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Genes for all metals—a bacterial view of the Periodic Table
The 1996 Thom Award Lecture

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Bacterial chromosomes have genes for transport proteins for inorganic nutrient cations and oxyanions, such as NH_4^+, K^+, Mg^{2+}, Co^{2+}, Fe^{3+}, Mn^{2+}, Zn^{2+} and other trace cations, and PO_4^{3-}, SO_4^{2-} and less abundant oxyanions. Together these account for perhaps a few hundred genes in many bacteria. Bacterial plasmids encode resistance systems for toxic metal and metalloid ions including Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, TeO_3^{2-}, Tl^+ and Zn^{2+}. Most resistance systems function by energy-dependent efflux of toxic ions. A few involve enzymatic (mostly redox) transformations. Some of the efflux resistance systems are ATPases and others are chemiosmotic ion/proton exchangers. The Cd^{2+}-resistance cation pump of Gram-positive bacteria is membrane P-type ATPase, which has been labeled with ^{32}P from [γ-^{32}P]ATP and drives ATP-dependent Cd^{2+} (and Zn^{2+}) transport by membrane vesicles. The genes defective in the human hereditary diseases of copper metabolism, Menkes syndrome and Wilson's disease, encode P-type ATPases that are similar to bacterial cadmium ATPases. The arsenic resistance system transports arsenite [As(III)], alternatively with the ArsB polypeptide functioning as a chemiosmotic efflux transporter or with two polypeptides, ArsB and ArsA, functioning as an ATPase. The third protein of the arsenic resistance system is an enzyme that reduces intracellular arsenate [As(V)] to arsenite [As(III)], the substrate of the efflux system. In Gram-negative cells, a three polypeptide complex functions as a chemiosmotic cation/protein exchanger to efflux Cd^{2+}, Zn^{2+} and Co^{2+}. This pump consists of an inner membrane (CzcA), an outer membrane (CzcC) and a membrane-spanning (CzcB) protein that function together.

Keywords: bacterial plasmids; toxic metal resistances; mercury; cadmium; arsenic

Charles Thom

Charles Thom, whose achievements we honor in this lecture series, was the first President of the Society of Industrial Microbiology, from 1949–1951. He had been earlier President of the Society of American Bacteriologists (later renamed the American Society for Microbiology) and was a member of the National Academy of Sciences. Charles Thom was born and raised on a farm near Peoria, Illinois, and after bachelors and masters degrees from a small college north of Chicago, he obtained the first PhD awarded at the University of Missouri (in 1899). Dr Thom worked essentially his entire professional career with the US Department of Agriculture (USDA), from 1902 until retirement in 1942, mostly in Washington, DC, but he then continued to work with the penicillin development program at the USDA Northern Regional Research Laboratories (NRRL) at Peoria for another 10 years.

During this long service, Dr Thom had several major achievements—more than most scientists expect. His first assignment was to bring a quality mold-ripened cheese industry from France to the USA. This required fungal taxonomy and he identified and named *Penicillium camerberti* and *Penicillium roqueforti*. Dr Thom became the major mycologist/taxonomist of his time and wrote (with collaborators) monographs on the genera *Penicillium* and *Aspergillus* in the 1920s that were updated and remained standard into the 1940s. Although he remained a mycologist with strong applied and theoretical interests, the USDA used Dr Thom's abilities for quite different enterprises. He helped develop the pure food and drug standards that became the basis for the Food and Drug Administration (FDA), which grew out from the USDA. Dr Thom appeared in court hearings, laying down production standards for the food processing industry. He wrote an early standard text on food microbiology. Next he helped to place soil microbiology on a solid scientific footing. With a colleague, he developed the first industrial fermentation of citric acid (by *Aspergillus niger*). This led to the establishment of a Fermentation Division and Fungal Culture Collection at the NRRL in Peoria. When during World War II, a British delegation trying to involve the American fermentation industry in developing penicillin ran into disinterest and lack of understanding, they visited Dr Thom in Washington DC with their problems. The USDA NRRL played a major role in the history of penicillin production. He maintained interests in fungal applications until his death at age 84 in 1956.

Although he worked within government, Charles Thom was well known for encouraging younger co-workers. This section was written from material of his best-known 'junior assistant', Kenneth B Raper [58,59], who was associated with Dr Thom from college graduation through the remainder of Dr Thom's time in the laboratory.

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Introduction

Bacteria have genes specific for transport of needed nutrients and for resistances to the toxic ions of most heavy

metal elements. Required inorganic nutrients include the cations NH_4^+, K^+, Mg^{2+}, Co^{2+}, Cu^{2+}, Fe^{3+}, Mn^{2+}, Ni^{2+}, Zn^{2+} and other trace cations [67], and the oxyanions PO_4^{3-}, SO_4^{2-} and less abundant anions. Figure 1 presents a current summary of inorganic nutrient transport systems and Figure 2 a listing of inorganic ion resistance systems and their biochemical mechanisms. Toxic inorganics with genetically-defined resistances include Ag^+, AsO_2^-, AsO_4^{3-}, Cd^{2+}, Co^{2+}, CrO_4^{2-}, Cu^{2+}, Hg^{2+}, Ni^{2+}, Pb^{2+}, Sb^{3+}, TeO_3^{2-}, TI^+ and Zn^{2+}. The first theme for this review is that for almost each and every inorganic cation or anion that is encountered in normal environments, there are corresponding genes and proteins that govern every movement [67]. For required nutrients there are highly specific membrane transport systems that concentrate needed nutrients from dilute media. Once inside the cell, some inorganic nutrients are sequestered (for example with the protein metallothionein) or enzymatically incorporated into specific proteins (for example using the enzyme ferrochelatase). Excess ions are either stored (for example iron in ferritin) or excreted (again by highly specific membrane transporters).

A similar situation occurs with regard to most toxic heavy metals and metalloids, as microbes have been exposed to these cations and oxyanions periodically since the origin of cellular life [68,72]. Most frequently, toxic metal resistance systems are found on plasmids, which facilitates movement from cell to cell. However, related systems are frequently determined by chromosomal genes in other organisms (for example, arsenic, mercury and cadmium resistance in *Bacillus*, cadmium resistance in methicillin-resistant *Staphylococcus aureus*, and arsenic resistance with chromosomal *E. coli* genes). Some cations such as Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+} are essential nutrients at low levels, but toxic at higher levels. For these cations there exist separate transport systems for uptake and for efflux in order to maintain cellular cation homeostasis [67,69].

Cations of related elements are often associated in pairs, with one needed for nutrition such as the Group Ia element K (with several parallel uptake transporters) and the other Na not used intracellularly, but frequently coupled for co-transport or cellular signaling, and needing a specific efflux mechanism. A similar situation occurs with the Group IIA (Mg as an intracellular nutrient and Ca maintained at low intracellular levels). For some higher atomic number elements of the Periodic Table (the lanthanides and actinides, including uranium and trans-uranium elements), there appear to be no specific genes or proteins for metal ion resistances, presumedly as these were not encountered in natural environments at levels that were toxic. Similarly, I am unaware of genes or proteins specific for Al^{3+}, since although Al is abundant in nature, the low solubility and low toxicity of aluminum cations does not require specific resistance systems. There also are no resistance genes for halides, although halides are abundant in the environment and toxic in higher concentrations.

A long-time hypothesis of this laboratory is that toxic metal resistance systems arose shortly after life started, in an already metal-polluted world. As with nutrient organic compounds, whatever was available in the environment provided selection for transport and metabolism. Recent activities of humans create local polluted environments of high selection for resistance, but toxic heavy metal resistance determinants predate these. For industrial and environmental microbiologists, inorganic cations and anions must

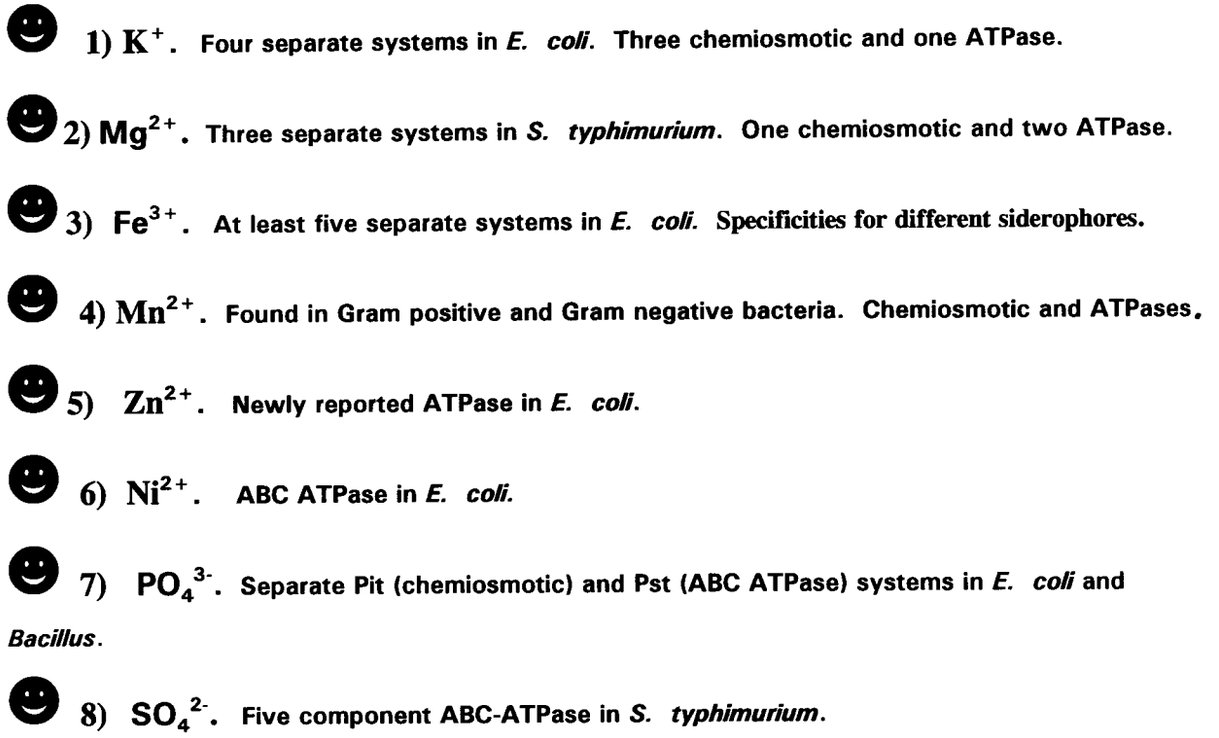

Figure 1 Nutrient cation and anion transport systems.

☹ 1. Hg^{2+}. *mer*. Hg^{2+} and organomercurials are enzymatically detoxified.

☹ 2. AsO_4^{3-}, AsO_2^-, SbO^+. *ars*. Arsenate is enzymatically reduced to arsenite by ArsC Arsenite and antimonite are "pumped" out by the membrane protein ArsB that functions chemiosmotically alone or with the additional ArsA protein as an ATPase.

☹ 3. Cd^{2+}. *cadA*. Cd^{2+} (and Zn^{2+}) are pumped from Gram positive bacteria by a P-type ATPase with a phospho-aspartate intermediate.

☹ 4. Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+}. *czc*. Cd^{2+}, Zn^{2+}, Co^{2+}, and Ni^{2+} are pumped from Gram negative bacteria by a three polypeptide membrane complex that functions as a divalent cation/2 H^+ antiporter. The complex consists of an inner membrane protein (CzcA), an outer membrane protein (CzcC) and a protein associated with both membranes (CzcB).

☹ 5. Ag^+. *sil*. Ag^+ resistance results from pumping from bacteria by three polypeptide chemiosmotic exchanger plus a P-type ATPase.

☹ 6. Cu^{2+}. *cop*. Plasmid Cu^{2+} resistance results from a four polypeptide complex, consisting of an inner membrane protein, an outer membrane protein, and two periplasmic copper-binding proteins. In *Pseudomonas*, Cop results in periplasmic sequestration of Cu^{2+}. In addition, chromosomally-encoded P-type ATPases provide partial resistance by effluxing Cu^{2+} or Cu^+.

☹ 7. CrO_4^{2-}. *chr*. Chromate resistance results from a single membrane polypeptide that causes reduced net cellular uptake, but efflux has not been demonstrated.

☹ 8. TeO_3^{2-}. *tel*. Tellurite resistance results from any of several genetically-unrelated plasmid systems. Although reduction to metallic Te^0 frequently occurs, this does not seem to be the primary resistance mechanism.

☹ 9. Pb^{2+}. *pum*. Lead resistance appears to be due to an efflux ATPase in Gram negative and accumulation of intracellular $Pb_3(PO_4)_2$ in Gram positive bacteria.

Resistance systems await understanding for bismuth (Bi), boron (B), thallium (Tl) and tin (Sn).

Figure 2 Plasmid toxic metal resistance systems and mechanisms (modified from Ref. [68]).

be understood and controlled in the same way as organics and gases are controlled.

From the time when it was shown that mercury-resistant bacteria volatilize mercury as mono-atomic elemental Hg^0 [79], the mechanisms of bacterial plasmid-determined resistances to many toxic inorganic cations and anions have been studied. Initially the production of mercury 'gas' as a resistance mechanism seemed strange. Now it is accepted. Here, one can only overview the range of mechanisms and a few newer aspects. However, many reviews appeared in a 1995 issue of *Journal of Industrial Microbiology* (vol 14), and in separately published reviews that are cited below. In addition to mercury-resistance determinants, there are highly specific resistance systems for arsenic (and antimony), copper, cadmium (and zinc in Gram-positive bacteria, but a different system shared with cobalt, nickel and zinc in plasmids of Gram-negative bacteria), tellurite, silver and other toxic metal and metalloid ions (Figure 2).

Three generalizations may be made: (i) Plasmid-determined metal resistance systems are very specific, as much so as those for antibiotic resistances, or sugar or amino acid metabolism. There is no general mechanism for resistance to all heavy metal ions. (ii) Metal-ion resistance systems have been found on plasmids of every eubacterial group tested, from *Escherichia coli* to *Streptomyces*. (iii) The mechanisms of resistance are generally efflux 'pumping' (removing toxic ions that entered the cell by means of transport systems evolved for nutrient cations or anions) and enzymatic detoxification (generally redox chemistry) converting a more toxic to a less toxic or less available metal-ion species. It would seem easier to keep toxic ions out (with highly specific uptake transport systems), rather than to expend metabolic energy bringing in toxic ions and then more energy pumping them out. Apparently, the metabolic penalty for having uptake pumps more specific is greater than the genetic cost of having plasmid genes in the population that can spread when needed. The efflux pumps that are the major groups of plasmid resistance systems can be either ATPases (as the Cd^{2+} ATPase of Gram-positive and the arsenite ATPase of Gram-negative bacteria) or chemios-

motic (as the divalent cation efflux system of soil *Alcaligenes* and the arsenite efflux system of the chromosome of *Bacillus* and *E. coli* and of plasmids in Gram-positive bacteria). The mechanisms are not precisely the same in all bacterial types: while the mercury-resistance and arsenic-resistance systems are highly homologous in all bacteria studied, cadmium resistance involves ATPases in Gram-positive bacteria and unrelated chemiosmotic antiporters in Gram-negative bacteria. These systems appear to be of independent evolutionary origin. There is also a well-described bacterial metallothionein, found so far only in cyanobacteria, and conferring resistances to Cd^{2+} and Zn^{2+} [85].

Genomics: a view from the top

While we and others have been cloning and sequencing the specific genes for heavy metal resistances in bacteria for over a decade, total genome sequencing projects have recently uncovered related systems, sometimes functionally equivalent or sometimes merely homologous in sequence. Consideration of these new untested genes provides an additional view of the generality of metal resistance systems and their ancient origin. For example, the 1.8-megabase total genome of *Haemophilus influenzae* [23] includes predicted genes for arsenate reductase (*arsC*) and mercury transport (*merT* and *merP*) homologous to those we had earlier sequenced from bacterial plasmids. We do not know as yet whether these genes are functional, as are the chromosomal *ars* operons of *E. coli* [7,8,20,74] and *Bacillus* [80]. However, our best guess is that such MerP homologs are not periplasmic mercury-binding proteins, but rather intracellular sensors for detecting and regulating levels of other (non-mercury) thiol-binding cations. The basis for predicting beyond mere sequence homologies is too uncertain to merit discussion. However, Clayton *et al* [14] recently summarized homologous transport systems in bacterial, Archae and yeast genomes, trying to draw conclusions for a wide range of cation and oxyanion transporters.

The custom has developed for the initial attribution of open reading frames of newly sequenced genomes of naming the single closest related protein sequence in available libraries [6,23,32]. It seems likely that these first assignments, although reasonable will be sometimes incorrect. For example, the first cyanobacterial genome, the 3.6-megabase sequence of *Synechocystis* includes many open reading frames listed as homologous [32] to ones that we initially sequenced as part of resistance systems, including slr1849 listed as mercuric reductase, sll1957 listed as 'ArsA arsenical resistance operon repressor' (already a mistake since ArsA is the ATPase protein and the transcriptional repressor is ArsR), slr0946 listed as 'ArsC arsenate reductase' (note that the *arsA* and *arsC* homologs are 600 kb apart and not linked as with all known *ars* operons), sll0142 listed as the cation efflux pump CzcZ, and slr1457 as the chromate resistance protein ChrA. slr0798 and sll0789 are divergently transcribed closely located homologs of the CadA cadmium resistance ATPase, with a single gene for a histidine kinase component of a two-component signal transduction complex in between, and slr0701 is a homolog to the *merR* mercury regulatory gene. The first point to be made is that any newly sequenced genome is likely to include a sizeable number of open reading frames whose predicted protein products are closer in sequence with proteins for heavy metal resistances than for any other known functions. The next point to be made is that some of these initial assignments are likely to be incorrect, and will be corrected when analyzed in more sophisticated ways, including consideration of key amino acid residues involved in function and of neighboring genes organized into operons. For example, the predicted mercuric reductase sequence of *Synechocystis* has the closest percent identity overall to the mercuric reductase of Gram-positive bacteria, but when the sequence is analyzed carefully, the carboxyl-terminal mercury-binding region that is tightly conserved in all mercuric reductases is missing in the *Synechocystis* sequence. In the carboxyl region, the new *Synechocystis* sequence is more like that of *E. coli* lipoamide dehydrogenase—perhaps a better candidate now for the enzymatic role of the gene product.

No one has reported resistance mechanisms such as described here with Archaea. Whether this reflects a difference in biology or a lack of research effort is hard to tell. Archaea grow in environments with high levels of toxic heavy metal ions and therefore are expected to have the same or alternative mechanisms of metal resistances. A step in this direction was the publication [6] of the 1.66-megabase genome of the methanogen *Methanococcus jannaschii*, which contains only two open reading frames that are reported as homologous to those we earlier reported. These encode homologs for ArsA (the ATPase subunit of the arsenite effluxing membrane transport system) and ChrA (the membrane protein required for chromate resistance). Why there are so few such recognizable genes in a deep sea vent-dwelling organism (expected to have been exposed to a range of toxic heavy metals), compared with the larger number for the human pathogen *Haemophilus influenzae* is unclear. An *arsA* homolog has also been identified with the sequencing project for the leprosy microbe, *Mycobacterium leprae* (DR Smith, GenBank listing MSGB577COS) listed in [35] and in sequenced human genes (see below).

Plasmid mercury resistance

Closely related systems for resistance to inorganic mercury have been found on plasmids of Gram-negative and Gram-positive bacteria. In the collection of some 800 antibiotic-resistance plasmids that had been mobilized from various Gram-negative bacteria into *E. coli*, 25% carried mercury resistance [66]. In most cases, the order and approximate number and functions of the genes are the same [39,72,73]. The sequences are similar enough that almost all crossreact by DNA/DNA hybridization [39]. Most *mer* systems start with a regulatory gene, *merR*, whose product is a unique positively-acting activator protein that in the presence of Hg^{2+} twists and bends the operator DNA region, allowing RNA polymerase to synthesize mRNA [54,72,78]. In the *mer* systems of Gram-negative bacteria, *merR* is transcribed separately from the remaining *mer* genes, allowing tighter control of the *mer* operon than possible with Gram-positive bacteria, with which *merR* is the first gene on the multi-

gene *mer* operon. There then follows one to three genes whose products are involved in transport of toxic Hg^{2+} across the cell membrane to the intracellular detoxifying enzyme mercuric reductase, the product of the long *merA* gene. In some *mer* operons, *merA* is followed immediately by *merB*, which encodes the enzyme organomercurial lyase, which breaks the carbon-mercury bond in highly toxic organomercurials such as phenyl- and methyl-mercury. It is clear that more variability in mercury resistance systems is to be found. In one example from *Pseudomonas*, the *merB* gene is found between *merR* and *merT*, together with an extra operator/promoter site. In *Bacillus mer* operons, there was a 1.1-kb gap in the published sequence between *merA* and *merB*, which we sequenced and where we found a second transcriptional start site, a second gene homologous to *merR*, and a second *merB* gene (Figure 3). The MerR2 amino acid sequence is sufficiently different from that of MerR1 as to make it questionable as to whether it functions to activate transcription in the same manner. However, the two organomercurial lyase gene products, MerB1 and MerB2, appear functional. The DNA sequence of the mercurial resistance region of the first well-studied mercury resistant strain, *Pseudomonas* K-62, was recently completed [34; M Kiyono and H Pan-Hou, personal communication] and also contains two *merB* genes determining two organomercurial lyase enzymes.

The structure of the *Bacillus* mercuric reductase was solved by X-ray diffraction [65]. As anticipated from their related biochemical mechanisms, the structure is very similar to that of glutathione reductase from humans, and is a homo-dimer, with each subunit containing a highly conserved active site with two critical cysteine residues. FAD is bound to each subunit, and an NADPH-binding site is found for electron transfer from NADPH to FAD to the substrate Hg^{2+}. The active site includes the redox-active disulfide region on one subunit and the substrate-binding site at the C-terminal (including conserved vicinal Cys residues) of the other subunit [21]. The crystallographic solution for mercuric reductase starts, however, after the first 160 amino acids [65], which appear to lack a fixed position in the crystals. This N-terminal region of *Bacillus* mercuric reductase contains a duplication and fusion of two 'metal-binding domains' for which homologous sequences have been identified at the N-terminus of mercuric reductases, at the N-teminus of soft-metal translocating P-type ATPases, and as a periplasmic mercury-binding protein (MerP) in mercuric resistance systems of Gram-negative bacteria [71–73]. An NMR-based structure for MerP with and without bound Hg^{2+} [77] has been determined as a $\beta\alpha\beta\beta\alpha\beta$ folded polypeptide with Hg^{2+} bound by cysteine thiols in a conserved GMTCXXC motif.

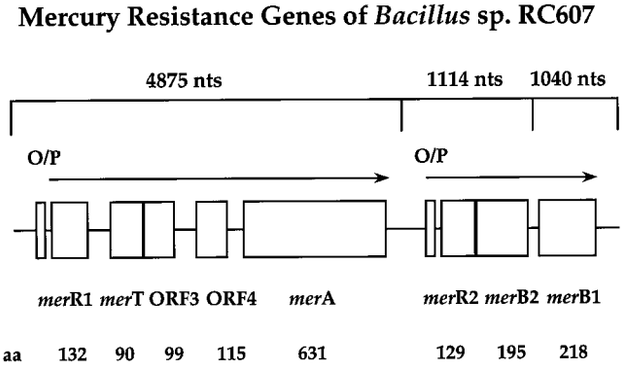

Figure 3 Genetic organization of the mercury resistance system in *Bacillus* sp RC607. Nts, nucleotides previously or newly sequenced. Two promoters are shown. The lengths of gene products in amino acids are shown (from A Gupta *et al*, in preparation).

Two conclusions concerning mercury resistance genes in the environment come from recent experience with mercury-resistant *Bacillus* isolates from the methylmercury-polluted area at Minamata Bay, Japan [44] and mercuric resistance genes from Gram-negative bacteria from North American mercury-polluted sites [61]: (a) The genetic determinants that have been extensively studied in the laboratory are representative of what is found in Nature; and (b) what occurs in Nature is moderately complex, so that understanding from laboratory studies is useful for understanding heavy metal resistance genes in real environments. Other recent developments on bacterial mercury resistance have been covered elsewhere [31,68,72,73].

Arsenic resistance

Understanding of bacterial arsenic resistance systems is more recent than that of mercury resistance. Fundamentally the same genes (and encoded biochemical mechanism) are found on plasmids in Gram-negative and Gram-positive bacteria [29,31,63,72] (Figure 4). However, the number of genes can vary and the details of their functions can differ. Seven *ars* operons have been sequenced and are shown in Figure 4. The two *Staphylococcus* sequences are 96% identical at the DNA level. The two Gram-negative plasmid systems (from plasmids R773 and R46) are also very similar. This is important since the Gram-negative plasmid systems as shown in Figure 4 contain two extra genes, *arsA* and *arsD*, that are missing from the Gram-positive plasmids and the chromosomal systems recently identified during genome sequencing of *E. coli* [74] and *Bacillus subtilis* [80] (Figure 4). The ArsD protein is a secondary regulator of *ars* operon transcription [13], so its presence or absence might have little effect on resistance.

The ArsA protein, by contrast, is a membrane-associated ATPase [33] attached to the ArsB inner-membrane protein [81,91] and energizing the arsenite efflux pump by ATP hydrolysis [18,72]. That an essential energy-coupling protein might be missing is puzzling. The answer is that the ArsB protein can function alone as a chemiosmotic (membrane-potential driven) arsenite efflux transporter or together with ArsA as an ATP-driven primary membrane pump [3,18,37]. Such alternative energy coupling is unique among known bacterial uptake or efflux transport systems. Other systems are either chemiosmotic or ATP-driven transporters. *arsA* homologs have recently been found in *Mycobacterium leprae* (GenBank listing MSGB577COS, listed in Ref. [35]) and even in the human genome [36]. Additional *arsA* homolog genes have been identified in the nematode *C. elegans* [35] and in yeast. For each of these hypothesized eukaryotic gene products, no function is known. Differences in lengths and amino acid sequences between the bacterial ArsA and its eukaryotic homologs are

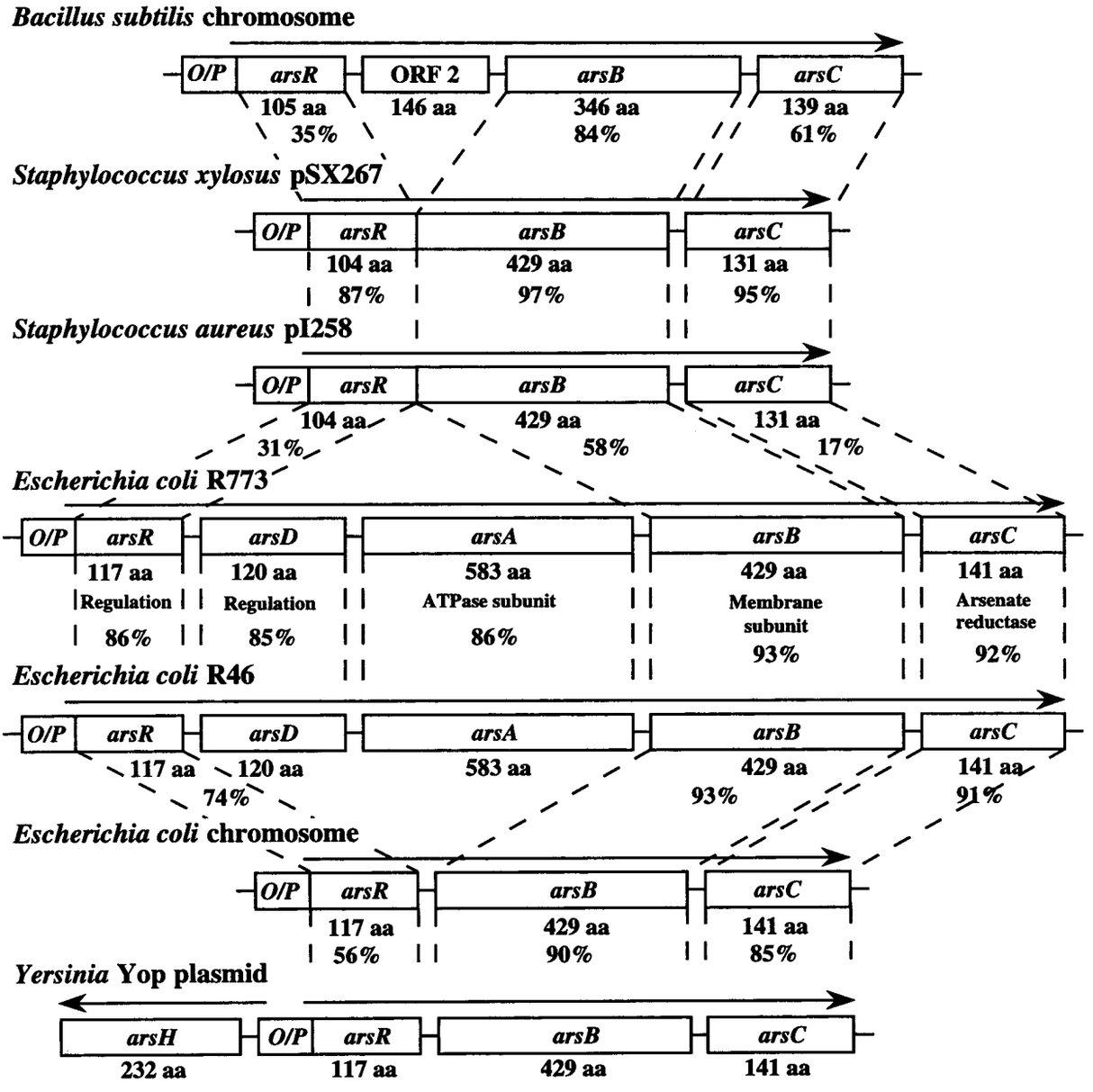

Figure 4 Genes and products for arsenic resistance in bacteria. Alignment and functions (below) of arsenic resistance genes (boxes) with aa sizes of predicted products (above and below genes) and percent identities between amino acid products.

such that it seems unlikely that the plant and animal products are actually components of arsenite transport systems. More likely, they are involved in alternative oxyanion metabolism involving an ATP-dependent process, and it is only a temporary situation where their closest available homolog for which a physiological or biochemical function is known is the bacterial ArsA transport ATPase. The point is clear, however: what we study in depth in bacterial cells with regard to inorganic ion metabolism will lead the way sometimes and be of use for research in higher organisms including man. That has been a gratuitous bonus of this work.

The final gene product common to all *ars* operons is ArsC, the enzyme that reduces less toxic arsenate [As(V)] to more toxic arsenite [As(III)] [25,28,30], the substrate for the ArsB transport protein. It seems illogical to convert a less toxic compound to a more toxic form, but ArsC activity is closely coupled with efflux from the cells [30] so that intracellular arsenite never accumulates. Arsenate reductases from plasmids pI258 [28] and R773 [25] both reduce arsenate and both confer arsenate resistance. However, their *in vitro* measured K_ms differ by 1000 times and their energy coupling is different. Arsenate reductase of pI258 derives reducing power from thioredoxin (glutaredoxin will not work), presumedly by recycling oxidized dithiol to two reduced cysteine residues (of the four found in the sequence). In contrast arsenate reductase of plasmid R773 uses glutaredoxin (but not thioredoxin) and probably only one of the two cysteines found in the protein sequence is required. The arsenic resistance system is suf-

ficiently novel that we expect radical changes of understanding in the future. It seems clear, however, that basically the same *ars* operon conferring resistances to As(III), As(V) and Sb(III) occurs widely in Gram-negative and Gram-positive bacteria [9,20,70]. The new *Yersinia* plasmid *ars* operon (Figure 4) includes an additional gene, *arsH*, which is not found on the other known systems, but which is required for arsenic resistance [45]. This gene is transcribed separately and in the opposite direction, and although the function of ArsH is unknown, it is postulated to be involved in regulation of the other three genes.

In addition to plasmid arsenic resistance that is well understood and for which clusters of genes have been isolated and sequenced, there is bacterial arsenic metabolism that involves oxidation of arsenite to arsenic (eg Ref. [2]), reduction of arsenate to arsenite as part of an oxyanion-coupled anaerobic respiration [1,41] or the coupled cleavage of carbon-arsenic bonds with oxidation to arsenate [57]. These systems are of major environmental concern in arsenic-containing settings, where they may be involved in mobilization or immobilization of environmental arsenic. These additional systems have not as yet been studied by molecular genetics.

Plasmid copper resistance in Gram-negative bacteria

Copper resistance has been described with plasmids of several Gram-negative bacteria [4,5,16,17]. These systems are homologous [4,16,17] and contain the same genes, but the genetic basis for plasmid copper resistance in Gram-positive bacteria remains to be determined. The *E. coli* structural genes are *pcoABCDE* and the regulatory genes *pcoR* and *pcoS* [4,5] (Figure 5). For *Pseudomonas*, the comparable genes are called *copABCD* and *copR*, *copS*. There is no *pcoE* homolog in the pseudomonad system. The CopR/PcoR and CopS/PcoS proteins were the first known metal resistance systems with transcriptional regulation by a classical two-component regulatory system. (The second and third examples will be described below.) The sensor protein PcoS is found in the membrane and probably can be labeled by an auto-kinase activity at a specific conserved histidine residue with ^{32}P from [γ-^{32}P]ATP (Figure 5). The DNA-binding responder protein PcoR is probably trans-phosphorylated on a specific aspartate residue by ^{32}P-labeled PcoS [4,5,17]. The four structural proteins determining copper resistance in *Pseudomonas* have been characterized and are the inner membrane protein CopD, the outer membrane protein CopB, and two periplasmic blue copper proteins CopA and CopC [11,16,17] (Figure 5). It is thought that storage of excess copper in the periplasmic space protects the cell from toxic copper. How CopD and CopB are involved in movement of copper across the membranes is not understood. However, a mutant *cop* operon containing *copD* but lacking one or more of the other genes confers hyper-sensitivity and hyper-accumulation of cellular copper [12], indicating a role for CopD in copper uptake by the cell. A major source of confusion in the understanding of copper resistance is that colonies of the copper-resistant *Pseudomonas* turn blue when grown in high copper-containing media, while those of *Xanthomonas* and *E. coli* turn brown, and show no sign of periplasmic copper storage [4,5]. Furthermore, there is preliminary evidence for copper efflux (not uptake) associated with *E. coli* copper resistance [5]. Resolution of this problem awaits further work. The *E. coli* plasmid system [4,5] includes an additional gene *pcoE*, the product of which is a periplasmic copper-binding protein, that is highly produced and separately regulated [64]. Models for these gene functions have changed from year to year (eg Ref. [4] and earlier papers quoted there) and more experimental work is needed. However, all models of copper homeostasis and resistance involve the control of cellular uptake and efflux of copper by chromosomal genes, as well as plasmid systems for additional resistance in high copper environments [17,22].

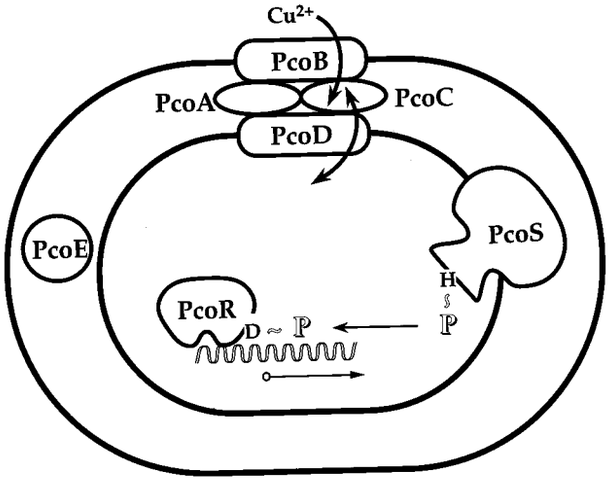

Figure 5 Plasmid copper resistance. The proposed locations and functions of the *pco* gene products from *E. coli* (see Refs [4,5,16] for specifics).

Chromosomal copper resistance in *Enterococcus hirae*

The best understood copper transport and resistance system is that of the Gram-positive *Enterococcus hirae* [51,76]. Remarkably, the two genes, *copA* and *copB*, that determine respectively uptake and efflux P-type ATPases, are found in a single operon [51]. The system is regulated in response to both copper-starvation (when the CopA uptake ATPase is needed) and copper-excess (when the CopB efflux ATPase is needed) [53]. *Enterococcus* CopA and CopB have the same names but different structures and functions from the *Pseudomonas* plasmid genes for copper resistance [17]. *E. hirae* mutants lacking the CopA uptake ATPase become somewhat copper-resistant and require higher levels of medium copper for growth. Bacterial mutants lacking the CopB efflux ATPase become copper-hypersensitive [12].

Regulation of the *cop* operon is governed by the products of the first two genes in the operon, *copY* (which determines a repressor protein) and *copZ* (which determines an 'anti-repressor' [53], a currently unique regulatory protein pair. The CopY apo-repressor is thought to be inactive and fails to bind to the operator/promoter DNA in the absence of intracellular Cu^+. A moderate level of intracellular Cu^+ binds to CopY converting it to a DNA-binding repressor

[53]. At higher intracellular Cu^+ levels, the CopZ antirepressor binds Cu^+, and CopZ-Cu^+ binds CopY-Cu^+, forming an inactive (that is non-DNA-binding) complex. This explains the simultaneous induction of synthesis of both ATPases by Cu^{2+} [52].

The CopB copper efflux ATPase of *E. hirae* has been studied by ATP-labeling and transport experiments. Solioz and Odermatt [75] isolated inside-out, subcellular, membrane vesicles from *E. hirae* cells and the vesicles required ATP in order to accumulate $^{64}Cu^+$ and $^{110m}Ag^+$. The *in vitro* substrate for CopB is thought to be Cu^+ rather than Cu^{2+}, but whether copper is taken up initially as Cu^{2+} and subsequently reduced to Cu^+ or whether copper is reduced at the cell surface (before or concomitant with transport) is not known. However, this is similar to the situation with eukaryotic cells where monovalent Cu^+ is thought to be the intracellular form [75].

Bacterial metallothionein in cyanobacteria

Bacterial metallothioneins, functionally homologous to the approximately 60-amino acids long, thiol-rich (perhaps 20 of those 60 amino acids are cysteines) mammalian metal-binding proteins, have rarely been reported and have been studied in detail only for the cyanobacterium *Synechococcus* [26,27,85]. The 58-amino acid polypeptide product of the *smtA* gene contains nine cysteine residues, which are clustered in groups of 4 and 5 respectively, as are the cysteines in animal metallothioneins. Metallothionein cysteines in the two domains bind divalent cations independently. The synthesis of metallothionein is regulated at three levels [85]. Firstly, the SmtB repressor protein binds divalent cations and dissociates from the target DNA. Secondly, there is gene amplification so that tandem multiple copies of the metallothionein locus are produced in metal-stressed cells [26]; and thirdly, deletion between repeated sequences on the DNA removes most of the *smtB* gene for the repressor protein [27].

Cadmium resistance in Gram-positive bacteria results from a P-type ATPase

The Cd^{2+} efflux ATPase from staphylococcal plasmid pI258 was the first of a system now found widely in Gram-positive bacteria [71–73], including soil bacilli and clinical *Listeria* (see Ref. [72] for references). The protein structure as diagramed (Figure 6a) is typical of P-type ATPases: it starts with a metal-binding motif, including a vicinal cysteine pair. One or two such motifs are found with different CadA ATPases, while six metal-binding motifs occur in the mammalian copper transporting efflux ATPases that are defective in the hereditary diseases Menkes and Wilson's [71,73,87]. The remarkable similarity of these ATPases between animals and bacteria is a major recent finding. There follows a membrane ATPase region closely homologous to other P-type ATPases. This includes the eight predicted membrane-spanning regions shown in Figure 6a for the heavy metal-translocating ATPases [76,87], the sixth of which is thought to be involved in the cation translocation pathway. It concludes a conserved proline residue (as shown), between cysteines that are found in the Cd^{2+} ATPases and related proteins. Earlier models [71,73] predicted six transmembrane segments rather than eight. However, Melchers *et al* [42] recently provided detailed data using protein fusions supporting the eight-segment model for a presumed-copper effluxing ATPase from *Helicobacter pylori*. Whereas the bacterial potassium ATPase KdpB has been modeled as having six membrane-spanning segments in the comparable region, the Mg^{2+} uptake ATPases and (postulated) Ca^{2+} efflux ATPases have an additional four segments towards the carboxyl end for a total of 10 as shown in Figure 6b. Thus P-type ATPases differ in membrane topology and length, depending more on cation specificity than on the difference between uptake and efflux directions.

Two intracellular domains shown in the model and common to P-type ATPases are the aspartyl kinase domain (including the conserved DKTGT sequence which includes the site of aspartyl-phosphorylation) and the phosphatase domain (with TGES), involved in removing the phosphate from the aspartate residue during the reaction and transport cycle. This class of ATPases is called 'P-type' since they are the only transport ATPases that have a covalent phospho-protein intermediate. The CadA cadmium ATPase is one of the few bacterial examples for which direct experimental data showing phosphorylation is available [82]. Tsai *et al* [83] showed directly ATP-dependent transport of cadmium by vesicles containing the CadA ATPase.

CadC, the product of the second gene of this operon, is a DNA-binding transcriptional regulatory repressor [22] and is a member of a new sub-family of metal-binding repressor proteins, with the arsenic system repressor ArsR [29,62,90,92] and the cyanobacterial metallothionein repressor SmtB [72,84]. Recently the first structure for this family of regulatory proteins has been obtained for SmtB [15]. The structure shows the dimeric protein with a 'helix-turn-helix' domain positioned so as to fit into the DNA grove and also with two cations bond per monomer, one in the helix-turn-helix region and the other at the interface between subunits. SmtB is a 'winged helix-turn-helix' protein [15] in that antiparallel beta sheet sequences follow the alpha-helix-turn-alpha-helix common to the larger group of DNA-binding proteins of bacteria and eukaryotes.

Cadmium resistance in Gram-negative soil bacteria results from a three-protein chemiosmotic antiporter

Large plasmids of the soil chemilithotrophic autotroph *Alcaligenes* have numerous heavy metal resistance determinants, including for mercury, chromate resistance, and three related ones for divalent cations called *czc* (for Cd^{2+}, Zn^{2+} and Co^{2+} resistances), *ncc* (for Ni^{2+}, Cd^{2+} and Co^{2+} resistances), and *cnr* (for Co^{2+} and Ni^{2+} resistances). The DNA sequences of *czc* and *cnr* have diverged sufficiently that Southern blot hybridization fails. Nevertheless, the predicted protein amino acid sequences [19,49,50] show closely related systems with basically the same three structural proteins [19,68,72]. Indeed, mutations of the Cnr system give additional Zn^{2+} resistance, showing that the two systems are fundamentally the same. CzcCBA is an efflux pump [49] that functions as a chemiosmotic divalent

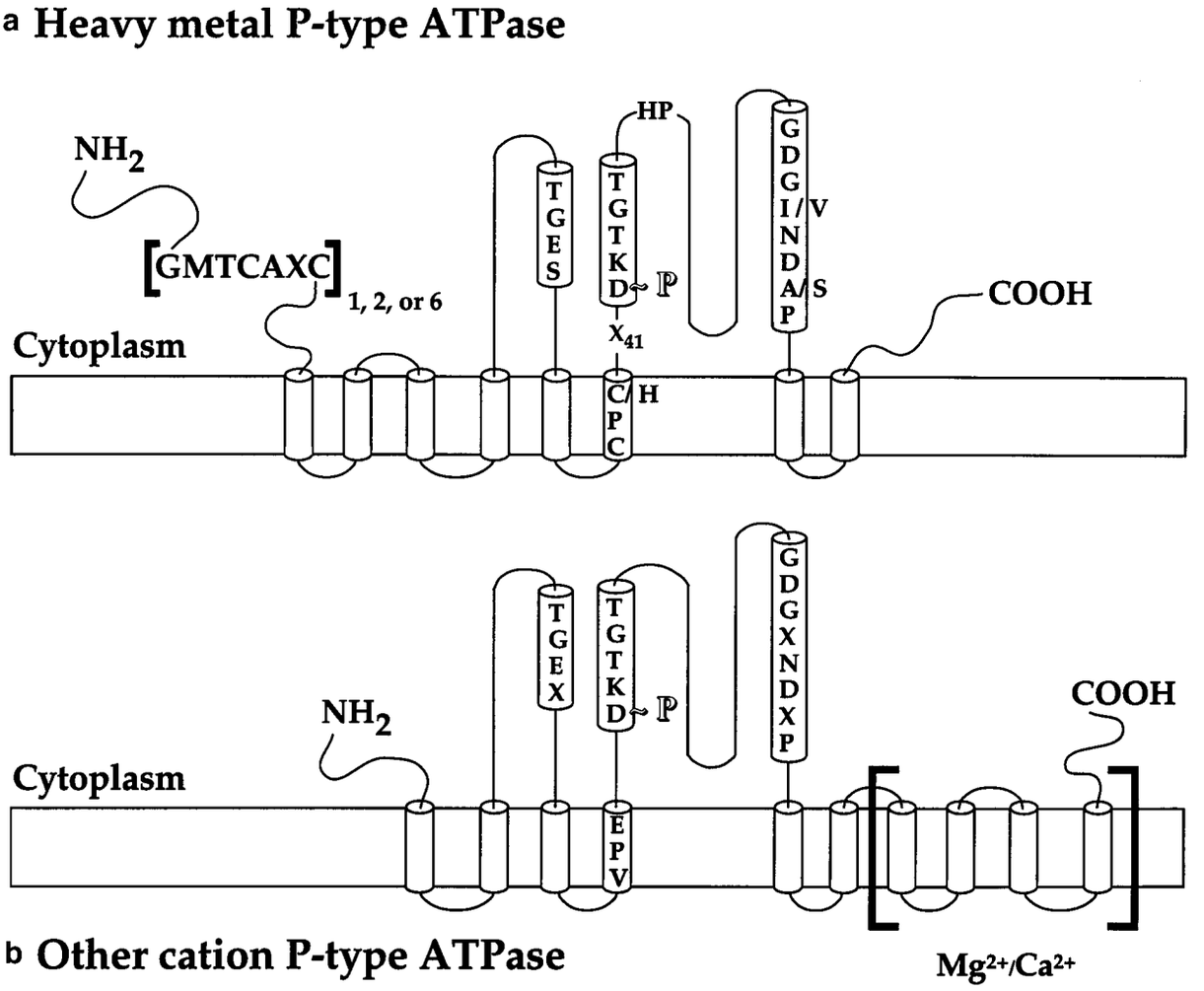

Figure 6 (a) Heavy-metal cation (eg Cd^{2+}) and (b) nutrient cation (eg Ca^{2+} and Mg^{2+}) P-type ATPases. The predicted motifs (cation-binding, phosphatase, membrane channel, and aspartyl kinase) regions are shown. Modified after Ref. [76] and earlier models.

cation/proton antiporter [48,49]. The proteins involved have become the paradigm for a new family of three-component chemiosmotic exporters [19], called CBA systems for the order of transcription of the genes and to contrast them with the ABC ('ATP-binding cassette' multicomponent ATPases). CzcC is thought to be an outer membrane protein [19]. CzcB appears to be a 'membrane fusion protein' that bridges the inner and outer cell membrane of Gram-negative bacteria (References in Refs. [19,31]). And CzcA is the basic inner membrane transport protein of over 1000 amino acids in length. Several regulatory genes are involved, but there is incomplete understanding of their number or functions [19,50]. Two of these, *czcR* and *czcS* encode a pair of cation-sensing sensor kinase (CzcS) and transphosphorylated responder (CzcR) proteins [86], homologous to PcoS and PcoR for plasmid copper resistance.

Chromate resistance and chromate reduction in Gram-negative bacteria

Resistance to chromate governed by bacterial plasmids appears not to involve chromate reduction. Furthermore, it is not clear whether chromate reduction that has been found with several bacterial isolates confers resistance to CrO_4^{2-} [56]. Plasmid-determined chromate resistance results from reduced uptake of CrO_4^{2-} by the resistant cells [55]. The DNA sequences of the *P. aeruginosa* [10] and *A. eutrophus* [47] chromate resistance systems share homologous *chrA* genes, which encode membrane proteins. A third *chrA* gene was found on a plasmid of a cyanobacterium [46], so more examples of chromate resistance operons will probably be found.

Tellurite resistance in Gram-negative bacteria

There are several well-studied and sequenced determinants of plasmid-governed tellurite resistance, but in each case the mechanism of tellurite resistance is unknown [31,88,89]. Tellurite resistance does not involve reduction to black metallic tellurium. This has been a surprise, since tellurite reduction to metallic Te(0) is found, especially when resistance allows cell growth [89]. However, sensitive cells when tested appear able to reduce tellurite to Te(0) as well as resistant cells.

Silver resistance in enteric bacteria

Ag^+ cations are exceedingly toxic to bacteria and have been used to treat drinking water and in hospitals as antiseptics on burnt skin and in inplanted catheters. Bacteria specifically resistant to Ag^+ have periodically been reported from such sources, as well as from mineral ores and the waste water of film processing plants. The genetic basis of plasmid-determined silver resistance is now actively under study (K Matsui, A Gupta and S Silver, in preparation) and Figure 7 represents a summary of current understanding—and the first presentation of as yet unpublished results. It requires 14 kb of DNA to encode the eight genes apparently involved in bacterial silver resistance and the functions of seven of these are diagramed in Figure 7. Two of these, *silS* and *silR* encode another pair of cation-sensing sensor kinase (SilS) and transphosphorylated responder (SilR) proteins (Figure 7), homologous to PcoS/PcoR for copper resistance and CzcS/CzcR for cadmium, zinc and cobalt resistances. As with the *pco* system, *silRS* is followed by *silE*, the determinant of a periplasmic Ag^+-binding protein, homologous to PcoE. However, the remainder of the silver resistance determinant is transcribed in the opposite direction (unlike the situation in *pco*) and encodes both a three-component CBA system, weakly homologous to CzcCBA, and a P-type ATPase that is generally speaking in the family of heavy metal-responding enzymes diagramed in Figure 6a, except for the absence of a CX_2C metal-binding motif. In the same position, a series of histidine residues may be functioning to recognize and function with Ag^+. After cloning and sequencing the first such silver resistance determinant, gene-specific DNA probes were used to show that similar (but not identical) systems occur in a wide range of enteric bacteria from clinical sources, including burn patients, where silver salts were used as antiseptics, and metallic silver catheters (A Gupta *et al*, in preparation). While silver resistance is being studied in clinical isolates, as had been the case for mercury and arsenic resistances in earlier years, this resistance system is expected to be important in film processing processes and for the mining industry, where developing metal-resistant microbes is becoming useful applied microbiology [60]. Charles Thom would have agreed that interesting topics have practical uses. During the next few years, the molecular genetic and biochemical basis for highly specific Ag^+ resistance should be understood in some detail, and comparable systems may be found in other groups of bacteria. It is already clear, however, that reduction of Ag^+ to metallic Ag^0 is not involved.

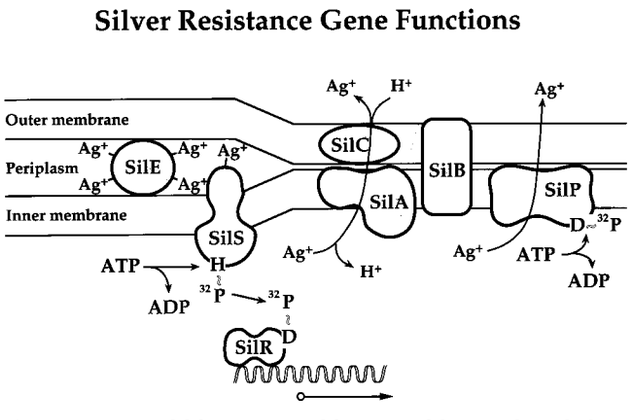

Figure 7 First model for proteins and functions of the new plasmid silver resistance determinant (from S Silver *et al*, in preparation).

Other toxic metal resistances

In addition to the specific resistances discussed above, other resistances are listed in the Figure 2. These have been studied still less and are material for future research. Lead (Pb^{2+}) resistance on plasmids appears to have a different basis in soil *Alcaligenes*, where still another cation-specific P-type ATPase has been found (D van der Lelie, personal communication) and in *Staphylococcus*, where a process involving precipitation in intracellular lead-phosphate granules has been proposed [38]. Plasmid resistance to tributyltin (used as anti-fouling compounds for ship hulls) has been reported [43].

A final comment

This brief summary of how microbial cells cope with the inorganic cations and anions from elements of the Periodic Table is not directly applied microbiology. However, Charles Thom frequently made the point that practical problems were the only ones worth studying and that the distinction between 'basic science' and applied science is artificial and generally harmful to both sides of a continuous spectrum of understanding. The approach here has been from the gene to the physiological process. Such an approach reflects the success of microbial genetics in the last generation: a genetic approach is usually the most powerful. A quite different approach to understanding the biology of inorganic elements is called 'bioinorganic chemistry' (eg Refs [24,40]) and is based more on chemical principles, minimizing the powers of microbiology and genetics. A middle ground is what is needed for fundamental understanding of problems of universal and practical importance. Looking at the absence of overlap between bioinorganic chemistry (eg Refs [24,40] and the approach to understanding here, perhaps this article should be expanded to a comparable monograph to be titled 'Systems of Bioinorganic Genetics!' The need shows how far we still have to go before a satisfactory understanding of the physical principles that allow microbes (and higher living forms) to discriminate between closely related cations and anions, and then to harness specific inorganic ions to precise biological functions.

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The research in our laboratory on these topics has involved the participation of many colleagues and friends (listed as authors below) for 30 years. The reference section is frequently limited to reviews or most recent articles, and apologies are given here for the inability to cite each primary paper for these topics.

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