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Bacterial Resistances to Mercury and Copper

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Abstract Heavy metals are toxic to living organisms. Some have no known beneficial biological function, while others have essential roles in physiological reactions. Mechanisms which deal with heavy metal stress must protect against the deleterious effects of heavy metals, yet avoid depleting the cell of a heavy metal which is also an essential nutrient. We describe the mechanisms of resistance in *Escherichia coli* to two different heavy metals, mercury and copper. Resistance of *E. coli* to mercury is reasonably well understood and is known to occur by transport of mercuric ions into the cytoplasmic compartment of the bacterial cell and subsequent reductive detoxification of mercuric ions. Recent mutational analysis has started to uncover the mechanistic detail of the mercuric ion transport processes, and has shown the essential nature of cysteine residues in transport of Hg(II). Resistance to copper is much less well understood, but is known to involve the increased export of copper from the bacterial cell and modification of the copper; the details of the process are still being elucidated.

Expression of both metal resistance determinants is regulated by the corresponding cation. In each case the response enables the maintenance of cellular homeostasis for the metal. The conclusions drawn allow us to make testable predictions about the regulation of expression of resistance to other heavy metals.

Key words: copper, heavy metal, ion transport, mercury, resistance

Bacteria have evolved to live in a wide variety of ecological niches, and contain genetic determinants of specialised function for these environments. Some determinants allow bacteria to metabolise a variety of unusual compounds as potential nutrients, and others eliminate or detoxify antimicrobial compounds [1]. Some environments contain toxic levels of heavy metal salts due to natural geochemical processes or human activities. As a result, bacteria in these environments have evolved sophisticated mechanisms for preventing damage by heavy metals. Separate determinants have been discovered that confer resistance to antimony, arsenic, bismuth, cadmium, chromium, cobalt, copper, lead, mercury, nickel, silver, tellurium, thallium, tin, tungsten, and zinc salts [2]. Interest in these resistances stems from the intrinsic complexity of the biochemical mechanisms of resistance, and from their possible applications, for example in bioremediation of polluted soil and water, in metal beneficiation, or as heavy metal biosensors.

Heavy metals can be classified into two groups. Those in the first are purely toxic and have no known beneficial biological function; these include cadmium, lead, and mercury. Those in the second group—which includes copper, zinc, and nickel—are required for the growth and maintenance of living organisms but are toxic in excess. In the study of the biological effects of heavy metals it is necessary to distinguish between these two groups. In the former case it is sufficient to prevent the toxic metal from coming into contact with the appropriate targets in the cell; but in the latter case the organism must maintain essential supplies of the heavy metal while protecting itself against the toxic effects. In both cases homeostatic mechanisms are required. These mechanisms are defined as specifying the maintenance of the intracellular metal concentration at levels that allow optimal cell growth under varying environmental condi-

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tions, particularly varying metal concentrations. In the case of a purely toxic metal the optimal level will be below a threshold usually close to zero concentration. In contrast, a toxic metal with a beneficial role in metabolism must have an optimal level that is significantly above zero in order to facilitate metabolism, while avoiding toxicity. In this article we describe current knowledge of the mechanism of resistance in *Escherichia coli* to one example from each of these classes of heavy metal.

Mercury and its salts are often released into the environment in biologically available form by geochemical processes and by human intervention [3]. Mercuric ions are highly toxic to all living cells due to the peculiar chemical properties of mercuric salts, especially their high affinity for thiol groups in proteins [4] and for other substituent groups in proteins, lipids, nucleic acids, and polysaccharides [3]. They bind to substituent groups at extremely low concentrations and often inactivate the biological molecule to which they are bound. Thiol groups are often responsible for the structural integrity of proteins and thiol or histidine groups may be required for catalytic activity, so that covalent modification of these groups by mercuric ion is often highly detrimental.

Copper is an extremely common metal in the environment. Its redox potential makes it an important constituent of electron-transfer proteins and enzymes involved in other redox reactions [5]. Copper is toxic in excess due to its capacity to catalyse adverse redox reactions, such as hydroxyl radical generation [6]. This may cause, for example, the peroxidation of lipids. Other biological macromolecules such as DNA and proteins may also be damaged by free radical reactions. Copper can also prevent adequate functioning of proteins by direct binding of the metal to amino acid side chains (especially histidine and cysteine).

MECHANISM OF RESISTANCE TO MERCURY SALTS

The mechanism of resistance to mercuric ions in Gram-negative bacteria is better understood than that of any other heavy metal. Detailed genetic and biochemical evidence has been obtained over several years, and has been reviewed elsewhere [2,3,7,8].

Mercuric ion resistance is the most widespread determinant of resistance to antimicrobial agents (including any single antibiotic); and several different determinants are known which confer resistance to mercuric ions in Gram-positive and Gram-negative bacteria. The major mechanism of resistance to mercuric salts is reductive detoxification to elemental mercury [2,7,8]. Studies by several groups [9–11] have shown that there are two processes involved in the resistance mechanism: transport of mercuric ions into the cell, and enzymatic reduction catalysed by mercuric reductase, converting Hg(II) to Hg(0).

Genetic and biochemical studies of two closely related mercury resistance determinants, of Tn*501* (from the *Pseudomonas* plasmid pVS1) and Tn*21* (from *Shigella* plasmid R100) have allowed the genes and proteins involved in mercuric ion resistance to be identified [12–16]. The genes for the transport proteins (*merT* and *merP*) and for mercuric reductase (*merA*) are transcribed in an operon. The MerT, MerP, and MerA proteins are inducible by mercuric ions acting via the *merR* gene product. The mercury-resistance proteins of Tn*501* and plasmid R100 have been identified by the specific labelling of newly synthesised proteins following induction with mercuric ions and by their absence in the appropriate mutants [14,16].

The MerT, MerP, and MerA proteins are distinguished by the presence of paired thiol groups in their amino acid sequences. The model for mercuric ion resistance in Gram-negative bacteria suggests that these thiol residues play a critical role in the binding and transport of mercuric ions during the detoxification process. The outline mechanism for mercuric ion resistance is shown in Figure 1 [7,17]. This involves ligand transfer of the mercuric ion between paired thiol groups. A model for thiol-pair to thiol-pair transfer is shown in Figure 2. Transfer is suggested to occur in four steps via two 3 ligand intermediates, which alternate with 2 ligand states, the preferred status for bound Hg(II).

The ligand transfer model shown in Figure 1 explains the data available on the detoxification of mercuric salts, but it is still some way from a detailed molecular mechanism of mercuric ion uptake and resistance. Recent work using site-directed mutagenesis [A.P.M. and N.L.B., in preparation] has shown that the first pair of cysteine residues in the MerT protein (Fig. 3; Cys24, Cys25) is essential for mercuric ion transport, although the Cys76, Cys82 pair in MerT is not. However, substitution of Cys76 and Cys82

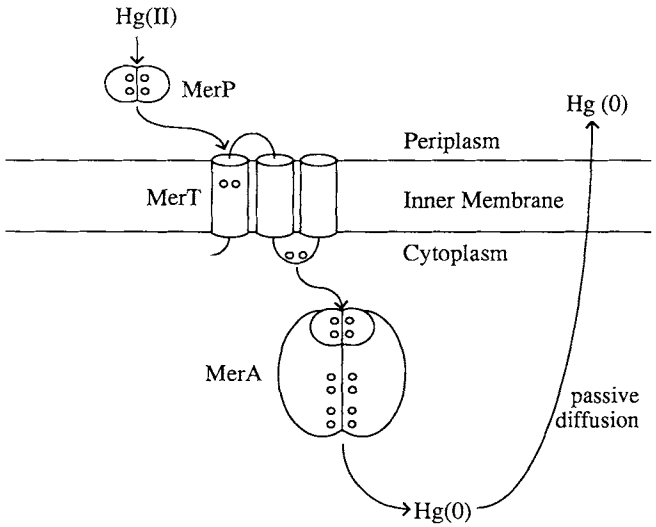

Fig. 1. Model for the mechanism of mercury resistance encoded by Tn*501*. The outline particulars of this model are as follows: A) Mercuric ions can pass freely into the periplasmic space (due to the cation-selective porins in the outer membrane) where they are sequestered by the pair of thiol groups (Cys33,Cys36) on the periplasmic protein, MerP. B) The first of a series of ligand exchange reactions occurs, in which the Hg(II) ion is passed to the pair of thiol groups (Cys24,Cys25) in the first transmembrane helix of the MerT protein. It is possible that MerT functions as a homo-dimer, such that the Cys24,Cys25 pairs of two MerT monomers interact to promote transport. C) The Hg(II) ion is passed through the membrane to the Cys76,Cys82 pair on the cytoplasmic face of MerT. D) Mercuric reductase receives the Hg(II) directly from MerT, thus protecting the cytoplasmic constituents from Hg(II). This may be by interaction of the N-terminal domain, which is conserved in all known Gram-negative mercuric reductases. E) Hg(II) bound to the C-terminal cysteine pair (Cys558,Cys559) of mercuric reductase is reduced to Hg(0), and the non-toxic product, Hg(0), diffuses out of the cell.

by serine residues does give a significant reduction in resistance [A.P.M. and N.L.B., in preparation]. This suggests that Cys76 and Cys82 may be involved in the coupling of the transport system to mercuric reductase, which in any case may be relatively loose in order to allow the reductase to capture mercuric ions entering the cell by other means. For example, mercuric ion as $HgCl_2$ is uncharged (being covalent rather than ionic) and can pass through the lipid por-

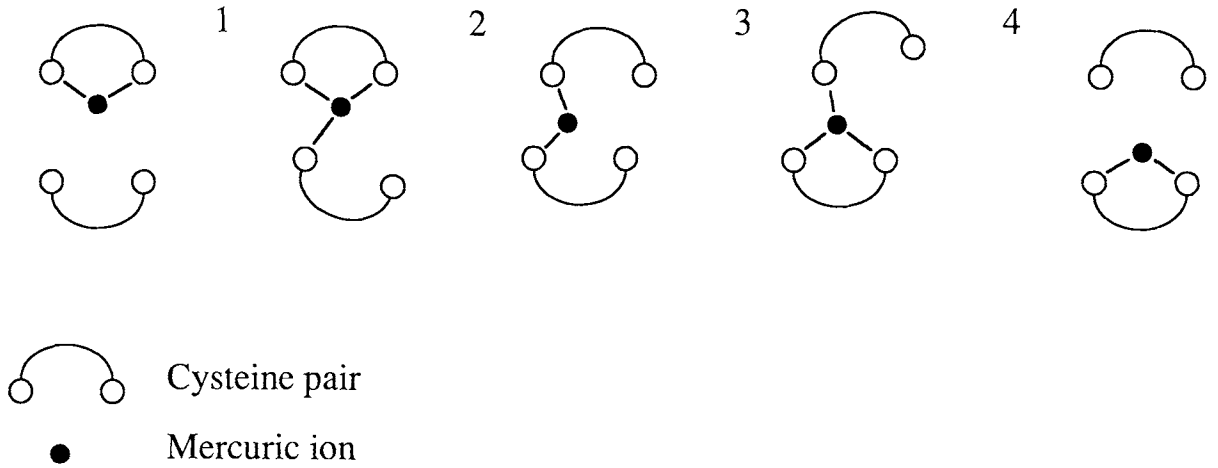

Fig. 2. Model for mercuric ion transfer between dithiol pairs. This model is based on the observation that mercuric ion exchange among glutathione ligands involves a transient 3:1 mercuric ion:glutathione complex (Cheesman, B.V. and Rabenstein, D.L. unpublished, quoted by Rabenstein [38]) and the identification of a tridentate mercury binding site involving cysteine residues in MerR [36,37]. Although Hg(II)-thiolate complexes have an extremely high thermodynamic stability these complexes are quite labile, allowing for rapid ligand exchange [38]. Thus the use of cysteine residue pairs in the resistance mechanism is likely to facilitate rapid controlled flow of mercuric ions through the resistance pathway.

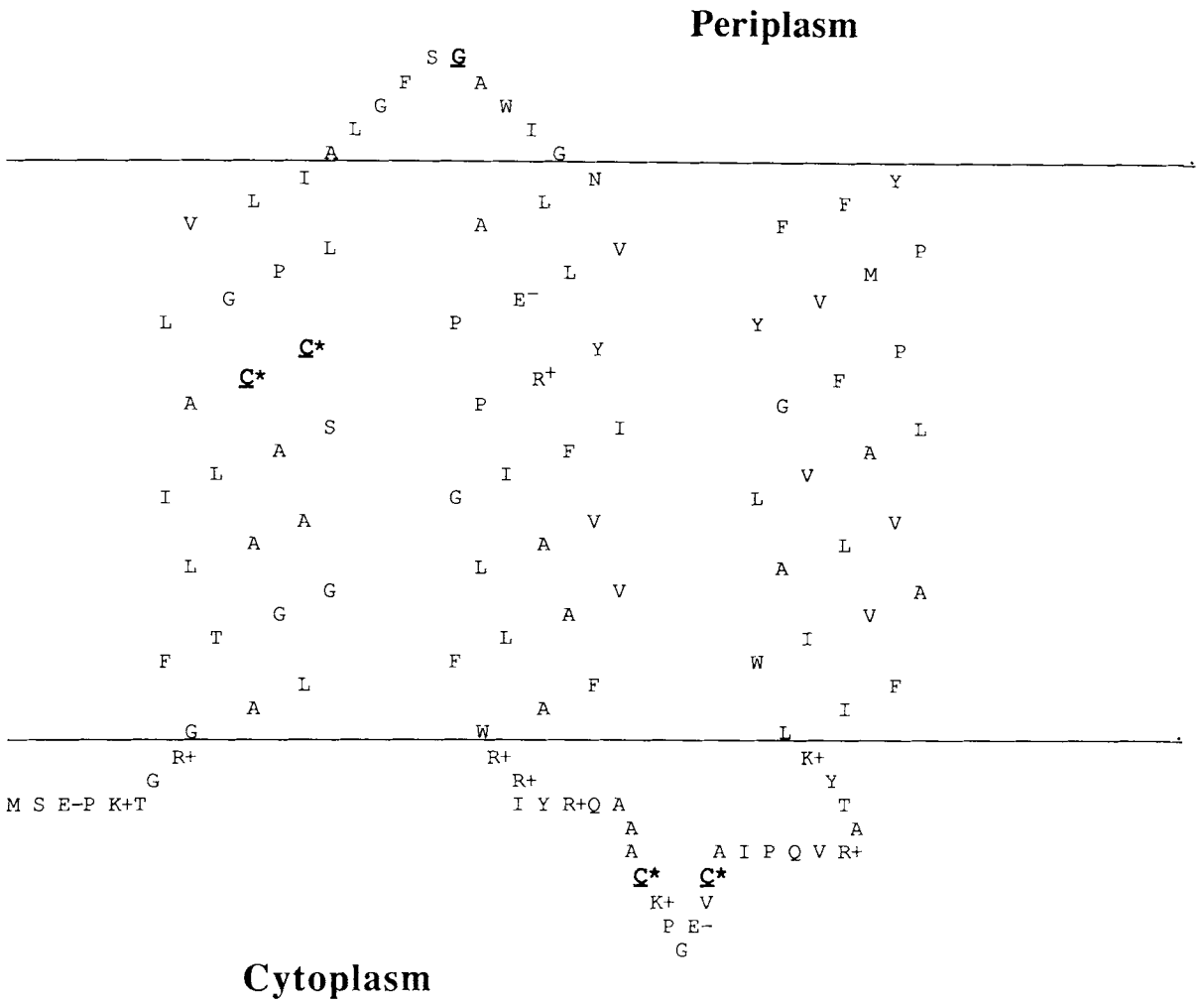

Fig. 3. Predicted folding of the Tn*501* MerT protein through the bacterial cytoplasmic membrane. The positions investigated by site-specific mutagenesis are indicated in bold, underlined type. Cys24 and Cys25 are in the transmembrane helix; Cys76 and Cys82 are in the cytoplasmic loop; Gly38 is in the periplasmic loop.

tion of the membrane directly [18]; once inside the cell it will generate ionic Hg(II).

The MerP product itself is not essential to transport and resistance. Deletion of the complete *merP* gene causes a partial loss of resistance, and it is thought that the MerP protein increases resistance due to its efficient scavenging of mercuric ions in the periplasmic space. There are only two cysteines in MerP. Alteration of Cys36 to serine causes no change in transport or resistance; however, a Cys33 to serine mutation in MerP does decrease both transport and resistance [A.P.M. and N.L.B., in preparation]. The mutant is proposed to have an inhibitory affect on MerT function due to a faulty interaction between the two proteins. Further, a substitution in the putative periplasmic loop of MerT, Gly38 to aspartate, produced a phenotype equivalent to that produced by deletion of MerP [A.P.M. and N.L.B., in preparation]. This suggests that this mutation disrupts the interaction of MerP with MerT. Thus, the results support the notion that a specific binding of MerP to MerT is involved in mercuric ion uptake. Since a complete deletion of the *merP* gene results only in a partial loss of resistance, it appears that the coupling of mercuric ion transfer between MerP and MerT is not essential for MerT function. This contrasts with binding-protein dependent transport systems, such as those for maltose and histidine [19]. In these cases interaction of the periplasmic binding protein with the membrane transport unit is an absolute requirement for uptake to occur.

There is an extremely high concentration of the *merA* gene product, mercuric reductase, in

the cytoplasm of the bacterial cell when expression of the resistance genes is induced by mercuric ions. In *Escherichia coli* containing a high copy number plasmid, mercuric reductase constitutes 6% of the soluble protein of the cell. This level of expression presumably allows MerA to capture mercuric ions that enter the cell via both the MerT-dependent and -independent pathways, and is therefore highly protective of the cell contents. This elevated degree of enzyme production is also seen in other antimicrobial resistance mechanisms, for example chloramphenicol resistance [1]. High expression overcomes the disadvantage of having enzyme-mediated modification of the antimicrobial which is wholly or partly uncoupled to uptake. This is particularly relevant in cases where a large number of intracellular sites require protection, as with mercury and ribosome inhibitors such as chloramphenicol. (In contrast, it is envisaged that for cases where a unique uptake pathway occurs, the transfer of the toxic substrate from uptake protein to detoxifying enzyme can be tightly coupled. In such a system the production of enzyme will be minimized.)

Why does the cell have such a complex mechanism for handling a toxic non-essential metal? Why cannot mercuric ions be kept away from the cellular constituents by a permeability barrier, or simply pumped out again, as happens with cadmium and arsenate? There are several reasons why the complex mechanism is the most effective and efficient. First, the transport system depletes the periplasmic space of mercuric ions, thus protecting the periplasmic constituents and the outer face of intrinsic inner membrane proteins from Hg(II). Second, the MerP protein is thought to make the transport and ultimate detoxification of mercuric ions much more efficient at low mercury concentrations and when the genes are present on low copy number plasmids. Third, reduction is a better means of protection than an export pump, as the elemental mercury produced is non-toxic and there is no continual export and re-entry of mercuric ions; reduction has to take place intracellularly, because the necessary cofactor (NADPH) occurs only within the cytoplasm.

A similar mechanism of mercuric ion resistance probably occurs in other bacteria, including *Bacillus, Staphylococcus, Streptomyces, Thiobacillus,* and other Gram-positive and Gram-negative genera [7,20]. All contain mercuric reductase enzymes which, in the cases where sequence data is available, show marked sequence similarity to those of Tn*501* and Tn*21*. In all the Gram-negative examples the transport genes are similar to those of Tn*501,* except that Tn*21* encodes an additional protein, *merC* which has recently been shown to mediate uptake of mercuric ions. [A.P.M. and N.L.B., unpublished data]. The determinant from *Thiobacillus* contains only *merC* and *merA* genes [21]. It is possible that MerC might confer more efficient transport at high levels of mercury than MerT/P, particularly at low copy number. This is supported by observations of Olsen and co-workers [22] which showed that in natural isolates of mercury-resistant bacteria, species isolated from higher mercury concentrations tend to contain mercury-resistance determinants of the Tn*21* type, with both MerT/P and MerC transport systems, whereas those from lower mercury concentrations have only the MerT/P system. The transport genes in Gram-positive genera are different from those of the Gram-negatives, as might be expected because of the intrinsic differences in the cell surfaces; but in all cases studied, there are predicted gene products with some sequence homology to MerT. This homology is in the region of Cys24, Cys25 and the periplasmic loop of MerT (Fig. 3).

MECHANISM OF RESISTANCE TO COPPER SALTS

Copper salts are toxic but they are also essential. A mechanism of resistance cannot therefore merely exclude or detoxify the metal ion, but must be integrated with the mechanisms of copper homeostasis in the cell. An understanding of the mechanism of copper resistance must therefore include an understanding of the mechanisms of normal copper metabolism in the bacterial cell. We have identified a number of mutants in *Escherichia coli,* which respond differently to the wild type to change in external copper concentration. Some mutants are copper-sensitive, being unable to grow on the higher copper concentrations (*ca* 4 mM in Luria broth) tolerated by normal *E. coli;* other mutants are dependent on small amounts of copper added to the media. These mutants mapped to seven complementation groups, *cutA–cutF* (copper *u*ptake and *t*ransport) and *cutR* (regulation). Analysis of the genetic and biochemical properties of these mutants was undertaken [23]. The properties of the copper uptake, copper accumulation, and copper efflux systems were measured in mutant

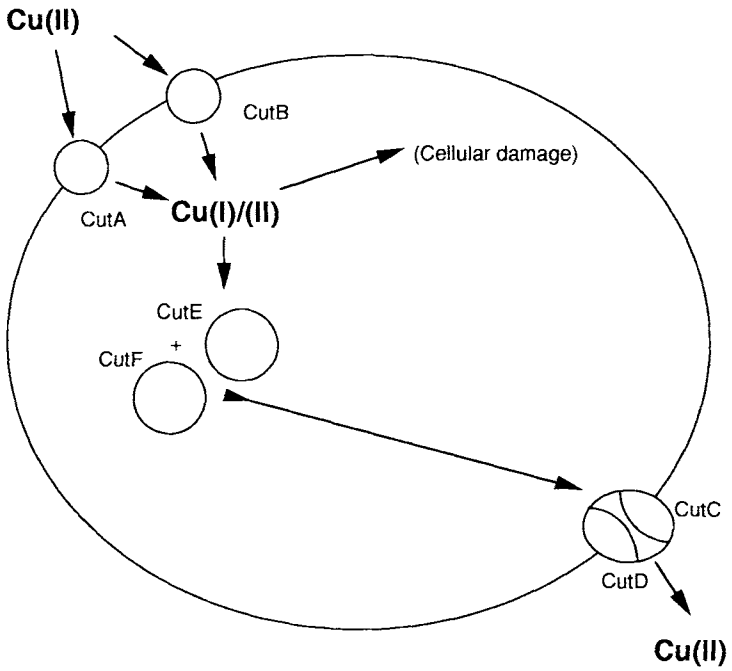

Fig. 4. Model for copper metabolism in *E. coli*. Copper is taken up into the cell through one of at least two transport systems, encoded by the *cutA* and *cutB* genes. The CutA system also transports Zn(II) and may be the main zinc transport system; the CutB system is thought to transport Ni(II) as a minor substrate in addition to copper. Once inside the cell, the Cu(II)—or possibly Cu(I)—ions cannot remain free in solution, due to their toxicity; they are proposed to be protein-bound, and there are proposed to be both storage proteins and intracellular transport proteins. The CutE protein is proposed to be an intracellular copper storage protein, and CutF is likely to be an intracellular copper transport protein. The intracellular transport proteins are responsible for delivering copper to the sites of synthesis of Cu-containing proteins, and to the export system. The products of the *cutC* and *cutD* genes constitute a copper-export system, which helps regulate the intracellular concentration of copper. CutR is a regulatory protein that responds to copper concentration and is assumed to alter the expression of some or all of the *cut* structural genes; the *cutR* gene was identified by its effect on the copper resistance genes.

and wild type cells. These data allowed a model for copper metabolism in *E. coli* to be proposed in which CutA–CutF were the products of structural genes responsible for copper metabolism in the cell, and CutR was the protein regulating their expression. The limited data available, together with *a priori* assumptions of the properties of a homeostatic mechanism, have led to a model for the mechanism of copper metabolism in *E. coli*, shown in Figure 4.

A plasmid-borne copper-resistance determinant was identified in isolates of *E. coli* from an Australian piggery [24]. *E. coli* strains carrying the *pco* (*p*lasmid-borne *co*pper resistance) determinant can tolerate approximately fivefold higher concentrations of cupric ions than wild-type strains. This resistance determinant has been characterised [23,25,26]. Genetic studies using transposon mutagenesis have shown that pco contains at least four genes, *pcoARBC*, that are required for copper resistance. At high copper concentrations the *pco* gene products are proposed to modify the action of the proteins for normal copper metabolism to both increase the export of copper, in an energy-dependent manner, and to modify the copper, such that it is no longer biologically available; the PcoA and PcoB genes may be involved in this. The PcoC product is a cytoplasmic copper binding protein. Two of the gene products required for normal copper metabolism are required for resistance also; namely, CutA (uptake) and CutD (efflux). The current outline model for the mechanism of *pco*-mediated copper resistance in *E. coli* [23,27,28] is shown in Figure 5.

As copper is essential, there must also be an uptake system so that the cell obtains sufficient copper for the synthesis of copper dependent enzymes even at low copper concentrations. Two mechanisms prevent this specific uptake system from becoming detrimental at very high copper concentrations in the presence of the resistance determinant. The first is that both chromosomal and plasmid encoded genes are regulated,

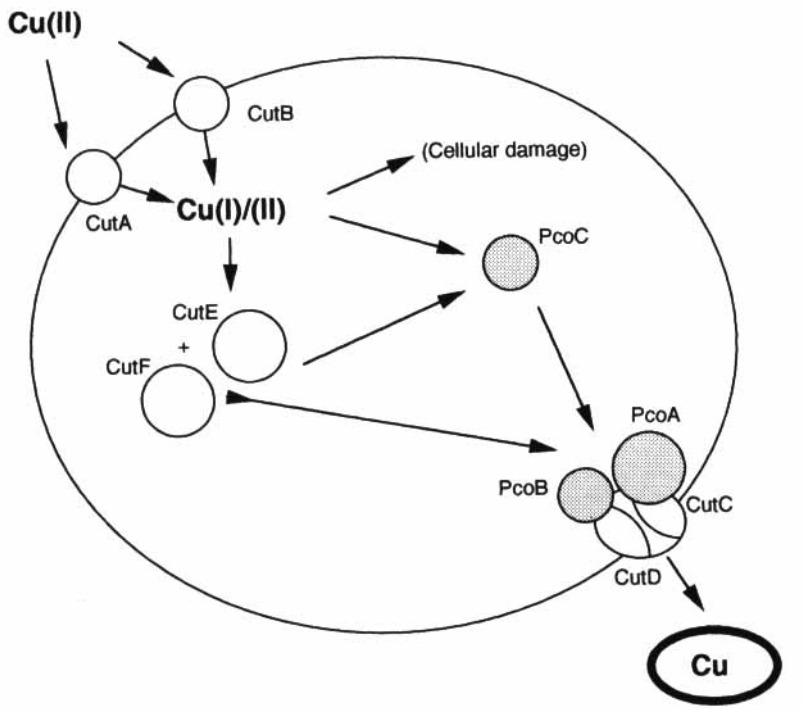

Fig. 5. Model for the mechanism of *pco*-encoded copper resistance. At high copper concentrations the *pco* genes are induced, and modify copper metabolism. The PcoC protein is an intracellular copper-binding protein that may provide internal protection against high copper concentrations, and may act as an intracellular transport protein. The increased export of copper and its modification to a biologically refractory form are proposed to be functions of the PcoA and PcoB proteins. The PcoR protein alters expression of the *pco* genes, and may also alter expression of the *cut* genes, thus modifying the flux of copper through the cell.

such that the resistance genes are switched on at high copper concentrations. The second mechanism is that of copper modification on export, such that the excess copper exported from the cell becomes unavailable to the uptake system.

REGULATION OF EXPRESSION OF METAL RESISTANCE GENES

Both the metal resistance systems discussed in this paper are inducible by their respective cations. The regulation of expression of the resistance genes is an integral part of the bacterial response to the presence of a toxic metal, and general deductions about metal homeostasis and the role of regulation of expression of metal resistance genes can be made.

The mechanism of induction of the *mer* genes by mercuric ion is now quite well understood. This involves a single protein, MerR, the product of the *merR* gene, which acts as a repressor of transcription of the *mer* operon in the absence of mercuric ions, and as an inducer in the presence of Hg(II) ions [29]. Remarkably, the protein does this by binding at the same site in the *mer* operator-promoter region [30]. Evidence is accumulating that the mechanism of induction involves the binding of a single mercuric ion to a MerR dimer bound at the *mer* operator, which in turn causes a conformational change in the promoter [31,32], and this activates transcription. RNA polymerase appears to be bound at the *mer* promoter in vivo in the absence of Hg(II), and activation of the preexisting DNA-MerR-RNA polymerase complex with a single Hg(II) ion allows the promoter to respond in an "ultrasensitive" manner to small changes in Hg(II) concentration, as befits a mechanism intended to remove totally a toxic compound from the bacterial cell [33].

Little is known about the regulation of expression of the copper resistance genes in *E. coli*. A copper-responsive promoter has been isolated from the *pco* determinant and preliminary data show that it responds in an approximately linear manner to external Cu(II) concentrations, whereas the *mer* promoter has a strong sigmoidal response to external Hg(II) [B.T.O.L. and T.W., unpublished data]. This could be predicted from homeostatic considerations, because internal concentrations of copper must be

maintained within certain limits to allow the cell to maintain copper-dependent functions of the cell, whereas mercuric ions can be totally eliminated [D.A.R., T.W., B.T.O.L., J.P., and N.L.B., in preparation]. The same considerations apply in the regulation of resistance to other non-beneficial toxic metals, such as lead and cadmium, which will have a low threshold of induction and will be ultrasensitive to changes in metal concentration about this threshold; whereas the induction of resistance to toxic essential metals, such as zinc and nickel, will have a more linear response to metal concentration in order to maintain at least the minimum intracellular concentration of the essential metal.

The *pco* determinant contains three structural genes (*pcoA–C*) that have been identified genetically, and a regulatory gene, *pcoR*. An additional putative gene (*pcoD*) has been identified from DNA sequence information, and a mutant is being sought to determine whether it has a function in copper resistance. The genes map in the order *pcoARBCD*. The PcoR protein mediates copper-inducible expression of the remaining *pco* genes. The *pco* genes in $pcoR^-$ mutants are still regulated, indicating that there is a chromosomal regulatory gene that can affect expression of the *pco* genes. This is the gene designated *cutR* and it is assumed to regulate the expression of chromosomal genes required for copper metabolism. The *pcoR* gene has been sequenced [N.L.B. and D.A.R., in preparation] and the predicted amino acid sequence of the PcoR protein shows sequence similarity to regulatory proteins from the two-component bacterial regulatory systems [34,35]. All these systems have, in addition to the regulatory protein which interacts directly with DNA, a sensor protein that responds to an environmental stimulus. In the cases where information is available, the environmental signal is transduced from the sensor by covalent modification (phosphorylation) of the regulatory protein. PcoR is presumed to be the regulatory protein, but the sensor for the copper regulatory system has not yet been identified. We have recently isolated mutants [B.T.O.L. and T.W., unpublished] which may be in the gene encoding the sensor protein.

CONCLUSIONS

The successful site-specific mutational analysis of the mercury resistance mechanism encoded by Tn*501* has confirmed the outline model of this mechanism, and has improved the detailed understanding of the system. The usefulness of such analysis is heavily dependent on established knowledge of the biochemistry of the system. In elucidation of the less well-studied mechanism for copper resistance specified by the *pco* determinant, a more general mutation analysis proved necessary, due to the genetic complexity of the system, which involves chromosomal- as well as plasmid-encoded products. A combination of genetic and biochemical methods has allowed the mechanism of copper resistance to be modelled.

Consideration of the role of both the mercury and copper resistance mechanisms in cellular homeostasis predicts different shapes for the induction curves for expression of the resistance genes. This prediction can be extended to other inducible systems for resistance to non-essential toxic metals, such as cadmium and lead (which would have ultrasensitive induction kinetics with a low threshold), and to systems for resistance to essential toxic metals, such as nickel and zinc (which would be more linear with metal concentration). These predictions are experimentally testable.

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