Biodegradation **8:** 113–124, 1997.

Detection of heavy metal ion resistance genes in Gram-positive and Gram-negative bacteria isolated from a lead-contaminated site

Suzana Trajanovska[1], Margaret L. Britz[2] & Mrinal Bhave[1,*]
[1]*Department of Biological and Food Sciences and* [2]*Centre for Bioprocessing and Food Technology, Victoria University of Technology, PO Box 14428, MCMC, Melbourne, Australia 8001 (* Author for correspondence)*

Received 10 February 1997

Key words: lead-resistant bacteria, metal-resistant Gram-positive bacteria, PCR, probing, *mer, pco, czc, chr* genes

Abstract

Resistance to a range of heavy metal ions was determined for lead-resistant and other bacteria which had been isolated from a battery-manufacturing site contaminated with high concentrations of lead. Several Gram-positive (belonging to the genera *Arthrobacter* and *Corynebacterium*) and Gram-negative (*Alcaligenes* species) isolates were resistant to lead, mercury, cadmium, cobalt, zinc and copper, although the levels of resistance to the different metal ions were specific for each isolate. Polymerase chain reaction, DNA-DNA hybridization and DNA sequencing were used to explore the nature of genetic systems responsible for the metal resistance in eight of the isolates. Specific DNA sequences could be amplified from the genomic DNA of all the isolates using primers for sections of the *mer* (mercury resistance determinant on the transposon Tn*501*) and *pco* (copper resistance determinant on the plasmid pRJ1004) genetic systems. Positive hybridizations with *mer* and *pco* probes indicated that the amplified segments were highly homologous to these genes. Some of the PCR products were cloned and partially sequenced, and the regions sequenced were highly homologous to the appropriate regions of the *mer* and *pco* determinants. These results demonstrate the wide distribution of mercury and copper resistance genes in both Gram-positive and Gram-negative isolates obtained from this lead-contaminated soil. In contrast, the *czc* (cobalt, zinc and cadmium resistance) and *chr* (chromate resistance) genes could not be amplified from DNAs of some isolates, indicating the limited contribution, if any, of these genetic systems to the metal ion resistance of these isolates.

Introduction

Many agricultural and industrial practices have led to environmental pollution by heavy metal ions. Metals such as zinc, cobalt, cadmium, nickel, copper and chromium are used for several industrial applications such as production of steel and other alloys, galvanization of iron, electroplating, manufacture of batteries, TV tubes and pigments. Copper has been used for a number of years as an active ingredient of bactericides and fungicides and as a growth enhancer of pigs. Mercury is a byproduct of burning coal and petroleum products and it is also used in household and hospital disinfectants. Contamination of soil by lead occurs following the use of lead arsenate insecticides. High levels of lead in waste waters may originate from combustion of leaded gasoline, coal burning or metal smelting. Mining and leaching from natural deposits also contribute to environmental contamination. A number of sites contaminated by heavy metals around the world are associated with human activities such as discharge of wastes into natural waterways, various metallurgical industries, accidental spills or mining [1–8].

Low concentrations of certain transition metals like cobalt, copper, nickel and zinc are essential for many cellular processes of bacteria, however, higher concentrations of these can be cytotoxic. Other heavy metals, including lead, cadmium, mercury, silver and chromium have no known beneficial effects to bacterial cells and are toxic even at low concentrations [9, 10]. Natural bacterial populations are very important in the major elemental and organic metabolism cycles

[1]. As environmental pollutants pose a great risk to natural bacterial populations, the adaptive responses of bacteria in contaminated environments have been studied extensively. Such investigations have indicated that bacteria which survive, and, indeed, flourish in such environments have developed or acquired genetic systems that counteract the effects of high metal ion concentrations. For example, resistance to mercury is encountered commonly in bacteria from areas of mercury deposits [11, 12], hospital samples [13] and contaminated terrestrial and aquatic environments [1, 5–7, 14, 15]. The genes encoding mercury resistance are found to be located frequently on plasmids and transposons [13-18], although a chromosomally-encoded determinant has been identified also [19]. Bacteria resistant to cadmium, zinc, cobalt, chromium, copper, arsenic and nickel have been isolated from several contaminated sites and natural deposits. Their genetic systems have been studied extensively and the resistance-encoding genes also appear to be predominantly plasmid-located [4, 20–31]. The observations that metal resistance determinants are located most frequently on plasmids and transposons have led to suggestions that these determinants are probably spread by horizontal transfer [11, 32]. Such genetic systems are useful tools to investigate the nature and extent of horizontal transfer of adaptive genes across natural bacterial populations.

Investigations of adaptive responses commonly involve studying phenotypic changes. However, a more basic understanding of adaptation can be achieved if the molecular mechanisms were understood also. Approaches used recently have included the use of molecular techniques such as polymerase chain reaction (PCR), DNA-DNA hybridization and/or restriction fragment length polymorphism (RFLP) determination [2, 5–7, 32–35]. These techniques are, in general, more sensitive and quicker than some of the traditional microbiological methods and can be aimed precisely at a particular genetic determinant; they thus provide a useful means of investigating bacterial responses to environmental stress and the molecular mechanisms of adaptation.

Several bacteria had been isolated previously from heavily lead-contaminated soil samples taken from a discontinued and rezoned lead battery manufacturing site [36]. We are interested in investigating the nature of the genetic systems encoding resistance to lead and other heavy metals in these isolates, which will enable comparison with similar genes isolated from other locations disparate from Australia. The broader context of this work is the application of the genetic systems in biomonitoring and potential use of the bacteria in pollution abatement. This paper describes the heavy metal resistance properties of eight soil bacterial isolates from Ardeer, Melbourne, and the analysis of genes encoding metal resistance in these isolates using PCR, DNA-DNA hybridization, RFLP and DNA sequencing. In the absence of extensive genetic information on lead resistance in bacteria, the approach used involved analysis of genes known to occur more broadly in nature and where the genetic mechanisms of resistance are well characterised (mercury, copper, cadmium resistance), to obtain genetic tools for future analysis of the genetics of lead resistance mechanisms especially in the Gram-positive isolates.

Materials and methods

Soil description, bacterial strains and plasmids Soil samples had been collected from a discontinued battery manufacturing site at Ardeer, an outer suburb of Melbourne, where the lead content of soil was up to 260 mg/g. Several bacteria had been isolated from the soil homogenates previously, either following enrichment for lead-resistant bacteria or by screening individual isolates for lead resistance [36]. Eight of these isolates, which showed different degrees of lead tolerance, were characterized by Gram staining and determining their biochemical properties including acid-alcohol fastness, catalase production, oxidase activity, glucose fermentation, citrate utilization, casein hydrolysis, hydrogen sulphide production, indole test, gelatine hydrolysis and nitrate reduction according to Reade [37]. Table 1 summarizes the properties, applications and sources of all bacterial strains and plasmids used in this study.

Estimation of bacterial tolerance to metals. Analytical grades of metal salts were used to prepare 0.5 M stock solutions, which were filter-sterilized and added to 50 mM Tris-buffered Nutrient Agar (Oxoid) (NAT) media [36] for determination of the Minimum Inhibitory Concentrations (MICs) of the metal ions for each isolate. The range of concentrations used was 0.5 to 4 mM for lead nitrate, 0.5 to 8 mM for cupric nitrate, 0.01 to 0.1 mM for mercuric chloride, 0.5 to 5 mM for zinc nitrate and cobalt nitrate and 0.1 to 3 mM for cadmium chloride. Overnight cultures grown in Nutrient Broth (Oxoid) (NB) were diluted to 10^5 cells/ml then spotted onto metal-salt-containing NAT plates. Dupli-

Table 1. Bacterial strains and plasmids

Bacterium/plasmid	Properties and applications	Reference
E9	Gram-positive, *Arthrobacter*	Present work
E11	Gram-positive, *Arthrobacter*	As above
AB12	Gram-positive, *Corynebacterium*	As above
AB18	Gram-positive, *Corynebacterium*	As above
AO5	Gram-positive, *Corynebacterium*	As above
AO17	Gram-negative, *Alcaligenes*	As above
AO21	Gram-positive, *Corynebacterium*	As above
AO22	Gram-negative, *Alcaligenes*	As above
Alcaligenes eutrophus CH34[a]	Contains pMOL28 (Ni^r, Co^r, $Chr_4{}^r$, Hg^r) and pMOL30 (Co^r, Zn^r, Cd^r, Pb^r, Hg^r). Positive control for amplification of *czc* and *chr* operons and for determining resistance levels to different metal ions under our conditions.	4, 44
E. coli AB1157 (pACYC184::Tn*501*)[b]	Hg^r, Cm^r. Transposon Tn*501* encodes *mer* (Hg^r) operon. Used as positive control for amplification of a section of the *mer* operon and for isolation of *mer* probe.	17
E. coli JM105 (pACYC184::Tn*21*)[b]	Hg^r, Cm^r. Transposon Tn*21* encodes *mer* operon (Hg^r) which is highly homologous to the Tn*501 mer* operon. Used as a positive control for amplification of a section of the *mer* operon.	17
E. coli ED8739 (pPA87)[c]	Amp^r, Cu^r. A 12.5 kb *Hin*dIII fragment encoding the *pco* (Cu^r) genetic system of pRJ1004 subcloned into pBR322. Used as positive control for amplification of *pcoR* and *pcoA* genes of the *pco* operon, for isolation of *pco* probe and for copper resistance phenotype.	31, 42
Corynebacterium glutamicum AS019	Rif^r. Plasmid-free. No known plasmid- or transposon-encoded metal-resistance genes. Used as a reference for estimating levels of resistance to different metals.	39
C. glutamicum ATCC 13032	Rif^s. Plasmid. No known plasmid or transposon-encoded metal-resistance genes. Same application as above.	ATCC
pBluescript SK+	Plasmid cloning vector.	Stratagene
E. coli XL-1 Blue	Host strain for pBluescript plasmids.	Stratagene

[a] Obtained from Dr. M. Mergeay, VITO, Mol, Belgium.
[b] Obtained from Dr. M. Osborn, School of Biological Sciences, Donnan Laboratories, The University of Liverpool, Liverpool, UK.
[c] Obtained for Dr. B. T. O. Lee, Department of Genetics, University of Melbourne, Australia.

cate plates of each isolate were incubated at 28 °C for 5 days before growth was scored and MIC (the lowest concentration of metal ion which completely inhibited growth) determined.

Isolation of total genomic and plasmid DNA. Total genomic DNA was isolated from cell pellets obtained following centrifugation of 10 ml of cultures grown overnight in NB at 28 °C. The extraction protocol followed for Gram-negative bacteria was similar to that in Sambrook et al. [38], with the following modifications. The cell pellets were suspended in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.6) containing 0.5% SDS, 10 μg/ml proteinase K, 10 μg/ml lysozyme and 10 μg/ml DNAase-free RNAase A and incubated at 37 °C for one hour. The lysate was extracted twice with Tris-equilibrated phenol-chloroform-isoamyl alcohol (25:24:1) and the DNA was precipitated with ethanol. The Gram-positive bacteria were grown in NB containing 2% glycine to achieve cell lysis and an extraction protocol that involved extensive digestions with lysozyme was followed [39]. Plasmid DNA was isolated by alkaline lysis of cells [38].

Oligonucleotide primers. Table 2 provides details of the primers and the expected size of products from the known genes. The positive controls used were Tn*501* and Tn*21* for *mer* (mercury resistance), pPA87 for *pco* (copper resistance), and total genomic and plasmid DNA of *A. eutrophus* CH34 for *czc* (cadmium-zinc-

Table 2. Oligonucleotide primers used for PCR amplification

Resistance determinant amplified	Upstream primer	Downstream primer	Exact length of amplified region (bp)	Reference
merRTΔ*P* region of Tn*501*	*mer*1 5' GAGATCTAAAGCACGCTAAGGC 3'	*mer*2 5' GGAATCTTGACTGTGATCGGG 3'	1011	18
*pco*R gene of *pco* operon	*pco*R1 5' CAGGTCGTTACCTGCAGCAG 3'	*pco*R2 5' CTCTGATCTCCAGGACATATC 3'	636	9, 42 and B. Lee, personal communication.
pcoA gene of *pco* operon	*pco*A1 5' CGTCTCGACGAACTTTCCTG 3'	*pco*A2 5' GGACTTCACGAAACATTCCC 3'	1791	9, 42 and B. Lee, personal communication.
czcA gene of *czc* determinant of *A. eutrophus* CH34	*czc*A1 5' GTTTGAACGTATCATTAGTTTC 3'	*czc*A2 5'GTAGCCATCCGAAATATTCG 3'	1885	26
czcB gene of above	*czc*B1 5' CTATTTCGAACAAACAAAAGG 3'	*czc*B2 5' CTTCAGAACAAAACTGTTGG 3'	1520	26
czcD gene of above	*czc*D1 5' CAGGTCACTGACACGACCAT 3'	*czc*D2 5' CATGCTGATGAGATTGATGATC 3'	398	26
chrA gene of above	*chr*A1 5' CTTATACGCTACGCCAACTG	*chr*A2 5' GTAATGGCATTCAGTCGCTTG	1292	27
chrB gene of above	*chr*B1 5' GTCGTTAGCTTGCCAACATC 3'	*chr*B2 5' CGgAAAGCAAGATGTCGATCG 3'	450	27

cobalt resistance) and *chr* (chromate resistance) systems. Primer pair *mer*1-*mer*2 was designed to amplify a 1 kb (kilo base pair, 1000 base pairs) conserved region containing the *merR* and *merT* genes and 5′ end of the *merP* gene (*merRT*Δ*P* region) of Tn*501* and Tn*21* (18). The other operons (*pco*, *czc* and *chr*) consist of several genes, the total size of which was too large to be amplified together by PCR. For these operons, several primer pairs were constructed to allow amplification of individual genes. All primers were at least 20 bases long to allow only specific binding to templates, had no self-complementary regions, and the base composition was chosen such that they had similar annealing temperatures (58–64 °C) so several amplifications could be carried out simultaneously.

PCR amplification. Templates for PCR amplification included the following: total genomic DNA from the eight test isolates and *A. eutrophus* CH34; plasmids encoding Tn*501* or Tn*21*; plasmid pPA87 and a plasmid isolated from strain AO21. Amplifications were performed in 100 μl reaction volumes for 35 cycles, using the GeneAmp kit and AmpliTaq DNA polymerase (Perkin-Elmer). After the initial denaturation at 95 °C for 5 min, each cycle consisted of denaturation (95 °C, 90 sec), annealing (57 °C, 90 sec) and extension (72 °C, 2 min). All reactions included a negative (sterile water) control.

Size determination and restriction digestion of PCR products. Aliquots (10 μl) of the amplification reactions were electrophoresed on 1% agarose gels made and run in 1X TAE buffer containing 0.5 μg/ml ethidium bromide [38]. The amplification products of the *mer*1-*mer*2 primer pair were digested with the restriction enzyme *Ava*I (New England Biolabs), electrophoresed as above and photographed.

Blotting and filter hybridization of PCR products. The gels containing *mer*, *pco*R and *pco*A PCR products were Southern-blotted [40] onto Hybond N filters (Amersham), which were then hybridized with the *mer* and *pco* probes labelled as below. Hybridizations were carried out overnight at 55 °C using Church buffer [41], filters were washed in wash buffer (40 mM Na_2HPO_4, pH 7.5, 1% SDS) 2X30 min at room temperature and

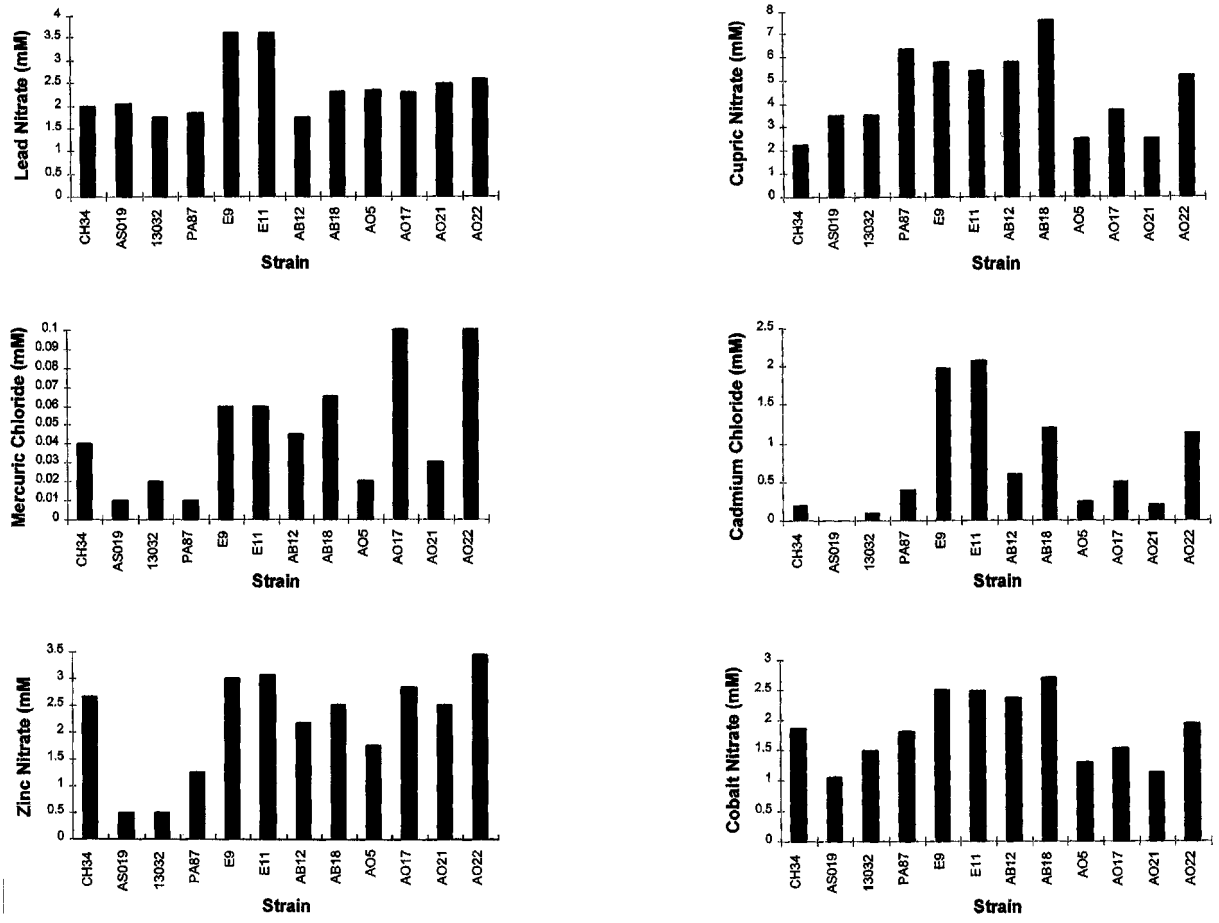


Figure 1. MICs of metal ions for control strains and selected soil bacterial strains. Details of the ranges of metal salt concentrations used are given in Materials and methods and details of the bacterial strains are given in Table 1.

1X30 min at 55 °C and exposed to Kodak XAR-5 X-ray film for 1–2 days at −70 °C.

Probe preparations. The *mer* probe was a 2.2 kb *Hin*dIII-*Eco*RI fragment of Tn*501* containing the *merRT*△*P* region [18] and the *pco* probe was a 12.5 kb *Hin*dIII insert of pPA87 encoding the *pco* operon [31, 42]. These were purified from the respective restriction digests run on gels by using the Bandpure DNA purification kit (Progen), and labelled by nick-translation using α-^{32}P-dCTP and the Nick-Translation System (Promega).

Purification, cloning and sequencing of PCR products. Three of the PCR products (amplification products of *Alcaligenes* strains AO17 and AO22 DNAs with *mer*1-*mer*2 primer pair and *Arthrobacter* strain E9 DNA with *pcoR*1-*pcoR*2 primer pair) were purified on Microspin^{TM} columns (Pharmacia), cloned into the *Eco*RV site of pBluescript SK+ (Stratagene) and sequenced manually by the chain termination method [43], using the T7 DNA Polymerase Sequencing kit (Pharmacia).

Results

Identification of soil isolates and determination of relative levels of resistance of the isolates to metals. Preliminary taxonomic classification of the eight selected Ardeer isolates was performed using classical biochemical and staining properties to provide a basis for differentiating strains which were otherwise distinct on the basis of colony morphology. The Gram-positive,

non-acid-fast bacteria were keyed out as belonging to the genera *Arthrobacter* and *Corynebacterium*, which were differentiated on the basis of glucose utilisation, and the remaining two Gram-negative strains were *Alcaligenes* species (Table 1), where all of these genera are commonly found in soil microflora. Further designation of these strains to species level was not possible based on their biochemical reactions and taxonomic determination is being undertaken currently by 16S RNA sequencing. The E and AB series of isolates were obtained following enrichment in lead-containing broths whereas the AO series were isolates randomly selected from the microflora recovered on non-selective media without enrichment for metal resistance.

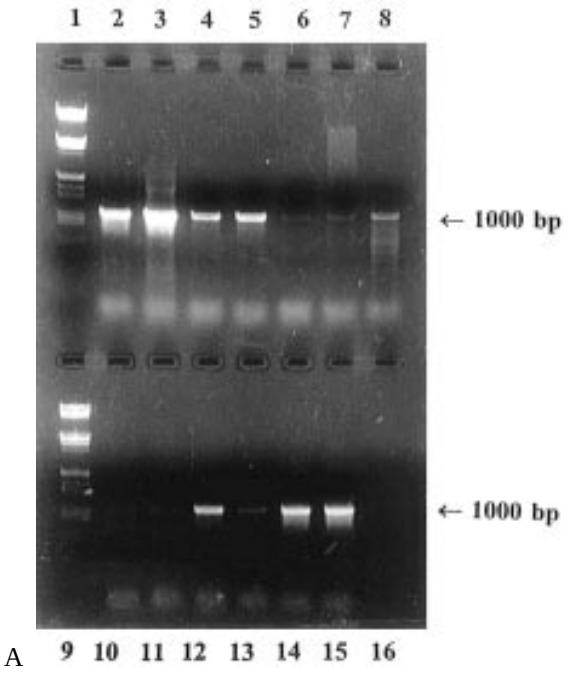
Previous experimental work in our laboratory [36] had shown that some of the Ardeer isolates grew poorly on minimal media which have been used by other researchers to determine MICs for several heavy metal ions, including the minimal medium used previously to determine lead resistance of *A. eutrophus* CH34. Consequently, MICs were determined using a rich medium which supported the growth of all isolates, although it was recognised that data generated would not be immediately comparable to previously published research. In particular, resistance to lead is difficult to determine because of non-specific binding of lead to media components, including polypeptides. However, by using a range of 20 strains with known resistance or presumed sensitivity to lead which served as controls, we could clearly differentiate between lead sensitiveand resistant strains using NAT media [36]. These strains included the lead resistant *A. eutrophus* strain CH34 and lead-sensitive variants, although it was noted that the level of lead resistance of strain CH34 was only subtly different to that seen for the sensitive *A. eutrophus* strains when screened on NAT plates [36]. Data presented in Figure 1 shows MICs determined following growth on NAT plates for six metal ions, including lead: *C. glutamicum* strains AS019 and ATCC 13032 served as controls with no known resistance to heavy metal ions, to provide a basis of comparison with MICs determined for the Gram-positive isolates, and strain CH34 is included in this data set as a Gram-negative strain with known resistance to the metal ions tested. Comparison of MICs of lead, zinc, copper, mercury, cadmium and cobalt indicated that several of the isolates were resistant to these metal ions, but each isolate exhibited a characteristic level of resistance to each metal (Figure 1). The Gram-positive isolates E9 and E11 were the most resistant of the test strains to lead, and the MICs for these metals were approximately two-fold that of *A. eutrophus* CH34 which is known to be lead-resistant (Table 1) and showed similar MICs to strain LH101, a lead-resistant *Enterobacter* strain (M. Mergeay, personal communication) [36]. These isolates also showed the highest level of resistance to cadmium and were also moderatly resistant to zinc, mercury, copper and cobalt. The MICs for copper for the isolates E9, E11, AB12 and AB18 were similar to those of pPA87 (which encodes Cu^r in *E. coli*, see Table 1) and *A. eutrophus* CH34 which is also known to encode Cu^r genes [44].

The Gram-negative isolates AO17 and AO22 (*Alcaligenes* species) were most resistant to mercury and were also resistant to high levels of zinc and cobalt. The levels of resistance to mercury were approximately 1.5 to 2-fold greater than those of the other test isolates as well as *A. eutrophus* CH34, which is known to have mer^r transposons [44].

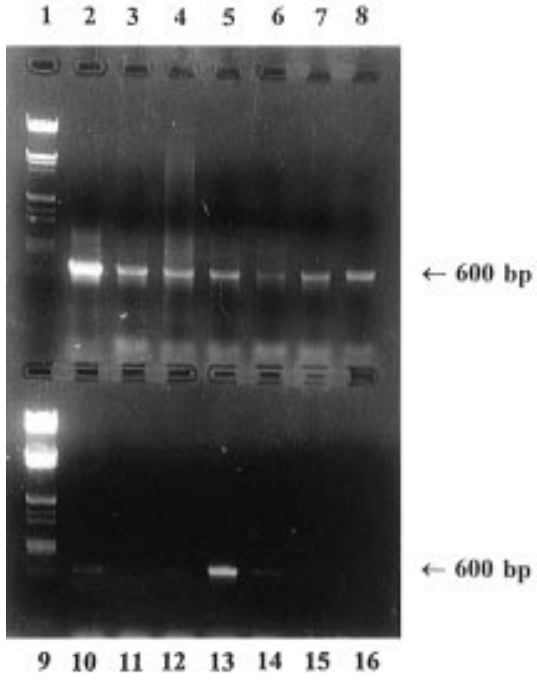
Amplification of mer and pco genes from soil bacteria. By using the primer pair *mer*1-*mer*2, amplification of a ~ 1 kb fragment was detected reproducibly in all eight soil bacterial isolates, in total genomic and plasmid DNA of *A. eutrophus* CH34 and in plasmid DNA of the isolate AO22 (Figure 2A). The size of the product was identical to that in the positive controls Tn*501* and Tn*21*, and corresponded to the expected 1 kb fragment encompassing the *merRT*△*P* region of the Tn*501 mer* operon [18]. Additional minor bands were seen for some isolates.

Using the primers *pcoR*1 and *pcoR*2, all isolates reproducibly showed the presence of a ~ 600 base pair (bp) fragment, which was identical to the expected fragment in the positive control pPA87 (Figure 2B). This fragment corresponds to the *pcoR* gene of the *pco* operon [9, 42 and B. Lee, personal communication]. Primer pair *pcoA*1-*pcoA*2, which spans a ~ 1.8 kb region encompassing the *pcoA* gene [9, 42 and B. Lee, personal communication], could amplify the expected fragment successfully from pPA87 (positive control) (Figure 2C). The isolates E9, AO5 and AO21 also showed this band and some additional smaller ones. Other test isolates showed either no amplification or smaller bands only. The 1.8 kb product, together with some additional bands, was also amplified from *A. eutrophus* CH34.

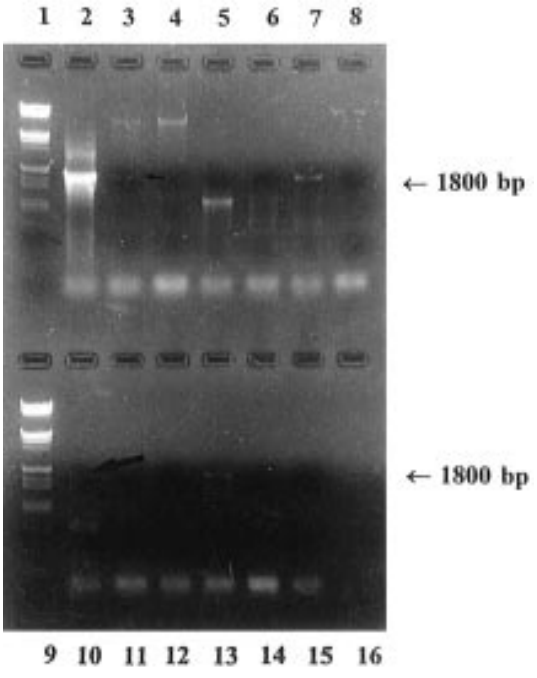
Amplification of czc and chr genes. Experiments involving amplification with *czcD*1-*czcD*2 primer pair yielded the expected ~ 400 bp products for the *A. eutro-*



A



B

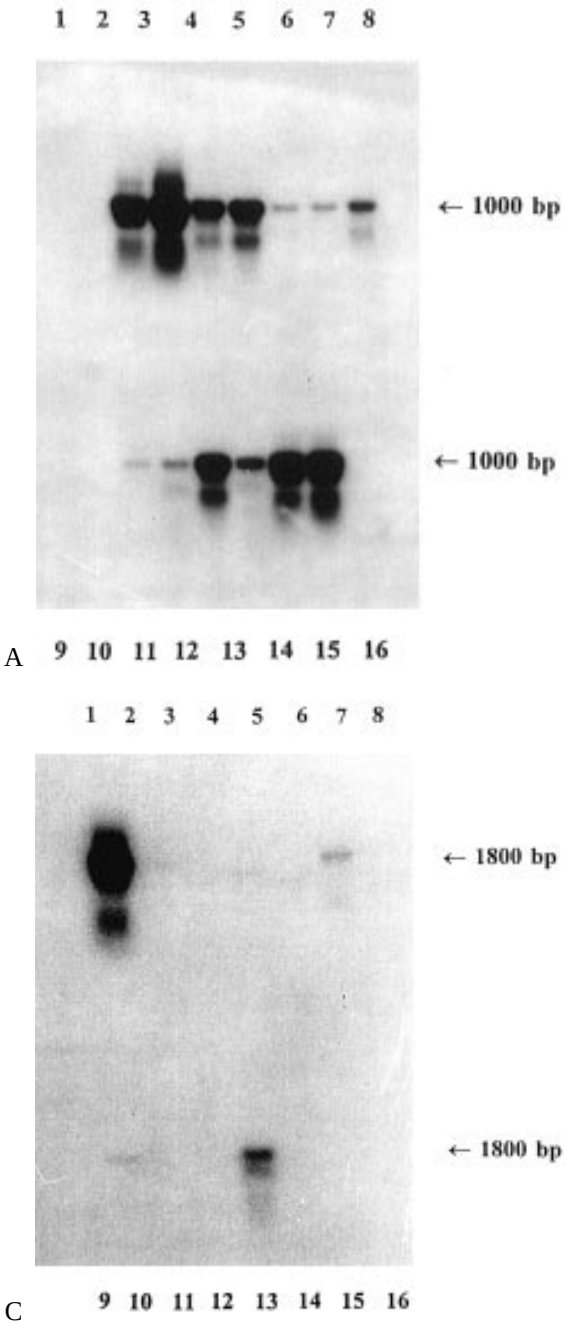


C

Figure 2. Agarose gel electrophoresis of PCR products of total genomic DNAs and/or plasmid DNAs with primer pair *mer*1-*mer*2 (Figure 2A), primer pair *pcoR*1-*pcoR*2 (Figure 2B) and primer pair *pcoA*1-*pcoA*2 (Figure 2C). Lanes for Figure 2A: 1 and 9, DNA marker (Lambda DNA digested with *Hin*dIII and *Eco*RI); 2, plasmid DNA of Tn*21*; 3, plasmid DNA of Tn*501*; 4, *A. eutrophus* CH34 genomic DNA; 5, *A. eutrophus* CH34 plasmid DNA; 6-8, 10-14, genomic DNA of isolates E9, E11, AB12, AB18, AO5, AO17, AO21 and AO22 respectively; 15, plasmid DNA of isolate AO22; 16, sterile water control. Lanes for Figure 2B and 2C: 1 and 9, DNA size marker (Lambda DNA digested with *Hin*dIII and *Eco*RI); 2, plasmid DNA of pPA87; 3-8, genomic DNA of isolates E9, E11, AB12, AB18, AO5, AO17 respectively; 10, genomic DNA of isolate AO21; 11, genomic DNA of isolate AO22; 12, plasmid DNA of isolate AO22, 13, *A. eutrophus* CH34 genomic DNA; 14, *A. eutrophus* CH34 plasmid DNA, 15, sterile water control; 16, blank (no sample loaded).

phus CH34 positive control and many other additional bands for the test isolates (data not shown). Most isolates yielded no amplification products with the primer pairs *czcA*1-*czcA*2 and *czcB*1-*czcB*2, one isolate (AO21) yielded a product of size other than expected with the primer pair *czcB*1-*czcB*2 (data not shown) and these results could not be reproduced.

Primer pair *chrA*1-*chrA*2 yielded the expected ~ 1.3 kb band in *A. eutrophus* CH34 (positive control) but only multiple smaller bands in some isolates (E11, AB18, AO17, AO21) or no amplification in others (E9, AB12, AO5, AO22) (data not shown). Similar results were obtained with primer pair *chrB*1-*chrB*2, where expected fragment of 450 bp could be produced from *A. eutrophus* CH34, but the test isolates yielded either no amplification (E9, E11, AB12, AO17, AO21, AO22), a very faint band of appropriate size (AB12) or a slightly larger band (AO5) (data not shown). Due to the poor amplification and reproducibility of results



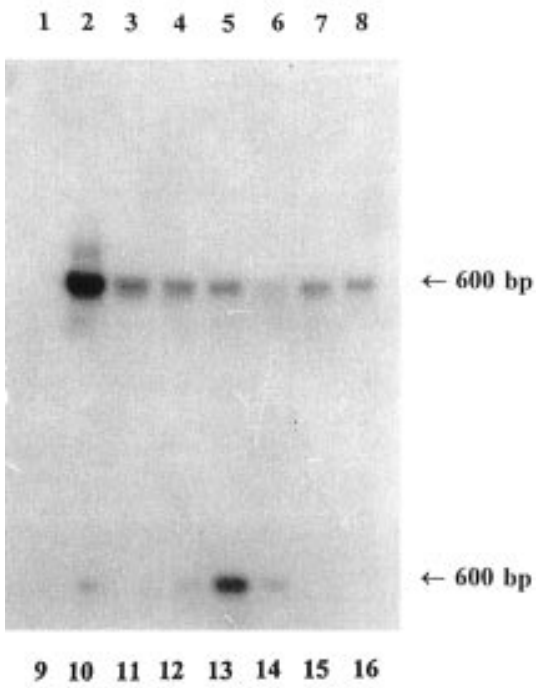


Figure 3. Southern blot hybridizations of the PCR products shown in Figure 2. Figure 3A shows results of hybridization of samples shown in Figure 2A with the *mer* probe. Figure 3B and 3C show hybridization of samples shown in Figure 2B and 2C respectively with the *pco* probe.

of most test isolates with the *czc* and *chr* primers, PCR and hybridization experiments for these genes were not pursued further.

Hybridization of PCR products with mer and pco probes. The amplification products from the gels illustrated in Figure 2A, 2B and 2C were transferred onto membranes and hybridized with *mer* (for samples shown in Figure 2A) and *pco* (for samples shown in Figure 2B and 2C) probes, to test whether the PCR products were the result of true amplification of homologous sequences or whether they were spurious amplifications of an unrelated area of the genome. Hybridization with the Tn*501 mer* probe produced strong hybridization signals for the 1 kb fragments (Figure 3A). Similarly, samples illustrated in Figure 2B showed strong hybridization signals for the 600 bp bands with the *pco* probe. (Figure 3B). None of the other minor bands seen in Figure 2A or 2B hybridized with the respective probes. These results indicate the presence of high homology between the probe and the appropriate fragments on the filters, as the hybridization and washing conditions would have allowed only a limited extent of mismatch between the two. The only hybridizations seen for the *pcoA* amplification products were the ~ 1.8 kb bands in the samples AO5, AO21 and *A. eutrophus* CH34 (Figure 3C). As expected, the *mer* probe hybridized with the sequences originating from Tn*21*, as the two Hg^r genetic systems are highly homologous [18]. The hybridization of the *mer* and *pco*

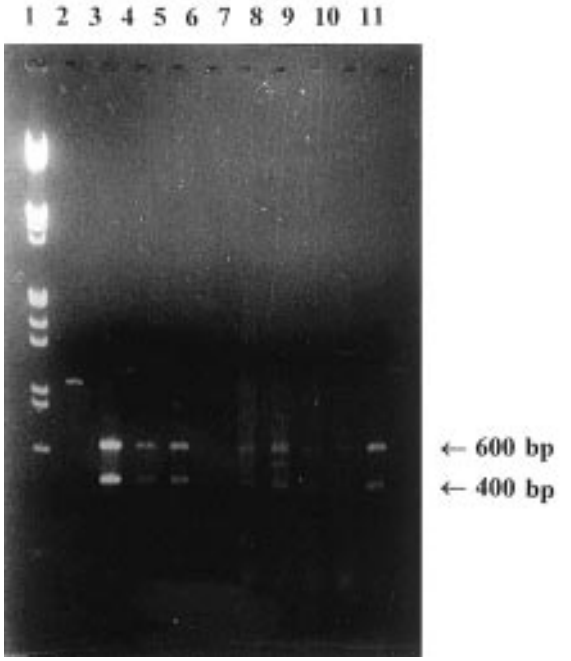


Figure 4. Agarose gel electrophoresis of *mer*1-*mer*2 PCR products digested with *Ava*I. Lanes: 1, DNA size marker (Lambda DNA digested with *Hin*dIII and *Eco*RI); 2, undigested *A. eutrophus* CH34 PCR product; 3-5: *Ava*I-digested PCR products of Tn*501*, *A. eutrophus* CH34 genomic DNA and *A. eutrophus* CH34 plasmid DNA respectively; 6-11, digested PCR products of genomic DNAs of E9, E11, AB12, AB18, AO5 and AO17 respectively.

probes to *A. eutrophus* CH34 is expected also, as this strain is Hg^r, Cu^r and encodes homologous sequences [2 and M. Mergeay, personal communication].

RFLP analysis of amplified mer sequences. The restriction map of the 1 kb *mer* RTΔP region of Tn*501* indicates the presence of an *Ava*I restriction site, dividing it into a 0.6 kb and a 0.4 kb fragment [18]. Since the *mer* amplification was aimed at this region, it was decided to subject the *mer* PCR products of the test isolates to restriction digestion by *Ava*I, to detect any polymorphisms present at this site. All digests revealed a 0.6 and a 0.4 kb band, indicating the presence of the *Ava*I site at a position similar to that in Tn*501* (Figure 4).

Cloning and sequencing of mer and pcoR amplification products. As PCR and probe hybridization had indicated similar results for all the test isolates, two samples (AO17 and AO22) from the *mer* and one (E9) from the *pcoR* amplification products were chosen for cloning and sequencing. Approximately 300–400 bases were sequenced from each of these (data not shown). Comparison of the AO17 and AO22 *mer* sequences with the appropriate 1 kb region of the Tn*501* mer operon [18], and of E9 *pcoR* sequence with the corresponding region of the *E. coli* pRJ1004 *pcoR* sequence [42 and B. Lee, personal communication] revealed >90% identity.

Discussion

The present investigation was aimed at determining whether the bacteria isolated from soil heavily contamined with lead had adapted to the high lead content in their microenvironment, and if so, which genetic factors could have contributed to such a phenotype. With the rapid developments in the recombinant DNA techniques, molecular methods are being applied more and more to investigations of this nature, as these methods often provide quick and accurate answers to the problems. A few investigations of this nature have been carried out in the last 5–10 years, however, we believe this is one of the first reports of application of molecular techniques to studying metal ion polluted environments in Australia.

Resistance to a variety of metal ions was examined in addition to lead because the literature had indicated that resistance often occurs for a range of metal ions rather than for a specific metal only [3, 4, 8]. MIC determination indicated that the soil bacteria used in the present investigation have developed resistance to several of the metal ions tested and some of the isolates showed high resistance to certain ions. The observation of high levels of mercury resistance in AO17 and AO21 is notable, as several other laboratories have identified mercury-resistant Gram-negative isolates from contaminated sites and these often have mercury-resistance encoding plasmids or transposons [1, 5–7, 14, 15]. A direct comparison of our MIC estimates with other studies was not attempted, as the differences in the metal-binding capacities of our media and those used by other investigators can result in discrepancies in the MIC results. *A. eutrophus* CH34 [4] was included in our study for comparative purposes and was indeed more resistant to Co, Cr, Zn, Pb, Cd and Hg when compared to the two *Alcaligenes* controls, as was expected due to the multiple resistance markers on pMOL28 and pMOL30 carried in strain *A. eutrophus* CH34.

In the absence of any genetic systems identified to encode lead resistance specifically, molecular investigations concentrated on looking for other heavy-metal

resistance genetic systems, particularly those that have been identified previously in bacteria isolated from contaminated environments. The *mer* system was chosen as it has been reported widely in different geographic locations and environments in the Northern hemisphere [1–3, 6, 11, 12, 34] and *czc* and *chr* systems were chosen to detect resistances to these ions in bacteria found at industrial sites [4, 5, 30]. The *pco* system was chosen even though it was isolated from an enteric bacterium, as it was discovered locally [9] and would provide a model for possible horizontal transfer of genes.

mer and *pcoR*-related sequences could be amplified successfully from the eight test isolates and positive hybridization of these to respective probes indicates that they were true amplification products corresponding to the endogenous *mer* or *pco* sequences in the genomic DNA of the isolates and not the results of amplification priming at unrelated locations in the genomes. The presence of mercury-resistance phenotype in lead-contaminated soil is not unexpected, since mercury-resistant microbes have been isolated previously from a variety of environments. However, the observation of Tn*501*-related sequences in the Gram-positive isolates is important, as these have been reported so far only for Gram-negative bacteria [reviewed in Ref. 17]. The observation that the two Gram-negative isolates (AO17 and AO22) were the most resistant ones to mercury prompted us to clone and obtain preliminary sequence data for their *mer* PCR products. The data, although partial, showed >90% identity to the appropriate region of Tn*501*, thus indicating that these two isolates probably contain Tn*501* or a similar genetic system.

The *pco* operon has been reported so far only in copper-resistant enteric bacteria [9, 31], so the amplification of *pcoR* in the soil isolates is an interesting observation. Its presence in the soil bacteria may suggest that the *pco* system could be either more widespread and not just restricted to enteric microorganisms, or it could have been spread by horizontal transfer between enteric and soil bacteria. Such modes of transmission, especially through animals, have been suggested to play a role in the dissemination of metal-resistance phenotypes. The *pcoR* PCR product from only one test isolate (E9) was sequenced and found to be very similar to the appropriate section of the *pcoR* system; thus it is not possible to determine at present whether the PCR products in the other isolates are authentic *pcoR* sequences or their homologues. *pcoR* has been shown recently to encode a regulatory protein with homologies to other regulators [42]. Thus the *pco*R amplification products observed in the present study could represent regulatory proteins with homology to *pcoR*. More widespread amplification of *pcoR* sequences compared to the *pcoA* sequences suggests that the *pcoR* (or related) sequences are more conserved than the *pcoA* which is a structural gene in the *pco* operon [9, 42]. Further work is needed to determine whether the entire *pco* system or only parts of it are present in these soil bacteria.

The lack of uniform amplification of various *czc*- and *chr*-related sequences in all test isolates, or the presence of fragments with sizes other than those obtained with positive control samples, suggests that either these sequences are present but highly diverged in the test isolates, or they are absent in, at least, some of the test strains. In either case, these sequences appear to have a limited role, if any, in contributing to metal resistance in the soil bacteria studied here. It is possible that these bacteria have other genetic systems such as *cnr* [24], *cadA* [28], *ncc* [8], or some as yet unidentified genes that contribute resistance to cadmium, cobalt, zinc, lead and nickel. Investigations are underway to test this possibility.

These and future studies should provide some insight into the molecular mechanisms of heavy metal ion resistance in these soil microorganisms and may demonstrate their utility in detecting environmental pollution by heavy metals.

Conclusions

Several Gram-positive and Gram-negative soil bacteria isolated from a lead-contaminated site exhibited resistance to a range of metal ions including lead, zinc, copper, cadmium, cobalt and mercury. PCR, DNA-DNA hybridization, RFLP and DNA sequencing were used to analyse the genetic systems involved in metal resistance in these bacteria. All isolates showed DNA sequences homologous to sections of the transposon-encoded *mer* and plasmid-encoded *pco* genetic systems of resistance to mercury and copper respectively. This is the first report of detection of *mer*-related sequences in Gram-positive isolates, and *pcoR*-related sequences in soil bacteria. The *czc* (cadmium-zinc-cobalt resistance) and *chr* (chromate resistance) genetic systems, on the other hand, could not be detected as uniformly in these bacterial isolates.

Acknowledgements

We are indebted to Drs. Barry T. O. Lee, Max Mergeay and Mark Osborn and Ms. Jill Williams for their generous gifts of various bacterial strains and plasmids, for sharing with us some of their unpublished results and for some very useful discussions and exchange of ideas. Technical suggestions and help from Mr. Scott Currie and Mr. Ki-Hyo Jang is also acknowledged.

References

1. Barkay T & Olson BH (1986) Phenotypic and genotypic adaptation of aerobic heterotrophic sediment bacterial communities to mercury stress. Appl. Environ. Microbiol. 52: 403–406
2. Diels L & Mergeay M (1990) DNA probe-mediated detection of resistant bacteria from soils highly polluted by heavy metals. Appl. Environ. Microbiol. 56: 1485–1491
3. Dressler C, Kues U, Nies D & Friedrich B (1991) Determinants encoding resistance to several heavy metals in newly isolated copper-resistant bacteria. Appl. Environ. Microbiol. 57: 3079–3085
4. Mergeay M, Nies D, Schlegel HG, Gerits J, Charles P & Van Gijsegem F (1985) *Alcaligenes eutrophus* CH34 is a facultative chemolithotroph with plasmid-bound resistance to heavy metals. J. Bacteriol. 162: 328–334
5. Nakamura K & Silver S (1994) Molecular analysis of mercury-resistant *Bacillus* isolates from sediment of Minamata Bay, Japan. Appl. Environ. Microbiol. 60: 4596–4599
6. Osborn AM, Bruce KD, Strike P & Ritchie DA (1993) Polymerase chain reaction-restriction fragment length polymorphism analysis shows divergence among *mer* determinants from Gram-negative soil bacteria indistinguishable by DNA-DNA hybridization. Appl. Environ. Microbiol. 59: 4024–4030
7. Rochelle PA, Wetherbee MK & Olson BH (1991). Distribution of DNA sequences encoding narrow- and broad-spectrum mercury resistance. Appl. Environ. Microbiol. 57: 1581–1589.
8. Schmidt T & Schlegel HG (1994) Combined nickel-cobalt-cadmium resistance encoded by the *ncc* locus of *Alcaligenes xylosoxidans* 31A. J. Bacteriol. 176: 7045–7054
9. Brown NL, Rouch DA & Lee BTO (1992) Copper resistance determinants in bacteria. Plasmid 27: 41–51
10. Gadd GM (1992) Heavy metal pollutants: environments and biotechnological aspects. In: Lenderberg L. (Ed) Encyclopaedia of Microbiology. Academic Press Inc., CA
11. Bogdanova ES, Mindlin SZ, Kalyaeva ES & Nikiforov VG (1988) The diversity of mercury reductases among mercury-resistant bacteria. FEBS Lett. 234: 280–282
12. Khesin RB & Karasyova EV (1984) Mercury-resistant plasmids in bacteria from a mercury and antimony deposit area. Mol. Gen. Genet. 197: 280–285
13. Griffin HG, Foster TJ, Silver S & Misra TK (1987) Cloning and DNA sequence of the mercuric- and organomercurial-resistance determinants of plasmid pDU1358. Proc. Natl. Acad. Sci. USA. 84: 3112–3116
14. Hobman J, Kholodii G, Nikiforov V, Ritchie DA, Strike P & Yurieva O (1994) The sequence of the *mer* operon of pMER327/419 and transposon ends of pMER327/419, 330 and 05. Gene 146: 73–78
15. Jobling MG, Peters SE & Ritchie DA (1988) Restriction pattern and polypeptide homology among plasmid-borne mercury resistance determinants. Plasmid 20: 106–112
16. Laddaga RA, Chu L, Misra TK & Silver S (1987) Nucleotide sequence and expression of the mercurial-resistance operon from *Staphylococcus aureus* plasmid pI258. Proc. Natl. Acad. Sci. USA. 84: 5106–5110
17. Misra TK (1992) Bacterial resistances to inorganic mercury salts and organomercurials. Plasmid 27: 4–16
18. Misra TK, Brown N, Fritzinger DC, Pridmore RD, Barnes WM, Haberstroh L & Silver S (1984) Mercuric ion-resistance operons of plasmid R100 and transposon Tn*501*: the beginning of the operon including the regulatory region and the first two structural genes. Proc. Natl. Acad. Sci. USA. 81: 5975–5979
19. Wang Y, Moore M, Levinson HS, Silver S, Walsh C & Mahler I (1989) Nucleotide sequence of a chromosomal mercury resistance determinant from a *Bacillus* sp. with broad-spectrum mercury resistance. J. Bacteriol. 171: 83–92
20. Bender CL, Malvick DK, Conway KE, George S & Pratt P (1990) Characterization of pXV10A, a copper resistance plasmid in *Xanthomonas campestris* pv. *vesicatoria*. Appl. Environ. Microbiol. 56: 170–175
21. Cervantes C, Ohtake H, Chu L, Misra TK & Silver S (1990) Cloning, nucleotide sequence and expression of the chromate resistance determinant of *Pseudomonas aeruginosa* plasmid pUM505. J. Bacteriol. 172: 287–291
22. Chen C-M, Misra TK, Silver S & Rosen BP (1986) Nucleotide sequence of the structural genes for an anion pump. The plasmid-encoded arsenical resistance operon. J. Biol. Chem. 261: 15030–15038
23. Cooksey DA, Azad HR, Cha J-S & Lim C-K (1990) Copper resistance gene homologs in pathogenic and saprophytic bacterial species from tomato. Appl. Environ. Microbiol. 56: 431–435
24. Liesegang H, Lemke K, Siddiqui RA & Schlegel HG (1993) Characterization of the inducible nickel and cobalt resistance determinant *cnr* from pMOL28 of *Alcaligenes eutrophus* CH34. J. Bacteriol. 175: 767–778
25. Nies DH (1992) Resistance to cadmium, cobalt, zinc and nickel in microbes. Plasmid 27: 17–28
26. Nies DH, Nies A, Chu L & Silver S (1989) Expression and nucleotide sequence of a plasmid-determined divalent cation efflux system from *Alcaligenes eutrophus*. Proc. Natl. Acad. Sci. USA. 86: 7351–7355
27. Nies A, Nies DH & Silver S (1990) Nucleotide sequence and expression of a plasmid-encoded chromate resistance determinant from *Alcaligenes eutrophus*. J. Biol. Chem. 265: 5648–5653
28. Nucifora G, Chu L, Misra TK & Silver S (1989) Cadmium resistance from *Staphylococcus aureus* plasmid pI258 *cadA* gene results from a cadmium-efflux ATPase. Proc. Natl. Acad. Sci. USA. 86: 3544–3548
29. Silver S & Walderhaug M (1992) Gene regulation of plasmid- and chromosome-determined inorganic ion transport in bacteria. Microbiol. Rev. 56: 195–228
30. Stoppel R-D & Schlegel HG (1995) Nickel-resistant bacteria from anthropogenically nickel-polluted and naturally nickel-percolated ecosystems. Appl. Environ. Microbiol. 61: 2276–2285
31. Williams JR, Morgan AG, Rouch DA, Brown NL & Lee BTO (1993) Copper-resistant enteric bacteria from United Kingdom and Australian piggeries. Appl. Environ. Microbiol. 59: 7027–7033

32. Barkay T, Fouts DL & Olson BH (1985) Preparation of a DNA gene probe for detection of mercury resistance genes in Gram-negative bacterial communities. Appl. Environ. Microbiol. 49: 686–692
33. Barkay T, Liebert S & Gillman M (1989) Hybridization of DNA probes with whole-community genome for detection of genes that encode microbial responses to pollutants: *mer* genes and Hg^{2+} resistance. Appl. Environ. Microbiol. 55: 1574–1577
34. Bruce KD, Hiorns WD, Hobman JL, Osborn AM, Strike P & Ritchie DA (1992) Amplification of DNA from native populations of soil bacteria using the polymerase chain reaction. Appl. Environ. Microbiol. 58: 3413–3416
35. Holben WE, Jansson JK, Chelm BK & Tiedje JM (1988) DNA probe method for specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54: 703–711
36. Manovski S, Roddick FA & Britz ML (1992) Isolation of lead-tolerant microbes from a contaminated site in Melbourne, Australia. In: Soil Decontamination Using Biological Processes, EFB Task Group on Soil Decontamination Using Biological Processes (pp 689–695). DECHEMA, Frankfurt
37. Reade E (1981) Microbial techniques. School of Microbiology, University of Melbourne, Australia
38. Sambrook J, Fritsch EF & Maniatis T (1989) Molecular cloning: a laboratory manual. (Ed. II). Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
39. Haynes JA & Britz ML (1990) Effects of growth conditions on electro-transformation frequencies of *Corynebacterium glutamicum*. J. Gen. Microbiol. 136: 255–263
40. Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503–517
41. Church GM & Gilbert W (1984) Genomic sequencing. Proc. Natl. Acad. Sci. USA. 81: 1991–1995
42. Brown NL, Barrett SR, Camakaris J, Lee BTO & Rouch DA (1995) Molecular genetics and transport analysis of the copper-resistance determinant (*pco*) from *Escherichia coli* plasmid pRJ1004. Mol. Microbiol. 17: 1153–1166
43. Sanger F, Nicklen S & Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74: 5463–6467
44. Diels L, Dong Q, van der Lelie D, Baeyens W & Mergeay M (1995) The *czc* operon of *Alcaligenes eutrophus* CH34: from resistance mechanism to the removal of heavy metals. J. Ind. Microbiol. 14: 142–153