brown *et al.*, 1986; Griffin *et al.*, 1987; Inoue, 1991; Kiyono, 1997; Schelert *et al.*, 2004). An exception to this is the marine bacterium *Pseudoalteromonas haloplanktis*, where the *merR* gene is co-transcribed with the *merTPCAD* genes (Iohara *et al.*, 2001). In the Gram-negative bacteria *Shewanella putrefaciens* plasmid pMERPH, the *mer* operon lacks *merR* and *merD* genes (Osborn *et al.*, 1996). In Gram-positive bacteria *mer* operons, the *merR* genes are generally transcribed in the same direction as the rest of the operon (Laddaga *et al.*, 1987; Wang *et al.*, 1987; Ravel *et al.*, 1998; Huang *et al.*, 1999a).

In the absence of Hg(II), the MerR protein binds as a homodimer to the promoter, which is a region of dyad symmetry, located just upstream of the *merT* gene (Ross *et al.*, 1989; Park *et al.*, 1992; Parkhill *et al.*, 1998; Rother *et al.*, 1999). When Hg(II) enters the cell,

it binds with high specificity to MerR to provoke an allosteric change in the protein, which is attached to the DNA of this operator region, leading to an unwinding of the operator DNA (Heltzel *et al.*, 1990; Ansari *et al.*, 1992; Ansari *et al.*, 1995; Parkhill *et al.*, 1998; Caguiut *et al.*, 1999; Song *et al.*, 2004). This leads to improved access of RNA polymerase, which is bound simultaneously along with MerR to the promoter, to the transcriptional start site (Lee *et al.*, 1993; Livrelli *et al.*, 1993). MerR can be cross linked to the α , β and σ^{70} subunits of RNA polymerase, whether in the absence of the DNA or when both MerR and Hg(II) are bound to the operator DNA (Kulkarni and Summers, 1999).

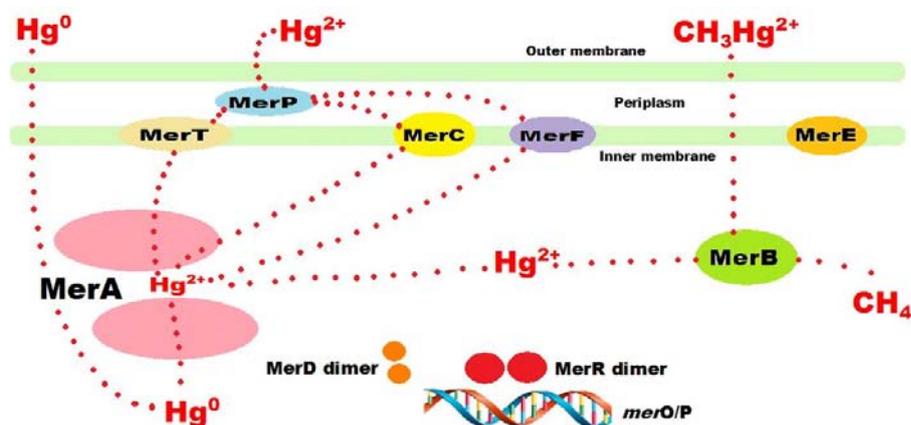


Figure 1.3 Diagrammatic representation of the protein products of the *mer* operons.

The MerR proteins of Tn501 and Tn21 are 144 amino acids long and differ in nine residues, three of which are conservative substitutions. Three cysteine residues are conserved in all MerR proteins, which have been confirmed to be the site for Hg(II) binding. In the Tn21 MerR, mutation of the three cysteines (Cys82, Cys117 and Cys126) caused a loss of Hg(II)-inducible activation (Ross *et al.*, 1989). The binding site of MerR to Hg(II) lies at the interface of the homodimer and involves Cys82 from one monomer and Cys117 and Cys126 from the other (Helmann *et al.*, 1990; Caguiut *et al.*, 1999). *In vitro* metal binding studies have shown that MerR binds only one atom of Hg(II), although there are two potential binding sites in the dimer. The binding of a

single Hg(II) ion to one site causes an allosteric change that renders the other site less able to bind Hg(II) in competition with other thiols (O'Halloran and Walsh, 1987; Shewchuk *et al.*, 1989; Helmann *et al.*, 1990; Zeng *et al.*, 1998; Caguiut *et al.*, 1999).

Mutants which affected the DNA binding (Glu22Lys and Arg25His) helped define the DNA binding region (Parkhill *et al.*, 1998), and indicated that the N-terminal helix-turn-helix motif, rather than a similar motif more centrally in the protein, was responsible for DNA binding (Ross *et al.*, 1989). It has been shown that only residues 80-128 were required for stable dimer formation and retained a high affinity for Hg(II) (Zeng *et al.*, 1998).

1.4.1.2 The OP region of the *mer* operon

The *mer* operator/promoter (OP), in the case of the *mer* operons of most Gram-negative bacteria, is a 19 bp hyphenated sequence with 7 bp palindromes flanking a 4 bp AT-rich center (Barrineau *et al.*, 1984; Brown *et al.*, 1986; Parkhill and Brown, 1990; Park *et al.*, 1992). An unusual feature of the *mer* OP region is that it lies within the spacer region between the -10 and -35 regions of the P_T promoter, slightly overlapping the -35 hexamer. The P_T promoter has consensus -10 and -35 hexamers, but is unusual in that it is 19 bp in length rather than the typical length of 17 bp found in most σ^{70} promoters in bacteria (Figure 1.4). MerR binding in the absence of Hg(II) in this region does not prelude, but rather fosters RNA polymerase occupancy of P_T, albeit in a transcriptionally inactive state until Hg(II) is present (O'Halloran *et al.*, 1989; Frantz and O'Halloran, 1990; Heltzel *et al.*, 1990; Kulkarni and Summers, 1999). Deletion mutants have shown that the -35 and -10 sequences must be correctly separated by 19 bp for normal promoter activity (Lund and Brown, 1989; Parkhill and Brown, 1990). When MerR, already bound to the OP region, binds to Hg(II), an increased reactivity of bases occurs near the operator centre which leads to the unwinding of the operator DNA (Ansari *et al.*, 1992), making the -10 region available to RNA polymerase (Condee and Summers, 1992). MerR requires a distinct operator contact for repression and activation of P_T (Park *et al.*, 1992). Further studies show that another gene, *merD*, may play an additional role in the regulation of the *mer* operon (discussed below).



Figure 1.4 The two divergent promoters of the Tn501 *mer* operon. P_T controls the expression of the *merTPAD* genes and P_R is the promoter for the regulatory *merR* gene.

1.4.1.3 MerD

In Tn501, the *merD* gene is a small cysteine-rich open reading frame that lies just downstream of *merA* (Brown *et al.*, 1986). Its protein product (from Tn21) has been observed to have an N-terminal region with a predicted helix-turn-helix motif similar to that of MerR and has been shown to be translated in very small amounts (Lee *et al.*, 1989). Deletions of this gene, from pDU1358, have shown to have no effect on the mercury resistant phenotype. (Nucifora *et al.*, 1990; Mukhopadhyay *et al.*, 1991). *In vitro*, MerD from *Cupriavidus metallidurans* (formerly *Ralstonia metallidurans*) CH34 has been shown to form a ternary complex with MerOP and MerR (as described in Section 1.4.1.2). It has been postulated that MerD displaces Hg-bound MerR from the operator, allowing the synthesis of Hg(II)-free MerR, which switches off the induction of *mer* genes in the absence of mercury (Champier *et al.*, 2004).

1.4.2 Transport of the Hg(II) ions

1.4.2.1 MerP

The gene *merP* of Tn21 encodes a small periplasmic mercury binding protein, with the 72 (12 kDa) amino acid long mature MerP being processed from a 91 (13 kDa) amino acid precursor (Jackson and Summers, 1982; Summers, 1986). This protein has been shown to function as a monomer and binds a single Hg(II) ion via two cysteines, at positions 14 and 17 (relating to Tn501 and Tn21) (Steele and Opella, 1997), which form part of novel metal binding motif Gly-Met-Thr-Cys-X-X-Cys, found singly or as

multiple repeats on the N-terminus of P-type ATPases, involved in influx/efflux of transition metal cations in prokaryotes and eukaryotes (Bull and Cox, 1994). Loss of either Cys14 or Cys17 has been shown to lead to an inability for MerP to bind Hg(II) (Sahlman and Skarfstad, 1993). The Tn21 MerP protein has been shown to exist in an oxidized (disulphide) or a reduced (dithiol) form, however, only in its reduced form, with the Cys14 and Cys17 residues as free thiols, can the protein act as a receptor of mercuric ions (Qian *et al.*, 1998). NMR studies of MerP show that in the absence of Hg(II), Cys14 of the reduced form is surface exposed and Cys17 is buried, however when Hg(II) is bound, both cysteines are surface exposed (Steele and Opella, 1997). It has been suggested that an electrostatic attraction between the buried Cys17 and Hg(II), triggers a structural change upon Hg(II) binding (Powlowski and Sahlman, 1999). Studies on the crystallized form of MerP from *C. metallidurans* CH34 propose that the side-chain of Tyr66, which is a conserved residue in MerP proteins, and the main-chain amide of Cys14 may play a role in the maintaining of Cys17 in an anionic form in the reduced form of the protein. This study also suggests that Tyr66 and Phe38, also conserved in MerP proteins, may be important in the mercury-binding reaction and transfer of Hg(II) to MerT (Serre *et al.*, 2004). MerP is believed to transfer Hg(II) to the amino-terminal cysteines Cys24 and Cys25 (corresponding to Tn21 and Tn501) of MerT (Hamlett *et al.*, 1992; Morby *et al.*, 1995). MerT is described below.

1.4.2.2 MerT

The gene *merT* encodes a product of 116 amino acids (12.4 kDa) and is an inner (cytosolic) membrane protein strongly predicted to have three transmembrane helices, the first of these having a cysteine pair which is thought to be accessible from the periplasmic side (Sahlman *et al.*, 1997; Liebert *et al.*, 2000; Brown *et al.*, 2002). In Tn501, Cys24 and Cys25 in the first transmembrane domain have been found to be essential for the transport of mercury ions through the cytoplasm (Morby *et al.*, 1995; Hobman and Brown, 1996). The second pair of cysteines is thought to lie on the cytoplasmic face of the inner membrane between the second and third transmembrane helices. Hg(II) may be transferred from the N-terminal-proximal cysteine pair to form a di-coordinate protein complex with these cysteines, and then transferred to MerA (Jackson and Summers, 1982; Schue *et al.*, 2007). Mutations of Cys76Ser, Cys82Ser or Gly38Asp (in Tn501) led to a reduction of resistance and transport (Morby *et al.*, 1995).

Mutations of Gly14Arg, Gly15Arg, Gly27Arg and Ala18Asp (also in Tn501) in the first predicted transmembrane helix have been shown to cause a loss of mercury resistance (Hobman and Brown, 1996).

1.4.3 Mercuric reductase (MerA)

The most widely observed mechanism of eubacterial mercury resistance is by the reduction of the highly reactive cationic form of mercury, to volatile, relatively inert monoatomic mercury vapor. This reduction is mediated by MerA (mercuric reductase), encoded by the gene *merA*, which is a flavoprotein with a redox-active cysteine at the active site (Fox and Walsh, 1982) which is a minimum of 1600 amino acids in length. This cytoplasmic protein (Summers and Sugarman, 1974) is a homodimer (Fox and Walsh, 1982) which catalyses the conversion of thiol-avid Hg(II) to volatile, uncharged Hg(0), utilizing NADPH as a source of electrons (Furukawa and Tomomura, 1972). When reduced by MerA, volatile Hg(0) diffuses through the cell membrane without the need for a dedicated efflux system (Barkay *et al.*, 2003).

MerA contains 8 cysteine residues, two of which (Cys135 and Cys140 in Tn501) are located in the active site (Brown *et al.*, 1983). In Tn501, the C-terminal cysteines (Cys558 and Cys559) of one monomer lie near the redox-active cysteines of the other monomer and could assist with Hg(II) binding at the active site (Brown *et al.*, 1983). Mutagenesis of two conserved pairs of cysteines in the N-terminus (Cys10 and Cys13) and the C-terminus (Cys558 and Cys559) in Tn501 indicated no essential roles for Cys10 and Cys13, but did identify a role in Hg(II) reduction for the latter two (Moore and Walsh, 1989). Further, when compared to the wild-type enzyme, the Cys558Ala and Cys559Ala mutants demonstrated 200-fold and 10-fold reductions in catalytic activity respectively (Moore *et al.*, 1992). It was also found in MerA from *Bacillus cereus* RC607 that in the absence of the C-terminal cysteines, HgX₂ substrates with small ligands can access the redox-active cysteines, while those with large ligands could not, indicating that the C-terminal cysteines play a crucial role in removing high-affinity ligands before Hg(II) reaches the redox-active cysteines (Cys135 and Cys140) in the inner active site (Engst and Miller, 1999). Approximately 77 amino acids in the N-terminal domain of MerA are homologous to MerP (Misra *et al.*, 1985; Schiering *et al.*, 1991). Proteolysis of the first 85 N-terminal amino acids (Fox and Walsh, 1982) and

site-directed mutagenesis of the N-terminal cysteines (Moore and Walsh, 1989) did not have any influence on the catalytic properties *in vitro*.

In *C. metallidurans* CH34, the MerA protein contains an N-terminal sequence of 62 amino acids, referred to as MerAa, which contains a motif (Gly-Met-Thr-Cys-X-X-Cys) homologous to part of MerP from the same organism. This MerAa N-terminal sequence was expressed independently and two cysteine residues, found in the motif, were found to be involved in the binding of one mercury atom, with an affinity comparable to MerP, indicating that MerAa may play a role in mercury transport (Rossy *et al.*, 2004). Amino acid sequence analysis indicates that homologies within this Gly-Met-Thr-Cys-X-X-Cys motif also exist between the N-terminal sequences of the MerA proteins and the MerP proteins of Tn501 and Tn21 (Rossy *et al.*, 2004). The MerAa domain and the catalytic core of the Tn501 MerA were expressed as two separate proteins. Results indicated that MerAa may be expressed as a soluble, monomeric protein capable of binding Hg(II) and delivering it to the catalytic core of MerA. However, in cells containing small molecular weight thiols (such as GSH), MerAa appears to serve little function (Ledwidge *et al.*, 2005).

1.4.4 Additional mercury transport genes

1.4.4.1 MerC

The *merC* gene of Tn21, pKLH2 of *Acinetobacter* sp. (Kholodii *et al.*, 1993), Tn5041 of *Pseudomonas* sp. (Kholodii *et al.*, 1997) and pMERPH of *Pseudomonas putrifaciens* (Osborn *et al.*, 1996) for example, is located between the *merP* and *merA* genes. It encodes the MerC protein, which ranges in size between 129 and 144 amino acids (Peters *et al.*, 1991; Kholodii *et al.*, 1993; Yurieva *et al.*, 1997; Liebert *et al.*, 1999), has four predicted transmembrane helices and is the largest of the *mer* operon-encoded membrane proteins (Summers, 1986). Deletion analysis of the *merC*-encoding Tn21 operon indicated that the loss of MerP and MerT had some phenotypic effect on Hg(II) resistance, while the lack of MerC did not change this or its Hg(II) volatilization capabilities (Hamlett *et al.*, 1992). Topological predictions suggest that the first cysteine pair (Cys22 and Cys25) of the MerC of Tn21 lies just within the membrane on the cytoplasmic side and that the second pair lies in the cytosol (Cys127 and Cys132)

(Sahlman *et al.*, 1997). It has been shown that an excess of MerP can inhibit Hg(II) binding to MerC indicating that an affinity of MerP for Hg(II) is greater than that of MerC (Sahlman *et al.*, 1997). Also the addition of MerT diminished uptake by MerC of Hg(II) to a level equal to that of MerT alone, suggesting that there is no synergy between these two proteins and that there may even be competition (Sahlman *et al.*, 1997). All MerC proteins conserve a Cys-Ala-X-Cys-Phe-Pro-Ala motif in the N-terminal cysteine pair, which is divergent from the Gly-Met-Thr-Cys-X-X-Cys motif of MerP and the N-terminal region of MerA (Liebert *et al.*, 2000). Site-directed mutagenesis of the four cysteines of MerC showed that both Cys22 and Cys25, which are predicted to lie in a canonical Gly-Met-X-Cys-Cys-Cys-Cys metal binding motif, just inside the cytoplasmic face of the membrane, are essential for Hg(II) binding to vesicles (Sahlman *et al.*, 1997). In the MerC of *Acidothiobacillus ferrooxidans*, this cysteine pair was shown to be involved in Hg(II)-recognition and uptake (Sasaki *et al.*, 2005).

1.4.4.2 MerE

The gene *merE* is a predicted open reading frame, of 78 amino acids, immediately following *merD* in many Gram-negative bacteria *mer* operons. It is predicted to contain a cysteine pair (Cys-X-X-Cys), positioned in a similar fashion to that of the N-terminus of MerF, suggesting a role in mercury transport, although this is yet to be elucidated (Liebert *et al.*, 2000).

1.4.4.3 MerF

The gene *merF* was first noted between *merP* and *merA* genes in the plasmid borne (pMER327/419) *mer* operon in an environmental *Pseudomonas* strain (Hobman *et al.*, 1994). MerF functions as a monomer (Wilson *et al.*, 2000) and is an 81 amino acid hydrophilic protein with two predicted transmembrane helices. It contains an N-terminal cysteine pair (Cys20 and Cys21), resembling the Cys-X-X-Cys motif found in MerC, and is predicted to lie near the middle of the first helix, and a C-terminal cysteine pair (Cys70 and Cys71) (Liebert *et al.*, 2000). These cysteine pairs have been shown to play a role in the transport of mercury across the cell membrane (Wilson *et al.*, 2000) and are exposed to the cytoplasm (Howell *et al.*, 2005).

1.4.5 Organomercurial resistance

Some mercury resistance operons contain additional genes, giving the bacteria broad-spectrum resistance, enabling them to detoxify not only inorganic forms of mercury, but also organomercurials such as methylmercury chloride and phenylmercury acetate. Most Gram-positive bacteria mercury resistance operons are broad-spectrum, whilst in Gram-negative bacteria operons, the occurrence of broad-spectrum resistance is approximately 20% (Liebert *et al.*, 1997). Broad-spectrum resistant strains of both Gram-negative bacteria and Gram-positive bacteria often have two *mer* operons, a broad-spectrum locus and a narrow-spectrum locus (Liebert *et al.*, 1997). The genes involved in broad-spectrum mercury resistance are discussed below.

1.4.5.1 MerB

The gene *merB*, encoding the protein organomercurial lyase (MerB), was first described in the *Pseudomonas* strain K-62, which was able to degrade organomercurial compounds such as phenylmercury, ethylmercury and methylmercury (Furukawa and Tonomura, 1971). MerB is responsible for splitting the C-Hg bond in the organomercurial, then releasing the protonated organic moiety and the Hg(II) cation, which is then reduced by MerA (Schottel, 1978). MerB, encoded by R831 (a conjugative IncM plasmid in *Escherichia coli*), was found to be a 22.4 kDa protein functioning as a monomer (Begley *et al.*, 1986) with no known homologs (Pitts and Summers, 2002). MerB is a cytosolic enzyme (Pitts and Summers, 2002) with no disulphide bonds (Barkay *et al.*, 2003) and consists of a novel protein fold, containing three non-interacting β -sheets surrounded by six α -helices (Di Lello *et al.*, 2004). Cys117 has been shown to play a structural role, while Cys96, Cys159 and Cys160 appear to be part of the active site (Di Lello *et al.*, 2004). Three of these cysteines are highly conserved at positions 96, 117 and 159 (numbering relating to MerB of R831) (Barkay *et al.*, 2003). Asp99 has recently been implicated in playing a role in the proton transfer step, which is required for the cleavage of the carbon-mercury bond (Lafrance-Vanasse *et al.*, 2009).

The *merB* enzyme has a broad substrate tolerance, being able to handle both alkyl and aryl mercurials. A model pathway for the function of MerB, encoded by R831, based on the current knowledge has been described by Barkay *et al.* (2003). Firstly, a cysteine (probably Cys159) of the fully reduced enzyme displaces the organic component from the organomercurial and a second cysteine (probably Cys96) forms a bis-coordinate structure with the mercurial component. Once the organic component is removed, MerB remains bound to Hg(II) until two solvent monothiols can remove it (Barkay *et al.*, 2003). It has been suggested that mercuric ions are transferred from the active site of MerB to the active site of MerA by a direct transfer mechanism (Benison *et al.*, 2004). Figure 1.3 provides an overview of the proposed mechanisms involved in narrow- and broad-spectrum mercury resistance.

1.4.5.2 MerG

This gene was first identified as an open reading frame with a possible signal sequence between *merA* and *merB* on the broad-spectrum resistance plasmid pMR26 of *Pseudomonas* strain K-12 (Kiyono and Pan-Hou, 1999). It has been suggested that the MerG protein is 217 amino acids long and is thought to be located in the periplasm. Deletion of the *merG* gene in the broad-spectrum operon of pMR26 did not impair the activities of MerA and MerB, however it made the cells more sensitive to phenylmercury, indicating that MerG is specific for phenylmercury. It is thought that MerG acts as an efflux mechanism rather than performing mercury biotransformation (Kiyono and Pan-Hou, 1999).

1.4.6 Diversity and organization of the *mer* operons

While the above information has described the functions and characteristics of each of the mercury resistance genes, it is important to outline the great diversity that is present between the *mer* operons of different bacteria. The general formula for a *mer* operon is *merRTP(C)A(B)D*, however, many variations exist. Table 1.3 outlines some of these.

It has been established in the literature that the *mer* operons generally share a number of genes, with the exception of some operons containing additional genes, such as *merC* and *merB* (as described in Sections 1.4.4.1 and 1.4.5.1). A detailed analysis of the

homologies shared between the *mer* genes and proteins is required to understand the true extent of their relatedness and diversity. Using the published genes in the ANGIS database (www.angis.org.au), extensive alignments of a number of Gram-negative bacteria and Gram-positive bacteria *mer* operons was performed. The information below summarizes the range of homologies between individual genes and the similarities in protein structures. Table 1.4 was constructed based on this analysis.

For the *merR* gene, the DNA homologies ranged from 100% between Tn501 and *Pseudomonas stutzeri* plasmid pPB and 72.18% between Tn501 and *Pseudomonas* sp. Tn5041 in Gram-negative bacteria. When comparing the degree of homology between Tn501 and the *merR* genes from Gram-positive bacteria operons, the levels of homology ranged from 45.54% to 35.71% for *B. cereus* RC607 *merR1* and *Streptomyces lividans* respectively. When the Gram-positive bacteria *merR* genes were compared to each other, the levels of homology were varied, with 100% homology observed between two *Bacillus* sp. for both the *merR1* and *merR2* genes (*Bacillus megaterium* MB1 and *B. cereus* RC607). The lowest homology between the *merR* genes from Gram-positive bacteria was 25.3% between *Staphylococcus aureus* pI258 and *B. cereus* RC607. Performing a comparison of the MerR proteins indicated that Cys82, Cys117 and Cys126 (corresponding to Tn501) were present in all the Gram-negative bacteria from Table 1.4. When the MerR proteins from the Gram-positive bacteria were compared to Tn501, Cys82, Cys117 and Cys126 were only present in *B. cereus* RC607 MerR1 and *B. megaterium* MB1 MerR1, while Cys126 was also present in *S. lividans* and *Streptomyces* sp. CHR28. The MerR protein also contains a helix-turn-helix motif (positions 5-75 in Tn501) and a DNA-binding region within (positions 9-27 in Tn501). Within the 71 amino acid long helix-turn-helix motif of the Gram-negative bacteria MerR proteins, the maximum number of amino acid differences compared to Tn501 was 7 in both *Xanthomonas campestris* Tn5044 and *Pseudomonas* sp. Tn5041. From the 19 amino acid-long DNA-binding motif, there was one amino acid difference between Tn501 and *S. marcescens* pDU1358 and two differences in amino acid sequence for both *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041. When comparing these regions in Tn501 to the Gram-positive bacteria MerR proteins and amongst the Gram-positive bacteria MerR proteins, very little homology exists.

Table 1.3 Diversity and organization of the *mer* operons

Organism	Gram staining type	Environment from which it was isolated	Location of genes	Operon organization	Operon features	Reference
<i>P. aeruginosa</i>	negative	Environment	plasmid	Tn501	<i>merRTPAD</i>	Misra <i>et al.</i> , 1984 Barrineau <i>et al.</i> , 1984 Brown <i>et al.</i> , 1986
<i>S. flexneri</i>	negative	Clinical	plasmid	Tn21	<i>merRTPCAD</i>	Nakaya <i>et al.</i> , 1960
<i>S. marcescens</i>	negative	Hospital isolate	plasmid	pDU1358	<i>merRTPABD</i>	Griffin <i>et al.</i> , 1987 Nucifora <i>et al.</i> , 1989a Yu <i>et al.</i> , 1994
<i>Acinetobacter</i> sp.	negative	Mercury ores in Kyrgizia	plasmid	pKLH2	<i>merRTPCAD</i>	Kholodii <i>et al.</i> , 1993
<i>Pseudomonas</i> sp.	negative	Mercury mine in Kyrgizia	chromosomal	Tn5041	<i>merRTPCAorfYD</i>	Kholodii <i>et al.</i> , 1997
<i>Xanthomonas</i> sp.	negative	Khaidarkan mercury mine, Kyrgizia	chromosomal	Tn5053	<i>merRTPFAD</i>	Kholodii <i>et al.</i> , 1995
<i>Xanthomonas</i> sp.	negative	Downstream of a hot spring on the Kamchatka Peninsula, Russia	plasmid	Tn5044	<i>merRTPsigYAD</i>	Kholodii <i>et al.</i> , 2000
<i>Pseudomonas fluorescens</i>	negative	River Mersey, UK	plasmid	pMer327/419	<i>merRTPFAD</i>	Hobman <i>et al.</i> , 1994 Wilson <i>et al.</i> , 2000
<i>P. putrificiens</i>	negative	River Mersey, UK	plasmid	pMERPH	<i>merTPCA</i> No <i>merR</i>	Osborn <i>et al.</i> , 1996

Table 1.3 continued

Organism	Gram staining type	Environment from which it was isolated	Location of genes	Operon organization	Operon features	Reference
<i>P. stutzeri</i>	negative	Originally isolated for its ability to degrade o-xylene	plasmid	pPB	2 operons- <i>merRTPAD</i> <i>merRBTPCAD</i>	Reniero <i>et al.</i> , 1995 Reniero <i>et al.</i> , 1998
<i>Pseudomonas</i> strain K-62	negative	Phenylmercury-polluted soil	plasmid	pMR26	<i>merRTPAGB</i>	Kiyono <i>et al.</i> , 1997 Kiyono, <i>et al.</i> , 1999
<i>Thiobacillus ferrooxidans</i>	negative	Not specified	chromosomal	No name	<i>merRTPA</i>	Inoue <i>et al.</i> , 1989 Inoue <i>et al.</i> , 1991 Inoue <i>et al.</i> , 1996 Velasco <i>et al.</i> , 1999
<i>Thiobacillus</i> T3.2	negative	Uranium mineral heap, Cuidad Rodrigo, Spain	chromosomal	No name	<i>merRTPA</i>	Velasco <i>et al.</i> , 1999
<i>B. cereus</i> RC607	positive	Boston Harbour sediment	chromosomal	No name	<i>merR1Torf3orf4AR2B2B1</i>	Wang <i>et al.</i> , 1989 Helmann <i>et al.</i> , 1989 Gupta <i>et al.</i> , 1999
<i>B. megaterium</i> MB1	positive	Preserved sediment sample from Minimata Bay, Japan		Tn <i>MER11</i>	MerB3R1ETPAR2B2B1	Huang <i>et al.</i> , 1999b
<i>S. aureus</i>	positive		plasmid	pI258	merRorf3orf4TAB	Laddaga <i>et al.</i> , 1987 Skinner <i>et al.</i> , 1991
<i>S. lividans</i> 1326	positive	Spontaneous chloramphenicol-sensitive, arginine auxotrophic (Arg ⁻) mutant	chromosomal		<i>merRTPorfX</i> in one direction. <i>merAB</i> in the opposite direction. Two divergent promoters. ORFIVmerPTRAB	Sedlmeier and Altenbuchner, 1992 Brunker <i>et al.</i> , 1996
<i>Streptomyces</i> sp. CHR28	positive	Mercury-contaminated sediment, Baltimore Inner Harbour	plasmid			Ravel <i>et al.</i> , 1998 Ravel <i>et al.</i> , 2000

For the *merT* gene, the DNA homologies ranged from 100% between Tn501 and *P. stutzeri* plasmid pPB and 78.34% between Tn501 and *Pseudomonas* sp. Tn5041 for Gram-negative bacteria. When comparing the degree of homology between Tn501 and the *merT* genes from Gram-positive bacteria operons, the levels of homology ranged from 45.23% to 35.22% in *S. lividans* and *S. aureus* pI258 respectively. When the Gram-positive bacteria *merT* genes were compared to each other, the levels of homology were varied, with 100% homology observed between *B. megaterium* MB1 and *B. cereus* RC607. The lowest homology in the *merR* genes from Gram-positive bacteria was 26.96% between *S. lividans* and both *B. megaterium* MB1 and *B. cereus* RC607. Performing a comparison of the MerT proteins indicated that Cys24, Cys25, Cys76 and Cys82 (corresponding to Tn501) were present in all Gram-negative bacteria MerT proteins compared. When comparing the Tn501 MerT protein with those from the Gram-positive bacteria, it is evident that Cys24 and Cys25 are present in all Gram-positive bacteria MerT proteins analyzed. The Cys76 was not present in any of the Gram-positive bacteria proteins compared, while the Cys82 was present in *S. aureus* pI258, *S. lividans* and *Streptomyces* sp. CHR28. The MerT protein contains three potential transmembrane regions in positions 16-35, 44-64 and 94-108 (corresponding to Tn501). When comparing the regions of other Gram-negative bacteria MerT proteins to Tn501, the differences range from one amino acid (in *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041) and two amino acids (in *E. coli* Tn21 and *Pseudomonas* sp. K-62 pMR26) in the first region. In the second region, the differences range from one amino acid (in *Xanthomonas* sp. Tn5053), two amino acids (in *S. marcescens* pDU1358) and three amino acids (in *S. flexneri* Tn21, *Pseudomonas* sp. K-62 pMR26, *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041). In the third region, the differences range from one amino acid (in *S. flexneri* Tn21, *Pseudomonas* sp. K-62 pMR26 and *S. marcescens* pDU1358), two amino acids (in *Xanthomonas* sp. Tn5053 and *X. campestris* Tn5044) and three amino acids (in and *Pseudomonas* sp. Tn5041). As was the case when comparing the MerR genes from Gram-positive bacteria, when comparing the potential transmembrane regions in Tn501 to the Gram-positive bacteria MerT proteins and amongst the Gram-positive bacteria MerT proteins, very little homology exists.

A comparison between the *merP* of Tn501 and the *merP* genes from other Gram-negative bacteria reveals homologies ranging from 98.91% for *P. stutzeri* pPB to

79.71% for *Pseudomonas* sp. Tn5041. Of the Gram-positive bacteria used for comparison, only *B. cereus* RC607, *B. megaterium* MB1 and *Streptomyces* sp. CHR28 possess the *merP* gene, with the levels of homology when compared to Tn501 being 40.44%, 40.44% and 41.3% respectively. When these Gram-positive bacteria genes were compared to each other, 100% homology exists between the two *Bacillus merP* genes and a 30.63% homology exists when comparing *Streptomyces* sp. CHR28 to both the *Bacillus* species. The cysteines of Tn501 (Cys14 and Cys17) were found to be present in all Gram-negative bacteria MerP proteins compared, as well as in the three Gram-positive bacteria compared. It is thought that Phe38 and Tyr66 may be involved in the mercury-binding reaction and transfer of Hg(II) to MerT (Serre *et al.*, 2004). These amino acids were found to be present in all Gram-negative bacteria compared, however these were not present in the Gram-positive bacteria. The potential signal sequence of MerP, between positions 1-18 (in Tn501), differs from one amino acid (in *S. flexneri* Tn21, *Pseudomonas* sp. K-62 pMR26 and *S. marcescens* pDU1358), three amino acids (in *Xanthomonas* sp. Tn5053 and *X. campestris* Tn5044) and four amino acids (*Pseudomonas* sp. Tn5041). In MerP, the HMA domain of the periplasmic component spans positions 22-89, with differences ranging from 1 amino acid to 20 amino acids between the Gram-negative bacteria. Very little homology exists between these regions of the MerP protein when comparing Gram-negative bacteria and Gram-positive bacteria proteins.

For the *merA* gene, when comparing Tn501 to other Gram-negative bacteria, the levels of homology ranged from 99.63% for *P. stutzeri* pPB to 41.39% for *Xanthomonas* sp. Tn5053. Of the Gram-positive bacteria used for comparison, the levels of homology with Tn501 ranged from 57.52% for *S. lividans* to 45.72% for both *B. cereus* RC607 and *B. megaterium* MB1. When these Gram-positive bacteria genes were compared to each other, 100% homology exists between the two *Bacillus merA* genes and a 57.47% homology exists when comparing *Streptomyces* sp. CHR28 to both the *Bacillus* species. A total of 8 cysteines exist in the Tn501 MerA protein (Cys10, Cys13, Cys135, Cys140, Cys236, Cys404, Cys558 and Cys559). All of these cysteines are present in all the compared Gram-negative bacteria MerA proteins. The Cys10, Cys13, Cys135, Cys140, Cys558 and Cys559 were all present in the Gram-positive bacteria MerA proteins used for comparison. In place of Cys404, all compared Gram-positive bacteria possessed a Valine, while in the place of Cys236, all Gram-positive bacteria MerA proteins

contained a Phenylalanine, with the exception of *S. lividans* and *Streptomyces* sp. CHR28, which possessed a Tyrosine. The MerA protein of Tn501 contains an HMA domain which spans positions 1-66, which is variable between Gram-negative bacteria MerA proteins with the total amino acid differences ranging between 2 amino acids (in *P. stutzeri* pPB) and 34 amino acids (in *Pseudomonas* sp. K-62). When comparing this region in Tn501 to the corresponding region in the Gram-positive bacteria MerA proteins, very little homology can be noted. A FAD-binding site is present in Tn501 between positions 128-136. Of the Gram-negative bacteria, only *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041 contain one amino acid difference each when compared to Tn501. The corresponding regions in the Gram-positive bacteria MerA proteins also contain only one amino acid difference when compared to Tn501. A disulphide bond is present in Tn501 between positions 136 and 141. With the exception of one amino acid in *P. stutzeri* pPB and *Xanthomonas* sp. Tn5053, this region is identical in all the Gram-negative bacteria analysed. With the exception of *S. aureus* pI258, which also contains only one amino acid difference compared to Tn501, the corresponding region in the Gram-positive bacteria are identical to that of Tn501.

The *merD* gene of Tn501 is also only present in the Gram-negative bacteria used for this analysis. The levels of homology between these when compared to Tn501 range from 97.26% in *P. stutzeri* pPB to 61.94% in *X. campestris* Tn5044. Although cysteines have not been implicated as yet in the literature to play a role in MerD protein function, three conserved cysteines were determined in the Gram-negative bacteria MerD proteins. Corresponding to Tn501, Cys34 was present in all of the MerD proteins, with the exception of *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041, while Cys51 was present in all, with the exception of *Pseudomonas* sp. Tn5041. Cys70 was present in all of the analysed Gram-negative bacteria MerD proteins.

The *merE* gene and URF-1 have been compared together in this analysis, as in each of the operons, this is the gene directly following the *merD* gene when present. This gene is present in the Gram-negative bacteria *P. stutzeri* pPB, *Xanthomonas* sp. Tn5053, *S. marcescens* pDU1358, *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041 and the Gram-positive bacteria *S. aureus* pI258, *B. cereus* RC607 and *B. megaterium* MB1. The levels of homology when comparing the Tn501 *merE* to the other Gram-negative bacteria ranged from 100% in *P. stutzeri* pPB to 58.69% in *X. campestris* Tn5044.

When comparing the Gram-positive bacteria *merE*/URF-1 genes to Tn501 *merE*, the levels of homology range from 40.58% for both *B. cereus* RC607 and *B. megaterium* MB1 to 34.61% in *S. aureus* pI258. Although cysteines have not been implicated as yet in the literature to play a role in MerE/URF1 protein function, two cysteines (Cys28 and Cys30 corresponding to Tn501) have been found in all Gram-negative bacteria and Gram-positive bacteria proteins analysed.

In the absence of the *merB* gene in Tn501, that of *S. marcescens* pDU1358 was used for comparison with the *merB* genes of *Pseudomonas* sp. K-62 (*merB1* and *merB2*) and *P. stutzeri* pPB. The homologies between these genes and that of pDU1358 ranged from 99.21% in *Pseudomonas* sp. K-62 *merB2* to 43.45% in *P. stutzeri* pPB. The *merB* genes from Gram-positive bacteria were also compared to pDU1358, including *B. cereus* RC607 and *B. megaterium* MB1 (*merB1*, *merB2* and *merB3*), *S. lividans* and *Streptomyces* sp. CHR28. The homologies between pDU1358 and these genes ranged from 50.33% in *Streptomyces* sp. CHR28 and 36.81% in both the *merB3* genes of *B. cereus* RC607 and *B. megaterium* MB1. The MerB protein of pDU1358 contains four cysteines (Cys96, Cys117, Cys159 and Cys160). Cys96, Cys117 and Cys159 were present in all MerB proteins analysed, with the exception of Cys159 from the MerB3 proteins of *B. cereus* RC607 and *B. megaterium* MB1. Cys160 was present in all Gram-negative bacteria with the exception of *P. stutzeri* pPB, while it was not present in any of the Gram-positive bacteria. An additional cysteine pair is located in positions 213/214 in *S. aureus* pI258 MerB, which is also found in corresponding positions in the MerB1 proteins of *B. cereus* RC607 and *B. megaterium* MB1 and also in *S. lividans* and *Streptomyces* sp. CHR28.

From the Gram-negative bacteria used for this analysis, the *merC* gene is present in *S. flexneri* Tn21, *P. stutzeri* pPB, *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041, while no *merC* was present in any of the Gram-positive bacteria used in this analysis. Using Tn21 for comparison, the homologies ranged between 72.857% for *X. campestris* Tn5044 and 67.83% for *X. campestris* Tn5044. There are four cysteines found in the MerC protein of Tn21 (Cys22, Cys25, Cys127 and Cys132) which are also present in all the MerC proteins used for this analysis. Tn21 also contains an N-terminal motif Cys-Ala-Ala-(X)-Cys-Phe-Pro-Ala, which is substituted with Threonine, Valine and

Methionine in *P. stutzeri* pPB, *X. campestris* Tn5044 and *Pseudomonas* sp. Tn5041, respectively.

From the above analysis, it is suggested that the *merR*, *merT* and *merP* genes show the greatest level of homology amongst the operons analyzed. Within these genes, it appears that the Gram-negative bacteria collectively share greater levels of homology than do the genes from Gram-positive bacteria, while a comparison between Gram-negative bacteria and Gram-positive bacteria show even less degrees of homology. Overall, it would appear that the *merA* gene is more divergent than the smaller *merR*, *merT* and *merP* genes in both the Gram-negative bacteria and Gram-positive bacteria examples, as are the less common genes, *merD* and *merE/URF1*. The additional genes, *merB* and *merC*, also demonstrate less similarities across all genes analysed. These observations indicate that while the *mer* operons are widespread in nature, the genes have evolved quite differently amongst the different species within both Gram-negative bacteria and Gram-positive bacteria.

1.5 Lead resistance

As described in Section 1.1, lead contamination occurs in the environment due to activities such as the manufacture of batteries, ammunition, solder and pipes. Lead contamination can occur in food and water sources, leading to health problems associated with the nervous system, kidneys, reproductive system and blood (ATSDR(b)). Bacteria have been isolated from contaminated environments and have shown lead resistant phenotypes and include *S. aureus* (Levinson *et al.*, 1996), *Citrobacter freundii* (Levinson and Mahler, 1998), *Pseudomonas marginalis* and *B. megaterium* (Roane, 1999). Further characterisation of these isolates indicated that *P. marginalis* showed extracellular lead extrusion, while *B. megaterium* demonstrated intracellular cytoplasmic accumulation of lead (Roane, 1999).

While lead is an extensive environmental contaminant, lead resistance systems have not been as widely studied as for mercury resistance, with the genetic mechanisms of lead resistance having only been identified from pMOL30 of *C. metallidurans* CH34. This isolate, formerly known as *Alcaligenes eutrophus*, has been studied for its resistance to

Table 1.4 Homologies (%) of various *mer* genes and proteins

Organism/ Operon	merR		merT		merP		merA		merD		merE/URF1		URF2	
	gene	protein	gene	protein	gene	protein								
<i>P. aeruginosa</i> Tn501														
<i>P. stutzeri</i> pPB	100.0	100.0	100.0	100.0	99.63	98.91	96.85	98.75	97.26	98.36	100.0	100.0	99.69	99.09
<i>Xanthomonas</i> sp. W17 Tn5053	89.42	91.66	95.15	93.16	88.40	93.47	41.39	36.23	95.08	97.54	79.32	88.60		
<i>S. flexneri</i> Tn21 R100	87.81	95.86	82.90	90.59	86.59	91.30	82.29	88.59	83.19	84.29				
<i>Pseudomonas</i> sp. K-62	85.28	93.10	82.90	90.59	72.10	81.52	66.84	76.97	88.25	86.88				
<i>S. marcescens</i> pDU1358	85.05	88.96	90.88	94.87	93.47	95.65	86.38	90.05	91.25	91.80	76.79	83.54	88.73	88.96
<i>X. campestris</i> Tn5044	74.02	78.67	80.91	86.32	80.43	93.69	73.55	77.54	61.94	62.39	58.69	53.24	65.74	58.57
<i>Pseudomonas</i> sp. Tn5041	72.18	76.55	78.34	88.30	79.71	80.43	74.76	80.32	63.53	63.33	63.23	65.67	60.71	50.44
<i>S. aureus</i> pl258	38.85	35.29	35.22	36.84			47.74	53.37			34.61	26.66		

Organism/Operon	merC		Organism/Operon	merB	
	gene	protein		gene	protein
<i>E. coli</i> Tn21 R100			<i>S. marcescens</i> pDU1358		
<i>X. campestris</i> Tn5044	74.048	72.857	<i>Pseudomonas</i> sp. K-62 pMR26 -B2	99.218	99.531
<i>P. stutzeri</i> pPB	71.631	69.504	<i>Pseudomonas</i> sp. K-62 pMR26 -B1	59.524	61.722
<i>Pseudomonas</i> sp. Tn5041	66.512	67.832	<i>S. aureus</i> pl258	48.738	52.427
			<i>P. stutzeri</i> pPB	43.457	30.882

References: as per Table 1.3

a number of heavy metals, including lead and mercury (as mentioned briefly in Section 1.4.1.3) (Diels *et al.*, 1989). Pb(II) resistance clones were isolated from a pMOL30 library and the sequence and orientation of the *pbr* operon was determined (refer to Figure 1.5 for the orientation for the *pbr* genes) (Borremans *et al.*, 2001). DNA sequence analysis of these clones revealed a *merR* homologue, referred to as *pbrR*, which regulates the expression of the other genes involved in Pb(II) resistance (Borremans *et al.*, 2001). The promoter P_{pbrA} , which is located between the *pbrR* and *pbrA* genes, has a 19 bp spacer (as does P_{merT}) between the -35 and -10 sequences and there is a conservation of the three cysteine residues known to be important in the metal binding of MerR. The transcription of P_{pbrA} is induced by Pb(II) ions and PbrR responds to Pb(II), but not significantly to other metals, indicating a high level of specificity (Corbisier *et al.*, 1999). A PbrR homologue (PbrR691) has been identified from the chromosome of *C. metallidurans* CH34. This protein displays selectivity towards Pb(II) over other metals and has been shown to bind one Pb(II) ion per PbrR691 dimer (Chen *et al.*, 2005).

The remainder of the genes involved in lead resistance combine the functions involved in uptake, efflux and accumulation of Pb(II). A Pb(II) uptake protein is encoded by the *pbrT* gene, is transcribed along with *pbrR*, divergently from *pbrABCD*. Experiments have shown that expression of *pbrT* in the absence of *pbrABCD* results in Pb(II) sensitivity, probably due to increased Pb(II) uptake in the cytoplasm (Borremans *et al.*, 2001). The *pbrA* gene encodes the PbrA protein, an efflux ATPase that possesses two Heavy Metal-Associated (HMA) motifs with the amino acids sequence Cys-Pro-Thr-Glu-Glu instead of the Cys-X-X-Cys consensus sequence seen in other ATPases (as mentioned in Section 1.4) (Borremans *et al.*, 2001). The gene *pbrB* encodes a predicted integral protein, of unknown function, while a prolipoprotein signal peptidase is encoded by the gene *pbrC*. It is hypothesised that the PbrC prolipoprotein peptidase is required for the processing of the PbrB prolipoprotein. Downstream from the *pbrC* gene is the *pbrD* gene, which encodes a Pb(II) binding protein, essential for lead sequestration (Borremans *et al.*, 2001). Cells lacking PbrD show a decreased accumulation of Pb(II) compared to wild-type cells. It has been suggested that this protein may protect against free exported Pb(II) in the cell. Once Pb(II) has entered the cytoplasm, it is exported by the PbrA efflux ATPase or it may be

bound by the PbrD, which may function as a chaperone, transferring Pb(II) to PbrA. It has also been found that PbrA is functional and able to compensate for full Pb(II) uptake driven by PbrT, however for full Pb(II) resistance, PbrB and PbrC are required (Borremans *et al.*, 2001).

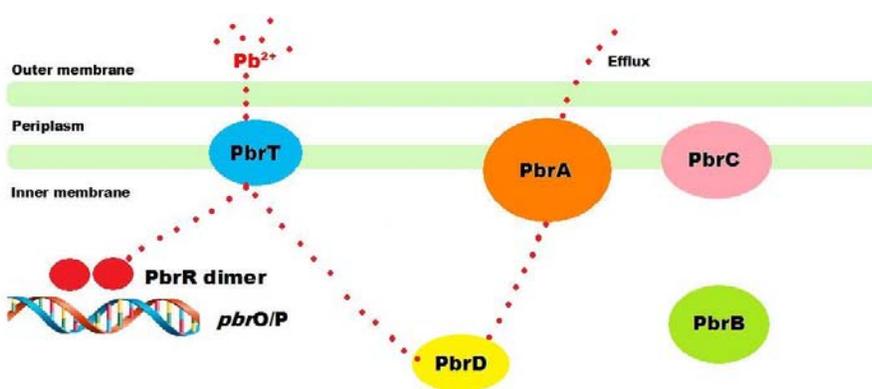


Figure 1.5 Model for *pbr* Pb(II) resistance operon-encoded lead resistance of *C. metallidurans* CH34.

1.6 Cadmium, zinc and cobalt resistance

As mentioned in Section 1.1, cadmium, zinc and cobalt may contaminate the environment through a number of industrial activities such as the manufacture of batteries, plastics, alloys, magnets, paint and rubber. Such activities may lead to contamination of the air, water and foods and target organs include lungs, stomach, heart and kidneys. Bacteria have been isolated that exhibit resistances to these contaminants and their genetic mechanisms determined. These include the *cad* operon of *S. aureus* pI258 which encodes cadmium resistance and the *czc* operon of *C. metallidurans* CH34, which encodes resistance to cadmium, zinc and cobalt. These genetic systems are described further in Sections 1.6.1 and 1.6.2.

1.6.1 The *cad* operon

Cadmium resistance has been determined in the Gram-positive bacteria species *S. aureus*, in which cadmium resistance determinants in two overlapping open reading frames have been identified on plasmid pI258 (Nucifora *et al.*, 1989b). The first open reading frame, *cadC*, encodes a soluble protein and the second, *cadA*, encodes the integral membrane protein, which is a soft metal ion-translocating P-type ATPase (Silver *et al.*, 1989; Lutsenko and Kaplan, 1995; Rensing *et al.*, 1999) and confers resistance to Cd(II), Zn(II) and Pb(II) (Nucifora *et al.*, 1989b; Yoon and Silver, 1991; Yoon *et al.*, 1991; Rensing *et al.*, 1998).

CadA catalyses the ATP-dependent efflux of Cd(II) from the cell cytosol in an electro-neutral exchange that transfers one Cd(II) ion out of the cell whilst accumulating two protons (Nucifora *et al.*, 1989b; Tsai *et al.*, 2002). The CadA ATPase has been shown to contain 8 transmembrane segments, the first comprising of a cytosolic domain that includes the Cys-X-X-Cys motif and the sixth comprising a Cys-Pro-Cys motif, both of which are distinguishing features of P-type ATPases. The phosphorylation site and ATP binding domain, conserved in all P-type ATPases, is situated within the large cytoplasmic loop, between the sixth and seventh transmembrane segments (Tsai *et al.*, 2002).

The CadC protein is a DNA-binding, negatively acting regulatory protein (Endo and Silver, 1995) and is a 27.6 kDa homodimer, composed of two 122 amino acid monomers, which must both have metal binding sites to be functional (Sun *et al.*, 2002). The binding of thiophilic divalent cations, including Cd(II), Zn(II) and Pb(II), to this *trans*-acting repressor protein allosterically regulates the DNA-binding activity of CadC to the *cad* operon region, with CadC binding to the proposed region as a DNA-CadC dimer in the absence of metal ions and dissociating in the presence of inducers allowing transcription of the *cadCA* genes (Yoon *et al.*, 1991; Yoon and Silver, 1991; Corbisier *et al.*, 1993; Endo and Silver, 1995; Tauriainen *et al.*, 1998; Busenlehner *et al.*, 2001; Sun *et al.*, 2001). When produced, CadC chelates metal ions using at least three cysteine residues (Cys7, Cys58, Cys60), which are conserved in all CadC homologues, and potentially a single carboxylic acid group (Sun *et*

al., 2001). This has been further strengthened by data suggesting that the two Cd(II) binding sites in the CadC homodimer are composed of Cys7 and Cys11 from one monomer and Cys58 and Cys60 from the other monomer (Wong *et al.*, 2002). *In vivo* CadC repression from the *cad* OP may be alleviated by the addition of Cd(II), Pb(II), Bi(II) and Zn(II) (Yoon *et al.*, 1991, Rensing *et al.*, 1998). DNaseI footprinting experiments have shown that the binding of CadC protects a 20 bp region between the –10 hexamer and the translational start site of the *cad* OP (Endo and Silver, 1995). Figure 1.6 provides an overview of the transcription and expression of the *cadCA* operon.

A system for resistance as described for the *cadCA* operon of pI258 has also been described in *Listeria monocytogenes*. From plasmid pLm74, a 3.1 kb *Eco*RI fragment hybridised to the *cadCA* genes from pI258. When this fragment was introduced into cadmium-sensitive strains of *L. monocytogenes* and *Bacillus subtilis*, cadmium resistance was observed. DNA sequencing of this fragment indicated two open reading frames and the deduced amino acid sequences were similar to those of CadA and CadC of pI258 (Lebrun *et al.*, 1994). CadA in *L. monocytogenes* contains 4 cysteine residues as described for pI258, two in the Cys-Thr-Asn-Cys sequence of the metal-binding domain (MBD) and two in the Cys-Pro-Cys sequence in the membrane domain. It has been shown that all four cysteine residues are required for phosphorylation (Bal *et al.*, 2003).

Examples of other bacteria that possess genes similar to the *cadCA* genes mentioned above include the *cadA* gene of *P. putida* (Lee *et al.*, 2001) and the *cadCA* genes of *Bacillus firmus* (Ivey *et al.*, 1992), *Bacillus stearothermophilus* (Vasquez *et al.*, 2000) and *Lactococcus lactis* (Liu *et al.*, 1997). The homologies of these genes were compared to those from pI258. For the *cadA* genes, these were 48.81%, 76.94%, 5.59% and 65.86% for *P. putida*, *B. firmus*, *B. stearothermophilus* and *L. lactis* respectively. When the CadA proteins were compared, all with the exception of *P. putida* contained the Cys-X-X-Cys motif in the cytosolic domain (corresponding to residues 23-26 in pI258) and all contained the Cys-Pro-Cys motif of the sixth transmembrane segment. The *cadC* genes showed 84.28%, 55% and 55.27% for *B. firmus*, *B. stearothermophilus* and *L. lactis*, respectively, when compared to *S. aureus* pI258. The protein comparisons of the CadC protein revealed

that all contained the Cys7, Cys11, Cys58 and Cys60 residues which form the homodimer, with the exception of *B. stearothersophilus*, which contains a Serine rather than Cys11.

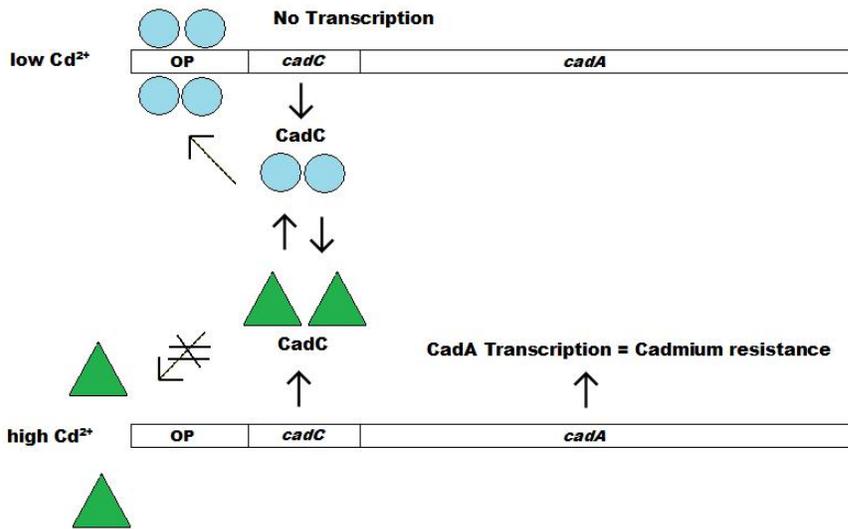


Figure 1.6 Mechanisms of transcription and expression of the *cadCA* operon.

1.6.2 The *czc* operon

While the *cad* operon encodes resistance to cadmium, the *czc* operon of pMOL30 of *C. metallidurans* CH34, which encodes multiple resistances to cadmium, zinc and cobalt (as mentioned in Section 1.4.1.3), has been cloned and characterised.

The products of *czcA*, *czcB* and *czcC* form a membrane-bound protein complex that catalyses an energy-dependent efflux of the three metal ions (Nies, 1992a). CzcA acts as an anion/cation antiporter and contains 12 transmembrane α -helices and two large periplasmic domains. There are two conserved Aspartate residues and a Glutamate residue in one of the transmembrane segments, which is essential for heavy metal resistance and

proton/cation antiport, but not for facilitated diffusion of cations. CzcB functions as a cation-binding subunit, while the CzcC protein acts as a modifier, to extend the substrate specificity to Co(II) and Cd(II) ions (Nies, 1992a). This model suggests that CzcA is a two-channel pump (Goldberg *et al.*, 1999). The CzcB protein contains two Histidine-rich motifs and CzcC is thought to be a periplasmic protein. It has been shown that CzcB and CzcC are bound to the membrane and do not require CzcA or each other to act as anchoring proteins (Rensing *et al.*, 1997a). Upstream of *czcCBA*, referred to as the upstream regulatory region (URR) are two genes of unknown function, *czcN* and *czcI* and the promoters *czcNp*, *czcIp* and *czcCp* (Grobe *et al.*, 1999). Figure 1.7 provides an overview of the model for the function of the CzcCBA efflux complex.

Downstream of *czcCBA*, is the downstream regulatory region, (DRR), comprising of the *czcDRS* genes, transcribed from the *czcDp* promoter (van der Lelie *et al.*, 1997; Große *et al.*, 1999). CzcD is the first prokaryotic protein to be described as a cation diffusion facilitator (CDF) (Nies, 1992b; Paulsen and Saier, 1997; Anton *et al.*, 1999; Kunito *et al.*, 1996). CzcD is a membrane bound protein containing at least four transmembrane α -helices. Deletion of *czcD* in *C. metallidurans* CH34 led to partially constitutive expression of the *czc* system due to an increased transcription of the *czcCBA* genes (Anton *et al.*, 1999). The *czcR* and *czcS* genes transcribe a two-component regulatory system, composed of a histidine kinase CzcS and a response regulator, CzcR (Grobe *et al.*, 1999). A newly identified gene, *czcE* has been identified and it is believed that CzcE acts with CzcR and CzcS to form a regulation pathway for the optimal expression of the CzcCBA efflux pump (Grobe *et al.*, 2004).

It has been suggested by van der Lelie *et al.*, (1997) that there may be four stages involved for the induction of the *czc* operon. The first stage occurs only in the presence of trace levels of cadmium, zinc or cobalt. This involves very little transcription of the URR and no expression of *czcCBA* or *czcRS*, with only *czcD* and *czcI* being expressed. In this case, CzcD detects the presence of heavy metal cations in the periplasm. At this point (stage two) CzcD interacts with CzcI, resulting in low level expression of *czcCBA*. With an increase in intracellular levels of heavy metal cations (stage 3), *czc* expression becomes

activated due to the actions of CzcR and CzcS. When the intracellular concentration of heavy metals lowers (stage four), some basal expression of *czc* remains as a result of CzcD and CzcI.

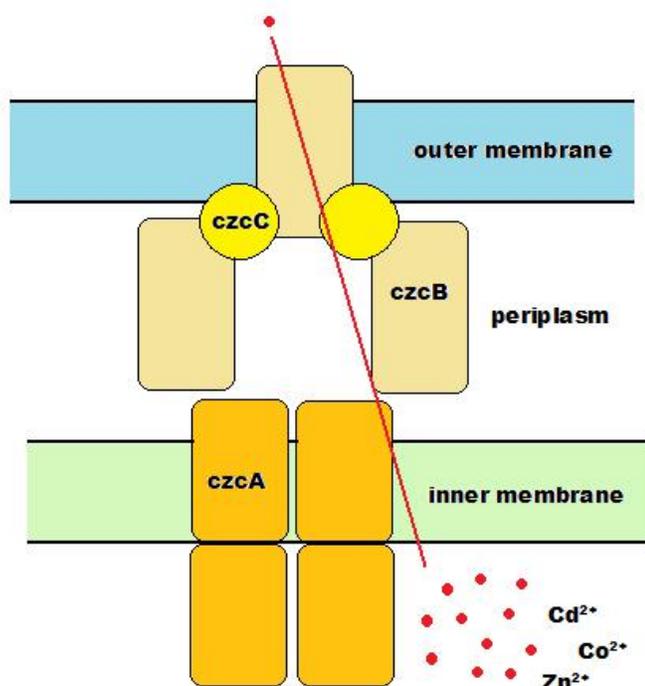


Figure 1.7 Model for the function of the Czc efflux complex. • denotes divalent cations of zinc, cobalt and cadmium; CPM denotes cytoplasmic membrane; OM denotes outer membrane.

The genes *czcA*, *czcB*, *czcC* and *czcD* have been published from both *C. metallidurans* CH34 (Nies *et al.*, 1989; Nies, 1999; Nies, 1992b; van der Lelie *et al.*, 1997) and *Alcaligenes* sp. (Kunito *et al.*, 1996). These genes were aligned and found to share homologies of 99.78% (*czcA*), 99.8% (*czcB*), 99.36% (*czcC*) and 99.79% (*czcD*). The genes *czcI*, *czcN*, *czcR* and *czcS* have to date only been published for *C. metallidurans* CH34.

1.7 PAH degradation

Along with heavy metals, another toxic contaminant found in the environment are the PAHs (Section 1.3), which are used in the production of coal tar, crude oil, creosote and roofing tar. PAHs are also used in the manufacture of dyes, plastics and pesticides. The release of PAHs into the environment may lead to air, water and food contamination, potentially having carcinogenic effects. In animals, PAH exposure has been shown to have detrimental effects on the skin, immune systems and reproductive system (ATSDR(g)). From soil, Gram-positive bacteria and Gram-negative bacteria can be readily isolated, which have the ability to degrade naphthalene, phenanthrene and anthracene. Microorganisms capable of degrading or co-metabolising the higher molecular weight PAHs have also been identified. Such genera include *Pseudomonas* sp., *Alcaligenes* sp., *Rhodococcus* sp., *Beijerinckia* sp., *Mycobacterium* sp., *Staphylococcus* sp., and *Arthrobacter* sp. (Cerniglia, 1992). The following sections will address some of the genetic mechanisms responsible for the degradation of some PAHs.

1.7.1 The *nah* operon

The degradation of the simplest PAH, naphthalene, has been studied extensively. In *P. putida* PpG7, the NAH7 plasmid carries a number of genes collectively responsible for naphthalene degradation on two separate operons (Kurkela *et al.*, 1988; Tsuda and Iino, 1990; Sota *et al.*, 2006). The first operon (*nahAaAbAcBFCQED*) encodes the enzymes responsible for the oxidation of naphthalene to salicylate, while the second operon (*nahGTHINLJKM*) (sometimes referred to as the *sal* operon), encodes the breakdown of salicylate to central metabolites via the meta-pathway (Williams and Sayers, 1994). The two *nah* operons are regulated by a *trans*-acting positive regulator, encoded by the gene *nahR*, the product of which is required for the high level of expression of the *nah* genes (Kurkela *et al.*, 1988). The same two-operon structure has been reported on a second plasmid, pWW60-1 from *P. putida* NCIB9816, although their relative positions and orientations differ (Williams and Sayers, 1994).

1.7.1.1 Regulation of the *nah* operon

The *nahR* gene of plasmid NAH7 binds specifically to and activates transcription of the *nah* and *sal* operons in response to salicylate. The transcription start site of the *nahR* gene was found 60 bp upstream of the transcription start site of *sal*. The *sal* and *nahR* promoters overlap at -35, however they are transcribed in opposite directions (Schell, 1986). DNaseI protection assays show that NahR protects both *nah* and *sal* promoter sequences between -82 and -47. Comparison of the two protected by NahR indicates a “consensus NahR-binding site” consisting of a 21-bp highly homologous region centered around -70 and a 12 bp A + T-rich region (>85%) between -60 and -48 (Schell and Poser, 1989). It is suggested that the *nahR* gene is constitutively expressed at a low level, where its product remains bound to both the *nah* and *sal* promoters at the -82 to -47 region. In the absence of the inducer, the NahR protein has little effect on transcription, but may repress its own expression as one of the NahR-protected regions also contains the transcription start site and the -10 sequence of the *nahR* gene. The inducer, salicylate, is thought to bind to the NahR protein to alter its structure, allowing it to change the promoter structure or that of RNA polymerase, which results in the transcription of the *nah* and *sal* operons (Schell and Poser, 1989). Both in the presence and absence of salicylate, NahR has close contact with two guanines in a 4-bp symmetrical interrupted dyad, which is part of a highly conserved 16 bp sequence found in both the *nah* and *sal* promoters. In the presence of salicylate, several additional guanines between -35 and -52 become enhanced or inhibited from methylation by NahR, which suggests a conformational change in the NahR-DNA complex, leading to transcriptional activation (Huang and Schell, 1991).

Sequence analysis of NahR has shown that amino acid substitutions clustered in an NH₂-terminal helix-turn-helix motif (residues 23-45) or a COOH-terminal domain (239-291) eliminated DNA-binding activity. Analysis of *nahR* termination mutants showed that the NH₂-terminal HTH motif was not sufficient for DNA binding alone, while a NahR protein lacking in the last nine amino acids was completely inactive, suggesting the COOH-terminal domain is also vital for DNA binding activity. The lack of a typical DNA-binding motif within the COOH-terminal domain suggests that this region may be involved in

maintenance of the structure necessary for positioning of the NH₂-terminal HTH motif (Schell *et al.*, 1990).

It has been shown that residues Arg248, Asn169, Arg132 and Met116 are important in maintaining the specificity of salicylate action on NahR for the induction of the *nah* and *sal* promoters. Mutations in the central domain of NahR were shown to increase the range of aromatic compounds that can activate NahR into a transcriptionally competent form. By performing the following mutations; Arg248Cys, Asn169Asp, Arg132Cys, Met116Ile, Met116Thr, Met116Val, inducer specificity was expanded to not only include salicylate, but also benzoate, salicylamide, 2-OH benzylalcohol, 2-chloro-benzoate, 3-chlorobenzoate and 4-chlorobenzoate (Cebolla *et al.*, 1997). Further studies on Asn169 and Arg248 showed that while the wild-type NahR bound to the promoter in the absence of salicylate, various mutant forms of NahR showed altered binding affinities to the *sal* promoter. Mutant forms Asn169Gln, Asn169Glu, Asn169Glu/Arg248Cys and Asn169Glu/Arg248Lys showed weaker binding affinity to the *sal* promoter, while Asn169Asp and Asn169Asp/Arg248Lys showed an increased affinity to the *sal* promoter. These results indicate that residues 169 and/or 248 have a greater effect on DNA binding than on activation (Park *et al.*, 2005a). Park and co-workers (2005b) have also presented data suggesting that the integral DNA binding domain of NahR may be required for NahR binding to the promoter and the presence of salicylate may bind to rebound NahR for transcriptional activation by RNA polymerase.

1.7.1.2 The upper *nah* operon (*nah*)

In the *nahAaAbAcBFCQED* operon (upper pathway), of the NAH7 plasmid, a multicomponent enzyme naphthalene dioxygenase is encoded by four genes; *nahAa* (reductase), *nahAb* (ferredoxin), *nahAc* (iron sulphur protein component-large subunit) and *nahAd* (iron sulphur protein component-small subunit) (Simon *et al.*, 1993). In the NAH7 plasmid, both *nahB* and *nahF* encode dehydrogenase enzymes, with *nahB* specifically encoding a *cis*-1,2-dihydroxynaphthalene-1,2-dehydrogenase and *nahF* specifically encoding a salicylaldehyde dehydrogenase, while *nahC* encodes 1,2-dihydroxynaphthalene

dioxygenase (Harayama and Reikik, 1989; Tsuda and Iino, 1990). Also within this operon, *nahD* encodes 2-hydroxychromene-2-carboxylate isomerase, *nahE* encodes a *trans-o*-hydroxybenzylidenepyruvatehydratase aldolase and *nahQ* encodes a protein of unknown function (Tsuda and Iino, 1990). It has been shown that the expression of at least two genes (*nahB* and *nahF*) is regulated by the presence of salicylic acid, a product of the conversion of naphthalene (Schell, 1983).

1.7.1.3 The lower *nah* operon (*sal*)

The *nahGTHINLOMJKXY* (lower pathway) of NAH7 plasmid encodes a number of enzymes for the breakdown of salicylate to central metabolites (Williams and Sayers, 1994). The *nahI* and *nahO* genes both encode for dehydrogenases, with *nahI* encoding 2-hydroxyomuonic semialdehyde dehydrogenase and *nahO* encoding acetaldehyde dehydrogenase. *nahG* encodes salicylate hydroxylase, *nahT* encodes a ferredoxin and *nahH* encodes catechol-2,3-dioxygenase (Tsuda and Iino, 1990). *nahN* encodes 2-hydroxyomuonic semialdehyde hydrolase, *nahL* encodes 2-oxypent-4-enoate and *nahM* encodes 4-hydroxy-2-oxovalerate aldolase (Tsuda and Iino, 1990). *nahK* encodes 4-oxalocrotonate decarboxylase, *nahJ* encodes 4-oxalocrotonate tautomerase and *nahX* encodes a protein of unknown function (Tsuda and Iino, 1990). *nahY* encodes a 538 amino acid protein with membrane topology and a C-terminal region with similarities to those of chemotaxis transducer proteins. Studies have suggested that NahY functions as a chemoreceptor, which is likely to bind naphthalene on its periplasmic face to initiate chemosensory signaling (Tsuda and Iino, 1990; Grimm and Harwood, 1999).

Figure 1.8 provides an overview of the naphthalene metabolic pathway, encoded by the *nah* operon of the NAH7 plasmid.

1.7.2 Diversity of genes relating to PAH degradation

Examples of other bacteria that possess genes for PAH degradation, similar to those of the NAH7 plasmid, include those of the *nah* operon of *P. stutzeri* (Bosch *et al.*, 1999a), the *phn* operon of *Burkholderia* sp. RP007 (Laurie and Lloyd-Jones, 1999), the *pah* operon of *Commonas testosteroni* (Moser and Stahl, 2001) and the *nag* operon of *Ralstonia* sp. U2 (Fuemayor, *et al.*, 1998). Table 1.5 summarizes some of the bacteria reported to possess PAH degradation genes and their relevant operons and the encoded proteins.

The homologies of the documented genes from these isolates were compared with those of NAH7 and this analysis is outlined in Table 1.6. It was determined that the degrees of homology between these related genes ranges from 89.94% between *nahF* from NAH7 and from *P. stutzeri* to 35.83% between the *nahH* gene of NAH7 and the *pahH* gene of *C. testosteroni*. The *nahR* genes were compared and the gene homologies were found to be 77.96%, 42.49% and 65.67% for the *nahR* gene of *P. stutzeri*, the *phnR* gene of *Burkholderia* sp. RP007 and the *nagR* gene of *Ralstonia* sp. U2 respectively, when compared to NAH7. The regulatory gene of the *pah* operon of *C. testosteroni* has not as yet been identified. These regulatory proteins were compared and the NH₂-terminal helix-turn-helix motif (residues 23-45) or a COOH-terminal domain (239-291) believed to be responsible for DNA-binding activity (Schell *et al.*, 1990) were analyzed. Various degrees of homology were noted in these regions with the NH₂-terminal helix-turn-helix motif of NAH7 NahR showing 86.95%, 15.00% and 78.26% homology and the COOH-terminal domain showing 72.91%, 29.16% and 60.46% homology for NahR of *P. stutzeri*, PhnR of *Burkholderia* sp. RP007 and NagR of *Ralstonia* sp. U2 respectively. It has been suggested that residues Arg248, Asn169, Arg132 and Met116 are important in maintaining the specificity of salicylate action on NahR for the induction of the *nah* and *sal* promoters (Cebolla *et al.*, 1997). It was found in the NahR proteins compared that all contained Arg132 and Arg248, while *P. stutzeri* contained an Isoleucine and *Burkholderia* sp. contained an Alanine in place of Met116, *Burkholderia* sp. contained an Alanine and *Ralstonia* sp. contained Histidine in place of Asn169.

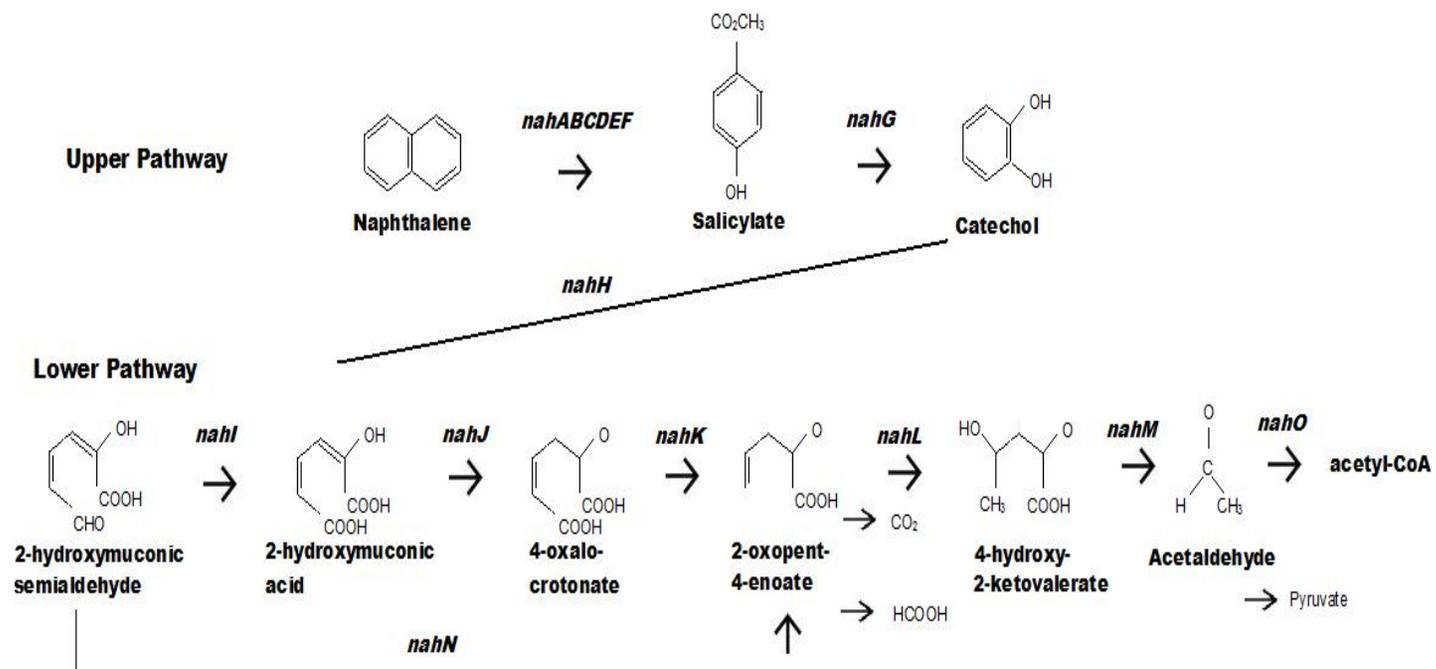


Figure 1.8 The naphthalene degradation pathway of *P. putida* G7.

Table 1.5 Diversity and organization of genes relating to PAH degradation

Organism	Operon structure	Gene products	References
<i>P. stutzeri</i>	Upper pathway: <i>nahAaAbAcAdBFCED</i> naphthalene→salicylate	Naphthalene dioxygenase reductase (<i>nahAa</i>), Naphthalene dioxygenase reductase (<i>nahAb</i>), Naphthalene dioxygenase Fe-S large subunit (<i>nahAc</i>), Naphthalene dioxygenase Fe-S small subunit (<i>nahAd</i>), <i>cis</i> -naphthalene dihydrodiol dehydrogenase (<i>nahB</i>), Salicylate dehydrogenase (<i>nahF</i>), 1,2-dihydroxynaphthalene dioxygenase (<i>nahC</i>), 1,2-dihydroxybenzylpyruvate aldolase (<i>nahE</i>), 2-hydroxychromene-2-carboxylate dehydrogenase (<i>nahD</i>)	Bosch <i>et al.</i> , 1999a Bosch <i>et al.</i> , 1999b
	Lower pathway: <i>nahGTHINLOMKJ</i> salicylate→pyruvate and acetyl-CoA	Salicylate hydroxylase (<i>nahG</i>), XylIT-like ferredoxin (<i>nahT</i>), Catechol 2, 3-dioxygenase (<i>nahH</i>), Hydroxymuconic semialdehyde dehydrogenase (<i>nahI</i>), Hydroxymuconic semialdehyde hydrolase (<i>nahN</i>), 2-oxopent-4-enoate hydratase (<i>nahL</i>), Acetaldehyde dehydrogenase (<i>nahO</i>), 2-oxo-4-hydropentanoate aldolase (<i>nahM</i>), 4-oxalocrotonate decarboxylase (<i>nahK</i>), 4-oxalocrotonate isomerase	
	<i>nahR</i> - transcribed divergently from <i>nahG</i>	Regulatory protein (<i>nahR</i>)	

Table 1.5 continued

Organism	Operon structure	Gene products	References
<i>P. aeruginosa</i>	<i>pahAA2A3A4BFCQE</i>	Reductase (<i>pahA</i>), Ferredoxin (<i>pahA2</i>), a large subunit of iron-sulfur protein (<i>pahA3</i>), a small subunit of iron-sulfur protein (<i>pahA4</i>), cis-1,2-dihydro-1,2-dihydroxynaphthalene dehydrogenase (<i>pahB</i>), Salicylaldehyde dehydrogenase (<i>pahF</i>), 1,2-dihydroxynaphthalene dioxygenase (<i>pahC</i>), a factor that facilitates naphthalene metabolism (<i>pahQ</i>), trans-o-hydroxybenzylidenepyruvate hydratase-aldolase (<i>pahE</i>)	Takizawa <i>et al.</i> , unpublished
<i>P. fluorescens</i>	<i>ndoC1C2C3</i>	naphthalene dioxygenase ferredoxin (<i>ndoC1</i>), naphthalene dioxygenase iron sulfur protein, large subunit (<i>ndoC2</i>), naphthalene dioxygenase iron sulfur protein (<i>ndoC3</i>)	Hamann,C, unpublished
<i>C. testosteroni</i>	<i>phnHAbAcAdBF</i> degradation of naphthalene and phenanthrene	Salicylate-5-hydroxylase small subunit-like protein (<i>pahH</i>), Ferredoxin (<i>pahAb</i>), PAH dioxygenase subunit (<i>pahAc</i>), PAH dioxygenase subunit (<i>pahAd</i>), cis-naphthalene dihydrodiol dehydrogenase-like protein (<i>pahB</i>), Salicylate dehydrogenase-like protein (<i>nahF</i>)	Moser and Stahl, 2001

Table 1.5 continued

Organism	Operon structure	Gene products	References
<i>Ralstonia</i> sp. strain U2	<i>nagRAaGHAcAdBFCQEDJIKLMN</i>	Regulator of the <i>nag</i> operon (<i>nagR</i>), Ferredoxin reductase (<i>nagAa</i>), Salicylate-5-hydroxylase large oxygenase component (<i>nagG</i>), Salicylate-5-hydroxylase small oxygenase component (<i>nagH</i>), Naphthalene 1,2-dioxygenase large oxygenase component (<i>nagAc</i>), Naphthalene 1,2 dioxygenase small oxygenase component (<i>nagAd</i>), <i>cis</i> -naphthalene dihydrodiol dehydrogenase (<i>nagB</i>), Salicylate dehydrogenase (<i>nagF</i>), 1,2-dihydroxynaphthalene dioxygenase (<i>nagC</i>), Putative aldolase (<i>nagQ</i>), <i>trans-o</i> -hydroxybenzylidenepyruvatehydratase-aldolase (<i>nagE</i>), 2-hydroxychromenecarboxylate isomerase (<i>nagD</i>), Glutathione-S-transferase-like protein (<i>nagJ</i>), Gentisate 1,2-dioxygenase (<i>nagI</i>), Fumarylpyruvate hydrolase (<i>nagK</i>), Maleylpyruvate isomerase (<i>nagL</i>)	Fuenmayor <i>et al.</i> , 1998 Zhou <i>et al.</i> , 2001

Table 1.6 Homologies (%) of the *P. putida* NAH7 PAH degradation genes compared to similar genes of other bacterial isolates

	<i>Aa</i>	<i>Ab</i>	<i>Ac</i>	<i>Ad</i>	<i>B</i>	<i>F</i>	<i>C</i>	<i>Q</i>	<i>E</i>	<i>D</i>	<i>R</i>
<i>P. stutzeri (nah)</i>	85.309	86.032	88.889	85.395	89.103	89.945	39.691		88.153	78.595	77.962
<i>Ralstonia sp.U2 (nag)</i>	62.918	74.921	81.101	75.726	79.103	81.887	79.868	67.299	76.908	68.855	65.673
<i>P. aeruginosa (pah)</i>	84.904	86.032	88.963	85.567	88.718	91.46	94.389	86.572	94.177		
<i>C. testosteroni (pah)</i>		74.921	39.658	73.846	79.231	80.357					
<i>P. fluorescens (ndo)</i>		97.368	96.593	95.57							
	<i>G</i>	<i>T</i>	<i>H</i>	<i>I</i>	<i>N</i>	<i>L</i>	<i>O</i>	<i>M</i>	<i>K</i>	<i>J</i>	
<i>P. stutzeri</i>	82.989	84.709	83.333	89.665	80.733	40.51	90.26	89.914	88.679	79.688	
<i>Ralstonia sp.U2</i>	40.652		39.669	42.884	43.566	43.429		40.417	41.594	38.542	
<i>P. fluorescens (ndo)</i>											
<i>C. testosteroni</i>			35.833								
<i>P. fluorescens (ndo)</i>											

References: as per Table 1.5.

1.8 The use of bacterial resistance genes in environmental biotechnology

Bacteria possessing heavy metal resistant and/or PAH degradative capabilities may be used and/or manipulated in a number of ways for the degradation, sorption or detection of contaminants in the environment. A number of processes and applications, including bioremediation, biosorption, biosensors and the formation of biofilms, will be discussed in the following sections with respect to heavy metals and PAHs.

1.8.1 Bioremediation

Bioremediation is described as “the use of biological treatment systems to destroy or reduce the concentrations of hazardous wastes from a contaminated site” (Caplan, 1993). It may also be described as “an application of microbial capacity to transform complex organic molecules into simpler inorganic elements” (Purohit, 2003). The benefits of using bioremediation over chemical waste removal methods is that it can be less expensive, can achieve complete detoxification of organic pollutants without destruction of either site material or its flora or fauna, and it can be performed *in situ* (Timmis and Peiper, 1999).

Various bacteria have been utilised and/or constructed to aid in the process of bioremediation. These may include biosensors and bacteria capable of biosorption or of forming biofilms. Such bacteria are discussed in the sections below with respect to heavy metals and PAHs. Such bacteria are of interest to this project as using organisms known to possess heavy metal resistance and PAH degradation may be of particular interest to the applications mentioned above in the processes of detecting and degrading such contaminants which can commonly occur in contaminated matrices.

1.8.2 Biosensors

Biosensors can be described as analytical tools, which use biological specificity in sensing target molecules. They are designed using a specific bioactive component for the desired molecule, to yield a signal that can be monitored. For example, a biosensor based on the production/activity of an enzyme may generate a signal either via by-product formation, the disappearance of a substrate or by co-enzyme conversion

(Purohit, 2003). If used in an environmental setting, the biosensor should have no detrimental effect on the indigenous microbial community and its specific monitoring should not be influenced by changes in the condition of the environment. Molecular biosensors generally contain a specific promoter, for which the expression is sensitive to the target molecule, along with a reporter to generate a signal, which is proportional to the expression of the promoter. Such reporter systems have been reviewed by Vollmer and Van Dyk (2004) and include the following:

lacZ: encodes β -galactosidase from *E. coli*. Produces chemiluminescent, fluorescent or coloured molecules with the addition of an appropriate enzymatic substrate and is detected by a variety of methods such as luminometry, fluorometry, spectrophotometry, visually or electrochemically.

gfp: encodes a green fluorescent protein from *Aequorea victoria*, which is able to be detected without the need to lyse cells or add substrates. This feature of GFP makes it useful in protein localization in microbial cells and as a marker of individual microbial species in mixed populations. This protein is detected using a number of methods including fluorometry with a fluorescence-activated cell sorter, fluorescence microscopy and visually.

luc: encodes insect luciferase which catalyses the oxidation of benzothiazolyl-thiazole luciferin in the presence of ATP, oxygen and magnesium, resulting in light production. The *luc* gene was first isolated from the firefly *Photinus pyralis* and is more commonly used in mammalian cells than in bacterial cells. Luciferase activity is measured upon the addition of luciferin by a number of methods including luminometry, scintillation counting, CCD imaging, photographic film or visually.

lux: a total of five *lux* genes encode proteins that form the heterodimeric luciferase (*luxAB*) and that result in the synthesis of a long chain aldehyde (*luxCDE*), which is oxidised in association with the reduced flavin mononucleotide (FMNH₂), which is the luciferin molecule. Sources of the *lux* genes may include *Vibrio fischeri*, *Photobacterium phosphoreum* and *Photorhabdus luminescens*. In the presence of oxygen, ATP and reducing power, expression of the five *lux* genes in cells results in

continuous light production, which may be detected without cell disruption or enzymatic assay. Detection of light emission produced by the *lux* system may be detected by methods such as luminometry, scintillation counting, CCD imaging, photographic film or visually.

The potential advantages of biosensor use include their economical advantages, along with their portability and the possibility of identifying and quantifying specific compounds directly in mixtures found in the air, soil, water and in biological samples (Ramanathan *et al.*, 1997).

A number of studies have been performed investigating the potential of a number of constructs for the sensing of heavy metals and PAHs using the reporter systems described above, with genes for heavy metal resistance and PAH degradation. Table 1.8 outlines a number of studies performed, including the host organisms, the reporter systems and resistance/degradation genes used along with the detection of heavy metals or PAHs. This table highlights the variations that may be used to construct a biosensor with respect to reporter systems used and demonstrates that it is not necessary in some cases to utilise whole resistance/degradation operons, as it can be seen that the use of regulatory genes and/or O/P sequences may be sufficient.

1.8.3 Biosorption

The broad-term ‘biosorption’ refers to a metabolism-independent, physico-chemical binding of a compound, while the term ‘bioaccumulation’ refers to the active immobilization of a compound, including enzymatically-mediated mechanisms, leading to ‘bioprecipitation’ or ‘biocrystallisation’ (Pumpel and Schinner, 1997). Biosorption uses inexhaustible, inexpensive, non-hazardous materials that show significant specificity for the targeted contaminants, thus generating low volumes of non-hazardous wastes. The secondary waste from biosorption-based processes may be readily treated and can be easily disposed of by incineration (Seki *et al.*, 1998). An alternative method for metal removal is ‘microbial leaching’ in which valuable metals are extracted from low-grade ore and mineral concentrates (Bosecker, 2001). Table 1.8 summarises examples of biosorption systems for the accumulation of mercury.

Table 1.7 Summary of biosensor constructs designed for the detection of heavy metals and PAHs

Host organism	Reporter genes	Resistance/Degradation genes	Detection levels	Reference
Not specified	<i>luxCDABE</i>	Tn21 operonpRB28 (<i>merR_c/pT⁺</i>) - pOS14 (active transport) - pOS15 (intact <i>mer</i> operon)	Hg(II) -1 nM -0.5 nM -25 nM	Selifinova <i>et al.</i> , 1993
<i>E. coli</i>	<i>luc</i>	Mercury inducible promoter of Tn21 operon	1.67 X 10 ⁻¹³ to 1.67 X 10 ⁻⁷ M.	Roda <i>et al.</i> , 2001
<i>P. fluorescens</i> 058	<i>luc</i>	Plasmid pTP11 containing the mercury resistance promoter	0.003 µg/kg	Petanen and Romanatschuk, 2002
<i>S. aureus</i> RN4220	<i>luc</i>	<i>cadC</i> , regulatory unit of <i>cadA</i> (ATPase)	10 nM Cd 33 nM Pb	Taurianen <i>et al.</i> , 1998
<i>B. subtilis</i> BR151			3.3 nM Cd 33 nM Pb	

Table 1.7 continued

Host organism	Reporter genes	Resistance/Degradation genes	Detection levels	Reference
<i>E. coli</i>	<i>lacZ</i> (pY52)	Co-transformed with pY5C1, containing <i>cadC</i> under the control of <i>cad</i> OP	pM levels of Cd, Pb, Zn	Shetty <i>et al.</i> , 2003
	<i>gfp</i> (pY5G1)		sub-nM levels of Cd, Pb, Zn	
<i>E. coli</i>	<i>gfp</i>	<i>CadC</i> under the control of <i>cad</i> OP	0.1 mM/L Cd 10 nM/L Pb	Liao <i>et al.</i> , 2006
<i>E. coli</i> RFM443	<i>luxCDABE</i>	<i>NagR</i> gene of <i>Ralstonia</i> sp. U2 and the upstream region of <i>nagAa</i>	2 µM salicylic acid	Mitchell and Gu, 2005
<i>P. putida</i>	<i>luxAB</i>	<i>nahR</i> gene, the <i>sal</i> promoter	50 nM naphthalene	Werlen <i>et al.</i> , 2004
<i>P. putida</i> and <i>E. aerogenes</i>	<i>luxCDABE</i>	<i>merR</i>	100 pM Hg	Fu <i>et al.</i> , 2008

1.8.4 Biofilms

In both the environment and industry, bacteria residing in a biofilm can be advantageous in treating areas of contamination. A bacterial biofilm may be described as a “microbially-derived sessile community characterised by cells that are irreversibly attached to a substratum or interface or to each other, are imbedded in a matrix of extracellular polymeric substances that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription” (Donlan and Costerton, 2002). Biofilms may be composed of a population derived from a single species or as a community derived from a number of bacterial species and are capable of forming on a wide range of abiotic and biotic surfaces (Davey and O’Toole, 2000). A number of organisms have been studied for their abilities to form biofilms, including the Gram-negative bacteria *P. aeruginosa*, *P. fluorescens*, *E. coli* and *Vibrio cholerae* and the Gram-positive bacteria *S. aureus*, *Staphylococcus epidermidis* and enterococci (O’Toole *et al.*, 2000).

1.8.4.1 Biofilm structure, development and mechanisms of resistance

In the formation of biofilms, the bacteria undergo a transition from the planktonic form to a community-based existence, where interaction may occur between various species in close proximity (O’Toole *et al.*, 2000). Biofilm formation has been shown to occur in distinct developmental steps (Davey and O’Toole, 2000) that include:

- initial attachment to a surface upon the detection of a change in environmental parameters.
- formation of microcolonies.
- maturation of microcolonies into an (extrapolymeric substance) EPS-encased mature biofilm.

Bacterial biofilms that have been found growing in natural and industrial environments have been found to be resistant to bacteriophage, amoebae and to a wide range of chemically diverse biocides (Costerton *et al.*, 1999). It has been shown in nature that bacterial communities perform many processes, such as the production and degradation of organic matter, the degradation of environmental pollutants, such as heavy metals, and the cycling of nitrogen, sulfur and a variety of metals (Davey and O’Toole, 2000).

Table 1.8 Summary of biosorption constructs designed for the uptake of mercury using various applications

Host organism	Features	Application	Accumulation levels	Reference
<i>E. coli</i>	<i>S. marcescens</i> pDU1358 Hg transport system Pea metallothioneins	Hollow fiber reactor	Reduction of Hg levels 400-fold, from 2 mg/L to 5 µg/L	Chen <i>et al.</i> , 1998
<i>E. coli</i>	<i>merT</i> and <i>merP</i> <i>Saccharomyces cerevisiae</i> metallothionein fused with glutathione-S-transferase	Not specified	5-fold increase in accumulation of Hg in cells containing (GST-MT) compared to those containing only <i>merT</i> and <i>merP</i>	Chen and Wilson, 1997
<i>Pseudomonas</i> sp.	Naturally resistant to Hg	Packed bed bioreactor flowing with continuous chloralkali electrolysis wastewater	Inflow Hg concentration: 3-10 mg/L Outflow Hg concentration: <50 µg/L, down to 10 µg/L when combined with a carbon filter	Wagner-Dobler <i>et al.</i> , 2000
<i>E. coli</i>	<i>B. cereus</i> RC607 and <i>Pseudomonas</i> sp. K-62 <i>merP</i>	Aqueous solution	Able to absorb Zn ²⁺ and Cr ³⁺ at levels up to 144% higher than cells lacking <i>merP</i>	Kao <i>et al.</i> , 2008.

Several bacteria of clinical importance are also able to form biofilms, leading to problems such as cystic fibrosis pneumonia, periodontitis and biliary tract infections, and of the infections of medical aids, such as urinary catheters, arteriovenous shunts and mechanical heart valves (Costerton *et al.*, 1999).

By residing in a biofilm, bacteria are provided with a degree of shelter and homeostasis, due to the surrounding extrapolymeric substance matrix, which comprises of EPS, proteins, nucleic acids and other substances (Davey and O'Toole, 2000). The mechanisms responsible for these resistances are generally due to one or more of the following:

- delayed penetration of the antimicrobial agent through the biofilm matrix.
- altered growth rate of the biofilm organisms.
- other physiological changes due to the biofilm mode of growth (Donlan and Costerton, 2002).

1.8.4.2 Environmental biofilms and heavy metals

Bacteria residing in biofilms have shown to be able to reduce levels of heavy metal concentrations or accumulate high levels of heavy metals from heavy metal-contaminated samples. It has also been shown in some cases that bacteria residing in biofilms are significantly more resistant to heavy metals than those in planktonic form. Many different strains of biofilm-forming bacteria have been investigated for the potential to remediate contaminated samples, such as wastewater. Such examples are described below in Table 1.9.

1.9 Studies previously undertaken at Victoria University related to this project

Separate investigations have been previously undertaken at Victoria University based on heavy metal resistant isolates and those capable of PAH degradation. These studies are summarised below.

Table 1.9 Examples of the use of biofilms to reduce levels of heavy metals in contaminated samples

Strain	Biofilm capabilities	Reference
<i>C. metallidurans</i> CH34	Grown on a filter membrane, within a reactor for the treatment of wastewater -zinc was reduced from 60 ppm to 1 ppm -copper was reduced from 8 ppm to 0.05 ppm -nickel was reduced from 8 ppm to 0.05 ppm	Diels <i>et al.</i> , 1995
<i>C. metallidurans</i> , <i>Pseudomonas mendocina</i> and <i>Arthrobacter</i> sp.	Grown together on sand within a moving bed sand filter. Removal of metals from wastewater: -zinc and copper between 95-100% -cobalt between 80-90% -arsenic by at least 80%	Diels <i>et al.</i> , 2003
<i>Desulfobacteriaceae</i> sp.	Concentrations of ZnS within a biofilm were 10 ⁶ times the concentration of ZnS in associated groundwater.	Labrenz <i>et al.</i> , 2000
<i>P. aeruginosa</i> PAO1	Compared to planktonic form, these cells in biofilm were: -600 times more resistant to copper -32 times more resistant to zinc -3.2 times more resistant to lead	Teitzel and Parsek, 2003
<i>P. putida</i> Spi3	Grown as a biofilm on porous carrier material in laboratory column reactors, allowed for the removal of between 90-98% of Hg from wastewater passed through	Von Canstein <i>et al.</i> , 1999

1.9.1 Work based on heavy metal resistance

Trajanovska *et al.*, (1997), studied a number of bacteria isolated from a discontinued battery-manufacturing site in Ardeer, an outer suburb of Melbourne. The soil from which these samples were isolated contained up to 260 mg lead per gram of soil (Manovski *et al.*, 1992). Isolates that were identified from this soil sample included the *Arthrobacter* sp. isolates E9 and E11 and the *Alcaligenes* sp. isolates AO17 and AO22. E9 and E11 were isolated following enrichment in lead-containing broth and were found to be non-acid fast and differentiated from *Corynebacterium* on the basis of glucose utilisation. AO17 and AO22 were randomly selected from the microflora recovered on non-selective media without enrichment for metal resistance. *C. metallidurans* CH34 and *Corynebacterium glutamicum* strains AS109 and ATCC13032, were used as controls. Minimum Inhibitory Concentration Assays (MICs) were performed and these strains were found to be resistant to varying levels of heavy metals (up to 3.5 mM Lead, 0.1 mM mercury, 3.5 mM zinc, 6 mM copper, 2.1 mM cadmium and 2.5 mM cobalt).

PCRs were performed to detect the presence of the *merR*, *merT* and *merP* genes. A 1 kb fragment corresponding to these genes was found in the chromosomal DNA of E9, E11, AO17 and AO22, as well as in the plasmid and chromosomal DNA of CH34. PCR was also used in an attempt to characterise the *czc* genes in these strains. The lack of uniform amplification of various *czc*-related fragments along with the presence of fragments of sizes different to the control, suggests that either the sequences are present, but highly divergent in the test isolates, or they are absent in the test strains (Trajanovska *et al.*, 1997). It has also been previously determined that these isolates possess genes relating to the *pco* operon of *E. coli*, which encodes copper resistance (Brown *et al.*, 1995). PCR analysis revealed the occurrence of expected fragment sizes indicating the presence of the *pcoA*, *pcoB*, *pcoC*, *pcoD* and *pcoR* genes. Partial sequence analysis and Southern blot hybridisation suggested that these isolates contain a copper resistance system highly homologous to the *pco* system of *E. coli* (Leong and Bhave, 2001).

1.9.2 Work based on PAH degradation

Stenotrophomonas maltophilia strain VUN 10010 was isolated from a PAH-contaminated soil sample and was able to degrade pyrene as a sole carbon and energy source, along with other HMW PAHs, with the addition of surfactants enhancing this process (Boonchan *et al.*, 1998). The co-metabolism of PAHs by VUN 10010 and *Penicillium janthinellum* VUO 10201, also isolated from contaminated soil was investigated. VUN 10010 alone could use pyrene as a sole carbon and energy source and can mineralise significant amounts of benzo[*a*]pyrene when pyrene is present. *P. janthinellum* VUO 10201 could not utilise PAHs as sole carbon and energy sources, but was found to be able to partially degrade them if cultured in nutrient broth. When VUN 10010 and VUO 10201 were combined as a fungal-bacterial co-culture, there was a significant degradation of and microbial growth on pyrene, chrysene, benz[*a*]anthracene and dibenz[*a,h*]anthracene. When this co-culture was inoculated into PAH-contaminated soil, there was a significant decrease in the HMW PAHs present. This was demonstrated by the removal of 53% of added [¹⁴C] benzo[*a*]pyrene, which was recovered as ¹⁴CO₂ within 100 days (Boonchan *et al.*, 2000).

1.9.3 Basis for investigating both heavy metal resistant and PAH degrading organisms

The bacteria used in this thesis have been studied previously from different perspectives as described in sections 1.9.1 and 1.9.2. For this study, it was decided to investigate these organisms together as the literature indicates that soil may often be contaminated with both heavy metals and PAHs and bacteria can be isolated from such sites that are resistant to both contaminant types. Soils and soil run-offs have been identified that are high in a variety of heavy metals and PAHs, such as cobalt, zinc, cadmium, lead and mercury, while some sites have been reported to contain up to 20 different PAHs (Mikkelsen *et al.*, 1996; Wilcke *et al.*, 2000; Monarca *et al.*, 2002; Muniz *et al.*, 2003; Khodadoust *et al.*, 2004). Bacterial species including *Clavibacter* sp., *P. putida*, have been isolated from contaminated soils and have been found to possess both heavy metal resistance and PAH degradative capabilities (Riha *et al.*, 1993; Fija *et al.*, 1998; Dore *et al.*, 2003; Kozlova *et al.*, 2003). Due to such correlations between heavy metal and

PAH contamination, it has been decided to investigate the above heavy metal resistant and PAH degrading organisms in this thesis.

The current project was designed to focus on further characterising the heavy metal MICs of the isolates *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9, along with determining the MICs of *S. maltophilia* VUN 10010. Further genetic characterisations will be performed on these isolates based on the information provided previously in this chapter. Comprehensive aims are outlined below.

1.10 Aims of this project

1.10.1 Overall aim

The aim of this project is to investigate resistance to and/or degradation of environmental contaminants by indigenous soil bacteria, isolated from soil contaminated with heavy metals (*Alcaligenes* sp. AO17 and AO22 and *Arthrobacter* sp. E9 and E11) and PAHs (*S. maltophilia*). This will involve an initial confirmation/characterisation of the Minimum Inhibitory Concentrations of each isolate, followed by an investigation of the genetic mechanisms involved in the resistance to and/or degradation of environmental contaminants (*mer*, *pbr*, *cad*, *pah*) by indigenous soil bacteria. Preliminary genetic analysis has previously been performed on the presence of heavy metal resistance genes in the bacteria isolated from the heavy metal-contaminated soil. No investigations into the existence of PAH degradative genes has previously been performed on *S. maltophilia*. With an interest in whether these bacteria show any potential for bioremediation or biosorption applications, an investigation into the capabilities of these bacteria to form biofilms in the presence of contaminants will be performed also.

1.10.2 Specific aims

The specific aims of this project are as follows:

1. To confirm and further characterise the minimum inhibitory concentrations (MICs) of *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 in a range of

concentrations of mercury, lead, cadmium, cobalt and zinc and to characterise the MICs of *S. maltophilia* in these heavy metals. An analysis of all three isolates will also be performed for the organomercurials methylmercury chloride and phenylmercury acetate.

2. To establish the growth curve characteristics of the soil isolates, in the absence of contaminants to enable the design and direction of subsequent gene expression experiments.
3. To identify *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 to the species level by analysis of the gene encoding 16S rRNA.
4. To determine the presence and sequence of genes involved in mercury, lead and cadmium resistance. The sequence of all obtained genes will be analysed for comparison with other published heavy metal resistance genes.
5. To determine the presence and sequence of genes involved in the degradation of low molecular weight (LMW) PAHs, such as naphthalene and Phenanthrene, in *S. maltophilia*. The sequence of all obtained genes will be analysed for comparison with other published PAH degradative genes.
6. To correlate the expression of the *mer* genes by time course analysis making comparisons between cells exposed to mercury and those grown in the absence of mercury.
7. To determine the soil isolates capabilities of forming biofilms in the presence of heavy metal contaminants in order to consider their potential for biosorption/biofilm applications.

1.11 Significance of this project

The isolates chosen for this project have been mentioned very little, if at all in the literature, and the fact that they were isolated directly from contaminated soil makes them of particular interest. It is also of interest to determine whether the chosen isolates

may be capable of additional activities, for example, do the PAH degrading organisms also have heavy metal resistance capabilities and visa versa? Such multiple capabilities may make an organism more useful for remediation activities. This project will also be investigating different genera isolated from the same contaminated soil. These bacteria share common resistances albeit at different levels. It is of interest to determine whether they share identical genes or somewhat different ones. When considering the use of bacteria to degrade environmental pollutants, it is not only necessary to determine if they possess the genes responsible for this, but also whether these genes are functional and how they are expressed when exposed to contaminants. Very little literature exists on the expression of heavy metal resistance genes or PAH degradation genes. By understanding the physiological characteristics of these isolates, along with the structure and regulation of genes for both heavy metal resistance and PAH degradation, we may gain information that will be useful in the design of biosensor and biosorption systems. Carrying out this work in the laboratory will aid to obtain the information that may allow for these isolates to be further considered for the design of on-site experiments.

Chapter 2

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MATERIALS

2.1 Kits, reagents, enzymes and chemicals

2.1.1 Commercial kits and other reagents

A range of commercially available kits and reagents were utilised in this project for a variety of applications. These, their suppliers and the appropriate application are outlined in Appendix 1.

2.1.2 Enzymes

A range of commercially available enzymes were utilised in this project. Many of these enzymes were provided with the kits outlined in Appendix 1, while others were obtained individually. These enzymes, their suppliers and the appropriate application are outlined in Appendix 1. Enzymes prepared in-house were prepared according to the methods outlined in Appendix 2 (Sambrook *et al.*, 1989; Ausubel *et al.*, 1995).

2.1.3 Buffers and general stock solutions

Analytical grade reagents were used for all buffers and solutions. The methods for preparing these were based on Sambrook *et al.* (1989) and Ausubel *et al.* (1995). These and the suppliers of chemicals used are outlined in Appendix 2.

2.1.4 Microbiological media and components

The details of all media used are outlined in Appendix 3. Media were prepared as per manufacturers' instructions.

2.1.5 Sterilisation of microbiological media, reagents, glassware, consumables and antibiotic stocks

All items requiring sterilisation were autoclaved at 121°C for a minimum of 15 minutes. Antibiotic stocks were filter-sterilised (0.2 µM) and stored in a sterile tube.

2.1.6 Disposal methods

All microbial waste was autoclaved at 121°C for a minimum of 40 minutes, prior to disposal. All acid, heavy metal and phenol waste was disposed of into appropriate Winchester bottles for disposal by the Laboratory Manager.

BACTERIA

2.2 Bacteria used in this investigation

Of the microorganisms utilised in this study, the heavy metal resistant soil bacteria were previously isolated by Sylvia Manovski (Manovski *et al.*, 1992) and the PAH-degrading isolates were previously isolated by Sudarat Boonchan (PhD Thesis, 1998). An *Escherichia coli* strain carrying the plasmid pACYC 184::Tn501, which has the Tn501 transposon housing the *mer* operon encoding mercury resistance, was kindly supplied by Dr. M. Osborn (University of Liverpool, UK). Table 2.1 outlines the bacterial isolates used, their origins, traits and purpose in this investigation.

Table 2.1 Bacteria used in this investigation

Organism	Isolate	Origin	Purpose in this investigation
<i>Alcaligenes</i> sp.	AO17	Lead contaminated soil sample* VU culture collection**	Heavy metal resistant
<i>Alcaligenes</i> sp.	AO22	Lead contaminated soil sample* VU culture collection**	Heavy metal resistant
<i>Arthrobacter</i> sp.	E9	Lead contaminated soil sample* VU culture collection**	Heavy metal resistant
<i>Arthrobacter</i> sp.	E11	Lead contaminated soil sample* VU culture collection**	Heavy metal resistant
Bacterial consortium (<i>Mycobacterium</i> sp. and <i>Stenotrophomonas maltophilia</i>)	VUN 10010	PAH contaminated soil sample# VU culture collection**	PAH degrader
<i>Escherichia coli</i>	AB1157	Obtained from Dr. M. Osborn.	Contains plasmid pACYC 184:: Tn501 (mercury resistance), positive control for <i>mer</i> PCRs
<i>Escherichia coli</i>	JM109	Promega	Competent cells for transformation of ligation products
<i>Pseudomonas aeruginosa</i>	NA	VU Culture Collection**	Positive control for biofilm assays

Manovski *et al.*, 1992; Trajanovska *et al.*, 1997

#Boonchan, PhD Thesis, 1998

**VU culture collection (Victoria University, Werribee Campus)

MICROBIOLOGICAL METHODS

2.3 Growth, storage and the investigation of the properties of AO22, E9 and VUN 10010 microorganisms in response to heavy metals and PAHs

2.3.1 Revival and growth of bacterial stocks

The bacterial isolates used in this investigation were previously stored either as glycerol stocks at -80°C or as lyophilised (freeze-dried) stocks at -20°C . To revive cultures stored as a glycerol stock, the stocks from -80°C were thawed on ice. A loopful of this stock was used to inoculate either 10 mL nutrient broth plus 0.05 mM Tris pH 7.5 (NBT) or to streak a nutrient agar plus 0.05 mM Tris pH 7.5 (NAT) plate (Appendix 3), the former being incubated at 30°C , 150 rpm and the latter being incubated at 30°C until visual microbial growth. Alternatively, to revive cultures stored in lyophilised form, approximately 100 μL of the medium was added to lyophilised cells, which was then used to inoculate 10 mL of the desired medium (NBT or basal salts medium plus 250 mg/L pyrene (BSM/PYR), followed by growth at 30°C , 150 rpm for 1-2 days for NBT cultures and at least one week for BSM/PYR cultures. Spray plates were also used, wherein BSM, containing no carbon source, was sprayed with 2% pyrene in diethylether. The above cultures were then used to subculture into new medium, as required. This was performed at a concentration of 1:100 and the subsequent culture was grown at 30°C , 150 rpm, overnight for growth curve analysis/RNA extractions (Sections 2.3.6/2.12) and DNA extractions (Section 2.4). To provide storage stocks, glycerol stocks were prepared from overnight cultures, adapted from the method described by Sambrook *et al.*, (1989). 250 μL of sterile 60% glycerol was added to 750 μL culture and stocks were stored at -20°C and -80°C .

2.3.2 Gram-staining

This technique was performed as described by Reade (1985) wherein a loopful of culture or a colony, resuspended in a loopful of water, was spread on a glass microscope slide. The bacteria were dried onto the slide by holding it over the Bunsen burner. 1% crystal violet was then applied to the slide and left for 30 seconds. The slide was rinsed in running tap water and Jensen's iodine was added and left for 30 seconds. It was then

rinsed again in running tap water and decolourised with acetone for 2 seconds. It was then washed again in running tap water, then counter-stained with neutral red for 30 seconds, washed in water, blotted with tissue paper and dried over the Bunsen burner prior to observing under a microscope. If the bacteria stained purple, it was taken as an indication of the presence of a Gram-positive organism, if they stained red, it was taken as an indication of the presence of a Gram-negative bacteria.

2.3.3 Minimum inhibitory concentration assays (MICs)

MIC tests were performed to confirm the heavy metal resistances of the *Alcaligenes* sp. AO17 and AO22 and *Arthrobacter* sp. E9 and E11 isolates, as were previously published (Trajanovska *et al.*, 1997) and to determine whether VUN 10010 possessed similar resistances, as this was not previously determined. The MICs were tested in the presence of varying concentrations of mercuric chloride (HgCl_2 , anhydrous) (0.01, 0.025, 0.05, 0.075 and 0.1 mM), lead nitrate ($\text{Pb}(\text{NO}_3)_2$, anhydrous) (0.1, 1.0, 2.0, 3.0 and 4.0 mM), zinc nitrate ($\text{Zn}(\text{NO}_3)_2 \cdot 4\text{H}_2\text{O}$) (0.1, 1.0, 2.0, 3.0 and 4.0 mM), cadmium chloride ($\text{CdCl}_2 \cdot 2\text{H}_2\text{O}$) (0.1, 0.5, 1.0, 1.5 and 2.0 mM) and cobalt nitrate ($\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$) (0.1, 0.5, 1.0, 1.5 and 2.0 mM) in NAT and NBT for the methods using spot plates, spread plates and microtitre plates. The concentrations of heavy metals were increased for the MIC experiments using liquid broth to 0.2 mM HgCl_2 , 8 mM $\text{Pb}(\text{NO}_3)_2$, 8 mM $\text{Zn}(\text{NO}_3)_2$, 6 mM CdCl_2 and 6 mM $\text{Co}(\text{NO}_3)_2$. The MICs of the isolates to the organomercurial compounds, methylmercury chloride and phenylmercury acetate, were tested at concentrations of 0.001, 0.0025, 0.005, 0.0075 and 0.01 mM in NBT. NAT/NBT containing various concentrations of heavy metals was prepared from stock solutions (Appendix 2). 10 mL liquid cultures were inoculated from an overnight culture at an inoculum ratio of 1:1000 and incubated at 30°C, 150 rpm. Cultures were also grown on NAT plates (spot and spread plates) containing the above concentrations of heavy metals at 30°C and were observed daily for visible signs of growth. Microtitre plates were also utilised for MICs and biofilm assays (Section 2.3.9).

2.3.4 Growth curve analysis

Growth curve analysis was performed to establish the approximate replication times of isolates in the absence of heavy metals and PAHs. This information was required in

subsequent experiments designed to study the effect of environmental challenges on bacterial growth (Section 2.3.6). From the initial culture (Section 2.3.1), three serial transfers of bacteria were performed to increase culture size. From the third culture, an optical density reading (OD_{600}) was taken to allow for the overnight “parent” culture to be inoculated to an OD_{600} of 0.1. When this culture reached an OD_{600} of 1.0, the cells were harvested by centrifuging at 9000 rpm for 15 minutes, resuspended in a smaller volume (i.e. 10 mL) of new NBT, from which 1 mL was used to inoculate the test cultures to achieve an OD_{600} of 0.1. These new culture flasks were incubated at 30°C, 150 rpm. A sample was taken directly immediately from the freshly inoculated culture and at subsequent hourly intervals for an OD_{600} reading: These results were plotted and the growth curve determined for each isolate.

2.3.5 Growth curve analysis in the presence of mercuric chloride

To investigate the effects of presence of mercuric chloride on physiology and gene expression, a growth curve analysis on *Alcaligenes* sp. AO22 and *A. woluwensis* E9 was performed in the presence of mercuric chloride. A glycerol stock was streaked onto an NAT plate containing 0.025 mM $HgCl_2$ (a maximum of 25% of the MIC obtained), to ensure a mercury resistant colony was selected, which was subsequently inoculated into 10 mL NBT. After two serial transfers of this culture in the absence of $HgCl_2$, a 400 mL NBT culture was inoculated to an OD_{600} of 0.1 (Section 2.3.5), which was grown at 30°C, 150 rpm, to an OD_{600} of 1.0 to provide a “parent” culture. The cells of this culture were harvested by centrifuging at 9000 rpm for 15 minutes and resuspended in new medium (18 ml of NBT) and 1 mL was added to each of 18 flasks of 100 mL NBT, 9 of which were supplemented with 0.05-0.075 mM $HgCl_2$ (a minimum of 75% of the MIC value obtained) to an OD_{600} of 0.1. As this experiment was focusing on adaptation to the presence of $HgCl_2$, cells were harvested at 0, 0.5, 1, 2, 4, 6 and 9 hours. At each time point, a flask of each of the NBT and NBT + $HgCl_2$ was taken and 1 mL was used for OD_{600} readings and cell counts. The remaining 100 mL of the culture was centrifuged at 9000 rpm for 15 minutes at 4°C, the supernatant discarded and the cell pellet stored at -20°C until RNA extraction (Section 2.12). It was, however, found that *A. woluwensis* E9, cells were not able to adapt as rapidly as *Alcaligenes* sp. AO22. To investigate the adaptation times, six 100 ml flasks of NBT (containing NBT only and 0.01-0.05 mM $HgCl_2$) were inoculated “parent culture (as described above) to an OD_{600}

of 0.1. These cultures were grown for up to 96 hours with OD₆₀₀ readings taken every 2 hours for 8 hours, then at 24, 48, 72 and 96 hours.

2.3.6 Determination of viable cell counts by plating

To determine viable cell numbers in the growth curve analysis experiment, 100 µL of each of the control and induced cultures was diluted with 900 µL of peptone water. 100 µL of the dilutions from 10⁻³ to 10⁻⁷ were plated in duplicate onto NAT plates and incubated at 30°C. Estimates of cell populations in the original culture were determined from plates with colony populations between 20 and 200. The number of viable cells per mL of culture was determined as: # of colonies X dilution factor X 10.

2.3.7 Biofilm assays

Biofilm assays were performed as described by O'Toole *et al.*, (2000). Microtitre plates (Polystyrene Nunc F96 MicroWell™ Plates) were used as the substratum. Each isolate and a *Pseudomonas* control was grown from a glycerol stock overnight in 10 mL NBT at 30°C, 150 rpm). The overnight cultures were diluted at 10⁻³ in either in NBT or NBT containing heavy metals (0.01, 0.025, 0.05, 0.075, 0.1 mM HgCl₂; 0.1, 1, 2, 3, 4 mM Pb(NO₃)₂; 0.1, 1, 2, 3, 4 mM Zn(NO₃)₂; 0.1, 0.5, 1, 1.5, 2 mM CdCl₂ and 0.1, 0.5, 1, 1.5, 2 mM Co(NO₃)₂) and inoculated into six individual wells of a microtitre plate (150 µL per well) as shown in Figure 2.1 (A total of 26 plates per experiment). Each separate plate contained the three isolates plus a negative (no inoculum) control in the presence of a single heavy metal at one of the concentrations tested or NBT only.

The plates were incubated for 48 hours at 30°C. To stain the cells, 10 µL of 0.1 % crystal violet was added to each well, the plates incubated at room temperature for 15 minutes and then rinsed with distilled water to remove any cells not attached to the wall of the well. Ethanol (70%, 180 µL) was added to each well to dissolve the crystal violet and resuspend the attached cells and incubated at room temperature overnight. The microtitre plates were then read using the plate reader (Kinetic Microplate Reader) at an absorbance of 570 nm to determine the proportion of attached cells. The results were

analysed on the basis of a higher absorbance reading being indicative of a higher proportion of cells forming biofilms.

To perform a statistical analysis, the absorbance readings (excluding the highest and lowest reading) for the negative controls and at each heavy metal concentration were averaged. The averages obtained in the presence of the heavy metals were then calculated as a percentage of the negative control. This was performed for two separate experiments and these percentages averaged to obtain a final result.

	1	2	3	4	5	6
A						
B						
C						
D						
E						
F						
G						
H						

Figure 2.1 Set up of biofilm assays in a microtitre plate. The biofilm plate for negative controls (no heavy metal) and for each heavy metal at a single concentration was set up as follows; 1A-F, negative (no inoculum) control; 2A-F, AO22; 3A-F, E9; 4A-F, VUN 10010; 5A-F, *P. aeruginosa*; 6A-F, negative (no inoculum) control. Wells G1-6 and H1-6 remained empty.

2.3.8 Removal of Gram-negative bacteria from the Consortium VUN 10010

The antibiotic, lincomycin, according to information provided by Sigma Aldrich, specifically targets Gram-negative bacteria. This antibiotic was used in an attempt to eliminate *S. maltophilia* from the Consortium VUN 10010 (refer to Section 3.4). A stock of 1 mg/mL lincomycin was prepared (filter sterilised through a 0.2 µM filter) and added to the growth medium at concentrations of 100 mg/L, 250 mg/L and 500 mg/L. Streak plates were used to determine the purity of the cultures.

MOLECULAR TECHNIQUES

DNA TECHNIQUES

2.4 Isolation of genomic DNA from bacterial cells

Separate methods were used for the large-scale isolation of genomic DNA from Gram-positive and Gram-negative bacterial cells, while a commercial kit was used for small-scale isolation of genomic DNA from both cell types.

2.4.1 Large scale isolation of genomic DNA from Gram-negative bacteria

This method was based on Ausubel *et al.*, (1995) with some modifications. 200 mL of overnight bacterial culture grown in NBT was centrifuged at 14000 rpm for 10 minutes at 4°C. The supernatant was discarded and the cell pellet resuspended in 9.5 mL TE buffer (pH 8) and 0.5 mL SDS (10%). 50 µL proteinase K (20 mg/mL) and 20 µL RNaseA (10 mg/mL) was added to the cell suspension and incubated at 37°C for one hour. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Appendix 2) was added and the mixture centrifuged at 14000 rpm for 10 minutes. The aqueous layer was transferred to a clean tube and a further phenol-chloroform-isoamyl alcohol extraction performed. The aqueous layer was collected and a DNA precipitation was performed by the addition of 1/10 the volume of 3M sodium acetate (pH 5.2) and 1.5 times the volume of pre-chilled 100% ethanol to it, then chilling the mixture at -80°C for 30 minutes, then centrifugation at 14000 rpm for 10 minutes at 4°C. The supernatant liquid was removed and the DNA pellet air-dried for 10 minutes at room temperature and resuspended in 200 µL of 10 mM Tris (pH 7.5).

2.4.2 Large scale isolation of genomic DNA from Gram-positive bacteria

This method was based on Ausubel *et al.*, (1995) with some modifications. 200 mL of overnight culture was grown in NBT as above and the cell pellet was resuspended in 3.2 mL of lysing solution and 0.6 mL of fresh lysozyme (20mg/mL). The cell suspension was incubated on ice for 5 minutes prior to the addition of 2 mL SDS (10%) and 50 µL

proteinase K (20 mg/mL). The cell suspension was then incubated at 55°C overnight with gentle shaking (40 rpm). An equal volume of Tris-equilibrated phenol was added and the mixture incubated for 3 hours at room temperature with gentle shaking (40 rpm). 5 mL of TE buffer (pH 8) was added and the phases mixed by inversion prior to centrifugation at 14000 rpm for 15 minutes at room temperature. The aqueous layer was removed to a clean tube and two phenol-chloroform-isoamyl extractions and DNA precipitation were performed as described above. 2 mL of 70% ethanol was added to wash the pellet, the DNA then repelleted by centrifugation at 15000 rpm for 10 minutes at 4°C. The liquid was decanted, the DNA pellet air-dried for 10 minutes at room temperature and resuspended in 200 µL of 10 mM Tris-Cl (pH 8).

2.4.3 Isolation of genomic DNA from Gram-negative and Gram-positive bacteria using the Wizard Genomic DNA Purification Kit (Promega)

The Wizard Genomic DNA Purification Kit and the accompanying protocol (Promega) were used for this method. 1 mL of overnight culture was centrifuged at 13000 rpm for 2 minutes to pellet the cells. For Gram-positive cells only, an extra initial step was performed, whereby the pelleted cells were resuspended in 480 µL of 50 mM EDTA and 120 µL 10 mg/mL lysozyme. The sample was incubated at 37°C for 30-60 minutes and centrifuged for 10 minutes at 14000 rpm. For both Gram-negative and Gram-positive cells, the cells were resuspended in 600 µL of 'nuclei lysis solution', incubated at 80°C for 5 minutes and then cooled on ice. 3 µL of RNase solution was added and the sample was inverted several times to mix, followed by incubation at 37°C for 15-60 minutes. The sample was cooled to room temperature and 200 µL of 'protein precipitation solution' was added, followed by vortexing for 20 seconds. The sample was incubated on ice for 5 minutes followed by centrifugation at 14000 rpm for 2 minutes. The supernatant was transferred to a new tube containing 600 µL of isopropanol. The sample was mixed by gentle inversion and centrifuged at 14000 rpm for 2 minutes. The supernatant was poured off gently, 600 µL of chilled 70% ethanol was added to the DNA pellet, the tube was inverted several times to wash the DNA pellet, prior to centrifugation at 14000 rpm for 2 minutes. The ethanol was aspirated carefully, the pellet air-dried for 15 minutes and then resuspended in 100 µL DNA rehydration solution and incubated at 65°C to dissolve the DNA.

2.5 Extraction of plasmid DNA from Gram-negative bacterial cells

For large-scale plasmid DNA extraction from Gram-negative bacterial cells an amended version of the procedure of Birnboim and Doly (1989) was used. 250 mL of overnight culture grown in NBT was centrifuged at 10000 rpm for 10 minutes at 4°C. The cell pellet resuspended in 6 mL of solution I and held in ice water for 20 minutes prior to the addition of 12 mL of solution II. The solution was mixed by inversion, held in ice water for 10 minutes, 7.5 mL of 3 M sodium acetate (pH 4.6) then added, the solution mixed by inversion and held in ice water for 20 minutes prior to centrifugation at 14000 rpm for 15 minutes at 4°C. The supernatant was transferred to a clean tube avoiding the white precipitate (containing denatured and chromosomal DNA and cellular debris) and 5 µL of RNase A (10 mg/mL) added to it, followed by incubation for 20 minutes at 37°C. A phenol-chloroform extraction was performed and the DNA precipitated with isopropanol as described above. The DNA pellet was resuspended in 100 µL of sterile dH₂O.

2.6 Quantitation of DNA samples

To quantitate DNA, a 1:20-1:200 dilution in water was carried out and the absorbance of the samples were measured at 260 nm and 280 nm using quartz cuvettes. To determine the concentrations of each sample the following formula was used:

$$\text{Absorbance (OD) reading (260 nm)} \times \text{dilution factor} \times 50 = \mu\text{g/mL DNA.}$$

The OD_{260nm}/OD_{280nm} ratio was used for determination of the purity of the DNA preparation. Pure preparations of DNA and RNA have ratios of 1.8 and 2.0 respectively, lower values being indicative of protein or phenol contamination (Sambrook *et al.*, 1989). If necessary, additional proteinase treatments, phenol extractions and precipitations were performed on the DNA preparations.

2.7 Visualisation of DNA samples by agarose gel electrophoresis

All DNA samples were electrophoresed by TAE (Tris/acetate/EDTA) gel electrophoresis Sambrook *et al.*, (1989). The agarose was dissolved in 1X TAE buffer by heating for 40 seconds to one minute in the microwave, then ethidium bromide was added to a concentration of 0.5 µg/mL. Generally, 5 µL of a DNA or RNA preparation

was electrophoresed. To run the sample, 2 μL of 10X loading dye and 3 μL of water were added to a final volume of 10 μL . The sample was electrophoresed by running at 70-100 V, the gel was visualised and photographed by placing on the UV platform (LKB Bromma 2011 Macrovue Transilluminator).

2.8 PCR analysis

The polymerase chain reaction (PCR) (Mullis and Faloona, 1987) was used in an attempt to amplify genes from AO22, E9 and VUN 10010 corresponding to the ones previously reported to be involved in heavy metal resistance and PAH degradation (outlined in Chapter 1) and characterisation of the isolates to a species level. For this purpose it was first necessary to design oligonucleotide primers corresponding to published genes. The following sections outline the design of primers and the setting up of PCR.

2.8.1 Criteria for design of oligonucleotide primers

As outlined by Sambrook *et al.*, (1989), the following criteria were considered:

- The length of each primer was at least 18 bases to allow for specific binding to the template.
- The primers contained no self-complementary regions and are not complementary to each other either
- The base composition chosen such that the primer pairs had similar annealing temperatures.

Where possible, primers were designed to amplify overlapping regions of the operon of interest (i.e. *mer*, *pbr*). In some cases, a number of related genes were aligned and areas of relative homology selected for primer design (i.e. *pah* genes) internally to the individual genes. It was sometimes necessary (i.e. for *mer* and *pah* genes) to design degenerate primers (more than one nucleotide in a particular position). These are represented by the following: R=A+G, Y=C+T, M=A+C, W=A+T, V=G+A+C. All primers were commercially synthesised by Sigma Genosys (Castle Hill, NSW), with cartridge purification. All primers were obtained as a dried powder and were

resuspended in sterile dH₂O at a concentration of 2 µg/µL, prior to dilution at appropriate concentrations for PCR and sequencing (refer to Sections 2.8.2 and 2.11.1).

2.8.1.1 Primer design for the amplification of mercury resistance genes

Primers were designed to determine the presence of the *mer* operon in *Alcaligenes* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010, using a number of reported *mer* gene sequences. The majority of the primers were designed based on the *P. aeruginosa* Tn501 *mer* operon (Z00027) which carries the archetypal *merRTPADE* genes (Misra *et al.*, 1984; Brown *et al.*, 1986; Barrineau *et al.*, 1984) (Section 1.4). Additional primers were designed based on the alignment of the genes from plasmid pDU1358 of *Serratia marcescens* (M24940) (Nucifora *et al.*, 1989a) and the Tn21 operon of *Shigella flexneri* (AP000342) (Barrineau *et al.*, 1984; Misra *et al.*, 1984; Misra *et al.*, 1985), to address possible heterogeneity of the genes in our isolates, compared to these ones. The primer pair mercP-cA, which corresponds to the *merP*, *merC* and *merA* genes, was designed based on the plasmid NR1 sequence (NM1MER) (Holt *et al.*, 1999). Details of the *mer* operons are outlined in Section 1.4. Figure 2.2 provides an overview of the primer locations relating to the *merRTPADEorf-2* genes. Further details for each primer are provided in Table 2.2.

2.8.1.2 Primer design for the amplification of lead resistance genes

Primers for the detection of lead resistance (*pbr*) genes were designed based on the only published lead resistance operon to date, from *Cupriavidus metallidurans* CH34 (X71400) (Borremans *et al.*, 2001) (Section 1.5). Figure 2.5 provides an overview of the primer locations and further details for each primer are provided in Table 2.2.

2.8.1.3 Primer design for the amplification of cadmium resistance genes

Primers for the detection of the cadmium resistance gene (*cadA*) and regulatory gene (*cadC*) were designed based on the pI258 plasmid of *Staphylococcus aureus* PI25CADA) as described in Section 1.6.2 (Nucifora *et al.*, 1989b). Figure 2.6 provides

an overview of the primer locations and further details for each primer are provided in Table 2.2.

2.8.1.4 Primer design for the amplification of genes relating to PAH degradation

Unlike the mer and pbr primer pairs, which were designed to span two or more genes, the primers targeting PAH degradation genes were all designed to amplify individual genes, due to the wide inter-species variation of operon structures (see Section 1.7). The primers were designed based on the regions of highest homology in a number of PAH degradation genes. The genes used were from the operons of *Ralstonia* sp. U2 (AF036940) (Fuenmayor *et al.*, 1998; Zhou *et al.*, 2001), *Pseudomonas stutzeri* (AF039533) (Bosch *et al.*, 1999a), *P. putida* (PSENAPDOXA) (Simon *et al.*, 1993), *Pseudomonas fluorescens* (AF004283) (unpublished), *Comamonas testosteroni* (AF252550) (Moser and Stahl, 2001) and *P. aeruginosa* (PSEORF1). Figures 2.7-2.13 provide overviews of the primer locations relating to PAH degradation. Further details of primers are provided in Table 2.2.

2.8.2 PCR conditions

PCR mixes were set up by adding: 1 µg genomic DNA template (prepared as described in Section 2.4), 5 µL 5X buffer, 2 µL 50 mM MgCl₂, 1 µL dNTPs (10 mM mix of dATP, dCTP, dGTP, dTTP), 3.5 µL of each forward and reverse primer (0.1 µg/µL), and 0.5 µL DNA polymerase enzyme (5 U/ µL) and dH₂O to a total volume of 50 µL. AmpliTaq[®] DNA polymerase (Perkin Elmer) and Taq DNA polymerase (Gibco BRL Life Technologies) enzymes were used for standard PCRs while Expand Long Template PCR system (Roche) was used longer fragments (>1000 bp) and Platinum[®] Taq DNA polymerase (Invitrogen) was used for reactions that required high levels of sensitivity. Negative control PCRs were set up as above, without the DNA template. PCRs were

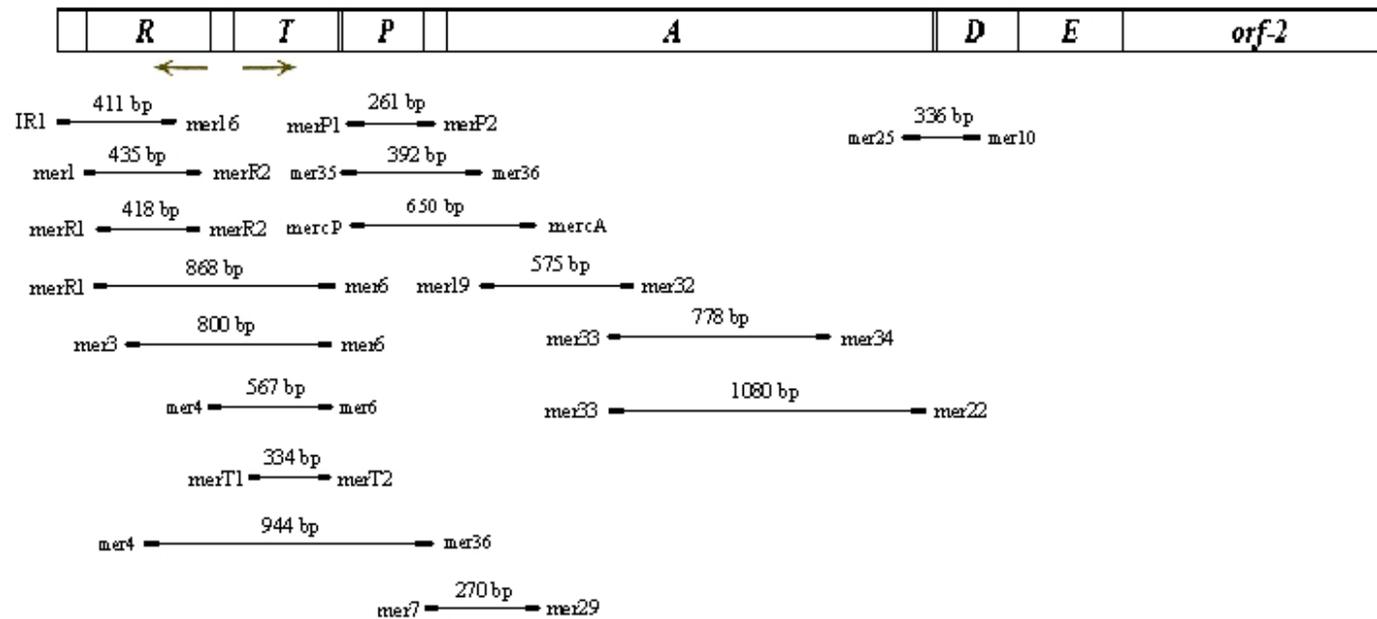


Figure 2.2 Location of primers designed to amplify the *merRTPADEorf-2* genes. This figure indicates the location of and expected size of amplified products for each primer pair. These primers were designed based on the *mer* operons of Tn501 (Z00027), Tn21 (AP000342), pDU1358 (M24940) and pNR1 (NM1MER).

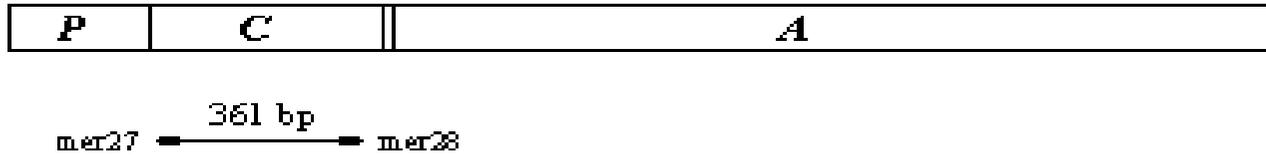


Figure 2.3 Location of primers designed to amplify the *merC* gene. This figure indicates the location of and expected size of amplified products for each primer pair. These primers were designed based on the *mer* operon of Tn21 (AP000342).

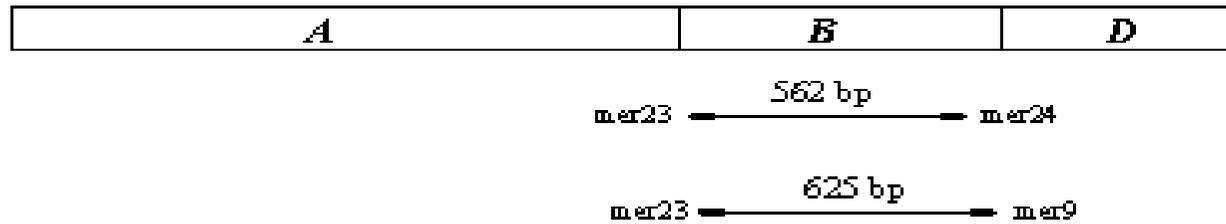


Figure 2.4 Location of primers designed to amplify the *merB* gene. This figure indicates the location of and expected size of amplified products for each primer pair. These primers were designed based on the *mer* operon of pDU1358 (M24940). The additional primer, *mer9* was designed to further amplify the 3' section of this gene beyond *mer24*.

run in the PTC-100 or PTC-200 Peltier Thermal Cycler (MJ Research) using the following typical cycles: an initial denaturation step at 95°C for 5 minutes followed by 35 cycles of: 94°C for 90 seconds (denaturation); 55-58°C for 1 minute (annealing); 70°C for 3 minutes (elongation); followed by a final elongation step of 70°C for 7 minutes. All amplification reactions were electrophoresed as described in Section 2.7.

2.9 Purification of PCR products

The PCR products of expected size, when required for further analysis such as sequencing, needed to be purified from PCR buffers, unincorporated dNTPs and any non-specific DNA fragments. Two methods were used depending on whether the PCRs yielded a single product or multiple bands due to non-specific amplification.

2.9.1 Microspin Columns

The S-400 Microspin columns (exclusion limit ≥ 200 bp) containing Sephacryl™ HR resin were used according to the suppliers' instructions (Amersham Pharmacia Biotech) for PCRs containing a single band. The resin was resuspended by vortexing for 10 seconds to remove the TE buffer from the resin and the tubes were centrifuged at 3000 rpm for 1 minute. The columns were transferred to a new microcentrifuge tube and the PCR mix was loaded onto the resin bed. The columns were centrifuged at 3000 rpm for 5 minutes and the flow-through containing the purified PCR product was confirmed by agarose gel electrophoresis.

2.9.2 Concert™ Gel Extraction System

The Concert™ Gel Extraction System was used according to the manufacturer's instructions (Invitrogen) for separation of a particular PCR product from a mix. The fragment of interest was excised from the gel, the gel slice was cut into small pieces and placed in a microfuge tube. To dissolve the gel slice, 30 μ L of gel solubilisation buffer (L1) was added for every 10 μ g of gel slice and the tube incubated at 50°C for at least

Table 2.2 PCR primers used in this investigation

Primer name	Orientation	Sequence (5'-3')	T _m (°C)*	Genbank Reference
IR1	Forward	GGGGGAACCGCAGAATTC	66.6	Z00027
Mer1	Forward	GGAAAATAAAGCACGCTAAGGC	65.0	
Mer3	Forward	GCATGGCAGGCGCACACC	69.5	
Mer4	Forward	CGGATGCTGCCATAGGGC	68.2	
Mer6	Reverse	CATGGTGAACCTCCGATCAG	61.8	
Mer7	Forward	GTCACGCTGTCCGTACCG	68.2	Z00027 PDUMER
Mer9	Reverse	CCTAGATGACATGGTCTGC	64.6	PDUMER
Mer10	Reverse	CATGGCAGACTCTCCGCG	68.5	Z00027
Mer16	Reverse	TGGCGAGGCGGATGTAACG	71.5	
Mer19	Forward	AATCACCGGCATGACTTGC	66.1	Z00027 AP000342
Mer22	Reverse	TGTTGAAGGTCTGCGCCG	68.8	Z00027
Mer23	Forward	GCTCGCCCATATATTTTAG	59.9	PDUMER
Mer24	Reverse	GACACTGACGATCGCCAAT	63.8	
Mer25	Forward	GGCTCTTGATGCCGGGG	69.3	Z00027
Mer27	Forward	CTGATGACACGATTGCCGA	70.7	AP000342 AF120959
Mer28	Reverse	CGGCGACACGAAGTCCAG	71.5	AP000342 AF120959
Mer29	Reverse	AGSGCAACCTTGACGTGCA	66.4	Z00027
Mer32	Reverse	ATGCCTTCGTA CTGGCGTG	67.1	AP000342
Mer33	Forward	CACGCCAAGTACGAAGGCAT	67.1	
Mer34	Reverse	ACGGTCGCCACTTGCGGAT	72.3	
Mer35	Forward	TGATCGGAGTTCACCATG	60.7	Z00027
Mer36	Reverse	GCAAGTCATGCCGGTGATT	66.1	Z00027 AP000342
MerR1	Forward	AGGCATAGCCGAACCTGC	65.3	Z00027
MerR2	Reverse	GAGAACCTGACCATGGC	60.7	
MerT1	Forward	TCTGAACCAAAACCGGGC	67.0	
MerT2	Reverse	GGCATGACGTAGGAAATC	61.9	
MerP1	Forward	CTGTTTGCCTCCCTCGC	64.7	
MerP2	Reverse	TGCTTGACGCTGGACGG	67.8	
MercP	Forward	CCCGATCACWGTCAAGMAVGC	64.4	NM1MER
MercA	Reverse	CGCTCGATCAGCCGWGACVYG	69.5	
Pbr8	Forward	ATCGGGGAGGCGCCAGAAT	72.1	X71400
Pbr9	Reverse	CGCCAGTCGCGAGATGA	67.9	
Pbr10	Forward	AGGACAGCTTCGCCTTCA	63.8	
Pbr11	Reverse	CCTTGTTAGCCAGACCT	56.7	
Pbr12	Forward	TGAGGTACGCGGTCAGTT	62.0	
Pbr13	Reverse	CTGCGTCTCCTTTCGATT	60.5	
Pbr14	Forward	TTGTCTTGCGTGGCGAGA	67.1	

Table 2.2 continued

Primer name	Orientation	Sequence (5'-3')	T _m (°C)*	Genbank Reference
Pbr15	Reverse	TGCCCGGTGGTGACCAT	69.1	X71400
Pbr16	Forward	CAACAGCCCTTCTTGTTTC	58.3	
Pbr17	Reverse	GAGCCAGTACACGACCT	55.9	
Pbr18	Forward	AGTTCAATCTGGTGACGC	58.9	
Pbr19	Reverse	GATCCGCGCCAATGTTGA	69.5	
Cad1	Forward	GAATGAAGATGGGATGATAA	56.3	PI25CADA
Cad2	Reverse	GATTCGCTAGTTTTTCAGGA	58.5	
Cad3	Forward	GCCCTAGCACATAAGAAAG	56.5	
Cad4	Reverse	CAGCAACCAAGGCTACAA	60.0	
Cad5	Forward	CGAAGTATTTGCAGGTACG	58.3	
Cad6	Reverse	CCCATATCGGAAAGAATCG	61.8	
PahAa1	Forward	TCACCCGGCGCGMATCRTCAA	73.5	AF036940
PahAa2	Reverse	CCGCTGGGATAGAASGCATC	66.5	AF039533
PahAc1	Forward	GGGCTGACSCAAAAARCACCT	63.0	AF036940 PSENAPDOXA AF039533
PahAc2	Reverse	CTGTTGTTCCGGAAAACGGTG	68.4	AF004283 AF252550 PSEORF1
PahAd1	Forward	CCACGACGCCGAAGAGTTTC	69.1	PSENAPDOXA AF039533
PahAd2	Reverse	AGAAGACATCGACTTGATTGCC	64.3	PSEORF1
PahC1	Forward	CATGGGCATCTCGGTCAAGG	70.0	AF036940 AF039533
PahC2	Reverse	TCAATGAGCCAGCCGAAGG	71.7	PSEORF1
PahE1	Forward	GATGCTTCTGAYTGCGCAG	55.0	AF036940 AF039533
PahE2	Reverse	AACTCCGAAAAGTCGCCACG	54.0	PSEORF1
PahF1	Forward	AAGCACCCYGTCAGTGGYGAG	66.6	AF036940 AF039533
PahF2	Reverse	TTGCCGCAGACCAGCGGATA	56.0	PSEORF1 AF252550
27f (16S)	Forward	AAGAGTTTGATCCTGGCTCA	62.0	AFARGSSA
1387r (16S)	Reverse	ACGGGCGGTGTGTACAAGAC	62.0	
SP6	Forward	ATTTAGGTGACAGTATAGAATAC	50.22	
T7	Reverse	GTAATACGACTCACTATAGGGC	56.02	

*calculated T_m values: provided by Sigma Genosys with the manufacture of primers (<http://www.sigma-genosys.com/calc/DNACalc.asp>)

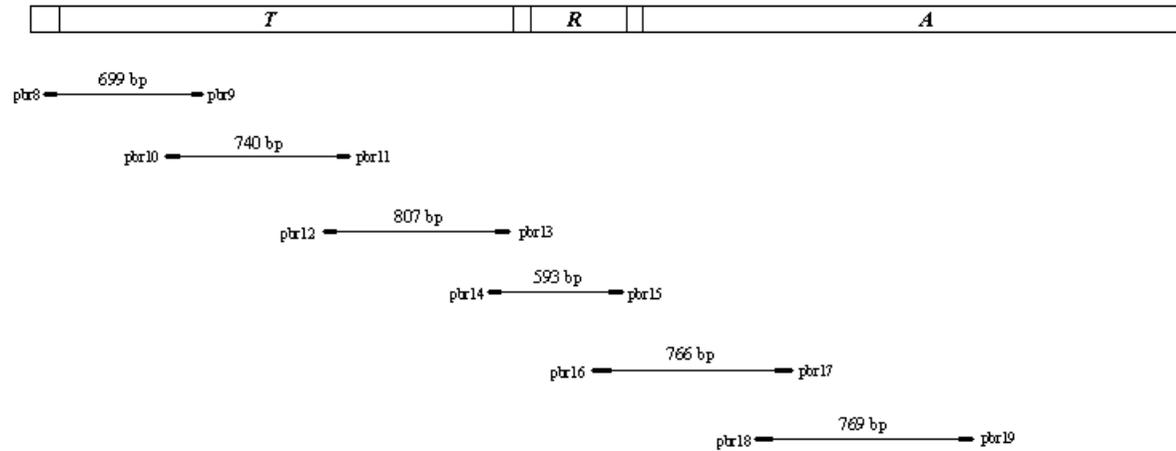


Figure 2.5 Location of primers designed to amplify the genes of the *pbr* operon. This figure indicates the location of and expected size of amplified products for each primer pair. These primers were designed based on the *pbr* operon of *C. metallidurans* CH34 (X71400)

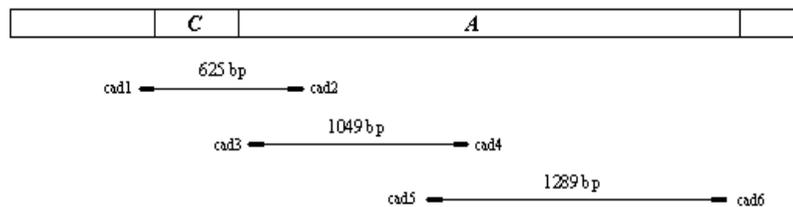


Figure 2.6 Location of primers designed to amplify the *cad* operon. This figure indicates the location of and expected size of amplified products for each primer pair. These primers were designed based on the *cadCA* genes of pI258 (PI25CADA).

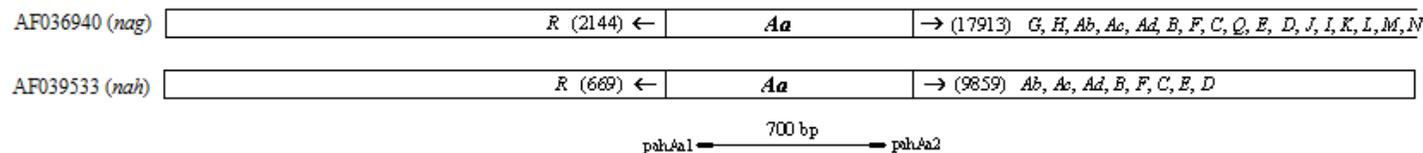


Figure 2.7 Location of primers designed to amplify the *nahAa* and *nagAa* genes. Primers were designed based on the alignment between the *nagAa* gene of *Ralstonia* sp. U2 (AF036940) and the *nahAa* gene of *P. stutzeri* (AF039533). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

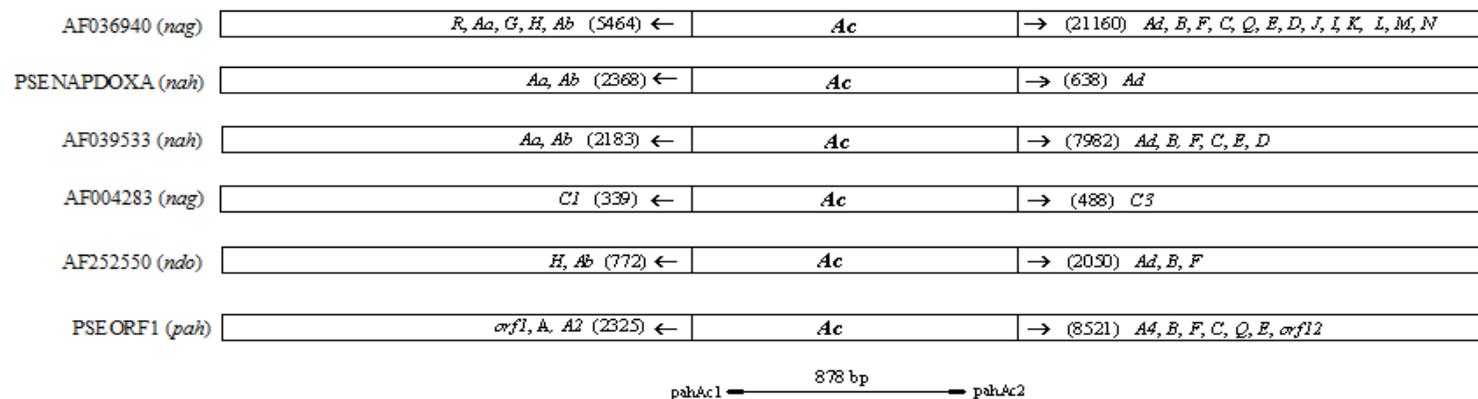


Figure 2.8 Location of primers designed to amplify the *nahAc*, *nagAc*, *ndoAc* and *pahAc* genes. Primers were designed based on the alignment between the *nagAc* gene of *Ralstonia* sp. U2 (AF036940), *nahAc* of *P. stutzeri* (AF039533), *nahAc* of *P. putida* (PSENAPDOXA), *ndoC2* of *P. fluorescens* (AF004283), *pahAc* of *C. testosteroni* (AF252550) and *pahA3* of *P. aeruginosa* (PSEORF1). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

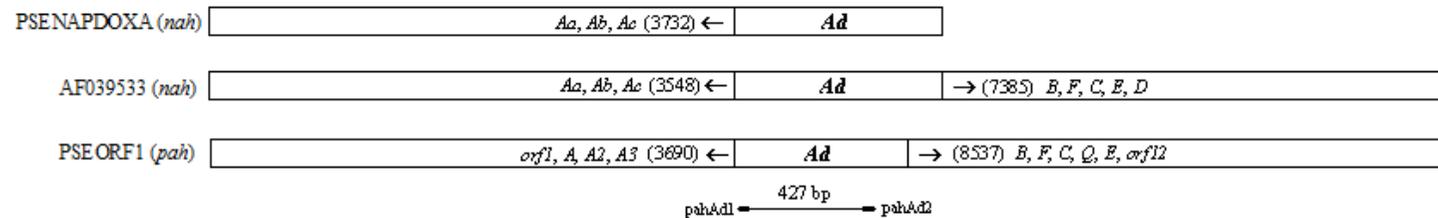


Figure 2.9 Location of primers designed to amplify the *nahAd*, *nagAd* and *pahA4* genes. Primers were designed based on the alignment between the *nahAd* gene of *P. putida* (PSE NAPDOXA), the *nagAd* gene of *P. stutzeri* (AF039533) and the *pahA4* gene of *P. aeruginosa* (PSE ORF1). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

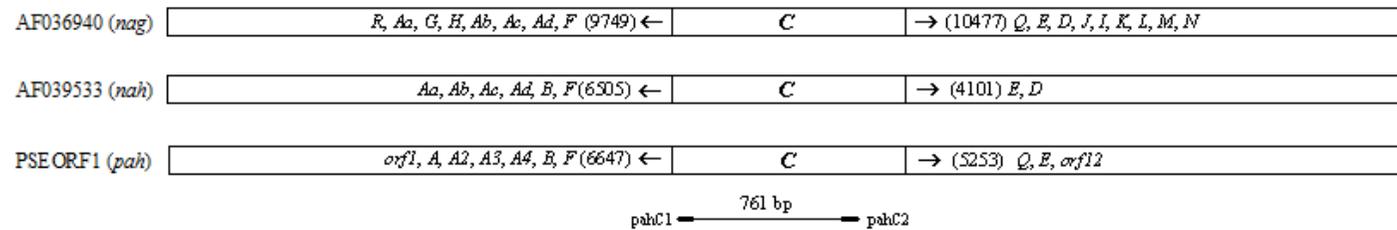


Figure 2.10 Location of primers designed to amplify the *nahC*, *nagC* and *pahC* genes. Primers were designed based on the alignment between the *nagC* gene of *Ralstonia* sp. U2 (AF036940), the *nahC* gene of *P. stutzeri* (AF039533), and the *pahC* gene of *P. aeruginosa* (PSE ORF1). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

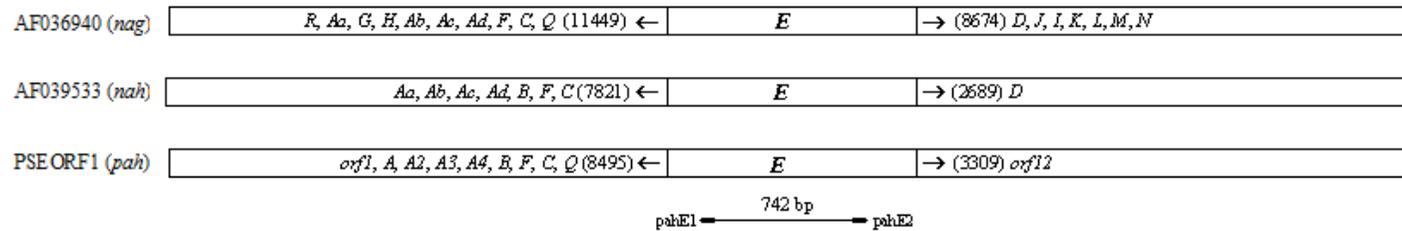


Figure 2.11 Location of primers designed to amplify the *nahE*, *nagE* and *pahE* genes. Primers were designed based on the alignment between the *nagE* gene of *Ralstonia* sp. U2 (AF036940), the, the *nahE* gene of *P. stutzeri* (AF039533), and the *pahE* gene of *P. aeruginosa* (PSEORF1). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

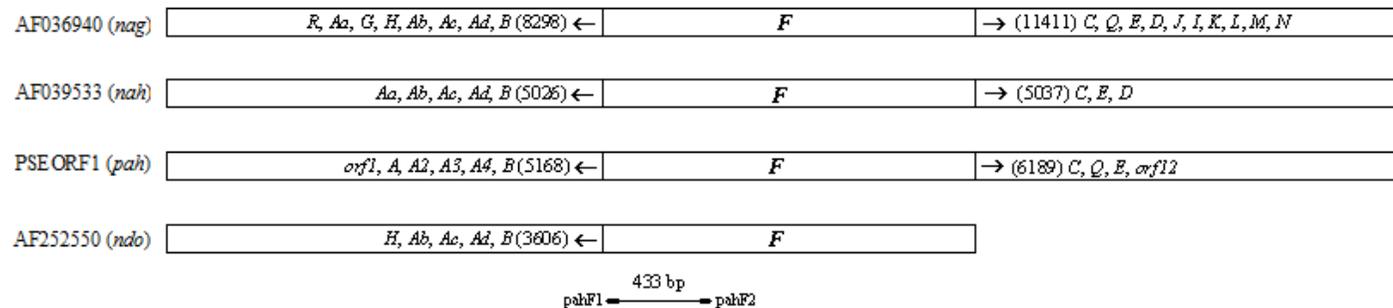


Figure 2.12 Location of primers designed to amplify the *nahF*, *nagF* and *pahF* genes. Primers were designed based on the alignment between the *nagF* gene of *Ralstonia* sp. U2 (AF036940), the, the *nahF* gene of *P. stutzeri* (AF039533), the *pahF* gene of *P. aeruginosa* (PSEORF1) and the *pahB* gene of *C. testosteroni* (AF252550). Indicated on either side of the gene of interest are the remaining genes in the applicable operon and the total bp flanking this gene within the operon.

15 minutes, with vortexing every 3 minutes. A further 5 minute incubation was performed once the gel slice had melted. The spin cartridge (containing a silica matrix) was placed in a 2 mL wash tube (provided with the kit) and the gel solution pipetted into the cartridge, which was then centrifuged at 12000 rpm for 1 minute. The flow-through liquid was discarded, 500 μ L of gel solubilisation buffer was added to the cartridge and incubated for 1 minute at room temperature prior to centrifugation at 12000 rpm for 1 minute. The flow-through liquid was again discarded and 700 μ L of wash buffer was added to the cartridge and incubated at room temperature for 5 minutes, then centrifuged for 1 minute. The flow-through was discarded and the cartridge centrifuged again to remove residual wash buffer. The spin cartridge was then placed in a 1.5 mL recovery tube and 50 μ L of warm TE buffer was loaded onto the center of the cartridge for elution of the matrix-bound DNA. After incubation for 1 minute at room temperature, the purified DNA was eluted in TE buffer was collected by centrifugation of the cartridge at 12000 rpm for 2 minutes. The size of the purified PCR product was then confirmed by agarose gel electrophoresis.

2.10 Cloning of PCR products

PCR products, purified by one of the above two methods, were cloned into the pGEM[®]-T Easy Vector (Promega), which contains single 3'-T overhangs to aid in the ligation process (Appendix 4). This high copy vector contains T7 and SP6 RNA polymerase promoters, which flank a multiple cloning region within the α -peptide coding region of the enzyme β -galactosidase (*lacZ*). The insertion of a DNA fragment into this cloning region results in the inactivation of the α -peptide and allows for cells containing recombinant plasmids (white) to be differentiated from cells containing non-recombinant plasmids (blue) when plated on colour-selection plates containing IPTG and X-Gal. The vector contains multiple restriction sites, which allow for the excision of the DNA insert by restriction.

2.10.1 Ligation of purified PCR products into cloning vectors

For the cloning and sequencing of PCR products, the pGEM[®]-T Easy Vector System (Promega) was used. Reactions were set up as per supplier's protocols as follows:

Component	Standard Reaction	Positive control	Negative control
2X rapid ligation buffer, T4 DNA Ligase	5 µL	5 µL	5 µL
pGEM [®] -T Easy Vector (50 ng)	1 µL	1 µL	1 µL
PCR product (50 ng)	X µL	-	-
control insert DNA	-	1 µL	-
T4 DNA ligase (3 Weiss units/µL)	1 µL	10 µL	1 µL
dH ₂ O water to a final volume of	10 µL	10 µL	10 µL

The reaction mixes were incubated at 4°C overnight.

2.10.2 Transformation of ligation reactions

2 µL of ligation reaction was added to 50 µL of just-thawed chemically competent *E. coli* JM109 cells, supplied with the pGEM[®]-T Easy Vector System (Promega). This mix was held on ice for 20 minutes and heat shocked for 45 seconds at exactly 42°C. The tubes were returned to the ice for 2 minutes prior to the addition of 950 µL of NB. The cells were then incubated at 37°C at 150 rpm for 90 minutes and 100 µL of the transformation mix was plated, in duplicate, onto colour selection plates. The plates were incubated overnight at 37°C. Cell containing recombinant plasmids appear as colourless colonies, while cells that contain non-recombinant plasmids appear blue.

2.10.3 Preparation of recombinant plasmids

The method was based on the protocol described by Sambrook *et al.* (1989). A single colourless colony was inoculated into 4 mL of LB broth containing 50µg/mL ampicillin and the culture incubated at 37°C overnight. 3 mL (2 X 1.5 mL in the same microfuge tube) of overnight culture was pelleted by centrifugation at 14000 rpm for 2 minutes at room temperature and the cell pellet was resuspended in 80 µL of solution I (Glucose/Tris-Cl/EDTA) and 20 µL of lysozyme solution (solution I containing 10 mg/mL lysozyme) was added to it. The tube was inverted to mix the contents and 200 µL of solution II (NaOH/SDS) was added. The tube was inverted to mix and placed on

ice for 5 minutes followed by the addition of 150 μL of 3 M sodium acetate (pH 5.2), then placed on ice again for 5 minutes. The tube was then centrifuged at 14000 rpm for 5 minutes to remove cellular debris, such as proteins and cell wall components, and 5 μL of DNase-free RNase A (10 mg/mL) was added to the supernatant and incubated at 37°C for 30 minutes. An equal volume of phenol-chloroform-isoamyl alcohol (25:24:1) (Appendix 2) was added and inverted prior to centrifuging at 14000 rpm for 5 minutes at room temperature. 2 volumes of pre-chilled 100% ethanol was added to the clear supernatant, and the mixture was held at -80°C for 10 minutes. The DNA was pelleted by centrifugation at 14000 rpm for 10 minutes at room temperature, the pellet air-dried for 10 minutes and resuspended in 50 μL of dH_2O .

Alternatively, recombinant plasmids were extracted using the Wizard® *PlusSV* Minipreps DNA Purification System (Promega). 3 mL of overnight culture was pelleted as described above, resuspended in 250 μL cell resuspension solution and vortexed followed by the addition of 250 μL cell lysis solution. The tube was inverted and incubated for 5 minutes at room temperature followed by the addition of 10 μL alkaline protease solution and a further incubation for 5 minutes at room temperature. 350 μL of neutralization solution was added and the tubes inverted and centrifuged at 14000 rpm for 10 minutes at room temperature. The clear lysate was transferred to a spin column contained in a 2 mL collection tube and centrifuged at 14000 rpm for 1 minute. The spin column was transferred to another collection tube and 750 μL of column wash solution was added followed by centrifugation at 14000 rpm. This wash step was repeated with 250 μL column wash solution. The spin column was then transferred to a new collection tube, 100 μL of nuclease-free water was added and the plasmid DNA was eluted by centrifugation at 14000 rpm.

2.10.4 Determination of the sizes of inserts by restriction digestion of clones of recombinant plasmids

To confirm the success of cloning, an *EcoR*I restriction digest was performed as follows:

Recombinant plasmid (5 μ L); *Eco*R1-10U/ μ L (2.5 μ L); 10X Buffer (2 μ L); dH₂O (up to 20 μ L). The reaction was incubated at 37°C for a minimum of 1 hour and the results examined by agarose gel electrophoresis.

2.11 Sequencing of DNA

Sequencing was performed using the dideoxynucleotide sequencing method, initially described by Sanger *et al.*, (1977). Sequencing reactions were performed either on recombinant plasmids (as described above) using the pGEM-T Easy vector-based primers for the T7 and SP6 RNA polymerase promoter sequences (Appendix 4, Table 2.2) or directly on the purified PCR products, using internal primers corresponding to the particular DNA fragment. Sequence reactions were set up and precipitated according to the protocol provided ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit v.3 (Applied Biosystems).

Sequencing reactions were set up as follows: BigDye reagent (Applied Biosystems) (2 μ L); 10X reaction buffer (10 mM MgCl₂, 250 mM Tris-Cl (pH 9.0) (2 μ L); 1 μ L SP6 or T7 primer (3.2 pmol/ μ L); template (X μ L); dH₂O (to 20 μ L). The amount of template DNA used was dependant on its concentration. Approximately 50ng of PCR product or 200ng of plasmid DNA was used. Reactions were run on the following cycle:

45 cycles of: 96°C for 30 seconds; 55°C for 15 seconds; 60°C for 4 minutes

5 cycles of: 96°C for 30 seconds; 60°C for 4 minutes; 25°C for 1 minute

Sequence reactions were precipitated according to the methods provided with the BigDye reagent (Applied Biosystems), by mixing 20 μ L of sequencing reaction with 20 μ L dH₂O, 6 μ L 3M sodium acetate (pH 5.2) and 80 μ L 100% ethanol. The mix was vortexed and incubated at room temperature for 15 minutes, then what was pelleted by centrifugation at 14000 rpm for 30 minutes. The supernatant was removed, 1 mL 70% ethanol was added to the pellet to rinse the DNA, which was then repelleted by centrifugation at 14000 rpm for 10 minutes. The DNA pellet was dried and sent to Micromon DNA Sequencing Facility, Department of Microbiology, Monash University, Clayton 3168, Australia, where it was analysed using the ABI 373A automated sequencer.

2.11.1 Sequence data analysis

Sequence data obtained was edited and analysed using the Bionavigator interface of the Australian National Genomic Information Service (ANGIS) (www.angis.org.au). The following programs were utilised to assist with this analysis:

Edit: used to modify a selected input file e.g. sequence, or alignment file.

Reverse: used reverse and/or complement the symbols in a sequence.

BlastN: used to compare a nucleotide query sequence to a nucleotide sequence database.

BlastX: used to compare the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

Gap: used to find the alignment of two complete sequences. The default conditions included a gap creation penalty of 8.0 and a gap extension penalty of 2.0. The results of this alignment provided the levels of sequence identity and protein similarities.

ClustalW (accurate): used to perform multiple sequence alignments, whereby the default conditions of a gap opening penalty of 10.0 and a gap extension penalty of 0.1 was used. The output created a visual of the areas of homology between the multiple sequences.

The BioEdit software package version 5.0.6 (www.mbio.ncsu.edu/BioEdit/bioedit.html; Hall, 1999) is a biological sequence editor that runs in Windows 95/98/NT/2000 and is intended to provide basic functions for protein and nucleic sequence editing, alignment, manipulation and analysis. This program was primarily used was also used for this study for DNA sequence alignments, and also aided in the design of primer sequences. This program also allowed data to be collated and edited in a format (Graphic View) to display as figures for the results sections of this thesis.

RNA TECHNIQUES

RNA was utilised to compare gene expression differences between samples based on time and the presence/absence of mercuric chloride in the media. Extracted RNA was used to produce cDNA, which was used for Reverse-Transcriptase PCR and Real-Time PCR analysis. The following sections detail these methods.

2.12 Isolation of RNA from bacterial cells

Prior to performing any RNA extractions, it was necessary to inactivate all RNAses from solutions and glassware. All solutions and water required for RNA work were first treated with diethyl pyrocarbonate (DEPC), as per manufacturer's instructions. 100 μL of DEPC was added per 1000mL of solution or water. The solutions were left at room temperature overnight in the fume hood, followed by autoclaving at 121°C for 15 minutes. Tris buffer solutions were prepared with DEPC-treated dH₂O followed by autoclaving. All glassware to be used for RNA extractions was baked at 240°C for 24 hours.

2.12.1 Determination of fixed cell numbers for RNA extractions

RNA was extracted from the cells collected during the growth curve/induction experiments (Section 2.3.6), to study the expression of the *mer* genes at various time points and in the presence or absence of mercuric chloride. RNA was extracted from equal cell numbers (approximately 1.75×10^8) as described by Emslie (2002). To do this, the cell numbers from each sample were determined (Section 2.3.7). The cell pellet that was determined to contain the lowest cell number was resuspended in 10 mL protoplasting buffer. To account for differences in cell numbers within the pellets, the amount of protoplasting buffer added to resuspend each additional pellet was increased 10 ml of the resuspended volumes (corresponding in cell numbers to the cell pellet containing the lowest number of cells) was used for RNA extraction.

2.12.2 Isolation of RNA from Gram-negative bacteria

The method was based on that described by Ausubel *et al.*, (1995). The cell pellet was resuspended in an appropriate volume of protoplasting buffer, 80 μL of lysozyme solution (50 mg/mL) was added to it, followed by incubation on ice for 15 minutes. The protoplasts were centrifuged at 5900 rpm for 5 minutes at 4°C, then resuspended in 0.5 mL Gram-negative lysing buffer and 15 μL DEPC-treated dH₂O was added. The solution was mixed and incubated at 37°C for 5 minutes, then cooled on ice followed by the addition of 250 μL saturated NaCl. The solution was mixed and incubated on ice for 10 minutes followed by centrifugation at 14000 rpm for 10 minutes at 4°C. The

supernatant was removed to two new microfuge tubes and 1mL ice-cold 100% ethanol was added to each. RNA was precipitated at -80°C for 30 minutes and pelleted by centrifuging at 14000 rpm for 15 minutes at 4°C. The RNA pellets were rinsed in 500 µL of ice-cold 70% ethanol, air-dried at room temperature for 10 minutes and resuspended in 100 µL of DEPC-treated dH₂O. To remove any residual DNA, the samples were later DNaseI treated (see below).

2.13 Quantitation of RNA samples

Extracted RNA samples were diluted and their absorbance taken as described for DNA quantification (Section 2.6). To determine the RNA concentration the following formula was used:

$$\text{RNA: Absorbance reading} \times 200 \text{ (dilution factor)} \times 40 = \mu\text{g/mL RNA.}$$

All RNA samples were electrophoresed as described in Section 2.7.

2.14 Reverse Transcription-PCR analysis

The following methods describe the DNase treatment of RNA samples, the synthesis of cDNA from the RNA and the amplification of the gene of interest from the cDNA.

2.14.1 Removal of residual DNA from RNA samples

RNA samples (prepared in Section 2.12.2) were DNase I treated to remove any residual DNA, by using the method based on that provided by New England Biolabs. Reactions were set up by adding: 5 µL (generally up to 10 µg) RNA, 1 µL DNase I (10U/µL) 1 µL RNasin, (20U/ µL) 1 µL 10X DNase buffer and 2 µL DEPC-treated water. These reactions were incubated at 37°C for 30 minutes, then stopped by adding 1 µL of 25 mM EDTA (pH 8.0) and incubating at 65°C for 15 minutes.

2.14.2 cDNA synthesis

The details of this method were provided with the SuperscriptII reverse transcriptase enzyme (Invitrogen). To produce cDNA, 1 µL of antisense strand primer (i.e., merR2,

merT2, merP2, mer32) or random primer (10 μ M, 6 bases; Promega) and 5 μ L of DEPC-treated dH₂O was added to 5 μ L of DNase-treated RNA and the mixture was incubated at 70°C for 15 minutes. The sample was then split into duplicates (5.5 μ L per tube) and 0.5 μ L RNasin, 4 μ L 5X first strand buffer, 2 μ L 0.1 M DTT and 1 μ L 10 mM dNTP mix was added to each tube. These were incubated at 42°C for 2 minutes. 1 μ L of Superscript II RT was added to one of the duplicate tubes and both tubes incubated at 42°C for 50 minutes, followed by 70°C for 15 minutes. The tube containing no Superscript II enzyme serves as a negative control, to determine whether any amplification products in the RT-PCR were as a result of any residual DNA in the RNA sample after DNaseI treatment.

2.14.3 Reverse Transcription-PCR

PCR amplifications were set up using 2 μ L of the above cDNA reaction mix, 5 μ L 10X buffer, 2 μ L 50 mM MgCl₂, 1 μ L 10 mM dNTP mix, 3.5 μ L (0.1 μ g/ μ L) of each forward and reverse primer for the gene to be amplified (Table 2.2), 25.5 μ L water and 0.5 μ L *Taq* polymerase (5 U/ μ L). The samples were then amplified as described in Section 2.8.2. 5 μ L of each PCR mix was electrophoresed to determine the presence of specific gene transcripts in the RNA samples (Section 2.7).

Chapter 3

Establishing the Heavy Metal Resistance of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

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3.1 Introduction

Minimum inhibitory concentration (MIC) may be defined as the lowest concentration of an antimicrobial compound that will inhibit the growth of a microorganism after overnight incubation (Nelson, 1991; Andrews, 2001). MICs are commonly used to determine the susceptibility of organisms to antimicrobials such as antibiotics, or resistances to environmental challenges, such as heavy metals (Andrews, 2001).

Several methods have been reported to determine MICs, including the use of liquid medium or solid medium (Rensing *et al.*, 1997b; Filali *et al.*, 2000; Lee *et al.*, 2001; Zeroual *et al.*, 2001). The latter may include streak plates (Barbieri *et al.*, 1996; Hassan *et al.*, 1999; Yilmaz *et al.*, 2003) and agar diffusion plates (Amoroso, 2000). A number of bacteria have previously been examined for heavy metal resistances from a range of environments including contaminated sludge (Zeroual *et al.*, 2001), sewage (Filali *et al.*, 2000), rhizosphere (Lee *et al.*, 2001), soil surrounding a contaminated river (Yilmaz *et al.*, 2003), contaminated river water (Hassan *et al.*, 1999) and soils polluted from manufacturing sites (Manovski *et al.*, 1992; Amoroso *et al.*, 2000). Bacterial genera isolated from the above samples include *Pseudomonas*, *Enterobacter*, *Proteus*, *Klebsiella*, *Staphylococcus*, *Bacillus*, *Streptomyces*, *Alcaligenes*, *Arthrobacter* and *Corynebacterium* (Manovski *et al.*, 1992; Hassan *et al.*, 1999; Amoroso *et al.*, 2000; Filali *et al.*, 2000; Lee *et al.*, 2001; Zeroual *et al.*, 2001; Yilmaz *et al.*, 2003). Some bacterial strains, including *Pseudomonas stutzeri*, *Pseudomonas fluorescens* and *Pseudomonas putida*, have been isolated based on their ability to degrade aromatic compounds and these strains are also found to be resistant to some heavy metals (Barbieri *et al.*, 1996).

This chapter focuses on the confirmation of the MICs to a range of heavy metals of the previously isolated bacteria, *Alcaligenes* sp. AO17 and AO22 and *Arthrobacter* sp. E9 and E11 using a previously used method, along with alternative methods. An additional isolate, Consortium VUN 10010 will also be investigated for heavy metal resistance capabilities. Growth curve characteristics of all isolates will be determined to allow for the design of subsequent gene expression experiments and the isolates *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 will be characterised to a species level by analysis of the gene encoding 16S rRNA.

3.2 Determination of heavy metal MICs by the spot plate method

Previous work performed by Trajanovska *et al.* (1997) provided an overview of the heavy metal resistances of a number of bacteria which had been obtained from a lead-contaminated soil sample (Manovski *et al.*, 1992) (Section 1.9.1). Of these, a Gram-negative bacterium (*Alcaligenes* sp.) and a Gram-positive bacterium (*Arthrobacter* sp.) were selected for this study, based on their previously determined levels of heavy metal resistances. Each genus included two isolates, *Alcaligenes* sp. isolates AO17 and AO22 and *Arthrobacter* sp. isolates E9 and E11. To determine that these isolates had maintained heavy metal resistances during the long-term storage at -80°C , the same method used by Trajanovska *et al.*, (1997) was utilised (Section 2.3.4). Each isolate was tested in levels of heavy metals up to: 0.1 mM for HgCl_2 , 3.5 mM for $\text{Pb}(\text{NO}_3)_2$, 3.5 mM for $\text{Zn}(\text{NO}_3)_2$, 2 mM for CdCl_2 and 2 mM for $\text{Co}(\text{NO}_3)_2$. A positive control strain, *Cupriavidus metallidurans* CH34 ([source: Mergeay *et al.*, 1985](#)), was included, as this strain has been reported to carry two endogenous megaplasmids, encoding resistances to mercury, lead, zinc, cadmium, cobalt, copper, nickel and chromium (Diels *et al.*, 1989) (Section 1.5). Comparisons between the previously reported MICs and those obtained in the current study are presented in Figure 3.1 and Appendix 5.

The purpose of performing this experiment was to confirm that heavy metal resistance had been retained by the chosen isolates during storage. The genes responsible for heavy metal resistance are often located on extrachromosomal plasmids, which may be lost by the cells under varying conditions. *C. metallidurans* CH34 maintained the reported resistance to metals, with the results being higher in this experiment for HgCl_2 , $\text{Pb}(\text{NO}_3)_2$, $\text{Zn}(\text{NO}_3)_2$ and $\text{Co}(\text{NO}_3)_2$ (25%, 75%, 34% and 5% increases in MIC respectively). A discrepancy was, however, found in the presence of CdCl_2 , with the previous experiment indicating a lack of resistance (0.1 mM), while the current study indicated a 20-fold increase in MIC. In the current experiment, the MICs obtained for *C. metallidurans* CH34, in the presence of $\text{Pb}(\text{NO}_3)_2$, $\text{Zn}(\text{NO}_3)_2$, CdCl_2 and $\text{Co}(\text{NO}_3)_2$ were equivalent to the highest concentration tested on this occasion.

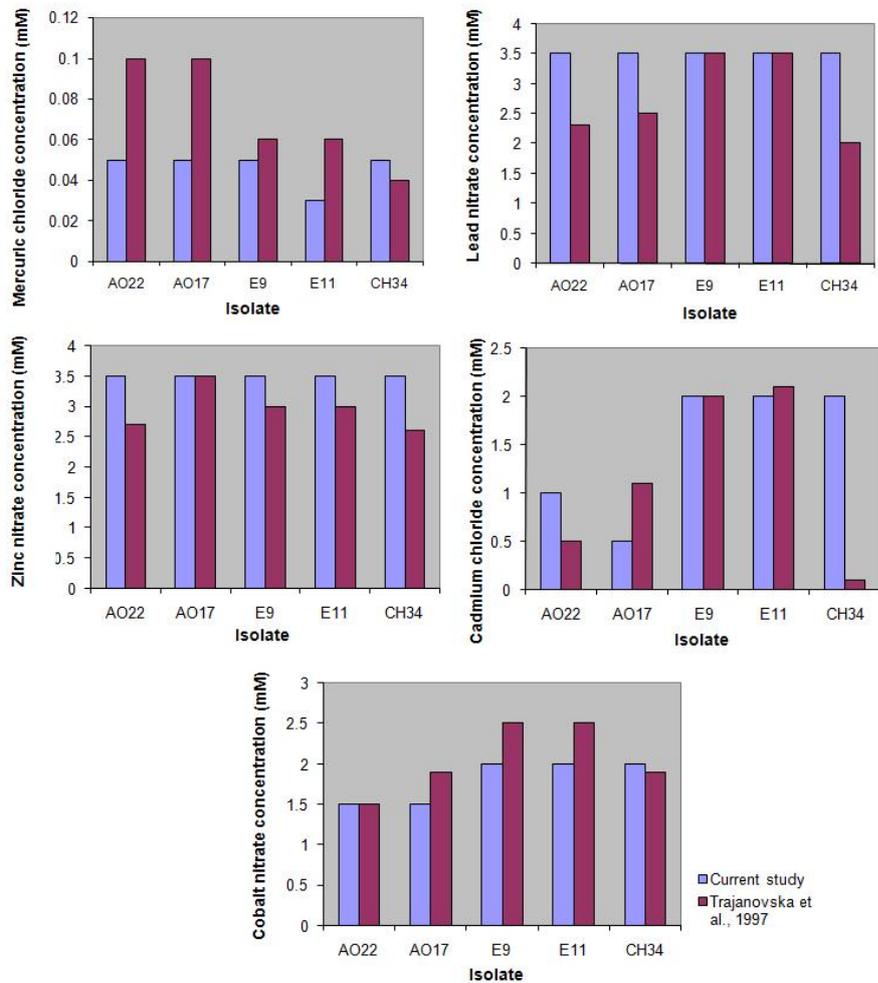


Figure 3.1 Comparison of MIC results obtained in the current study with those obtained by Trajanovska *et al.*, (1997). Experiments were performed as outlined in Section 2.3.4. Cells were inoculated from a 10 ml overnight NBT culture onto NAT plates containing 0-0.1 mM HgCl₂, 0-3.5 mM Pb(NO₃)₂, 0-3.5 mM Zn(NO₃)₂, 0-2 mM CdCl₂ and 0-2 mM Pb(NO₃)₂. Plates were incubated at 30°C for 72 hours.

The *Alcaligenes* sp. isolates AO17 and AO22 had also maintained varying levels of resistance to the metals tested. However, the MICs for both in the presence of HgCl₂, were half that reported in Trajanovska's experiment. For AO17, the MICs were higher than previously reported for Pb(NO₃)₂, Zn(NO₃)₂ and CdCl₂, while the value obtained in the presence of Co(NO₃)₂ was identical for AO17. For AO22, the results were identical for Zn(NO₃)₂, higher for Pb(NO₃)₂ and lower for Co(NO₃)₂ and CdCl₂. As seen for *C. metallidurans* CH34, AO17 and AO22, the MICs in the presence of Pb(NO₃)₂ and Zn(NO₃)₂ were equivalent to the highest concentrations tested. The *Arthrobacter* sp. isolates E9 and E11 demonstrated identical results to those obtained by Trajanovska in the presence of Pb(NO₃)₂ and for E9 in the presence of CdCl₂, while a small decrease in MIC was seen for E11. Both E9 and E11 showed an increase in the presence of Zn(NO₃)₂. An MIC of 2 mM was detected for both E9 and E11 in the presence of Co(NO₃)₂ while the previously reported results showed an MIC of 2.5 mM, but 2 mM was the highest level of Co(NO₃)₂ tested in the current experiment. Decreases in MICs were also seen for E9 and E11 in the presence of HgCl₂ while in the presence of Pb(NO₃)₂ and Zn(NO₃)₂ they were equivalent to the highest concentration tested on this occasion.

3.3 Comparison of the spot plate and spread plate methods for MIC analysis

While the spot plate method provides a broad scope of the MIC levels of the isolates, it would not have been appropriate for further experiments to be conducted in this work, as it does not allow for individual colonies to form. For such reasons, further MIC analysis was also performed using the spread plate method (Section 2.3.4). Only one of the *Alcaligenes* sp. isolates (AO22) and one of the *Arthrobacter* sp. isolates (E9) was used, due to the similar levels of resistances seen above between AO17 and AO22; and between E9 and E11. As described previously, each isolate was tested in levels of heavy metals up to 0.1 mM HgCl₂, 3.5 mM Pb(NO₃)₂, 3.5 mM Zn(NO₃)₂, 2 mM CdCl₂ and 2 mM Co(NO₃)₂. These results are compared to those in Figure 3.2 and Appendix 5.

The comparisons in Figure 3.2 illustrate several differences. In the presence of Pb(NO₃)₂ and Zn(NO₃)₂, at the highest concentration tested, the results remained constant for both methods. For HgCl₂, the MIC for AO22 using the spread plate

method grew to the highest concentration tested (0.1 mM) and was double that obtained using the spot plate method, while the MIC of E9 increased by 62% using the spread plate method. In the presence of CdCl₂, a 3-fold MIC values was seen using spread plate method AO22, while the results obtained for E9 using the spread plate method was identical. In the presence of Co(NO₃)₂, a 33% decrease was seen for AO22, while a 25% decrease was seen for E9 using the spread plate method. Some of these differences are significant (25-50% increases or decreases), and are most likely due to differences in growth conditions and the integrity of the colony used for inoculation. Further MIC analysis was performed using liquid medium with increased levels of the heavy metals and the inclusion of the isolate Consortium VUN 10010. These results are outlined in Section 3.5.

3.4 VUN 10010

As explained in Sections 1.9.2 and 2.3.9, VUN 10010 was originally isolated from a PAH-contaminated soil sample and identified as a pure culture of *S. maltophilia* (Boonchan, PhD thesis, 1998). During the course of this project, however, it was found that it was a mixed culture of *S. maltophilia* and a *Mycobacterium* sp. (C. Dandie, personal communication, Dandie *et al.*, 2004). Attempts were made to isolate the *S. maltophilia* from the *Mycobacterium* sp. in this consortium to determine whether one, or both, of these bacteria were responsible for PAH degradation and whether any heavy metal resistances were evident. These attempts are outlined in Sections 3.4.1-3.4.4.

3.4.1 Gram-staining

Gram-stains were performed on a variety of cultures and glycerol stocks to determine whether the mixed culture or only the Gram-negative bacteria *S. maltophilia* could be detected. The results are summarised in Table 3.3 below:

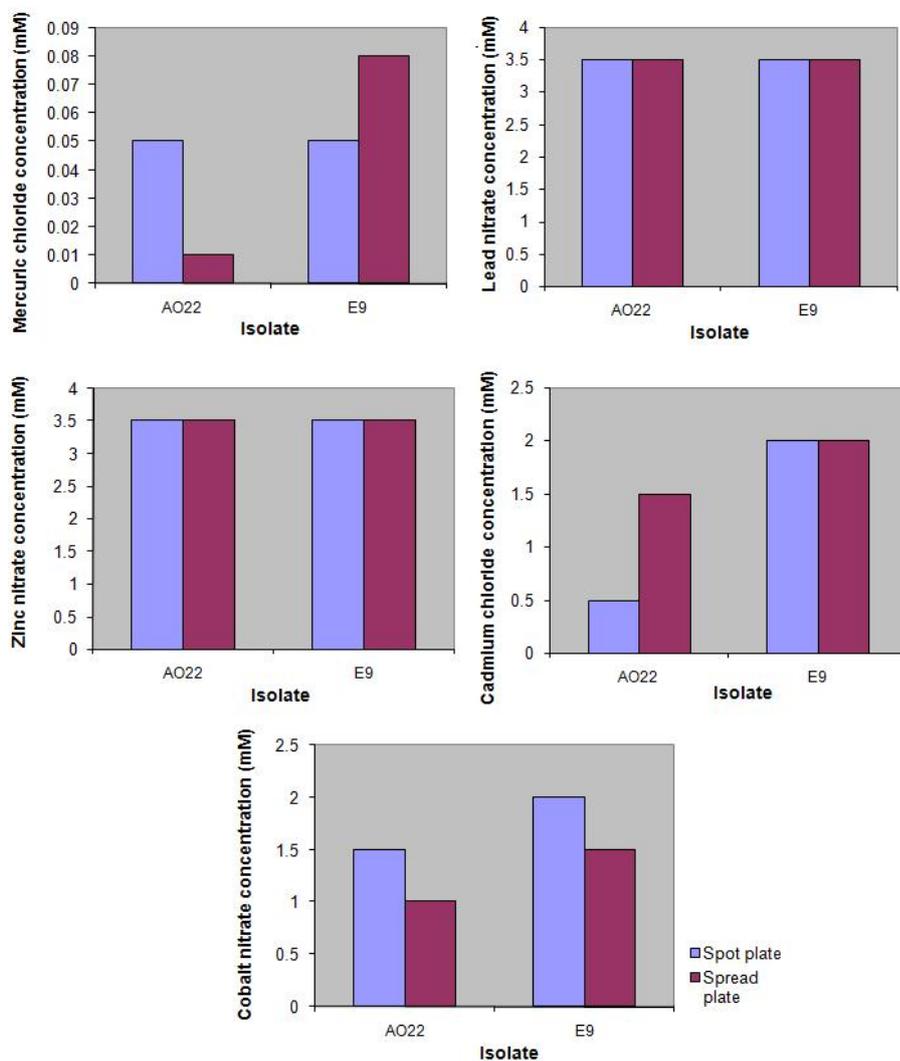


Figure 3.2 Comparison of MIC results obtained using the spot plate and spread plate methods. Experiments were performed as outlined in Section 2.3.4. Cells from a 10 ml overnight NBT culture were spotted or streaked onto NAT plates containing 0-0.1 mM HgCl_2 , 0-3.5 mM $\text{Pb}(\text{NO}_3)_2$, 0-3.5 mM $\text{Zn}(\text{NO}_3)_2$, 0-2 mM CdCl_2 and 0-2 mM $\text{Pb}(\text{NO}_3)_2$. Plates were incubated at 30°C for 72 hours.

Table 3.1 Gram-stain results of various cultures and glycerol stocks of VUN 10010

Culture/glycerol stock description	Gram-stain result
Culture growing in BSM containing 1000 mg/L pyrene	Gram-negative bacteria Gram-positive bacteria
Glycerol stock #1 (from culture grown in NBT)	Gram-negative bacteria
Glycerol stock #2 (from culture grown in NBT)	Gram-negative bacteria
Glycerol stock #3 (from culture grown in NBT)	Gram-negative bacteria
Culture from a BSM/PYR culture, grown in NBT	Gram-negative bacteria

It thus appears that both strains are present in the BSM/PYR culture, while only the *S. maltophilia* is present when the culture is grown in NBT from glycerol stocks, which may have initially lead to the assumption that the glycerol stocks are pure. However, when nutrient broth is inoculated from a BSM/PYR culture, a Gram stain indicates that only *S. maltophilia* is present, although it originated from a mixed culture.

3.4.2 Spray plates

An aliquot of VUN 10010 was spread onto BSM plates which were sprayed with 2% pyrene in diethylether, and BSM plates containing 250 mg/L pyrene (Section 2.3.1; Appendix 3). After a two-week incubation, no growth was detected on the spray plates, while the BSM plates containing pyrene showed cream coloured colonies mixed with small pink colonies. Four of the cream colonies were inoculated into 10 mL BSM containing 250 mg/L pyrene. Subsequent streak plates from cultures again indicated the presence of both types.

3.4.3 Use of Lincomycin

The antibiotic lincomycin was used in an attempt to eliminate the Gram-positive bacterium (*Mycobacterium* sp.) (see Section 2.3.9). Three separate cultures were set up: 1 g/L pyrene + 500 mg/L lincomycin, 1 g/L PYR + 100 mg/L lincomycin and 250 mg/L pyrene + 100 mg/L lincomycin. Plating of the cultures demonstrated that lincomycin was ineffective at removing the *Mycobacterium* sp. from the mixed culture.

3.4.4 *Mycobacterium* stab cultures: further attempts at separation of the two organisms

A stab culture was used that was believed to be *Mycobacterium* sp. isolated from the VUN 10010 consortium (provided by C. Dandie, Flinders University). This was inoculated into BSM + 250 mg/L pyrene. A Gram-stain of this culture showed it to be a Gram-positive bacteria, but after growing it for a further two weeks the Gram-stain indicated that both organisms were present. Dr. Lila Serwincinska, a visiting scientist from Poland, also isolated what was believed to be separate pure cultures of *S. maltophilia* and *Mycobacterium* sp. from VUN10010. Both were sub-cultured and checked again, but neither maintained purity with two different colony types being seen in each culture after streak plating.

Based on the above observations and results of all attempts to purify *S. maltophilia* and *Mycobacterium* sp. from VUN 10010, it was evident that both strains had PAH resistance capabilities, with at least one of these using the pyrene in the media as a sole carbon source, and perhaps one was dependent on the other. It was decided at this point to work with this isolate as a Consortium in all further experiments.

3.5 MIC analysis of AO22, E9 and VUN 10010 using liquid broth

MICs using 10 mL NBT aliquots containing varying concentrations of heavy metals, were performed for the AO22, E9 and VUN 10010 isolates. Based on the results obtained in Section 3.3 the levels of heavy metals added was increased up to: 0.2 mM HgCl₂, 8 mM Pb(NO₃)₂, 8 mM Zn(NO₃)₂, 6 mM CdCl₂ and 6 mM Co(NO₃)₂. These results are compared in Table 3.2.

Table 3.2 Heavy metal MICs of VUN 10010, AO22 and E9 using the liquid broth method

Isolate	Heavy metal (mM)				
	HgCl ₂	Pb(NO ₃) ₂	Zn(NO ₃) ₂	CdCl ₂	Co(NO ₃) ₂
AO22	0.15	4	5.5	1	3
E9	0.125	6	4	1.5	3
VUN 10010	0.175	4	5.5	susceptible	2.5

VUN 10010 appears resistant to levels of heavy metals comparable to those of AO22 and E9 using the liquid broth method, and that AO22 and E9 are capable of growing in levels of heavy metals higher than previously tested. AO22 was able to grow up to 0.15 mM HgCl₂ and E9 up to 0.125 mM and VUN 10010 up to 0.175 mM. The MICs were found to be 4 mM for AO22 and VUN 10010 and 6 mM for E9 in Pb(NO₃)₂, while in the presence of Zn(NO₃)₂, both AO22 and VUN 10010 showed MICs up to 5.5 mM and E9 up to 4 mM. . AO22 had recorded an MIC up 1 mM for CdCl₂, E9 up to 1.5 mM and, while VUN 10010 demonstrated no resistance. For Co(NO₃)₂, both AO22 and E9 recorded an MIC of 3 mM, while VUN 10010 was found to have a MIC of 2.5 mM. With the exception of CdCl₂, levels of MIC for both AO22 and E9 were determined to be higher than determined using the spot and spread plates. The MICs of VUN 10010 could be considered comparable to those obtained by AO22 and E9 using liquid broth.

3.6 Determination of heavy metal MICs by the microtitre plate method

An additional method, using microtitre plates was tested, as this is was to be used to test the isolate's ability to form biofilms (see Chapter 6). Each isolate was aliquotted into six individual wells with media containing varying levels of each heavy metal: up to 0.1 mM HgCl₂, 4 mM Pb(NO₃)₂, 4 mM Zn(NO₃)₂, 2 mM CdCl₂ and 2 mM Co(NO₃)₂ (Section 2.3.8). MICs were determined from the three separate experiments and statistical analysis was performed (Figure 3.3, Appendix 5).

The levels used to test the MICs using the microtitre plate method were generally equivalent to those used for the spot and spread plates, therefore lower than those used for with liquid broth. Considering this, in the presence of HgCl₂, whereby all isolates demonstrated growth in most experiments up to 0.1 mM, that the MIC obtained from this method is comparable to previously obtained results. In the presence of Pb(NO₃)₂ and Zn(NO₃)₂ the MICs obtained for all three isolates was 4 mM, again comparable to results achieved in previous experiments. The results obtained in the presence of CdCl₂ were a little inconsistent, with results ranging from 1-1.5 mM for AO22 and E9, which was comparable to most of the previous experiments. VUN 10010 had only been previously tested using liquid broth and was found to not exhibit resistance, however

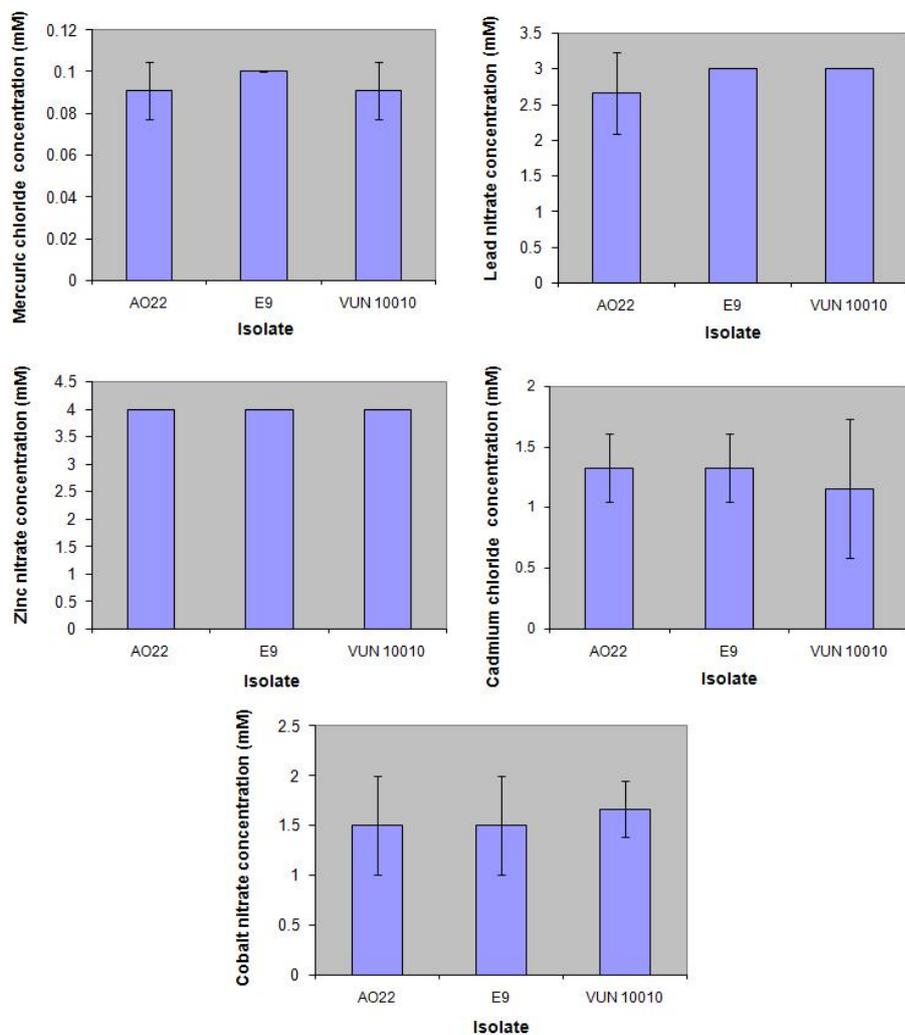


Figure 3.3 Heavy metal MICs of VUN 10010, AO22 and E9 using microtitre plates. Experiments were performed as outlined in Section 2.3.7. Cells from a 10 ml overnight NBT culture were diluted 10^{-3} in NBT or NBT containing 0-0.1 mM HgCl_2 , 0-4 mM $\text{Pb}(\text{NO}_3)_2$, 0-4 mM $\text{Zn}(\text{NO}_3)_2$, 0-2 mM CdCl_2 and 0-2 mM $\text{Pb}(\text{NO}_3)_2$. Diluted cells were inoculated into microtitre plates (six replicates) and plates were incubated at 30°C for 48 hours. Statistical analysis was performed from two experiments. No error bar indicates consistent results across all replicates.

using the microtitre plate method, an MIC ranging between 0.5-1.5 mM was seen. In the presence of $\text{Co}(\text{NO}_3)_2$, MICs comparable to those obtained using spot and spread plates were seen for AO22 and E9, however these were lower than those achieved using liquid broth. The MIC obtained for VUN 10010 was between 1.5-2 mM which was also lower than the MIC seen using liquid broth. A summary of all MIC results obtained is summarized in Table 3.3.

Table 3.3 Summary of heavy metal MICs of VUN 10010, AO22 and E9 using various methods

Isolate	Method	Heavy metal (mM)				
		HgCl ₂	Pb(NO ₃) ₂	Zn(NO ₃) ₂	CdCl ₂	Co(NO ₃) ₂
AO22	Spot plate	0.05	3.5	3.5	0.5	1.5
	Spread plate	0.1	3.5	3.5	1.5	1.0
	Liquid broth	0.15	4	4	1.5	2.5
	Microtitre plate*	0.091	2.66	4	1.33	1.5
E9	Spot plate	0.03	3.5	3.5	2	2
	Spread plate	0.08	3.5	3.5	2	1.5
	Liquid broth	0.125	6	4	1.5	3
	Microtitre plate*	0.1	3	4	1.33	1.5
VUN 10010	Spot plate	Not performed				
	Spread plate	Not performed				
	Liquid broth	0.175	4	5.5	susceptible	2.5
	Microtitre plate*	0.091	3	4	1.16	1.66

*The results obtained using microtitre plates are the average of 3 separate experiments. Raw data and standard deviations can be found in Appendix 10.

3.7 Organomercurial MICs

Since it was now established that *Alcaligenes* sp. AO22, *Arthrobacter* sp. E9 and Consortium VUN 10010 were resistant to mercuric chloride, it was of interest to determine whether they also demonstrated broad-spectrum resistances to the more toxic organomercurials (ATSDRa). The resistances of these strains to methylmercury chloride (MMC) and phenylmercury acetate (PMA) were tested using the liquid broth method at concentrations of 0.001, 0.0025, 0.005, 0.0075 and 0.01 mM. The results are outlined in Table 3.3.

Table 3.4 Organomercurial MICs of AO22, E9 and VUN 10010 using the liquid broth method

Isolate	MMC	PMA
AO22	0.001 mM	0.0025 mM
E9	No growth	No growth
VUN 10010	No growth	0.005 mM

The results indicated that the isolates demonstrated little, if any, resistance to these compounds. According to Sadhukhan *et al.*, (1997), any bacterium that is able to grow above 0.003 mM MMC and PMA may be considered resistant to these compounds. The above results thus indicate that AO22 and E9 there were not resistant, with the exception of a borderline result for AO22 (0.0025 mM PMA). VUN 10010 was able to grow in PMA at a concentration of 0.005 mM, which may be considered resistant, but it was unable to grow in the presence of MMC.

3.8 Growth Curve Analysis

Growth curve analysis involves the identification of the lag, exponential, stationary and death phases of any given isolates growth (Tatora *et al.*, 1992). The lag phase is representative of the inoculated cells adjusting to a new environment, and growth of the cells is retarded. The exponential phase involves the cells rapidly dividing, where growth is occurring at a fast rate while during the stationary phase, the cells are dividing at a rate equivalent to that at which cells are dying. The last phase, the death phase, indicates that the cells in the culture are dying faster than they are able to multiply (Tatora *et al.*, 1992). It was important to determine the growth characteristics of these isolates prior to performing experiments such as induction studies, wherein gene expression was monitored in heavy metal-stressed cells, where the cells from the parent culture are required to be in late exponential phase before inoculating test cultures. The growth curves were performed on all three isolates by inoculating two separate cultures (A and B) with equal cell numbers and monitoring the OD₆₀₀ readings at hourly intervals (Section 2.3.5). The results were plotted exponentially to determine the phases of growth (Figure 3.4, Appendix 6).

The results obtained indicate that the lag phase of AO22 was short, with the culture entering the exponential phase within an hour and continuing to grow up to approximately the 10-hour time point. Beyond this point the OD₆₀₀ readings leveled off indicating the stationary phase had commenced and continued up to at least 32 hours, after which the experiment ceased. The cultures of E9 demonstrated a relatively short lag phase, with the cells entering the exponential phase within one hour and entering the stationary phase after 18 hours. No distinct lag phase could be noted in VUN 10010, and the culture grew exponentially up to approximately 8 hours, beyond which its growth rate begins to decline. No distinct death phase was noted within the 32 hour time frame for any of the isolates.

3.9 16S sequencing

Alcaligenes sp. AO22 and *Arthrobacter* sp. E9 were initially isolated and characterised to the genus level by biochemical tests by Manovski *et al.*, (1992). These isolates were characterised to the species level by sequencing of the genes encoding 16S ribosomal RNA in the present work. DNA was isolated from these bacteria as described in Sections 2.4.1 and 2.4.2 and PCR was set up using 16S specific primers. The products were visualised by agarose gel electrophoresis and the PCR fragments purified using column purification and cloned into the pGEM-T Easy vector. Plasmids were prepared and DNA sequencing was performed on two recombinant plasmids using the T7 and SP6 primers (Table 2.2) and analysis of the sequence data was performed using the BlastN program.

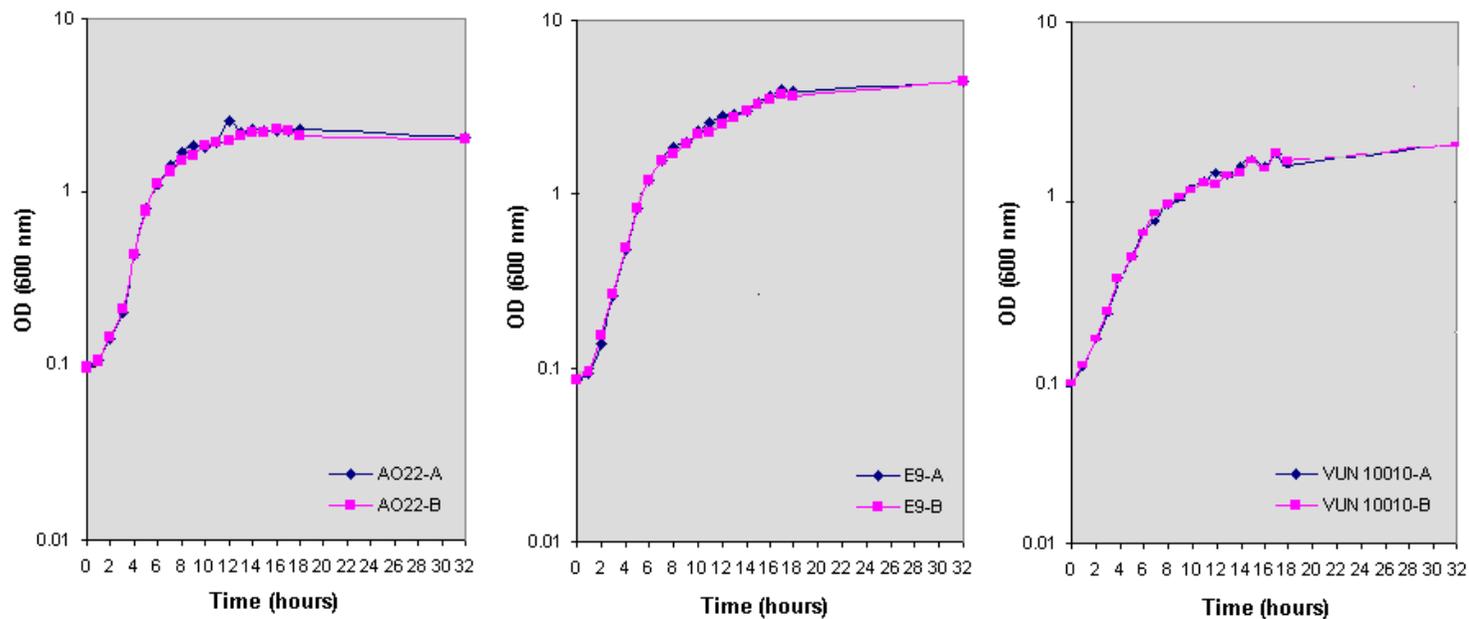


Figure 3.4 Growth curve analysis of AO22, E9 and VUN 10010. Experiments were performed in duplicate (A and B) as described in Section 2.3.5. Cultures were inoculated from a parent culture at an OD₆₀₀ of 0.1 and were incubated at 30°C, 150 rpm.

Using the BlastN program, the 1000 bp of sequence obtained from AO22 was compared and showed greater than 99% identity to the sequences of the gene encoding 16S rRNA of *Alcaligenes faecalis* 5959H (99.9%) (AJ509012, Figure 3.5) and *Achromobacter xylosoxidans* (99.1%) (AF411021). This result confirmed further and submitted to GenBank by Shee Ping Ng from Swinburne University (Accession number EU696789) (Ng *et al.*, 2009). It has been documented by Wellinghousen *et al.* (2006) that the NCBI GenBank entry of strain 5659-H is actually the 16S rRNA gene sequence of *A. xylosoxidans* subsp. *xylosoxidans*. A phylogenetic tree was performed by S. Ng based on the alignment of the gene encoding 16S rRNA of AO22 with a number of *Achromobacter* sp., *Alcaligenes* sp. and β -Proteobacteria. This phylogenetic tree revealed that AO22 and 5659-H belong to a cluster of *Achromobacter* sp., which are relatively distant to *Alcaligenes* spp. (Ng *et al.*, 2009). Comparisons were also made between the sequence of AO22 with genes encoding 16S rRNA from other *Alcaligenes* and *Achromobacter* species and it showed 98.8%, 98.7% , 94.0% and 93.6% identities to *Achromobacter xylosoxidans* DSM10340 (Y14908), *Achromobacter xylosoxidans* subspecies *denitrificans* (AF232712), *Alcaligenes faecalis* IAM (D88008) and *Alcaligenes faecalis* ATCC8750 (M22508) (Appendix 7). Based on the level of relatedness of AO22 to a number of *Achromobacter* spp., AO22 is now referred to as *Achromobacter* sp. DNA-DNA hybridisation may be required to further characterise AO22 to a species level (Ng *et al.*, 2008).

The 1000 bp of sequence from E9 showed 99.9% identity to the sequence of the gene encoding 16S rRNA of *Arthrobacter woluwensis* (AY112986) (Figures 3.5 and 3.6). Likewise, E9 showed 93.88%, 93.8%, 93.474% and 93.4% identity to *Arthrobacter agilis* (AJ577725), *Arthrobacter sulfureus* (AB046358), *Arthrobacter atrocyaneus* (X80746) and *Arthrobacter ruscicus* (AB071950), respectively (Appendix 6). The sequence obtained for the gene encoding 16S rRNA was submitted to NCBI GenBank and assigned the accession number GQ856043. 16S sequencing has been previously performed for the VUN 10010 consortium, by Boonchan (PhD thesis, 1998) and Dandie *et al.*, (2004). This is described further in the discussion (Section 3.9).

3.10 Discussion

The focus of this chapter was to confirm the heavy metal resistances of the bacterial isolates from the contaminated soil, *Alcaligenes* sp. isolates AO17 and AO22 and the *Arthrobacter* sp. isolates E9 and E11 (Trajanovska *et al.*, 1997). The same method used in the previous study, the spot plate method, was initially used. Some differences could be noted, with some MICs obtained in the current investigation being lower or higher, however, the results confirmed that the heavy metal resistances in these isolates had been maintained. One of the most surprising differences noted was using the positive control strain *Cupriavidus metallidurans* CH34. Trajanovska *et al.* (1997), indicated an MIC of only 0.1 mM in the presence of CdCl₂, while the current study indicated an MIC of 2 mM, which was closer to the 1 mM MIC for cadmium as was reported by Hassan *et al.*, (1999) for *C. metallidurans* CH34, although the compound tested was not indicated.

A number of alternative methods were investigated for the investigation of MIC to assess the range of MICs obtained under varying conditions and in preparation for other types of planned experiments (i.e. growth curve analysis, biofilm analysis). At this point in the investigation, it was decided to limit the testing of the isolates to one of the *Alcaligenes* sp. isolates (AO22) and one of the *Arthrobacter* sp. isolates (E9) for further investigations such as the presence of heavy metal genes and those mentioned above. This was based on similarities of the results obtained using the spot plate method, when comparing the *Alcaligenes* sp. isolates AO17 and AO22, and when comparing the *Arthrobacter* sp. isolates E9 and E11. While other researchers have reported little difference in results obtained using both solid and liquid medium (Filali *et al.*, 2000; Zeroual *et al.*, 2001), variations in MIC results were noted between the different methods used in this investigation, which may be attributed to adaptability of the cells

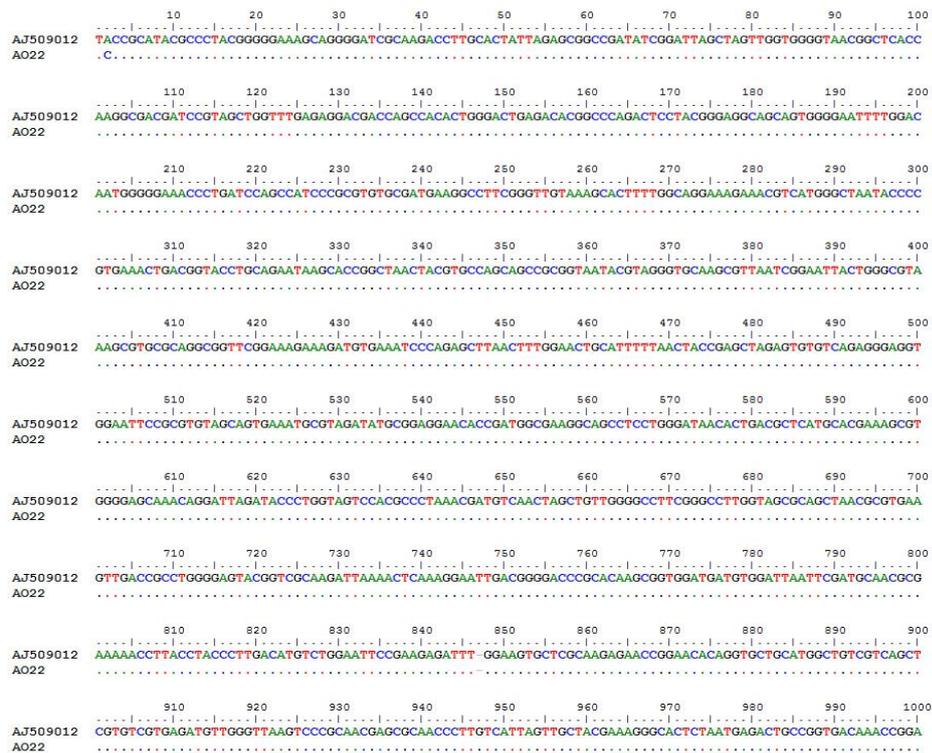


Figure 3.5 Comparison of the 16S RNA gene sequence of AO22 with the 16S RNA gene sequence of *A. xylosoxidans* (AJ509012). Sequencing was performed as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).



Figure 3.6 Comparison of the 16S RNA gene sequence of E9 with the 16S RNA gene sequence of *A. woluwensis* (AY112986). Sequencing was performed as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

under varying conditions or the integrity of the stock used for inoculations for each experiment. However, using all three methods described, adequate degrees of resistance were observed by all isolates.

An additional isolate was included in this study, VUN 10010, as it had been previously determined that it was capable of PAH degradation (Boonchan, PhD thesis, 1998). It was initially believed that this strain was *S. maltophilia*, however, early in this investigation, it was determined that VUN 10010 also contained a *Mycobacterium* sp. (C. Dandie, School of Biological Sciences, Flinders University, personal communication). All attempts to purify these two strains were unsuccessful in our

laboratory; therefore VUN 10010 was investigated further as a Consortium. It is yet to be determined whether some form of symbiotic relationship exists between *S. maltophilia* and *Mycobacterium* sp. The soil sample from which VUN 10010 originated was documented to contain a number of heavy metals, including lead (570 ppm), cadmium (<1 ppm), copper (190 ppm) and zinc (260 ppm) (Boonchan, PhD thesis, 1998). On this basis, the isolate was investigated for heavy metal resistance and was found resistant to varying levels of mercury, lead, zinc, cadmium and cobalt, many being comparable to the degree of resistance shown by AO22 and E9. The heavy metal resistance as well as PAH degradation capabilities make VUN10010 of particular interest for potential for applications such as bioremediation of sites with multiple contaminants.

It has been reported in literature that some bacteria possess resistance to organomercurial compounds such as MMC and PMA (Furukawa and Tonomura, 1971; Sudhukhan *et al.*, 1997; Hines *et al.*, 2000) (Section 1.4.5). However, amongst our test isolates, only VUN 10010 demonstrated MICs at levels considered “resistant” (0.003 mM) for PMA and MMC respectively. There are no reports in literature of any *Alcaligenes*, *Achromobacter*, *Arthrobacter*, *Stenotrophomonas* or *Mycobacterium* species showing organomercurial resistances.

A number of other bacteria have been reported to possess broad-spectrum mercury resistances, e.g., the study performed by Sudhukhan *et al.* (1997), on bacteria isolated from the gills and gut of fish from wetland fisheries around Calcutta, India, which were contaminated with mercuric compounds. A number of papers have reported the presence of the *merB* gene, which encodes organomercurial resistance, in a number of isolates. These include the genera *Pseudomonas* (Kiyono *et al.*, 1997; Kiyono and Pan-Hou, 1999; Reniero *et al.*, 1995; Reniero *et al.*, 1998), *Bacillus* (Helmann *et al.*, 1989; Wang, *et al.*, 1989; Gupta *et al.*, 1999; Huang *et al.*, 1999b), *Staphylococcus* (Laddaga *et al.*, 1987; Skinner *et al.*, 1991), *Streptomyces* (Ravel *et al.*, 1998; Ravel *et al.*, 2000; Sedlmeier and Altenbuchner, 1992; Brunker *et al.*, 1996) and *Serratia* (Nucifora *et al.*, 1989a; Yu *et al.*, 1994; Griffin *et al.*, 1997). To date, there are no reports of the *merB* gene, which encodes organomercurial lyase, being present in genera *Achromobacter*, *Arthrobacter*, *Stenotrophomonas* or *Mycobacterium*. This result was followed up by

searching for genetic evidence (*merB* gene) or a relic of organomercurial resistance in our isolates. The results of this investigation are outlined in Chapter 4.

The combined results for the MICs of a range of heavy metals, including organomercurials, provided strong grounds to further investigate the heavy metal resistance on a molecular level. In preparation for induction studies of gene expression, growth curve analysis of all three isolates in the absence of any heavy metals, was performed. All three isolates demonstrated very short lag periods before the cells entered exponential growth. The results obtained indicated that for further experiments, it would be ideal to inoculate cells from a parent culture that had reached that late-exponential phase at an OD₆₀₀ reading of 1.

Alcaligenes sp. AO22 and *Arthrobacter* sp. E9 had only previously been identified by biochemical tests. Using 16S rRNA gene sequencing, *Alcaligenes* sp. AO22 was characterised here as *Alcaligenes faecalis* 5959H, which has been subsequently classified by Wellinghoausen *et al.* (2006) as *Achromobacter xylosoxidans* (AJ509012). Further analysis by S. Ng indicated a level of relatedness of AO22 to a number of *Achromobacter* spp., therefore AO22 is now referred to as *Achromobacter* sp (Ng *et al.*, 2008). *Arthrobacter* sp. E9 was identified as most similar to *A. woluwensis* (AY112986). VUN 10010 was previously identified as *S. maltophilia* by 16S rRNA gene sequencing (AF068008, Boonchan, PhD thesis, 1998), however as mentioned previously, this was determined to be a mixed culture. Results have shown that growing VUN 10010 in nutrient broth is favourable for the growth of the *S. maltophilia* component. 16S sequencing was performed from the DNA of VUN 10010 grown in nutrient broth, hence the likelihood of the DNA extracted being from *S. maltophilia* (Boonchan, PhD thesis, 1998). Dandie *et al* (2004) performed 16S sequencing on DNA extracted from what they believed to be a pure isolate of *Mycobacterium* sp. from VUN 10010. This analysis revealed two distinct rRNA sequences, with significant differences (21 bp) mostly within the hypervariable regions common to the 16S gene. Phylogenetic analysis of both sequences (AY227355 and AY227356) indicated the *Mycobacterium* from VUN 10010 was most closely related to a fast-growing species the *Mycobacterium tuberculosis* subgroup. This related strain, SM7.6.1, was isolated with phenanthrene-degrading capabilities under low bioavailability conditions (Friedrich *et al.*, 2000). Having established the varying levels of heavy metal resistances of AO22,

E9 and VUN 10010, further investigations will concentrate on the molecular investigations of genes relating to heavy metal resistance and PAH degradation and expression patterns of heavy metal resistance genes, and the biofilm formation potentials, for use in bioremediation applications.

Chapter 4

Molecular basis, growth and expression patterns of the mercury (*mer*) resistance operons of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

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4.1 Introduction

As described in Chapter 1, the mercury resistance (*mer*) operon is comprised of a number of genes, each encoding proteins of different functions. In brief, *merR* encodes the negative regulator of the operon in the absence of mercury, while *merD* encodes a second regulatory protein. *merT* and *merP* encode Hg(II) transport proteins, while *merA* encodes the NADPH-dependent, FAD-containing mercuric reductase which reduces Hg(II) to Hg(0). Some *mer* operons contain additional genes such as *merC*, which encodes an additional transport protein, and *merB*, which encodes the enzyme organomercurial lyase, conferring resistance to organomercurial compounds. It was established in Chapter 3 that the soil bacterial isolates *Achromobacter* sp. AO22, *Arthrobacter woluwensis* E9 and Consortium VUN 10010 are resistant to mercuric chloride. This chapter explores the molecular basis of mercury resistance in these bacteria by determining the presence of *mer*-related genes and comparing the sequences obtained from these isolates with those published in the literature. This chapter also investigates the adaptation of *Achromobacter* sp. AO22 and *A. woluwensis* E9 in the presence of mercuric chloride and expression of these *mer* genes upon induction with mercuric chloride in *Achromobacter* sp. AO22.

4.2 PCR analysis of the presence of mercury resistance genes based on the Tn501, Tn21 and pDU1358 operons

To test for the presence of a typical *mer* operon, primers were designed to encompass the *merRTPADEurf2* genes. The sequence of the Tn501 operon (Misra *et al.*, 1984, Brown *et al.*, 1986, Barrineau *et al.*, 1984) was used as a basis for primer design, although sequences from the *mer* operons of *Shigella flexneri* Tn21 (Nakaya *et al.*, 1960) and/or *Serratia marcescens* pDU1358 (Nucifora *et al.*, 1989a) *mer* operons were also used in the design of some primers (Misra *et al.*, 1984; Brown *et al.*, 1986; Griffin *et al.*, 1987). Primer pairs were specifically designed to amplify overlapping fragments based on these *mer* operons for full sequence coverage of any amplified sections (Figures 2.2-2.4). Primer details are outlined in Table 2.2. Tables 4.1-4.8 show the results of various amplifications. The size and numbers of PCR fragments produced using the various primer pairs provided a preliminary estimation of which genes may be present or absent.

4.2.1 Amplification of the *mer* genes of Tn501

The primer pairs described above amplified fragments of expected size for the Tn501 positive control (pACYC 184:: Tn501) (Figure 4.1 and Table 4.1) using all primer pairs. These results provided a basis for use of these primers for detection of the *mer* sequences in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 isolates.

4.2.2 Amplification of the *mer* genes from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

The results obtained for amplification of the *mer* genes from *Achromobacter* sp. AO22 were identical to those obtained for Tn501 (Figure 4.2, Table 4.2), suggesting that the structure of its *mer* operon is similar to that of the Tn501. The amplification of *mer* genes from *A. woluwensis* E9 isolate (Figure 4.3, Table 4.2) led to amplified fragments of expected size with the primer pairs IR1-16, mer35-36 and mer7-29, indicating some similarities with the *mer* operons of Tn501. However, mer3-6 did not produce a fragment of expected size, while no products were amplified using mer19-32 and mer33-34. The same primer pairs were used to amplify the *mer* operon from the genomic DNA of Consortium VUN 10010. All primers produced amplicons of expected sizes, with the exception of mer3-6 (Figure 4.4, Table 4.2), suggesting some similarities between the sequence of Consortium VUN 10010 and the *mer* operon of Tn501.

Changes to reaction conditions included using differing quantities of template and different concentrations of MgCl₂ (2 mM and 2.5 mM), but this did not result in amplification products from *A. woluwensis* E9.

A number of additional primer pairs were designed to amplify other regions (Figure 2.2, Table 2.2). The design of primer pairs merR1-R2 and merP1-P2, were based on different areas of the Tn501 operon. An additional primer, mer4, was used with mer6 and the primer pair, mercP-cA (Holt *et al.*, 1999) was also tested. Tn501 was used as a positive control. These results are summarised in Figure 4.5 and Table 4.3.

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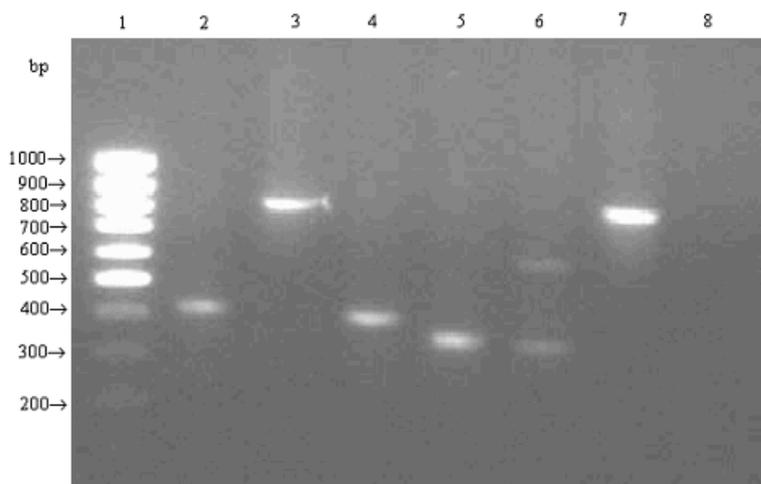


Figure 4.1 PCR amplification of *mer* gene sections from the positive control plasmid pACYC 184::Tn501. PCRs were set up as described in Section 2.8 using *Taq* DNA polymerase and were run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. IR1-mer16; 3. mer3-6; 4. mer35-36; 5. mer7-29; 6. mer19-32; 7. mer33-34; 8. negative control (mer3-6).

Table 4.1 Results obtained from the PCR analysis of the positive control plasmid pACYC 184::Tn501 using the *mer* primers

Primer pair	Genes targeted	Expected fragment size (bp)	Approx. fragment size obtained (bp)
IR1-16	<i>merR</i>	411	410
Mer3-6	<i>merR, merT, merP</i>	792	800
Mer35-36	<i>merP, merA</i>	394	390
Mer7-29	<i>merP, merA</i>	342	350
Mer19-32	<i>merA</i>	575	580, 300
Mer33-34	<i>merA</i>	779	780

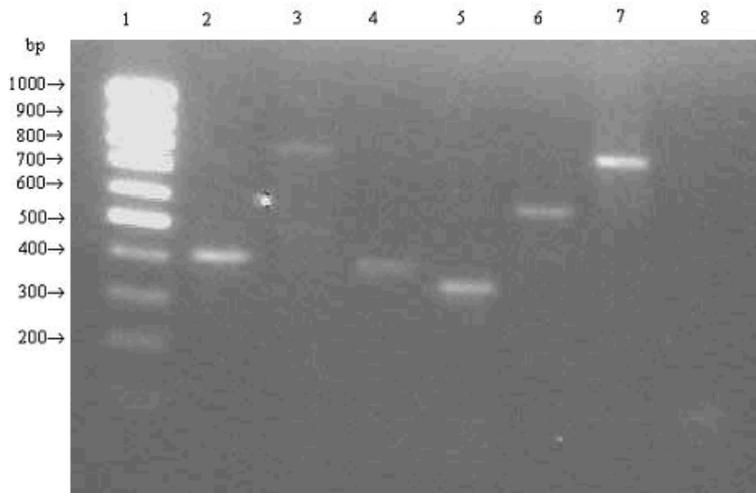


Figure 4.2 PCR amplification of *mer* genes from the genomic DNA of *Achromobacter* sp. AO22. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. IR1-mer16; 3. mer3-6; 4. mer35-36; 5. mer7-29; 6. mer19-32; 7. mer33-34; 8. negative control (mer33-34).

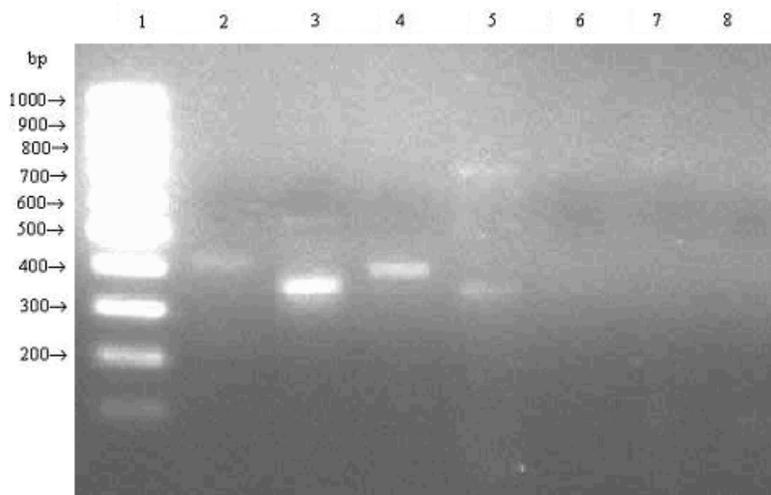


Figure 4.3 PCR amplification of *mer* genes from the genomic DNA of *A. woluwensis* E9. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and were run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. IR1-mer16; 3. mer3-6; 4. mer35-36; 5. mer7-29; 6. mer19-32; 7. mer33-34; 8. negative control (mer3-6).

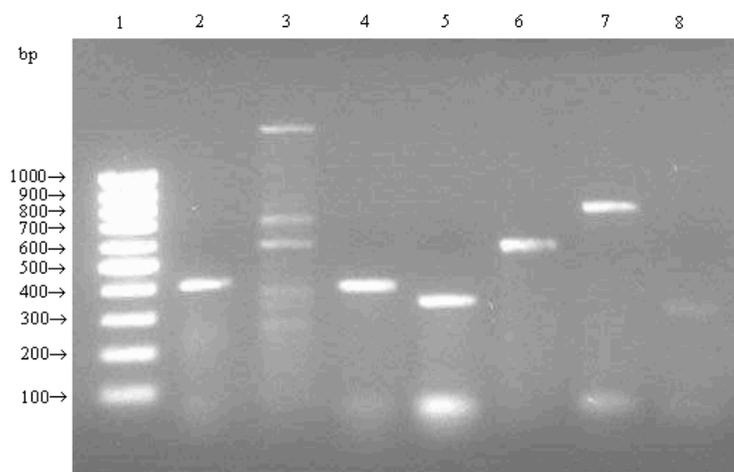


Figure 4.4 PCR amplification of the *mer* genes from the genomic DNA of Consortium VUN 10010. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. IR1-mer16; 3. mer3-6; 4. mer35-36; 5. mer7-29; 6. mer19-32; 7. mer33-34; 8. negative control (mer3-6).

Table 4.2 Results obtained from the PCR analysis of the genomic DNA from *Achromobacter* sp. AO22 using *mer* specific primers

Primer pair	Genes targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)		
			AO22	E9	VUN 10010
IR1-16	<i>merR</i>	411	410	410	410
Mer3-6	<i>merR</i> , <i>merT</i> , <i>merP</i>	792	800	350	600, 750
Mer35-36	<i>merP</i> , <i>merA</i>	394	390	390	390
Mer7-29	<i>merP</i> , <i>merA</i>	342	350	350, 750	350
Mer19-32	<i>merA</i>	575	580	NA	580
Mer33-34	<i>merA</i>	779	780	NA	780

NA: no amplification products observed.

Further reactions were established using mer3-6, mer19-32 and mer33-34 for E9. Changes to reaction conditions included using differing quantities of template and different concentrations of MgCl₂ (2 mM and 2.5 mM), but this did not result in amplification products from *A. woluwensis* E9.

A number of additional primer pairs were designed to amplify other regions (Figure 2.2, Table 2.2). The design of primer pairs merR1-R2 and merP1-P2, were based on different areas of the Tn501 operon. An additional primer, mer4, was used with mer6 and the primer pair, mercP-cA (Holt *et al.*, 1999) was also tested. Tn501 was used as a positive control. These results are summarised in Figure 4.5 and Table 4.3.

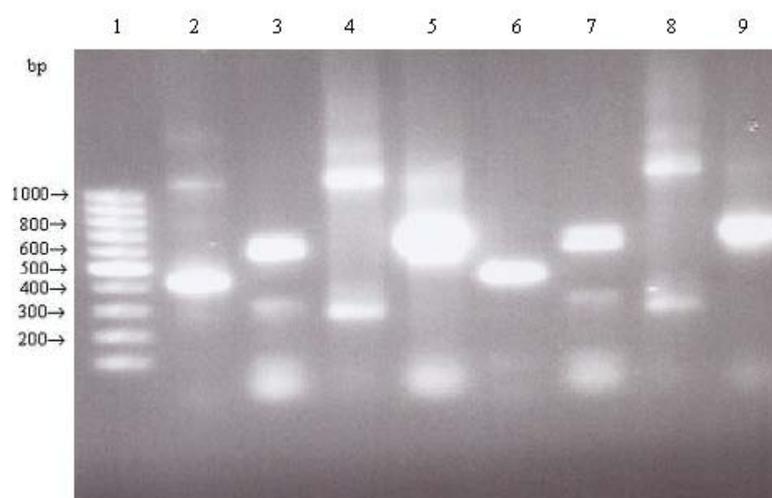


Figure 4.5 PCR amplification of Tn501 and genomic DNA of *A. woluwensis* E9 using additional *mer* primers. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. merR1-R2 (Tn501); 3. mer4-6 (Tn501); 4. merP1-P2 (Tn501); 5. mercP-cA (Tn501); 6. merR1-R2 (E9); 7. mer4-6 (E9); 8. merP1-P2 (E9); 9. mercP-cA (E9); negative control not shown.

Table 4.3 Results obtained using additional *mer* primers from Tn501 and the genomic DNA of *A. woluwensis* E9

Primer pair	Genes targeted	Expected fragment size (bp)	Approx. amplicon size obtained (bp)	
			Tn501	E9
merR1-R2	<i>merR</i>	418	420	420
mer4-6	<i>merR</i> , <i>merT</i> , <i>merP</i>	567	570	570
merP1-P2	<i>merP</i>	261	260, >1000	260, >1000
mercP-cA	<i>merP</i> , <i>merA</i>	650	650	650

Other reactions were run for Consortium VUN 10010 using the mer3-6 pair with different quantities of template and different MgCl₂ concentrations (2 mM and 2.5 mM), but the amplification of a fragment of expected size was not observed. Three additional primer pairs, mer1-R2, merR1-6, and mer4-36, were then used to provide overlapping regions with the fragments previously amplified (Table 4.2). Tn501 was used as a control and the results are summarised in Figure 4.6 and Table 4.4.

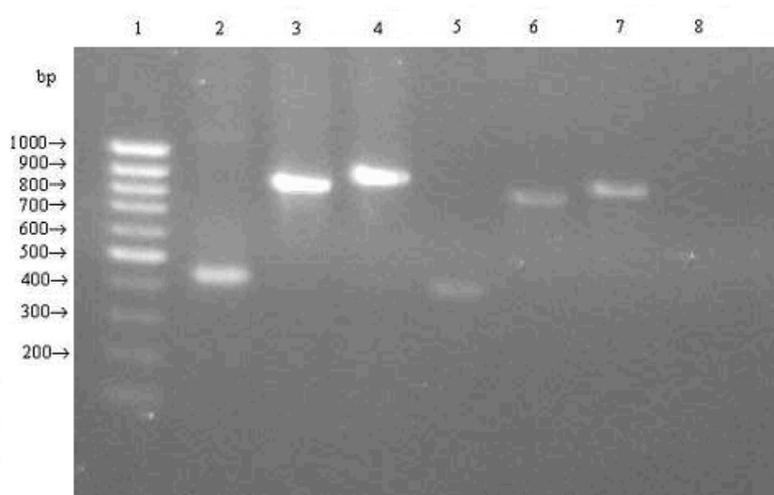


Figure 4.6 PCR amplification of Tn501 and the genomic DNA of Consortium VUN 10010 using additional *mer* primers. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. mer1-R2 (Tn501); 3. merR1-6 (Tn501); 4. mer4-36 (Tn501); 5. mer1-R2 (VUN 10010); 6. merR1-6 (VUN 10010); 7. mer4-36 (VUN 10010); 8. negative control (mer4-36).

Table 4.4 Results obtained using additional *mer* primers from Tn501 and the genomic DNA of Consortium VUN 10010

Primer pair	Genes targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)	
			Tn501	VUN 10010
mer1-R2	<i>merR</i>	435	420	400
merR1-6	<i>merR</i> , <i>merT</i> , <i>merP</i>	868	850	800
mer4-36	<i>merR</i> , <i>merT</i> , <i>merP</i>	944	900	900

4.2.3 Further amplification of the 3' end of the *merA* gene and the *merD* gene from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

The previous section (4.2.2) describes the amplification of fragments relating to the *merR*, *merT* and *merP* genes of all three isolates. Using mer7-29, mer19-32 and mer33-34, approximately 80% of *merA* was amplified from *Achromobacter* sp. AO22 and Consortium VUN 10010 compared to Tn501 (Z00027). Only mer7-29 was successful for *A. woluwensis* E9, along with merC-P-cA, which resulted in the amplification of approximately 22% of *merA* compared to Tn501 (Z00027). Two additional primer pairs, based on the sequence of Tn501, mer33-22 and mer25-10 were used also (Figure 2.2, Table 2.2) to amplify the *merA* and *merD* genes. These reactions were initially conducted using a final MgCl₂ concentration of 1.5 mM (Figure 4.7 and Table 4.5).

Primer pair mer33-22 did not amplify fragments of expected size from the Tn501 control or from *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010. A fragment of approximately 720 bp was amplified from Tn501, however, this was shorter than the expected size. Using this primer pair, no amplification was seen for any of the three test isolates. The pair mer25-10 produced a fragment from Tn501, however it was more than twice the expected size. A fragment was also amplified from *A. woluwensis* E9, however, this was longer than expected. No fragments were amplified from *Achromobacter* sp. AO22 or Consortium VUN 10010 using mer25-10. Additional reactions were run using increased MgCl₂ concentrations as described in Figure 4.8 and Table 4.6.

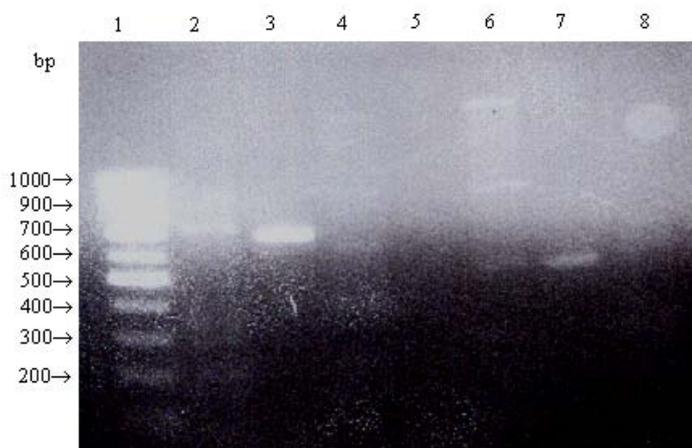


Figure 4.7 PCR amplification of Tn501 and the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 using additional *merA* and *merD* primers. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. mer33-22 (Tn501); 3. Mer25-10 (Tn501); 4. mer33-22 (AO22); 5. mer25-10 (AO22); 6. mer33-22 (E9); 7. mer25-10 (E9); 8. mer33-22 (VUN 10010); 9. mer25-10 (VUN 10010) 10. negative control (mer25-10).

Table 4.5 Results obtained using additional *merA* and *merD* primers from Tn501 and the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

Primer pair	Genes targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)			
			Tn501	AO22	E9	VUN 10010
Mer33-22	<i>merA</i>	1080	720	NA	NA	NA
Mer25-10	<i>merD</i>	336	700	NA	500	NA

NA: no amplification products observed.

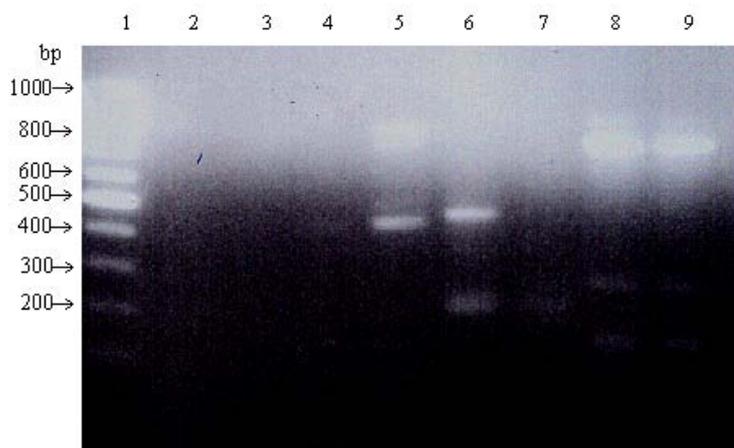


Figure 4.8 PCR amplification of Tn501 and the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 using additional *merA* and *merD* primers and increased MgCl₂ concentration. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. mer33-22, 2 mM (E9); 3. mer33-22, 2.5 mM (E9); 4. Mer25-10, 2 mM (E9); 5. Mer25-10, 2.5 mM (E9); 6. mer33-22, 2 mM (VUN 10010); 7. mer33-22, 2.5 mM (VUN 10010); 8. Mer25-10, 2 mM (VUN 10010); 9. Mer25-10, 2.5 mM (VUN 10010); negative control not shown.

Table 4.6 Results obtained using additional *merA* and *merD* primers with increased concentrations of MgCl₂, from Tn501 and the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

Primer pair	MgCl ₂ Conc.	Genes targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)			
				Tn501	AO22	E9	VUN 10010
Mer33-22	2	<i>merA</i>	1080	NA*	NA*	NA	480, 200
	2.5			NA*	NA*	NA	200
Mer25-10	2	<i>merD</i>	336	NA*	NA*	NA	750, 280, 150
	2.5			NA*	NA*	450, 850	750, 280, 150

NA: no amplification products observed.

*Agarose gel not shown

Using primer pair mer33-22, no bands were observed at MgCl₂ concentrations of 2 mM and 2.5 mM for Tn501 *Achromobacter* sp. AO22 or *A. woluwensis* or for VUN 10010 at 2.5 mM; a band of approximately 480 bp was observed for VUN10010 at 2 mM, which is considerably smaller than the expected size. Using primer pair mer25-10, no bands were observed for Tn501 or AO22 at either MgCl₂ concentration. Amplification products from E9 were not observed at 2 mM MgCl₂, while a fragment of approximately 450 bp (larger than expected) was observed at 2.5 mM MgCl₂. For VUN 10010, fragments of approximately 750 bp were amplified at both MgCl₂ concentrations which was more than twice the expected size.

4.3 PCR analysis for detecting the presence of *merC* and *merB* genes

As described in Chapter 1, some *mer* operons possess additional genes associated with mercury transport (*merC*) and organomercurial resistance (*merB*). Supplementary primers were designed based on internal regions of the *merC* gene (mer27-28) of the Tn21 *mer* operon (Misra *et al.*, 1985) and *E. coli* 1349B (Liebert *et al.*, 1997) and *merB* gene (mer23, mer24 and mer9) of pDU1358 *mer* operon (Griffin *et al.*, 1987), to determine whether these genes are present in the *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 isolates (see Figures 2.3 and 2.4 and Table 2.2).

4.3.1 Amplification of *merC* from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

The results presented earlier (Section 4.2.2) suggest that *merC* was not located between *merP* and *merA* in *A. faecalis* AO22 (using mer35-36, mer7-29), *A. woluwensis* E9 (using mer35-36, mer7-29, merC-pA) or VUN 10010 (using mer35-36, mer4-36, mer7-29). Therefore, the internal primer pair mer27-28 was utilised to determine if *merC*-like sequences were present elsewhere in the DNA sequence. Three different concentrations of MgCl₂ were used for these reactions (Figure 4.9 and Table 4.7).

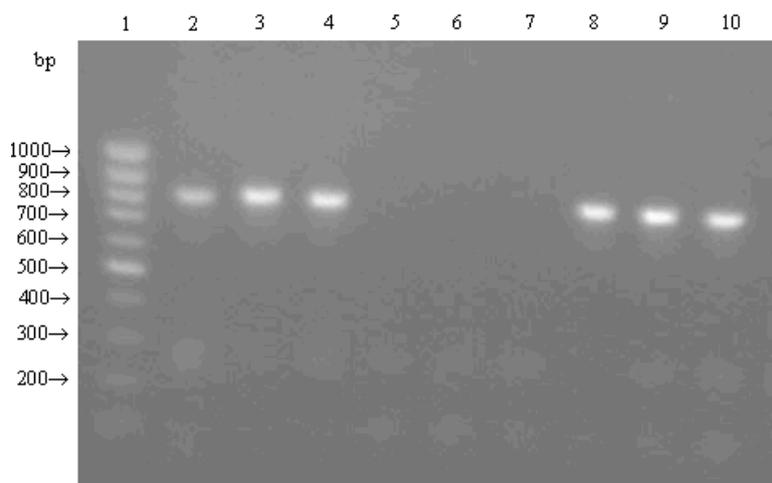


Figure 4.9 PCR amplification of *merC* using *mer27-28* with genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. 1.5 mM (AO22); 3. 2 mM (AO22); 4. 2.5 mM (AO22); 5. 1.5 mM (E9); 6. 2 mM (E9); 7. 2.5 mM (E9); 8. 1.5 mM (VUN 10010); 9. 2 mM (VUN 10010); 10. 2.5 mM (VUN 10010); 11. Negative control (1.5 mM).

Table 4.7 Results obtained using *merC* primer pair *mer27-28* from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010

Primer pair	MgCl ₂ Conc.	Genes targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)		
				AO22	E9	VUN 10010
Mer27-28	1.5	<i>merC</i>	360	800	NA	750
	2			800	NA	750
	2.5			800	NA	750

NA: no amplification observed

No fragments of expected size were amplified from any isolate, however, products of larger size were observed for *Achromobacter* sp. AO22 and Consortium VUN 10010. The results suggest that the *merC* gene used to design the primers is most likely not present in these isolates and an unrelated sequence may have been amplified. No further analysis, such as sequencing, of these fragments was undertaken.

4.3.2 Amplification of *merB* from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

A similar approach to that used in Section 4.3.1 was applied for the detection of an internal section of *merB* gene, using primer pairs mer23-24 and mer 23-9 (Figure 4.10 and Table 4.8). This approach was used due to the lack of success in amplifying the 3' section of *merA* and the *merD* gene.

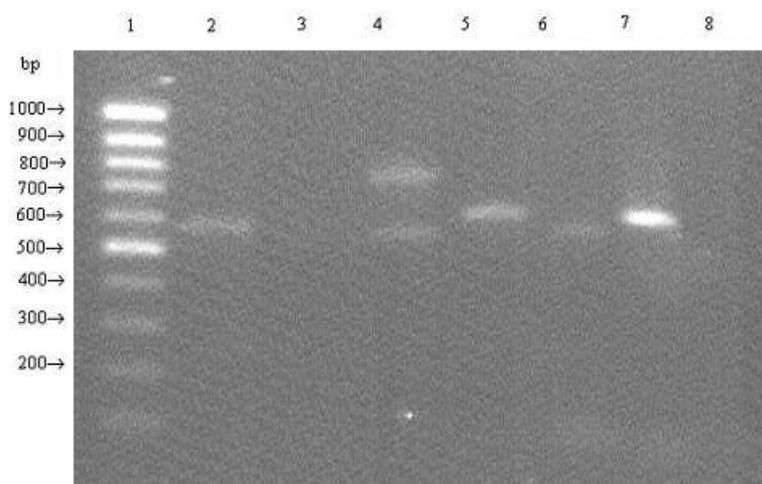


Figure 4.10 PCR amplification of *merB* from genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 using internal primers. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. mer23-24 (AO22); 3. mer 23-9 (AO22); 4. mer23-24 (E9); 5. mer 23-9 (E9); 6. mer23-24 (VUN 10010); 7. mer 23-9 (VUN 10010); 8. Negative control (mer23-24).

Table 4.8 Results obtained using internal *merB* primer pairs to amplify genomic DNA regions of *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010

Primer pair	Gene targeted	Expected amplicon size (bp)	Approx. amplicon size obtained (bp)		
			AO22	E9	VUN10010
mer23-24	<i>merB</i>	540	580	550, 750	550
mer23-9	<i>merB</i>	620	NA	600	600

NA: no amplification observed

Bands were observed for most isolates with exception being mer23-9 for *Achromobacter* sp. AO22. The latter was rerun using 2 mM and 2.5 mM MgCl₂ but no amplification products were observed. These results suggest that the *merB* gene is present in *A. woluwensis* E9 and VUN 10010, but an incomplete *merB*, or a *merB* with sequence diversity in the 3' region, may be present in *Achromobacter* sp. AO22, making the mer9 primer unsuitable for *merB* detection. An additional amplicon was observed for *Achromobacter* sp., a potentially longer *merB*-like sequence, although it is possible that the product is a result of non-specific primer binding.

4.4 Sequence analysis of the genes associated with *mer* operons

Amplified gene sections of interest were either cloned and sequenced, or sequenced directly, and the sequence data aligned using the Bioedit program (Section 2.11). The sequence of the *mer* operon from each of the isolates was deduced from the constructed contigs and compared with the Tn501 or other *mer* operons. Figures 4.11-4.20 show the sequence alignments of the *merR*, *merT*, *merP*, *merA* and *merB* genes and the putative protein products from the three test isolates.

4.4.1 *merR* and O/P sequence analysis

The sequencing results of the PCR products of *Achromobacter* sp. AO22 (primer pairs IR1-16, mer3-6), *A. woluwensis* E9 (IR1-16, merR1-R2, mer4-6) and Consortium VUN 10010 (IR1-16, mer1-R2, mer4-36) showed the presence of regulatory gene, *merR*, which is identical to that associated with the Tn501 operon (Z00027). It has been reported that this gene may either be transcribed divergently from the remainder of the

mer genes, as is the case with *Tn501*, or co-transcribed with the remainder of *mer* genes (see Section 1.4.4.1). Mer O/P sequence data was also obtained from the amplified sections of *Achromobacter* sp. AO22 (mer3-6), *A. woluwensis* E9 (mer4-6) and VUN 10010 (merR1-6, mer 4-36), showing 100% commonality to the mer O/P region of *Tn501* and the *merR* genes of all three are transcribed divergently from this region (Figure 4.11). The predicted MerR protein is 100% identical to that of *Tn501* (Figure 4.12) and contains the helix-turn-helix motif, DNA binding region and the cysteines reported to serve as binding sites for Hg²⁺ ions (Misra *et al.*, 1984). The sequence from *Achromobacter* sp. AO22 was confirmed by Ng *et al.*, 2009, submitted to NCBI GenBank and assigned accession number EU696790. The sequences from *A. woluwensis* E9 and Consortium VUN 10010 were also submitted to NCBI GenBank and assigned the accession numbers GQ415452 and GQ415453, respectively.

4.4.2 *merT* sequence analysis

The *merT* gene encodes a mercury transport protein (Section 1.4.2.2). Sequence data for this gene was obtained by sequencing the sections produced using primers mer3-6 (*Achromobacter* sp. AO22), mer 4-6 (*A. woluwensis* E9) and merR1-16 and mer 4-36 (VUN 10010). *merT* was present in all three isolates and the sequences for *A. woluwensis* E9 and VUN 10010 were 100% identical to *merT* in *Tn501*(Z00027), while *Achromobacter* sp. AO22 showed a one base pair difference, due to a change from glutamic acid in *Tn501*, to aspartic acid in *Achromobacter* sp. AO22 (Figures 4.13 and 4.14). This change falls within the second of three potential transmembrane regions within the *Tn501* protein (Morby *et al.*, 1995). The sequence from *Achromobacter* sp. AO22 was confirmed by Ng *et al.*, 2009, submitted to NCBI GenBank and assigned the accession number EU696790. The sequences from *A. woluwensis* E9 and Consortium VUN 10010 were also submitted to NCBI GenBank and assigned the accession numbers GQ415452 and GQ415453, respectively.

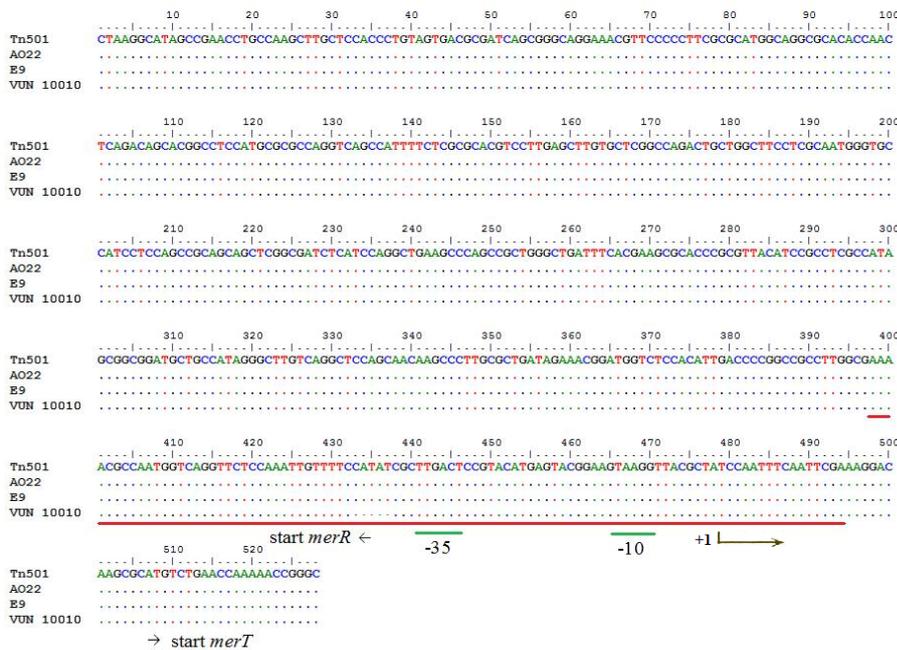


Figure 4.11 DNA sequence alignment of *merR* and OP regions from Tn501 (Z00027) with sequences of amplified fragments from *Achromobacter* sp. AO22, *A. wuluwensis* E9 and VUN 10010. Sequencing was performed as described in Section 2.11. Reactions were conducted using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).



Figure 4.12 Alignment of the MerR protein from Tn501 (Z00027) with the predicted MerR protein sequences from *Achromobacter* sp. AO22, *A. wuluwensis* E9 and VUN 10010.

- ↑ Binding site for mercury ions
- Helix-turn helix
- DNA-binding region (of the H-T-H motif)

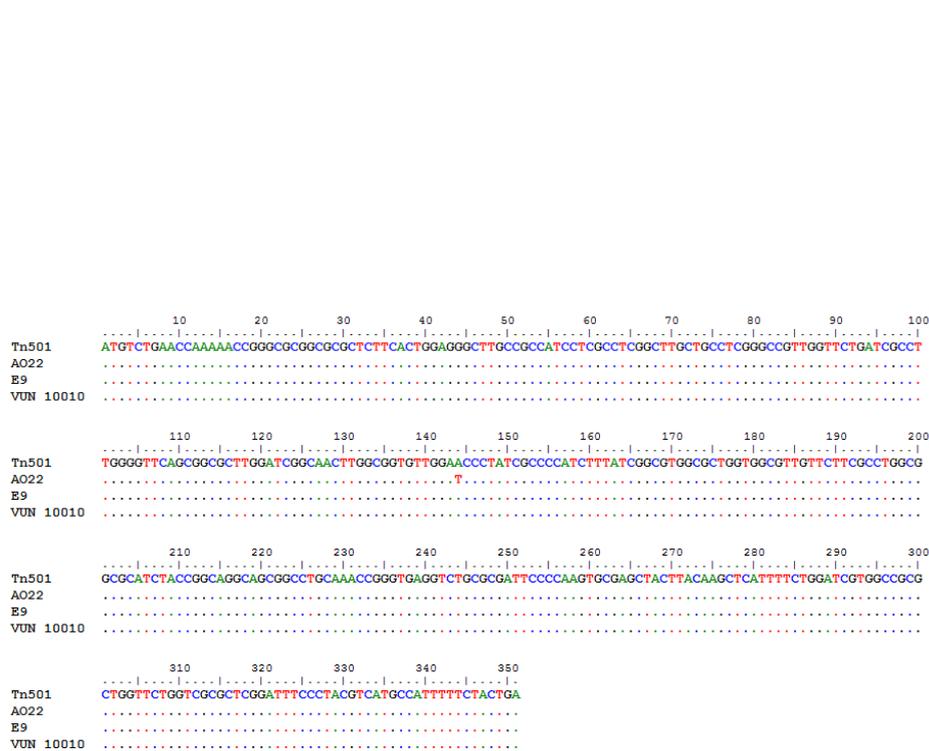


Figure 4.13 DNA sequence alignment of *merT* from Tn501 (Z00027) with sequences obtained from the amplified fragments from *Achromobacter* sp. AO22, *A. wulwensis* E9 and VUN 10010. Sequencing was performed as described in Section 2.11. Reactions were conducted using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

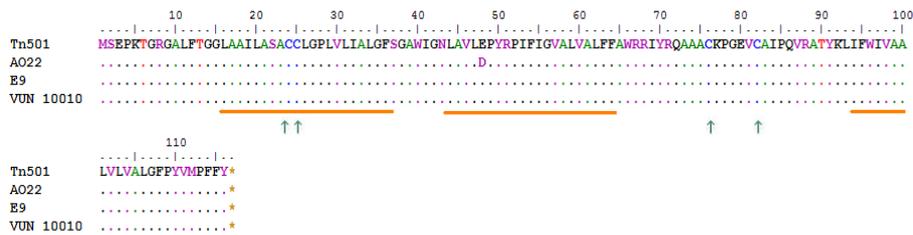


Figure 4.14 Alignment of the MerT protein from Tn501 (Z00027) with the predicted MerT protein sequences from *Achromobacter* sp. AO22, *A. wulwensis* E9 and VUN 10010.

↑ Binding site for mercury ions
 — Potential transmembrane regions

4.4.3 *merP* sequence analysis

The *merP* gene, which also encodes a mercury transport protein, was found to be present in all three isolates by sequencing PCR fragments using the mer35-36 and mer7-29 primers. The sequence of this gene in *A. woluwensis* E9 and Consortium VUN 10010 was 100% identical to *merP* in Tn501 (Z00027), while the *merP* gene of *Achromobacter* sp. AO22 differed by one base pair (A→G) (Figure 4.15), resulting in a change from glutamic acid to lysine in the predicted protein (Figure 4.16). This substitution is located within both the HMA (heavy-metal-associated) domain and periplasmic component relating to the Tn501 MerP protein (Steele and Opella, 1997). The sequence from *Achromobacter* sp. AO22 was confirmed by Ng *et al.*, 2009, submitted to NCBI GenBank and assigned accession number EU696790. The sequences from *A. woluwensis* E9 and Consortium VUN 10010 were submitted to NCBI GenBank and assigned the accession numbers GQ415452 and GQ415453, respectively.

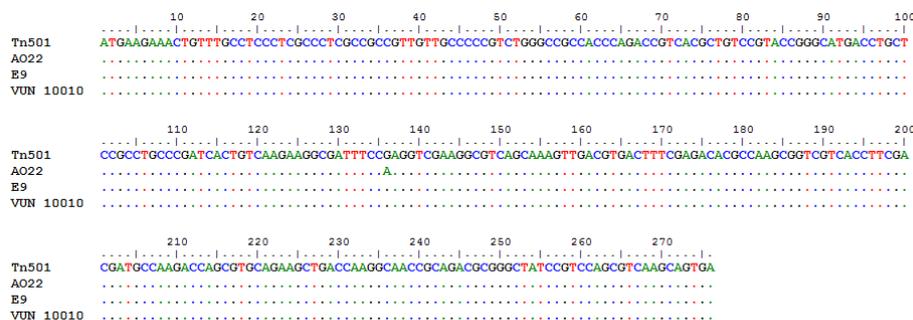


Figure 4.15 DNA sequence alignment of *merP* from Tn501 (Z00027) with sequences of amplified fragments from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. Sequencing was performed as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

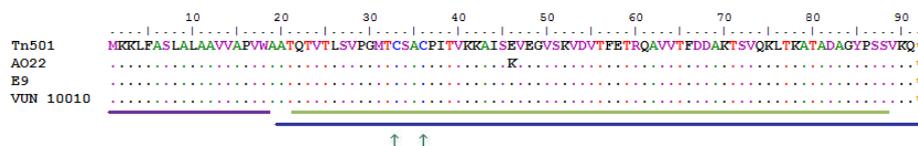


Figure 4.16 Alignment of the MerP protein from Tn501 (Z00027) with the predicted MerP protein sequences from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010.

- ↑ Binding site for mercury ions
- Potential signal sequence
- HMA domain
- Periplasmic component

4.4.4 *merA* sequence analysis

The *merA* gene has only been partially sequenced in the three isolates; approximately 80% from *Achromobacter* sp. AO22 and Consortium VUN 10010 and 22% from *A. woluwensis* E9 compared to Tn501 (Figure 4.17). Of the regions sequenced, only one base substitution (G→C) was detected in both *Achromobacter* sp. AO22 and VUN 10010, which was determined to be silent when an amino comparison was made. The remaining portion of the *merA* gene in *Achromobacter* sp. AO22 and Consortium VUN 10010 could not be sequenced because amplification products were not observed. The full *merA* gene was sequenced by Shee Ping Ng (Ng *et al.*, 2009), submitted to NCBI GenBank and assigned the accession number EU696790. The full *mer* operon of *Achromobacter* sp. AO22 was isolated on a transposon (TnAO22), which was captured by the introduction of a broad-host-range IncP plasmid into *Achromobacter* sp. AO22. The full *merA* gene was shown to have >99% identity to Tn501 (Ng *et al.*, 2009). The partial *merA* sequence obtained from Consortium VUN 10010 was submitted to NCBI GenBank and assigned the accession number GQ415453.

Approximately 22% of the *merA* gene of *A. woluwensis* E9 could be amplified and sequenced (Figure 4.17), which showed no differences compared to *merA* of Tn501 (Misra *et al.*, 1984). The HMA domain relating to *merA* in Tn501 was identified in the amino acid sequence obtained for *A. woluwensis* E9, however no sequence was available to determine whether the FAD binding site was present (Misra *et al.*, 1984).

The partial *merA* sequence obtained from *A. woluwensis* E9 was submitted to NCBI GenBank and assigned the accession number GQ415452. Sequencing the PCR products for this gene from *A. woluwensis* E9 was largely unsuccessful. Two possibilities may explain this outcome; i) *A. woluwensis* E9 possesses a truncated version of *merA*; or ii) it may possess an intact *merA* gene, which displays significant sequence variation compared to the sequences used to design the primers (Section 2.8.1.1). These results do however suggest that differences exist between the *merA* genes of *Achromobacter* sp. AO22 and VUN 10010, compared to *merA* from *A. woluwensis* E9.

4.4.5 *merB* sequence analysis

Earlier, some amplification products using *merB* specific primers were achieved for *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 (Section 4.3.2). A sequence was only obtained internally to the *merB* gene since the 3' and the 5' ends of *merB* could not be amplified in all three isolates (Figure 4.19). From the sequence data for AO22 (position 6 to 652), two base changes and a base deletion were detected, the substitutions not resulting in a change to the predicted amino acid sequence compared to *merB* of *S. marcescens* pDU1358 (PDUMER; Griffin *et al.*, 1987), but the deletion resulted in an amino acid change from serine to valine. The same two substitutions were seen for *A. woluwensis* E9 and VUN 10010 (Figure 4.20), while an additional substitution was seen for VUN 10010 (G→C) resulting in an alanine to proline change in the predicted protein. Sequences from *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 were submitted to NCBI Genbank and assigned the accession numbers GQ415454, GQ415455 and GQ415456, respectively.

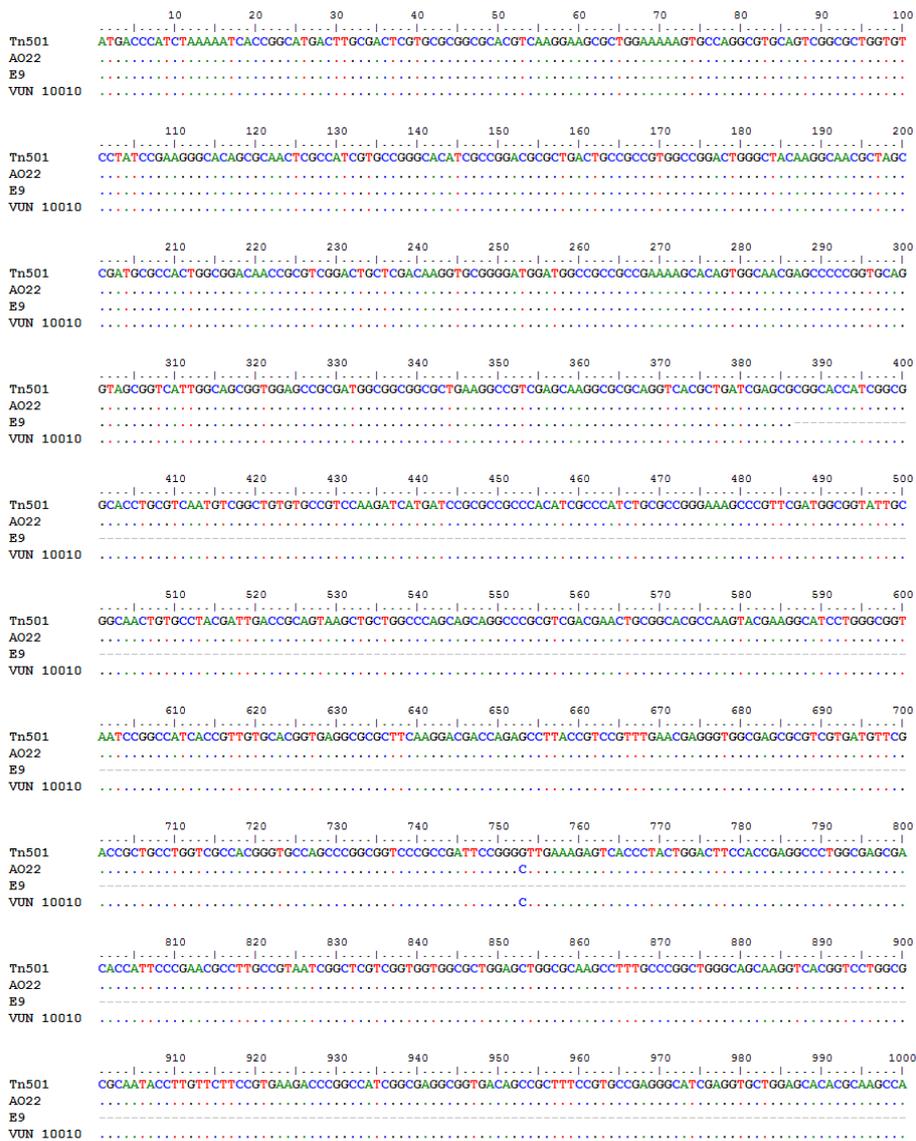


Figure 4.17 DNA sequence alignment of *merA* from Tn501 (Z00027) with sequences obtained from amplified fragments from *Achromobacter* sp. AO22, *A. wulwensis* E9 and VUN 10010. Sequencing was conducted as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

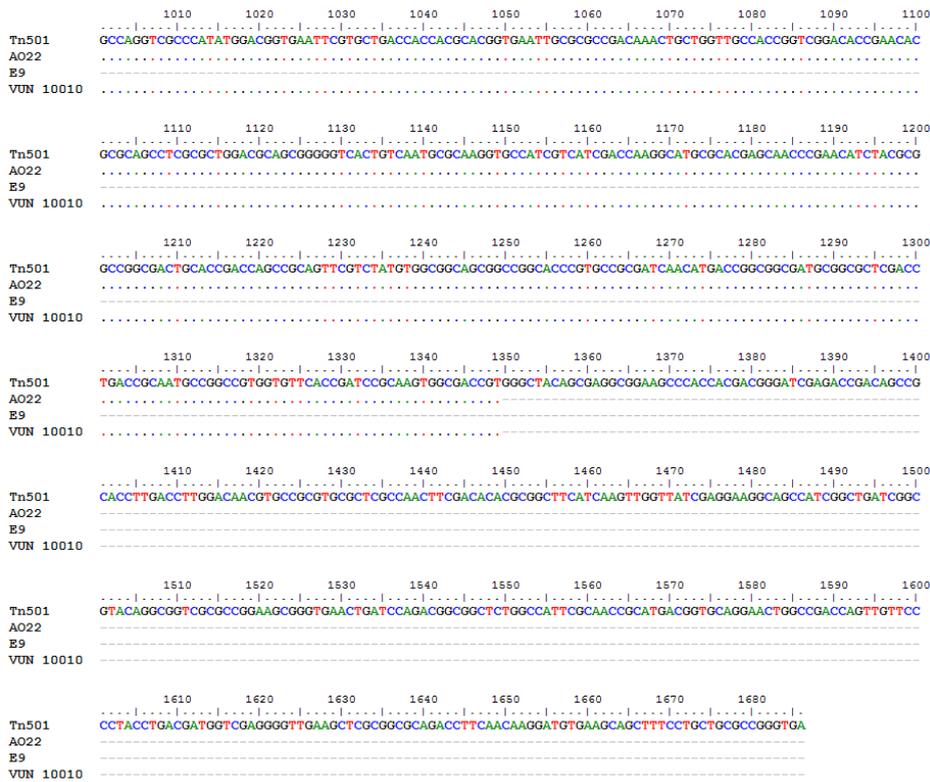


Figure 4.17 cont. DNA sequence alignment of *merA* from Tn501 (Z00027) with sequences obtained from amplified fragments from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. Sequencing was conducted as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

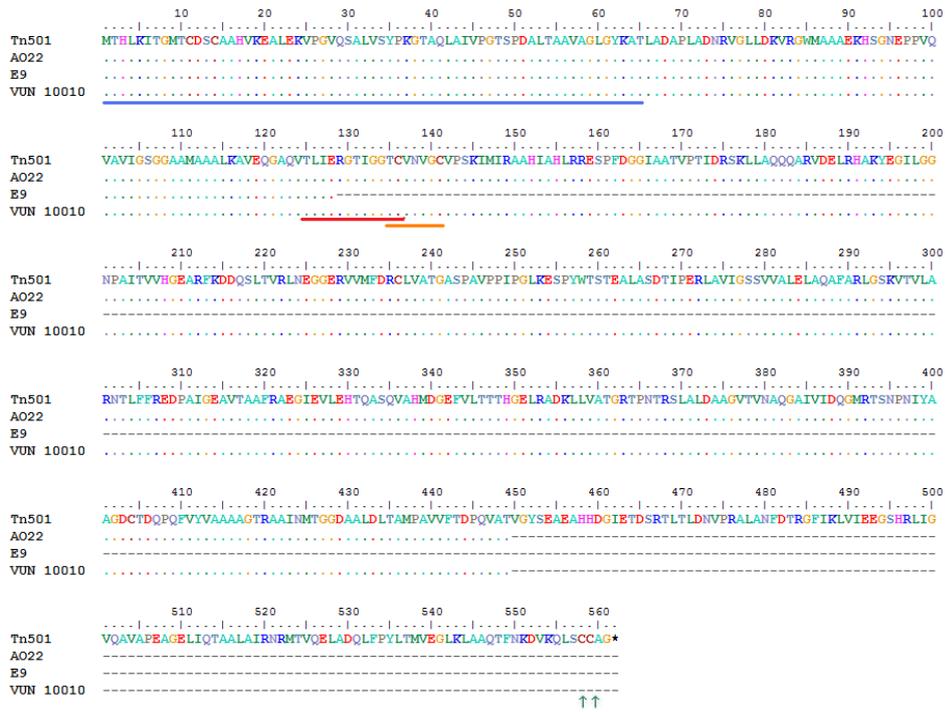


Figure 4.18 Alignment of the MerA protein from Tn501 (Z00027) with the predicted MerA protein sequences from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010.

- ↑ Binding site for mercury ions
- HMA domain
- FAD Binding site
- Disulphide bond

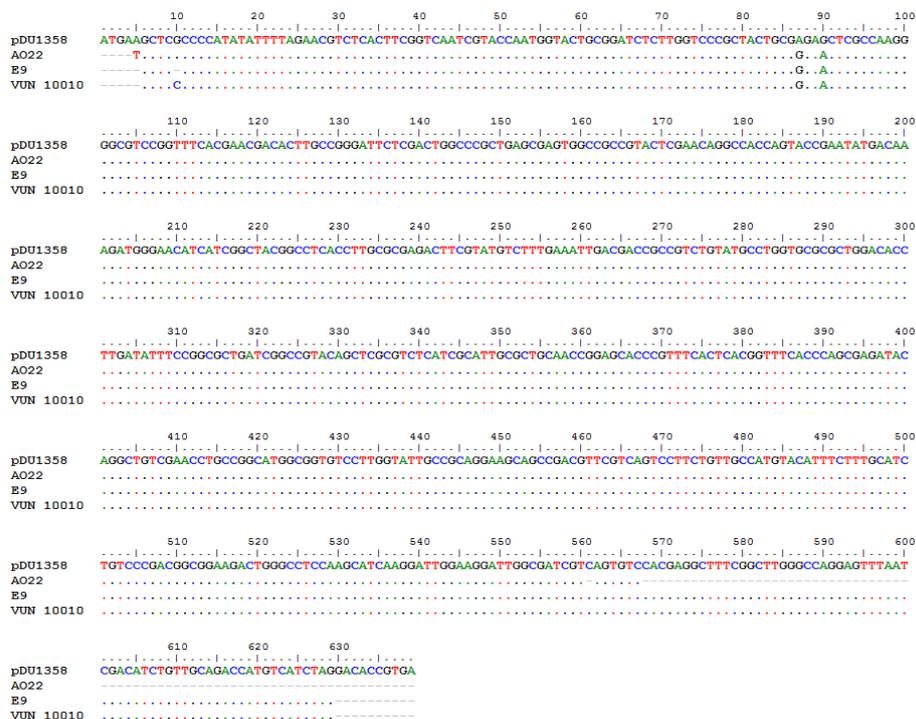


Figure 4.19 DNA sequence alignment of *merB* from pDU1358 (PDUMER) with sequences obtained from amplified fragments from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. Sequencing was conducted as described in Section 2.11. Reactions were performed using Applied Biosystems BigDye reagent and analysed on the ABI 373A Automated Sequencer (Micromon DNA Sequencing Facility).

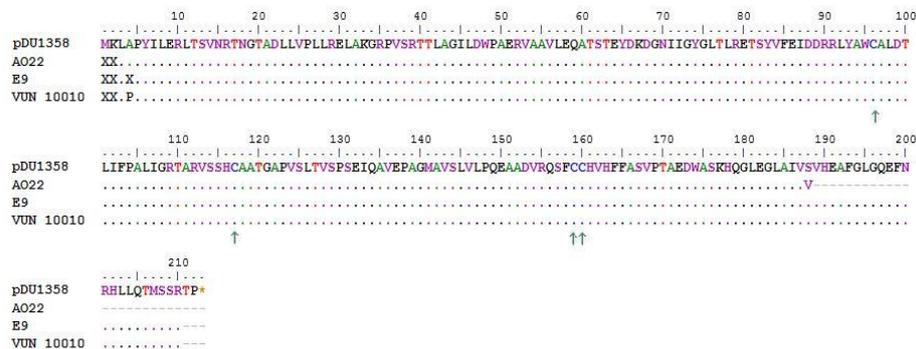


Figure 4.20 Alignment of the MerB protein from pDU1358 (PDUMER) with the predicted MerA protein sequences from *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010.

↑ Binding site for mercury ions

Table 4.9 Summary of *mer* genes detected in *Arthrobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010

Isolate	Gene							
	<i>merR</i>	O/P	<i>merT</i>	<i>merP</i>	<i>merA</i> [#]	<i>merD</i>	<i>merB</i> *	<i>merC</i>
AO22	Present	Present	Present	Present	80%	ND	Present	ND
E9	Present	Present	Present	Present	22%	ND	Present	ND
VUN 10010	Present	Present	Present	Present	80%	ND	Present	ND

[#]*merA* was not fully detected due to inability to amplify 3' areas. This table represents the portion of *merA* that was amplified and sequenced.

**merB* was only detected using internal primers. Was not identified with respect to location to the remainder of the *mer* operon.

4.5 Mercuric chloride induction and growth curve analysis of *Achromobacter* sp. AO22

To determine the influence of HgCl₂ on cells in culture, a time course experiment was performed (as described in Section 2.3.6) in which the growth profile of *Achromobacter* sp. AO22 was compared in the presence and absence of 0.075 mM HgCl₂ (refer to Section 2.3.6). A glycerol stock was initially plated onto an NBT plate containing 0.025 mM HgCl₂, to ensure that a colony with mercury resistance was being selected. From this colony, cells were grown overnight to an OD₆₀₀ of 1.0 in NBT without HgCl₂ to obtain a parent culture. This OD₆₀₀ was chosen as it has been determined that the cells are in the late exponential phase (Chapter 3). The cells were inoculated into fresh medium to OD₆₀₀ of 0.1 containing NBT only or NBT with 0.075 mM HgCl₂; OD₆₀₀ readings were taken regularly (Figure 4.21, Appendix 8) and cell counts were performed (Figure 4.22, Appendix 8). The remaining cells in culture at each time point were pelleted and stored at -20°C for RNA extraction.

Some differences in OD_(600nm) readings were observed between the induced and control *Achromobacter* sp. AO22 cells, with the HgCl₂-containing culture having a slightly lower exponential growth rate; both were inoculated to an initial OD₆₀₀ of 0.108. By the nine hour time point, both cultures had reached an OD₆₀₀ reading of >1.0, indicating the cells had entered early stationary phase. The results suggest that although stressed by the presence of 0.075 mM HgCl₂, the *Achromobacter* sp. AO22 cells rapidly acclimated to the presence of the mercuric chloride in the medium.

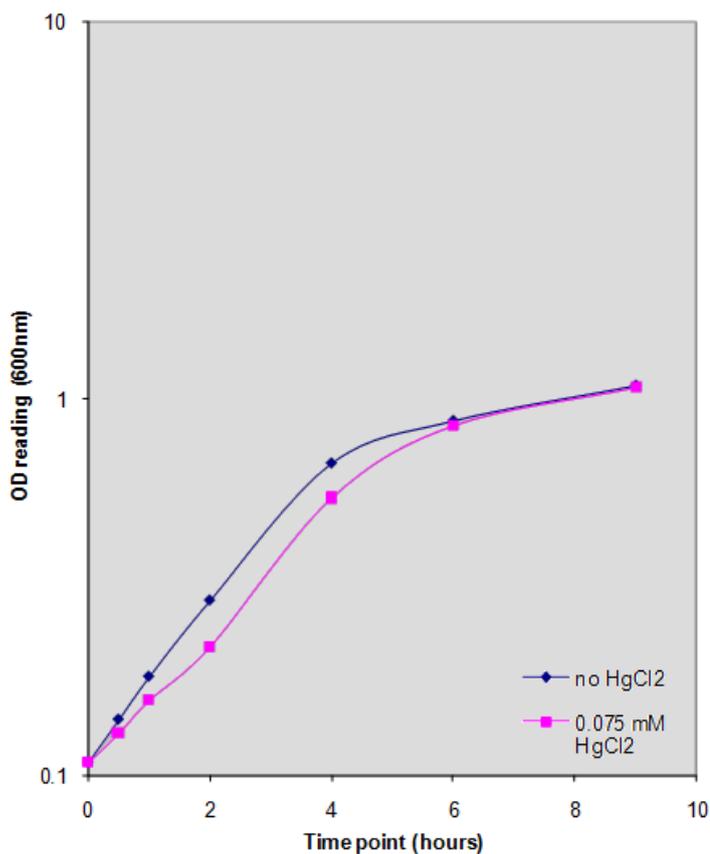


Figure 4.21 OD₆₀₀ readings of *Achromobacter* sp. AO22 cultures in the presence or absence of 0.075 mM HgCl₂. Experiments were conducted as described in Section 2.3.5. Late exponential phase cells from the same parent culture were inoculated into flasks containing NBT or NBT plus 0.075 mM HgCl₂ and incubated at 30°C/150 rpm.

OD₆₀₀ readings do not separately recognise living and dead cells, the latter potentially having a significant effect on viable cell population in stressed cultures (*i.e.* containing HgCl₂). Viable cell counts were performed so that the viable cell population profile of each culture could be determined (Section 2.3.7). Cell counts from the *Achromobacter* sp. AO22 cultures induced with 0.075 mM HgCl₂ and the control cultures were performed (Figure 4.22, Appendix 8). Both cultures experienced a growth lag period

however the control culture commenced exponential growth earlier than the stressed cultures (containing HgCl₂). Although the error in the results make it difficult to determine if differences exist in exponential growth rate, it does appear that both cultures were growing at approximately equivalent rates, with the data suggesting that both cultures were still in the exponential growth phase after 9 hours of incubation.

4.6 Growth profile of *A. woluwensis* E9 in the presence or absence of mercuric chloride

Initially, the approach used to study the impact of HgCl₂ on the growth of *A. woluwensis* E9 was similar to that used for the *Achromobacter* sp. AO22 cells (Section 2.3.6). This experiment was performed using cultures in the presence or absence of 0.03 mM HgCl₂, and OD₆₀₀ readings were taken (Table 4.10). The *A. woluwensis* E9 control cultures commenced growth with 30 minutes of inoculation, however, the culture containing 0.03 mM HgCl₂ did not commence growth within a 24 hour time period, with the biomass level appearing to decline.

Further experiments were performed to determine the impact of various HgCl₂ concentrations (0.01-0.05 mM) on the biomass profile of *A. woluwensis* E9 cultures (Figure 4.23; Appendix 8). The only culture that grew within the first 24 hours was the control sample; after 8 hours, these cells were in late exponential phase. No noticeable biomass increase was observed for any cultures exposed to HgCl₂ during this 8-hour period. After 24 hours, the control culture had reached stationary phase, while none of the HgCl₂-containing cultures had commenced growth, rather the biomass levels in all stressed cultures had declined. The cultures containing 0.01-0.03 mM HgCl₂ began to grow between 24-48 hours post-inoculation, while cultures containing 0.04 and 0.05 mM HgCl₂ only commenced growth 48 hours post-inoculation.

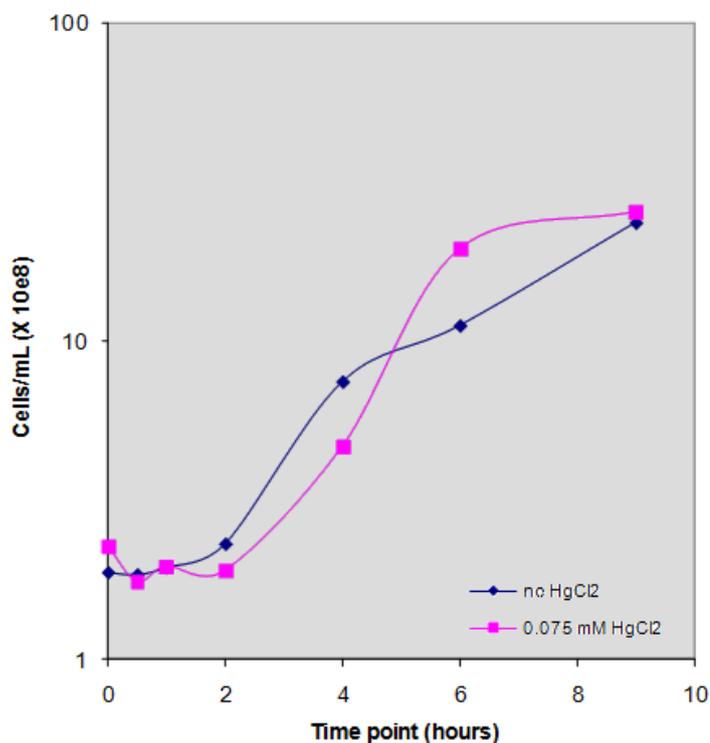


Figure 4.22 Viable cell population of *Achromobacter* sp. AO22 cultures in the presence and absence of 0.075 mM HgCl₂. Experiments were conducted as described in Section 2.3.5. Late exponential phase cells from the same parent culture were inoculated into flasks containing NBT or NBT plus 0.075 mM HgCl₂ and incubated at 30°C/150 rpm.

The results obtained for the growth profiles of *Achromobacter* sp. AO22 and *A. woluwensis* E9 in the presence of HgCl₂ demonstrated the different sensitivities of these strains to HgCl₂, with E9 being considerably more sensitive to mercuric chloride than AO22. The MIC results (Chapter 3) suggest that *Achromobacter* sp. AO22 can grow in the presence of HgCl₂ concentrations up to 0.15 mM, with *A. woluwensis* E9 being growth tolerant up to 0.125 mM HgCl₂. The relative sensitivities of these two strains to HgCl₂ were the same in both sets of experiments (i.e. E9 being less HgCl₂ tolerant than AO22), however it is difficult to comment on the differences in HgCl₂ concentrations observed to affect these two strains given the different conditions used in the two experimental platforms. MIC experiments were performed either on solid medium or in liquid cultures up to 10 ml over 3 day periods.

Table 4.10 OD₆₀₀ readings from the *A. woluwensis* E9 cultures in the presence or absence of 0.03 mM HgCl₂

Time (h)	Control (no HgCl ₂) (cells/mL)	Stressed Cultures (Containing 0.03 mM HgCl ₂) (cells/mL)
0	0.097	0.098
0.5	0.124	0.097
1	0.139	0.099
2	0.222	0.101
3	0.443	0.093
5	0.691	0.089
8	1.023	0.082
24	1.558	0.077

The results described in this chapter on the tolerance of *Achromobacter* sp. AO22 and *A. woluwensis* E9 to mercuric chloride demonstrate that both isolates potentially possess genes associated with mercury resistance, however, the effectiveness of their response to HgCl₂ stress are quite different, *A. woluwensis* E9 being considerably more growth sensitive compared to *Achromobacter* sp. AO22. Although HgCl₂ acclimation is slower for E9 than AO22, the cells are capable of acclimating to mercuric chloride in the medium at concentrations up to 0.05 mM, such that exponential growth commences. Consortium VUN 10010 was not considered for this part of the study due to the fact that two isolates, *S. maltophilia* and *Mycobacterium* sp. were present in this culture. This would have firstly made RNA extraction difficult as one is a Gram-negative and the other a Gram-positive, each of which could not be isolated from each other. From what RNA that may have been extracted, it would not be possible to entirely determine if any *mer* gene expression determined was coming from the *S. maltophilia* or the *Mycobacterium* sp., or both. Given the results obtained for *Achromobacter* sp. AO22 and *A. woluwensis* E9, and the challenges associated with Consortium VUN 10010, it was decided to extract RNA from *Achromobacter* sp. AO22 control cells and HgCl₂-exposed cells for analysis of *mer* genes expression. *Achromobacter* sp. AO22 was chosen for expression studies due to its greater HgCl₂ tolerance.

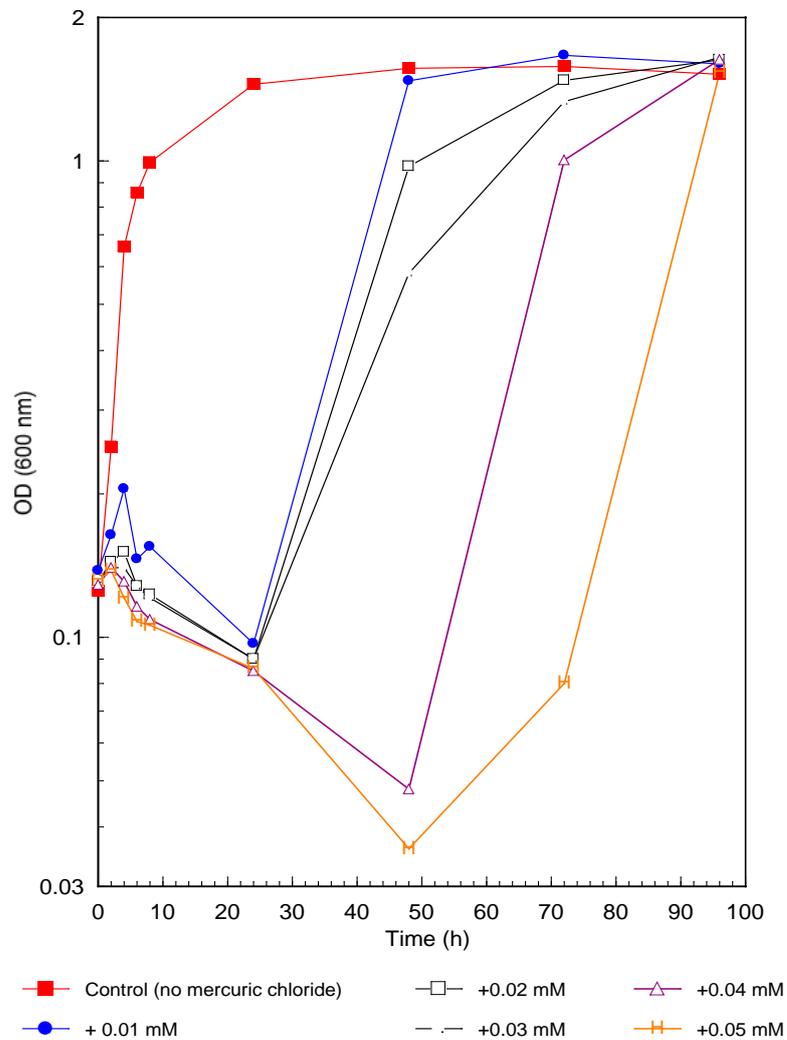


Figure 4.23 Biomass profiles of *A. woluwensis* E9 cultures in the presence of 0.01 - 0.05 mM HgCl₂ and in the absence of HgCl₂. Experiments were conducted as described in Section 2.3.5. Late exponential phase cells from the same parent culture were inoculated into flasks containing NBT or NBT plus 0.01-0.05 mM HgCl₂ and incubated at 30°C/150 rpm.

4.7 Expression of *mer* genes in *Achromobacter* sp. AO22 during HgCl₂ stress

RNA was extracted from the frozen cell pellets obtained from the experiment described in Figure 4.22, over a 9 hour period; extractions were based on equal cell number for both stressed and control cultures (Sections 2.12.1 and 2.12.2). Prior to performing RNA extractions, cell populations were determined to enable equal cell numbers to be extracted (approximately 1.75×10^8 cells per extraction), as the basis for detection of differences in mRNA levels in control cells and HgCl₂-induced cells (Emslie, 2002). Figure 4.24 shows the agarose gel electrophoresis (Section 2.7) of 5 μ L of the RNA prepared from the *Achromobacter* sp. AO22 cells exposed to 0.075 mM HgCl₂ and from the control *Achromobacter* sp. AO22 cells.

Intact RNA of a high yield was extracted from the *Achromobacter* sp. AO22 cells (Figure 4.24). In addition to the 23S and 16S rRNA fragments, genomic DNA is also evident on the gel. The RNA samples were treated with DNaseI to remove remnant DNA that may result in spurious amplification (Section 2.14). The effectiveness of DNaseI treatment was confirmed by the use of negative controls (omitting reverse transcriptase) in subsequent experiments (see below). Equal volumes (2 μ L of the total preparation) of sample from the DNaseI-treated RNA were used to prime cDNA synthesis. As specific *mer* genes were examined in this study, the upstream primer of each of these genes was used to prepare the cDNA. Internal primers to the *merR* (merR1-16), *merT* (merT1-T2), *merP* (merP1-P2) and *merA* (mer19-32) genes were utilised in Section 2.8.1.1. The process was performed for the *merR*, *merT*, *merP* and *merA* mRNA transcripts separately.

A negative control reaction (lacked the Reverse-Transcriptase enzyme, SuperScript II) was performed in the cDNA synthesis step, which to test for PCR products derived from genomic DNA. For all subsequent experiments, negative controls were conducted and in all cases there was no evidence of *mer* fragment amplification, demonstrating the effectiveness of DNaseI treatment for all samples. Agarose gel figures of the negative controls are shown in Appendix 9.

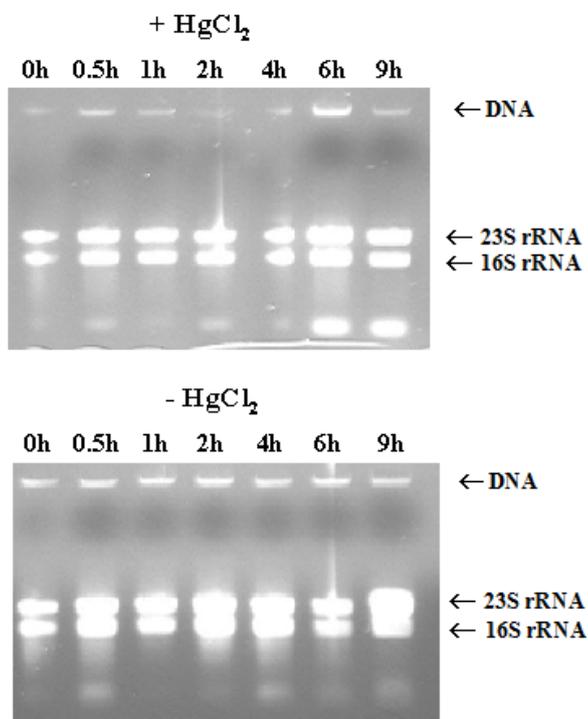


Figure 4.24 RNA prepared from *Achromobacter* sp. AO22 cells exposed to 0.075 mM HgCl₂ and from control AO22 cells. RNA was extracted as described in Section 2.12.2 and visualised on a 1% Agarose gel.

No *merR* transcript was detected in samples taken at time zero (Figure 4.25). After 30 minutes of HgCl₂ exposure, a transcript of expected size (295 bp) was observed in the *Achromobacter* sp. AO22 cells exposed to HgCl₂, however, this was not present in the control cells (-HgCl₂). At the one-hour time point, the *merR* transcript was observed for both stressed and control samples, with greater amounts appearing in the former compared to the latter. This expression pattern continues up to the two-hour time-point. After four hours, there appears to be more transcript in the control cells compared to the HgCl₂-stressed cells and at the six-hour time point the amount of transcript appears to be diminishing. By the nine-hour time-point no *merR* mRNA is observed for both the control and stressed cells.

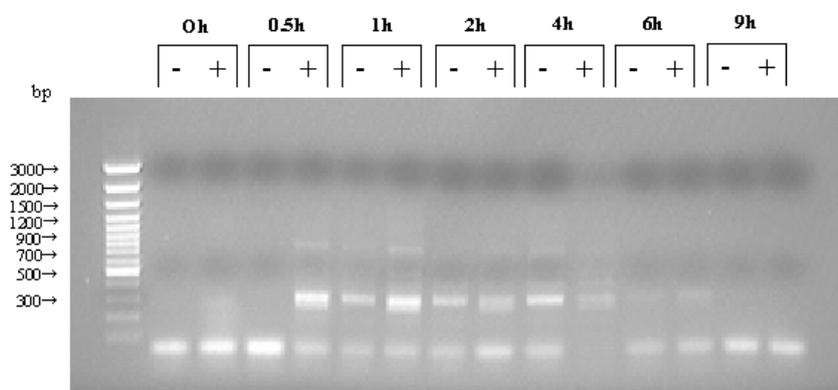


Figure 4.25 RT-PCR amplification of *Achromobacter* sp. AO22 cDNA using the merR1-16 primer pair. cDNA was produced using SuperScript II Reverse Transcriptase as described in Section 2.14. PCRs were visualized on a 1% Agarose gel. The Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas) was used to determine the sizes of the products. The time-point is indicated for each sample. (+) represents cells grown in the presence of 0.05 mM HgCl₂, (-) represents cells grown in the absence of HgCl₂.

Both the *merT* and *merP* transcripts (expected sizes of 335 bp and 280 bp respectively) were detected at time-zero for the HgCl₂-stressed and control cells, however, there appears to be more transcript present in the stressed samples. For all subsequent time points (30 minutes to 9 hours), *merT* and *merP* gene expression is observed in all samples. At each time point there appears to be more transcript present in the stressed samples compared to the control samples, although the levels vary at different time points.

Unlike the *merR*, *merT* and *merP* transcripts, the *merA* transcript (expected size of 575 bp) was detected only in HgCl₂-exposed cells and only for the time period, 0 - 2 hours, with expression levels at the one and two hour time points being relatively small.

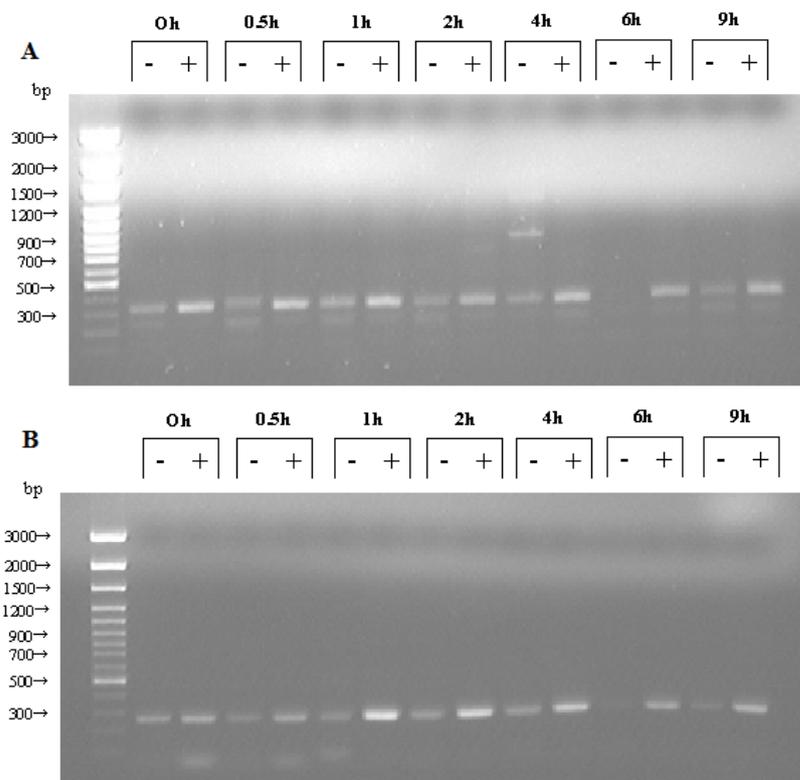


Figure 4.26 RT-PCR amplification of *Achromobacter* sp. AO22 cDNA using the merT1-T2 (A) and merP1-P2 (B) primer pairs. cDNA was produced using SuperScript II Reverse Transcriptase as described in Section 2.14. PCRs were visualized on a 1% Agarose gel. The Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas) was used to determine the sizes of the products. The time-point is indicated for each sample. (+) represents cells grown in the presence of 0.05 mM HgCl₂, (-) represents cells grown in the absence of HgCl₂.

4.8 Discussion

This chapter addressed the isolation and characterisation by sequencing of the *mer* genes present in *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. The O/P regions of all three were found to be 100% identical to the corresponding region of the Tn501 *mer* operon. The *merR* genes and predicted proteins of *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 were 100% identical to the *merR* gene and

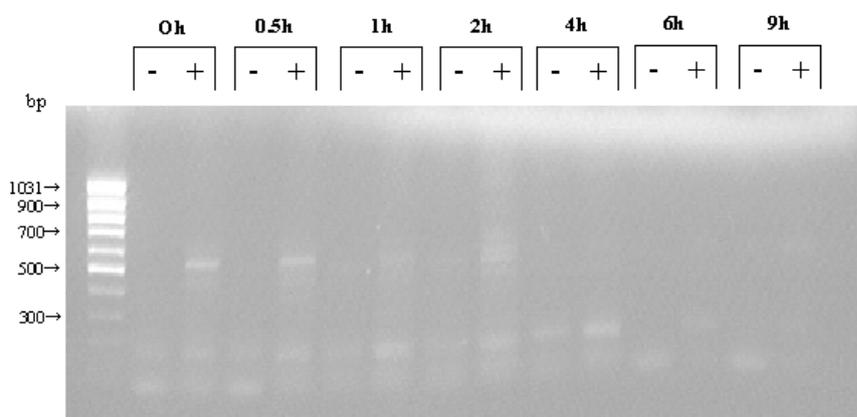


Figure 4.27 RT-PCR amplification of *Achromobacter* sp. AO22 cDNA using the mer19-32 primer pair. cDNA was produced using SuperScript II Reverse Transcriptase as described in Section 2.14. PCRs were visualized on a 1% Agarose gel. The Gene Ruler™ 100 bp DNA Ladder Plus (MBI Fermentas) was used to determine the sizes of the products. The time-point is indicated for each sample. (+) represents cells grown in the presence of 0.05 mM HgCl₂, (-) represents cells grown in the absence of HgCl₂.

protein of Tn501 (Z00027) and the results suggest that *merR* is transcribed divergently to the O/P region, as for Tn501. In other Gram-negative bacteria, the degree of homology between the MerR proteins compared to Tn501 ranges from 95.86% for *S. flexneri* Tn21 (AP000342) to 76.55 % for *Pseudomonas* sp. Tn5041 (X98999) (Table 1.5). When the MerR proteins of Gram-positive bacteria were compared to that of Tn501, the homology ranges from 46.15% for both *B. cereus* RC607 MerR1 (AB066362) and *B. megaterium* MB1 MerR1 (Y09907) to 28.57% for *Streptomyces* sp. CHR28 (AF222792). It is interesting that the sequence of *A. woluwensis* E9 is more similar to the reported sequences for Gram-negative bacteria *mer* genes than those identified for Gram-positive bacteria. It may be that given *A. woluwensis* was isolated from the same site as *Achromobacter* sp. AO22 (Section 1.9.1), the evolution of these genes within bacteria from this particular site may have included some form of horizontal gene transfer.

The *merT* genes and predicted proteins of *A. woluwensis* E9 and VUN 10010 were 100% identical those of Tn501. However, the *Achromobacter* sp. AO22 *merT* gene has

a single base difference (A→T) at position 144, leading to a glutamic acid → aspartic acid change in the predicted second transmembrane sequence. Based on the results obtained in Chapter 3, where the HgCl₂ MIC of *Achromobacter* sp. AO22 is comparable to that of *A. woluwensis* E9 and VUN 10010, it would appear that this change in the protein sequence has little if any impact on the functionality of the protein. Glutamic acid and aspartic acid are charged R group amino acids, which may account for the merT protein maintaining function. The MerT of Tn501 contains four cysteines (Cys24, Cys25, Cys76 and Cys82) implicated in the binding of mercury ions all of which are present in *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010. As with the *merR* gene and protein, the *merT* genes and proteins in each strain are similar (>99%) to *merT* of Tn501. As noted above, it is unusual that *A. woluwensis* E9, a Gram-positive bacterium, has high *merT* homology with Gram negative bacteria given that most Gram-positive species do not.

The *merP* genes and predicted proteins of *A. woluwensis* E9 and VUN 10010 were 100% identical to the *merP* gene and protein of Tn501 (Z00027), while *merP* from *Achromobacter* sp. AO22 had one base substitution (G136→A), resulting in a Glutamic acid→Lysine change in the HMA domain/periplasmic component of predicted protein. This A has also been reported at the corresponding position within all the other Gram-negative bacteria compared in this project and also in the *merP* of the Gram-positive bacteria *Streptomyces* sp. CHR28 (AF222792). As with the *merT* protein, it would appear that this change in the protein sequence has no impact on the protein functionality, based on the MIC results reported in Chapter 3 and given that both glutamic acid and lysine are charged R group amino acids. The cysteines in the MerP protein of Tn501 that are implicated in the binding of mercury ions (Cys14 and Cys15 of the periplasmic component/HMA domain) are present in all three isolates. Residues corresponding to two other amino acids (Phe38 and Tyr66 periplasmic component/HMA domain) implicated in the mercury binding reaction and transfer of Hg(II) to MerT (Serre *et al.*, 2004) were also detected in all three isolates. As with the *merR* and *merT* genes and proteins, the *merP* gene and protein of Gram-positive bacterium *A. woluwensis* E9 was more closely related to those from Gram-negative bacteria isolates, compared to other Gram-positive bacteria isolates. In the amplified sections of *merA*, one substitution (G→C) was observed in both *Achromobacter* sp.

AO22 and Consortium VUN 10010 which does not result in an amino acid change but is also present in *merA* genes from *Xanthomonas* sp. Tn5053 (RP1W17A), *S. flexneri* Tn21 (AP000342), *S. marcescens* pDU1358 (PDUMER; PDUMERR), *S. lividans* (X65467) and *Streptomyces* sp. CHR28 (AF222792).

The MerA protein of Tn501 contains eight cysteine residues, of which Cys10 and Cys13 have not been shown to have an essential role; however, they are both present in *Achromobacter* sp. AO22, Consortium VUN 10010 and all reported MerA proteins searched in this project, as are Cys135 and Cys140, located in the active site. Cys558 and Cys559, which play a role in Hg(II) reduction, were not determined for *Achromobacter* sp. AO22 and VUN 10010 in this thesis, however they were detected in *Achromobacter* sp. AO22 by Shee Ping Ng (Ng *et al.*, 2009). Two other Cys residues (Cys236 and Cys404) of Tn501 are also present in *Achromobacter* sp. AO22, VUN 10010 and in all the Gram-negative bacteria used for comparison (as outlined in Table 1.4). The *merA* gene from *A. woluwensis* E9 was only partially sequenced; the first 386 bp (130 amino acids) were determined, encompassing the HMA region yet it ceased at the beginning of the FAD binding site, hence the presence of this site, and the cysteines comprising the binding site for mercury ions were not determined. The *merA* gene of *Achromobacter* sp. AO22 was however sequenced by another investigator (Ng *et al.*, 2009) suggesting that the primers and/or conditions used in the current project may not have been suitable. To further sequence the *merA* gene from *A. woluwensis* E9 and Consortium VUN 10010, similar methodologies to those used by Shee Ping Ng should be applied to the DNA of these isolates before concluding that the full *merA* gene is not present (Ng *et al.*, 2009). Based on the differences in results obtained from *Achromobacter* sp. AO22 and Consortium VUN 10010, to those obtained for *A. woluwensis* E9, it is suggested that sequence differences exist in the *merA* gene of *A. woluwensis* E9. It may be that *A. woluwensis* E9 does not possess a full complement of the *merA* gene since the sequence did not contain a stop codon, however this would need to be further investigated. Each sequence obtained ceased at the location of the primer that was used to amplify a particular fragment. In light of this, the most likely explanation is that the undetermined portions of these *merA* genes were not amplified due to sequence variations, which prevented the primers from annealing.

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The *merB* gene, which encodes organomercurial lyase, was partially amplified from all three isolates and all shared the A→G and G→A substitutions, compared to the *S. marcescens* pDU1358 *merB* gene, neither of which result in an amino acid change. This A→G substitution is also present in *Pseudomonas* sp. K-62 *merB2* (AB013925), *B. cereus* *merB2* and *merB3* (AB066362), *B. megaterium* *merB2* (AB027307) and *merB3* (AB027306) and *Streptomyces* sp. CHR28 (AF222792), while the G→A substitution is also seen in *Pseudomonas* sp. K-62 *merB2* (AB013925) and *P. stutzeri* pPB (PSU90263). Four cysteines (Cys96, Cys117, Cys159 and Cys160) are associated with the function of MerB protein (Section 1.4.5.1). All four cysteines were identified in the three isolates. From the results obtained in Chapter 3, *Achromobacter* sp. AO22 and *A. woluwensis* E9 did not demonstrate resistance to organomercurials, with VUN 10010 showing some resistance to phenylmercury acetate. Without having elucidated the full sequence of the *merB* gene and its location, it is not possible to conclude whether the lack/low level of organomercurial resistance is due to changes that may be present within the protein, whether it is associated with its location within the *mer* operon or if a *merB*-like sequence is present elsewhere in the genome.

The *merC* gene was not detected in any of the isolates, and no additional gene was detected between *merP* and *merA*. Analysis of reported *merC* genes shows that the homologies are highly variable, ranging up to 100% between MerC proteins of *K. pneumoniae* Tn5073 (AF461013) and *A. calcoaceticus* pKLN201 (ACA251307) (Essa *et al.*, 2003, Kholodii *et al.*, 2004) and as low as 66.512% between *S. flexneri* Tn21 (AP000342) and *Pseudomonas* sp. Tn5041 (X98999). Due to this, it is difficult to design appropriate primers for detecting *merC* genes using PCR. The *mer* operons of these isolates are very similar to that of Tn501, and to Tn21, therefore any *merC* gene, if present, would occur between *merP* and *merA*. The results from this project suggest it is unlikely that these three possess a *merC* gene.

Chapter 3 of this thesis described the experimental determination of minimum inhibitory concentrations for *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 to a range of heavy metals, including mercuric chloride. The current chapter also investigated on the growth of *Achromobacter* sp. AO22 and *A. woluwensis* E9 in the presence of HgCl₂, along with a time course profile of *mer* gene

expression in *Achromobacter* sp. AO22 cells exposed to HgCl₂. Differences were noted between the growth and adaptation rates of *Achromobacter* sp. AO22 and *A. woluwensis* E9, with *Achromobacter* sp. AO22 able to adapt and grow within nine hours at a concentration of 0.075 mM HgCl₂, while *A. woluwensis* E9 took up to 92 hours to grow in the presence of 0.05 mM HgCl₂. The investigation into the MICs of both *Achromobacter* sp. AO22 and *A. woluwensis* E9 indicated that E9 was slightly more sensitive to the presence of HgCl₂ compared to AO22, and the results of this chapter confirm this relative sensitivity to HgCl₂, with the growth of *Achromobacter* sp. AO22 being less affected than E9 by HgCl₂. The higher tolerance of AO22 may be related to *Achromobacter* sp. AO22 having a complete version of the *merA* gene, as described in Chapter 4. This would require confirmation by further investigation.

RNA was extracted from equal cell numbers obtained from *Achromobacter* sp. AO22 cultures exposed to HgCl₂ and from control cells (without HgCl₂ exposure). All RNA samples were DNase treated and in the first instance, cDNA was produced using upstream primers specific to the *merR*, *merT*, *merP* and *merA* genes for the purpose of Reverse Transcriptase-PCR. Negative controls were prepared, containing no Reverse Transcriptase enzyme, to ensure that amplification products were not generated from genomic DNA. The *merR* transcript was detected 30-minutes after inoculation, but only in cells exposed to HgCl₂, indicating the cells responded to the HgCl₂ stress via the stress regulatory mechanisms. After one hour, the *merR* transcript was observed in both HgCl₂-stressed and control samples, although the amount of transcript is greater in the stressed sample; this is also case at the two-hour time point. After 4 hours, more transcript was present in the control cells than in the induced cells and by six-hours the amount of *merR* transcript in both stressed and control cells is declining. No *merR* transcript could be detected in either the stressed or control samples after nine hours of incubation. These results show that *merR* is induced in both control and stress cells, however, induction occurs earlier for the stressed cells and that *merR* does not continue to be expressed for the duration of HgCl₂ exposure or through all stages of growth.

The transcripts of the *merT* and *merP* genes, both encoding mercury transport proteins, were similarly expressed. The expression both genes appear to be constitutive, with corresponding transcripts being detected at time zero in both the stressed and control samples. Expression of these genes continues throughout the experiment with

transcripts of each being seen up to the nine-hour time point, noting that transcript levels are higher in the stressed samples compared to the control samples for all time points. The higher expression level in the stressed sample was also observed at the zero time point, which is not expected for a constitutively expressed gene; it is expected that the expression levels at this time point would be the same. It is important to note that time-zero may not be a true reflection of instant HgCl₂ exposure in the stressed samples and the results seen here may reflect those reported by Jeffery *et al.*, (1994), whereby stationary phase *P. aeruginosa*, containing the Tn501 operon, showed *merA* gene expression within 30 seconds of HgCl₂ exposure. It is also important to note that it takes a small amount of time to prepare the samples for centrifugation, followed by a total of 15 minutes in the centrifuge. It may be that the time-zero stressed cells were exposed to HgCl₂ for a small time period, thus it may not be a true reflection of *merT* and *merP* expression instantly upon HgCl₂ addition. There was no evidence of trace levels of mercury in the medium prior to inoculation.

In contrast to the above, the *merA* gene only appears to be expressed in the stressed cells and the transcript was only detected between the zero and two hour time points. This may indicate that expression is required only for a short time to allow sufficient protein production. There was a considerably higher level mRNA present for the *merR*, *merT* and *merP* genes for both stressed and control cells, compared to the *merA* transcript in the cells exposed to HgCl₂ (no *merA* expression was evident in the control cells). This result correlates with results reported by Jackson and Summers (1982) who found that 80% of the *mer* mRNA transcripts terminate between the *merC* and *merA* genes in the Tn21 operon. In the case of *Achromobacter* sp. AO22, a similar event may be occurring between the *merP* and *merA* genes. They also found that the *merA* portion of the transcript never reaches a level equivalent to that of the *merT* and *merP* genes in the Tn21 operon, which is similar to the results obtained for *Achromobacter* sp. AO22.

The *mer* genes expression results for *Achromobacter* sp. AO22 was unexpected. The induction of the *merR* gene in the control samples was not expected, while the expression of the transport genes in both induced and control cells could be expected at minimal levels, to enable mercury ion transport. It appears that the presence of mercury lead to an up-regulation of both the *merT* and *merP* genes in AO22. The *merD* gene was not investigated since it could not be amplified. This gene has subsequently been

identified in *Achromobacter* sp. AO22 by Shee Ping Ng (Ng *et al.*, 2009). The MerD protein is reported to bind to the DNA operator region to act as a downregulator of the *merT*, *merP* and *merA* genes (Champier *et al.*, 2004). If a functional *merD* is present, the expression of *merR* in control cells would not be expected nor at time zero in stressed cultures, however without evidence of the behaviour of the *merD* gene, it is not possible to comment on why the *merR* transcript was observed in these samples". Whilst the growth profile was investigated in the isolate *Arthrobacter woluwensis* E9, gene expression was not due to the slow growth of the culture in the presence of mercuric chloride so it is not possible to propose mechanisms for the mercury resistance in this isolate other than it being dissimilar to that of *Achromobacter* sp. AO22 due to fewer genes being isolated and the differences in growth rate. Consortium VUN 10010 was not investigated for growth in the presence of mercuric chloride since this was a mixed culture. Based on gene isolation however it may be possible that the mechanisms of resistance would be similar to that of *Achromobacter* sp. AO22 given the genetic similarities.

While the results presented in this chapter provide an overview of the expression profile of the *mer* genes of *Achromobacter* sp. AO22, in the presence and absence of HgCl₂, the data is semi-quantitative and does not provide comparisons of transcript levels between genes and time points. Future directions for investigations following this project should include the use of Real-Time PCR. The use of this method allows for more concise quantification between samples, and it is possible to view the results at the completion of each PCR cycle rather than relying on the final result (*i.e.* an agarose gel). This method is more sensitive than Reverse-Transcriptase PCR and the results are based on fold-increase and -decrease of transcript present in each sample (relative quantification). Real-time PCR can also be performed to gain "absolute quantification", whereby results can be expressed as an absolute value, such as copy number per cell or µg/µl.

Chapter 5

Formation of biofilms by *Achromobacter* sp. A022, *A. woluwensis* E9 and Consortium VUN 10010 in the presence of a range of heavy metal contaminants

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5.1 Introduction

As described in Section 1.8.4, a bacterial biofilm may be described as 'a sessile community of microbially-derived cells, which are irreversibly attached to a substratum or interface, or to each other. These cells are imbedded in a matrix of extracellular material that they have produced, and exhibit an altered phenotype with respect to growth rate and gene transcription' (Donlan and Costerton, 2002). Biofilms may contain cells derived from a single species or as a community comprising two or more bacterial species. Many species of bacteria have been studied for their ability to form biofilms, including *Pseudomonas aeruginosa*, *Escherichia coli*, *Vibrio cholerae* and *Staphylococcus aureus* (O'Toole *et al.*, 2000).

Bacterial biofilms have been found to grow in natural, industrial and medical/surgical environments and to be resistant to attack by amoebae, bacteriophage and diverse biocides (Costerton *et al.*, 1999). Such resistance is generally due to a delayed penetration of the antimicrobial agent through the matrix produced by the biofilm and/or other physiological changes that occur due to the biofilms mode of growth, including an altered growth rate of the organism (Donlan and Costerton, 2002). Biofilms have been shown to cause major problems in the medical field, by either residing on indwelling medical devices (i.e. catheters) or causing persistent infections in immuno-compromised patients (Costerton *et al.*, 1999). Biofilms formed by environmental bacteria have the potential to remediate contaminated matrices, such as wastewater, e.g., *Cupriavidus metallidurans* CH34 (White and Gadd, 2000), *Desulfobacteriaceae* sp. (Labrenz *et al.*, 2000) and *P. aeruginosa* (Langley and Beveridge, 1999) (Section 1.8.4.2).

As established in Chapter 3, *Achromobacter* sp. isolate AO22, *Arthrobacter woluwensis* isolate E9 and VUN 10010 all possess resistance capabilities to mercury, lead, zinc, cadmium and cobalt. It was thought that if these isolates also exhibit biofilm-producing capabilities, this may increase the potential of these isolates for use in bioremediation processes. The work described in this chapter examines this further.

5.2 Biofilm formation in the absence of heavy metal contaminants

The average results of two separate experiments are outlined in Figure 5.1 and Appendix 10. *P. aeruginosa* was used as a positive control for biofilm formation and a negative control (no inoculum) was also included.

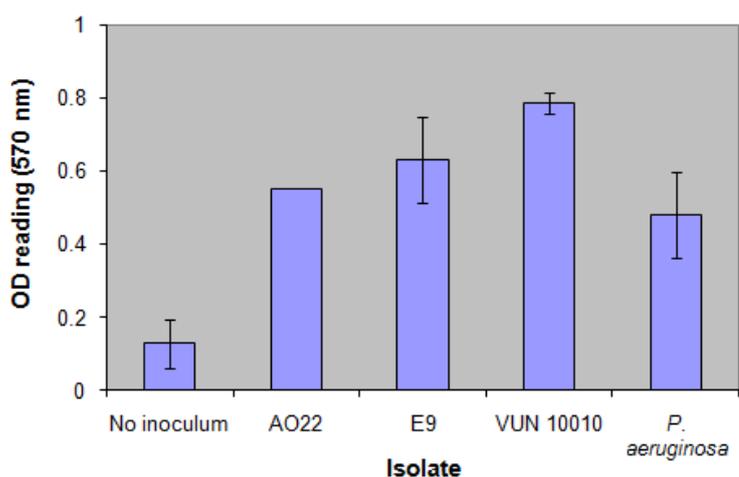


Figure 5.1 Biofilm formation in the absence of heavy metals. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT only and grown in microtitre plates for 48 hours at 30°C.

The results indicate that all three isolates, *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 are capable of forming biofilms in the absence of heavy metals. *Achromobacter* sp. AO22 was able to form biofilms at biomass levels approximately 15% higher than *P. aeruginosa*, while *A. woluwensis* E9 and Consortium VUN 10010 were approximately 31% and 61% higher, respectively. These results were also subsequently used as the benchmark for comparison with biofilm production in the presence of heavy metals.

5.3 Biofilm formation in the presence of mercuric chloride

The biofilm-producing potential of *Achromobacter* sp. AO22, *A. woluwensis* E9, VUN 10010 and *P. aeruginosa* were tested within the range of 0.01-0.1 mM HgCl₂ in microtitre plates. ~~Appendix 7 contains the OD₅₇₀ results and the statistical analysis for each isolate tested for each of the three experiments performed.~~ The results of the two replicate ~~experiments were averaged~~ and the extent of biofilm formation of each sample exposed to mercury was calculated as a percentage, relative to the extent of biofilm formation (% of attached cells) of a particular isolate in the absence of HgCl₂ (100%). These results are ~~and the remaining results, in the presence of HgCl₂, being adjusted according!~~ summarised in Figure 5.2 and Appendix 10.

In the presence of HgCl₂, the level of biofilm formation by *Achromobacter* sp. AO22 at 0.05 mM, is similar to that in the absence of HgCl₂; whereas at higher HgCl₂ concentrations ~~it decreases~~ to between 60-80% of the control. The biofilm formation for both *A. woluwensis* E9 and VUN 10010 is maintained above approximately 80% up to 0.1 mM HgCl₂. For *P. aeruginosa* the biofilm level increases initially and then decreases, compared to the results obtained in the absence of HgCl₂.

5.4 Biofilm formation in the presence of lead nitrate

The biofilm-producing potential of the isolates was tested within a range of 0.1-2 mM Pb(NO₃)₂ in microtitre plates. The results are shown in Figure 5.3 and Appendix 10.

All three isolates had similar biofilm formation patterns in the presence of Pb(NO₃)₂ when compared to *P. aeruginosa* in that beyond 3 mM, the biofilm levels are generally below 50% of those observed in the absence of Pb(NO₃)₂. Between the concentrations of 0.1 and 1 mM, the levels of biofilm formation are higher for *A. woluwensis* E9 and VUN 10010 and lower for *P. aeruginosa* compared to the levels seen in the absence of heavy metal result. For *Achromobacter* sp. AO22, once the Pb(NO₃)₂ concentration increased to 2 mM, a significant decrease in biofilm formation was observed.

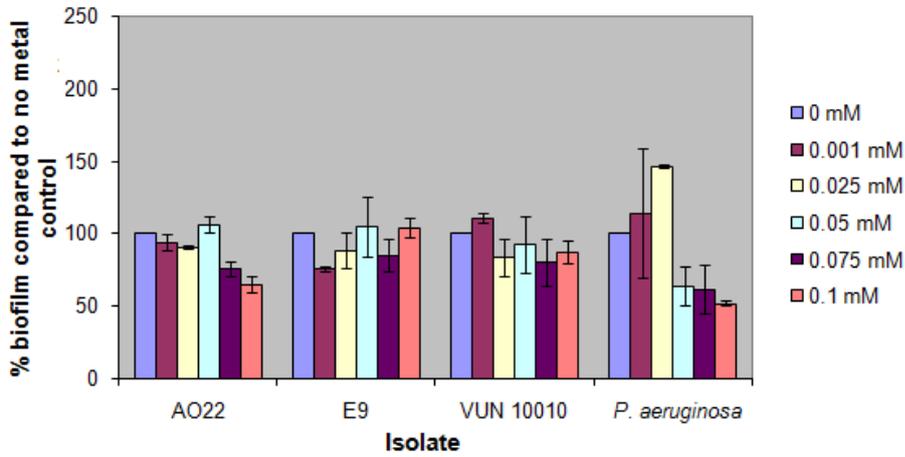


Figure 5.2 Biofilm formation in the presence of mercuric chloride. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT containing various levels of HgCl_2 and grown in microtitre plates for 48 hours at 30°C .

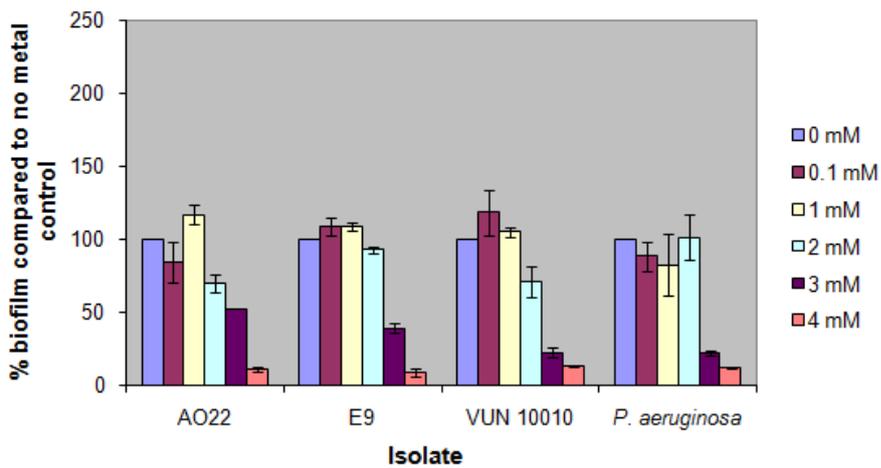


Figure 5.3 Biofilm formation in the presence of lead nitrate. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT containing various levels of $\text{Pb}(\text{NO}_3)_2$ and grown in microtitre plates for 48 hours at 30°C . Experiments were performed as outlined in Section 2.3.8.

5.5 Biofilm formation in the presence of zinc nitrate

The biofilm-producing potential of the isolates was tested within a range of 0.1-2 mM $Zn(NO_3)_2$ (Figure 5.4 and Appendix 10).

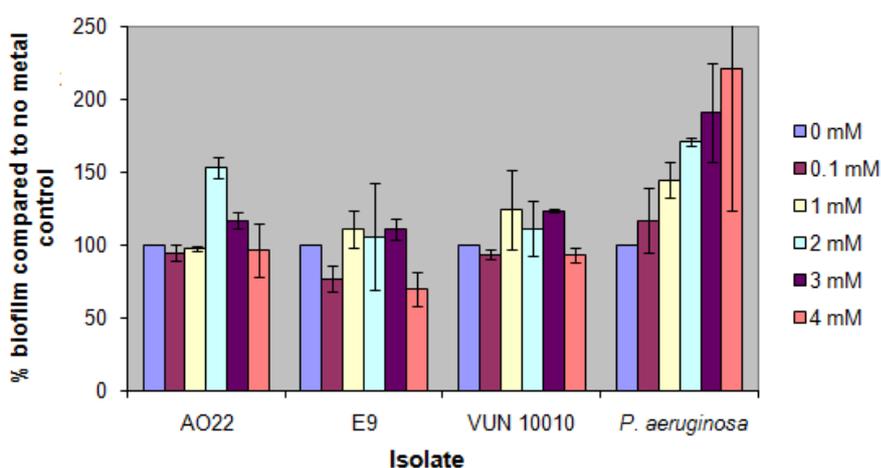


Figure 5.4 Biofilm formation in the presence of zinc nitrate. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT containing various levels of $Zn(NO_3)_2$ and grown in microtitre plates for 48 hours at 30°C.

The addition of $Zn(NO_3)_2$ did not significantly affect biofilm formation for *Achromobacter* sp. AO22 at concentrations of up to 1 mM, biofilm formation increased at 2 mM and 3 mM and then decreased around the same levels observed at 0.1 mM. A similar pattern was observed with *A. woluwensis* E9 with an increase between 1 and 3 mM, followed by a decrease at 4 mM. Biomass formation by VUN 10010 was not significantly affected in the presence of $Zn(NO_3)_2$ concentrations up to 4 mM. For *P. aeruginosa*, with each increase in $Zn(NO_3)_2$ concentration, an increase in biofilm formation was observed.

5.6 Biofilm formation in the presence of cadmium chloride

The biofilm-producing potential of the isolates was tested within a range of 0.1-2 mM CdCl₂ within microtitre plates. The results are shown in Figure 5.5 and Appendix 10.

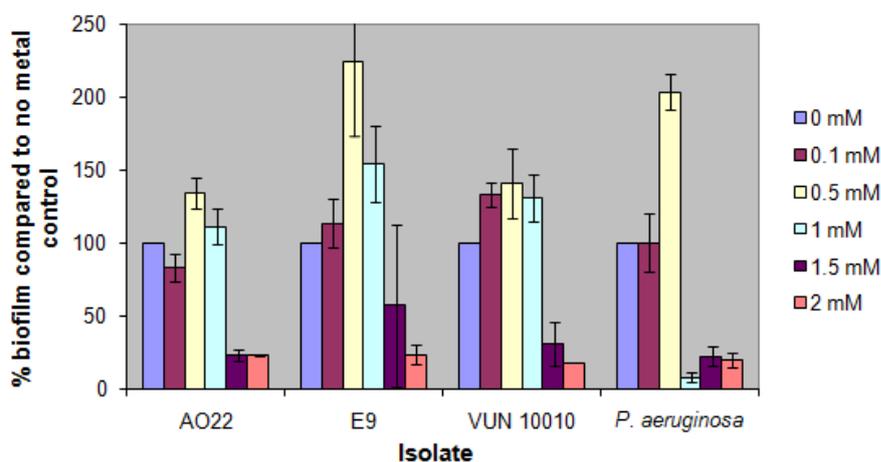


Figure 5.5 Biofilm production in the presence of cadmium chloride. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT containing various levels of HgCl₂ and grown in microtitre plates for 48 hours at 30°C.

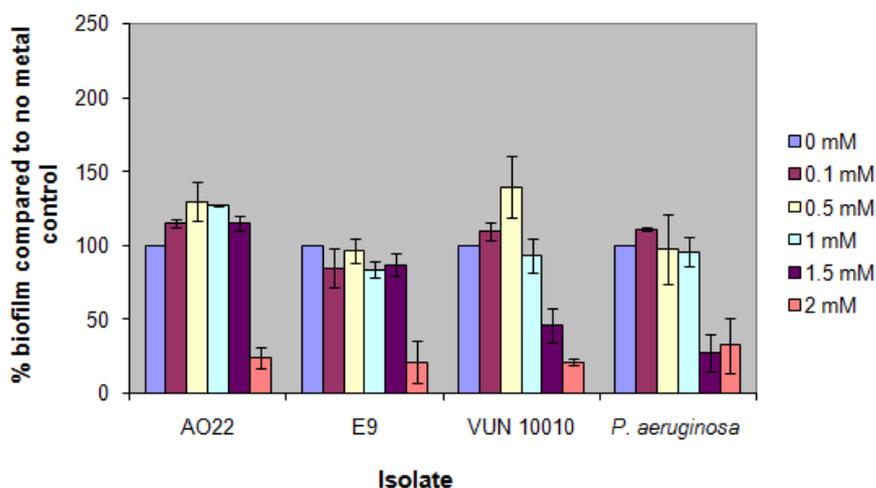
The addition of CdCl₂ resulted in a decrease in biofilm mass at 0.1 mM, when compared to the negative control, in the biofilm formation for *Achromobacter* sp. AO22, however an increase was detected up to 1 mM CdCl₂, which was followed by a marked decrease at 1.5 mM CdCl₂ and beyond. For *A. woluwensis* E9, an increase in biofilm formation was detected up to 1 mM, followed by a significant decrease at 1.5 mM. VUN 10010 showed an increase in biofilm formation relative to the negative control at up to 1 mM and at 1.5 mM a rapid decrease was observed. For *P. aeruginosa*, the level of biofilm

formation seen was equivalent to that of the negative control at 0.1 mM, while an increase was observed at 0.5 mM. At 1 mM and beyond, a rapid decrease was detected.

5.7 Biofilm formation in the presence of cobalt nitrate

The biofilm-producing potential of the isolates was tested within a range of 0.1-2 mM $\text{Co}(\text{NO}_3)_2$ within microtitre plates. The results are shown in Figure 5.6 and Appendix 10.

In the presence of $\text{Co}(\text{NO}_3)_2$ the level of biofilm formed for *Achromobacter* sp. AO22 is higher relative the negative control to a concentration of 1.5 mM, while it decreases at a concentration of 2 mM. For *A. woluwensis* E9, the levels of biofilm formation were similar to the negative control, up to 1.5 mM and decreases at 2 mM. For VUN 10010, the level of biofilm formed is above that of the negative control up to 0.5 mM, beyond which it decreases with each increase in $\text{Co}(\text{NO}_3)_2$ concentration. The level of biofilm formed by *P. aeruginosa* remains similar to the negative control up to 1 mM, beyond which a decrease is observed.



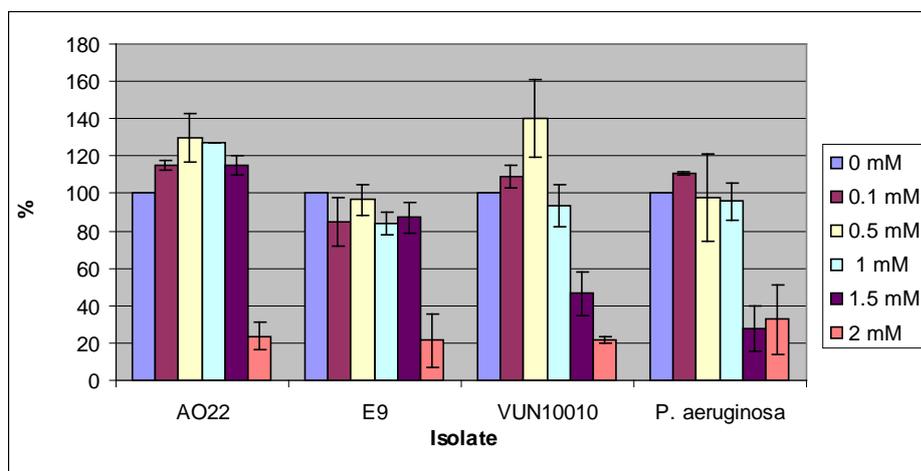


Figure 5.6 Biofilm formation in the presence of cobalt nitrate. Experiments were performed as described in Section 2.3.7. Cells were inoculated into NBT containing various levels of $\text{Co}(\text{NO}_3)_2$ and grown in microtitre plates for 48 hours at 30°C .

5.8 Discussion

The focus of the work presented in this chapter was to perform a preliminary study on whether *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 are capable of forming biofilms in the presence of various heavy metals. The results for these isolates were compared to *P. aeruginosa*, which has previously been reported to form biofilms (Langley and Beveridge, 1999); negative controls (no inoculum) were also included. All three soil isolates were able to form biofilms in the absence of heavy metals at levels comparable, if not higher, than those for *P. aeruginosa*. Comparing the results presented in this chapter to the MIC results obtained in Chapter 3, some variations between planktonic (liquid broth) and biofilm levels of heavy metal resistance are notable.

In the presence of HgCl_2 , growth was seen in liquid broth up to 0.125 mM, 0.125 mM and 0.175 mM for *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 respectively. Although biofilm growth was only tested to a concentration of 0.1 mM HgCl_2 , microbial growth was sustained up to this concentration, with biofilm levels starting to decrease (down to 65% compared to in the absence of HgCl_2 for *Achromobacter* sp. AO22) at 0.1 mM. In the presence of $\text{Pb}(\text{NO}_3)_2$, the three isolates

were able to grow in concentrations up to 6 mM in liquid broth. In the biofilm experiments, a rapid decrease in the number of biofilm-forming cells was observed between 3-4 mM for all three isolates, suggesting that such $\text{Pb}(\text{NO}_3)_2$ concentrations were inhibiting biofilm formation even though these isolates have MICs above these concentrations.

In the presence of $\text{Zn}(\text{NO}_3)_2$, all three isolates were able to grow in concentrations up to 5.5 mM in liquid broth. Although the biofilm experiments only tested biofilm formation up to a concentration of 4 mM, biofilm formation levels was sustained up to this concentration. In the presence of CdCl_2 , heavy metal tolerance MICs in liquid broth cultures were 1 mM, 1.5 mM and 0 mM for *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 respectively. Similar resistance levels were observed in the biofilm experiments, whereby a decrease in biofilm production was observed for *Achromobacter* sp. AO22 at 1.5 mM and for *A. woluwensis* at 2 mM. VUN 10010 was unable to grow at all in the presence of CdCl_2 , however it was able to produce biofilms up to 1.5 mM CdCl_2 . In the presence of $\text{Co}(\text{NO}_3)_2$, the three isolates were able to grow in concentrations up to 3.5 mM in liquid broth. In the biofilm experiments, the isolates appeared less tolerant with levels of biofilm formation decreasing at 2 mM for all three isolates.

Identical levels of heavy metal resistance between the liquid broth cultures and the biofilm cultures was not necessarily expected, given the difference in experimental such as culture size and different parent cultures. Also, the MIC results measure the ability of the cells to survive exposure to various heavy metal concentrations, whereas the biofilm experiments tested the ability of the cells to produce biofilms in the presence of heavy metals. Increased EPS production may account for cases where biofilm levels appear to be enhanced by the addition of heavy metals (in particular in the presence of $\text{Zn}(\text{NO}_3)_2$ and CdCl_2) when compared to the negative control. Previous studies have shown that metal exposure leads to an increase in EPS production in organisms such as the algae chlorophyte *Chlorococcum* sp. and the cyanobacterium *Phormidium* sp. (García-Meza *et al.*, 2005) and *Pseudomonas syringae* pv. *Syringae* (Kidambi *et al.*, 2005). To determine if this is also the case with the isolates in this study, further investigation would be required. The results of the MICs and biofilms combined

however do indicate that all three isolates have relatively high heavy metal resistance and are capable of forming biofilms in the presence of heavy metals.

Previous to this study there has been some evidence of *Achromobacter*, *Arthrobacter*, *Stenotrophomonas* and *Mycobacterium* species being capable of forming biofilms. *Achromobacter* species have been found associated with continuous-flow fixed-bed reactors used for the denitrification of drinking water (Mergaert *et al.*, 2001) and dental unit waterlines (Pankhurst *et al.*, 1998). *Achromobacter* has also been associated with mixed culture biofilms, with *Achromobacter* sp. being identified from dental unit waterlines along with *Sphingomonas paucimobilis*, *Xanthomonas maltophilia* and *Bacillus* sp. (Meiller *et al.*, 1999) and *A. xylosoxidans* has been associated with biofilms in sink drains which also contained *Aeromonas* sp., *Pseudomonas* sp., *Stenotrophomonas* sp. and *Alcaligenes* sp. (McBain *et al.*, 2003). Schabereiter-Gurtner *et al.*, (2001), investigated the correlation between bacterial colonization and rosy discolouration of masonry and lime wall paintings of historical buildings in Austria and Germany. *Arthrobacter* was found to be amongst the genera within these populations. *Stenotrophomonas maltophilia* was found to be capable of forming biofilms on a polystyrene surface within 2 hours of incubation (Di Bonaventura *et al.*, 2004); this species was also suggested to be involved in haemagglutinin biofilm formation and adherence to cultured mammalian cells (De Oliveira-Garcia *et al.*, 2003). *Mycobacterium* species that have been identified in biofilms include *Mycobacterium xenopi*, found to colonise in experimental drinking water (Dailoux *et al.*, 2003), *Mycobacterium fortuitum* and *Mycobacterium marinum* (Bardouniotis *et al.*, 2003).

As discussed in Chapter 3, Consortium VUN 10010 was isolated as a mixed culture, comprising *S. maltophilia* and *Mycobacterium* sp. Both *Stenotrophomonas* and *Mycobacterium* have been reported to be involved in biofilm formation within bacterial communities. Stoodley *et al* (1999) investigated the mixed species biofilm, containing *S. maltophilia*, *P. aeruginosa* and *Pseudomonas fluorescens*, grown in glass flow cells under laminar or turbulent flow. *S. maltophilia* has also been investigated with a number

of other cultures for its ability to grow as a binary culture biofilm with *Listeria monocytogenes*. Other isolates investigated along with *S. maltophilia* included *Bacillus* sp., *P. fluorescens*, *Kocuria varians*, *Staphylococcus capitis* and *Commons testosteroni*. Most of these isolates had been obtained from food processing plants after cleaning and disinfection (Carpentier and Chassaing, 2004). A number of bacteria were isolated from copper plumbing biofilms, including *S. maltophilia*, *Acidovorax delafieldii*, *Flavobacterium* sp., *Corynebacterium* sp. and *Pseudomonas* sp. (Critchley *et al.*, 2003). An extensive study was undertaken wherein *S. maltophilia* and *Fusarium oxysporum* were used as the first or second coloniser on PVC and glass surfaces. This study concluded that there was no distinction observed with using either strain as the first coloniser and the surface used had no impact (Elvers *et al.*, 2001).

The current work has established that the three soil isolates are capable of forming biofilms in the presence of heavy metals. It may now be possible to consider these isolates for further, larger scale analysis for biosorption applications. A factor in determining whether these isolates should be further investigated for bioremediation and/or biosorption applications, is how the results obtained in this study compare to examples in the literature. Okino *et al.*, (2000) reported that *P. putida* PpY101/pSR134 can grow in HgCl_2 concentrations up to 100 mg/L (0.36 mM) and can reduce the amount of HgCl_2 in the medium to between 92-98% within 24 hours. Heavy metal resistant bacteria were isolated in 10 mg/L HgCl_2 (0.036 mM). Of these, *P. aeruginosa* was able to grow in 75 mg/L HgCl_2 (0.27 mM), 100 mg/L CdCl_2 (0.45 mM), and 100 mg/L $(\text{CH}_3\text{COO})_2\text{Pb}$ (0.3 mM), while *A. xylooxidans* was able to grow at 50 mg/L HgCl_2 (0.18 mM), 100 mg/L CdCl_2 (0.45 mM) and 100 mg/ml $(\text{CH}_3\text{COO})_2\text{Pb}$ (0.3 mM). HgCl_2 removal from the medium by these isolates occurred up to 8 mg/L (0.03 mM), while in medium containing 100 mg/L Cd, the concentration dropped as low as 17.4 mg/L in 72 hours and in medium containing $(\text{CH}_3\text{COO})_2\text{Pb}$ at 100 mg/L the levels reached as low as 1.8 mg/L in 96h (Jayasanker *et al.*, 2007). A consortium was isolated from lake sediment and found at concentrations of 100 mg/L ZnSO_4 (0.34 mM) and PbSO_4 (0.32 mM), metal removal could be achieved to between 95-100%. *Ralstonia* sp. HM-1 was isolated from this consortium, and was able to achieve 97-100% removal of each metal at 200 mg/L (0.69 mM ZnSO_4 , 0.65 mM PbSO_4) (Lee *et al.*, 2008). Green-Ruiz (2006) demonstrated the ability of non-viable (by autoclaving) *Bacillus* sp.

to remove HgCl₂ from medium. At concentrations of between 0.25 mg/L HgCl₂ (0.75 uM) and 10 mg/L (0.03 mM) biosorption rates ranged between 91.9-68.1%.

When comparing isolates from the current study to those described above, the levels of resistance and biofilm capabilities are comparable in most instances. It is recommended the isolates from this project be considered for further study for biosorption and bioremediation experiments. To determine whether the heavy metal ions are entering the cells of our isolates, a number of methods may be utilised. These may include atomic absorption spectrophotometry (AA) (Chen and Wilson, 1997; Chen *et al.*, 1998; Seki *et al.*, 1998; Sousa *et al.*, 1998; Langley and Beveridge, 1999; El-Helow *et al.*, 2000; Pan-Hou *et al.*, 2001; Spriang *et al.*, 2002; Huang *et al.*, 2003) or inductively coupled plasma-mass spectrophotometry (ICP-MS) (Wang *et al.*, 1998; Webb *et al.*, 1998; Langley and Beveridge, 1999; Pagnanelli *et al.*, 2000; Valls *et al.*, 2000) whereby metal concentrations can be determined in cells and supernatants. Scanning electron microscopy (SEM) may be used as a means to observe the formation of metal carbonates within cells (Diels *et al.*, 1995; White and Gadd, 2000) as may uptake experiments such as those using ¹⁰⁹Cd with liquid scintillation analysis (Pazirandeh *et al.*, 1995; Byrnes Brower *et al.*, 1997). Biofilms may be utilised in bioreactors for the removal of heavy metals in a number ways as summarised by Singh *et al.*, 2006. Such applications include anaerobic-anoxic-oxic (A2O) biofilm process, moving bed sand filters, on granular activated carbon and in composite membrane reactors.

When considering whether *Achromobacter* sp. AO22, *A. woluwensis* E9 and VUN 10010 may be suitable for bioremediation or biosorption applications, further preliminary work would need to be conducted to determine growth characteristics on a number of different types of medium in a laboratory setting (i.e. glass, polystyrene, PVC) or a field setting (i.e. sand, soil). A further understanding would also need to be gained as to how these isolates behave in a mixed culture setting and under different growth conditions (i.e. nutrient availability, temperatures, presence of mixed contaminants). The determination of such characteristics would allow the design of further experiments for these isolates to determine their suitability for use in different types of processes and applications such as hollow fiber reactors (Chen *et al.*, 1998), packed bed bioreactors (Wagner-Dobler *et al.*, 2000), filter membranes (Diels *et al.*, 1995) and sand filters (Diels *et al.*, 2003).

Chapter 6

Preliminary investigations into the presence of genes relating to lead and cadmium resistance in the soil isolates *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 and genes relating to PAH degradation in the soil isolate Consortium VUN 10010

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6.1 Introduction

As described in Chapter 1, mercury resistance genes have been reported in a number of bacterial species. Chapter 4 outlines characteristics of these genes in the soil bacterial isolates, *Achromobacter* sp. AO22, *Arthrobacter woluwensis* E9 and Consortium VUN 10010, isolated from lead and PAH-contaminated soils. As described in Chapter 3, these isolates were confirmed to have resistances to the heavy metals lead, cadmium, cobalt and zinc, as previously reported by Trajanovska *et al* (1997) for *Achromobacter* sp. AO22 and *A. woluwensis* E9. This chapter will focus on determination of the presence of lead resistance genes related to the *pbr* operon and cadmium resistance genes related to the *cad* operon.

In brief, one authentic lead resistance operon (*pbr*) has been characterised to date, in the strain *Cupriavidus metallidurans* CH34, on the pMOL30 plasmid (Borremans *et al.*, 2001). The operon comprises of six genes, including a *merR* homologue (*pbrR*) which regulates the expression of the structural genes. The *pbrA* encodes an ATPase, *pbrT* encodes a Pb(II) uptake protein and *pbrB* encodes a predicted integral protein of unknown function. A prolipoprotein signal is encoded by *pbrC*, while *pbrD* encodes a Pb(II) sequestration protein (Borremans *et al.*, 2001). The operon has been detailed in Section 1.5.

A number of species have been reported to possess genes encoding resistance to cadmium, generally referred to as the *cad* genes. Of these, *cadC* encodes a *trans*-acting repressor protein, *cadA* encodes a soft metal ion-translocating ATPase (Silver *et al.*, 1989; Lutsenko and Kaplan, 1995; Rensing *et al.*, 1999), *cadB* has been reported to encode a putative cadmium binding protein and *cadD* confers low levels of cadmium resistance (Chaouni *et al.*, 1996; Crupper *et al.*, 1999; Lee *et al.*, 2001). The *cad* genes of *S. aureus* pI258 have been discussed Section 1.6.1.

The isolates *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 are of interest to investigate their lead and cadmium resistance genes as they have already demonstrated levels of resistance to compounds containing these heavy metals. The attempts to identify the genes responsible for lead and cadmium resistance in these isolates is summarised below.

As described in Chapter 1, the isolate Consortium VUN 10010, which was originally isolated from a PAH contaminated soil sample, was initially classified as *Stenotrophomonas maltophilia*. (Boonchan, PhD thesis, 1998; Boonchan *et al.*, 1998). However, during the course of this project Consortium VUN 10010 was identified as a mixed culture, containing *S. maltophilia* and a *Mycobacterium* sp. (C. Dandie, formerly from School of Biological Sciences, Flinders University, South Australia, personal communication) (as outlined in Chapter 3). Attempts to purify the two species proved unsuccessful and work was thus continued using Consortium VUN 10010 as a mixed culture. This isolate has been previously reported to possess PAH degradation abilities (Boonchan *et al.*, 1998) and this chapter focuses on attempts to elucidate the genetic mechanisms related to these capabilities.

Many bacterial species are reported to have operons encoding degradation pathways for low molecular weight PAHs such as naphthalene and phenanthrene and one or often even two operons have been identified as responsible for these. For example, *Pseudomonas stutzeri* AN10 possesses two operons, *nahA-F* facilitating the biotransformation of naphthalene to salicylate and *nahG-W* the biotransformation of salicylate to pyruvate (Bosch *et al.*, 1999a; b). Many other species such as *Ralstonia*, *Comamonas* and *Burkholderia* utilise similar pathways (Fuenmayor *et al.*, 1998; Zhou *et al.*, 2001; Moser and Stahl, 2001; Laurie and Lloyd-Jones, 1999). However, unlike the genes involved in mercury or cadmium resistance, the annotation of these genes is not kept constant between species; for example, the genes may be referred to as *nah*, *phn* and *nag* (Bosch *et al.*, 1999a; b; Fuenmayor *et al.*, 1998; Zhou *et al.*, 2001; Laurie and Lloyd-Jones, 1999). Also the actual gene denotations between operons may not be referring to the same protein product. Section 1.7 provides further details of these genes and the mechanisms of PAH degradation.

6.2 PCR detection of lead resistance genes in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010.

Primers were designed based on various genes of the *pbr* operon of the pMOL30 plasmid of *C. metallidurans* CH34 (Borremans *et al.*, 2001), as described in Section 2.8.1.2. Six primer pairs, targeting the potential *pbrT*, *pbrR* and *pbrA-related* genes, were used for the three test isolates. Table 6.1 summarises the primer pairs, the expected fragment sizes based on the *pbr* operon of pMOL30 and the results obtained for each test isolate.

All reactions were initially set up using 1.5 mM MgCl₂. *Achromobacter* sp. AO22 showed no fragments amplified using primer pairs pbr8-9, pbr10-11 and pbr12-13, all designed to target the *pbrT*, or with pbr14-15, spanning *pbrT* and *pbrA*. Larger than expected fragments of > 1000 bp and approximately 950 bp were amplified using pbr16-17, designed to span the *pbrR* and *pbrA* genes, instead of the expected size of 766 bp. The pbr18-19 pair, designed to target *pbrA* with an expected size of 769 bp, produced a fragment of approximately this size.

Figure 6.3 and Table 6.1 outline the results obtained for the *pbr* primers at 1.5 mM MgCl₂, from the genomic DNA of Consortium VUN 10010. No fragments were amplified using primer pairs *pbr*8-9, *pbr*10-11, *pbr*12-13 and *pbr*18-19. Primer pairs *pbr*14-15 and *pbr*16-17 both amplified single fragments, although neither of these were close to expected size.

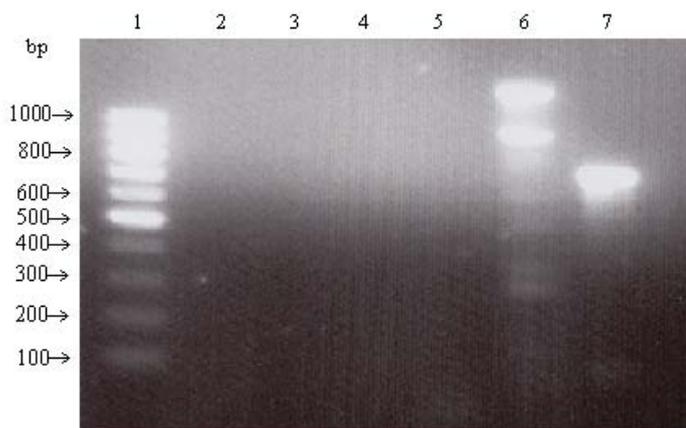


Figure 6.1 PCR amplification of *pbr* genes using *pbr* specific primers with genomic DNA of *Achromobacter* sp. AO22. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. *pbr*8-9; 3. *pbr*10-11; 4. *pbr*12-13; 5. *pbr*14-15; 6. *pbr*16-17; 7. *pbr*18-19.

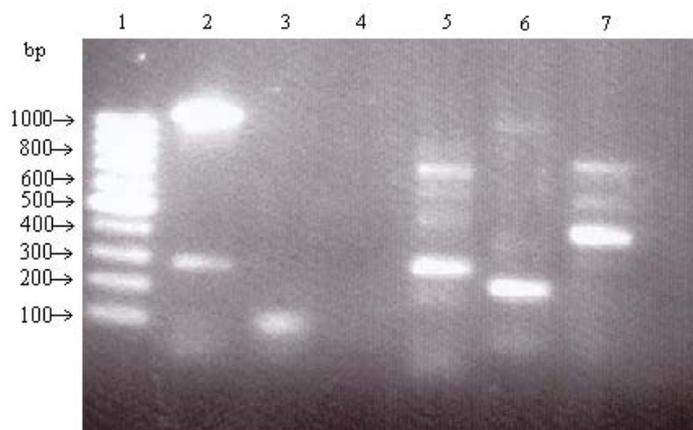


Figure 6.2 PCR amplification of *pbr* genes using *pbr* specific primers with genomic DNA of *A. woluwensis*. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. pbr8-9; 3. pbr10-11; 4. pbr12-13; 5. pbr14-15; 6. pbr16-17; 7. pbr18-19.

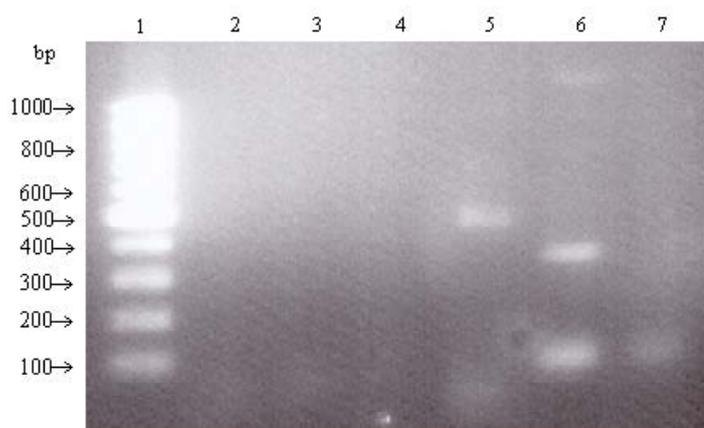


Figure 6.3 PCR amplification of *pbr* genes using *pbr* specific primers with genomic DNA of Consortium VUN 10010. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. pbr8-9; 3. pbr10-11; 4. pbr12-13; 5. pbr14-15; 6. pbr16-17; 7. pbr18-19.

Table 6.1 Results obtained from the PCR analysis of the genomic DNA from *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN10010 using *pbr* specific primers

Primer pair and gene targeted	Expected fragment size (bp)	MgCl ₂ Conc.	Approx. fragment size obtained (bp)		
			AO22	E9	10010
Pbr8-9 <i>pbrT</i>	593	1.5	NA	300, >1000	NA
		2	NA	>1000	NA
		2.5	NA	300, >1000	NA
Pbr10-11 <i>pbrT</i>	740	1.5	NA	NA	NA
		2	NA	NA	250, >1000
		2.5	NA	NA	250
Pbr12-13 <i>pbrT</i>	807	1.5	NA	NA	NA
		2	NA	NA	NA

		2.5	NA	NA	NA
Pbr14-15	593	1.5	NA	300, 700	500
<i>pbrTR</i>		2	550	300	200, 450
		2.5	NA	300	200, 300, 450
Pbr16-17	766	1.5	950, >1000	200	380
<i>pbrRA</i>		2	300, 950	200	NA
		2.5	300, 350, 950, >1000	200	350
Pbr18-19	769	1.5	770	400, 750	NA
<i>pbrA</i>		2	770	400	NA
		2.5	770	400	NA

NA: no amplification observed

All reactions using pbr primer pairs were repeated with increased concentrations of MgCl₂ (2 mM and 2.5 mM). From the gDNA of *Achromobacter* sp. AO22 (Table 7.1 and Figure 7.4), no fragments were amplified using primer pairs pbr8-9, pbr10-11 and pbr12-13, while primer pair 14-15 produced a fragment at 2 mM MgCl₂, which was about 50 bp smaller than expected and no product at 2.5 mM MgCl₂. Using the increased concentration of MgCl₂ with primer pair pbr16-17, two fragments of 300 and 950 bp were amplified at 2 mM MgCl₂, while four fragments of 300, 350, 950 and >1000 bp were amplified at 2.5 mM MgCl₂, none of which were close to the expected size of 766 bp. The primer pair pbr18-19 led to amplification of a single fragment of expected size (769 bp) at both 2 mM and 2.5 mM MgCl₂ as with 1.5 mM.

Table 6.1 and Figure 6.5 outline the results obtained using increased MgCl₂ concentration for the gDNA of *A. woluwensis* E9. No products were obtained using primer pairs pbr10-11 and pbr12-13, at these concentrations. The results using pbr8-9 at both these concentrations were similar to those at 1.5 mM MgCl₂, with fragments of >1000 being amplified at 2 mM MgCl₂ and 300 and >1000 bp at 2.5 mM MgCl₂, none of which are close to the expected size of 593 bp. Primer pair pbr14-15 yielded fragments which were smaller than the expected 593 bp. Primer pair pbr16-17 amplified a fragment of 200 bp at all concentrations, much smaller than the expected 766 bp. Using the increased concentrations of 2 mM and 2.5 mM MgCl₂, only the 400 bp fragment was produced.

Figure 6.6 and Table 6.1 outlines the results obtained using increased $MgCl_2$ concentration and the *pbr* primers from the genomic DNA of Consortium VUN 10010. No fragments were amplified using an increased $MgCl_2$ concentration with primer pairs *pbr*8-9, *pbr*12-13 and *pbr*18-19. Using primer pair *pbr*10-11, at 2 mM and 2.5 mM, fragments of 250 and >1000 bp and 250 bp were seen respectively. These fragments were not close to the expected size of 740 bp. Using primer pair *pbr*14-15 fragments of 200 and 450 bp being seen at 2 mM, while fragments of 450, 300 and 200 bp were seen at 2.5 mM, with all fragments amplified being smaller than the expected size of 593 bp. Primer pair *pbr*16-17 produced a fragment of 350 bp was seen at a 2.5 mM $MgCl_2$, while no fragment was amplified using 2 mM $MgCl_2$.

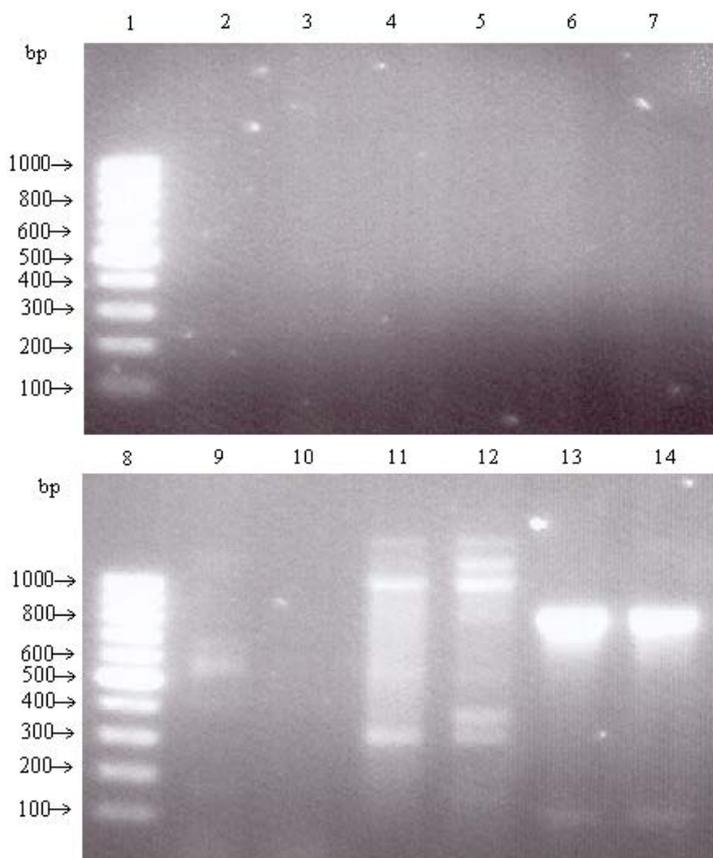


Figure 6.4 PCR amplification of *pbr* genes using *pbr* specific primers and increased concentrations of $MgCl_2$ with genomic DNA of *Achromobacter* sp. AO22. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run

using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. pbr8-9 (2 mM); 3. pbr8-9 (2.5 mM); 4. pbr10-11 (2 mM); 5. pbr10-11 (2.5 mM); 6. pbr12-13 (2 mM); 7. pbr12-13 (2.5 mM); 8. GeneRuler™ 100 bp marker; 9. pbr14-15 (2 mM); 10. pbr14-15 (2.5 mM); 11. pbr16-17 (2 mM); 12. pbr16-17 (2.5 mM); 13. pbr18-19 (2 mM); 14. pbr18-19 (2.5 mM).

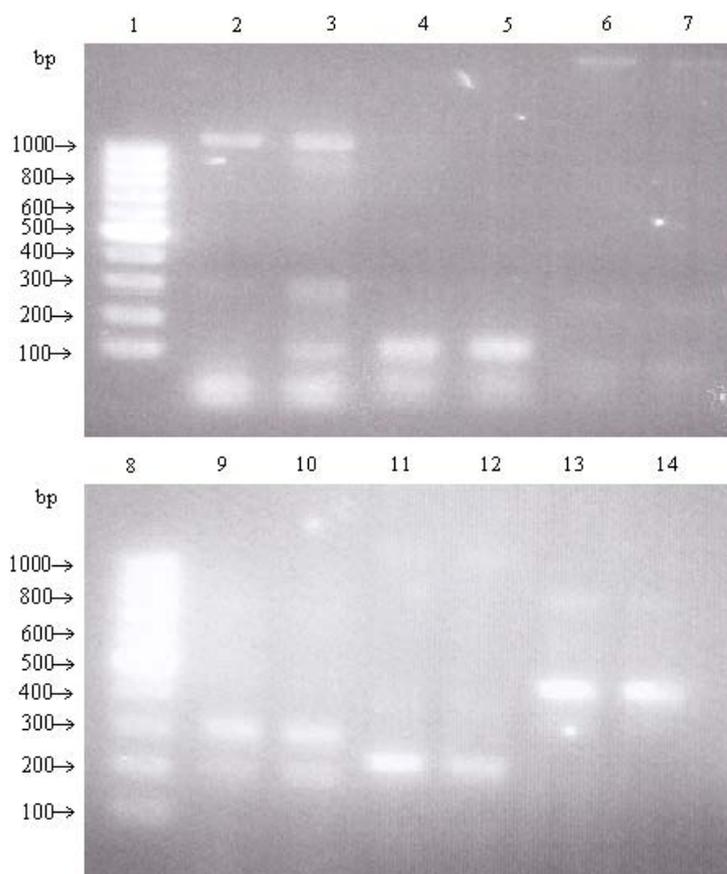


Figure 6.5 PCR amplification of *pbr* genes using *pbr* specific primers and increased concentrations of MgCl₂ with genomic DNA of *A. woluwensis* E9. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. pbr8-9 (2 mM); 3. pbr8-9 (2.5 mM); 4. pbr10-11 (2 mM); 5. pbr10-11 (2.5 mM); 6. pbr12-13 (2 mM); 7. pbr12-13 (2.5 mM); 8. GeneRuler™ 100 bp marker; 9. pbr14-15 (2 mM); 10. pbr14-15 (2.5 mM); 11. pbr16-17 (2 mM); 12. pbr16-17 (2.5 mM); 13. pbr18-19 (2 mM); 14. pbr18-19 (2.5 mM).

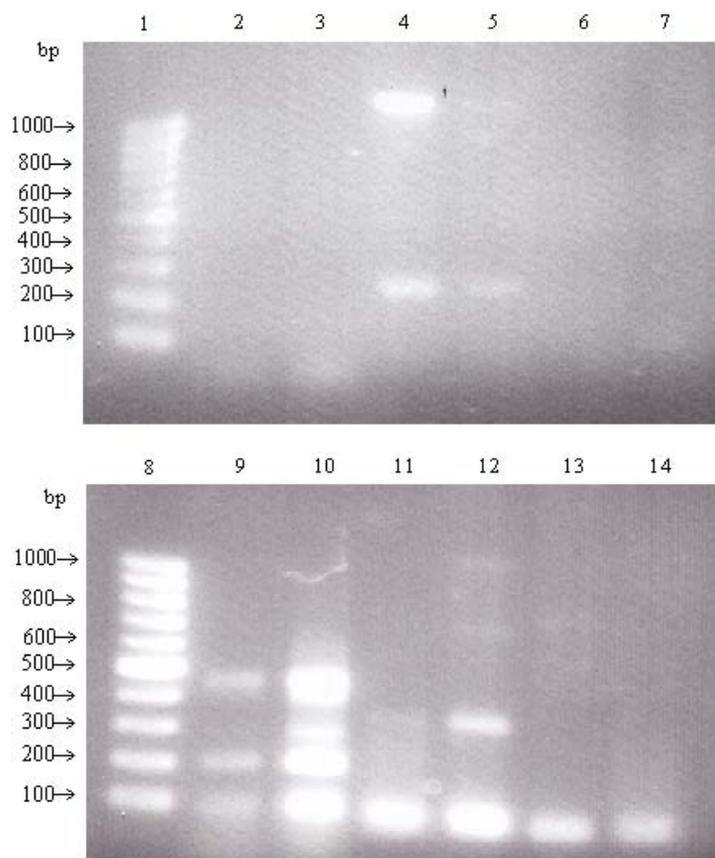


Figure 6.6 PCR amplification of *pbr* genes using *pbr* specific primers and increased concentrations of $MgCl_2$ with genomic DNA of Consortium VUN 10010. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. pbr8-9 (2 mM); 3. pbr8-9 (2.5 mM); 4. pbr10-11 (2 mM); 5. pbr10-11 (2.5 mM); 6. pbr12-13 (2 mM); 7. pbr12-13 (2.5 mM); 8. GeneRuler™ 100 bp marker; 9. pbr14-15 (2 mM); 10. pbr14-15 (2.5 mM); 11. pbr16-17 (2 mM); 12. pbr16-17 (2.5 mM); 13; pbr18-19 (2 mM); 14. pbr18-19 (2.5 mM).

6.3 Sequence analysis of a fragment obtained using *pbr* specific primers

Of the fragments obtained from the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010, using the *pbr* primers, only one reaction produced a single fragment, of expected size, which was reproducible using 1.5 mM, 2 mM and 2.5 mM MgCl₂, this being from *Achromobacter* sp. AO22 with the *pbr*18-19 primer pair. This fragment was cloned into pGEM-T Easy and sequenced (Sections 2.10 and 2.11). Two of these clones were used for sequencing and sequence data of 1203 bp and 1198 bp were obtained. The PCR fragment cloned was approximately 770 bp. The sequences obtained were analysed for the sequences relating to those corresponding to primers *pbr*18 and *pbr*19. Within the sequence 1203 bp, the *pbr*19 sequence was found between positions 823 and 841, while from the sequence of 1198 bp the *pbr*19 sequence was found between positions 826 and 843. For each of these alignments with *pbr*19, there was 1 bp difference in both the sequences obtained, that being a cytosine in the place of a guanine. The sequence that was closest to that of primer *pbr*18 was located between positions 110 and 127 and between 112 and 129 for the 1203 bp and 1198 bp sequence data respectively, however there was a 7 bp difference between the *pbr*18 primer and each of the sequences obtained. Based on the alignments of the sequences for *pbr*18 and *pbr*19 as described above, the edited sequence data is a 732 bp sequence, which is 37 bp shorter than the expected fragment size and the size estimated by agarose gel electrophoresis of the PCR fragment. The two sets of sequence data obtained were aligned with the sequence from *C. metallidurans* CH34 that corresponds to the *pbr*18-19 primer pair. No significant similarities were seen, indicating that the fragment amplified was not from a *pbr*-related sequence. A BlastN search was performed on the sequence data obtained to determine if there were any similarities to known sequences in the ANGIS database. Of the results were obtained, the greatest length of sequence similarity that was detected was to a maximum of 22 bp, in the positions between 3-23, 25-45 and 699-720 in the sequence data. The matches that were detected include *Homo sapiens* chromosome 8, clone RP11-379I19 (AC087664) and the 16S rRNA gene sequence of *Zobellella* sp. (EU14338), *Rheinheimera* sp. (AM888197), *Francisella* sp. (CP000608), Bacterium VFR5-3 (EU037278), Bacterium QLW23 (AY937393) and Marine sediment bacterium (AY93634). There were no other similarities to any known sequence for the remainder of the data obtained.

6.4 PCR detection of cadmium resistance genes in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010.

Primers were designed based on the *cad* genes of *S. aureus* pI258, encoding cadmium resistance, as described in Section 2.8.1.3. Three primer pairs were utilised, the first *cad1-2* targeting both the *cadC* and *cadA* genes, with an expected size of 625 bp, while the other two, *cad3-4* and *cad5-6*, each targeted different sections of the *cadA* gene, with expected sizes of 1066 and 1289 bp respectively. No fragments were amplified using the *cad* primers at a $MgCl_2$ concentration of 1.5 mM for any test DNA (agarose gel not shown) Further reactions were set up using increased $MgCl_2$ concentrations of 2 mM and 2.5 mM, but, no amplification was evident using these (agarose gel not shown).

6.5 PCR detection of PAH degradation-encoding genes in Consortium VUN 10010

Primers were designed based on a number of PAH degrading operons, as described in Section 2.8.1.4, Table 2.2 and Figures 2.7-2.12. It is important to note that it was determined during the course of this project that Consortium VUN 10010 was a mixed culture, which included *S. maltophilia* and *Mycobacterium* sp. Therefore, DNA was prepared using a Gram-positive bacteria extraction technique, which can also be applied to Gram-negative bacteria in an attempt to isolate DNA from all cells present in the culture. PCRs were set up using the above primers n for primer pairs at $MgCl_2$ concentrations of 1.5 mM, 2 mM and 2.5 mM.

Using primer pair *pahAa1-2*, at $MgCl_2$ concentrations of 1.5 mM, 2 mM and 2.5 mM $MgCl_2$, no amplification was seen, as was the case using primer pairs *pahAc1-2*, *pahAd1-2*, *pahC1-2* (data not shown) and *pahE1-2* using three different concentrations of $MgCl_2$. Primer pair *pahF1-2* produced a fragment of approximately 750 bp at all three concentrations of $MgCl_2$. This fragment was however much larger than the expected size of 433 bp. These results are shown in Figure 6.7.

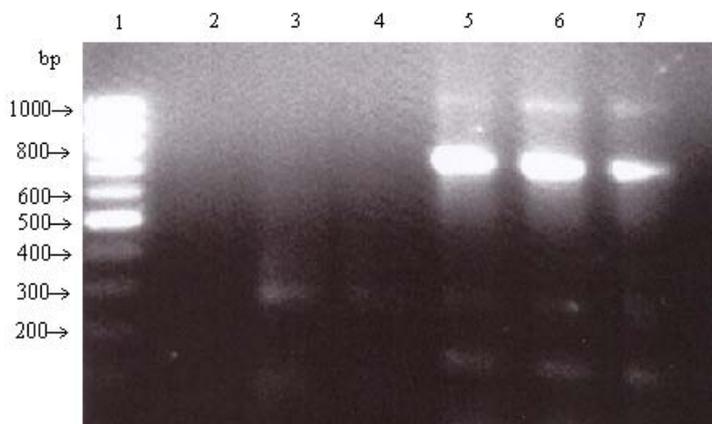


Figure 6.7 PCR amplification of *pah* genes using *pah* specific primers with genomic DNA of Consortium VUN 10010. PCR conditions were as described in Section 2.8 using *Taq* DNA polymerase and run using the PTC-100 or PTC-200 Thermal Cycler (Perkin Elmer). PCRs were visualized on a 1% Agarose gel. Lanes represent the following: 1. GeneRuler™ 100 bp marker; 2. *pahE1-2*, 1.5 mM; 3. *pahE1-2*, 2 mM; 4. *pahE1-2*, 2.5 mM; 5. *pahF1-2*, 1.5 mM; 6. *pahF1-2*, 2 mM; 7. *pahF1-2*, 2.5 mM.

6.6 Sequence analysis of fragments obtained using PAH specific primers

Of the six primer pairs that were designed to target some of the genes related to PAH degradation, only one amplified a fragment using PCR, producing a fragment of approximately 750 bp instead of the expected 433 bp. The fragment was cloned into pGEM T-Easy and sequenced to determine if there was any sequence similarities to any known PAH degradation genes. While the sequence of primer *pahF* was found within the sequence data, with 100% homology, the remainder of the data did not show any sequence similarities to any known PAH degradation genes, including those against which the *pahF1-F2* primer was designed. Some matches to other known sequences were determined using the BlastN program on the ANGIS database, however the longest stretch of sequence similarity determined was only 24 bp (data not shown). Based on these results and the fact that the PCR fragment being investigated was significantly larger than the expected size, it was decided not to pursue this fragment further.

6.7 Discussion

For the determination of the lead resistance (*pbr*) genes, only one published authentic lead resistance operon sequence exists, which has been characterised from *C. metallidurans* CH34, with a number of genes being identified. This genetic system was used to design the primers to target three of the six genes identified in the *pbr* operon, the *pbrR* gene, encoding a regulatory protein, the *pbrA* gene, encoding an ATPase and the *pbrT*, encoding a Pb(II) uptake protein. Aside from this being the only published *pbr* operon to date, the pMOL30 plasmid also contains the *mer* operon. As we know our isolates also contain *mer* operons, there is a chance that they may contain the *pbr* operon, whether on a shared operon or as a result of horizontal transfer. As two of our isolates (*Achromobacter* sp. AO22 and *A. woluwensis* E9) were initially isolated from soil identified as being contaminated with lead and all isolates demonstrated lead resistances (Chapter 3), it was expected that these isolates would contain some genetic mechanism for lead resistance.

Six different primer pairs were utilised in order provide overlapping fragments from any potential related genes in the test isolates. Very little success was achieved using these primer pairs with the genomic DNA of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010. A fragment of expected size was only obtained for *Achromobacter* sp. AO22 using primer pair pbr18-19, but sequencing of this fragment indicated that it was not related to the any sequence of the *pbr* operon of *C. metallidurans* CH34, nor any other known gene sequence. Fragments were also produced from *Achromobacter* sp. AO22 using primer pair pbr14-15 and pbr16-17, however these were either significantly larger or smaller than the expected size. Using the genomic DNA of *A. woluwensis* E9, no fragments were amplified using primer pairs pbr10-11 or pbr12-13 using a range of MgCl₂ concentrations. Using primer pairs pbr8-9, pbr14-15, pbr16-17 and pbr18-19 and a range of MgCl₂ concentrations, some fragments were amplified however, they were either significantly larger or smaller than the expected sizes. The genomic DNA of VUN 10010 amplified, with primer pairs pbr8-9, pbr12-13 and pbr18-19, and a range of MgCl₂ concentrations also did not result in any amplification. Primer pairs pbr10-11, pbr14-15 and pbr16-17 resulted in some amplifications, however, these were also either significantly larger or smaller than the expected sizes, based on the *pbr* operon of *C. metallidurans* CH34.

The identification of a lead resistance genetic system from our isolates proved difficult using the conventional method of designing primers based on one identified sequence. As was mentioned in Section 1.5, a number of bacteria have been isolated previously based on their lead resistant phenotypes. These include *Staphylococcus aureus* (Levinson *et al.*, 1996), *Citrobacter freundii* (Levinson and Mahler, 1998), *Pseudomonas marginalis* and *B. megaterium* (Roane, 1999), however, no genetic systems relating to lead resistance have been identified to date from these organisms. Chapter 3 established the ability of the three isolates to grow up to 6 mM lead nitrate. This would suggest that these bacteria possess some form of lead resistance genetic system, although this may not be related to the *pbr* operon of *C. metallidurans* CH34. Identification of a new genetic system using alternative approaches such as the use of degenerate primers designed from the amino acid sequence of the *pbr* proteins (McPherson *et al.*, 1995). Techniques using RNA expression to compare cells grown in the presence of lead, such as differential display (Liang *et al.*, 1997) and cDNA libraries (Dale and Park, 2004) could also be explored. This was not pursued further for these isolates as the work focused heavily on the *mer* operon based on the results obtained.

As with the *pbr* primers, *cad* primers were designed based only on one published sequence, the *cadC* and *cadA* genes of pI258 from *S. aureus*. The same logic was applied as with the *pbr* primers in that pI258 also contains *mer* genes, as does our isolates and the resistances to cadmium were shown in Chapter 3. When using the three sets of primer pairs, no amplification was achieved at a range of MgCl₂ concentrations from any of the three isolates. This was not pursued further due to the focus on the *mer* operon throughout this thesis. A comparison of the *cad* genes from other isolates to those from *S. aureus* pI258 was performed and revealed a varying level of identity. For example, the DNA identity of the pI258 *cadC* gene is 84.28%, 55.00%, 57.27% and 57.30% to these genes in *B. firmus* (BACTNPRAB), *B. stearothersophilus* (AF098974), *L. lactis* (LLU78967) and *L. monocytogenes* (LISCADTNP) respectively, and that of pI258 *cadA* is 45.81%, 76.94%, 57.67%, 65.86% and 66.35% to these genes in *P. putida* (AF333961), for *B. firmus* (BACTNPRAB), *B. stearothersophilus* (AF098974), *L. lactis* (LLU78967) and *L. monocytogenes* (LISCADTNP) respectively. If these genes were to be pursued further, degenerate primers would firstly be designed to include the above sequences. If using these primers was not successful, alternative

methods such as differential display and cDNA libraries as mentioned above may also be considered.

Another system, encoding resistance to cadmium, along with zinc and cobalt is the *czc* operon, has been characterised in *C. metallidurans* CH34 (Nies *et al.*, 1989; Nies, 1992b; van der Lelie *et al.*, 1997) and *Alcaligenes* sp. (Kunito *et al.*, 1996). PCRs were previously attempted using primers designed based on the *czc* operon of *C. metallidurans* CH34 and showed what (Trajanovska *et al.*, 1998). Using *Achromobacter* sp. AO22 and *A. woluwensis* E9 DNA, the present results showed a lack of uniform amplification between. These results suggested that the sequences may be present, although possibly highly diverged, or the fragments obtained may be a result of non-specific binding of primers. Compared to *cad* genes, the *czc* genes of what *C. metallidurans* CH34 (X98451) and *Alcaligenes* sp. (AFACZCCBAD) share greater similarities with identities of 99.78% (*czcA*), 99.8% (*czcB*), 99.36% (*czcC*) and 99.79% (*czcD*). The genes *czcI*, *czcN*, *czcR* and *czcS* have to date only been published for *C. metallidurans* CH34

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As mentioned in Section 6.3, the 750 bp fragment amplified using primer pair pahF1-F2 was sequenced, however a terminated sequence was obtained. This was repeated adding 1 µL DMSO, which may act as a DNA denaturant, however this did not result in obtaining the full sequence. Based on the results obtained from the partial sequence and the larger than expected size of the PCR fragment, it was decided not to pursue this PCR fragment further. The above information illustrates the difficulties faced with the determination of PAH degradation genes from Consortium VUN 10010. From the lack of amplification achieved, it can be strongly suggested that Consortium VUN 10010 does not possess any genes similar to the *nahAa*-like genes of *P. stutzeri*, *Ralstonia* sp. U2 or *P. aeruginosa*, to the *nahAc*-like and *nahAd*-like genes of *P. stutzeri*, *Ralstonia* sp. U2, *P. aeruginosa*, *C. testosteroni* or *P. fluorescens*. Previous studies, as outlined in section 1.9.2, have determined that VUN 10010 is capable of a number of PAH degradation activities, which would suggest the presence of some genetic system responsible for this. There are a greater number of published genes to potentially design further primers from, however, considering the diversity present (Section 1.7.2). This (these) organism(s) may have entirely new genetic system(s). There was also the added disadvantage in that Consortium VUN 10010 is not a pure culture and all attempts to

purify the *S. maltophilia* and *Mycobacterium* sp. were unsuccessful. However previous observations (Chapter 3) have suggested that *S. maltophilia* appears to be the dominant organism in a number of cultures observed. It may have also been possible that there was a higher proportion of DNA from *S. maltophilia* in the DNA preparations, whereby the PAH degradation genes may have been located in the DNA of the *Mycobacterium* sp. Many questions remain regarding the genetic mechanisms for PAH degradation. However, by the inclusion of Consortium VUN 10010 in this study, it is clear that this isolate is resistant to a number of heavy metals (Chapter 3), possesses sequences relating to mercury resistance (Chapter 4) and is capable of forming biofilms in the presence of heavy metals (presented in Chapter 6). This information gained provides a basis for the possible consideration of Consortium VUN 10010 for bioremediation applications.

Had the isolation of the *cad* and *pah* genes been pursued further in this project, a more structured approach to experimental design would have been taken to determine if these genes were present in the isolates. The experiments discussed above did not include a positive control, which would have indicated whether the primer pairs used were suitable for isolation of these genes. $MgCl_2$ concentration adjustments were made in the experiments performed; however annealing temperatures were not amended in an attempt to achieve amplification. Another potential issue is the presence of DNA-binding protein, which may still be present on the sample if DNA purity is was not adequate. In this case, the primer target might be occupied by such protein, requiring the initial denaturation of the PCR to be at a higher temperature for a given period of time to allow primers access to their binding site.

Chapter 7

General Conclusions and Future Directions

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7.1 Introduction

The aim of this project was to characterise, at a physiological and molecular level, soil isolates previously determined to be resistant to heavy metals and capable of PAH degradation. In studies prior to the current investigation, it had been determined that *Achromobacter* sp. AO22 and *Arthrobacter* sp. E9 were resistant to mercuric chloride, lead nitrate, zinc nitrate, cadmium chloride and cobalt nitrate. The information regarding the molecular basis of their resistance was however limited, with PCR experiments indicating the presence of genes associated with mercury and copper resistance. The third isolate included in this study Consortium VUN 10010, was previously reported to degrade pyrene as a sole carbon source and, in the presence of pyrene, it was also able to mineralise benzo[*a*]pyrene. When used as a co-culture with *Penicillium janthinellum* VUO 10201, there was a significant degradation of and microbial growth on pyrene, chrysene, benz[*a*]anthracene and dibenz[*a,h*]anthracene. The results obtained from this project improves our understanding of the heavy metal resistance mechanisms possessed by *Achromobacter* sp. AO22 and *Arthrobacter* sp. E9 and characterises the heavy metal resistance of Consortium VUN 10010. It was also found that the three isolates were capable of forming biofilms in the presence of a range of heavy metals. The mercury resistance (*mer*) operon of all three isolates was determined, although attempts to determine the lead (*pbr*) and cadmium (*cad*) resistances operons were not successful. Investigations were conducted to determine the presence of genes responsible for PAH degradation, however this was not successful. The growth of *Achromobacter* sp. AO22 and *Arthrobacter* E9 was investigated in the presence of mercuric chloride and gene expression of the *mer* genes was investigated for *Achromobacter* sp. AO22. *Achromobacter* sp. AO22 and *Arthrobacter* sp. E9 were also characterised to the species level. The information gained from this project can be used to increase our understanding in heavy metal tolerance and the potential of all three isolates in bioremediation applications.

7.2 General conclusions

7.2.1 Further characterisation of *Alcaligenes* sp. AO22 and *Arthrobacter* sp. E9 to the species level

The use of 16S rRNA gene primers and phylogenetic tree analysis further characterised *Alcaligenes* sp. AO22 to *Achromobacter xylosoxidans* (99.9%). 16S analysis revealed that *Arthrobacter* sp. E9 was most closely resembled *Arthrobacter woluwensis* (99.9%). These isolates were subsequently referred to as *Achromobacter* sp. AO22 and *A. woluwensis* E9.

7.2.2 Determination of *Stenotrophomonas maltophilia* VUN 10010 being a mixed culture

Work performed in this project and by researchers at Flinders University, Adelaide determined that the previously reported *S. maltophilia* VUN 10010 was a mixed culture, which also contained a *Mycobacterium* sp. most closely related to the *Mycobacterium tuberculosis* subgroup. Although efforts to purify these strains were unrewarded, experimental work continued with these isolates which were subsequently referred to as Consortium VUN 10010.

7.2.3 Confirmation of the heavy metal resistances of *Achromobacter* sp. AO22 and *A. woluwensis* E9

Previous work on these isolates used a single method for the determination of the heavy metal MICs of *Achromobacter* sp. AO22 and *A. woluwensis* E9. The current project included the use of liquid medium, solid medium and microtitre plates. Both isolates were found to have heavy metal resistances comparable to previous studies and when higher concentrations of heavy metals were tested, a higher level of resistance was observed. *Achromobacter* sp. AO22 was found to be resistant to heavy metal concentrations up to 0.15 mM mercuric chloride, 4 mM lead nitrate, 5.5 mM zinc nitrate, 2 mM cadmium chloride and 3 mM cobalt nitrate. *A. woluwensis* E9 was found to be resistant to heavy metal concentrations up to 0.125 mM mercuric chloride, 6 mM

lead nitrate, 4 mM zinc nitrate, 2 mM cadmium chloride and 3 mM cobalt nitrate. Most of these heavy metal tolerances were higher than previously reported.

7.2.4 Characterisation of the heavy metal resistances of VUN 10010

Although Consortium VUN 10010 was isolated based on its PAH degrading capabilities, it was investigated in this study for heavy metal resistance. It was found that Consortium VUN 10010 was resistant to heavy metal concentrations up to 0.175 mM mercuric chloride, 4 mM lead nitrate, 5.5 mM zinc nitrate, and 2.5 mM cobalt nitrate using the liquid broth method. VUN 10010 was found to be susceptible to cadmium chloride using this method, however when tested in a microtitre plate was found to be resistant up to 1.5 mM.

7.2.5 Determination of organomercurial resistance in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

An organism is considered to be organomercurial resistant if it can tolerate concentrations of 0.003 mM or higher. Consortium VUN 10010 was able to grow above this concentration in the presence of phenylmercury acetate, although it could not grow in the presence of methylmercury chloride. Neither *Achromobacter* sp. AO22 nor *A. woluwensis* E9 were able to grow in the presence of either methylmercury chloride or phenylmercury acetate.

7.2.6 Determination of the formation of biofilms in the presence of heavy metals by *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

The ability of the three isolates to form biofilms in the absence of heavy metals was initially compared to that of *P. aeruginosa*. All three isolates were able to form biofilm biomass levels higher than that formed by *P. aeruginosa*, with Consortium VUN 10010 forming the most biofilm biomass, followed by *A. woluwensis* E9, then *Achromobacter* sp. AO22. All three isolates were able to form biofilms in the presence of up to 0.1 mM mercuric chloride with slight decreases being observed as heavy metal concentrations increased. All three isolates were able to form biofilms in the presence of up to 4 mM lead nitrate, although significant decreases in the amount of biofilm formed were

observed in lead nitrate concentrations of 3 mM and 4 mM. All three isolates were capable of forming biofilms in the presence of zinc nitrate up to a concentration of 4 mM, with no significant decreases in biofilm biomass observed up to this concentration. All three isolates were also able to form biofilms in the presence of up to 2 mM cadmium chloride and cobalt nitrate. Significant decreases in biofilm formation were observed at both 1.5 mM and 2 mM cadmium chloride and at 2 mM cobalt nitrate.

7.2.7 Determination of the presence of the *mer* operon in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

1. The *merR* gene was present in all three isolates and is transcribed divergently to the remainder of the *mer* genes. The *merR* genes from all three isolates showed 100% homology to the *merR* gene of *P. aeruginosa* Tn501.
2. The O/P region of all three isolates was located between the *merR* and *merT* genes. These sequences showed 100% homology to the O/P region of the *mer* operon of Tn501, including the two separate promoters controlling the expression of the *merR* gene and the *merTPAD* genes separately.
3. The *merT* gene was present in all 3 isolates between the O/P region and the *merP* gene. This gene was found to have 100% homology in *A. woluwensis* E9 and Consortium VUN 10010 when compared to the *merT* gene of Tn501. There was one base pair difference between the *merT* gene of *Achromobacter* sp. AO22 and Tn501. This change led to an amino acid difference between the MerT proteins of *Achromobacter* sp. AO22 and Tn501, which was located in the second of three potential transmembrane regions within the Tn501 protein.
4. The *merP* gene was present in all 3 isolates between the *merT* and *merA* genes. This gene was found to have 100% homology in *A. woluwensis* E9 and Consortium VUN 10010 when compared to the *merP* gene of Tn501. There was one base pair difference between the *merP* gene of *Achromobacter* sp. AO22 and Tn501. This change led to an amino acid difference between the MerP proteins of *Achromobacter* sp. AO22 and Tn501, which was located within both the HMA

(heavy-metal-associated) domain and periplasmic component relating to the Tn501 MerP protein.

5. Approximately 80% of the *merA* gene of *Achromobacter* sp. AO22 and Consortium VUN 10010 was amplified and sequenced when compared to the full length *merA* of Tn501. The *merA* gene of both these isolates was located after the *merP* gene. From the sequence obtained, one base pair difference was determined from *Achromobacter* sp. AO22 and VUN 10010 when compared to Tn501, which did not result in an amino acid change and was not located in the regions corresponding to the HMA domain, the FAD binding site or the disulphide bond described in the Tn501 MerA. The remainder of the *merA* gene has been subsequently sequenced by another researcher showing >99% identity of the full *merA* gene to that of Tn501.
6. Approximately 22% of the *merA* gene was amplified and sequenced from *A. woluwensis* E9 compared to the full-length *merA* of Tn501, and it was located after the *merP* gene. No base pair differences in the sequence obtained were noted when compared to Tn501. From the sequence obtained from *A. woluwensis* E9, it was possible to determine the presence of the HMA domain relating to that of Tn501, while no sequence could be obtained identifying whether the FAD binding site and disulphide bond was present.
7. A sequence was obtained internally to the *merB* gene relating to *S. marcescens* pDU1358. It was only possible to use internal primers based on the inability to amplify and sequence the 3' of the *merA* gene and the *merD* gene in all three isolates. While sequence relating to the *merB* gene was obtained, the location of this gene was not determined with respect to the *merR*, *merT*, *merP* and *merA* genes of all three isolates. Of the sequence that was obtained relating to *merB* from *Achromobacter* sp. AO22, two base pair differences and a base pair deletion were detected, with only the deletion resulting in an amino acid change when compared to the sequence of pDU1358. The same two base pair changes were detected in both *A. woluwensis* E9 and Consortium VUN 10010, which did not result in an amino acid change; an additional base pair change was detected in Consortium VUN 10010, which resulted in an amino acid change when compared to the MerB protein of pDU1358.

8. The results of experiments using primers internal to the *merC* gene of *S. flexneri* Tn21 and those located within the *merP* and *merA* genes, suggested an absence of a *merC* gene in all three isolates.

7.2.8 Growth profile of *Achromobacter* sp. AO22 in the presence of mercuric chloride

Growth curves were performed in the presence of 0.075 mM mercuric chloride and compared to control cultures containing no mercuric chloride. Cells grown in the presence of mercuric chloride had a lag period of approximately 2 hours, followed by exponential growth to the level of the control culture by the 9 hour time point, indicating a rapid adaptation of *Achromobacter* sp. AO22 to the presence of mercuric chloride.

7.2.9 Growth profile of *A. woluwensis* E9 in the presence of mercuric chloride

A. woluwensis E9 was grown in a mercuric chloride concentration of 0.03 mM and its growth was compared to control cultures containing no mercuric chloride. This experiment indicated there was no adaptation of the *A. woluwensis* E9 cells to this concentration of mercuric chloride within 24 hours. In a further experiment, cells were grown at concentrations of 0.01 mM, 0.02 mM, 0.03 mM, 0.04 mM and 0.05 mM mercuric chloride and the cultures monitored for 96 hours. The cells grown in the presence of 0.01 mM took up to 48 hours to reach stationary phase, while those grown in 0.02 mM, 0.03 mM and 0.04 mM took up to 72 hours. Cells grown in the presence of 0.05 mM took up to 96 hours to reach stationary phase. These results show that while *A. woluwensis* E9 has tolerance to mercuric chloride, it takes a significantly longer period of time to adapt to the presence of this compound in its growth medium compared to *Achromobacter* sp. AO22.

7.2.10 *mer* gene expression in *Achromobacter* sp. AO22

RT-PCR was performed on RNA extracts of *Achromobacter* sp. to investigate the expression of the *merR*, *merT*, *merP* and *merA* genes. Using 16S rRNA gene-specific

primers to test the individual samples by RT-PCR, expression was found to be constant across the samples, indicating that the RNA extraction method was suitable for this experiment. The following expression patterns for each of these genes were determined as outlined below.

1. Using RT-PCR, the *merR* transcript was detected in *Achromobacter* sp. AO22 grown in the presence of 0.075 mM mercuric chloride following 30 minutes exposure and was detected in incubations up to six hours, although the level of transcript decreased over time, and no transcript was detected at the nine hour time point. The *merR* transcript was detected in the control cells at the one hour time point and expression was observed for up to six hours, although as with the stress-induced cells the level of transcript decreased over time and no transcript was observed at the nine hour time point. There appeared to be more transcript present in the induced samples compared to the control samples at each time point.
2. Using RT-PCR, the expression of the *mer* transport genes, *merT* and *merP*, was detected over the entire course of the nine hour experiment in both the stress-induced cells and the control cells. It was noted that at each time point, the level of expression appeared significantly higher in the induced cells, compared to the control cells, suggesting that while there may be some constitutive expression of these genes, there is an upregulation when in the presence of mercuric chloride.
3. Expression of the *merA* gene was only detected in *Achromobacter* sp. induced by 0.075 mM mercuric chloride. Expression was at its highest at the 30 minute time point and continued for two hours, with expression decreasing at each time point. When compared to the *merR*, *merT* and *merP* transcripts, the level of *merA* transcript produced was significantly lower.

7.2.11 Determination of the presence of the *pbr* operon in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

Using PCR primer design based on the sequence of the *pbr* operon of pMOL30 of *C. metallidurans* CH34, only one fragment was amplified, being from *Achromobacter* sp. AO22. Sequencing of this fragment did not show any similarities to the *pbr* operon. No

fragments were amplified from either *A. woluwensis* E9 or Consortium VUN 10010, suggesting there were no sequences similar to the *pbr* operon of *C. metallidurans* in any of the isolates.

7.2.12 Determination of the presence of the *cad* operon in *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010

Using PCR primer design based on the *cad* operon of *S. aureus* pI258, no amplification occurred, suggesting that there were no sequences similar to this *cad* operon in any of the three isolates.

7.2.13 Determination of the presence of genes relating to PAH degradation in Consortium VUN 10010

Using PCR primer design based on the sequence of a number of PAH degradation genes, only one fragment was amplified. Sequencing of this fragment did not show any similarities to any published PAH degradation operon. This result indicates that there were no sequences present in Consortium VUN 10010 that related to the sequences used for primer design. It must be noted however, that not all published PAH degradation operons were used for primer design and a high level of sequence variability exists between PAH degradation.

7.3 Overall conclusion

Although not all of the aims of this project were met, some important results were obtained. The initial heavy metal resistances of the isolates *Achromobacter* sp. AO22 and *A. woluwensis* E9 were confirmed using a number of different methods and these isolates were identified to the species level. It was found that Consortium VUN 10010, which was initially included in this study due to its PAH degradation capabilities, was also resistant to a number of heavy metals at levels comparable to *Achromobacter* sp. AO22 and *A. woluwensis* E9. A detailed analysis indicated that all three isolates possess the *mer* genes most likely responsible for their mercury resistances, however the genetic mechanisms underpinning their lead, cadmium, cobalt and zinc tolerances remains to be determined, as does the characterisation of PAH degradation genes from

Consortium VUN 10010. The expression of the *mer* genes in *Achromobacter* sp. AO22 was investigated, suggesting that this isolate possesses a functional *mer* operon. It was also found that all three isolates are capable of forming biofilms in the presence of a range of heavy metals. The results presented in this thesis suggest that these isolates should be studied further to determine their suitability in bioremediation applications. These isolates are indigenous to the soil, which would eliminate concerns of introducing recombinant organisms into the environment. Some potential further research with these organisms is discussed in Section 9.3.

7.4 Future directions

While this thesis presents some valuable information on the characterisation of *Achromobacter* sp. AO22, *A. woluwensis* E9 and Consortium VUN 10010 at a physiological and molecular level, these results raise a number of questions about these bacteria that need pursuing in further studies. Some of these further investigations are described below.

7.4.1 Further investigations of these isolates at a physiological level

The work described in this thesis determined the MICs of these isolates and their abilities to form biofilms in the presence of a number of heavy metals. Only *Achromobacter* sp. AO22 and *A. woluwensis* E9 however were investigated further (in culture) in the presence of mercuric chloride. Further investigations should include the analysis of all three isolates in the presence of the entire range of heavy metals tested for MICs and biofilm formation. This may also be extended to organomercurials, of which only two types were examined for MIC. The range of organomercurials tested should be extended to include more varieties and be examined for MIC, biofilms and growth curve analysis. The investigation into the formation of biofilms was only performed in microtitre plates, which can be limiting. Further investigations may include a number of different surfaces and larger scale cultures. Biofilms may also be investigated as mixed cultures and in mixed contaminants, which would be more reflective of a natural setting. Another area of investigation would involve analysis of the degradation of these heavy metals both in culture and soil samples. This would further assess these isolates potential for use in remedial applications.

7.4.2 Further investigations of these isolates on a molecular level

While this thesis presented the characterisation of most of the *mer* operon, the work performed failed to determine the location between the *merR* and *merT* genes. Other approaches that may be considered for the determination of the location of this gene may include “primer walking” whereby the sequence already determined may be used in a PCR to isolate the flanking regions. For the genes that remain undetermined in these isolates, differential display experiments may also be utilised whereby RNA from induced and control cells are random primed to find unique or up-/down-regulated transcripts. Transcripts of interest are then sequenced and compared to known sequences or analysed based on their features and potential protein structures. cDNA libraries may also be utilised for the identification of genes associated with heavy metal resistance, also examining expressed genes in the presence of contaminants. Another alternative for gene identification may be the development of whole genome microarrays, which may be used for hybridisation experiments with cell transcripts produced during exposure to the contaminants of interest. With very little published data available for primer design for the *pbr* and *cad* operons, further methodologies such as those described above are required at this point to determine the genetic mechanisms responsible for these resistances in the isolates investigated in this thesis.

Similar approaches to those described above could be applied to the investigation of PAH degradation genes in Consortium VUN 10010. However, prior to employing such methods, it may be simpler to perform a more thorough approach to PCR based on sequences published for VUN 10010. Sequence variation between related genes for PAH degradation was determined to be very high and the primers used in this thesis were designed based on only a small number of sequences that showed areas of homology. Further analysis could be performed investigating the more unique sequences available.

In our isolates, only the *mer* genes were determined, which allowed using the methods chosen to only investigate these genes at the RNA level. *Achromobacter* sp. AO22 was chosen for further analysis due to its ability to rapidly adapt in the presence of mercuric chloride. Other experiments may be designed however in the future to deal with the

problems faced with *A. woluwensis* E9 adaptation to mercury chloride in the large scale culture and the existence of two species in Consortium VUN 10010. Such further investigations could not be pursued in the time frame of this investigation. Some data was obtained relating to the expression of the *mer* genes in *Achromobacter* sp. AO22, however there is a significant scope for further investigation, as what has been presented in this thesis may be considered only preliminary. A wider range of time points may be considered, such as earlier in the experiments where gene expression may be at its most variable. As RT-PCR is generally considered to be semi-quantitative, further analysis may be performed using Real-Time PCR, allowing the amplification to be monitored at each cycle. Using Real-Time PCR, it is also possible to obtain results as specific as number of transcript copies per cell. Microarrays may also be developed based on mRNA expression as a means to examine the expression of multiple

7.4.3 Further investigations of these isolates using practical applications

Chapter 1 outlined a number of practical applications for detection and bioremediation processes that may use resistance and/or degradation genes or whole bacteria to detect, reduce or absorb contaminants. Genes detected in these isolates may be used to construct biosensors (as described in section 1.8.2 and table 1.7) whereby the isolated genes may be combined with reporter genes such as *lux*, *luc*, *lacZ* and *gfp* to detect contaminants in environmental samples. The genes, or the organisms themselves, may also be further investigated for biosorption applications (as described in section 1.8.3 and table 1.8) through either reduction or accumulation of contaminants using systems such as hollow fiber reactors, packed bed bioreactors or aqueous solutions. Another use for these organisms that may be considered is reduction of contaminants by used of biofilms (as described in section 1.8.4 and table 1.9) whereby these isolates may be grown in biofilm on mediums such as filter membranes, sand filters and porous carrier materials. Such investigations however would only be considered after the further work described in sections 7.4.1 and 7.4.2 was complete.

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**APPENDIX 1
COMMERCIAL REAGENTS**

A. Commercial kits used in this investigation

Kit	Supplier	Application
Ampli <i>Taq</i> [®] DNA Polymerase	Perkin Elmer	PCR
BigDye [®] Terminator v3.1 Sequencing Kit	Applied Biosystems	DNA Sequencing
Expand Long Template PCR System	Roche	PCR
pGEM [®] -T Easy Vector System	Promega	Cloning of PCR products
Microspin [™] Columns	Amersham Pharmacia Biotech	Purification of PCR products
Platinum [®] <i>Taq</i> DNA Polymerase	Invitrogen	“Hot Start” PCR
SuperScript [™] II RNase H ⁻ Reverse Transcriptase	Invitrogen	Reverse-Transcriptase PCR
<i>Taq</i> DNA Polymerase	GibcoBRL Life Technologies	PCR
Wizard [®] SV Genomic DNA Purification System	Promega	Genomic DNA extraction
Wizard [®] <i>Plus</i> SV Miniprep DNA Purification System	Promega	Plasmid DNA extraction
100 mM dNTP set	Invitrogen Life Technologies	PCR

B. Commercial reagents used in this investigation

Reagent	Supplier	Application
10 mM dNTP mix	MBI Fermentas, Invitrogen	PCR
Gene Ruler™ 100 bp DNA Ladder	MBI Fermentas	Agarose gel electrophoresis
Gene Ruler™ 100 bp Plus DNA Ladder	MBI Fermentas	Agarose gel electrophoresis
Lambda (λ) <i>EcoR</i> I+ <i>Hind</i> III DNA marker	MBI Fermentas	Agarose gel electrophoresis
RNA marker, 0.28-6.58 kb	Promega	Agarose gel electrophoresis
Diethyl Pyrocarbonate (DEPC)	Aldrich	Inactivation of RNases
Random primers	Promega	cDNA synthesis
RNase Erase	ICN Biochemicals	Inactivation of RNases

C. Commercial enzymes used in this investigation

Enzyme	Supplier	Application
Ampli <i>Taq</i> ® DNA Polymerase	Perkin Elmer	PCR
Expand Long Template PCR System (3.5U/μL)	Roche	PCR
Platinum® <i>Taq</i> DNA Polymerase (5U/μL)	GibcoBRL Life Technologies	PCR
<i>Taq</i> DNA Polymerase (5U/μL)	GibcoBRL Life Technologies	PCR
Big Dye Reagent		
Restriction endonuclease, <i>EcoR</i> I (2.5U/μL)	MBI Fermentas	Restriction digestion
Restriction endonuclease, <i>Hind</i> III (10U/μL)	Promega.	Restriction digestion
T4 DNA ligase (3 Weiss units/μL)	Promega	Cloning of PCR fragments
DNaseI, RNase-free (10U/μL)	Roche	DNA removal
RNasin® Ribonuclease Inhibitor (20U/μL)	Promega	RNase protection
Superscript™ II RNase H ⁻ Reverse Transcriptase (200U/μL)	Invitrogen	cDNA synthesis

APPENDIX 2 CHEMICALS AND REAGENTS

A. Chemicals used in this thesis.

This table outlines all chemicals used as components for the preparation of all buffers and media used in this thesis. Supplier details of each chemical are also provided.

Chemical	Supplier
Agarose	Progen
<i>p</i> -aminobenzoic acid	Sigma
Ammonium acetate	BDH
Ammonium Oxalate	Sigma
Ammonium sulphate	M&B
Ampicillin	Boehringer
d-Biotin (Vitamin H)	ICN
Boric acid	APS
Cadmium chloride	Sigma
Calcium acetate	BDH
Calcium chloride	BDH
D-calcium pantothenate	Sigma
Chloroform	BDH
Cobalt (II) chloride	BDH
Cobalt nitrate	H & W
Copper chloride	BDH
Crystal Violet	Sigma
[^α - ³² P]dCTP	Perkin Elmer
Diethyl ether	BDH
Diethyl pyrocarbonate (DEPC)	Sigma
Ethanol	CSR
Ethidium bromide	Sigma
Ethylenediamine tetra-acetic acid (EDTA)	Ajax
Ferrous sulfate	APS
Folic acid (Pteroglutamic acid)	Sigma
Formaldehyde	Sigma
Formamide	Unilab
Glacial Acetic Acid	BDH
D-Glucose	BDH
Glycerol	BDH
8-Hydroxyquinoline	BDH
Iodine Crystals	BDH
Iron (II) sulfate (ferrous sulfate)	Ajax
Isoamyl alcohol	BDH
Isopropyl-β-D-thiogalactopyranoside (IPTG)	Promega
Lead nitrate	BDH
Lincomycin hydrochloride	Sigma
Magnesium chloride	Merck
Magnesium sulphate	BDH
Manganese chloride	BDH

Mercury chloride	Sigma
Methylmercury chloride	Sigma Aldrich
Neutral Red	BDH
Niacin (nicotinic acid)	Sigma
Nickel chloride	Sigma
Phenol	Wako
Phenylmercury acetate	Sigma Aldrich
Potassium dihydrogen orthophosphate	Ajax
<i>di</i> -Potassium hydrogen orthophosphate	Ajax
Potassium Iodide	BDH
Pyrene	Aldrich
Pyridoxal HCl	Sigma
Riboflavin (B ₂)	Sigma
Sodium acetate	Ajax
Sodium chloride	AnalaR
Sodium citrate	BDH
Sodium dihydrogen orthophosphate	BDH
<i>di</i> -Sodium hydrogen orthophosphate	Merck
Sodium hydroxide	Merck
Sodium lauryl sulphate (SDS)	Sigma
Sodium molybdate (NaMoO ₂ ·2H ₂ O)	Sigma
Sucrose	BDH
Thiamine hydrochloride	BDH
Tris(2-amino-2-hydroxymethyl-1,3-propanediol) (Tris)	Amresco
Vitamin B ₁₂	Sigma
5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal)	Progen
Zinc sulfate	BDH, Ajax

B. Enzymes

DNase-free RNase A was dissolved at a concentration of 10 mg/mL in 10 mM Tris-Cl (pH 7.5) and 15 mM NaCl. This was heated at 100°C for 15 minutes to denature any DNase present. The solution was divided into aliquots and stored at -20°C.

Lysozyme, used for the disruption of bacterial cell walls, was prepared by dissolving a powdered stock at a concentration of 10 mg/mL in 10 mM Tris-Cl (pH 8.0) immediately before use.

Proteinase K, used for the removal of proteins from DNA preparations, was prepared by dissolving a powdered stock at a concentration of 20 mg/mL in sterile 50 mM Tris (pH 8.0) and 1.5 mM calcium acetate. Stocks were stored at -20 °C.

C. Buffers

10X DNase buffer: 100 mM Tris-HCl (pH 7.5), 25 mM MgCl₂, 5 mM CaCl₂

DNase digestion buffer: 20 mM Tris-Cl (pH 8.0), 10mM MgCl₂.

Gram-negative lysing buffer: 10 mM Tris-Cl (pH 8.0), 10 mM NaCl, 1 mM Sodium Citrate, 1.5% SDS.

Lysis buffer: 30 mM Tris-Cl (pH 7.4), 100 mM NaCl, 5 mM EDTA, 1% SDS, 100 µg/mL Proteinase K.

Lysing solution: 50 mM Tris-Cl (pH 8.0), 0.7 M Sucrose.

Protoplasting buffer: 15 mM Tris-Cl (pH 8.0), 0.45 M Sucrose, 8 mM EDTA.

Solution I (large scale plasmid preps): 25 mM Tris-HCl (pH 7.5), 10 mM EDTA, 15% Sucrose, 2 mg/mL lysozyme.

50X TAE Buffer: 242 g Tris base, 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0) per 1000 mL dH₂O.

TE Buffer: 10 mM Tris pH 8.0, 1 mM EDTA pH 8.0

D. General stocks and solutions

50 mg/mL Ampicillin: 200 mg ampicillin was dissolved in 4 mL dH₂O, filter sterilized and stored at -20°C.

0.5 M Cadmium chloride: 4.582 g was dissolved in 50 mL dH₂O.

100 mM Calcium acetate: 15.8 g was dissolved in 100 mL dH₂O.

Chloroform:isoamyl alcohol: 10 mL Isoamyl alcohol was added to 240 mL Chloroform (24:1).

0.5 M Cobalt nitrate: 7.275 g was dissolved in 50 mL dH₂O.

10X DNA loading dye: 0.42% Bromophenol blue, 0.42% Xylene cyanol FF and 50% glycerol in dH₂O.

0.5 M EDTA: 186.1 g was dissolved in 800 mL dH₂O. The pH was adjusted to 8.0 with NaOH and the solution made up to 1000 mL with dH₂O.

Ethidium bromide stock solution (10 mg/mL): 0.1 g was dissolved in 10 mL dH₂O. The solution was stored in a dark bottle at 4°C.

20% IPTG: 2 g of Isopropyl-β-D-thiogalactopyranoside (IPTG) was dissolved in 10 mL dH₂O, filter sterilized and stored at 4°C.

0.5 M Lead nitrate: 8.28 g was dissolved in 50 mL dH₂O.

1M Magnesium chloride: 203.3 g was dissolved in 1000 mL dH₂O.

0.5M Mercuric chloride: 6.788 g was dissolved in 50 mL dH₂O.

100 mM Methylmercury acetate: 0.1255 g was dissolved in 50 mL diethylether.

Phenol (Tris equilibrated): Phenol was thawed at 68°C and 8-Hydroxyquinoline was added to a final concentration of 1%. Phenol was saturated several times with an equal volume of 1M Tris (pH 8.0), followed by 0.1 M Tris (pH 8.0) until the pH of the aqueous phase was >7.6.

Phenol:Chloroform mixture: An equal volume of chloroform was added to Tris-equilibrated phenol (1:1).

Phenol-Chloroform-Isoamyl-Alcohol Mixture: A mixture of Chloroform and Isoamyl Alcohol (24:1) was added to the equilibrated phenol (25:24:1).

100 mM Phenylmercury acetate: 0.1682 g was dissolved in 50 mL dH₂O.

100 mg/mL Pyrene: Pyrene was dissolved in dimethylformamide and the prepared solution was stored in a dark bottle at 4°C.

10% SDS: 50 g SDS was dissolved in 500 mL dH₂O.

5M Sodium chloride: 73.05 g was dissolved in 500 mL dH₂O.

Saturated Sodium chloride: 40 g NaCl was dissolved in 100 mL DEPC-treated dH₂O. The solution was stirred until it reached saturation.

10N Sodium hydroxide: 400 g NaOH pellets were dissolved in 1000 mL dH₂O.

Solution I (plasmid minipreps): 50 mM Glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0).

Solution II: 0.2 M NaOH, 1% SDS.

20X SSC: 175.3 g NaCl and 27.6 g C₆H₅Na₃O₇ were dissolved in 1000 mL dH₂O.

1M Tris: 121 g was dissolved in 1000 mL dH₂O.

20 mg/mL X-Gal: 100 mg of 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-gal) was dissolved in 5 mL dimethylformamide. The prepared solution was stored in the dark at -20°C.

0.5 M Zinc nitrate: 4.734 g was dissolved in 50 mL dH₂O.

APPENDIX 3 MICROBIOLOGICAL MEDIA AND REAGENTS

A. Suppliers of microbiological media

Media	Supplier
Bacteriological agar	Merck
Luria Bertani Broth	Amyl Media (Cat. #AM755)
Nutrient Agar	Amyl Media (Cat. #AM130)
Nutrient Broth	Amyl Media (Cat. #AM131)
Peptone Water	Amyl Media (Cat. #AM148)

B. Preparation of microbiological media

Nutrient Broth + 0.05 mM Tris-Cl pH 7.5 (NBT): 13g/L of powder was used to provide the following quantities: Bacteriological peptone (5 g/L), Sodium chloride (5 g/L), Yeast extract (2 g/L) and Beef extract (1 g/L). Tris-Cl (pH 7.5) was added to a final concentration of 0.05 mM.

Nutrient Agar + 0.05 mM Tris-Cl pH 7.5 (NAT): 26g/L of powder was used to provide the following quantities: Bacteriological peptone (5 g/L), Sodium chloride (5 g/L), Yeast extract (2 g/L), Beef extract (1 g/L) and Bacteriological agar (13 g/L). Tris-Cl (pH 7.5) was added to a final concentration of 0.05 mM.

Luria Bertani Broth (LB): 20 g/L of powder was used to provide the following quantities: Casein peptone, pancreatic (10 g/L), Sodium chloride (5 g/L) and Yeast extract (5 g/L).

Colour Selection Plates: These plates were prepared as described for the preparation of Luria Bertani Broth. Bacteriological agar was also added to a concentration of 15 g/L. After sterilisation by autoclaving, the agar was left to cool to approximately 55°C, and the following components were added (per 400 mL agar):

Ampicillin (100 µg/mL): 400 µL (100 mg/mL stock)

IPTG (100 mM): 2 mL (0.5 mM stock)

X-gal (80 µg/mL): 640 µL (50 mg/mL stock)

Peptone Water: 15 g/L of powder was used to provide the following quantities: Bacteriological peptone (10 g) and Sodium chloride (5 g).

Basal Salt Medium (BSM): This carbon-free media was prepared using the following formula: Basal salt solution (985 mL/L), Trace element solution (5 mL/L), Vitamin solution (5 mL/L) and Mg/Ca solution (5 mL/L). The Trace Element, Vitamin and Mg/Ca solutions were filter sterilized and added to the Basal Salt Medium solution after autoclaving. To prepare BSM agar, 15 g of bacteriological agar was added to the BSM solution prior to autoclaving. The required solutions were prepared as follows:

Basal Salt Solution (*formula per litre*): K_2HPO_4 (0.4 g), KH_2PO_4 (0.4 g), $(NH_4)_2SO_4$ (0.4 g) and NaCl (0.3 g).

Trace element solution (*formula per litre*): $FeSO_4 \cdot 7H_2O$ (200 mg), $ZnSO_4 \cdot 7H_2O$ (10 mg), $MnCl_2 \cdot 4H_2O$ (3 mg), $CoCl_2 \cdot 6H_2O$ (20 mg), $CuCl_2 \cdot 2H_2O$ (1 mg), $NiCl_2 \cdot 6H_2O$ (2 mg), $NaMoO_4 \cdot 2H_2O$ (500 mg) and H_3BO_3 (boric acid) (30 mg).

Vitamin solution (*formula per 100 mL*): Biotin (2 mg), Folic acid (2 mg), Thiamine HCl (B_1) (5 mg), D-calcium pantothenate (5 mg), Vitamin B_{12} (5 mg), Riboflavin (B_2) (5 mg), Niacin (nicotinic acid) (20 mg), Pyridoxal HCl (3 mg), *p*-aminobenzoic acid (2 mg).

Mg/Ca solution (*formula per litre*): $MgSO_4 \cdot 7H_2O$ (0.4 g) and $CaCl_2 \cdot 2 H_2O$ (0.4 g)

Pyrene was added to the complete BSM medium at concentration of 250 mg/L after the addition of Trace element, Vitamin and Mg/Ca solutions. It was important to ensure the pyrene was not added to the flask prior to the addition of BSM, as the solvent evaporates immediately upon contact with the glass surface of the flask.

BSM agar was prepared as described, without the addition of pyrene to the media. A 2% pyrene solution in diethylether was sprayed over the surface of the plates after the inoculation of culture. Once the diethylether had evaporated, the plates were sealed with parafilm and incubated at 30°C until colonies indicating pyrene-degrading microorganisms.

C. Preparation of Gram stain reagents

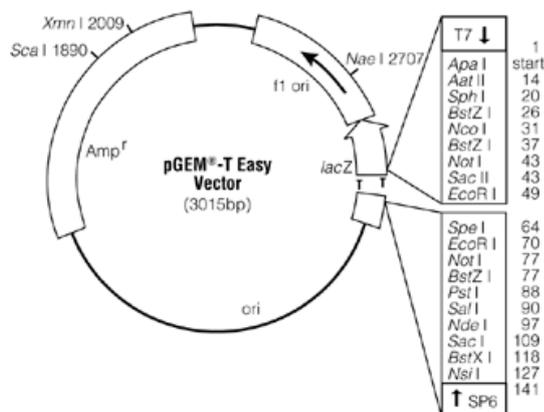
Crystal Violet (1%): Crystal violet (1 g), 95% Alcohol (20 mL) and Aqueous Ammonium Oxalate (1%). The solution was left to dissolve overnight and filtered through paper prior to use (Reade, 1985).

Iodine Solution (Jensen's): Iodine crystals (1g), Potassium iodide (2g) and Distilled water (100 mL). The iodine was dissolved in strong potassium iodide (2g in 2 mL water) and then diluted (Reade, 1985).

Neutral Red (0.5%): Neutral red (5.5g), Glacial acetic acid (2 mL), Distilled water: (1000 mL). The solution was left to boil for 1 hour and then filtered through paper (Reade, 1985).

APPENDIX 4

pGEM[®]-T Easy Vector



(Cat.# A1360)

Reference: <http://www.promega.com/figures/popup.asp?fn=1473va>

APPENDIX 5

MINIMUM INHIBITORY CONCENTRATION ASSAY DATA

Table A5.1 MIC results obtained by the spot plate method

Isolate	Heavy metal (mM)									
	HgCl ₂		Pb(NO ₃) ₂		Zn(NO ₃) ₂		CdCl ₂		Co(NO ₃) ₂	
	A	B	A	B	A	B	A	B	A	B
<i>Achromobacter</i> sp. Isolate AO17	0.05↓	0.1	3.5↑	2.3	3.5↑	2.7	1↑	0.5	1.5	1.5
<i>Achromobacter</i> sp. Isolate AO22	0.05↓	0.1	3.5↑	2.5	3.5	3.5	0.5↓	1.1	1.5↓	1.9
<i>Arthrobacter</i> sp. Isolate E9	0.05↓	0.06	3.5	3.5	3.5↑	3	2	2	2↓	2.5
<i>Arthrobacter</i> sp. Isolate E11	0.03↓	0.06	3.5	3.5	3.5↑	3	2	2.1	2↓	2.5
<i>C. metallidurans</i> CH34	0.05↑	0.04	3.5↑	2	3.5↑	2.6	2↑	0.1	2	1.9

A- current results

B- previously reported results (Trajanovska *et al.*, 1997)

↓ and ↑ indicate a lower or higher result in the current study compared to Trajanovska *et al.*, 1997.

Table A5.2 Comparison of MIC results obtained using the spot plate and, spread plate methods

	Heavy metal *	Spot plate (mM)	Spread plate (mM)
<i>Achromobacter</i> sp. isolate AO22	HgCl ₂	0.05	0.1
	Pb(NO ₃) ₂	3.5	3.5
	Zn(NO ₃) ₂	3.5	3.5
	CdCl ₂	0.5	1.5
	Co(NO ₃) ₂	1.5	1.0
<i>Arthrobacter</i> sp. isolate E9	HgCl ₂	0.05	0.08
	Pb(NO ₃) ₂	3.5	3.5
	Zn(NO ₃) ₂	3.5	3.5
	CdCl ₂	2	2
	Co(NO ₃) ₂	2	1.5

*The concentrations tested are outlined in Section 2.3.4.

Table A5.3 Heavy metal MICs of VUN 10010, AO22 and E9 using microtitre plates

Heavy metal	Experiment #	AO22	E9	VUN 10010
HgCl ₂	1	0.075	0.1	0.075
	2	0.1	0.1	0.1
	3	0.1	0.1	0.1
	Average	0.091	0.1	0.091
	Std. Deviation	0.014	0	0.014
Pb(NO ₃) ₂	1	3	3	3
	2	2	3	3
	3	3	3	3
	Average	2.66	3	3
	Std. Deviation	0.57	0	0
Zn(NO ₃) ₂	1	4	4	4
	2	4	4	4
	3	4	4	4
	Average	4	4	4
	Std. Deviation	0	0	0
CdCl ₂	1	1	1	0.5
	2	1	1	1.5
	3	1.5	1.5	1.5
	Average	1.33	1.33	1.16
	Std. Deviation	0.28	0.28	0.57
Co(NO ₃) ₂	1	1.5	1.5	1.5
	2	2	2	2
	3	1	1	1.5
	Average	1.5	1.5	1.66
	Std. Deviation	0.5	0.5	0.28

**APPENDIX 6
RAW GROWTH CURVE DATA**

Table A6 Growth curve analysis (in duplicate) of AO22, E9 and VUN 10010

Time Point (h)	OD ₆₀₀					
	AO22-A	AO22-B	E9-A	E9-B	VUN 10010-A	VUN 10010-B
0	0.1	0.096	0.085	0.086	0.101	0.104
1	0.107	0.106	0.092	0.095	0.127	0.130
2	0.141	0.144	0.137	0.154	0.180	0.180
3	0.202	0.215	0.258	0.263	0.248	0.255
4	0.443	0.441	0.481	0.493	0.365	0.376
5	0.796	0.791	0.822	0.820	0.509	0.506
6	1.11	1.14	1.21	1.21	0.700	0.680
7	1.42	1.33	1.57	1.56	0.780	0.860
8	1.70	1.52	1.86	1.72	0.970	0.970
9	1.84	1.62	1.99	1.92	1.02	1.06
10	1.82	1.87	2.31	2.22	1.19	1.16
11	1.92	1.93	2.56	2.26	1.31	1.26
12	2.59	1.99	2.79	2.52	1.43	1.24
13	2.23	2.10	2.88	2.78	1.38	1.37
14	2.30	2.21	3.00	3.0	1.56	1.43
15	2.25	2.21	3.32	3.27	1.71	1.69
16	2.25	2.30	3.69	3.54	1.56	1.52
17	2.28	2.27	4.03	3.73	1.81	1.84
18	2.30	2.13	3.89	3.62	1.59	1.65
32	2.08	2.02	4.5	4.42	2.07	2.06

APPENDIX 7 16S SEQUENCE COMPARISONS

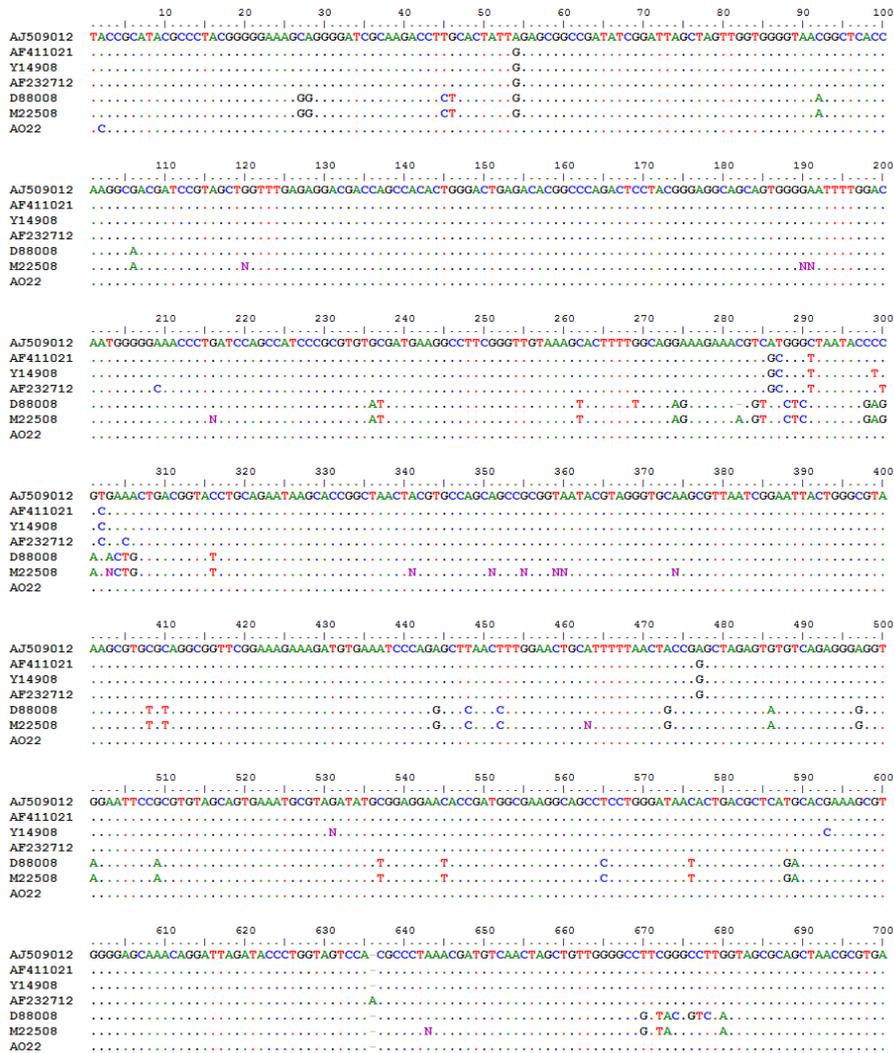


Figure A7.1 Comparison of the 16S RNA gene fragment from *Achromobacter* sp. AO22 with published 16S RNA genes.

```

      710      720      730      740      750      760      770      780      790      800
AJ509012 AGTTGACCCGCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAATTGACGGGGACCCGCAAGCGGTGGATGATGTGGATTAAATTCGATGCCAACCC
AF411021 .....
Y14908 .....
AF232712 .....
D88008 .....
M22508 .....
AO22 .....

      810      820      830      840      850      860      870      880      890      900
AJ509012 GAAAAACCTTACCTACCTTGACATGTCTGGAAATTCGGAAGAGATTGGAAAGTCTGCAAGAGAACCGGAACACAGGTGCTGCATGGCTGCTCAGC
AF411021 .....G.....C.....
Y14908 .....G.....C.....
AF232712 .....C.....A.....
D88008 .....AG.....CC.....
M22508 .....AG.....CC.....
AO22 .....

      910      920      930      940      950      960      970      980      990      1000
AJ509012 TCGTGTGCTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCAATTAGTTGCTACGAAAGG-GCACTTAATGAGACTGCCGGTGACAAACCG
AF411021 .....
Y14908 .....
AF232712 .....
D88008 .....G.....G...NA.....
M22508 .....C...A.....
AO22 .....

AJ509012 GAG
AF411021 ...
Y14908 ...
AF232712 ...
D88008 ...
M22508 ...
AO22 ...

```

Figure A7.1 cont. Comparison of the 16S RNA gene fragment from *Achromobacter* sp. AO22 with published 16S RNA genes.



Figure A7.2 Comparison of the 16S RNA gene fragment from *A. woluwensis* E9 with published 16S RNA genes.

```

      710      720      730      740      750      760      770      780      790      800
AY112986  GCTGAGGAGCGAAGCATGGGGAGCGAACAGGATTAGATACCCCTGGTAGTCCATGCCGTAAACGTTGGGCAC TAGGTGTGGGGGACATTCCACGTTTTCC
AJ5577725 .....
X80746    .....C.....G.....
AB046358  .....
AB071950  .....C.....
E9        .....

      810      820      830      840      850      860      870      880      890      900
AY112986  GCGCCGTAGCTAACGCATTAAGTGCCTCCCTGGGGAGTACGGCCGCAAGGCTAAAACCTCAAAGGAATTGACGGGGGCCCGCACAAAGCGGGAGCATGC
AJ5577725 .....
X80746    .....C.....
AB046358  .....
AB071950  .....A.....
E9        .....

      910      920      930      940      950      960      970      980      990      1000
AY112986  GGATTAAATTCGATGCAACGCGAAGAACCCTTACCAAGGCTTGACATGGACTGGATCGCATCAGAGATGGTGTTCCTCC TTCGGGGCTGGTTCACAGGTGGT
AJ5577725 .....A...C...AT..ATG.....T...CAG...AC..T...C...T...
X80746    .....TG..C...C...TCC.....G..C.....C.....C.....
AB046358  .....TG..CA..C...CGTG..A..CAG.....C...T.....
AB071950  .....A...C...AA..GCGT...A...AC...CC...AC..T...TC...T...
E9        .....

      1010
AY112986  GCATGGTTGTCGTCAGCT
AJ5577725 .....
X80746    .....
AB046358  .....
AB071950  .....
E9        .....

```

Figure A7.2 cont. Comparison of the 16S RNA gene fragment from *A. woluwensis* E9 with published 16S RNA genes.

APPENDIX 8
OD₆₀₀ READINGS AND CELL COUNTS FROM MERCURIC
CHLORIDE INDUCTION EXPERIMENTS

Table A9.1 OD₆₀₀ readings from *Achromobacter* sp. AO22 cells induced with 0.075 mM HgCl₂ and the control cells

Time point (h)	-HgCl ₂ (0 mM)	+HgCl ₂ (0.075 mM)
0	0.108	0.108
0.5	0.140	0.129
1	0.182	0.158
2	0.290	0.219
4	0.671	0.545
6	0.870	0.849
9	1.079	1.073

Table A9.2 Cell counts from *Achromobacter* sp. AO22 cells induced with 0.075 mM HgCl₂ and the control cells

Time point (h)	-HgCl ₂ (cells/mL)	+HgCl ₂ (cells/mL)
0	1.87 X 10 ⁸	2.26 X 10 ⁸
0.5	1.84 X 10 ⁸	1.75 X 10 ⁸
1	1.94 X 10 ⁸	1.96 X 10 ⁸
2	2.3 X 10 ⁸	1.90 X 10 ⁸
4	7.45 X 10 ⁸	4.65 X 10 ⁸
6	1.12 X 10 ⁹	1.94 X 10 ⁹
9	2.36 X 10 ⁹	2.54 X 10 ⁹

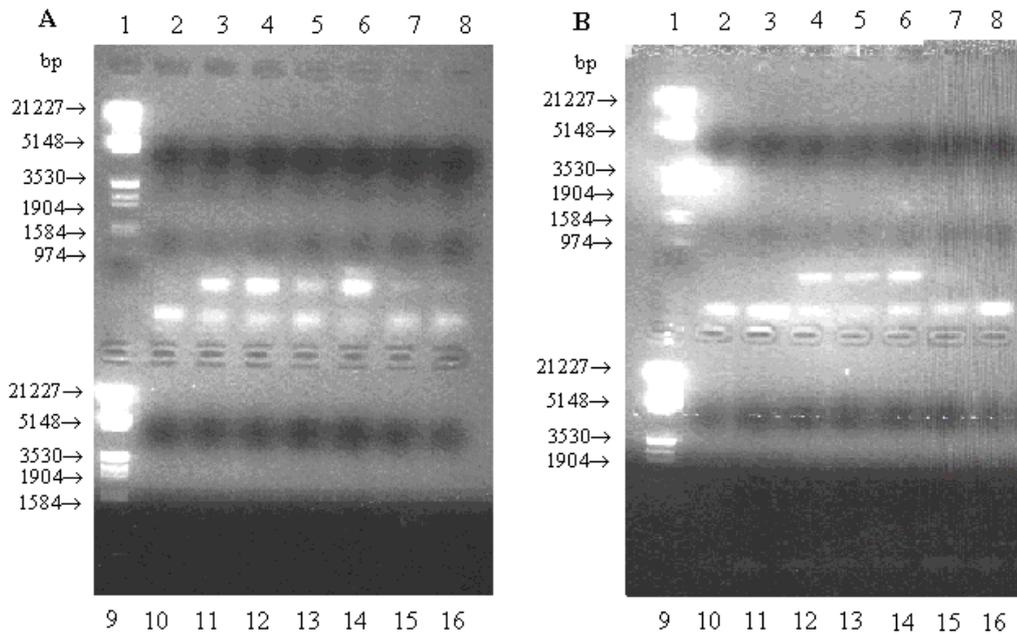
Table A9.3 OD₆₀₀ readings from the *A. woluwensis* E9 cells induced with up to 0.05 mM HgCl₂ and the control cells

Time (h)	0 mM	0.01 mM	0.02 mM	0.03 mM	0.04 mM	0.05 mM
0	0.125	0.138	0.131	0.132	0.129	0.132
2	0.251	0.164	0.144	0.140	0.140	0.138
4	0.660	0.205	0.151	0.140	0.131	0.121
6	0.857	0.146	0.128	0.128	0.116	0.108
8	0.993	0.155	0.123	0.121	0.109	0.106
24	1.448	0.097	0.090	0.090	0.085	0.086
48	1.565	1.473	0.974	0.581	0.048	0.036
72	1.579	1.666	1.476	1.332	1.004	0.080
96	1.520	1.599	1.630	1.650	1.632	1.530

APPENDIX 9 NEGATIVE CONTROL RT-PCR

The following figures show the RT-PCR experiments as outlined in Section 5.4. In brief, RNA from *Achromobacter* sp. AO22 cells exposed to 0.075 mM HgCl₂ and control *Achromobacter* sp. AO22 cells was DNAaseI treated to remove any residual DNA. RT-PCRs were set up using SuperScript II Reverse Transcriptase. Negative control reactions, containing no SuperScript II, were set up to ensure that any amplification seen was not due to the presence of residual DNA in the original sample. The agarose gels shown below are the original gels and show both the samples that contained SuperScript II and the negative controls.

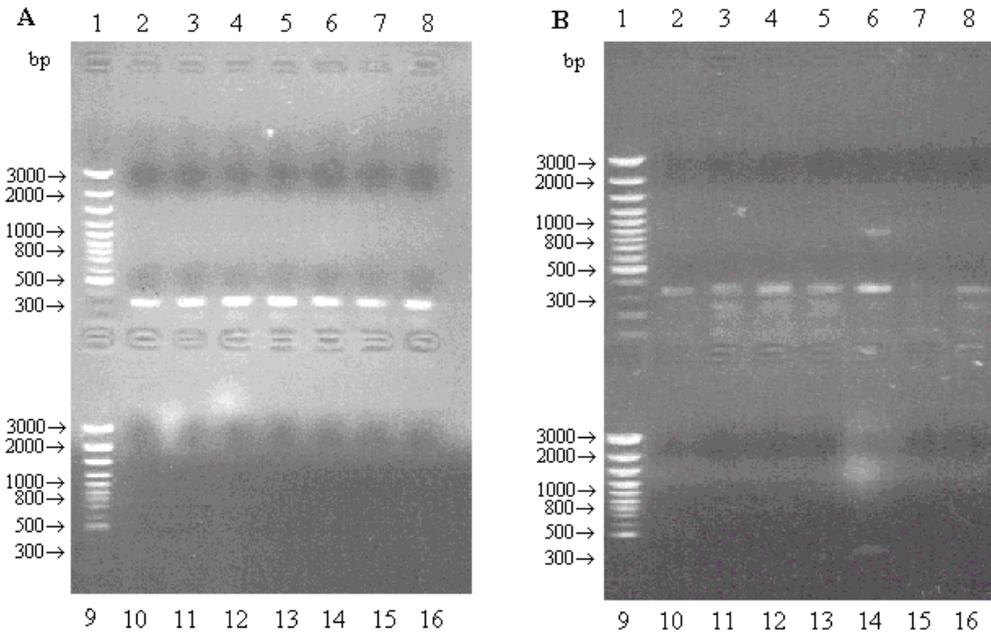
Figure A8.1 *MerR*



Gel A (+HgCl₂): 1. λ *HindIII/EcoRI* DNA marker; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. λ *HindIII/EcoRI* DNA marker; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

Gel B (-HgCl₂): 1. λ *HindIII/EcoRI* DNA marker; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. λ *HindIII/EcoRI* DNA marker; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

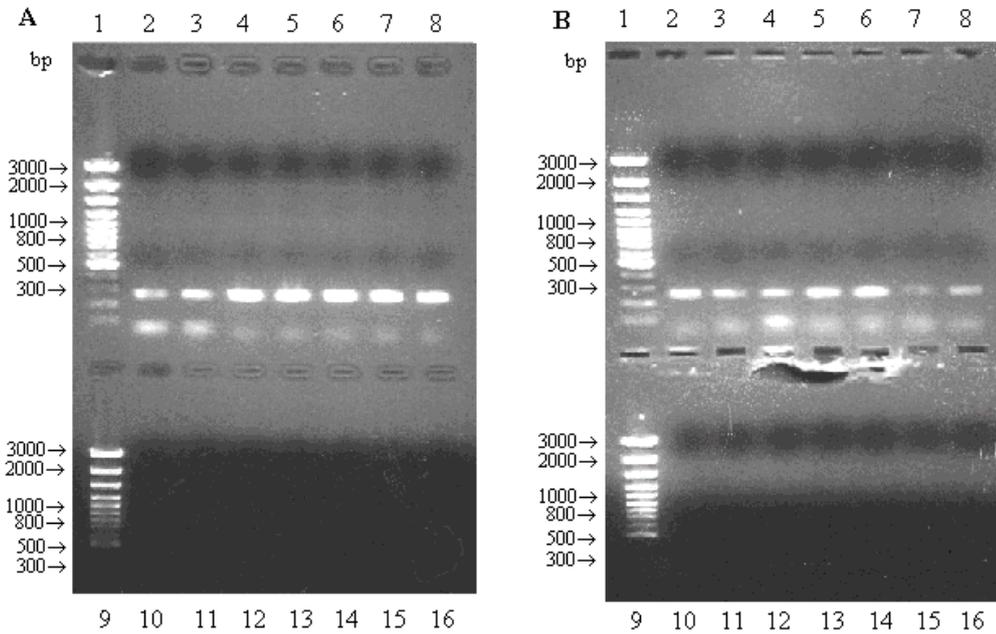
Figure A8.2 *MerT*



Gel A (+HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder Plus; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder Plus; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

Gel B (-HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder Plus; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder Plus; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

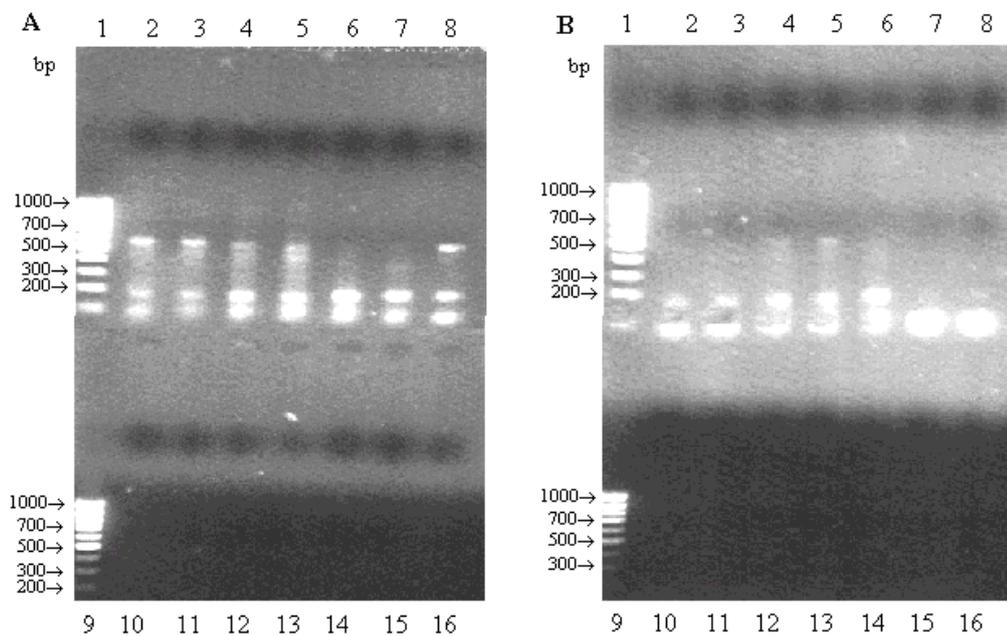
Figure A8.3 *MerP*



Gel A (+HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder Plus; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder Plus; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

Gel B (-HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder Plus; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder Plus; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

Figure A8.4 *MerA*



Gel A (+HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

Gel B (-HgCl₂): 1. Gene Ruler™ 100 bp DNA Ladder; 2. 0h, +SS; 3. 0.5h, +SS; 4. 1h, +SS; 5. 2h, +SS; 6. 4h, +SS; 7. 6h, +SS; 8. 9h, +SS; 9. Gene Ruler™ 100 bp DNA Ladder; 10. 0h, -SS; 11. 0.5h, -SS; 12. 1h, -SS; 13. 2h, -SS; 14. 4h, -SS; 15. 6h, -SS; 16. 9h, -SS.

APPENDIX 10 BIOFILM ASSAY DATA

Table A10.1 Biofilm formation in the absence of heavy metals (NBT only)

Experiment	OD reading (570nm)*				
	No inoculum	AO22	E9	VUN 10010	<i>P. aeruginosa</i>
A	0.082	0.55275	0.712	0.806	0.563
B	0.1745	0.5525	0.5485	0.767	0.39575
Average	0.12825	0.552625	0.63025	0.7865	0.479375
Std. Dev.	0.065407	0.000177	0.115612	0.027577	0.118264

Table A10.2 Biofilm formation in the presence of mercuric chloride

Isolate	HgCl ₂ concentration (mM)*						Statistical analysis
	0	0.001	0.025	0.05	0.075	0.1	
AO22	100	93.57	90.41	106.37	75.52	64.76	Average
		5.44	0.97	5.37	5.29	5.62	Std. Dev.
E9	100	75.64	88.64	104.75	84.98	103.7	Average
	0	1.46	12.34	20.47	10.81	7.02	Std. Dev.
VUN 10010	100	110.58	83.47	92.47	80.18	87.29	Average
	0	3.17	12.68	19.71	16.14	8.17	Std. Dev.
<i>P. aeruginosa</i>	100	113.98	146.36	63.81	61.46	51.80	Average
	0	44.45	1.33	13.75	16.62	1.86	Std. Dev.

*The results for each sample in the presence of each concentration of HgCl₂ were calculated as a percentage of the result obtained in the absence of HgCl₂.

Table A10.3 Biofilm formation in the presence of lead nitrate

Isolate	Pb(NO ₃) ₂ concentration (mM)*						Statistical analysis
	0	0.1	1	2	3	4	
AO22	100	84.66	117.16	70.09	52.38	10.91	Average
	0	13.88	6.25	6.4	0.05	1.19	Std. Dev.
E9	100	108.69	109	92.99	39.26	8.78	Average
	0	6.58	2.7	2.14	3.18	2.33	Std. Dev.
VUN 10010	100	118.38	105	70.97	22.47	13.3	Average
	0	15.67	3.43	11.04	3.49	0.93	Std. Dev.
<i>P. aeruginosa</i>	100	88.35	82.49	101.42	22.02	12.33	Average
	0	10.34	20.8	15.95	2.13	0.63	Std. Dev.

*The results for each sample in the presence of each concentration of Pb(NO₃)₂ were calculated as a percentage of the result obtained in the absence of Pb(NO₃)₂.

Table A10.4 –Biofilm formation in the presence of zinc nitrate

Isolate	Zn(NO ₃) ₂ concentration (mM)*						Statistical analysis
	0	0.1	1	2	3	4	
AO22	100	94.62	97.77	153.36	116.95	96.46	Average
	0	5.68	1.67	6.95	5.42	18.82	Std. Dev.
E9	100	77.01	110.83	105.59	110.91	69.6	Average
	0	8.52	12.48	36.59	7.68	11.92	Std. Dev.
VUN	100	93.46	124.12	111.66	123.75	93.15	Average
	0	2.911	27.05	18.78	1.27	5.06	Std. Dev.
<i>P. aeruginosa</i>	100	116.92	144.45	170.81	190.65	221.01	Average
	0	22.34	12.1	3.13	33.77	97.53	Std. Dev.

*The results for each sample in the presence of each concentration of Zn(NO₃)₂ were calculated as a percentage of the result obtained in the absence of Zn(NO₃)₂.

Table A10.5 Biofilm formation in the presence of cadmium chloride

Isolate	CdCl ₂ concentration (mM)*						Statistical analysis
	0	0.1	0.5	1	1.5	2	
AO22	100	83.19	133.9	111.46	23.09	23.09	Average
	0	9.55	10.53	12.05	4.05	0.99	Std. Dev.
E9	100	113.7	224.71	154.01	57.37	23.88	Average
	0	16.87	50.58	26.28	55.36	6.51	Std. Dev.
VUN	100	132.93	140.62	131.25	31.14	18.15	Average
	0	8.6	23.67	15.9	14.91	0.41	Std. Dev.
<i>P. aeruginosa</i>	100	100.21	203.38	8.12	22.59	19.46	Average
	0	20.33	12.1	3.33	6.22	5.12	Std. Dev.

*The results for each sample in the presence of each concentration of CdCl₂ were calculated as a percentage of the result obtained in the absence of CdCl₂.

Table A10.6 Biofilm formation in the presence of cobalt nitrate

Isolate	Co(NO ₃) ₂ concentration (mM)*						Statistical analysis
	0	0.1	0.5	1	1.5	2	
AO22	100	114.84	129.52	126.89	115.06	23.65	Average
	0	2.5	12.95	0.1	4.98	7.11	Std. Dev.
E9	100	84.77	96.68	83.88	87.09	21.36	Average
	0	13.33	8.31	5.7	8.07	14.46	Std. Dev.
VUN	100	109.16	139.77	93.23	46.33	21.38	Average
	0	6.07	20.76	11.31	11.54	1.88	Std. Dev.
<i>P. aeruginosa</i>	100	110.83	97.79	95.66	27.5	32.83	Average
	0	1.02	23.31	9.69	12.2	18.59	Std. Dev.

*The results for each sample in the presence of each concentration of $\text{Co}(\text{NO}_3)_2$ were calculated as a percentage of the result obtained in the absence of $\text{Co}(\text{NO}_3)_2$.