

Bacterial mercury resistance from atoms to ecosystems

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Abstract

Bacterial resistance to inorganic and organic mercury compounds (HgR) is one of the most widely observed phenotypes in eubacteria. Loci conferring HgR in Gram-positive or Gram-negative bacteria typically have at minimum a mercuric reductase enzyme (MerA) that reduces reactive ionic Hg(II) to volatile, relatively inert, monoatomic Hg(0) vapor and a membrane-bound protein (MerT) for uptake of Hg(II) arranged in an operon under control of MerR, a novel metal-responsive regulator. Many HgR loci encode an additional enzyme, MerB, that degrades organomercurials by protonolysis, and one or more additional proteins apparently involved in transport. Genes conferring HgR occur on chromosomes, plasmids, and transposons and their operon arrangements can be quite diverse, frequently involving duplications of the above noted structural genes, several of which are modular themselves. How this very mobile and plastic suite of proteins protects host cells from this pervasive toxic metal, what roles it has in the biogeochemical cycling of Hg, and how it has been employed in ameliorating environmental contamination are the subjects of this review.

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1. Introduction

Study of bacterial mercury resistance is in its fifth decade. This earliest discovered example of a bacterial defense system against this ubiquitous toxic metal and its organic derivatives has been studied for its novel biochemical and genetic aspects, for its natural and engineered role in environmental bioremediation and biomonitoring, as a model of horizontal gene exchange, and for the general insights it provides into how cells deal with redox-active thiophilic metals. The genes encoding the proteins of mercury resistance occur naturally on chromosomes, plasmids, and transposable elements in a striking diversity of arrangements, often involving duplications and distributions of the enzymes, transporters or regulators among several replicons in one cell. Moreover, two major *mer* genes, the regulator MerR and the major detoxification enzyme, MerA, the mercuric ion reductase, are each composed of discrete modules observed in paralogs with distinct but related roles in prokaryotic physiology.

This brief review touches on each of these aspects of this appropriately protean system which has evolved in response to an equally protean and unremittingly toxic element. We endeavor to place the coordinated workings of the *mer* proteins into a cellular context; a theme throughout will be the roles of cysteines in the *mer* proteins and their relationship to thiols in their respective cellular compartments. Cysteines are the most vulnerable targets of mercury-induced loss of function in any protein. Surprisingly, rather than dodging such interactions, every *mer* protein uses cysteines (perhaps exclusively) to interact with either inorganic or organic mercurials. Thus, *mer* proteins have evolved to carry out metalloregulation, transport, and enzyme catalysis but emerge unscathed from their interactions with mercury, converting the tenaciously reactive ionic or organic forms into the reduced and relatively inert, monoatomic elemental form, Hg(0). Since Hg(0) has a high vapor pressure (Henry's constant of 0.3) and very low aqueous solubility (6 µg per 100 ml of water at 25°C), it is volatile at a liquid:air interface but may coalesce into a liquid in a closed or diffusion-limited system. We are now beginning to understand how the *mer* proteins have evolved to exploit the internal cellular milieu to manage these feats.

Mercury resistance is also the only bacterial metal resistance system whose mechanism leads to large-scale transformation of its toxic target. The mechanisms of other cation and oxoanion resistances are based on efflux pumps or extracellular sequestration. Thus, there has long been interest in *mer*'s role in the global cycling of mercury and in employing the resistance mechanism for remediation

efforts. Most of these practical applications are as yet only demonstration projects, but a few have become economically viable.

1.1. A brief history of the study of mercury resistance

Moore [1] first reported bacterial resistance to inorganic and organic mercury compounds (HgR) in a clinical isolate of *Staphylococcus aureus* which was also penicillin-resistant. This was of concern because at the time mercurial compounds were extensively used as topical disinfectants and antiseptics in hospitals and in the community. Shortly thereafter groups in the UK [2] and the USA [3] demonstrated that both HgR and penicillin resistance in *S. aureus* strains were genetically linked on a newly discovered class of mobile genetic elements called plasmids. Similar findings were made in clinical strains of *Escherichia coli* [4]. About the same time owing to growing concern about the use of mercurial fungicides on grain in Sweden and elsewhere [5,6], there were several reports of non-clinical, environmental bacteria which could transform organomercurials into volatile, relatively inert monoatomic Hg(0) [7–9]. It was quickly recognized that some bacteria were resistant to and able to transform both inorganic and organic mercury compounds and other bacteria (even of the same genus) could only resist and transform inorganic mercury compounds [10]. The former phenotype was given the name 'broad-spectrum Hg resistance' and the latter 'narrow-spectrum Hg resistance' [11].

Work on the mechanism of HgR led to identification of the key detoxification enzyme, MerA, the mercuric ion reductase [12], and also of a second enzyme, MerB, which split the carbon–Hg bond in such compounds as the disinfectant phenylmercuric acetate (PMA) and the fungicide methylmercury chloride, a potent neurotoxic agent [13]. Membrane and periplasmic proteins involved in the seemingly paradoxical inward transport of ionic mercury [14,15] were also identified [16,17]. The first sequences of HgR loci revealed proteins corresponding to these biochemical and physiological functions as well as a candidate regulatory gene (*merR*). Somewhat surprisingly, each operon had distinct gene complements and operonic arrangements [18–21]. Later sequencing efforts showed even greater variety in contents and arrangements of *mer* operons [22–27]. At present, as detailed below, considerable recent progress has been made in understanding the structure–function relationships in many of these genes.

The population biology of HgR is more extensive than that for other bacterial metal resistances owing to concerns over the environmental dissemination of Hg. Aerobic and facultative HgR bacteria are easily isolated on a

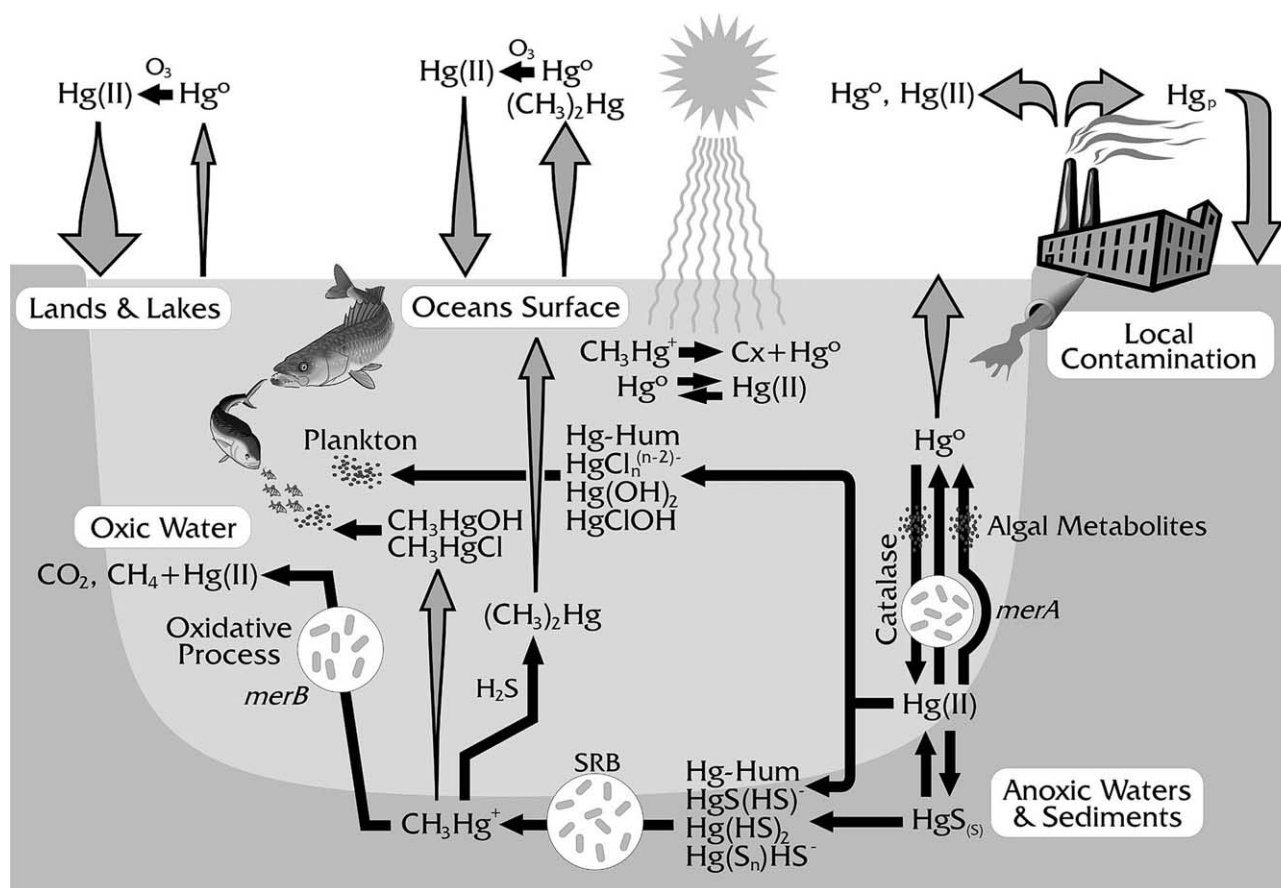


Fig. 1. The biogeochemical cycle of mercury in the environment (modified from [47]). Solid arrows represent transformation or uptake of mercury. Hollow arrows indicate flux of mercury between different compartments in the environment. The width of the hollow arrows is approximately proportional to the relative importance of the flux in nature. The speciation of Hg(II) in oxic and anoxic waters is controlled by chloride and hydroxide, and by sulfide respectively [47]. Transformations known to be mediated by microorganisms are represented by circles depicting bacterial cells. SRB stands for sulfate-reducing bacteria, and *merB* and *merA* refer to the activity of genes encoding the enzymes organomercurial lyase and mercuric reductase, respectively. A group of dots indicate the involvement of unicellular algae. Light-mediated water column transformations are positioned below the sun. Photodegradation of CH_3Hg^+ results in mostly Hg° and an unknown C_1 species depicted as C_x (D. Krabbenhoft, personal communication). Reprinted with permission from [77].

variety of media from soils, water, and sediments as well as from humans and other animals [23,25,28–36]. In cases where it has been examined these bacteria reduce Hg(II) to Hg(0). In general, but not always, DNA similar to either widely found Gram-negative or Gram-positive *mer* genes (typically MerA) can be found in the genomes or plasmids of these strains. Thus, it appears that while Hg(II) reduction is a common mechanism, there may be even greater divergence in the mechanisms of this reduction than is so far apparent. Details and general themes arising from this great phylogenetic variety are described below.

2. The global mercury cycle

2.1. Sources of Hg in the environment

Mercury is present in the Earth's crust at concentrations ranging from 21 (lower crust) to 56 (upper crust) ppb [37]

in the elemental form and as a variety of HgS binary minerals, such as cinnabar, metacinnabar and hypercinnabar [38]. Although these minerals have a low solubility (10^{-6} g per 100 ml water for cinnabar [39]), their solubilized mercury products are available for transformations between the +2, +1 and 0 oxidation states and for conversion to various organic forms by natural and anthropogenic processes.

Anthropogenic sources of Hg include input to the atmosphere resulting from burning fossil fuels and incineration or other disposal of products such as fluorescent light fixtures, batteries, dental restorations, and electrodes used in the chlor-alkali process. Deposition of Hg to land and natural waters can result from existing and defunct industries, from landfills and as a result of sludge applications [40]. Worldwide anthropogenic input is substantial, accounting for 75% of the global input of Hg to the environment [41]. Changes in atmospheric Hg concentrations over time [42,43] and enhanced accumulation rates in-

Table 1
Transformations in the Hg biogeochemical cycle

Transformation	Process	Mechanism	References
Hg(II) methylation	Biotic	Methyltransferase-mediated transfer of CH_3^- from methylcobalamin via the acetyl-CoA pathway in sulfate-reducing bacteria	[63]
	Abiotic	Methylation by fulvic and humic acids, carboxylic acids and methylated tin compounds	[72,73]
$\text{CH}_3\text{Hg(I)}$ demethylation	Biotic	Reductive demethylation by <i>merAB</i> yielding CH_4 and Hg(0) . Oxidative demethylation by (an) uncharacterized microbial process(es) yielding CO_2 and an unidentified Hg moiety	[75,238,239]
	Abiotic	Photodegradation at 200–400 nm	[81,82]
Hg(II) reduction	Biotic	Bacterial mercuric reductase and undefined pathways by algae during light and dark growth	[95,100]
	Abiotic	Photochemical and dark reactions by organic and inorganic free radicals. Disproportionation: $2\text{Hg(I)} \rightarrow \text{Hg(II)} + \text{Hg(0)}$	[83,104]
Hg(0) oxidation	Biotic	Oxidation by hydroperoxidases in microbes, plants and animals	[105,109]
	Abiotic	Photooxidation by various oxidants and free radicals and dark oxidation possibly by O_2 in saline conditions	[110,113]

ferred from sediment [44,45] and ice cores [46] clearly show increasing Hg input to the environment in the last two centuries.

Biotic and abiotic processes facilitate Hg cycling from soils and water to the atmosphere and back to the surface by both wet and dry deposition (Fig. 1 and Table 1) [47]. The following is a brief discussion of the contribution of these biotic and abiotic transformations that constitute the global Hg cycle.

2.2. Ionic mercury [*Hg(II)*] methylation

Owing to some devastating historical episodes of mass intoxication resulting from very high exposures to anthropogenic methylmercury (MeHg), current public health concerns focus primarily on this potent neurotoxin [40,48,49]. Although the bulk of Hg in the environment is in inorganic forms [50], natural biotic and abiotic processes can synthesize MeHg that is then subject to bioaccumulation unless degraded by other biotic or abiotic processes [51].

Discovery of Hg methylation by microorganisms in anaerobic sediments [52] focused attention on methylcorrinoids (vitamin B_{12}) as the most likely agent able to transfer CH_3^- to Hg^{2+} [53]. Research on corrinoid-producing anaerobes including methanogens [54] was followed by the discovery that many bacteria can methylate Hg(II) in lab culture [55]. Subsequent field and laboratory experiments with anaerobic sediments using specific metabolic inhibitors and substrates clearly implicated sulfate-reducing bacteria (SRB) as the principal methylators in natural anaerobic sediments [56–58].

More recent work, combining Hg chemical speciation with methylation rate measurements [59–61], showed that soluble, neutral HgS is the substrate for methylation by SRB. Richard Bartha and his students, studying methylation by crude cell extracts of *Desulfovibrio desulfuricans* strain LS, showed that the methyl group originated either from serine C3 [62] or from formate via acetyl-CoA [63]

via CH_3 -tetrahydrofolate (MeTHF) to methylcobalamin [64] followed by enzymatic methylation of Hg [65]. A positive correlation of Hg(II)-dependent transformation of MeTHF to THF in soil and sediment extracts with in situ MeHg concentrations [66] further supported the notion that enzymatic catalysis, rather than spontaneous transfer of CH_3 from methylcobalamin [53], is the mechanism of microbial MeHg synthesis. The microbial production of dimethylmercury (diMeHg), although proposed initially [54], has not been unequivocally demonstrated to date. The production of diMeHg from monoMeHg [67] by disproportionation in H_2S -rich environments [68] might be the mechanism by which SRB form diMeHg during sulfidogenic growth [69]. Recent reports of high levels of diMeHg in aerial fluxes from oceanic upwelling sites [70] (N. Bloom, personal communication) and terrestrial [71] sources warrant a closer look at the mechanisms by which diMeHg is formed.

Abiotic methylation of Hg occurs and its contribution to MeHg production in the environment is presently a contested issue [72]. Agents shown to be responsible for abiotic Hg methylation include humic and fulvic acids [72], carboxylic acids [73], and alkylated tin compounds used in agriculture as fungicides and as marine antifouling agents [74].

2.3. $\text{CH}_3\text{Hg(I)}$ demethylation

MeHg can be degraded reductively to CH_4 and elemental Hg, Hg(0) , by *mer* operon functions (see below). Alternatively, under certain conditions oxidative demethylation, the degradation of MeHg to CO_2 and a small amount of CH_4 , occurs, possibly as a cometabolic by-product of methylotrophic metabolism [75]. Like methylation, oxidative demethylation is mediated by anaerobic bacteria. Its mechanism is currently unknown, but may be analogous to monomethylamine degradation by methanogens or to acetate oxidation by SRB [76]. Whether MeHg is degraded reductively or oxidatively in anaerobic sediments may be

important. The reductive, *mer* operon-determined process results in net removal of Hg from the sediment as monoatomic elemental vapor. In contrast, Hg(II), the probable product of oxidative demethylation, is a substrate for remethylation within the sediment community. Thus, a methylation–demethylation cycle may exist in environments lacking the *mer*-mediated process [77]. Several environmental studies [75,78,79] suggest that reductive *mer*-mediated demethylation dominates at high Hg concentrations in more aerobic settings, whereas oxidative demethylation dominates at lower Hg concentrations in more anaerobic settings (see [77] for details). That this pattern might arise from the effect of oxygen on the induction of *mer* operon expression is suggested by the observation that expression of the broad-spectrum *mer* operon of a denitrifier, *Pseudomonas stutzeri* OX, [80], was induced at lower Hg(II) concentrations during aerobic growth than during anaerobic growth [77]. Thus, conditional inducibility of the *mer* operon may critically affect MeHg production in Hg-contaminated environments.

Abiotic degradation of MeHg can be effected by sunlight, specifically UV-A and UV-B, spanning a wavelength range of 280–400 nm, and this process is inhibited by singlet oxygen-trapping agents [81]. Dark incubations of lake water had 350-fold higher MeHg concentrations than light incubations [82]. Suda et al. [81] reported production of inorganic Hg from MeHg during photodegradation and Hg(0) was identified as the major product of photodegradation in wetlands (D. Krabbenhoft, personal communication). Thus, in light-exposed environments, such as wetlands and lakes, and especially at low total Hg concentrations, photodegradation may be the major mechanism for MeHg degradation [82]. In contrast, in sediments and bottom waters, where MeHg accumulates following methylation, photodegradation may have little impact on demethylation and either reductive or oxidative microbial processes will most likely dominate.

2.4. Ionic mercury [Hg(II)] reduction

Reduction of Hg(II) to Hg(0), which occurs in natural waters [83] and soils [84,85], results in partitioning of Hg into the air due to Hg(0)'s low aqueous solubility (60 $\mu\text{g l}^{-1}$ water at 25°C) and high volatility (Henry's coefficient of 0.3) [39]. Hg in rain and snow (the major means for the global redistribution of Hg) is largely in the ionic form [86] and is highly available for reduction. Reduction of the deposited Hg(II) to Hg(0) transports Hg back to the atmosphere [87] and therefore prevents its precipitation and settling to bottom sediments where it can be methylated [51,88].

Indeed, biological reduction contributes significantly to the flux of Hg from natural waters into the atmosphere. Nutrient-rich waters near the equatorial upwelling in the Pacific Ocean are supersaturated with Hg(0) [89] and, in latitudinal transects, Hg(0) concentration correlated posi-

tively with biological productivity [90]. Filtration [91] or autoclaving [92] inhibits Hg volatilization from natural waters. Environments highly contaminated with Hg(II) enrich for populations of resistant bacteria [92,93] and induce *mer* operon-mediated reduction [94–96]. However, Hg concentrations in most natural environments, including those where MeHg bioaccumulation occurs, are in the fM to pM range. Although *mer-lux* transcriptional fusions can be induced at < pM of Hg(II) in oligotrophic conditions [97], induction is likely to be inefficient [98]. In addition to the bacterial Hg(II) reductase, some algae reduce Hg(II) by both light-dependent [99] and light-independent [100] processes; the latter transformation may be mediated by extracellular metabolites rather than by a specific enzymatic activity.

Abiotically Hg(II) can be reduced by photochemical transformations or dark reactions. Photoreduction of Hg(II) is due to organic free radicals produced by photolysis of dissolved organic carbon [83], dissolved oxygen, organic carbon complexes, and Fe(III)–organic acid coordination compounds [101]. In the dark, Hg(II) can be reduced by fulvic [102] and humic [103] acid-associated free radicals. Lastly, Hg(0) is in equilibrium with Hg(I) and Hg(II); Hg(I) is favored unless there are ligands which strongly favor the Hg(II) state, i.e. thiols [104].

2.5. Elemental mercury [Hg(0)] oxidation

Biologically induced oxidation of Hg(0) is the least explored step in the Hg biogeochemical cycle. Smith et al. [105] showed Hg(0) oxidation by bacterial hydroperoxidases, KatG and to a lesser extent KatE, in *E. coli*. A double mutant, lacking both enzymes, retained a low level of Hg(0) oxidation, suggesting the existence of other bacterial Hg(0) oxidases. Aerobic soil bacteria, *Bacillus* and *Streptomyces*, had high levels of Hg(0)-oxidizing activity suggesting a potential role for microbial oxidation in the cycling of Hg in the environment [105]. Note that in all cases the rate of Hg(0) oxidation in these Hg(II)-sensitive, plasmid-free bacteria is at least 10-fold lower than the rate of MerA-mediated Hg(II) reduction (see below) observed in bacterial cells carrying a *mer* operon [106,107]; thus, hydroperoxidase-mediated oxidation does not generate a futile cycle within a HgR cell. Moreover, Siciliano et al. [96] recently showed a relationship between Hg(0) oxidase activities, measured by the accumulation of Hg(II) after incubation of protein extracts of lake microbial biomass with Hg(0)-saturated water, and the rate of accumulation of dissolved gaseous mercury, mostly Hg(0), in lake water. Plant catalases are also capable of oxidizing metallic mercury vapor [108], as are those of animals [109].

Abiotic oxidation of Hg(0) to Hg(II) occurs in the atmosphere [110], natural waters [96], and soils [111]. Since ionic Hg(II) is rapidly absorbed by rain, snow and airborne particles, oxidation of Hg(0) enhances atmospheric deposition of Hg. Attention to this important deposition

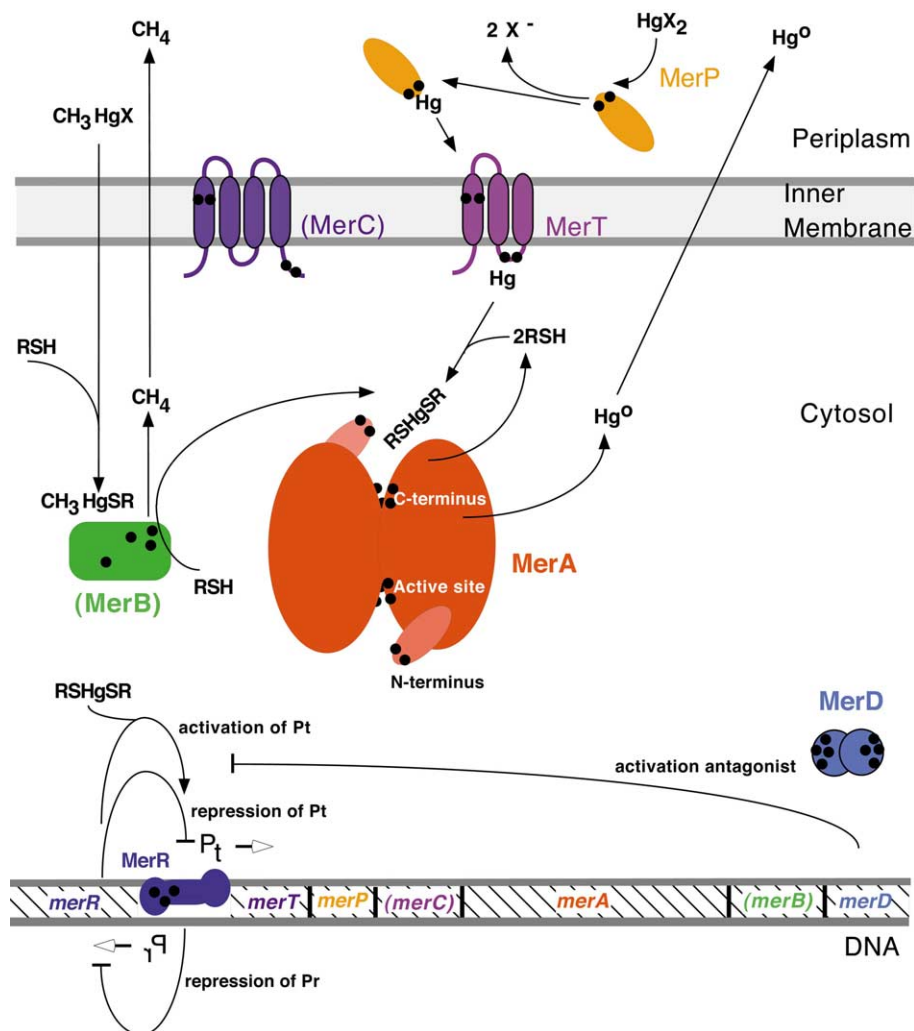


Fig. 2. Model of a typical Gram-negative mercury resistance (*mer*) operon. The symbol ● indicates a cysteine residue. X refers to a generic solvent nucleophile. RSH is the low-molecular-mass, cytosolic thiol redox buffer such as glutathione. Parentheses around gene or protein designations indicate proteins/genes that do not occur in all examples of the operon (see text for other sporadic *mer* genes).

process has resulted in delineation of several pathways for photochemical oxidation. Because research has focused on atmospheric transformations, little is known about the mechanisms of Hg(0) oxidation in natural waters and soils where this process may critically affect MeHg production by increasing Hg(II) concentrations. In fact, it has been suggested that oxidation is the sink for Hg(0) in natural waters [112,113].

Photooxidation of Hg(0) is mediated by O₂ in the presence of excess chloride [114], by hydrogen peroxide, ozone [115,116], sulfhydryl compounds [117], the free radicals BrO•, Br• and Cl• [110,118], and by UV-B in the presence of Cl₂ and photoreactive compounds such as benzoquinone [112]. Dark oxidation of Hg(0) in the presence of chloride, most likely by O₂, in seawater is also known [113].

The large body of knowledge briefly summarized above resulted from concerns that arose in the late 1960s following the discovery of MeHg biomagnification in the aquatic food chains. Today, marine and freshwater fish remain the

chief source of dietary Hg exposure, constituting approximately half of non-occupational Hg exposure. The balance of non-occupational Hg exposure for humans comes from amalgam ('silver') dental fillings and merthiolate used as a preservative in vaccines [49]. Since the same biotransformations that constitute the Hg biogeochemical cycle can take place inside the human body, understanding these external transformations and transport processes will help in figuring out which of these processes can exacerbate or ameliorate Hg intoxication in humans.

3. Functional components of Hg resistance operons

With one exception all of the foregoing biotic processes transforming mercury are adventitious side reactions of normal physiological redox and methylation reactions. That exception is the widely distributed phenomenon of specific resistance to and transformation of mercury compounds embodied in the *mer* operon. In this section we

describe the regulatory and the structural genes of the typical and more extensively studied Gram-negative *mer* operons. In the next section we consider the varieties of operon structure and their dissemination on plasmids and transposons in Gram-negative and Gram-positive bacteria of environmental and clinical origin.

3.1. The generic *mer* operon

Since the *mer* operons of Gram-negative bacteria are the most extensively studied examples of bacterial mercury resistance, we will use this model (Fig. 2) as a brief primer to the more detailed information and corresponding references which follow. Gram-positive bacteria have similar sets of genes, generally arranged in similar order into operons (see Section 4). This most widely observed mechanism of eubacterial mercury resistance involves the reduction of the highly reactive cationic form of mercury to volatile, relatively inert monoatomic mercury vapor. The agent of this reduction is MerA, the mercuric reductase, a cytosolic flavin disulfide oxidoreductase (homodimer ~120 kDa) which uses NAD(P)H as a reductant. In Gram-negative bacteria and in all but one genus of Gram-positive bacteria that have been studied, MerA has a flexible amino-terminal domain which is homologous to MerP, a small periplasmic mercury binding protein. MerP itself uses its two cysteine residues to displace the nucleophiles (likely Cl⁻) to which Hg(II) is coordinated in typical aerobic microbial growth media. MerP is thought to exchange Hg(II) to two cysteines in the first of three transmembrane helices of MerT, an inner (cytosolic) membrane protein. How MerT transfers Hg(II) into the cytosol is not understood, but may involve its second cysteine pair predicted to lie on the cytosolic side of the inner membrane.

Once on the inner side of the cytoplasmic membrane, millimolar concentrations of cytosolic thiol redox buffers such as glutathione in Gram-negative bacteria or cysteine in some Gram-positive bacteria compete with MerT's cytosolic cysteines to remove Hg(II) as a dithiol derivative, a form in which it is a substrate for MerA. Alternatively, the MerP-like N-terminal domain of MerA may directly remove Hg(II) from MerT's cytosolic cysteines. When reduced by MerA, volatile, lipid-soluble Hg(0) diffuses through the cell membranes without the need for any dedicated efflux system.

In addition to a Hg(II) uptake system and the Hg(II) reductase, most mercury resistance loci also have some means of controlling expression of these proteins. This function is carried out by MerR, a metal-specific activator-repressor of the operon that encodes the MerT, MerP, and MerA structural genes. These three components (transport, catalysis, and regulation) are the central functions of mercury resistance found in both Gram-negative and Gram-positive eubacteria.

Both Gram-positive and Gram-negative operons fre-

quently encode several other proteins not all of which are depicted in Fig. 2. Some of these are membrane proteins; MerC is the most common of these in Gram-negative bacteria, but MerF and MerE are increasingly observed as sequence data accumulate. MerC and MerF, which replaces MerC in some operons, can each function solo in Hg(II) uptake although, with the exception of *mer* in the thiobacilli, they are always found with MerT. The only other *mer*-related enzyme, the organomercurial lyase MerB, transfers a proton to the C–Hg bond in alkyl- and aryl-mercurials, yielding ionic mercury which is then reduced to Hg(0) by MerA. Bacteria with MerB are resistant to inorganic and organic mercurials. An apparently periplasmic protein, MerG, found recently in some Gram-negative bacteria, provides a modest increase in organomercurial resistance even to strains lacking MerB. Finally, in many Gram-negative bacteria there is an additional protein, MerD, which appears to antagonize MerR's activation of *mer* operon transcription. We now consider each of these aspects of this general detoxification mechanism in its most current detail.

3.2. Regulation

3.2.1. MerR

The metal-responsive regulatory protein MerR and its growing family of novel transcriptional activators are considered in detail in Brown et al. [119]. Here we briefly describe this novel regulatory protein, its cognate DNA binding site, and its putative antagonist, MerD, and also note other unusual aspects of control of gene expression in the *mer* operon.

MerR is the first described of a growing family of novel transcriptional activator-repressors with similar properties [120]. MerR binds (apparently as a homodimer) to a region of dyad symmetry called MerO (operator) just upstream of the *merT* gene [121,122]. MerO lies between the -10 and -35 RNA polymerase recognition sites for the promoter of the structural genes (Pt). MerR's own promoter (Pr) reads divergently from Pt and overlaps with it so extensively that when MerR occupies MerO, it prevents RNA polymerase from transcribing the *merR* gene, i.e. MerR is a negative autoregulator.

Despite binding right between the RNA polymerase recognition hexamers at -10 and -35, MerR attracts RNA polymerase to the Pt promoter even in the absence of metal ion inducer [123,124] and also bends the promoter DNA away from the polymerase, establishing a stable, non-transcribing pre-initiation complex [125,126]. When Hg(II) is present, it binds to MerR provoking an allosteric change in the protein which is propagated to the DNA of the operator region, leading to an underwinding of the operator DNA [123,125,126] and consequent improved access of RNA polymerase to the transcriptional start site [127,128]. MerR can be crosslinked to the α , β , and σ^{70} subunits of RNA polymerase whether free or when

both are bound to operator DNA [124]. Deletion analysis indicates that these interactions are specific to domains within MerR (Kulkarni and Summers, in preparation). This novel mechanism of repression and activation constitutes a third major type of bacterial regulatory mechanism [129] which is mimicked in the regulator/operator relationships of several related proteins responsive to other metals, oxidative shock, or to bulky, toxic organic molecules [119]. By the very unusual strategy of forming a stable pre-initiation complex with RNA polymerase, MerR establishes conditions for an instantaneous response to the presence of Hg(II) in the cytosol. This ‘ready to rock’ status in the machinery for transcriptional initiation is echoed in the NADPH-primed over-reduced ‘EH₄’ state that MerA, the mercuric reductase, maintains (see below).

The manner in which MerR binds Hg(II) is also unusual in that the binding site actually lies at the interface of the homodimer and involves one cysteine from one monomer and two distinct cysteines from the other monomer [130]. Unlike metalloenzymes which are built to have stable, long-term relationships with their metal cofactors, MerR has evolved to have a significant but transient relationship with a metal ion. Much was learned about the structure of MerR from genetic, biochemical, and biophysical data [122,130–134] even before the first 3D structures of MerR family members BmrR and MtaN were published [135,136]. These earlier studies identified Cys82, Cys117, and Cys126 (numbering based on Tn21 *merR*) as being involved in a very stable tri-coordinate Hg–SR₃ complex. Biochemical and genetic data predicted a dimer structure in which symmetrical metal binding sites lie at each end of an antiparallel coiled-coil which constitutes the dimer interface [134]. Given the prediction and observation of a long α -helical domain between Cys82 and Cys117, such an arrangement is the only one which would allow juxtaposition of Cys82 with Cys117 and Cys126 of the other subunit. This prediction was subsequently proved correct in the 3D structure determination of the MerR family member BmrR [136]. With in vitro metal binding studies of MerR at low concentrations (1–10 μ M) [130,133,134,137], MerR bound only one atom of Hg(II), although it has two potential binding sites in the dimer. This low stoichiometry of MerR has been attributed to a putative allosteric change in the protein which (at least when not bound to DNA) renders the second potential metal binding site unavailable for Hg(II) binding once the first site has bound Hg(II) [134]. Although ¹⁹⁹Hg nuclear magnetic resonance (NMR) indicates no change in the MerR–Hg coordination geometry upon addition of DNA [138], neither the structure nor the stoichiometry of Hg added to MerR pre-bound to DNA (as would occur naturally in the cell) has been examined.

At the protein sequence level MerR’s closest relative involved in an essential physiological process is ZntR, one of two regulators of Zn(II) homeostasis in *E. coli* and many other eubacteria [139–142]. In response to

Zn(II), Cd(II), and Pb(II), ZntR controls expression of a metal ion efflux pump, ZntA. Interestingly, although in vivo [120] and in transcriptional runoff assays [143] MerR has strong specificity for Hg(II) as an inducer, as a purified protein it will bind several other transition metals ([144–146]; Song, Caguiat, and Summers, unpublished). Thus, the basis for MerR’s metal-specific transcriptional activation lies not only in its metal binding ability but in other, as yet undefined, aspects of this process.

3.2.2. *MerO*, *Pt*, and *Pr*

The cognate DNA sites through which MerR interacts with RNA polymerase to control its own expression and that of the *mer* operon structural genes are also unusual. MerO itself is, in most Gram-negative occurrences, an 18-bp hyphenated dyad with 7-bp palindromes flanking a 4-bp AT-rich center [19,20,121,147]. What is unusual about MerO is not this fairly common motif of dyad symmetry itself, but rather the fact that it lies within the spacer region between the –10 and –35 regions of the Pt promoter, slightly overlapping the –35 hexamer. The Pt promoter has consensus –10 and –35 hexamers, but is unusual in that this spacer region is 19 bp long rather than the typical 17-bp length found in most σ^{70} promoters in bacteria. Surprisingly, as noted above, MerR binding in this region does not preclude, but actually fosters RNA polymerase occupancy of Pt, albeit in a transcriptionally inactive state until Hg(II) is present [123,124,148]. Increased reactivity of bases near the operator center with nucleases and with permanganate occurs when MerR binds Hg(II) and the consequent underwinding of the operator DNA [125] makes the –10 region available to RNA polymerase; activation of the *mer* operon is consequently dependent on the supercoiled state of the DNA [149]. Genetic evidence indicated that MerR requires a distinct operator contact for repression and activation of Pt [121] and that alterations in the C-terminal domain of σ^{70} can influence activation [150]. In contrast, the divergent Pr promoter has a shorter than typical 15-bp spacer and good consensus –10 and –35 hexamers. MerR represses the Pr promoter less strongly than the Pt promoter [121,124,151], but the details of the simultaneous control of these two tightly overlapped promoters are unknown.

3.2.3. *MerD*

A small, cysteine-rich open reading frame (ORF) lying just beyond the *merA* gene of Tn501 [19] was observed to have an N-terminal region with a predicted helix-turn-helix motif similar to that of MerR. Given the name MerD, it was subsequently shown to be translated in very small amounts [152]. Although deletions of it did not affect the Hg resistance phenotype, they appeared to allow continued expression of the structural genes even after MerA had eliminated Hg(II) from the medium [153,154]. Purified MerD binds to MerO although with a

lower apparent affinity compared to MerR. Thus, MerD appears to be an antagonist of MerR function, perhaps replacing it at MerO although other mechanisms or roles for MerD have not been ruled out. MerD is also a unique protein with no apparent homologs with identified functions.

Why would a MerR antagonist be necessary? It was initially assumed that the intracellular concentration of Hg(II) would decrease due to its volatilization by MerA and the Hg(II) bound to MerR would exchange to cellular thiols, returning it to a non-activated state. However, *in vitro* experimental evidence of the very high affinity of MerR's trigonal coordination site for Hg(II), even in the presence of 5 mM thiol [134], indicated that MerR does not give up its bound Hg(II) quickly enough to account for the observed rapid restoration of repression *in vivo* [155]. It may be important for MerA expression to be repressed as soon as its substrate Hg(II) is exhausted because, like other flavin oxidoreductases in the absence of substrate, MerA has an oxidase activity which results in production of toxic hydrogen peroxide [156].

3.2.4. Transcriptional elongation and termination in Gram-negative *mer* operons

In addition to repression and activation of transcriptional initiation, expression of the Tn21 *mer* operon is subject to attenuation and natural polarity [155]. Messenger RNA corresponding to *merTPC* can be observed within 30 s of induction, but 80% of the transcripts terminate in the junction between *merC* and *merA*. Paradoxically, the *merA* portion of the transcript never reaches a level equivalent to that of the transport genes. No precise position for the C-A attenuation was identified and the operonic terminator itself is not distinct. The behavior of the *mer* transcript is consistent with prior minicell measurements of proteins expressed by the Tn21 *mer* operon [17]. MerP is the most abundant protein with ca. 10–20-fold more copies per cell than MerA; MerT occurs at about half the abundance of MerP and MerC's abundance is similar to that of MerA. Neither MerR or MerD was visualized in minicells as might be expected for regulatory proteins. At present, neither the functional relevance nor the generality of this curious mid-operon attenuation and strong transcriptional polarity in Tn21 *mer* has been examined further.

With regard to locus composition and gene arrangement, Gram-negative *mer* operons have a fairly high GC content, averaging 61% overall [157–159], regardless of the host bacterium in which any given *mer* locus was first isolated. While high GC is characteristic of some proteobacteria, the GC content of the Enterobacteriaceae, which are very common hosts for Hg resistance loci, averages 40–50%. The *mer* operon's high GC content might impede expression in a low-GC host; however, *merTP* and *merA* have apparently evolved to offset this possible impediment by employing a relatively limited codon set [157]. The

mosaic character of the Gram-negative *mer* operons makes it impossible to assign any of them or any single gene in them to a 'host of origin'; this is not surprising given their common location on plasmids and transposons. An alternative possible basis for the high GC in Gram-negative *mer* might be the fact that Hg(II) makes relatively stable intra-strand adducts at thymines which can disrupt replication [160]. Thus, there might be a selective advantage in minimizing thymines in the operon. A second unusual structural feature of Gram-negative *mer* operons is that, with one exception, the intergenic regions are short (the T-P intergenic region ranges from 12 to 15 bp [157]) or non-existent (MerE overlaps MerD [161]). The exception is the region between *merP* and *merA* that is influenced by unusual recombination events [157] (see MerC and MerF, below).

3.3. Transport

The seemingly paradoxical Hg(II) uptake system characteristic of both Gram-positive and Gram-negative mercury resistance loci is understandable in terms of the cellular biochemistry of the detoxification process. The energetically least expensive way to get rid of a potentially volatile toxic metal is to reduce it to a monoatomic gas as opposed to building carbon- and energy-intensive disposal devices like metallothionein or hydroxamate chelators which maintain homeostasis of other transition metals. The cell interior contains an abundant reservoir of low-molecular-mass thiols to coordinate ionic Hg(II) and reductants such as NAD(P)H necessary to convert it to the volatile metal.

Of course, Hg(II) can get into a cell without a dedicated transport system. Transcriptional induction of the repressed *mer* operon is detectable within 30 s after addition of Hg(II) to cells carrying a monocopy *mer* operon [155]. And in the complete absence of any *mer* operon-encoded transport proteins, MerR-directed expression of *merOP-lacZ* transcription is observable within 2–3 min after exposure to sub-micromolar Hg(II) [149]. At low [Hg], adventitious reduction by non-specific reductases might suffice for survival. However, given the vulnerability of the many cysteine-rich proteins involved in membrane-based energy generation and other significant biochemical reactions, *mer* operons have evolved to redirect such haphazard uptake into a specific, relatively high-affinity dedicated transport system consisting (in Gram-negative bacteria) of a small periplasmic protein 'Hg sponge', MerP, and one or more inner membrane proteins, MerT, MerC [162], MerF [163], and possibly MerE [157].

The existence of an operon-specific Hg(II) uptake system was first suggested on the basis of the Hg(II)-hypersensitive phenotype of *merA* mutants [14,15]; such variants were more sensitive to Hg(II) than cells lacking the operon altogether, consistent with there being some mechanism for bringing Hg(II) into the cell. In the absence of

the mercuric reductase, the internalized Hg(II) is not detoxified by reduction to Hg(0).

The *mer* operons of Gram-positive bacteria have a MerT-like predicted membrane-bound protein and also a MerP homolog [21,26,36,164]. The latter is a bit surprising since Gram-positive bacteria do not have an outer membrane and, thus, lack a periplasmic space. There are no functional studies on Hg(II) uptake by Gram-positive bacteria although their transport proteins will apparently function in *E. coli*.

3.3.1. MerP

The 72-amino acid periplasmic form of MerP is processed from a 91-amino acid precursor by removal of a typical *Sec*-type signal sequence by an energy-dependent process [17]. Structural studies [24] indicate that the protein functions as a monomer and binds a single Hg(II) ion via its two cysteine residues at positions 14 and 17 (Tn501 numbering). These two cysteines are key elements in defining a novel metal binding motif GMTcxC found singly and as multiple repeats on the N-termini of P-type ATPases involved in influx of beneficial or efflux of deleterious transition metal cations in prokaryotes and eukaryotes and also in intercompartmental flux in the latter [165]. Similar primary and secondary structural components are also found in small intracellular proteins, the so-called metal chaperones, which function in the insertion of metal cofactors in redox-active enzymes in eukaryotes [166]. Thus, although MerP does not resemble any other periplasmic transporter involved in normal metabolism, the MerP motif appears to be quite ancient and widely disseminated in proteins involved in both membrane transiting and cell interior trafficking of thiophilic metal cations.

The K_D of fully reduced MerP for Hg(II) is 3.7 μM [167]; as a disulfide MerP does not bind Hg(II) nor do mutants lacking either cysteine when there is competition from low-molecular-mass thiols [168]. Interestingly, MerP is not essential for Hg(II) uptake as MerT alone will suffice. However, a Cys14Ser mutation blocks uptake of Hg(II) by MerT (see below) [169]; the corresponding mutation at Cys17 of MerP does not have this effect. NMR shows that in the absence of Hg(II) Cys14 of reduced MerP is surface-exposed and Cys17 is buried; when bound to Hg(II) both cysteines are surface-exposed [24]. The oxidized structure more closely resembles that of the Hg(II)-bound form, and both show extensive structural changes compared to the reduced form [170]. It is interesting to note the differential effects of these two cysteines in their interaction with their transport partner, MerT; although Cys17 is buried in the reduced form of the protein, its position is unknown in the Cys14Ser mutation which can prevent Hg(II) uptake by wild-type MerT (see below).

MerP purified without added thiols is found in the disulfide form and can be reduced by mono- or dithiols. Although the periplasm is an oxidizing environment, buried Cys17 of MerP in intact cells reacts with iodoac-

tate (i.e. it is reduced) [171] owing to its having a low pK_a (5.5). Similar modulation of cysteine pK_a figures in the activity of members of cysteine pairs in proteins involved in redox homeostasis, specifically thioredoxin [172] and DsbA [173]. MerP's redox potential lies at -190 mV, about midway between DsbA and thioredoxin; the reduced form of the protein is considerably more stable than the oxidized form [171]. Interestingly, expression of the intact *mer* operon is required to maintain MerP in a reduced state in the periplasm; when MerP is expressed alone from a heterologous promoter, it is in the periplasm in an oxidized form. It is not known what components of the rest of the operon are required to maintain MerP in a reduced form in the periplasm.

It is noteworthy in this respect that intact cells bearing a *mer-lux* MerR-dependent transcriptional reporter equipped with both *merT* and *merP* genes (but not *merA*) responded to Hg(II) in minimal medium at subnanomolar levels, provided the cell density was kept to a minimum. Onset of the response was quite rapid (ca. 10 min using 10^6 cells ml^{-1}) [174]. It was also demonstrated that neutral HgCl_2 or Hg(OH)_2 species are more effective inducers than the negatively charged species HgCl_3^- and HgCl_4^{2-} which can arise in saline environments [175]. With respect to the affinity of MerP these observations suggest that the negatively charged outer surface of Gram-positive (due to teichoic acids) or Gram-negative (due to lipopolysaccharides) bacteria may repel negatively charged complexes of Hg(II). Thus, MerP will likely encounter Hg(II) as neutral chloride or hydroxide complexes whose ligands can readily be displaced by the sulfurs of MerP's cysteine residues. Since the periplasm is an oxidized environment lacking low-molecular-mass thiols, MerP's major competitors for Hg(II) will be proteins carrying out thiol-mediated redox activities such as those of the Dsb loci involved in correct formation of disulfide bonds in periplasmic and other secreted proteins [173]. Like periplasmic binding proteins involved in nutrient uptake, MerP is the most abundantly synthesized protein encoded by its operon [16,17,155], consistent with its role as an extracytoplasmic 'sponge' for Hg(II).

NMR studies show that MerP binds Hg(II) in a linear, bi-coordinate configuration. However, recent NMR studies of mutant peptides based on the immediate neighborhood of these two cysteines showed considerable flexibility in this region and concomitant acquisition of novel metal specificities [176]. Opella's group also showed that the wild-type model peptide has ca. two- to four-fold higher affinity for Hg(II) than for other thiophilic transition metals. It is hypothesized that MerP hands off the bound Hg(II) ion to the cysteine pair in the first transmembrane helix of MerT (or MerC or MerF, see below) via a three-coordinate intermediate [162].

3.3.2. MerT

MerT is the other central player in Hg(II) transport in

both Gram-positive and Gram-negative bacteria (excepting *Acidithiobacilli* which apparently use only *merC*) [107,177]. This 116-residue (12.4-kDa) inner membrane protein is strongly predicted to have three transmembrane helices, the first of which has a cysteine pair which would lie within the first hydrophobic helix and perhaps be accessible from the periplasmic side [157,162,178]. Mutations altering either of these cysteines decrease the Hg(II)-hypersensitive phenotype that is a surrogate for physical measurement of Hg(II) uptake [15]. As noted above, a Cys14Ser mutation in MerP blocks uptake of Hg(II) by wild-type MerT [169], perhaps by formation of a stable Hg(II)-bound trigonal structure involving the MerP Cys17 and MerT's N-terminus-proximal cysteine pair. It is not clear why the Cys17Ser mutant of MerP would not also get stuck; the difference may lie in the unusually low pK_a of MerP Cys17.

The second pair of cysteines in MerT is predicted to lie on the cytoplasmic face of the inner membrane between the second and third transmembrane helices. Hg(II) may be transferred from the N-terminus-proximal cysteine pair to form a di-coordinate protein complex with these cysteines and then transferred to cytoplasmic low-molecular-mass thiols and/or directly to MerA. Direct evidence on this point is presently lacking. MerA's N-terminal domain, which is a homolog of the secreted portion of MerP (see NmerA, below), may be involved in removing Hg(II) directly from this cytosolic cysteine pair of MerT, although this is not an obligatory step since *in vivo* and *in vitro* Hg(II) complexes with low-molecular-mass thiols are apt substrates for MerA (see below). Although MerA is a soluble enzyme, there is evidence for a small amount of it associating stably with the inner membrane [17].

MerT is the locus of an interesting polymorphism found in a large number of occurrences of the *mer* operon on Tn21-like transposons from both animal and environmental bacteria. Possibly owing to a sharp transition in GC content (from ca. 40% to ca. 80%) which lies at the border of the operator–promoter region with the *merT* gene, single and multiple tandem iterations of 15 bases can occur in the immediate N-terminal region of MerT. The beginning of these insertions is always offset to the +1 frame so the amino acids encoded are entirely novel, not a duplication of existing amino acids in MerT. The duplicated region is high in charged residues, has a predicted propensity to form a random coil, and would tend to remain in the cytosol. This polymorphism is apparently without effect on MerT function because cells bearing it have a Hg(II) resistance phenotype indistinguishable from those which lack it [161,179].

There are no reported physical studies on MerT, largely owing to the difficulty of such studies on membrane proteins. Possible homologs of the *mer* inner membrane proteins with known functions have not yet been spotted, although doing so might be difficult. The closest thing to a motif in these very small proteins (two, three, or four

generic membrane-spanning helices equipped with a couple of thoughtfully deployed cysteine pairs and the odd proline) is not a promising substrate for incisive phylogeny. But, there must be more here than meets the eye; how Hg(II) gets from the cysteine pair of MerP into the cytosol is one of the biggest mechanistic mysteries in the entire subject of Hg(II) resistance and is ripe for biochemical and biophysical studies. Moreover, since we know Hg(II) can enter the cell even without the *mer* transport proteins, this question is a key to understanding why all described *mer* operons have a dedicated Hg(II) transport system.

3.3.3. *MerC*

This 161-residue membrane-bound protein with four predicted transmembrane helices is the largest of the generally small *mer* operon-encoded membrane proteins. Its appearance in only one of the first two, otherwise very similar *mer* operons sequenced was the first hint of the mosaic character of the operon [159]. A careful deletion analysis of the *merC*-encoding Tn21 operon revealed that while loss of MerP or MerT had some phenotypic consequences, lack of MerC did not diminish the Hg resistance phenotype or Hg(II) volatilization ability when the relevant operons were carried on small, mono- or oligo-copy plasmids [107]. These data suggested that even though it carries *merC* none of the other features of the Tn21 *mer* operon is altered so as to make the operon's function dependent on having the MerC protein. Thus, it was initially considered to be vestigial, possibly a decaying variant of a *merT* duplication.

The discovery in *Thiobacillus* isolated from mercury mines of operons that apparently had only *merC* and no *merT* genes forced reconsideration of the view that *merC* is vestigial. Cloning the *Thiobacillus merC* gene into *E. coli* revealed that its product was able to function in Hg(II) uptake [180], although not as effectively as MerT. The additional findings of the widespread occurrence of plasmid-borne Tn21-like and other *mer* operons carrying *merC* ([23] and Section 4) made it clear that there must be some functional relevance for the MerC protein. Subsequent studies [157] concluded that *merC* is evolving differently than genes immediately adjacent to it in the operons where it occurs and maybe also evolving differently in different hosts. It has been suggested that MerC may be needed under conditions of very high Hg(II) exposure [181], but this point has not been explicitly tested. *merC* is not uniquely associated with *merB* [23] or *merG* [182].

Despite the fact that it also is a membrane protein and, thus, refractory to work with, there are more biochemical studies of this ambiguous membrane protein, MerC, than of the unequivocal Hg(II) transporter, MerT. In two very informative studies [171,178], Lena Sahlman and co-workers established important fundamentals of MerC structure and function. First, Sahlman et al. [178] observed that membrane vesicles prepared from cells expressing MerC

took up on average six-fold more Hg(II) than vesicles from a strain carrying the vector plasmid alone. This uptake was completely inhibited by the hydrophobic thiol-modifying reagents *N*-ethylmaleimide and benzophenone-4-maleimide, and partially inhibited by the hydrophilic thiol reagents iodoacetate, iodoacetamide, and fluorescein maleimide, indicating that the essential cysteines of MerC are inaccessible from the periplasmic surface of the inner membrane in vesicles. The total amount of Hg(II) taken up was proportional to the amount of MerC incorporated into the vesicles and thus indicated simple binding of the Hg(II) to the thiols of MerC, not uptake and release into the vesicle lumen. The minimal effects of an electrochemical gradient, changes in osmotic pressure and detergent exposure also indicated that Hg(II) was simply held by MerC, not pumped into the vesicles.

Further studies by Sahlman et al. [171] with purified MerC showed that it has a tendency to dimerize even when purified with cysteine present and that even as a fully reduced monomer only two of its cysteines react with Ellman's reagent, suggesting that its other two are buried or otherwise non-reactive. Topological predictions suggest that MerC's first cysteine pair lies just within the membrane on the cytoplasmic side and that the latter pair lies in the cytosol. So, MerC presents no cysteines to the periplasm, consistent with a lack of effect of MerP on whole cell binding of Hg(II) to MerC. Nonetheless, in the vesicle system an excess of MerP can inhibit Hg(II) binding to MerC indicating that the affinity of MerP for Hg(II) is greater than that of MerC. Addition of MerT diminished uptake by MerC to a level equal to that of MerT alone, suggesting that there is no synergy between these two proteins and that there may even be competition.

Site-directed mutagenesis of the four cysteines of MerC revealed that both Cys22 and Cys25, predicted to lie in a canonical GMxCxxC metal binding motif just inside the cytoplasmic face of the membrane, are essential for Hg(II) binding to vesicles [171,178]. Interestingly, elimination of the C-terminal cytoplasmic cysteine pair at positions 127 and 132 had minimal effect on Hg(II) binding. Moreover, each of the single Cys127 and Cys132 variants had a higher tendency to dimerize but this apparently does not affect Hg(II) uptake. The greater significance of the N-terminus-proximal, membrane-embedded cysteine pair of MerC compared to its C-terminal cytosolic pair is similar to findings with analogous mutants in MerT and suggests that it is the membrane-embedded cysteine pairs in these proteins which carry Hg(II) from the periplasm to the cytosol. A precise role for the cytosolic cysteine pair remains to be determined. Possible roles include facilitating multimerization of MerC and/or MerT or docking with MerA.

3.3.4. MerF

The *merF* gene was first noted between the *merP* and

merA genes in a plasmid-borne *mer* operon in an environmental pseudomonad [31]; this region is occupied by *merC* in Tn21 and is much shorter and lacks any ORF in Tn501 [157]. The occurrence of *merF* affirmed the idea of the *mer* operon as a mosaic of swappable protein modules. MerF was predicted to be a membrane-bound protein [31] with a vicinal cysteine pair near the center of the first of its two transmembrane helices and another vicinal cysteine pair near its C-terminus in the cytosol. Thus, in its predicted structure, MerF resembles the first two transmembrane helices of MerC, although MerC's N-terminal cysteine pair is CXXC and MerF's is CC. All other small membrane-related *mer* proteins have one or two amino acid spacers between their paired cysteines; only MerF has two vicinal CC motifs. The only other vicinal cysteines in *mer* proteins occur in the organomercurial lyase, MerB (see below).

Nigel Brown's group [163] demonstrated that MerF is located in a crude membrane fraction derived from radiolabelled maxicells. Expression of MerF facilitated volatilization of Hg(II) but this activity was not enhanced by MerP. MerF, like MerC and MerT, has a proline and a single charged residue in its second transmembrane helix. The relevance of this motif to Hg(II) uptake is not known, although the occurrence of the helix-breaker proline in the middle of a transmembrane α -helix is reminiscent of the P-type ATPases which often figure in metal ion transport systems [183].

3.3.5. MerE

The *merE* gene is a predicted small ORF immediately following *merD* in many Gram-negative *mer* operon sequences. Whether it makes a protein and, if so, how much, has never been examined. Structure predictions suggest that MerE would locate in a membrane and have a CXC motif lying within the membrane region spanned by its first predicted hydrophobic α -helix [157]. No specific *merE* mutants have been made and it has been assumed but never demonstrated that MerE locates in the inner membrane.

3.3.6. Transport of organomercurials and merG

It has been assumed that organomercurial compounds are sufficiently lipid-soluble that, with neutralizing monovalent counterions such as chloride or acetate from the growth medium, they could pass through the cell membrane(s) by simple diffusion. However, when cloned into *E. coli* the *merT* and *merP* genes of the *Pseudomonas* strain K62 plasmid pMR26 will confer hypersensitivity and hyper-uptake of PMA [184] although not of methylmercury [185]. Both Hg(II) uptake and PMA uptake are slightly diminished by loss of MerP, but unlike Hg(II) uptake, PMA uptake is not eliminated by mutation of Cys24 and Cys25 of MerT's first transmembrane helix [186]. Thus, it appears that both MerT and MerP play some role in the uptake of arylmercurials and that role

does not have the same structural requirements as the uptake of Hg(II).

The product of the *merG* gene appears to prevent organomercurial uptake in soil pseudomonads. This gene was first observed as an ORF with a possible signal sequence between *merA* and *merB* on the broad-spectrum-resistant plasmid pMR26 of *Pseudomonas* strain K-62 [187]. Maxicell protein synthesis revealed a polypeptide of ca. 20 kDa, the processed form of the predicted protein. Deletion of *merG* had no effect on Hg(II) resistance but the mutant became more sensitive to PMA. *E. coli* bearing the plasmid with the *merG* deletion took up more PMA than the cells with the intact plasmid gene and loss of *merG* did not affect the activity of MerB. The present model is that *merG* lies in the periplasm and reduces cell permeability to phenylmercury; other mercurials were not examined. Recent work shows that the *merG* gene is frequently found in broad-spectrum resistance operons borne by Tn5041 and carried by soil and water pseudomonads [182]. So far, it has not been observed in the Enterobacteriaceae. Its apparent action in preventing entry of organomercurials is consistent with the idea noted above that organomercurials are sufficiently lipid-soluble to enter the cell efficiently without a specific uptake system.

3.3.7. Energy source for mercury transport

Only a few studies directly address the energetic basis of Hg(II) and organomercurial uptake and these have all used Gram-negative operons. Such studies are challenging because net uptake of Hg(II) can only be observed when the mercuric reductase has been deleted; otherwise MerA's reduction of Hg(II) to volatile Hg(0) is so rapid that no transient accumulation of mercury can be detected. Thus, studies of Hg(II) uptake are only done in hypersensitive mutants lacking the reductase but capable of expressing the inducible transport genes. Not surprisingly, these strains are limited to taking up only as much $^{203}\text{Hg(II)}$ as can saturate their intracellular Hg(II) binding capacity without poisoning themselves [15,188]. However, initial rates of uptake by induced hypersensitive *merA* mutants are considerably faster than $^{203}\text{Hg(II)}$ uptake by cells lacking the *mer* operon and it is on such rates that work has been done to dissect the energetic basis of uptake.

Several studies report that uptake of Hg(II) is inhibited by NaCN [15,188] and the uptake of PMA is inhibited by NaCN and also by the uncoupler mCCCp [184,186]. Work using *mer-lux* transcriptional fusions suggested that the Na^+ gradient might stimulate Hg(II) transport [189]; however, the fact that *mer* expression is sensitive to the supercoiled state of the DNA [149] which can be altered by osmotic changes [190] complicates this conclusion.

Thus, at present no clear picture of the exact energy source for Hg(II) or organomercurial uptake has emerged. It remains possible that the apparent energy dependence of Hg(II) or PMA uptake results, not from any *mer*-specific

energy coupling, but simply from the collapse of the proton gradient effected by any energy poison. Consequent oxidation of the cytoplasm would decrease available intracellular thiols resulting in a diminished intracellular 'sink' for Hg(II) and a corresponding decline in a process as simple as facilitated diffusion mediated by MerT or MerC [188]. Certainly a facilitated diffusion mechanism responding to the cytoplasmic redox status would be a very phylogenetically cosmopolitan mechanism, appropriate for a highly peripatetic locus.

In addition to resolution of the energy dependence of Hg(II) uptake, perhaps the most important puzzle to be resolved in this area is the structural one: what does MerT (or MerC or MerF) do to get the Hg(II) it receives from MerP moved to the cytosolic side of the inner membrane?

3.4. Enzymes of mercury resistance

3.4.1. MerA

The heart of the *mer* HgR mechanism is mercuric ion reductase (MerA), an enzyme that catalyzes the conversion of the thiol-avid Hg(II) to volatile, uncharged Hg(0) that lacks significant affinity for any liganding functional groups. The enzyme utilizes NADPH as source of electrons [191] and is located in the cytoplasm [12] where this substrate is plentiful. However, thiols of proteins and smaller molecules that are the primary target for tight binding by Hg(II) are also plentiful in this location. Consequently, the efficiency of the reductase at competing with these cellular thiols to scavenge and reduce the incoming metal ion is critical to the survival of the cell, and significant research has focused on understanding the features of the protein that are essential for this process.

Although MerA was purified in the early 1970s from both *Pseudomonas K62* and *E. coli* [191–194], significant mechanistic studies [195–198] were not initiated until after the discovery of a FAD cofactor [194] in the enzyme. Fox and Walsh [195] reported the first large-scale purification of the Tn501-encoded MerA and showed that the homodimeric enzyme exhibited spectral and biochemical properties similar to those of the pyridine nucleotide disulfide oxidoreductase family whose most common members include glutathione reductase (GR) and lipoamide dehydrogenase. Partial peptide sequences [196] confirmed the relationship by the presence of a highly homologous active site region with the two cysteines that form the characteristic redox-active disulfide/dithiol of the family. However, unlike the other family members that use this cysteine pair for catalysis and are inhibited when Hg(II) binds to them [199], MerA has additional structural features to help it avoid such inhibition.

Two unique regions, each with a pair of cysteines, were identified in the Tn501 *merA* gene: a short C-terminal extension, and a lengthy N-terminal extension of ca. 95 amino acids [200]. On the basis of the X-ray structure of human GR [201], and the MerA/GR homology, it was

predicted that the C-terminal cysteines of one monomer would lie near the redox-active cysteines of the other monomer and could assist in Hg(II) binding at the active site [200], a prediction proven true by a wide variety of biochemical and structural studies described below. On the other hand, the lack of homology in the N-terminal extension suggested that this region would form a separate domain with its two cysteines involved in acquiring Hg(II) and handing it off to the cysteines in the catalytic core [200]. Early studies provided no evidence for a function of the N-terminal extension, and further analysis of this domain was delayed until very recently. The latter will be discussed in Section 3.4.2.

Elucidation of the structural organization and roles of the four core cysteines in the catalytic mechanism has been a major focus of research on the protein. As noted above, the redox-active cysteines in other disulfide reductases cycle between the disulfide (E_{ox}) and dithiol (EH_2) states in each round of catalysis. Pyridine nucleotide substrates for those enzymes reduce the disulfide in E_{ox} to the dithiol in EH_2 but need not be bound to EH_2 during the reduction of the disulfide substrate by the enzyme dithiol [199]. Although an analogous mechanism was considered a possibility for MerA [195], chemical precedent for the interaction of thiols with Hg(II) suggested a role for high-affinity complexation of Hg(II) by these thiols rather than a role in its reduction. The first evidence in favor of the simple binding role came from the observation that Hg(II) could bind to the inner (i.e. redox-active) cysteines of EH_2 in MerA but was not reduced unless another molecule of NADPH was also bound [202]. Consistent with this, it was found that the EH_2 form of MerA binds NADPH very tightly [197,203,204], such that in the cell the enzyme would be primed with its binding site and bound reductant waiting for Hg(II) to enter the cell. Although these results suggested that the inner cysteines participate only in Hg(II) binding, the catalytic competence of the EH_2 -Hg complex (in terms of rate of reduction) was not tested and left open the possibility of a mechanism involving binding of Hg(II) to only one of the inner cysteines in EH_2 -NADPH followed by oxidation of the pair concomitant with reduction of Hg(II) and then rapid reduction of the disulfide by the bound NADPH [205,206].

Further distinction between these possibilities as well as analysis of the roles of the C-terminal cysteines in the process has relied heavily on studies using enzymes with single, double or triple site-directed mutations of the four cysteines. Mutation of the redox-active cysteines to either serine [207] or alanine [208,209] confirmed an orientation of these cysteines relative to FAD identical to that found in GR, as well as an essential, but still ambiguous role for the pair in catalysis.

Mutation of the C-terminal cysteines to alanines provided several key observations in the elucidation of the roles of all four cysteines. The double mutant (CCAA,

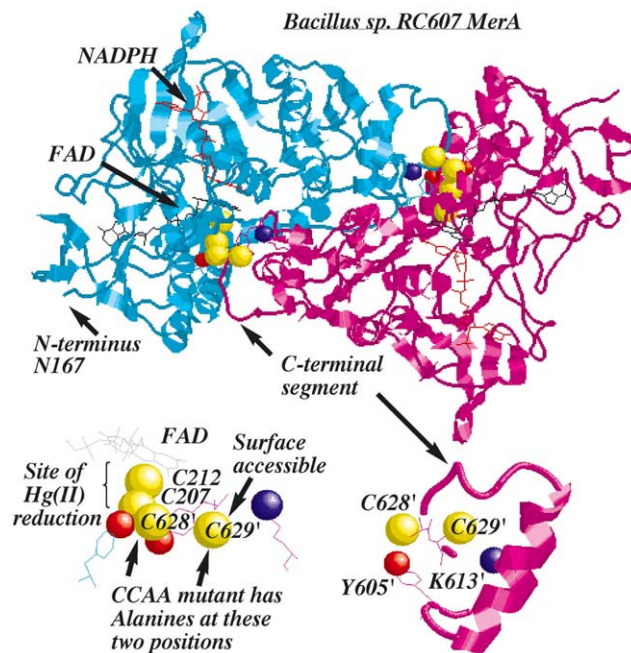


Fig. 3. Model of MerA from crystal structure of *Bacillus* enzyme [206]. Functional groups of conserved residues in binding pathway and active site are shown in spacefill model: yellow-Cys sulfur, red-Tyr hydroxyl, blue-Lys amino.

cysteines listed in order of primary sequence) showed no activity in steady-state assays even though it could form the EH_2 -NADPH complex and bind Hg(II) [210,211]. This suggested that the Hg(II) complex of the inner cysteines alone was not a reducible complex [212]. At the same time, careful reductive titrations of the wild-type protein demonstrated that most preparations required four electrons to reach a stable EH_2 state (instead of the expected two electrons) and that each two electrons gave rise to the appearance of two new cysteine thiols in the protein for a total of four [212] rather than the two previously reported [195]. The CCAA mutant however took only two electrons to form the stable EH_2 indicating that the extra disulfide in the 'hyperoxidized' wild-type enzyme was at the C-terminus [210]. Consistent with this, the hyperoxidized wild-type enzyme showed no initial activity when enzyme was added last to steady-state assays [212]. However, it was fully active after a short preincubation with NADPH [212,213] suggesting that the C-terminal disulfide was in sufficient proximity to the inner cysteines to undergo reduction via disulfide interchange with them. This was corroborated by the observation that reaction of the hyperoxidized E_{ox} enzyme with one equivalent of NADPH led first to rapid reduction of the inner cysteines followed by reoxidation concomitant with the appearance of two new cysteine thiols [212]. The combination of these results confirmed the original proposal by Brown et al. [200] that the C-terminal cysteines lie near the active site and play some role in binding Hg(II).

In a clever subsequent study, equal amounts of the inactive double mutant of the inner cysteines (AACC) and

the inactive double mutant of the C-terminal cysteines (CCAA) were mixed under conditions allowing dissociation, scrambling and reassociation of the monomers into dimers. The resulting mix exhibited 25% of the wild-type activity as expected for a statistical mix of 25% of each inactive homodimer and 50% heterodimers with only one of two active sites reconstituted with four cysteines [214], a result consistent with the structural prediction that the C-terminal cysteines from one monomer form an active site with the inner cysteines of the other monomer in the homodimeric enzyme [200]. Final confirmation of these structural predictions appeared in the X-ray crystal structure of MerA from *Bacillus* sp. RC607, a model of which is shown in Fig. 3 [206].

Single Cys → Ala mutations of the C-terminal cysteines were used to further probe their individual roles. In vivo evaluation of either single or the double Cys → Ala mutants demonstrated an essential role for both for in vivo resistance [210,215]. In spite of this, only the double mutant showed no activity in vitro [210]. While one of the single mutants was more impaired, the other exhibited ca. 50% of the wild-type k_{cat} . However, both had significantly lower k_{cat}/K_M values than wild-type indicating that both C-terminal cysteines are important in the acquisition of Hg(II) from solution and suggesting that a lowered efficiency at scavenging Hg(II) was the major cause of lost resistance for these two mutants [215]. The retention of activity in the single mutants but complete lack of any steady-state activity in the CCAA mutant, in spite of its ability to bind Hg(II), also suggested that one of the C-terminal cysteines might be involved in formation of the actual reducible Hg(II) complex along with one or both of the inner cysteines [212,215]. A two-coordinate complex involving one C-terminal and one inner cysteine would be consistent with the mechanism involving reduction of Hg(II) via initial oxidation of the inner cysteines [205,206], while a three-coordinate complex would be consistent with the simple binding role for the inner cysteines.

Some support for the mechanism involving the two-coordinate complex came from the crystal structure of a Cd(II) complex of the *Bacillus* MerA [206]. Cd(II), which has too low a potential to be reduced but inhibits the enzyme, was found to bind in a distorted tetrahedron involving one inner and one C-terminal cysteine and two conserved tyrosines in the active site [206]. As the C-terminal cysteines are clearly involved in the acquisition of Hg(II) in vivo, such a complex for Hg(II) would certainly be expected to occur at some point in the binding process. However, the observation of this binding site for Cd(II) in MerA may simply reflect both its higher affinity for oxygen ligands and greater preference for tetrahedral coordination than found for Hg(II) [216].

The critical evidence that finally established a simple binding role for the inner cysteines and ruled out any participation of the C-terminal cysteines in formation of the reducible Hg(II) complex came from pre-steady-state

kinetic studies of the wild-type and CCAA enzymes [217–219]. In these studies, three HgX₂ complexes with ligands of varying size and basicity were examined as substrates in the reaction with the NADPH complex of EH₂, the competent species for reduction of Hg(II) [202]. The most important finding was that with HgBr₂ as substrate, the CCAA enzyme bound and reduced Hg(II) as rapidly as the wild-type enzyme in both single and multiple turnovers [217,219]. This result alone demonstrated that neither C-terminal cysteine is required to form the reducible Hg(II) complex and, together with the spectral changes during the course of the reaction, ruled out the mechanism involving reduction of Hg(II) via initial oxidation of the inner cysteines. The data clearly indicate that Hg(II) forms a complex with the two inner cysteines and then is reduced by electrons transferred through the flavin from the bound NADPH [217,219].

The reason for the discrepancy between this surprising rapid pre-steady-state turnover by the CCAA enzyme versus the non-existent steady-state activity of this mutant became clearer when the pre-steady-state reactions were examined with Hg(CN)₂ and Hg(Cys)₂, the latter one being the substrate in the steady-state assays. Although these two substrates reacted with drastically different rate constants (see below), they formed spectrally identical Hg(II) complexes with the enzyme in which the bound Hg(II) was not reduced [218]. The spectrum of this non-reducible Hg(II) complex differs from that of the reducible complex formed using HgBr₂ as substrate in a way that suggests buildup of negative charge in the active site dur-

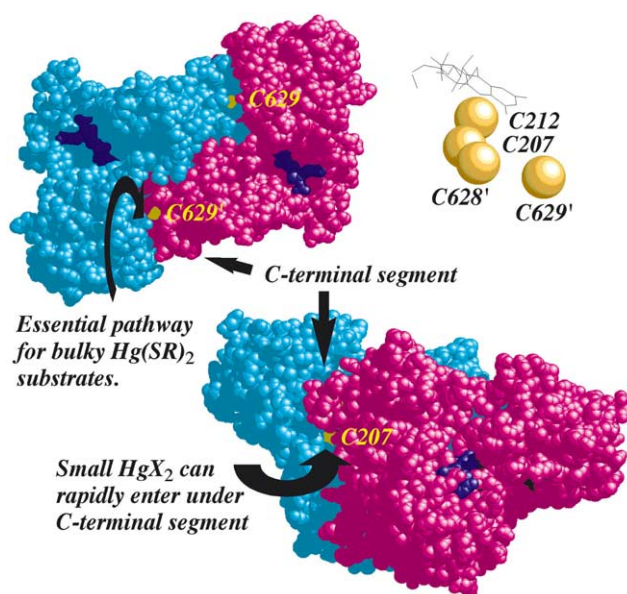
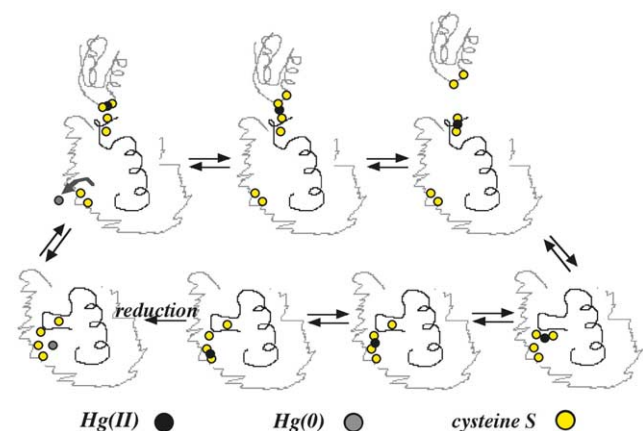


Fig. 4. Space-filling model of *Bacillus* sp. RC607 MerA. The main pathway is shown for entry of bulky Hg(SR)₂ substrates via C-terminal cysteines and the alternative pathway that small non-physiological Hg(II) substrates such as HgBr₂ and Hg(CN)₂ can enter. The alternative pathway is too small for bulky Hg(SR)₂ substrates, so the segment must move out of the way when the C-terminal cysteines are absent in the CCAA mutant.



Scheme 1. Proposed role of NmerA.

ing displacement of the more basic CN^- and thiolate ligands from the respective HgX_2 substrates. The presence of a negative charge in the vicinity of the bound Hg(II) would be expected to inhibit the input of electrons needed to reduce it. These results suggested then that the C-terminal cysteines provide an essential ligand exchange pathway that removes the basic thiolate ligands before Hg(II) reaches the inner active site thereby avoiding buildup of negative charge at the site of reduction [218].

As expected, the wild-type enzyme received and reduced Hg(II) from the Hg(Cys)_2 substrate at a catalytically competent rate in the pre-steady-state reaction. However, the reaction with Hg(CN)_2 varied with the number of equivalents used in the reaction. With excess Hg(CN)_2 the same inhibited complex was formed as observed in the CCAA enzyme, while with one equivalent, about half of the Hg(II) was reduced at a catalytically competent rate. These results suggested that Hg(CN)_2 can access the inner cysteines of the wild-type enzyme via two pathways with different outcomes. Direct reaction with the inner cysteines, as must occur in the CCAA enzyme, leads to the inhibited complex, while reaction via the C-terminal ligand exchange pathway, as must occur with Hg(Cys)_2 in the wild-type enzyme, leads to the reducible Hg(II) complex [218]. This result provided further evidence for the importance of the C-terminal pathway for removal of the more basic ligands from Hg(II) before reaching the site of reduction.

The HgX_2 studies also provided evidence for mobility in the C-terminal segment of the protein that may be important for catalysis. As alluded to above, the rate constants for Hg(II) binding to the CCAA enzyme were dramatically affected by the size of the ligand with delivery from Hg(CN)_2 with small ligands being ca. 10^4 -fold faster than from Hg(Cys)_2 with bulky ligands. As seen in the space-filling model of the enzyme (Fig. 4), the C-terminal segment fills the cavity adjacent to the active site and, in this static picture, leaves only a narrow pathway to the inner cysteines too small to accommodate the bulky Hg(Cys)_2 substrate if the C-terminal thiols are not present.

Thus, for reaction of Hg(Cys)_2 with the inner cysteines in the CCAA enzyme, this segment must move out of the way. Strict second-order kinetics observed in the reaction indicate that this motion is not rate-limiting but rather that the enzyme exists in an ensemble of conformational states most of which are closed to access by the bulky Hg(Cys)_2 but are in rapid equilibrium with a small fraction that are open to access by this substrate. The 10^3 -fold faster reaction of Hg(Cys)_2 with the wild-type enzyme via the C-terminal cysteines over the direct reaction with the inner cysteines in CCAA [218] indicates that full extension of the segment out of the active site should not be necessary for catalysis. However, the depth of the accessible C-terminal cysteine in a surface cleft and the orientation of the two C-terminal cysteines in the structure [206] do suggest that mobility of the segment within the cavity may be important to bring the C-terminal cysteines closer to the surface for exchange with substrate (while Hg(0) escapes underneath) and then closer to the inner cysteines for delivery as depicted in Scheme 1.

High-temperature factors in the crystal structure data at a hinge region of the segment are consistent with this prediction and also suggest that the motion toward the surface could bring the C-terminal cysteines closer to the amino group of a conserved lysine residue [206]. With a similar pK_a to thiol groups, this type of residue would be an appropriate choice to facilitate proton transfers from the enzyme thiols to the extraneous thiolate ligands during the initial steps of the exchange process. Additional residues would be expected to facilitate proton transfers between the enzyme thiols as Hg(II) is transferred down the path from the C-terminal to the inner cysteines. The only other conserved residues in this path are the two tyrosines identified as weak ligands to the Cd(II) in the crystal structure [206]. In vivo analysis of $\text{Tyr} \rightarrow \text{Phe}$ mutations of these in the *Bacillus* enzyme indicates that although one tyrosine is more critical for activity, both are required for high-level resistance [220]. Steady-state analysis again points to a lowered k_{cat}/K_M value as the most likely cause of the lost resistance, although both mutations also affect k_{cat} [220,221].

Although the crystal structure of the *Bacillus* enzyme has proved invaluable for interpretation of the biochemical data, most of those data have been obtained through studies of the Tn501 enzyme, which previously failed to form diffractable crystals [206,222]. Although the sequences of the two proteins are 60–65% identical [206], the alignment indicates that some residues in the C-terminal segment and hypothetical binding pathway are not conserved. Thus before proceeding with further investigation of the properties of the C-terminal segment and residues in the binding pathway, the Miller lab has recently subcloned the catalytic core region of the Tn501 MerA (residues 96–561) [223] in an effort to obtain homogeneous protein free of the problematic proteolysis of the full-length protein [195]. Both wild-type and mutant versions of this construct

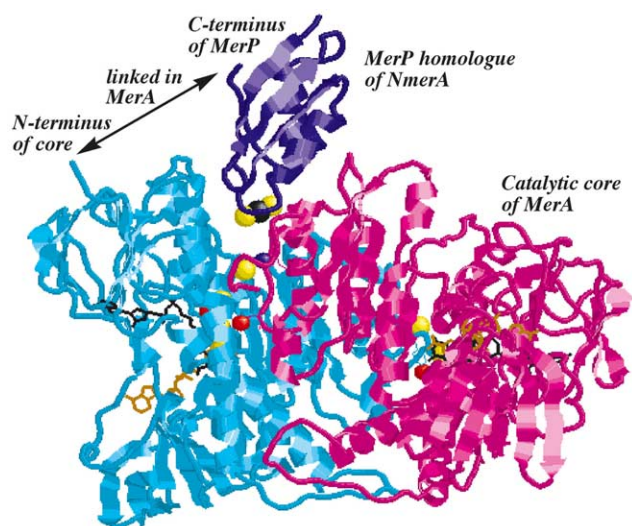


Fig. 6. Model of core of *Bacillus* MerA [206] and Hg-bound MerP [24]. Proteins are shown with MerP docked in trough where the surface-accessible C-terminal cysteine of MerA lies.

indicating that this motif has been highly conserved through both vertical and horizontal transfer evolutionary processes. As mentioned in Section 3.2.1, both MerP and the NmerA domains are homologous with an increasing number of small soluble proteins and domains of soluble and membrane-bound proteins that have been identified as components of intracellular trafficking pathways for soft heavy metal ions such as Cu(I), Zn(II), Cd(II), and Hg(II). Recently solved structures of several of these cloned domains and proteins including MerP [24,226–231] show that they all adopt a similar ‘ferredoxin-like’ $\beta\alpha\beta\beta\alpha\beta$ structural fold [232] and utilize the cysteines of the conserved XXCXXC motif to chelate their cognate metal ions. Since NmerA should behave similarly, the structure of Hg-bound MerP can be used as a model to evaluate how it might interact with the catalytic core of MerA. As shown by the model in Fig. 6, the complementary shapes of the metal binding region of MerP and the cleft on the MerA core with the surface-accessible C-terminal cysteine provide a compelling argument for a role for the NmerA domain in delivery of Hg(II) to the core [223].

To simplify analysis of the properties of the NmerA domain and its interactions with the catalytic core, the 69-amino acid N-terminal region and the catalytic core region including residues 96–561 of the Tn501 *merA* have recently been subcloned [223]. The nearly completed NMR structure of NmerA shows it to be quite similar to its homologues with the expected $\beta\alpha\beta\beta\alpha\beta$ fold (Ledwidge, Miller and Dötsch, unpublished results). As further expected, the protein binds a single equivalent of Hg(II) using the two cysteines of the XXCXXC motif, and the Hg(II) complex of the protein serves as an excellent substrate for the catalytic core in the absence of extraneous thiol-containing compounds, demonstrating that it definitely has the ability to serve as a facilitator for Hg(II)

acquisition and delivery to the core (Ledwidge, Falkowski and Miller, unpublished results).

With these results in hand, functional analysis of the domain in the full-length protein has also been re-examined under conditions ensuring that both the N-terminal and C-terminal cysteines were fully reduced. Low thiol titers for the proteins assayed in the original comparisons [195,196] indicate that the N-terminal cysteines were not initially reduced when the proteins were added to the assays. This and the later observation that a disulfide at the N-terminus does not undergo reduction in the timeframe of the assays [212] suggest that the cysteines in the NmerA domain of the full-length protein may not have been available to participate in the reaction in those studies [196]. In the new studies, steady-state profiles for fully reduced core and full-length proteins have been compared using cysteine and glutathione as small physiological thiol ligands for Hg(II), as well as *E. coli* thioredoxin as a protein dithiol chelator for Hg(II). The results show that the impact of the NmerA domain on the kinetics increases as the size of the thiol ligand on the Hg(II) substrate increases and as the excess concentration of the thiol ligand in the assay decreases (Ledwidge, Falkowski and Miller, unpublished results). This suggests that the NmerA domain may only become critical under conditions of depletion of the cellular thiol pool such that binding of Hg(II) to protein thiols would then become more problematic. Since the *in vivo* function of the N-terminal di-Ala mutant was tested [210] in otherwise unstressed *E. coli*, the normal intracellular thiol concentrations of GSH (ca. 6 mM) may have eliminated any benefit the NmerA domain could provide. This proposal is currently under investigation using GSH-depleted cells (Summers and Miller, unpublished results).

Another clue that the type and quantity of available thiol ligands may alter the impact of the domain on the reaction is provided by the species variation in the N-terminal appendage (Fig. 5). Comparison of the species from which MerAs have been isolated with published analyses of intracellular thiol content [233,234] suggests that the presence and number of repeats of the domain in the N-terminal appendage may be correlated with the major type and quantity of thiol synthesized in each species. Thus, cells that lack glutathione and have rather low intracellular concentrations of other thiols (some *Bacillus* and *Clostridium* sp.) have double repeats in their associated MerA appendages, while cells that synthesize glutathione (*E. coli*, *Pseudomonas* sp.) or have relatively high concentrations of other thiols such as cysteine or coenzyme A (*S. aureus*) have a single repeat in their MerA appendages. The correlation of double repeat sequences with low intracellular thiol concentration strongly suggests that the NmerA domains should be more critical for resistance in those organisms since they must serve both as the intracellular buffer to prevent inhibitory binding to other proteins in the cell and as the specific delivery agent to the catalytic core. This idea further suggests that in cells with

higher concentrations of thiols, the single domain would not normally be needed as the buffering agent, but might become important in this role if the cells are subjected to additional oxidative or other stresses that deplete the intracellular thiol concentration. Note that the one sequence lacking an N-terminal domain in the list is from an organism that utilizes the unusual sugar derivative of cysteine, mycothiol, instead of glutathione as its cellular thiol [234]. The implications of this curious observation require further investigation.

3.4.3. MerB

Kenzo Tonomura and his group first described the cell-free, substrate-inducible activity of a mercury-resistant soil *Pseudomonas*, strain K62, which degraded organomercurial compounds such as phenylmercury, ethylmercury, and methylmercury [79,235]. They posited a multi-protein complex involving one or more dehydrogenases and cytochromes as well as a specialized ‘decomposing enzyme’ and later characterized [191,192,236] two distinct organomercurial degrading activities from strain K62.

Simon Silver’s group showed that inducible genes encoding organomercurial resistance occurred on transferrable plasmids in approximately 20% of mercury-resistant Enterobacteriaceae [10] and in all mercury-resistant *Staphylococci* [237]. They also showed that in an *E. coli* carrying the conjugative IncM plasmid, R831, only two proteins were required for the organomercurial-degrading activity: a soluble enzyme which split the C–Hg bond in the organomercurial, releasing a protonated organic moiety (such as methane from methyl mercury, Fig. 2) and the Hg(II) cation which was then reduced to Hg(0) by the mercuric reductase, MerA [13] (for details on MerA, see above). The organomercurial-degrading enzyme whose size was estimated at 25 kDa was initially called a hydrolyase under the assumption that the proton added to the organic moiety arose from water. Inducible polypeptides in this size range were also observed in minicells programmed with plasmid R831 [16].

Meticulous characterization of the MerB encoded by R831 revealed it to have a molecular mass of 22.4 kDa, to lack any cofactors, and to function as a monomer [238,239]. The purified protein required a minimum two-fold molar excess of thiol over organomercurial substrate to exhibit any activity and preferred the physiological thiol cysteine to non-physiological mercaptans. The enzyme has a very broad substrate tolerance, handling both alkyl and aryl mercurials, with a slight preference for the latter. As is often the case with enzymes of low substrate specificity, MerB has very slow turnover rates ranging from 0.7 to 240 min⁻¹ on various substrates. Although relatively slow for an enzyme, these rates do represent a 10⁶–10⁷-fold acceleration over chemical protonolysis rates of organomercurials. Paradoxically for a protonolysis catalyst, MerB’s pH optimum was >9. Mechanistic studies revealed retention of the skeletal configuration of the substrate consistent

with the rare S_E2 mechanism rather than a radical-based mechanism. Deuterium isotope effects were consistent with a rate-limiting proton delivery step. Relevant chemical model studies [240] demonstrated a 1000-fold acceleration of aryl-Hg protonolysis by a compound capable of bis-coordination, whereas a monothiol reagent provided only a 50-fold rate acceleration, suggesting that in MerB two protein thiol groups might be involved in stabilizing a reaction intermediate.

Recent phylogenetic analysis makes it clear that MerB is a unique enzyme without known homologs which has evolved into several subgroups distinguishable on the basis of their primary and predicted secondary structures [241]. Consistent with its carriage by transferrable plasmids, the MerB phylogeny does not map onto the phylogeny of the bacteria in which those sequenced examples have occurred, i.e. several examples of MerB found in distantly related Gram-positive and Gram-negative bacteria cluster closely and are equidistant from examples found in other Gram-positive and Gram-negative bacteria. Three cysteines are highly conserved at positions 96, 117, and 159 (numbering as for MerB of R831) and consistent with its high requirement for thiols, MerB is a cytosolic enzyme with no disulfide bonds. Cys96 and Cys159 are essential for catalysis and Cys117 appears to have a structural role rather than a catalytic role. MerB works equally well with either of the physiological thiols, glutathione or cysteine.

Most described Gram-positive mercury resistance operons are broad-spectrum (i.e. have one or more MerB genes; see Section 4). However, in Gram-negative bacteria the prevalence is closer to 20% [23]. Broad-spectrum-resistance strains of both Gram-positive and Gram-negative bacteria frequently have two *mer* operons, a broad-spectrum locus and a narrow-spectrum locus, sometimes on the same plasmid as is the case with R831 [242] and

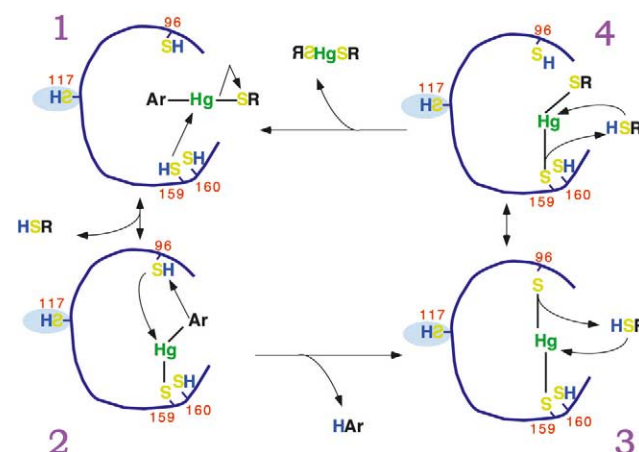


Fig. 7. Roles of Thiols in MerB protonolysis of organomercurials. Small red numbers designate positions of cysteine residues in the primary structure of MerB of plasmid R831b [241]. RSH is a low-molecular-mass, cytosolic thiol redox buffer such as glutathione. Ar refers to an aryl (aromatic) moiety in an organomercurial compound such as phenylmercuric acetate.

pDU1358 [243]. Interestingly, in many occurrences the operon bearing the *merB* gene in broad-spectrum-resistant Enterobacteriaceae has undergone deletions of varying lengths removing most of the intervening genes between *merB* and the operonic promoter [23] resulting in the *merB* gene being much closer to that promoter. In these internally deleted operons, the associated *merR* gene is retained, possibly because the MerR protein of the co-resident narrow-spectrum resistance operon will not respond to organomercurials [244].

Fig. 7 is an updated reaction model embodying the current state of knowledge concerning MerB. In step 1, a cysteine (probably Cys159 [241]) of the fully reduced enzyme displaces the solvent thiol adduct from the organomercurial and a second protein cysteine (probably Cys96) forms a bis-coordinate structure with the aryl mercurial (step 2). The proton may be donated to this activated bis-coordinated complex by Cys96 or perhaps by some other protonated residue in MerB such as the highly conserved Tyr93. Once the protonated organic moiety leaves, MerB is stuck with Hg(II) (step 3) until two solvent monothiols can remove it (step 4). Interestingly, dithiothreitol (DTT) actually inhibits MerB, possibly by forming a stable three-coordinate complex with the product Hg(II) and one of MerB's cysteines. DTT inhibition can be slowly reversed by either cysteine or glutathione.

While this model accounts for the minimum two-fold excess of thiol required for any catalytic turnover as observed by others, it does not explicitly address the paradoxically high (in vitro) pH optimum of MerB nor the requirement for at least a 15-fold molar excess of thiol for optimum activity [238,239,241].

4. Diversity of mercury resistance loci

4.1. Operon structure

The *mer* locus is widely distributed among eubacterial lineages and *mer*-like sequences have been identified in several archaean genomes, such as *Sulfolobus solfataricus*, *Thermoplasma volcanicum* and *Halobacterium* sp., though to the best of our knowledge functional *mer* in Archaea have not been described to date. Several variations on the structure and organization of *mer* operons are known (Fig. 8) reflecting the mosaic nature of the operon [23,245]. There seem to be some characteristic differences between the operons of Gram-positive [26,246–249] and Gram-negative bacteria [161,243,250–254]. These differences are: (i) *merB* is more common in Gram-positive *mer* operons described to date than in Gram-negative operons; (ii) *merR* in low-GC Gram-positive operons is transcribed in the same direction as the rest of the operon's genes but in the high-GC Gram-positive *Streptomyces* operons and all Gram-negative operons *merR* is transcribed divergently from the structural genes. The Gram-negative marine bacterium *Pseudoalteromonas haloplanktis* is the exception with *merR* cotranscribed with *merTPCAD* [253]. Low homology with other Gram-negative *mer* genes and additional molecular characteristics further distinguish *P. haloplanktis*'s *mer* from other Gram-negative operons described to date [253]. The *merR-merD*-less operon on plasmid pMERPH from *Shewanella putrefaciens* is another exception to the Gram-negative *mer* operon pattern though the plasmid does carry an unidentified regulator of *merTPCA* [254].

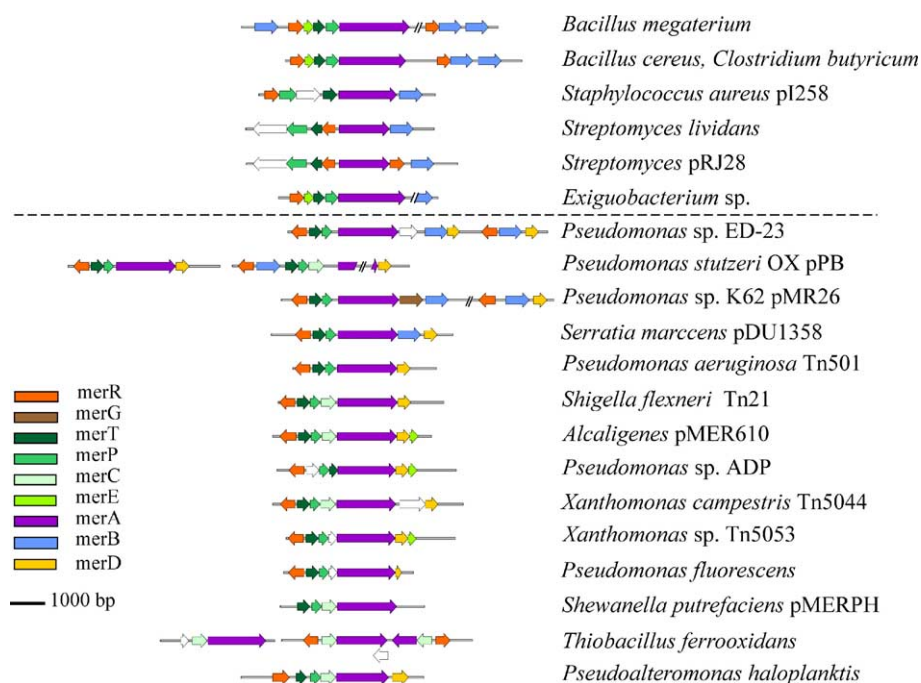


Fig. 8. Diversity of *mer* operons. Sequenced *mer* operons from Gram-positive (above line) and Gram-negative (below line) bacteria (see text for citations). Arrows indicate the direction of translation of each gene product. Colorless arrows indicate ORFs with unknown functions.

4.2. *mer* as a part of mobile genetic elements

The mercury resistance (*mer*) operon is the metal resistance locus whose dissemination by lateral gene transfer is best established [23,25,245,255]. One archetypal *mer* operon is carried by Tn21, a composite transposon originally isolated on plasmid NR1 from *Shigella flexneri* in Japan [256]. Tn21 is a Tn3-like class II replicative transposon that carries genes for a transposase (*tnpA*), a resolvase (*tnpR*), a *res* site for cointegrate resolution, and a class I integron, and is flanked by 38-bp inverted repeats [161,257]. Mercury resistance operons are often part of group II transposons in both Gram-negative [258,259] and Gram-positive [164,260] bacteria and these transposons often carry integrons with one or more antibiotic resistance genes [259,261]. A recent survey of the literature revealed 98 independent examples of *mer* transposons (J. Coombs, personal communication). Two groups have examined the distribution and diversity of Tn21-type transposons and their associated *mer* operons in soil bacteria. Strike et al. used probes, polymerase chain reaction primers, restriction fragment length polymorphism (RFLP) and sequence data to show linkage of *mer* with *tnpR*, *tnpA*, and integrons in bacterial isolates from mercury-contaminated and pristine soils [262,263] and in DNA extracts from the same environments [264]. While most of the isolates carried both group II-type *tnpA* and *tnpR*, RFLP patterns indicated a high diversity of these genes [262]. Nikiforov et al., who have characterized *mer* transposons from soil bacteria by employing a transposon capture approach [265], showed a high occurrence of recombination resulting in chimeric structures of many transposons [258]. For example, recent, exciting work on *mer* loci carried by the widely distributed Tn5041 transposon in pseudomonads from soil, freshwater, and mercury mine tailings [245] has demonstrated the high level of recombinational promiscuity in this locus and in such elements in general. The basic Tn5041 *mer* operon has *merRTPCAD* in an arrangement like that of *mer* in Tn21 (Figs. 1 and 8). Variants of Tn5041 *mer* have suffered transposition of the entire Tn21 *mer* locus (lacking In2 integron) into the 5' region adjacent to Tn5041 *merA*. Other Tn5041 *mer* variants have experienced insertion of an extremely variegated locus called *mer2*, which encodes *merRTPAGBD*. This event appears to have arisen by Chi-mediated homologous recombination of a *mer* operon on a covalently closed circle that was generated by homologous recombination at its flanking IS1015 sequences. Such mosaic structures as recognized by Kholodii et al. [245] also exist in previously sequenced *mer* loci recovered from feces of monkeys exposed to very high levels of Hg(II) released from amalgam dental restorations [23]. Thus, *mer* loci reveal variation in structure of the operon and its individual genes and evolution by frequent horizontal gene transfer and recombination events [157]. It is likely that the use of antibiotics in the clinical setting and environmental Hg

contamination exert selective pressure that fosters the persistence of *mer* and its associated mobile elements. Consistent with this idea is the recent finding that *mer* transposons in clinical isolates from the preantibiotic era had >99.9% DNA sequence identity with Tn1696, Tn5036, Tn5053, and Tn21 [266].

5. Applied biology of mercury resistance

The exploitation of *mer*-mediated functions for the removal of mercury from wastewater was proposed and demonstrated nearly 20 years ago [267]. By now several approaches, employing engineered and naturally occurring *mer* operons, have been constructed and their application demonstrated in a variety of contaminated industrial streams and environments. The most advanced of these, and the only one in industrial use, is a packed bed bioreactor inoculated with a mixture of several natural isolates of mercury-resistant bacteria (mostly pseudomonads) for the treatment of chlor-alkali process wastewater [268]. The inoculated strains grow during the operation of the bioreactor to form a thick biofilm of cells embedded in exopolysaccharides (EPS) that fill the cavities of the pumice granules that constitute the packed bed [269]. Because the feed wastewater is not sterile invading indigenous mercury-resistant strains can also become established within the bioreactor [269–271]. The bioreactor is fed with a neutralized wastewater supplemented with low concentrations of sucrose and yeast extract, at a flow rate of up to 2000 l h⁻¹ of wastewater containing up to 10 mg l⁻¹ mercury. Stable operation of up to 240 days with 99% efficiency in mercury removal has been reported [269]. Residual mercury in bioreactor effluents is removed in an activated charcoal filter where both adsorption and further microbial reduction contribute to an additional six-fold decrease in effluent mercury concentrations to below 50 µg l⁻¹, the regulated level of Hg in wastewater streams [268,269]. EPS may limit diffusion in the reactor, resulting in accumulation of reduced Hg as droplets of elemental Hg(0) within the bed matrix [270]. This strategy can afford the safe disposal of the reduced mercury or, if commercially feasible, subsequent recycling. The long-term operation of the industrial-scale bioreactor is challenged by fluctuations in influent mercury concentrations and variations in other wastewater parameters. Von Canstein et al. [269,272] recently showed that stable bioreactor operation under varying conditions is facilitated by the diversity of the mercury-resistant community. Thus, at high mercury loads the zone of active reduction migrated up the bottom-to-top bioreactor where only a few and most resistant members of the community were present while at low mercury loads the active zone migrated down the bioreactor. Strains with lower levels of mercury resistance were abundant downstream of the reduction front and in the activated charcoal filter [269]. Experimental bioreactors inoc-

ulated with a single resistant strain and fed with sterile chlor-alkali wastewater collapsed when influent mercury concentrations increased to 10 mg l^{-1} , whereas a stable performance was achieved by a mixture of the strains [272].

The in situ bioremediation of mercury is more challenging than end-of-pipe treatment, because mercury persisting in the environment is associated with complex matrices and is usually in sulfidic forms [273]. Note that while HgS is highly insoluble and is considered an inert form of mercury, the formation of Hg(0) from HgS was reported for a *Thiobacillus ferrooxidans* strain that was isolated from a pyrite mine [274]. Organisms that carry complete and partial *mer* operons have been prepared and tested with the goal of remediating mercury-contaminated soil, sediment, and subsurface environments. An increased rate of Hg(0) evolution from microcosms simulating a contaminated pond by the application of active mercury-reducing strains (bioaugmentation) was reported [275]. The highly radiation-resistant strain *Deinococcus radiodurans*, to which Tn21 was transferred, reduced Hg(II) in the presence of 6000 rad h^{-1} [276]. Such strains were constructed with the prospect of remediating US Department of Energy subsurface sites containing radionuclides, metals, and organic contaminants [277]. Constitutive *mer* operons were prepared [278] with the prospect of improving wastewater treatment operations [279], but when tested they proved inferior to naturally isolated mercury-reducing strains [272]. The construction of recombinant *E. coli* with enhanced mercury accumulation due to combined *merTP* and cytoplasmic eukaryotic metallothioneins was reported [280]. Induced cells in hollow-fiber bioreactor retained 99% of the mercury from wastewater containing $> 2 \text{ mg l}^{-1}$ mercury, various other ions, and at an alkaline pH. Affinity for Hg(II) exceeded that of chelating agents suggesting the feasibility of a combined treatment whereby metals released by chelators are subsequently sequestered by the recombinant strain [281,282]. Finally, the *mer* system was used to engineer several species of plants for phytoremediation of inorganic [283,284] and organomercury [285,286]. In a recent development, the efficiency of Hg(0) evolution from hydrophobic organomercury was improved 10–70-fold by targeting recombinant MerB to the endoplasmic reticulum or the cell wall in *Arabidopsis* compared to activities of plants carrying cytoplasmic MerB [287]. However, the engineered plants emit Hg(0) into the air, a matter of concern since the atmospheric deposition of Hg(II), following the oxidation of Hg(0) in the atmosphere, remains a major source of Hg in the environment [40]. Although calculations suggest that plant-enhanced emissions would have little effect on the global atmospheric Hg(0) pool [283], public perceptions have slowed the implementation of this promising *mer*-based phytoremediation strategy.

Lastly, there is considerable interest in using MerR as a biosensor in vivo with various transcriptional fusions

[149,174,288,289] and in vitro to optical detection devices [145,146]. The value of such sensors is in distinguishing bioavailable from total Hg(II). Such tools are needed to improve our understanding of the mercury geochemical cycle and to facilitate regulatory action aimed at controlling human and wildlife exposures in contaminated environments [290]. Such constructs have been valuable for research on extra- and intracellular interactions of Hg(II) with bacterial cells [97,291], and on the environmental behavior of mercury [145,146,174,289] but, to date, have made only limited contributions to the analysis of bioavailable mercury in environmental [110,290] and industrial applications.

6. Future directions

Despite being the longest studied of the bacterial toxic metal resistance loci, *mer* continues to bring new insights to gene regulation and enzymology. There are also some large mysteries yet to be solved in the subject of Hg(II) transport as well as more discrete puzzles about how the various *mer* proteins tune their thiol groups to hold mercurial compounds in just the right way and just long enough to convert them into a less toxic and readily dissipated form. The population biology of ubiquitous *mer* loci is proving a rich resource for dissecting lateral gene transfer.

Study of this bacterial operon also provides insights into a much larger subject: ‘the biology of mercury’. This apparent oxymoron is apt because mercury’s protean chemical properties have made it a valuable element, exploited by humans for millennia. Although its toxicity has long been known, mercury’s chemical utility allowed humans to overlook its dangers. In the later half of the last century, discovery that organisms as tough as bacteria had evolved a specialized system to deal with Hg toxicity contributed, along with several high profile anthropogenic human exposure disasters, to increased concern for the dangers of Hg to more delicate creatures such as ourselves [292]. Since Hg(II) mimics reactive oxygen species (ROS) in its interactions with cellular constituents, the chronic nature of Hg intoxication in higher animals owes a lot to the fact that eukaryotes have evolved a wealth of strategies for dealing with ROS over the last 2.5 billion years. Recent revelations of the subtlety of the redox-sensing regulators OxyR and SoxR (a MerR family member) [293–295] suggest that examination of Hg(II) interactions with ROS-sensing and redox homeostasis systems in both prokaryotic and eukaryotic cells will be a timely and fruitful pursuit.

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