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Reviews

# Microbial Heavy Metal Resistance Transposons and Plasmids: Potential Use for Environmental Biotechnology

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## Introduction

Many genes on bacterial transposons and plasmids that encode specific resistance systems for toxic heavy metal ions and inorganic oxyanions have been identified. These include for resistances to $Ag^{+}$, $AsO_2^{-}$, $AsO_4^{3-}$, $Cd^{2+}$, $Co^{2+}$, $CrO_4^{2-}$, $Cu^{2+}$, $Hg^{2+}$, $Ni^{2+}$, $Sb^{3+}$, $TeO_3^{2-}$, $Tl^{+}$ and $Zn^{2+}$. A working hypothesis for our laboratories has been that microbial resistance systems arose shortly after prokaryote life started (in an already metal-polluted world) and therefore will be found in essentially all microbial types. In addition, human activities create local environments of high selection for heavy metal resistance. Since the discovery that mercury resistant bacteria could decompose organomercurials[25] and volatilize mercury, as metallic mercury[44,97], the subject of genetically-precise bacterial transposon- and plasmid-determined resistances to toxic inorganic cations and anions has burgeoned.

While the mercury resistance systems in all bacteria studied are related and have diversified by divergent evolution, the widely-found arsenic resistance systems have evolved more than once and show convergent evolution, generally with differences between Gram-positive and Gram-negative bacteria. Cadmium resistance uses a single-polypeptide ATPase in Gram-positive bacteria (including *Staphylococcus*, *Listeria*, and *Bacillus*) to pump $Cd^{2+}$ out from the cell, but a three-polypeptide chemiosmotic cadmium efflux pump functions in Gram-negative bacteria. These systems are of independent evolutionary origin. It follows that if we want to understand the environmental microbiology of toxic inorganic ions and to use microbial processes for Environmental Biotechnology, then we need to understand what is happening in the environment. In this paper, we can only briefly review the range and mechanisms of bacterial toxic inorganic ion resistances carried by transposons and plasmids. Horizontal gene transfer of the resistance determinants between different bacterial species is also covered. From the reviewing of recent science on bacterial heavy metal resistance, some overall concepts for cleaning up the environmental heavy metal pollution using bacterial gene transfer in the environment may be derived.

## Transposons for heavy metal resistace

### General Aspects of Transposons:

Three major classes of bacterial transposons have been identified. Class I transposons include insertion sequences (IS) that encode at least one transferase gene and composite IS structure(s) with plural IS elements[2,27,48] (Fig. 1a). On composite transposons, structural gene(s) and gene operon(s) are sometimes flanked between plural IS elements[41,55,100] (Fig. 1b). A second type of the transposons, class II transposons, encode a transferase gene, a resolvase gene and a resolution site[2,28,31] (Fig. 1c). Both class I and II transposons have short inverted repeat nucleotide sequences at each end of the transposon. A third type bacterial transposon is classified as conjugative transposons[23,24,79,83]. This class of transposons has an *int-Tn* gene which is essential for excision and integration and a *xis-Tn* gene which stimulates excision[62]. Fig. 1d shows a typical conjugative transposon, Tn*916*, found in the Gram positive bacterium *Enterococcus faecalis*. Conjugative transposons are self-transferable by bacterial contact (conjugation) without help by other conjugative

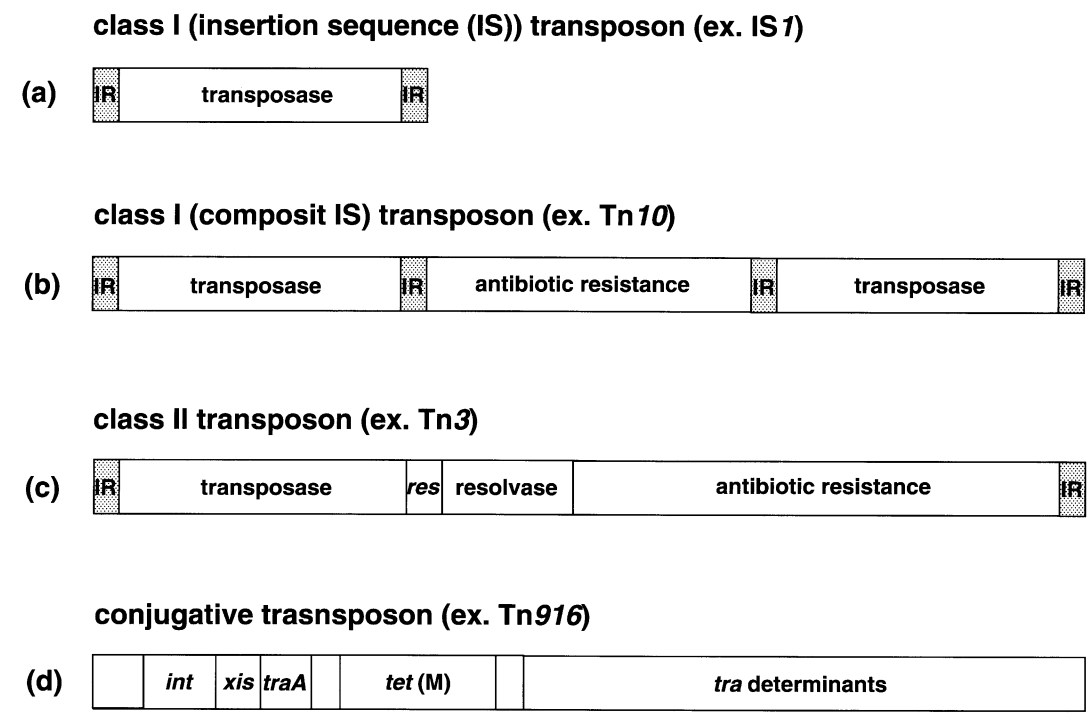


Fig. 1. General structure of three types of transposons. (a) class I (insertion sequence (IS)) transposon, (b) class I (composit IS) transposon, (c) Class II transposon, and (d) conjugative trasnsposon.

elements such as conjugative plasmids. Therefore, transposons are recognized as a means of intracellular and intercellular gene transposition.

**Transposons for Mercury Resistance:**

The first recognized mercury resistance transposon, Tn*501*, was isolated from Gram negative bacterium *Pseudomonas aeruginosa*[94]. Its mercury resistance determinants and transposition mechanisms[6,7] were analyzed. After Tn*501*, a different mercury resistance transposon, Tn*21* and its mercury resistance operon were recognized on the IncFII plasmid NR1 (also sometimes called R100.1 or R222)[1]. The wide distribution of mercury resistant transposons in Gram negative bacteria was studied by Osborn et al.[67,68] and Pearson et al.[71] with results indicating wide presence in natural populations of Gram negative bacteria from environmental and clinical sources. Recently, a novel class II transposon, Tn*MERI1*, that carries genes for resistance to mercurial compounds was isolated from a Gram positive bacterium[35]. Fig. 2 shows the structure of this transposon and the details of its mercury resistance module. Tn*MERI1* was found from on *Bacillus megaterium* strain MB1 that was isolated from sediment of Minamata Bay, Kyushu Japan, the site of an infamous mercury poisoning disaster. This transposon has a bacterial intron and two separate *mer* operons with two regulatory genes and three organomercurial lyase genes[34]. Closely related class II mercury resistance transposons from Gram positive bacteria were reported from non-marine soil isolated from Russia[3].

The *mer* operons of nearly 100 Minamata Bay *Bacillus* strains including *B. megaterium* MB1 showed high se-

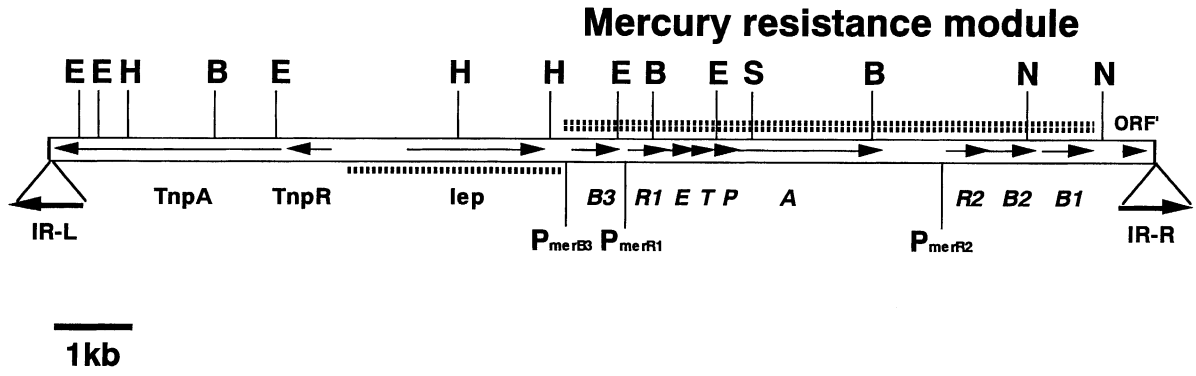


Fig. 2. Complete structure of the mercury resistance transposon, Tn*MERI1*, isolated from Gram negative bacterium *Bacillus megaterium* MB1.

quence similarity to one another, to the Russian isolates and to that of *B. cereus* RC607, that was isolated from Boston Harbor, USA. This was shown with restriction endonuclease maps, Southern hybridization patterns, PCR products and sequences[30,54].

Since the *mer* operon of *B. megaterium* MB1 is encoded in the transposon Tn*MERI1*, it was of interest whether this same transposon mediated gene transfer resulting in these geographically separated strains with essentially identical mercury resistance determinants. To amplify the complete region of the putative mercury-resistant transposon, the 38-bp nucleotide sequence of the class II transposon IR downstream the *mer* operons both of *Exiguobacterium* sp. TC38-2b and *B. cereus* RC607[4] was used to design a single primer for PCR. An 11.5-kb DNA fragment was amplified by PCR using the single primer and was mapped. By Southern hybridization restriction mapping, demonstrating that the *mer* operon region of *B. megaterium* RC607 was also encoded in an transposon region with terminal IR sequences. Within the *mer* operon module and the transposition module, the restriction map of strain RC607 was identical to those of *Exiguobacterium* sp. TC38-2b[4] and *B. megateruim* MB1. A mobile genetic element which is classified a member of bacterial group II introns[15,49] was located between resolvase gene and *mer* operon[35]. However, the intron did not exist in the transposon region of *B. cereus* RC607 and was transposed to other position of the chromosome[35]. The transposon encoded in the plasmid of *Exiguobacterium* sp. TC38-2b and encoded in chromosomes of other Minamata Bay *Bacillus* strains also have no introns. These similar transposons encoded in the chromosome of *B. cereus* RC607 and the plasmid of *Exiguobacterium* sp. TC38-2b were designated Tn*5084* and Tn*5085*, respectively[3] to distinguish them from the intron containing transposon Tn*MERI1*. By partial sequence of the Tn*5084* encoding on *B. cereus* RC607, it was confirmed that Tn*5084* is identical to the Tn*MERI1* except for the intron region. This result confirms the involvement of transposons in global dissemination of mercury resistance operon among Gram positive bacilli strains.

The bacterial gene for key enzyme of mercury detoxification, *merA* gene, encodes a NADPH-dependent FAD-containing mercuric reductase, reducing intracellular $Hg^{2+}$ to $Hg^0$, which then freely diffuses out from the cells[50,87,102]. The mercuric reductase MerA from *B. cereus* RC607 was crystallized and its structure was solved[81]. [This is the only protein of bacterial mercury resistance determinants to be solved by x-ray crystallography to date.] The secondary and tertiary structures of mercuric reductase are remarkably similar with that of glutathione reductase (the structure of the human enzyme ahs been solved), except for an additional N-terminal 160 amino acid residues in mercuric reductase, which do not occur in glutathione reductase. These 160 amino acids lack of a fixed position in the crystal[81] and have been postulated[86,87] to function much like a baseball mitt, taking $Hg^{2+}$ from the membrane protein MerT and passing it on to the vicinal cysteine pair at the carboxyl end of the reductase polypeptide. This carboxyl terminal region also differs between mercuric reductase and glutathione reductase (as is reasonable for differing substrate-binding determinants), whereas the redox active sites and regions involved with binding NADPH and FAD are highly homologous. The amino-terminal region is homologous with the small periplasmic mercury-binding protein MerP (of Gram negative bacteria) for which the structure has been solved by NMR spectroscopy[95]. MerP contains an additional vicinal cysteine pair[50,86,87] and folds in a compact alpha-helix beta-sheet form with the two cysteines forming a linear S-Hg-S structure on the protein side[95]. The *Bacillus* mercuric reductase sequence from the various Japanese and Russian bacteria as well as strain RC607 contain a fusion dimeric form of two MerP-like 80 amino acid long sequences forming the 160 amino acid N-terminus that is not in a fixed position in the crystal[81]. The MerA enzyme functions as a dimer[81] containing one NADPH site and one FAD in each subunit. $Hg^{2+}$ binds to the MerA dimer using four cysteine sulfurs (Cys135-Cys140 from one subunit and Cys558-Cys559 from the other)[18,20,81,86].

## Plasmids for heavy metal resistance

### Plasmids for Mercury Resistance:

Whereas the homologous *mer* mercury resistance determinants of all Bacilli studied appear to be chromosomal, those of other Gram positive bacteria such as *Staphylococcus aureus* and those of Gram negative bacteria are generally found on multiresistance plasmids that also have genes for antibiotic resistances[86,87]. Closely related plasmid systems for resistances to inorganic mercury and sometimes organic mercurial compounds have been found on plasmids of Gram negative. For example, in a collection of some 800 plasmids that had been mobilized from various Gram negative bacteria into *E. coli*, 25% expressed mercury resistance[49]. The mercury resistance plasmids from several different sources of bacteria have been actively studied and more than fifteen plasmids that contain *mer* operons have been isolated and their *mer* operons were partially or completely sequenced[50,87,102]. The *mer* operons identified from plasmids are shown in Fig. 3.

Mercury resistance (*mer*) operons are divided into two groups. Those that confer resistance to both organic and inorganic mercury are designated as broad spectrum *mer* operons and those confer resistance only to the inorganic mercuric ion are designated as narrow spectrum *mer* operons[82]. In most Gram negative bacteria, the order and approximate functions of the *mer* genes are the same[81,85,87] (Fig. 3(a)), with the exception of the *mer* determinant from *Acidothiobacillus ferrooxidans*. In most Gram positive bacteria, the order and approximate functions of the *mer* genes are also the same (Fig. 3(b)), with the

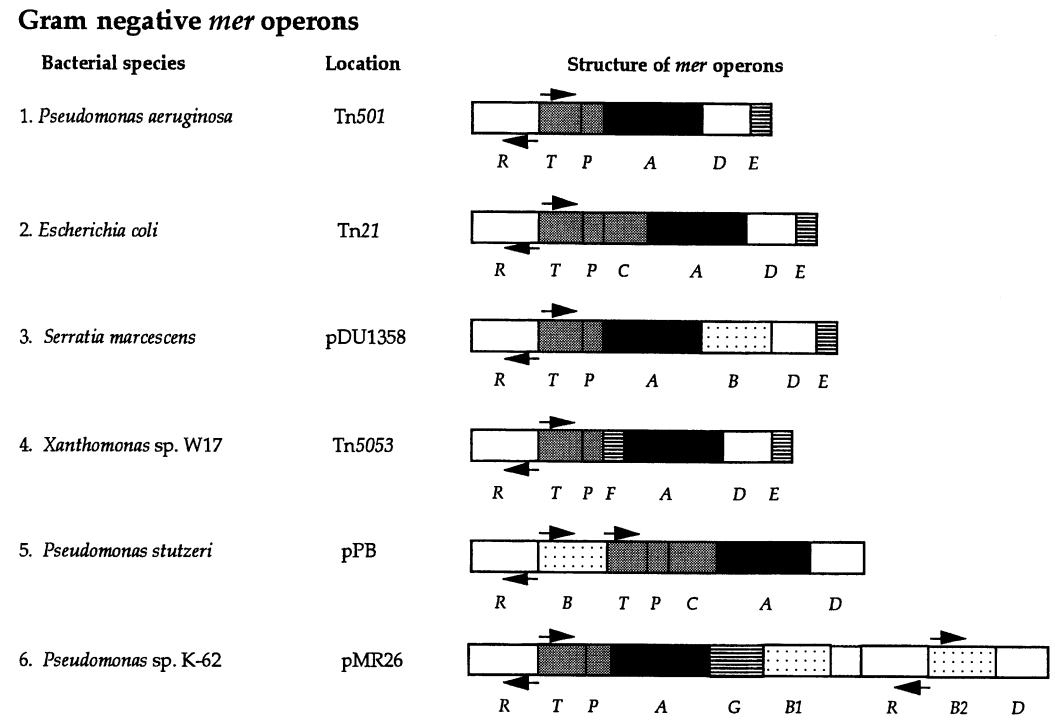


Fig. 3(a)

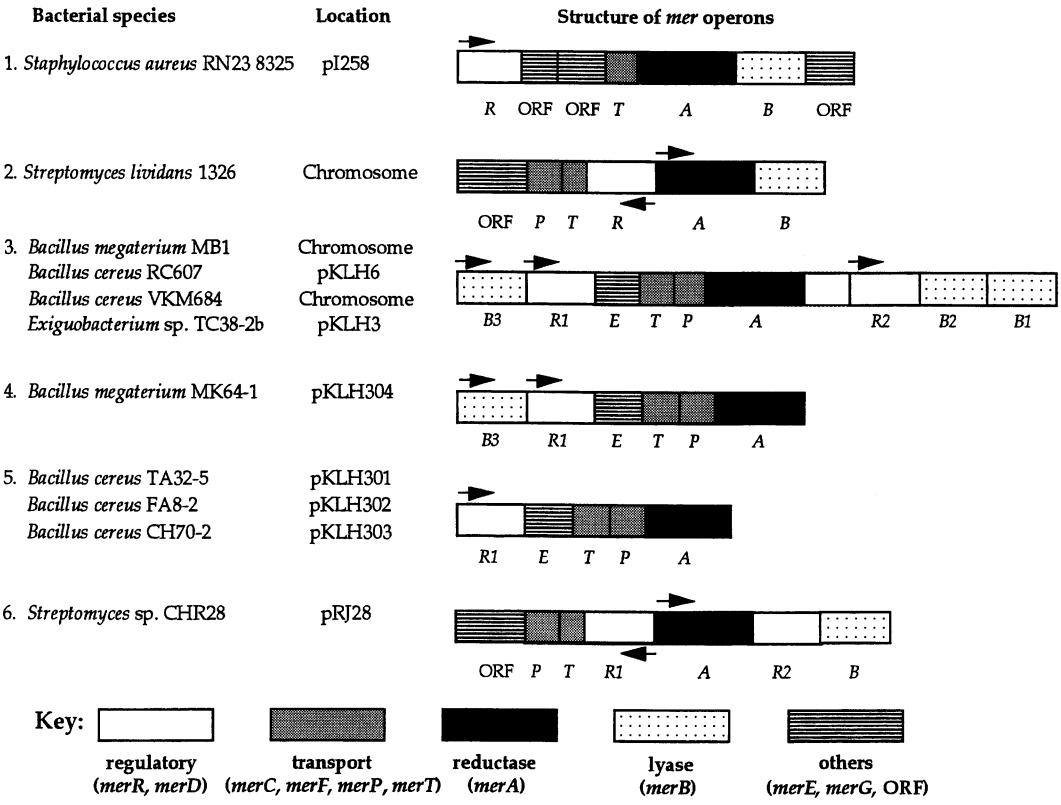


Fig. 3(b)

Fig. 3. Schematic representation of *mer* operons on plasmids from Gram negative bacteria (a), and Gram positive bacteria (b).

exception of the *mer* operon from *Streptomyces* spp. In the plasmids from Gram negative bacteria, these systems start with a regulatory gene, *merR*, whose product is a unique positively-acting activator protein. In addition of the *merR* regulatory gene, in almost all *mer* operons of Gram negative bacteria, there is a second regulatory gene,

merD (again an exception is the environmentally important acidophilic soil microbe *A. ferrooxidans*). The predicted MerD amino acid sequences are homologous with those of MerR, especially in the helix-turn-helix DNA-binding region. In vivo complementation analysis showed that MerD functioned as a trans-acting regulator[50,51] and in vitro experiments (gel retardation and footprinting) with purified plasmid pDU1358 MerD showed that it binds specifically to the same *mer* mRNA transcriptional start operator region and protects the same nucleotide residues as does MerR[51].

Except for the monocistronic *merR*, the remaining genes of most Gram negative *mer* operons are transcribed as a single mRNA in the opposite orientation. The first two genes, *merT* and *merP*, encode proteins involved in mercuric ion transport[32,33,47,50,87]. MerP is a $Hg^{2+}$ binding protein (one $Hg^{2+}$ per MerP monomer), utilizing a vicinal cysteine pair[78,95]. Located in the periplasmic space, MerP functions as a "shuttle", delivering $Hg^{2+}$ to the MerT inner membrane protein[33,50,87]. MerT has two vicinal cysteine pairs. Membrane topography analysis suggests that one vicinal cysteine pair faces the periplasmic space and the other faces the cytoplasm. $Hg^{2+}$ may be released from MerP to the periplasmic-side cysteine pair of MerT and then transferred to the cytoplasmic-side pair, and handed subsequently directly to the C-terminal cysteine pair of mercuric reductase[20], without being releasd into the cytoplasm. Another additional transport gene in a *Pseudomonas* plasmid pMR26, *merG*, was also reported (Fig. 3a). The MerG product protein appears to function as a phenylmercury-specific transporter[42].

In the plasmids of Gram positive bacteria, three $Hg^{2+}$ transport genes have also been defined. Three open reading frames in *Staphylococcus aureus* plasmid pI258 *mer* operon between *merR* and *merA* (Fig. 3(b)) appear to be involved in $Hg^{2+}$ transport and the three gene products have been directly identified[45,89]. The three transport genes were also identified on a plasmid pKLH3 from a Gram positive *Exiguobacterium* sp. TC38-2b, and those were designated as *merE*, *merT*, and *merP*, respectively[3,34]. The MerP protein sequence of Gram positive bacteria is weakly homologous to the MerP periplasmic protein of Gram negative bacteria.

All bacteria that are resistant to mercurial compounds have a *merA* gene in their *mer* operon. The *merA* gene encodes the NADPH-dependent FAD-containing mercuric reductase, reducing intracellular $Hg^{2+}$ to $Hg^0$, as mentioned above, and is essential for mercury resistance.

Sequenced *mer* operons encoded on broad-spectrum *mer* plasmids have *merB* genes determining the enzyme organomercurial lyases (that cleave the C-Hg bond to release $Hg^{2+}$ which is subsequently reduced by the mercuric reductase) following *merA*[43,87]. The frequency of broad spectrum *mer* operons among the total group of *mer* operons (narrow plus broad-spectrum) varies with bacterial groups, from essentially all in Gram positive isolates to date, to approximately 50% in plasmids found in pseudomonads to less than 5% with *mer* plasmids in Gram negative enterobacteriaciae. In Gram positive Bacilli, there can be three organomercurial lyase genes, *merB1*, *merB2* and *merB3* (Fig. 3(b)).

**Plasmids for Arsenic Resistance:**

Plasmid-mediated arsenic resistant bacteria have been widely found in various sources[12]. Increasinmg numbers of these plasmid systems are being sequenced as are comparable *ars* arsenic resistance systems on bacterial chromosomes as the number of sequenced genomes and plasmids is rapidly changing[52,75,85]. Basically the same arsenic resistance system is found on plasmids from *Escherichia coli*[8,14], *Staphylococcus aureus*[39] and *Staphylococcus xylosus*[77]. However, the number of arsenic resistance genes on plasmids of Gram negative and Gram positive plasmids is different (Fig. 4). The *ars* operons from *E. coli* plasmids R773 and R46 consist of total five genes, *arsR*, *arsD*, *arsA*, *arsB* and *arsC* in order[8,14,80,103], but only three genes (*arsR*, *arsB* and *arsC*) are found from the *ars* operons from *Staphylococcus* plasmidS[39,103]. Several summaries of understanding of plasmid-mediated arsenic resistance have appeared[12,40,52,74,84,90].

The *ars* operons are transcribed as a single polycistronic mRNA[39,40], regulated by the *arsR* gene[80] which encodes a trans-acting repressor protein, and can be induced by arsenate, arsenite, antimonite and bismuth in vivo[77,80,90,103]. In addition to *arsR*, the *E. coli* plasmids *ars* operons have a second regulatory gene, *arsD*. The *arsD* (and *arsA*) genes are mssing from the chromosomal versions of the *ars* operons that are found and functional[45] on the two *E. coli* strains for which the entire genomes have been sequenced, strain K-12, which is the standard laboratory strain, and strain O157H which causes serious human infections, but *arsD* is found on a series of new genomes including thjose from *Bacillus* and *Klebsiella*.

Following *arsD*, there is the *arsA* gene in the plasmids R773 and R64, that is missing from both staphylococcal plasmids[39,77] and the *E. coli* chromosome *ars* operon (GenBank U00039). The *arsA* gene encodes an ATPase subunit, as inferred initially from sequence homologies with the ATP-binding regions with other ATP-binding proteins[54] and subsequently studied in elegant detail[40,74].

Both *Staphylococcus* and *E. coli arsB* genes encode integral membrane proteins, 428 or 429 amino acids in length and 58% identical in sequence. This is an unusually high sequence conservation elvel when comparing proteins from Gram positive and Gram negative bacteia. The *E. coli* plasmid R773 ArsB has 12 transmembrane spans[104] and is the membrane anchor for the ArsA ATPase subunits[17]. The *S. aureus* ArsB alone is responsible for arsenite resistance and efflux with membrane potential as energy source[5]. Co-expression of the *E. coli* ArsA with the *S. aureus* ArsB dramatically increased the level of arsenite resistance, suggesting a direct physical interaction between

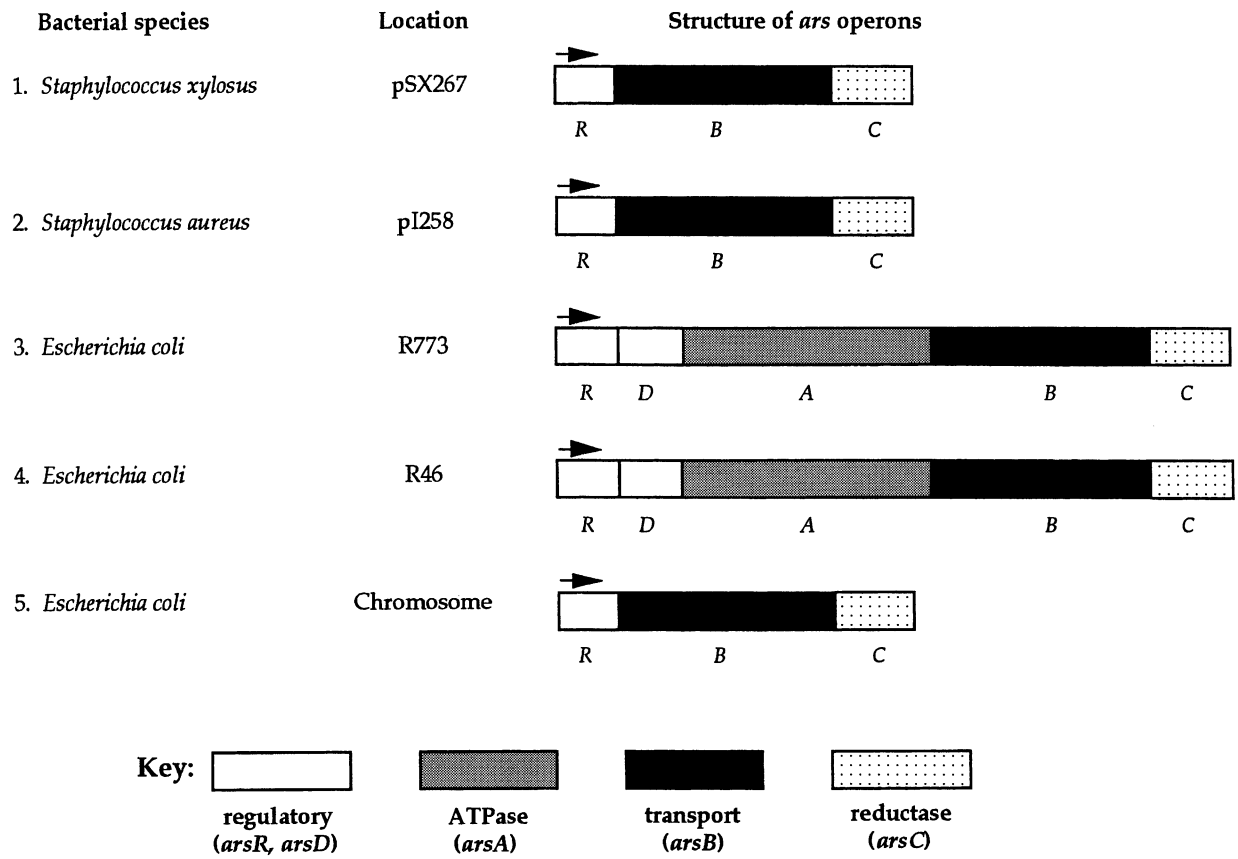


Fig. 4. Genes and products for arsenic resistance in *S. aureus* and *E. coli*. Alignment and functions (below) of arsenic resistance genes (shaded boxes). ArsD has no recognized DNA binding motif. Genetic analysis, however, showed that the ArsD protein is an inducer-independent trans-acting regulatory protein which controls the upper level of expression of the ars genes, and is not needed for regulating low level ars gene expression (Wu and Rosen, 1993).

these two proteins[5,8], one from a Gram negative bacterium and the other from a Gram positive bacterium.

The last gene in all four operons (*arsC*) encodes an arsenate reductase enzyme (131 amino acid residues for *S. aureus* pI258 and 141 for *E. coli* plasmid R773), that reduces intracellular arsenate to arsenite which is extruded out from the cells by the pump[26,39,52,76,84]. Although ArsC of staphylococci and *E. coli* catalyze the same reaction, reduction of $AsO_4^{3-}$ to $As(OH)_3$, the two proteins (anmd their encoding genes) lack significant sequence hoimology. Furthermore in the last year, x-ray crystal structures of Gram positive and Gram negative arsenate reductase have been published (summarized in ref.52)) and along with enzymatic assays establish that the two forms of arsenate reductase represent convergent evolution by separate pathways, much as the wings of birds and insects are unrelated except both provide flight[52]. Similarly, there appear to be two independently evolved clades of the ArsB membrane efflux proteins and the distinction between Gram positive bacteria for one clade and Gram negative bacteria for another seems to have broken down, with indication of much lateral transfer and rearrangmetns of gene orders. For example, the *Pseudomonas aeruginosa* genome has both types of arsenate reductases. These findings are been summarized in depth recently[52].

**Plasmids for Cadmium Resistance:**

The $Cd^{2+}$ efflux P-type ATPase (CadA) from staphylococcal plasmid pI258 (Fig. 5) was the first of a system now found widely in Gram positive bacteria, including *Staphylococcus*[38,63,82,86,92,93] and *Bacillus*[105]. The ATPase protein is inducibly synthesized[99,106] when resistant cells are exposed to $Cd^{2+}$, it extrudes intracellular $Cd^{2+}$ from the cells using ATP as energy source[91]. It is interesting to note that the subsequently cloned and sequenced genes for the human Menkes and Wilson's diseases show amino acid sequences predicted from the cDNA sequences that are more closely homologous to the bacterial P-type cadmium ATPases than to those of other P-type ATPases of eukaryotes[10,16,38,85]. Both Menkes' and Wilson's diseases result from defects in human $Cu^{2+}$ transport (and metabolism), but the diseases that result differ in symptoms and gene expression occurs in different tissues of the human body[19]. The adjacent gene *cadC* produces a repressor protein that binds to the operator region of DNA, preventing transcription. CadC dissociates from the DNA

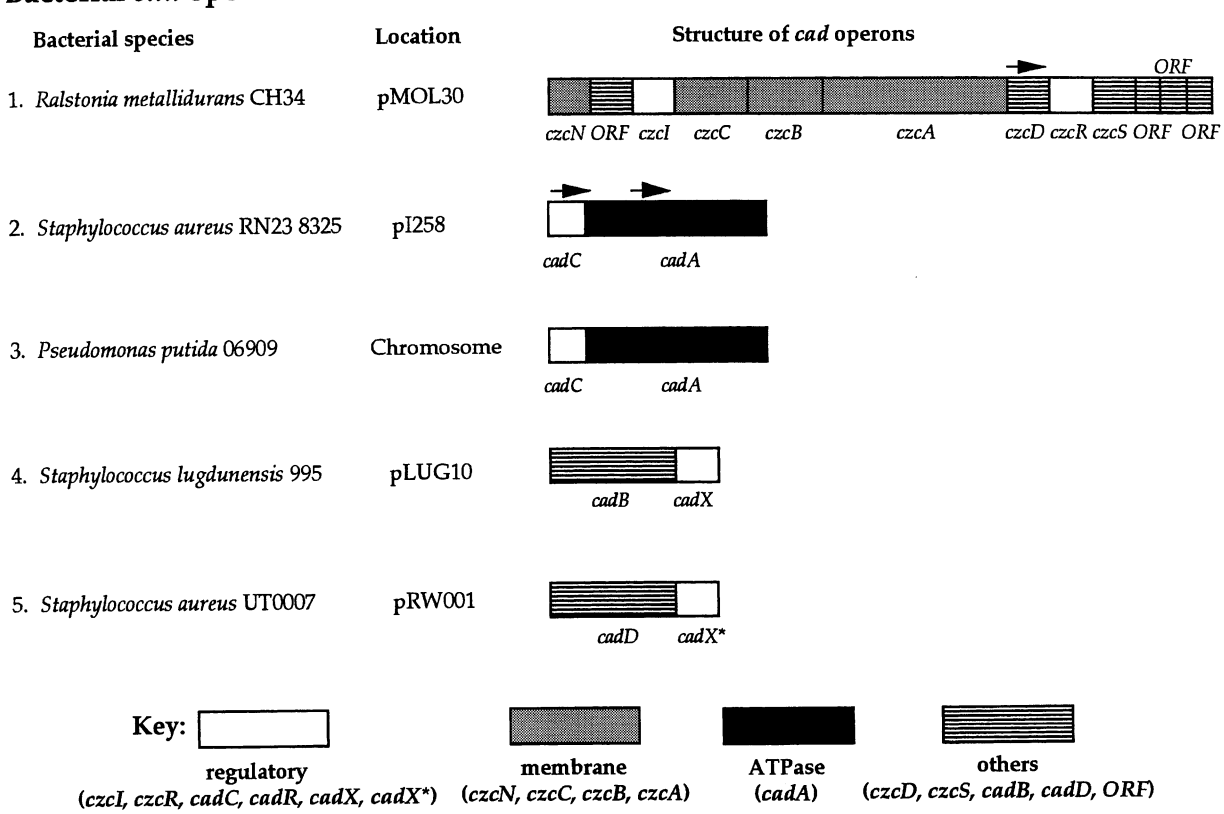


Fig. 5. The *cad* operon of *S. aureus* plasmid pI258, and the Czc (cadmium, zinc and cobalt) and Cnr (cobalt and nickel resistance) systems of *A. eutrophus* plasmids pMOL28 and pMOL30.

complex on adding of $Cd^{2+}$ [58].

A completely different $Cd^{2+}$ resistance system is found on a large plasmid of the soil chemolithotroph Gram negative bacteria *Ralstonia eutropha* strain CH34. This strain has two "mega-plasmids" with numerous heavy metal resistance determinants[46,57–61,73,85] with three *mer* operons that are related to those described above, and an additional determinant for chromate resistance (see below). Those resistances to divalent "soft" cations are called Czc (for $Cd^{2+}$, $Zn^{2+}$ and $Co^{2+}$ resistances) and Cnr (for $Co^{2+}$ and $Ni^{2+}$ resistances). DNA sequencing shows that the Czc and Cnr system products are closely related (Fig. 5), although the DNA sequences difference are sufficient so that Southern blot DNA/DNA hybridization analysis does not detect homology.

The Czc system confers resistance to $Cd^{2+}$, $Zn^{2+}$ and $Co^{2+}$ and functions as a cation/proton exchanger[61] (not an ATPase) effluxing cations from the cells[57,59–61,73]. The *czc* operon cloned from *R. eutropha* plasmid pMOL30 consists of three genes: *czcA*, *czcB* and *czcC* whose products form a complex membrane cation efflux pump, and *czcD* which is involved in *czc* operon expression[46,53,59,61]. The CzcABC membrane transport complex is homologous to the better-studied Acr acridine resistance efflux pump for which the homolog of CzcA has been solved by X-ray crystallography and consists of a camplex protein trimer, with each subunit containing a membrane embedded region (presumedly forming the membrane channel for divalent cation) plus a periplasmic region that forms a pore leading to the outer membrane protein. Both *czcA* and *czcB* gene encode hydrophobic membrane proteins; CzcC is an outer membrane channel protein, homologous to TolC of Acr system. The "membrane-fusion" protein CzcB is considered to anchor the outer membrane CzcC protein to the immer membrane, with the result that a channel forms through a funnel-like structure in CzcA to a similar opening in the outer membrane protein so that the substrate cation can be transferred from inside the cell to outside without needing to pass via the periplasmic space[53]. The *cnr* operon confers resistance $Co^{2+}$ and $Ni^{2+}$ and its sequence (from *R. eutropha* plasmid pMOL30) is closely homologous to that of the *czcABC* genes[59]. Like Czc system, the resistance mechanism for the Cnr system is also energy-dependent cation efflux[46]. The *cnr* operon has six identified genes (Fig. 5). Three structural gene products predicted from DNA sequence (CnrCBA) are homologous with those of CzcCBA, suggesting both systems function fundamentally in a similar manner. There are three additional potential genes (*cnrY*, *cnrR* and *cnrH*) upstream of the structural genes *cnrCBA* and these three genes are apparently involved in regulation of *cnr* operon expression[46].

**Plasmids for Chromate Resistance:**

Chromate resistance and chromate reduction both occur, but resistance to chromate governed by plasmids of Gram negative bacteria appears to have nothing to do with chromate reduction. Furthermore, it is not clear whether the chromate reduction ability found with several bacterial isolates confer resistance to $CrO_4^{2-}$ or not[64]. Plasmid-mediated chromate resistance is due to reduced cellular accumulation of chromate[13,59,65]. The reduced accumulation results from accelerated $CrO_4^{2-}$ efflux[72].

The two sequenced chromate resistance determinants (*chr* operons) from plasmids of *P. aeruginosa*[13] and *R. eutropha*[59] are quite similar. Both *chr* operons contain the long *chrA* gene. The two predicted ChrA proteins are highly hydrophobic and have 29% identical amino acid residues[13,59]. ChrA was identified as a membrane protein in *E. coli*, although the cloned *chr* operon does not confer chromate resistance in *E. coli*[59]. The role of ChrA in chromate efflux appears to be as a novel $CrO_4^{2-}$ efflux pump[11,72]. In both *chr* operons, an additional open reading frame (intact in *Pseudomonas* but partial in the cloned fragment from *Ralstonia*) was found and its function in chromate resistance is still unknown. For the *Ralstonia chr* operon, there is an additional gene, *chrB*, which is likely to a regulatory gene involved in *chr* operon expression[11,87].

## Possible use of heavy metal resistance transposons in environmental biotechnology

Heavy metals are toxic to mammalians and also for microbes. Microbes have developed these highly specific resistance systems for toxic heavy metals. The resistance are based generally on enzymatic exchange of heavy-metal cation or oxyanion species or more often on memberane pump efflux of heavy metals from the microbial cells. Molecular breeding of microorganisms has been performed by techniques using genetic engineering. However, application of genetically modified microorganisms (GMMOs) to environmental bioremediation uses has been slowed and strictly regulated to prevent unexpected propagation of GMMOs and resulting environmental impacts[21,96]. Bioremediation of environmental contamination of hazardous toxic heavy metals and carcinogen-containing materials needs effective microorganisms encoding genes that can decompose or remove those hazardous matters from the contaminated sites. In some cases, indigenous microorganisms in the polluted site are not sufficiently effective (in rapidity or completion of cleanup processes), and GMMOs are attractive alternatives for microbial engineering. In most cases, however, GMMOs are not acceptable by general public, because of concerns over environmental stability and human health.

Because mercury and its compounds have widely contaminated the environments as a result of geological and industrial activities, many mercury-resistant microbes have been isolated and investigated. The bacterial mercury resistance mechanism which is based on the clustered genes in an operon (*mer* operon) to detoxify Hg(II) to volatile metallic mercury, Hg(0), by enzymatic reduction has been intensively studied[68,88,98]. Although mercury resistance genetic determinants of Gram negative aerobic bacteria have been deeply studied, the comparable systems of Gram positive bacteria have also been clarified. Since spore forming Gram positive bacteria may be considered as better candidates for environmental biotechnology, mercury-resistant Gram positive bacteria were isolated from sediment samples of Minamata Bay, Japan, where is infamous as the site of a severe methylmercury-poisoning incident.[37] and the genes for mercury resistance of the bacterial isolates were analyzed[34,56]. These were studied in depth to develop a molecular basis for microbiological mercury detoxification. Gram positive anaerobic and aerobic mercury resistant bacteria were isolated from Minamata Bay samples and the genes for mercury resistance were analysed[34,56]. An anaerobic *Clostridium butylicum* strain and an aerobic *Bacillus megaterium* strain were found to possess the identical *merA* genes, determining mercury reductase[34,56]. This means that the mercury resistance determinants were transferred beyond the boarder of anaerobic and aerobic environments and across bacterial genera lines. This evidence of lateral gene transfer prompted the development of molecular breeding technology for bioremediation, employing natural gene transfer by transposons.

The results obtained from our study showed that the horizontal transfer of mercury resistance determinants in Gram-positive bacterial strains is mediated by the Tn*MERI1*-related transposons[34,35] and may be transferred beyond the boarder of species and the boarder of anaerobic-aerobic environments. The *mer* operons in Gram negative bacteria were also often found in class II (Tn*3*-like) transposons and in conjugative plasmids[68]. These results reveal the mobile nature of the bacterial heavy metal resistance determinants through transfer with transposons. This character might be useful as a good mediator for natural molecular breeding of the bacteria, without using the more restricted recombinaut DNA technology. Since microorganisms possessing *mer* operon are also useful in removing mercury compounds from the polluted site, characteristics of the mobile Tn*MERI1*-like transposons offers a novel opportunity for in situ molecular breeding for bioremediation. The bacterial strains such as *B. megaterium* MB1 and *C. butyricum* Mersaru may serve as the seed strains for molecular breeding basing on natural gene transfer beyond bacterial species or specific strains. Genetic dissemination of *mer* operon mediated by transposon transfer is taken place between the indigenous microorganisms and result in improvement of mercury removal capability of the environments.

From the experimental and analytical results obtained from our recent studies, we propose a novel protocol for

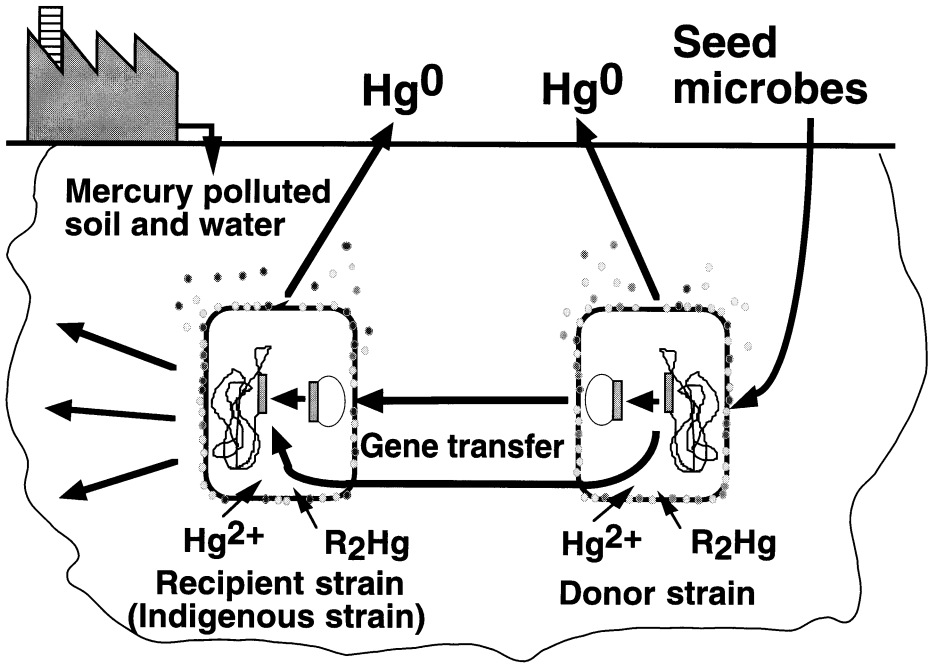


Fig. 6. Schematic diagram of the new concept on in situ molecular breeding of indigenous microorganisms for bioremediation of contaminated environments.

environmental biotechnology shown in Fig. 6. Genetic elements for environmental bioremediation carried by class II transposons or conjugative transposons on the chromosome or conjugative plasmids of the seed microorganisms are used as donor elements and first obtained from introduced into contaminated environments. The genetic elements would then transfer onto other conjugative transposon(s) or transposon(s) and horizontally transfer to indigenous microbial cells as recipients. When the genetic elements are stabilized in the recipient cells and functioning in the indigenous microorganism, the newly bred microorganisms can be used for effective bioremediation of contaminated environments.

Application of GMMOs in the open environmental sites is restricted by government or laws. However, the novel concept of in situ molecular breeding for environmental biotechnology is based on stimulation of natural gene transfer and recombination by mobile transposons, and therefore might be allowed when introduction of recombinant DNA organisms would not be. Such a process is considered potentially useful for application of environmental biotechnology, more effective in practice and acceptable by public, because the breeding technology of microorganisms is based on natural phenomena and not man-made technologies and because indigenous dominant microorganisms in the environment are utilized as the leading actresses and retrictions applied to some biotechnology would not apply.

## Possible use of heavy metal resistance plasmids in environmental biotechnology

Overall, mechanisms of plasmid determined heavy metal resistance are (a) efflux pumping of the toxic metal out from the bacterial cell, (b) bioaccumulation in a physiologically inaccessible compound, and (c) redox chemistry in which a more toxic ion species is converted to a less toxic ion. Redox chemistry perhaps affords the most promising systems for bioremediation of toxic heavy metals. Reduction and volatilization of mercury from inorganic mercury and organomercurials is the best understood of these resistance mechanisms. More likely efforts focus on retaining the reduced elemental mercury in bioreactors. von Canstein et al.[101] used natural bacterial isolates as proposed here and Brunke et al.[9], Okino et al.[66] and Pan-Hou and Kiyono[69] used genetically engineered bacteria having higher rates of mercury reduction or the ability to degrade organomercurials, and immobilized them on ceramic or glass beads or alginate beads. Especially with the work in refs.101), bioremediation of aqueous mercury pollution has moved from the laboratory to the realm of process engineers and long-term reactors functioning at a commecially useful level.

Chromate reduction and arsenite oxidation are other potential bioremediation systems using plasmids. The biochemistry and molecular biology behind these redox processes are less understood but rapidly approaching a stage where useful bioremediation efflorts based on genetic and enzymatic knowledge may be undertaken[52,64]. Efflux pumping mechanisms may be promising for practical use, but there the potential is more long-term, as membranes with highly specific permeabily to toxic metals based on protein pump channels would need to be developed. However, it is not possible to know the possibility of these processes being useful in cleaning up environmental pollution by heavy metals, until we understand the fun-

damental biochemical and genetic characteristics of microbial resistances to toxic metal ions.

Enforced bioaccumulation of cadmium, copper and zinc using recombinant plasmids also may be put to practical use. Some cyanobacterial strains have metallothioneins like heavy metal binding proteins of animals[29,36], which bind multiple metal cations using cysteine and histidine side groups. These bacterial metallothioneins appear to serve as an intracellular sink for toxic excess heavy metals. A *Citrobacter* strain has a cell-surface organophosphate hydrorase[22]. When the cells are fed organophosphate substrates, inorganic phosphate is released at cell surface and cations such as $Cd^{2+}$ are precipitated and bioaccumulated at the cell surface. An attractive recombinant plasmid system was developed using polyphosphate kinase gene (*ppk*). The fusion plasmid worked well to highly accumulate $Cd^{2+}$ (and also $Hg^{2+}$) in *E. coli* cells without toxicity[70].

It is expected that effective bacterial agents to remove heavy metals can be constructed by increasing copy number of the advanced genetic systems using plasmids. Therefore, molecular breeding of the heavy metal removing microorganisms by using plasmid technology is a possible way to improve the performance of heavy metal removal from polluted environments.

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