Applied Organometallic Chemistry (1989) 3 283–294

0268–2605/89/03401283/$03.50

REVIEW

Mercury resistance and detoxification in bacteria

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Received 8 March 1989 *Accepted 10 April 1989*

INTRODUCTION

Mercury and a number of other heavy metals, such as cadmium and lead, have received considerable attention due to their highly toxic nature, widespread distribution in the environment, and translocation through the food chain.[1–4] Unlike other heavy metals such as cobalt and nickel, which are required in trace amounts and are toxic only at higher concentrations, mercury has no known biological function.[5] Elementary mercury (Hg^0) is less reactive with biological systems than ionic or organic forms. Mercuric mercury (Hg^{2+}) is toxic to bacteria because it binds strongly to sulfhydryl groups of proteins in membranes and inhibits synthesis of macromolecules and enzyme activity.[6,7] Mercury compounds can also interact with carboxyl groups and imino nitrogens.[8] A number of enzymes have critical thiol groups which are inactivated when bound by mercuric ions *in vitro*. Transcription and translation are particularly sensitive to mercuric ions which may be due to inhibition of precursor synthesis or to mercuric ion binding to polynucleotides.[7,9] Also, Beppu and Arima[10] reported the induction by mercuric ions of extensive degradation of cellular ribonucleic acid (RNA) in *Escherichia coli*. Of the several forms of mercury found in the environment, including elemental mercury, mercuric or mercurous (Hg_2^{2+}) ions and organomercurial compounds, an organic form of mercury, methylmercury, is known to be the most toxic to higher life forms including humans. Methylmercury is 50 to 100 times more toxic than inorganic mercury,[11–13] and has been demonstrated to be mutagenic and teratogenic under experimental conditions.[5,14–17] The solubility of inorganic and organic mercury compounds in lipids accounts for their toxic nature.[2] Methylmercury also has a long retention time in biological tissues, particularly in the brain.[18]

The problems associated with mercury pollution became known after the discovery of high levels of methylmercury in fish and shellfish in Minamata Bay, Japan. As a result of eating fish and shellfish from the Minamata Bay area, many people suffered severe neurological disorders. This affliction, which became commonly known as 'Minamata disease', resulted in approximately 120 confirmed cases of methylmercury toxicity and 43 deaths between 1953 and 1970.[12,18] The source of the mercury contamination was found to be a fertilizer plant, upstream from the bay area, that utilized mercury as a catalyst for producing vinyl chloride. The discharged mercury compounds from spent wastes were shown to accumulate at various stages of the food chain; methylmercury was concentrated in fish and shellfish to such a degree that a continuous diet of the fish resulted in sufficient concentrations to reach toxic levels in the populace.[19] In Sweden, mercury levels in seed-feeding birds and in birds of prey increased after phenylmercuric acetate and methylmercury were introduced as fungicidal agents in seed dressings. Consequently, this practice resulted in a significant decrease in the population of these birds.[20] In the Great Lakes region of North America, high levels of methylmercury have also been measured in fish.[2,21]

OCCURRENCE OF MERCURY IN NATURE

There are many sources of mercury in the environment that are both natural and anthropogenic in origin.

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Mercury occurs in a variety of rocks and soils. The most abundant and important natural mercury ores are cinnabar (red mercuric sulphide, HgS), which is found in only a few places at the earth's surface, and metacinnabar (black mercuric sulphide[3,12,13]). The terrestrial abundance of mercury is approximately 50 μg kg^{-1} except in mercuriferous regions and anthropogenically polluted areas.[18] In soil the mercury content is in the range of 30–500 μg kg^{-1} with an average of about 100 μg kg^{-1}, and in rocks the mercury content ranges from 10 to 20 000 μg kg^{-1}.[18] High levels of mercury are found in rocks near recent volcanic activity, deep sea vents or hot spring areas, and in mineral veins or in fractures as impregnations.[3] The air above rocks and minerals high in mercury ranges from 1.2 μg dm^{-3} (i.e. μg/ℓ) above mineral ore deposits to 16 μg dm^{-3} over mercury mines.[13,22]

CHEMISTRY OF MERCURY AND THE MERCURY CYCLE

Mercury is one of the best understood examples of an element that undergoes a complex cycle in the environment and for which there is evidence for the geochemical and biochemical bases for the transformations. To understand how mercury cycles through the environment, it is important to understand the chemistry of mercury. Mercury is unique because it can exist as a metallic liquid or vapour, as a covalent organic derivative, or in several different ionic salts at standard earth temperatures and pressures.[5] Inorganic mercury exists in three valence states. Elemental mercury, Hg^0, is the only metal that occurs as a liquid at standard atmospheric temperatures and pressures, and because of its high vapour pressure is highly volatile.[23] Mercuric mercury (Hg^{2+}) and mercurous mercury (Hg_2^{2+}) are the other two valence states of mercury, and they exist in equilibrium due to chemical dismutation as follows: $Hg_2^{2+} \rightleftharpoons Hg^0 + Hg^{2+}$.[24] Mercury(II), Hg^{2+}, is methylated in nature to mono- and dimethylmercury, and in addition forms insoluble complexes with sulphide (S^{2-}) to form cinnabar (HgS) in sulphide-rich environments.[25–27] However, with other anions such as Cl^-, mercury forms soluble complexes, such as $HgCl_4^{2-}$; this contributes to its continued bioavailability.[28,29]

In surface waters, except where influences due to certain geological and anthropogenic sources prevail, mercury exists as mercuric hydroxide [$Hg(OH_2)$] and mercuric chlorides ($HgCl_2$ and $HgCl_4^{2-}$) at less than 0.1 μg dm^{-3}.[30] The average total mercury content of seawater has been shown to be in the range 0.1–1.2 μg dm^{-3}.[18] However, mercury found in fish, such as tuna and swordfish, from clear, uncontaminated seawater, is methylated. It is very likely that micro-organisms are involved with the transformation of mercury to methyl species, as will be discussed in detail below. The most common form of mercury in anoxic sediments is HgS.[20] Most of the mercury found in the atmosphere exists as mercury(O) or methylmercury, whereas much lower levels (less than 1%) of dimethylmercury are reported.[23] It is also very likely that micro-organisms play a role in the transfer of mercury to the atmosphere. In unpolluted air, mercury levels are in the range of 1–10 μg dm^{-3}, and the distribution is dependent on the content of mercury in the soil, water and mineral deposits in the area.[23] Natural degassing of the earth's crust releases an estimated 2.3×10^7 to 4.5×10^8 kg of mercury each year, whereas the total mercury content in the ocean is estimated at 1.8×10^{11} kg.[31]

In areas where mercury has been discharged from anthropogenic sources, local concentrations can be several thousand-fold higher than described above.[5] The use of organomercury compounds as fungicides and slimicides in agriculture and pulp and paper manufacturing, and as catalysts and disinfectants in the chlor-alkali, paint and pharmaceutical industries, has resulted in a world-wide estimated consumption of greater than 8×10^9 kg annually.[12,19,31–36] Over 11×10^6 kg of mercury are released from industrial mining activities, and over 3×10^6 kg are released from coal combustion.[12] Although the mercury content in fossil fuels is relatively low (approximately 180 μg dm^{-3}), an additional $(9–55) \times 10^6$ kg are released from the combustion of crude oils.[34]

Sewage treatment facilities are an important source of both inorganic and organic mercury compounds, including mercury(O), mercury(II), methylmercuric chloride and dimethylmercury.[2] Values of total mercury were shown to range from 0.5 to 105 μg dm^{-3} in sewage systems, and airborne mercury(O) and organomercurials introduced from urban sewage plants in notable quantities have also been reported.[3] Levels of mercury were shown to be highest near the facility and dropped rapidly several kilometres from the plant. A similar concentration–distance relationship was found for airborne mercury fallout from

chlor-alkali plants in Sweden which has released approximately 100–400 kg annually.[2,33]

Human activities account for an estimated release of (1.8–6.4) × 10^7 kg of mercury per year into the atmosphere and water resources.[2] Although humans have contributed to the levels of mercury in the environment, it is still a relatively small amount of the total mercury that is present in nature.

Since some environments, such as volcanic soils, hot springs and deep sea vents, naturally contain relatively high concentrations of mercury, micro-organisms have been exposed to mercury and other toxic metals long before humans began increasing local concentrations through industrial and agricultural practices. Therefore, it is not surprising that many micro-organisms, including bacteria, have evolved metal resistance mechanisms in order to minimize the toxic effects of these elements. In some instances [for example arsenic(V), silver(I) and cadmium(II)], bacterial metal resistances have been shown to be due to differences in uptake and/or transport of the toxic metal.[37] In other cases, as with mercury(II) ion, the metal is transformed by reduction, methylation or demethylation into a form which is either less toxic or more volatile than the original mercurial compound.[38] It is now apparent that these mechanisms of resistance not only protect the organism in the environment, but they also may play a crucial role in the cycling of mercury in the biosphere.

The biological cycle of mercury in the environment has received considerable attention to determine the contributions made by microbial activities. The roles of a number of different bacteria in the transformations of mercury have been demonstrated.[3] In addition, there appears to be a positive correlation between the distribution of mercury compounds and that of resistant micro-organisms in metal-contaminated sediments.[39] Many mercury-resistant bacteria have been detected in and isolated from other environmental sources such as soil and water.[2,3,5,7,40–42] In soil, the high incidence may be due to natural leaching of mercury compounds from rocks or to fallout of airborne mercury from industrial pollutants.[27,43] In waterways, this may be due to industrial pollution.[44,45] There is also a genetic linkage between mercury resistance and antibiotic resistance in clinical isolates.[46–49] However, in environmental isolates, linkage of mercury and antibiotic resistance is far less frequent.[2,7,44]

Bacteria have developed a diversity of resistance mechanisms for dealing with the many forms of mercury that occur naturally in the environment or that result from human activities. Many of these mechanisms involve direct transformation (methylation, demethylation, reduction) of mercury compounds, and may therefore play an important role in the cycling of mercury in the environment.

METHYLATION OF MERCURY

Methylation of inorganic mercury by micro-organisms in aquatic sediments and in animal faeces represents an important part of the mercury cycle.[50,51] Although methylated mercury is more toxic than inorganic mercury, due to its lipophilic nature, it is more volatile and therefore methylation may be considered to be a detoxification mechanism.[2,52–54] There are at least three known pathways involving the methylation of mercury: (1) photochemical methylation, an abiotic process involving the conversion of mercuric chloride to methylmercury by ultraviolet irradiation; (2) *via* methylcobalamin (Vitamin B_{12}), released by bacteria, which can act as a methyl donor to mercuric ions; and (3) methylation of mercuric ions by bacteria on the gills or in the gut of aquatic animals also, perhaps, utilizing methylcobalamin as the methyl group donor.[3,38] Although the latter was a hypothesis suggested by Summers and Silver,[3] it has never been tested.

Biological methylation of mercury has been experimentally demonstrated in aerobic and anaerobic bacteria, and in cell-free extracts of methanogenic bacteria.[2,26,51,55] Methyl groups are transferred to mercuric ions through electrophilic attack by Hg^{2+} on the C–Co bond of methylcobalamin.[56] Although Hg^{2+} appears to be the most likely substrate for methylation, Matsumura *et al.*[57] isolated soil and aquatic bacteria that were able to methylate phenylmercuric acetate to dimethylmercury. This could also proceed via mercury(II) ions.

The ecological significance of B_{12}-dependent methylation of mercuric ions is best illustrated using the results of Pan-Hou and Imura.[58] These authors described two strains of *Clostridium cochlearium*, one of which was B_{12}-dependent and the other B_{12}-independent. The B_{12}-dependent strain was capable of methylating mercuric ion to CH_3Hg^+, whereas the B_{12}-independent strain was unable to catalyse this reaction. Both strains transported mercuric ions into cells at equal rates, but growth of the B_{12}-dependent

strain was inhibited by 40-fold lower concentrations of mercuric ion than the B_{12}-independent strain. The authors interpreted these results as an indication that the B_{12}-dependent strain used methylation as a mechanism of mercury detoxification. In the natural environment, methylmercury released from the microbial system can enter the food chain as a consequence of its rapid diffusion rate.[56]

HYDROGEN SULPHIDE (H_2S) AND INTRACELLULAR SULPHIDE

Resistance to mercurial compounds involving the microbial production of hydrogen sulphide (H_2S) and intracellular acid-labile sulphide (SH^-) has been reported.[59]

In estuarine environments, the reduction of sulphate by *Desulfovibrio* species to produce hydrogen sulphide plays an important role in reducing methylmercury by S^{2-}-catalysed disproportionation to volatile dimethylmercury and insoluble HgS.[56] Hydrogen sulphide is very effective in the precipitation of mercury in the aqueous environment. Such chemical reactions occur where *Desulfovibrio* species have access to sulphate in anaerobic environments.[56] The disproportionation of methylmercury is shown in Eqn [1].

$$2CH_3Hg^+ + H_2S \rightarrow (CH_3)_2Hg \uparrow + HgS \downarrow \quad [1]$$

Once in the atmosphere, volatile dimethylmercury is unstable because the carbon–mercury (C–Hg) bond is susceptible to cleavage by ultraviolet irradiation.[56] The mercuric sulphide is highly insoluble and is therefore prevented from entering the food chain as a toxic agent.

Resistance to methylmercury in a strain of *Clostridium cochlearium* was found to involve both the demethylation of the organomercurial and the subsequent conversion of the Hg^{2+} product to mercuric sulphide by reaction with hydrogen sulphide.[59] The capacity to demethylate methylmercury and to produce hydrogen sulphide was believed to be controlled by an extrachromosomal genetic element (plasmid). These properties were lost upon curing and regained by conjugation experiments, but no plasmid deoxyribonucleic acid (DNA) analysis was reported. It is believed that this type of mercury transformation is probably not a specific response to mercury compounds in the environment, but is more likely to be a result of a metabolic process.[5]

Aiking *et al.*[60] isolated a strain of *Klebsiella aerogenes* that was able to tolerate inorganic mercury by a detoxification mechanism termed 'faciliated precipitation'. The process was aptly named because mercury probably accumulated and precipitated (as mercuric sulphide) near the cell due to relatively high local concentrations of intracellular sulphide.

PERMEABILITY BARRIERS AND ACCUMULATION

Other inorganic mercury-resistance mechanisms in micro-organisms include accumulation, and alteration of the membrane permeability to mercuric ions.[59,61] Pan-Hou and Imura[59] reported an *Enterobacter aerogenes* isolate that produced two outer membrane proteins which reduced mercuric ion uptake by altering the permeability of the cell wall. Although this is not a detoxification mechanism involved in the biological mercury cycle, it is a novel mechanism of resistance in some bacteria.

A number of bacteria and fungi are able to accumulate and immobilize mercuric ions intracellularly and extracellularly from solution (up to 1.37×10^{-4} mol g^{-1} bacteria). These species may make excellent candidates to recover inorganic mercury from industrial effluents and mine wastewater.[61] For further details on the biochemical basis for intracellular and extracellular metal traps developed by micro-organisms for metal ion removal, see the reviews by Shumate and Strandberg[62] and Wood and Wang.[56]

ENZYMIC DEMETHYLATION AND REDUCTION OF MERCURY COMPOUNDS

The earliest observation of mercury- and organomercurial-resistant bacteria came from Japan, where resistant bacteria were isolated from organomercurial-polluted soil[63] and from the United Kingdom, among hospital isolates of *Staphylococcus aureus*.[64,65]

The reduction of Hg^{2+} to Hg^0 and the decomposition of organomercurial compounds by mercury-resistant bacteria have since been demonstrated in a

wide variety of bacterial genera isolated from soil, sediment, and clinical sources.[27,39,44–49,66–78]

Our understanding of the biochemistry, genetics and molecular biology of bacterial resistance to inorganic mercury and organomercury has made mercury resistance the best characterized and understood heavy-metal detoxification mechanism to date.[79] The wide distribution of mercury resistance can be partially explained by the observation that the resistance determinants are usually plasmid-encoded, particularly in Gram-negative bacteria.[1] Resistance to mercury compounds is a common property in both Gram-positive and Gram-negative bacteria.[7] Several mercury resistance determinants were also shown to be borne on transposons in Gram-negative bacteria of soil and clinical origin.[80–82]

Two general classes of enzyme-mediated mercury resistance systems have been described: (1) narrow-spectrum resistance involves the reduction of mercuric ion (Hg^{2+}) to elemental mercury (Hg^0) and also provides resistance to several organomercurials including merbromin and fluorescein mercuric acetate, although this does not involve enzymatic transformation but rather a permeability barrier; and (2) broad-spectrum resistance provides resistance to all of the above compounds and includes the reductive transformation (involving hydrolysis and detoxification) of additional organo-mercurial compounds including phenylmercuric acetate and methylmercury.[5,7,83]

The reduction of Hg^{2+} to Hg^0 is catalysed by the intracellular, FAD-containing enzyme known as mercuric reductase. A considerable amount of interest has focused on this enzyme, primarily because of its structural and mechanistic similarities with dithiol oxidoreductases of mammalian systems including glutathione reductase and lipoamide dehydrogenases.[83] The detoxification of organomercurial compounds is a two-step process which involves the cleavage of the carbon–mercury bond by the intracellular enzyme organomercurial lyase followed by reduction of Hg^{2+} by the accompanying mercuric reductase.[84,85] Reductive mercury resistance is inducible by sub-inhibitory concentrations of Hg^{2+} or organomercurials. Both the mercuric reductase and the organomercurial lyase, if present, are induced, but not necessarily in the same fashion, as will be discussed below.[1] Gram-positive and Gram-negative bacteria in which the reductive biotransformation of mercury compounds involving volatilization has been described are summarized in Table 1.

As previously described, one common characteristic of mercury resistance determinants in Gram-negative bacteria isolated from the environment is their location on plasmids and the absence of linkage to antibiotic resistance genes,[2,44] in contrast to clinical isolates where mercury resistance is often linked to antibiotic resistance markers.[46–49,69]

Several surveys of clinical bacteria have consistently

Table 1 Mercury volatilizing bacteria

Bacterial species	Spectrum of resistance	Location	Reference
Gram-positive			
Bacillus cereus 5	Narrow	Not known	74
Bacillus sp. strain RC607	Broad	Chromosomal	86
Bacillus sp.	Narrow	Plasmid	39
Clostridium perfringens	Narrow	Chromosomal	87
Mycobacterium scrofulaceum	Narrow	Plasmid	88
Staphylococcus aureus	Broad	Plasmid and chromosomal	47, 49, 78
Streptococcus agalactiae	Narrow	Chromosomal	75
Streptomyces lividans	Narrow	Chromosomal	75
Streptomyces lividans	Broad	Chromosomal	89
Gram-negative			
Bacteriodes ruminocola	Narrow	Chromosomal	87
Enterobacteria	Narrow and broad	Plasmid	48, 90
Pseudomonads	Narrow and broad	Plasmid	46, 69, 90
Thiobacillus ferrooxidans	Narrow	Chromosomal	91
Yersinia enterocolitica	Narrow	Plasmid	67

reported the presence of mercury resistance determinants on drug resistance plasmids. In a plasmid study of over 800 strains from the Hammersmith Hospital in London, England, approximately 25% of a wide range of species, including *Proteus*, *Providentia*, *Salmonella*, *Serratia*, *Shigella* and *Klebsiella*, transferred mercury resistance determinants to an *E. coli* K12 host. About 30% of the mercury resistance plasmids also conferred resistance to antibiotics.[48] Broad-spectrum resistance to mercury was rare, with only a few plasmids carrying this trait.

Recent studies in the United States and Japan have shown that the incidence of mercury resistance among hospital staphylococci has declined, which may be due to the discontinuation of organomercurials such as phenylmercury and mercurochrome (merbromin) as hospital disinfectants.[47]

SPECTRUM OF RESISTANCE TO ORGANOMERCURIALS IN BACTERIA

Patterns of resistance to organomercurial compounds vary between organisms and are a complex combination of the toxicity of the organomercurial under study, its efficiency as an inducer, and its efficiency as a substrate of the organomercurial lyase.[1] The phenotypes of enteric bacteria and *Pseudomonas aeruginosa* differ even when different hosts harbour the same mercury resistance plasmid.[7] All staphylococci strains that are resistant to mercury confer a broad-spectrum phenotype which is different from that of Gram-negative bacteria. These phenotypes are summarized as follows.

Enteric bacteria volatilize mercuric ion and are also resistant to the organomercurials merbromin and fluorescein mercuric acetate. This latter resistance may be due to the permeability barriers mentioned above, although enzymatic detoxification of organomercurials was recently shown in static cultures of *Klebsiella aerogenes* harbouring narrow-spectrum resistance determinants.[7,92] The most thoroughly studied examples of plasmid-determined reductive mercury resistance (*mer*) are the narrow-spectrum systems encoded by the transposons Tn*21* (located on plasmid R100-1, a conjugative R plasmid originally isolated from *Shigella*) and Tn*501* (located on plasmid pSV1 and originally isolated from a clinical strain of *Pseudomonas aeruginosa*.[52,80,83,93–102]

Broad-spectrum resistance is limited to a small number of plasmids in the enteric bacteria. In this group, the narrow-spectrum phenotype is extended to include the organomercurials phenylmercuric acetate and thimerosol.[7] Although only resistance to thimerosol is conferred, Hg^0 is formed by volatilization from thimerosol, *p*-hydroxymercuribenzoate, methylmercury and ethylmercury.[48,90] This was attributed to a low level of organomercurial lyase activity and a greater toxicity of ethyl- and methyl-mercury.[7]

Well characterized examples of broad-spectrum resistance specifying organomercurial lyase include the plasmids R831b in *E. coli* (originally isolated from a strain of *Serratia marcescens*[48,96]) and pDU1358 in *Serratia marcescens*.[103,104] It is interesting to note that pDU1358 carries two *mer* determinants, one narrow-spectrum and one broad-spectrum, that can function independently.

Narrow-spectrum resistance to mercurials in *Pseudomonas aeruginosa* is different from that in *E. coli*, because resistance is conferred to *p*-hydroxymercuribenzoate without volatilization of Hg^0.[7,69] A host factor in *P. aeruginosa* may be responsible because broad-host-range mercury resistance plasmids impart resistance to *p*-hydroxymercuribenzoate in *E. coli*.[69,90]

Similarly to the narrow-spectrum determinations in *P. aeruginosa* described above, *p*-hydroxymercuribenzoate resistance occurs without hydrolysis or volatilization. In contrast, *E. coli* plasmids slowly volatilize Hg^0 from *p*-hydroxymercuribenzoate even though they are sensitive to the mercurial compound.[90]

All *S. aureus* mercury resistance determinants described to date confer broad-spectrum resistance.[47,49,78] Two phenotypic groups have been studied. The first group is different from Gram-negative broad-spectrum determinants in that *S. aureus* does not confer resistance to merbromin.[7] Resistance to *p*-hydroxymercuribenzoate and fluorescein mercuric acetate is conferred without volatilization. These strains are also sensitive to thimerosol although slow hydrolysis and volatilization of Hg^0 from this compound was detected.[7] The second group of resistance determinants confers resistance to thimerosol, merbromin and methylmercury. Thimerosol resistance was explained by the fact that this compound was a more effective inducer of the mercury resistance determinant, and that the lyase had greater hydrolytic activity.[78] The most thoroughly studied example of plasmid-determined resistance is plasmid pI258.[105] Not all mercury resistance determinants in *S. aureus* are located on plasmids.

Witte *et al.*[78] also located a mercury resistance determinant on the chromosome of a novel clinical isolate of *S. aureus* by using probe DNA from pI258 in hybridization experiments.

Both narrow- and broad-spectrum resistance have been described for soil and marine species of the genus *Bacillus*. Narrow-spectrum resistance in a *Bacillus cereus* strain No. 5 soil isolate was described by Izaki.[74] The organism was resistant to mercuric ions and was shown to volatilize Hg^0 from mercuric and mercurous ions. No attempt was made to determine whether the determinant was plasmid-encoded. This isolate was initially reported to be sensitive to phenylmercuric acetate and was thus believed to have no hydrolytic activity. It is now known that mercury resistance is carried on a 130 MDa plasmid in *B. cereus* No. 5.[106] A marine *Bacillus* species, strain RC607, was described by Mahler *et al.*[86] to confer resistance to Hg^{2+} and phenylmercuric acetate. Southern blot analysis using probe DNA from plasmid pI258 revealed that the broad-spectrum determinant was located on the chromosome.[107] Attempts to locate the mercury resistance determinant in *B. cereus* strain 5 using probe DNA from the plasmid R100 determinant[66] and the marine *Bacillus* sp.[107] have failed.

BIOCHEMISTRY OF MERCURY RESISTANCE

The enzyme responsible for the reduction of Hg^{2+} to volatile Hg^0 in mercury-resistant bacteria is mercuric reductase, an intracellular, cytoplasmic flavoprotein which belongs to the same class of enzymes as the pyridine nucleotide-disulphide oxidoreductases, lipoamide dehydrogenase and glutathione reductase.[1,7] Comparing the predicted amino-acid sequences of the mercuric reductase genes in Tn*501*, Tn*21*, pDU1358, pI258 and *Bacillus* sp. strain RC607 with those of lipoamide dehydrogenase (from pig heart, *E. coli* and *Bacillus stearothermophilus*) and glutathione reductase (from human erythrocytes, yeast, and *E. coli*) has revealed a close sequence relationship, particularly in the disulphide-containing peptides in the active sites.[96] Mercuric reductase uses NADPH as an electron donor and requires exogenous thiols such as mercaptoethanol for *in vitro* activity.[1] The thiols prevent the formation of inhibitory Hg^{2+}–NADPH complexes and may also prevent Hg^{2+} from forming inhibitory complexes with the active site which contains a redox-active disulphide and FAD. The added thiols also ensure that the Hg^{2+} is present as a dimercaptide, allowing for the *in vitro* reaction of mercuric reductase to proceed as follows (Eqn [2]):

$$RS{-}Hg{-}SR + NADPH + H^+ \rightarrow Hg^0 + NADP^+ + 2RSH \quad [2]$$

The reductase has redox-active cysteine residues at its active site located at residues Cys-135 and Cys-140 in the Tn*501* protein.[83] From kinetic, spectroscopic and fluorescence studies of the mercuric reductase protein it was shown that two-electron and four-electron reduced forms of the enzyme exist and that both the FAD and disulphide are redox-active. Electrons donated by NADPH are transferred via FAD to reduce the active-site cystine, converting it to two cysteine residues with titratable —SH groups. The Cys-140 residue forms a charge transfer complex with FAD, and the active-site cysteines then reduce Hg^{2+}, bound to the carboxy-terminal cysteines, forming Hg^0.[7]

Mercuric reductase is highly specific for mercuric and mercurous ions, and no significant oxidation or reduction of other metal ions has been shown.[1,7] Competitive inhibition of mercuric reductase binding sites was shown with copper(II) (Cu^{2+}), cadmium(II) (Cd^{2+}) and silver(I) (Ag^+) ions; noncompetitive inhibition was demonstrated with gold(III) (Au^{3+}). Chromate, vanadate and ferricyanide anions, as well as cobalt(II) (Co^{2+}), manganese(II) (Mn^{2+}), iron(II) (Fe^{2+}) and nickel (Ni^{2+}), have no *in vitro* effect on mercuric reductase.[74,102]

The predicted molecular weights of the Gram-negative Tn*501* and Tn*21* mercuric reductase enzymes are each 58 700 Da, which were estimated from DNA sequence analysis.[83,101] These estimates conformed with the estimates of the subunit molecular weights from SDS polyacrylamide gel electrophoresis.[96,102] *In vivo* the enzymes are believed to be active as dimers. However, earlier work reported that the molecular weights of Tn*21* and R831b reductases were 175 000 Da,[84] suggesting a monomer.[3,108]

Plasmid-encoded mercuric reductase enzymes from Gram-negative organisms are all closely related[7] even though they are classified into two groups based on immunological cross-reaction and inhibition studies. The first group, class I, represented by Tn*21* (plasmid R100), and the second, class II, represented by Tn*501* (pVS1), are 86% identical in amino-acid sequence homology, a difference of only 79 of the 561 (Tn*501*) or 564 (Tn*21*) amino-acid residues. In terms of nucleo-

tide sequence homology there is 82% identity between Tn*21* and Tn*501* mercuric reductase genes.[7]

Mercuric reductases from Gram-positive bacteria can differ considerably from those of other Gram-positive bacteria and from Gram-negatives.[89] No immunological cross-reaction exists between staphylococcal enzymes and those from Gram-negative bacteria.[7] Detailed immunological and biochemical studies have not been performed with Gram-positive mercuric reductases, although *B. cereus*[74] and *S. aureus*[49] enzymes are known to require NADPH as a cofactor, and are roughly the same size as the R831b enzyme from Gram-negative organisms. Much of what is known about *S. aureus* mercuric reductase was predicted from DNA sequence analysis.[105] There is about 48% nucleotide and amino-acid sequence homology between plasmid pI258 mercuric reductase and that of Tn*21*.[7] The mercuric reductase subunit (620 amino-acid residues) from *Bacillus* sp. strain RC607[86] is only 63% identical with the pI258 enzyme, which is 547 amino-acids long.[89,109] Another mercuric reductase enzyme from *Streptomyces lividans* is considerably different from those of the *Bacillus* sp. strain RC607 or pI258[89] and matches these sequences in only about 50% of the amino-acid positions predicted from DNA sequence analysis. A surprising finding was that the *Streptomyces* sequence totally lacks the amino-acid terminal putative Hg^{2+}-binding domain and is only 474 amino-acids long, similar to lipoamide dehydrogenase.[110]

Organomercurial lyase, specified by broad-spectrum resistance determinants, is an inducible, probably cytoplasmic, enzyme which hydrolyses carbon–mercury (C–Hg) bonds.[84] Several biochemical studies of organomercurial lyase proteins have been documented. Tezuka and Tonomura[85] purified two distinct lyase active fractions from *Pseudomonas* K-62. The proteins were estimated to be 19 000 and 20 000 Da, and were shown to have different substrate specificities.[85] It is not known whether these proteins are coded for by separate lyase genes.

Schottel[84] isolated a lyase enzyme specified by plasmid R831b which displayed two distinct activities, but two separate proteins were not purified. Recent work has shown that the R831b enzyme is a monomeric protein of 22 400 Da,[111] and this is supported by the DNA sequence analysis of the lyase gene of a related broad-spectrum determinant, pDU1358.[104]

Mechanistic studies with purified lyase specified by R831b[112] showed that it catalyses the protonolysis of C–Hg bonds in a number of organomercurials by an S_E2 pathway (Eqns [3], [4]).

$$CH_3HgCl \xrightarrow{RSH} CH_3HgSR \xrightarrow{Enz} EnzCH_3HgSR \rightarrow \quad [3]$$

$$CH_4 + EnzHgSR^+ \xrightarrow{RSH} Enz + (RS)_2Hg \quad [4]$$

Exogenous thiols are required for *in vitro* activity. L-Cysteine is the most effective in promoting decomposition compared with other reducing agents, including thioglycolate and 2-mercaptoethanol.

The DNA sequence of the pDU1358 lyase gene was determined and the amino-acid sequence (212 amino-acid residues) was predicted.[104] The lyase protein from pDU1358 is very similar to that specified by R831b with 45 of 46 *N*-terminal amino-acids being identical.[7] There are four cysteine residues in both the R831b enzyme (as determined by amino-acid composition analysis[111] and in the pDU1358 protein (as determined from the DNA sequence[105]). There is also significant homology between the pDU1358 enzyme and that of *S. aureus* pI258 (39% identical amino-acid residues as determined from DNA sequence analysis[105]). The homology is mainly concentrated in a region of 100 amino-acids, and three of four cysteines in the pDU1358 lyase are conserved in the pI258 enzyme, suggesting that they may be functionally important.[7] No data analyses of the marine *Bacillus* sp. strain RC607 or the *Streptomyces lividans* lyase enzymes have been published; therefore no comparisons of these enzymes with pI258 or pDU1358 lyase genes can be drawn.

GENETIC ORGANIZATION OF MERCURY RESISTANCE DETERMINANTS

The narrow-spectrum mercury resistance determinants discussed here (Tn*21*, Tn*501*, R831b, pDU1358 and pI258) also encode transport functions, which may carry mercurial compounds across the cell membrane and transfer the mercurials to the detoxifying enzymes. If, for example, the transport functions are expressed in the absence of mercuric reductase activity, then the cells become hypersensitive to mercurial compounds.[46]

Genetic and DNA sequence analysis of the narrow-spectrum determinants, Tn*21* and Tn*501*, have revealed an operon structure for both systems.[83,93,94,100,101,113] The operons consist of a regulatory gene (*merR*), an operator/promoter region, and at least four (*merT*,

merP, *merA*, and *merD*, in Tn*501*) or five (*merT*, *merP*, *merC*, *merA* and *merD*, in Tn*21*) structural genes.

The regulatory gene, *merR*, specifies a cytoplasmic protein (144 amino-acid residues long) which controls transcription of the *merTP(C)AD* operon both positively and negatively. The *merR* gene of Tn*21* is transcribed divergently from the *mer* structural genes, and it is believed to be the same in the other *mer* operons that have been sequenced, including Tn*501* and the broad-spectrum operon in pDU1358.[7,114] The regulatory protein normally represses the transcription of the *mer* structural genes;[70] however, in the presence of sub-inhibitory concentrations of mercuric ion, the *merR* product acts as an inducer of the mercurial resistance phenotype.[113] The *merR* product of some broad-spectrum *mer* operons can complement Tn*21 merR* mutants and regulate the Tn*21 mer* system.[114] In addition, *merR* appears to regulate its own transcription negatively.[114] The protein was shown to be a dimer with subunits estimated to be 15 76 Da (Tn*501*) to 15 906 Da (Tn*21*) from DNA sequence analysis.[7]

The *merT* gene is the most promoter-proximal of the structural genes in the *mer* operon, and has a predicted molecular weight of approximately 12 400 Da (116 amino-acid residues) from the DNA sequence analysis of both Tn*21* and Tn*501*.[101] The protein is very hydrophobic and is believed to span the inner membrane since it contains three potential membrane-spanning regions with no charged amino-acid residues (except a Glu-48, Arg-51 pair) within the membrane.[1] The *merT* gene product is expected to lie in the inner membrane rather than in the outer membrane because the latter apparently offers little barrier to divalent cations.[1] The predicted structure has a pair of cysteine residues (Cys-76, Cys-82) on one face of the membrane (probably the cytoplasmic face) and another pair (Cys-24, Cys-25) on or near the other face of the membrane by the periplasmic space.[1]

The second of the pair of genes involved in mercury transport, *merP*, specifies a protein which is probably located in the periplasmic space.[7] The DNA sequence analysis of Tn*21* and Tn*501* showed that the predicted primary translation product should be approximately 9 429 Da (91 amino-acid residues long), but is likely to be processed to a product that is 7 403 Da (Tn*21*) to 7 523 Da (Tn*501*). The processed product has a cysteine pair (Cys-14, Cys-17) in a sequence that is closely homologous to an *N*-terminal cysteine pair (Cys-10, Cys-13) in mercuric reductase.[100] No gene products other than those of *merT* and *merP* genes are required for the uptake and transport of mercury into bacterial cells.[1]

The *merC* gene is only present in the Tn*21 mer* operon.[93,101] There is some doubt about the role of *merC* since the Tn*501* operon lacks this particular open reading frame, yet still expresses normal resistance (at least in *E. coli* and *P. aeruginosa*[7]). The 14 000 Da protein (140 predicted amino-acid residues long from DNA sequence analysis) associates with the inner membrane and may play a role in mercury transport.[5] It may be required for optimum expression of resistance in different hosts or in different environments.[7]

Proceeding from left to right, the next gene in the *mer* operon is *merA*, which specifies the mercuric reductase enzyme. The primary translation product is predicted to be 58 905 (Tn*21*) and 58 727 Da (Tn*501*), containing 564 (Tn*21*) and 561 (Tn*501*) amino-acid residues from DNA sequence analysis.[7] The observed molecular weights were shown to be 69 000 (Tn*21*) and 66 000 Da (Tn*501*) but the primary product from each operon is cleaved post-translationally to remove the *N*-terminal 2 000–3 000 Da.[98,115] It is not clear whether this proteolytic cleavage has any functional significance *in vivo*. The protein is cytoplasmic although it may transiently associate with the inner face of the cytoplasmic membrane.[98]

Promoter-distal to, and downstream from, *merA* is another gene, *merD*. The protein product has never been isolated from cells although from the predicted DNA sequences of Tn*21* and Tn*501* the proteins are estimated to be 13 000 Da (approximately 121 amino-acid residues long[7]). The *merD* gene product may play a small role in resistance conferred by Tn*501* in *P. aeruginosa* and by Tn*21* in *E. coli* genes in a multi-copy state (for example, when present on high-copy-number plasmids). It may also be required when the *mer* operon is expressed in a particular host species or when the expression level and the mercury flux are particularly high.[1,7]

Much less information is available on the genetics of broad-spectrum mercury resistance determinants. Broad-spectrum determinants should be similar in organization to narrow-spectrum *mer* operons with an additional gene, *merB*, specifying organomercurial lyase.[104,105] The broad-spectrum *mer* operon in pDU1358 was thus shown to be organized similarly to the narrow-spectrum Tn*501* operon but with an additional lyase gene located promoter-distal to *merA*.[105] The *merB* gene in the pDU1358 operon was

shown to lie between the *merA* and *merD* genes. The primary translation product was predicted to be 212 amino-acids long from the DNA sequence analysis, similar to the R831b system.[104] In the R831b operon, *merB* was located approximately 13.5 kilobases (kb) downstream away from the other *mer* genes.[116]

The broad-spectrum determinant of the *S. aureus* pI258 plasmid has been cloned and sequenced.[105] Two genes (*merA* and *merB*) were identified by their homology with the equivalent genes in pDU1358. They are organized in the same fashion as the pDU1358 *mer* operon, with *merB* lying promoter-distal to *merA*. In addition, four open reading frames were identified promoter-proximal to *merA*, but no homology exists with any of the genes in the Gram-negative *mer* operons.[7] One open reading frame may specify a hydrophobic protein which could play a role in mercury transport.

The *mer* determinant in *Bacillus* sp. strain RC607 has also been cloned and is currently being sequenced.[107,109] The organization of the operon appears to be similar to the pI258 operon, but, surprisingly, the *merB* gene is not contiguous with the *merA* gene, lying 2 kb downstream from *merA*.[89,109] This situation is analogous to the R831b *mer* operon. The DNA sequence of the mercury resistance determinant in *Streptomyces lividans* has also been determined.[89] It contains four open reading frames apart from recognizable *merA* and *merB* genes. The four unidentified open reading frames are not homologous at the sequence level to those of *S. aureus* pI258. The *merB* gene sequence starts only 38 base pairs after *merA* ends.

MODEL FOR MERCURY DETOXIFICATION

The tentative model for mercuric ion and organomercurial resistance was proposed by Brown[1] and modified by Foster[7] and Silver and Misra.[89] Highly toxic mercury (Hg^{2+}) enters the periplasmic space where the *merP* gene product scavenges for Hg^{2+} ions. Mercury (Hg^{2+}) binds to the cysteine pair (Cys-14 and Cys-17) of *merP*, releasing two protons ($2H^+$). The binding should prevent Hg^{2+} from affecting sulphydryl groups in important proteins in the periplasmic space and on the surface of the cytoplasmic membrane (for example, lactose permease[1,7]).

Mercury (Hg^{2+}) is next transferred from the *merP* gene product to the outermost cysteine pair (Cys-24 and Cys-25) of the *merT* gene product, and a pair of protons are exchanged in the opposite direction (from *merT* to *merP*). All subsequent transfers of Hg^{2+} are believed to occur in a similar redox-exchange fashion.[1] The Hg^{2+} is then transferred to the cysteine pair (Cys-76 and Cys-82) on the inner face of the *merT* gene product.

The Hg^{2+} is then transferred to the cysteine pair (Cys-10 and Cys-13) in the *N*-terminal domain of the mercuric reductase (*merA*). In order for this to occur, mercuric reductase must transiently associate with the *merT* gene product in the cytoplasmic membrane.[1] Mercury (Hg^{2+}) is next transferred to the *C*-terminal cysteine pair (Cys-557 and Cys-558) where it is presented to the active site in the same or the other subunit of the dimer, where it is reduced. The less toxic mercury(0) is released to the cytoplasm, and diffuses from the cell. Elemental mercury(0) is presumed to pass out of the cell by simple diffusion due to its lipid solubility and, because of its high vapour pressure, will quickly volatilize away from the cell.

The *merD* gene product may be involved in facilitating the diffusion of mercury(0) under certain circumstances. If the rate of reduction is high, as can be expected in the case of a high-copy-number plasmid, the diffusion of mercury(0) may become limiting, thus causing product inhibition of mercuric reductase.[1] Mercury (Hg^{2+}) then entering the cell cannot be accepted from the *merT* gene product and detoxified, allowing the Hg^{2+} to interact with cytoplasmic constituents.

Organomercurial compounds are believed to cross the cytoplasmic membrane via the *merP*, *merT* system in a similar fashion to Hg^{2+}.[7] Once the organomercurial compound is transferred to the inner face of the cytoplasm, one of two events may occur. Firstly, the organomercurial may be transferred to the organomercurial lyase (*merB* gene product), and once Hg^{2+} is released, it may then be passed directly from the lyase to mercuric reductase. Alternatively, the organomercurial may be transferred directly from the *merT* gene product to mercuric reductase. If this occurred, the lyase could react with the organomercurial in a complex with the reductase. In either case, protein–protein and protein–membrane interactions would be involved to prevent the release of toxic Hg^{2+} into the cytoplasm.

The main feature conferred by the mechanism described in the model is that from the bacterial view-

point highly toxic Hg^{2+} is specifically and coordinately immobilized before it can react with cellular components, and it is not released until it has been detoxified.[1]

The chemistry and biology of mercury cycling in the environment are complex processes that are becoming better elucidated. Powerful analytical and genetic techniques have provided a great deal of knowledge about this toxic metal in the last 15 years. A better understanding of mercury cycling in the environment and microbial resistance/detoxification mechanisms are now available.

Acknowledgements This research was supported by an NSERC (Canada) operating grant to J T Trevors. B H Belliveau was the recipient of an NSERC post-graduate scholarship.

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