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MINI-REVIEW

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Microbial heavy-metal resistance

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Abstract We are just beginning to understand the metabolism of heavy metals and to use their metabolic functions in biotechnology, although heavy metals comprise the major part of the elements in the periodic table. Because they can form complex compounds, some heavy metal ions are essential trace elements, but, essential or not, most heavy metals are toxic at higher concentrations. This review describes the workings of known metal-resistance systems in microorganisms. After an account of the basic principles of homoeostasis for all heavy-metal ions, the transport of the 17 most important (heavy metal) elements is compared.

Introduction: heavy-metal toxicity, tolerance and resistance

Heavy metals are metals with a density above 5 g/cm^3, thus the transition elements from V (but not Sc and Ti) to the half-metal As, from Zr (but not Y) to Sb, from La to Po, the lanthanides and the actinides can be referred to as heavy metals. Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 (with As included) are heavy metals (Weast 1984).

Most heavy metals are transition elements with incompletely filled d orbitals. These d orbitals provide heavy-metal cations with the ability to form complex compounds which may or may not be redox-active. Thus, heavy-metal cations play an important role as "trace elements" in sophisticated biochemical reactions. At higher concentrations, however, heavy-metal ions form unspecific complex compounds in the cell, which leads to toxic effects. Some heavy-metal cations, e.g. Hg^{2+}, Cd^{2+} and Ag^{+}, form strong toxic complexes, which makes them too dangerous for any physiological function. Even highly reputable trace elements like Zn^{2+} or Ni^{2+} and especially Cu^{2+} are toxic at higher concentrations. Thus, the intracellular concentration of heavy-metal ions has to be tightly controlled, and heavy-metal resistance is just a specific case of the general demand of every living cell for some heavy-metal homoeostasis system.

To have any physiological or toxic effect, most heavy-metal ions have to enter the cell. At first glance, divalent heavy-metal cations are structurally very similar; the divalent cations Mn^{2+}, Fe^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+} and Zn^{2+} have ionic diameters between 138 pm and 160 pm (Weast 1984), a difference of 14%, and all, of course, carry a double positive charge. Oxyanions like chromate, with four tetrahedrally arranged oxygen atoms and two negative charges, differ mostly in the size of the central ion, so the structure of chromate resembles that of sulfate. The same is true for arsenate and phosphate. Thus, uptake systems for heavy-metal ions have to bind those ions tightly if they want to differentiate between a couple of structurally very similar ions. However, tight binding costs both time and energy.

Most cells solve this problem by using two types of uptake system for heavy-metal ions: one is fast, unspecific and, since it is used by a variety of substrates, constitutively expressed. These fast systems are usually driven only by the chemiosmotic gradient across the cytoplasmic membrane of bacteria. The second type of uptake system has a high substrate specificity, is slower and often uses ATP hydrolysis as the energy source, sometimes in addition to the chemiosmotic gradient, and these expensive uptake systems are only produced by the cell in times of need, starvation or a special metabolic situation; they are inducible (Nies and Silver 1995).

Ni^{2+}, Co^{2+}, Zn^{2+}, and Mn^{2+} are accumulated by the fast and unspecific CorA (metal inorganic transport,

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Table 1 Protein families important for heavy-metal transport. For references see the respective section in the "The microbiologist's walk through the periodic system". *CPM* cytoplasmic membrane, *ABC* ATP-binding cassette (Fath and Kolter 1993), *RND* resistance, nodulation, cell division (Saier et al. 1994; Saier 1994), *CHR* chromate transport (Nies et al. 1998), *MIT* metal inorganic transport (Paulsen et al. 1998), *CDF* cation-diffusion facilitators (Nies and Silver 1995; Paulsen and Saier 1997)

Family	Direction of transport	Energy	Metal ions	Composition
ABC	Uptake	ATP	Mn^{2+}, Zn^{2+}, Ni^{2+}, Fe^{2+}	2 membrane-integral parts[a] + 2 ATPase parts = ABC core + periplasmic binding protein
	Efflux	ATP	–	ABC core + membrane fusion protein and outer membrane factor
P-type[b]	Both	ATP	Mg^{2+}, Mn^{2+}, Ca^{2+}, K^{+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Pb^{2+}, Ag^{+}	1 membrane-bound protein as core
A-type[c]	Efflux	ATP	Arsenite	1 membrane-integral protein + a dimeric ATPase subunit
RND	Efflux	Proton gradient	Co^{2+}, Zn^{2+}, Cd^{2+}, Ni^{2+}, Cu^{2+}?, Ag^{+}?	1 CPM proton/cation antiporter + membrane fusion protein (dimer?) + outer membrane factor: CBA transport systems
HoxN	Uptake	Chemiosmotic	Co^{2+}, Ni^{2+}	Membrane-Integral protein
CHR	Antiport?	Chemiosmotic	Chromate	Membrane-integral protein (ChrA)
MIT	Uptake	Chemiosmotic	Most cations	Membrane-integral protein (CorA)
CDF	Efflux	Chemiosmotic	Zn^{2+}, Cd^{2+}, Co^{2+}, Fe^{2+}?	Membrane-integral protein (CzcD, ZRC1p, ZnT1)

[a] "Parts" are proteins or protein domains, depending on the specific transporter
[b] Fagan and Saier 1994
[c] Saier 1994

MIT, family; Table 1) magnesium uptake system in gram-negative bacteria (Smith and Maguire 1995; Tao et al. 1995), archaea (Smith et al. 1998) and baker's yeast (MacDiarmid and Gardner 1998). Arsenate is transported by the fast Pit (phosphate inorganic transport) system and chromate by the fast sulfate-uptake system (Nies and Silver 1995). In addition (Table 1), there are inducible P-type ATPases for magnesium uptake, ATP-binding cassette (ABC) transporters for Mn^{2+}, Zn^{2+} and Ni^{2+}, slow and specific chemiosmotic transporters of the HoxN family for Ni^{2+} and Co^{2+}, and also ABC transporters for sulfate and phosphate in bacteria (Table 1).

When a cell faces a high concentration of any heavy metal that is accumulated by such an unspecific system, the specific heavy-metal ion is transported into the cytoplasm in spite of its high concentration, because these unspecific transporters are constitutively expressed. Thus, the gate cannot be closed. This "open gate" is the first reason why heavy-metal ions are toxic (Nies and Silver 1995).

Of course, expression of the gene for the fast and unspecific transporter may be diminished by mutation, and the resulting mutants are metal-tolerant. In fact, *corA* mutants were found because they were cobalt-tolerant (Nelson and Kennedy 1971; Park et al. 1976), and *pit* mutants are tolerant to arsenate (Rosen 1996). However, tolerant mutants are less robust than the wild type in a growth medium without the toxic heavy-metal ion, and are thus rapidly overgrown by revertant strains.

Once inside the cell, heavy-metal cations, especially those with high atomic numbers, tend to bind to SH groups, e.g. Hg^{2+}, Cd^{2+} and Ag^{+}. The minimal inhibitory concentration (Table 2) of these metal ions is a function of the complex dissociation constants of the respective sulfides (data not shown). By binding to SH groups, the metals may inhibit the activity of sensitive enzymes. Other heavy-metal cations may interact with physiological ions, Cd^{2+} with Zn^{2+} or Ca^{2+}, Ni^{2+} and Co^{2+} with Fe^{2+}, Zn^{2+} with Mg^{2+} thereby inhibiting the function of the respective physiological cation. Heavy-metal cations may bind to glutathione in gram-negative bacteria and the resulting bisglutathionato complexes tend to react with molecular oxygen to form oxidized bisglutathione (GS-SG) (Kachur et al. 1998), the metal cation and hydrogen peroxide (H_2O_2). Since

Table 2 Toxicity of heavy-metal ions in *Escherichia coli*. The minimal inhibitory concentration (*MIC*) was determined on TRIS-buffered mineral salts medium (Mergeay et al. 1985), starting pH 7.0, containing 2 g sodium gluconate/l as carbon source, and 1 g yeast extract/l to complement *E. coli* auxotrophies. The plates were incubated for 2 days at 30 °C

MIC (mM)	Heavy-metal ions
0.01	Hg^{2+}
0.02	Ag^{+}, Au^{3+}
0.2	CrO_4^{2-}, Pd^{2+}
0.5	Pt^{4+}, Cd^{2+}
1.0	Co^{2+}, Ni^{2+}, Cu^{2+}, Zn^{2+}
2.0	Tl^{+}, UO_2^{2-}, (La^{3+}, Y^{3+}, Sc^{3+})[a], (Ru^{3+}, Al^{3+})[b]
5.0	Pb^{2+}, (Ir^{3+}, Os^{3+}, Sb^{3+}, Sn^{2+}, In^{3+}, Rh^{2+}, Ga^{3+}, Cr^{3+}, V^{3+}, Ti^{3+}, Be^{2+})[b]
10.0	(Cr^{2+})[b]
20.0	Mn^{2+}

[a] Weak acidification of the medium had to be allowed to keep the metal ion in solution
[b] Acidification of the medium had to be allowed to keep the metal ion in solution

the oxidized bisglutathione has to be reduced again in an NADPH-dependent reaction and the metal cations immediately bind another two glutathione molecules, heavy-metal cations cause a considerable oxidative stress. Finally, heavy-metal oxyanions interfere with the metabolism of the structurally related non-metal (chromate with sulfate, arsenate with phosphate) and reduction of the heavy-metal oxyanion leads to the production of radicals, e.g. in case of chromate.

This potential for heavy-metal ion toxicity in connection with the "open gate" situation has forced life in its early evolution to develop metal-ion homoeostasis factors and metal-resistance determinants. Since heavy-metal ions cannot be degraded or modified like toxic organic compounds, there are only three possible mechanisms for a heavy-metal resistance system. First, the accumulation of the respective ion can be diminished by efflux, an active extrusion of the heavy-metal ion from the cell (Nies and Silver 1995). Second, cations, especially the "sulfur lovers", can be segregated into complex compounds by thiol-containing molecules. Third, some metal ions may be reduced to a less toxic oxidation state. Finally, for many metals, resistance and homoeostasis involve a combination of two or three of the basic mechanisms mentioned.

To be detoxified by reduction, the redox potential of a given heavy metal should be between that of the hydrogen/proton couple (−421 mV) and that of the oxygen/hydrogen couple (+808 mV) [calculated from Weast (1984) at 30 °C and pH 7.0], which is the physiological redox range for most aerobic cells. Thus, Hg^{2+} (+430 mV), chromate (+929 mV), arsenate (+139 mV) and Cu^{2+} (−268 mV) may be reduced by the cell, but Zn^{2+} (−1.18 V), Cd^{2+} (−824 mV), Co^{2+} (−701 mV) and Ni^{2+} (−678 mV) may not. A metal compound that can be reduced should be able to diffuse out off the cell or it might re-oxidize itself; however, most reduction products are quite insoluble (Cr^{3+}) or even more toxic (AsO_2^-) than the educts. Thus, if the cell chooses to detoxify such a compound by reduction, an efflux system should be present to export the reduced products. Only in the case of mercury do reducibility and a low vapour pressure of the metallic reduction product fit together; mercury is thus detoxified by reduction of Hg^{2+} to Hg^0 with diffusional loss of the Hg^0.

If a heavy-metal compound cannot be reduced by cellular means or reduction is not desirable, the only choice is between complexation and efflux, or both. However, the cost of complexation is huge compared to efflux if a fast-growing cell is considered: assuming that an aerobic cell detoxifies Cd^{2+} by forming CdS, sulfate has to be taken up (1 ATP), PAPS (Phosphoadenosin-5′-phosphosulfate) has to be formed (3 ATP) and reduced to sulfite (2 electrons lost, which may yield 3 ATP during respiration) and finally sulfide (6 electrons = 9 ATP). This amounts to about 16 ATP for the formation of 1 sulfide, which complexes 1 Cd^{2+}. If glutathione, its derivatives or even a ribosomally synthesized protein like metallothionein is considered, these costs are immense.

The efflux of 1 Cd^{2+} by an efflux system only costs about 1 ATP, but a futile cycle of uptake and efflux may be formed. Complexation would only be "cheaper" than efflux if all the cadmium in the direct environment were complexed by the bacterial population in the end, which is usually not the case. Thus, complexation is only an efficient way of metal detoxification in cells exposed to low concentrations of heavy metals. Since reduction is not possible or may not be sensible as the sole mechanism of detoxification, heavy-metal ions have to be detoxified by efflux, alone or in combination, in any organism growing fast in an environment contaminated with high concentrations of heavy metals. Heavy-metal metabolism is therefore transport metabolism.

Ecology of heavy metals: which heavy metals are biologically important?

Before starting a microbiologist's walk through the periodic system, a few short cuts can be taken: fortunately, not all of the 53 heavy metals have a good or bad biological function. This is simply because some heavy metals are not available to the living cell in the usual ecosystems; they may be present in the earth's crust only in very low amounts, or the ion of a particular heavy metal may not be soluble.

To summarize these two factors, the composition of sea water may be used as a kind of "average environment". Depending on their concentration in sea water (Weast 1984), four classes of heavy metal can be easily differentiated as possible trace elements: frequent elements with concentrations between 100 nM and 1 μM (Fe, Zn, Mo), elements with concentrations between 10 nM and 100 nM (Ni, Cu, As, V, Mn, Sn, U), rare elements (Co, Ce, Ag, Sb) and finally elements just below the 1 nM level (Cd, Cr, W, Ga, Zr, Th, Hg, Pb). The remaining 31 elements, e.g. gold, present at 55.8 pM in sea water, are not likely to become trace elements; if an element has a concentration of 1 nM in an ecosystem containing a bacterial population of 10^9/ml, each cell would receive only 600 ions. Thus, elements at an average concentration smaller than 1 nM are very unlikely ever to be useful or toxic, and it would not pay to harbour metabolic genes for these metals.

Which of these 22 heavy metals is of some biological importance is simply based on the solubility function under physiological conditions and the toxicity, which involves its affinity to sulfur plus interaction with macrobioelements. Because of the low solubility of the tri- or tetravalent cations (Weast 1984), Sn, Ce, Ga, Zr and Th have no biological influence. Of the remaining 17 heavy metals, Fe, Mo and Mn are important trace elements with low toxicity and Zn, Ni, Cu, V, Co, W and Cr are toxic elements with high to moderate importance as trace elements; As, Ag, Sb, Cd, Hg, Pb and U have limited beneficial function, but have to be considered as toxins (Table 2). Thus, these 17 heavy metals will be discussed.

A microbiologist's walk through the periodic system of the elements

Vanadium (V): mostly toxic

Vanadium mostly exists as V(V), the trivalent oxyanion vanadate. Vanadate is structurally similar to phosphate and is thus known as an inhibitor of ATPases, and it may be taken up by phosphate-uptake systems (Mahanty et al. 1991; Rehder 1992). Because of its toxicity, the beneficial use of vanadate is an exception: bacteria like *Azotobacter chroococcum* are able to form a vanadate-dependent nitrogenase for nitrogen fixation if molybdate is not present in the ecosystem (Chatterjee et al. 1997; Eady 1995; Joerger and Bishop 1988; Pau 1989; Thiel 1996). Further trace-element functions are obscure (Nielsen 1991), but vanadate can be used as an electron acceptor for anaerobic respiration (Lyalikova and Yurkova 1992; Yurkova and Lyalikova 1990). Physiological work on vanadate resistance has only been done in *Saccharomyces cerevisiae* (Nakamura et al. 1995). *Sulfolobus* has a minimal inhibitory concentration of 20 mM vanadate (Grogan 1989). However, the detailed mechanism of vanadate resistance remains elusive.

Chromium (Cr): beneficial only as an exception

Chromium mainly occurs as Cr(VI) in the divalent oxyanion chromate and as Cr(III), the trivalent cation. Reduction/oxidation reactions between the two states are thermodynamically possible under physiological conditions (Weast 1984), thus chromate and Cr^{3+} are both biologically important ions. Chromate is more toxic than Cr^{3+} (Table 2), so beneficial functions of chromium can only be performed by Cr^{3+}. In man, the chromium cation binds to a low-molecular-mass binding substance, a small polypeptide, at a ratio of 4 Cr/peptide (Davis and Vincent 1997b). The resulting Cr-containing peptide is able to activate specifically the insulin receptor tyrosine kinase (Davis and Vincent 1997a). These new findings explain why chromium starvation in man leads to reduced glucose tolerance with a physiological condition similar to diabetes. Chromate, on the other hand, is toxic, carcinogenic and allergenic (mason's allergy) to man (Costa 1997).

In microorganisms, no beneficial influence of chromium was found. Chromate enters the cell of *Ralstonia* sp. strain CH34 (formerly *Alcaligenes eutrophus*) (Brim et al. 1999) by the sulfate-uptake system (Nies and Silver 1989a), which is normal for many microorganisms (Nies and Silver 1995). Chromate resistance is probably based on an interaction of chromate reduction and chromate efflux. The first bacterium found to be resistant to chromate, *Pseudomonas fluorescens* strain LB300, was shown to reduce chromate (Bopp and Ehrlich 1988), and a broad variety of bacteria able to reduce chromate have since been found (Cervantes and Silver 1992). Chromate resistance was then mainly thought to be based on chromate efflux; however, recent data for *Ralstonia* sp. CH34 suggest that both processes, efflux and reduction, are involved (Peitzsch et al. 1998).

Since chromate resistance in *Ralstonia* sp. CH34 is inducible by chromate, a biological chromate sensor has been developed on the basis of the luciferase system (Peitzsch et al. 1998). Any chromate remediation of soils or water that uses chromate-reducing bacteria has to take account of the fact that any chromate remaining in an ecosystem may be rapidly oxidized again (James et al. 1997), thus, any detoxification would not be permanent. Since chromium may be present in at least two oxidation states in an ecosystem (Aide and Cummings 1997; Armienta et al. 1996; Baron et al. 1996; Palmer and Wittbrodt 1991; Rinehart et al. 1997), plants may be better suited for biological leaching than bacteria (Kleiman and Cogliatti 1997). Because chromate was intensively used in tanneries, soils with quite high chromate contents (several grams of chromate per kilogram of soil) are "available" (Snyder et al. 1997), and an inexpensive bioremediation process may be interesting from a commercial point of view. Chromate, however, is immediately reduced to Cr^{3+} in the roots, which is rarely transported further into the shoots (Zayed et al. 1998). This makes phytoremediation of chromate a complicated process, and further research, on plants as well as on the physiology of chromium metabolism in bacteria, is required to develop a functional system for chromium detoxification.

Manganese (Mn): essential for oxygen production during photosynthesis

Manganese exists in various oxidation states; from Mn(II) to Mn(VII) every state is possible with the Mn^{2+} cation being the predominant form. Therefore, it seems logical that manganese is used by bacteria as an electron acceptor in anaerobic respiration processes (Langenhoff et al. 1997). The toxicity of manganese is very low (Table 2), but it has been shown to be toxic to the central nervous system (Ingersoll et al. 1995).

The power of manganese, and of all heavy-metal cations following manganese in the first transition group of the periodic system, lies in their ability to form complex compounds. Manganese complexes in the low-spin form are relatively inert as redox compounds, and manganese may thus be a substitute for magnesium in general. In a high-spin complex, manganese functions as a kind of "electron buffer". Its most important function is the catalysis of water cleavage during oxygenic photosynthesis (Abramowicz and Dismukes 1984). In photosystem II, responsible for this process, four manganese ions are bound in a tetranuclear complex (Brudvig 1987), together with calcium and chlorine (Yachandra et al. 1993). In this complex, manganese may alternate between the Mn(III) and Mn(IV) oxidation state, with Mn(II) probably also being present in the complex

(Ahrling et al. 1997). Ultimately driven by light energy, water is oxidized to molecular oxygen in a five-step cyclic process (Dekker and van Gorkom 1987). The manganese ions are bound to histidine (Tang et al. 1994) and are located close to a tyrosine radical residue, which may be required for the abstraction of protons from water (Gilchrist et al. 1995; Hoganson and Babcock 1997; Noguchi et al. 1997).

Mn^{2+} is taken up into *Ralstonia* sp. by the Mg-uptake system (Nies and Silver 1989a). In *Salmonella typhimurium*, heavy-metal cations are mainly accumulated by the fast and unspecific CorA system (MIT family, Table 1) and the inducible, slower, and more specific P-type ATPases MgtA and MgtB (Smith et al. 1993; Snavely et al. 1989a, b, 1991); all of them are magnesium uptake systems. These systems transport Mn^{2+} too, but under housekeeping conditions bacterial cells may be mainly supplied with Mn^{2+} by the CorA system (Smith et al. 1998; Smith and Maguire 1995). MIT systems also exist in *S. cerevisiae* (MacDiarmid and Gardner 1998), besides other manganese transport systems (Farcasanu et al. 1998; Paulsen et al. 1998). Under manganese starvation, Mn^{2+} uptake in bacteria may be mainly catalysed by transporters of the ABC (Table 1) family (Bartsevich and Pakrasi 1995, 1996; Dintilhac et al. 1997; Kolenbrander et al. 1998).

Iron (Fe) is biologically the most important heavy metal cation

Iron is the only macro-bioelement of the heavy metals. The dissociation constant of iron hydroxides is 1.8×10^{-15} for Fe(II) and 6×10^{-38} for Fe(III) (Weast 1984). Thus, Fe(III) became almost unavailable with the accumulation of oxygen on earth. The solution of this ''iron crisis'' was the evolution of specific iron-binding complex compounds which bind Fe(III) and shuttle it to the cell: the siderophores (Braun et al. 1998). Because of its low solubility, Fe^{3+} is not toxic to aerobic bacteria.

In addition to the siderophore-mediated uptake of Fe^{3+}, Fe^{2+} is also transported into bacterial cells. Fe^{2+} is similar in ionic diameter and charge to Mg^{2+}, thus, it is also accumulated by the fast and unspecific CorA magnesium transport system (MIT, Table 1) in *Escherichia coli* (Hantke 1997). However, *E. coli* possesses in addition a high-affinity ABC transport system (Table 1) for ferrous iron encoded by *feoABC* (Kammler et al. 1993). The presence of ferrous iron uptake systems seems to be important for bacteria that live, mostly or occasionally, under anaerobic conditions. Because anaerobic bacteria may use Fe^{3+} as an electron acceptor (Ehrenreich and Widdel 1994), Fe^{2+} should be the main ionic form of this metal under anaerobic conditions.

S. cerevisiae, which lives aerobically or anaerobically, uses a complicated mechanism for iron uptake: Fe^{3+} is first reduced by the ferric reductases, Fre1p to Fre6p. These genes are transcriptionally induced by iron depletion (Georgatsou et al. 1997; Martins et al. 1998). In *S. cerevisiae* and *Schizosaccharomyces pombe* (Askwith and Kaplan 1997), Fe^{2+} is then taken up by a copper-dependent ferrous iron oxidase, Fet3p, and the permease Ftr1p or related proteins (Dancis et al. 1994a, b). In addition, *S. cerevisiae* harbours a low-affinity Fe^{2+} uptake system, Fet4p (Liu et al. 1997; Paulsen et al. 1998). Fe^{2+} is transported into the mitochondria by MNT1p and MNT2p (Paulsen et al. 1998) [also called MFT1p and MFT2p (Li and Kaplan 1997)], which may belong to of the cation diffusion facilitator (CDF) transport protein family. From the many connections between yeasts, plants and man (Askwith and Kaplan 1998; Eide 1997), iron transport in eukaryotes becomes clear: it is probably a combination between low-affinity uptake of Fe^{2+} backed up by high-affinity uptake involving copper-dependent oxidation of Fe^{2+} to Fe^{3+}.

Cobalt (Co): always important

Cobalt is found mainly in the Co^{2+} form, Co^{3+} is only stable in complex compounds. Co^{2+} is of medium toxicity (Table 2), but cobalt dust may cause lung diseases (Nemery et al. 1994). Cobalt occurs mainly in the cofactor B_{12}, which mostly catalyses C—C, C—O and C—N rearrangements. In addition, a new class of cobalt-containing enzymes, nitrile hydratases, has been recently described (Kobayashi and Shimizu 1998).

Co^{2+} is rapidly accumulated by the CorA system in most bacterial cells (Smith et al. 1993; Snavely et al. 1989a, b, 1991). No inducible ATP-driven uptake system has yet been identified that is induced when the cobalt concentration is too low, but a system related to the nickel transporter HoxN from *Ralstonia eutropha* was found in *Rhodococcus rhodochrous* (Komeda et al. 1997), a bacterium containing a nitrile hydratase. Thus, this HoxN homologue seems to supply Co^{2+} for the production of a non-B_{12}-cobalt protein.

Resistance to cobalt in gram-negative bacteria is based on transenvelope efflux driven by a resistance, nodulation, cell division (RND) (Table 1) transporter. Cobalt resistance seems always to be the by-product of resistance to another heavy metal, either nickel (Liesegang et al. 1993; Schmidt and Schlegel 1994) or zinc (Nies et al. 1987). Members of the CDF protein family (Table 1) have also been found to transport cobalt. The COT1p protein from *S. cerevisiae* transports Co^{2+} across a mitochondrial membrane (Conklin et al. 1994, 1992) and the ZntA protein brings about Co^{2+} efflux in the gram-positive bacterium *Staphylococcus aureus* (Xiong and Jayaswal 1998). Thus, cobalt is taken up by CorA transporters or exceptionally by a HoxN-type transporter. Co^{2+} is detoxified by RND-driven systems in gram-negative bacteria and by CDF transporters in eukaryotes and gram-positive bacteria.

Nickel (Ni): used only for a few important reactions

Free nickel occurs mostly in the Ni^{2+} cationic form; Ni^{3+} is even more unstable than Co^{3+}. Nickel toxicity is comparable to that of cobalt (Table 2), but its toxic effect on man is better documented. Nickel allergy (contact dermatitis), especially to cheap jewellery, is very common; up to 20% of the population in industrially developed countries have positive results in epicutaneous testing (Savolainen 1996).

Nickel-mediated catalysis is the catalysis of complex rearrangements; small molecules are bound to the cation and split or, vice versa, two small molecules or atoms are fused. The best known examples for nickel catalysis are NiFe hydrogenases, which split molecular hydrogen into protons and electrons, urease, which splits urea into carbon dioxide and ammonia, cofactor F_{430} (Thauer and Bonacker 1994) in methanogenic bacteria, which releases methane from a methyl group, and the acetyl-SCoA synthase in anaerobic bacteria where nickel accepts a methyl group from B_{12} and fuses it together with CO and HSCoA to acetyl-SCoA (Goubeaud et al. 1997; Hausinger 1987; Thauer et al. 1983, 1980).

In the best-known nickel-containing enzymes, hydrogenase, urease and CO dehydrogenase, nickel is bound in the active site mainly to cysteine or histidine. In all three enzymes, nickel is added to the polypeptide by a complicated reaction involving GTPases (Maier et al. 1993). In addition, C-terminal processing of the pre-protein is required to form the mature enzyme (Cheesman et al. 1989; Gollin et al. 1992; Mulrooney and Hausinger 1990), as well as chaperones. The UreE protein, which binds six nickel cations, functions as the nickel donor for urease (Lee et al. 1993), and UreD as the chaperone (Park et al. 1994). The nickel donor HypB for hydrogenase binds four to nine Ni atoms (Rey et al. 1994) and is also a GTPase (Fu et al. 1995). The proteins involved in nickel incorporation into the CO dehydrogenase are homologous to the helper proteins for hydrogenase and urease (Kerby et al. 1997; Watt and Ludden 1998). A novel Ni-containing protein is a superoxide dismutase, and synthesis of this protein seems also to involve protein processing (Kim et al. 1998).

Nickel enters the cell (Fig. 1A) mainly by the CorA system, in bacteria and *S. cerevisiae* (Hmiel et al. 1989; MacDiarmid and Gardner 1998; Snavely et al. 1989a, b). An additional nickel transporter, part of the hydrogenase gene cluster, was identified in *R. eutropha* (Eberz et al. 1989; Eitinger and Friedrich 1991; Eitinger et al. 1997) and found to be an archetype of the new HoxN class of transport proteins. Uptake of nickel [and cobalt in the related protein (Komeda et al. 1997)] is probably driven by the chemiosmotic gradient. For hydrogenase formation in *E. coli*, nickel is supplied by an ABC transporter and a periplasmic nickel-binding protein (de Pina et al. 1995; Navarro et al. 1993; Wu et al. 1991). In its natural environment, the gut, the nickel concentration may be too small to allow sufficient nickel uptake by HoxN-type transporters, which are driven only by the chemiosmotic gradient.

Nickel is detoxified by sequestration and/or transport. It is bound to polyphosphate in *S. aureus* (Gonzalez and Jensen 1998) and to free histidine in nickel-hyperaccumulating plants (Kramer et al. 1996). In *S. cerevisiae*, nickel is disposed of and probably bound to histidine in the vacuole (Joho et al. 1992). The transport into the vacuoles requires a proton-pumping ATPase (Nishimura et al. 1998); thus, this kind of nickel transport may also be driven by a chemiosmotic gradient. Other yeasts and fungi probably detoxify nickel by similar mechanisms and also by mutation of the CorA uptake system (Joho et al. 1995; Ross 1995).

The best-known nickel resistance in bacteria, in *Ralstonia* sp. CH34 and related bacteria, is based on nickel efflux driven by a RND transporter (Fig. 1A). Two systems have been described, a nickel/cobalt resistance Cnr (Liesegang et al. 1993) and a nickel/cobalt/cadmium resistance Ncc (Schmidt and Schlegel 1994). Both are closely related to the cobalt/zinc/cadmium resistance system Czc from strain CH34, which will be described in the zinc section.

Nickel has an important function in the pathogenicity of *Helicobacter pylori*, a gram-negative bacterium causing gastritis and peptic ulcer disease in humans. For the colonization of the gastric mucosa, *H. pylori* needs to produce urease to deal with the acidic environment by producing ammonia from urea (Mobley et al. 1995b). Urease production and function depend on the availability of nickel (Evans et al. 1991; Hawtin et al. 1991; Hu and Mobley 1993; Mobley 1996; Mobley et al. 1995a). Thus, *H. pylori* has an extensive array of nickel transport proteins: Ni^{2+} is accumulated by NixA of the HoxN family (Bauerfeind et al. 1996; Fulkerson et al. 1998) and an ABC uptake system. It is specifically bound by heat-shock proteins (Amini et al. 1996; Gilbert et al. 1995; Kansau and Labigne 1996; Suerbaum et al. 1994). *H. pylori* harbours the genes for at least three RND transporters, which may drive Cnr-related nickel-efflux systems (Tomb et al. 1997). Moreover, a P-type ATPase (ATPase 439) was recently described, which binds Ni^{2+}, Cu^{2+} and Co^{2+} to its amino terminus (Melchers et al. 1998). This may be the first example of a nickel P-type ATPase in bacteria. Including CorA, *H. pylori* contains all nickel transport systems known for bacteria today (Fig. 1).

Copper (Cu): a sword with two edges

The electrochemical potential of Cu^{2+}/Cu^{+} is −268 mV, well within the physiological range. Copper easily interacts with radicals, best with molecular oxygen. Its radical character makes copper very toxic (Table 2), and many organisms are more sensitive to copper (Gordon et al. 1994) than *E. coli*. Copper toxicity is based on the production of hydroperoxide radicals (Rodriguez Montelongo et al. 1993) and on interaction with the cell

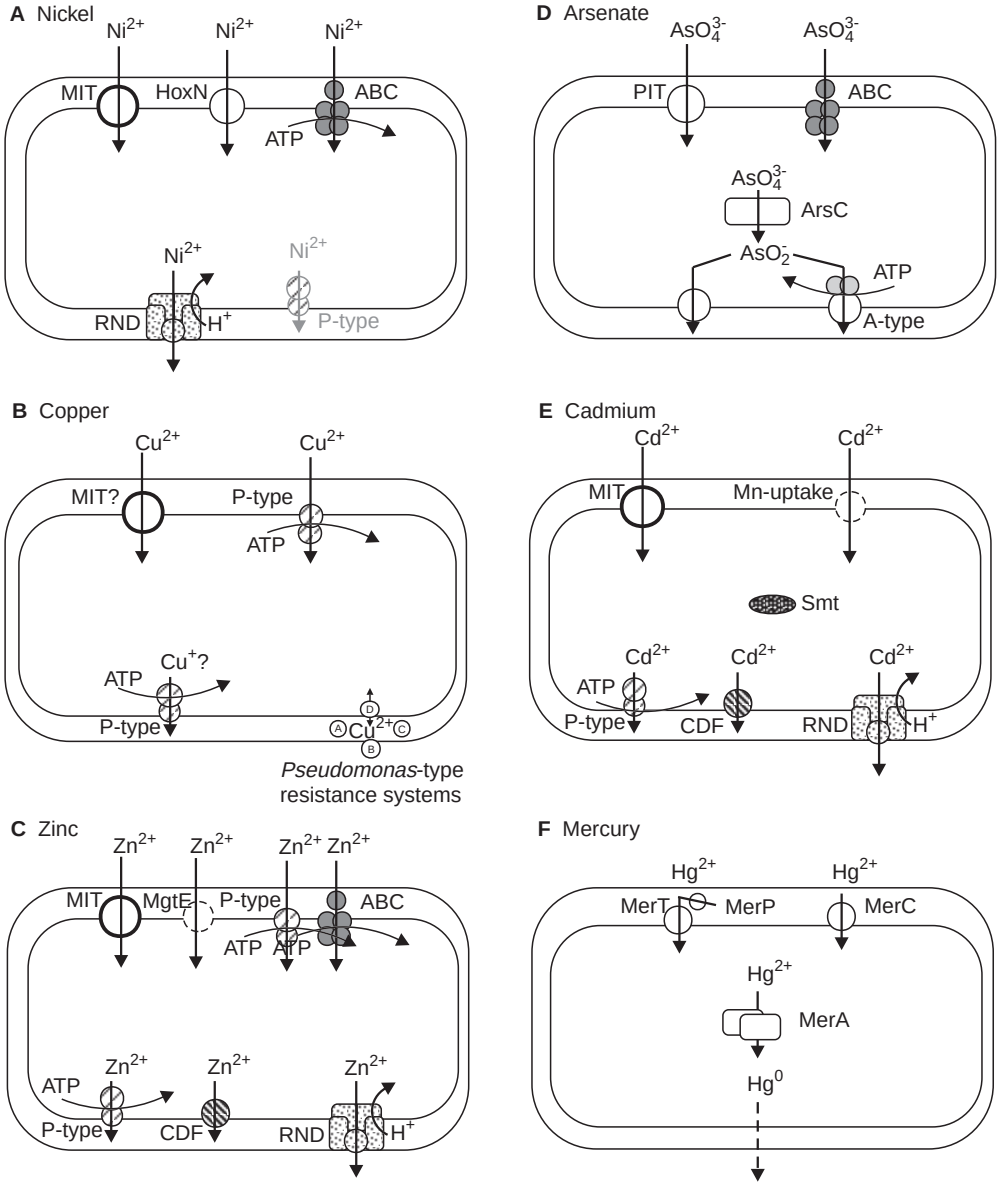

Fig. 1A–F Protein families involved in bacterial heavy-metal metabolism. **A** Ni^{2+} is accumulated by the fast and unspecific CorA (metal transport system, *MIT*) Mg^{2+} transport system. Highly specific nickel transporters are either HoxN chemiosmotic transporters or ATP-binding cassette (*ABC*) uptake transporters, which use a periplasmic nickel-binding protein, depending on the bacterial species. Characterized nickel resistance systems are based on inducible, resistance-, nodulation-, cell division (*RND*)-driven transenvelope transporters. Moreover, a nickel-efflux P-type ATPase (drawn in *grey*) may exist in *Helicobacter pylori*. **B** Cu^{2+} is possibly accumulated by the CorA-Mg^{2+} transporter, and additionally by P-type ATPases under copper starvation (shown in *Enterococcus hirae*). The mechanism of resistance systems similar to the *Pseudomonas* Cop system is still elusive but, in gram-positive bacteria, P-type ATPases seem to detoxify copper via efflux. The copper-resistance systems of the *Pseudomonas* type usually encode four proteins (circles with *A*, *B*, *C*, or *D*), which bind copper in the periplasm or close to the outer membrane. **C** Zn^{2+} is accumulated by the fast and unspecific CorA (MIT) Mg^{2+} transport system in some bacterial species, and by the fast and unspecific MgtE system in others. Inducible, high-affinity ABC transporters supply zinc in times of need. P-type ATPases may transport zinc in both directions, bringing about its uptake as a by-product of Mg^{2+}-uptake again, and its efflux as detoxification. Slow efflux is catalysed by cation-diffusion facilitator (*CDF*) transporters, high-efficiency transenvelope efflux by inducible RND-driven transporters like Czc. **D** Arsenate is accumulated by the constitutive, fast and unspecific Pit (phosphate inorganic transport) and the phosphate-inducible Pst (phosphate-specific transport) systems. Inside the cell, it is reduced by ArsC to arsenite, which is removed from the cell by ArsB, either acting alone or together with the A-type ATPase ArsA. **E** Magnesium (MIT) and/or manganese uptake systems are responsible for the uptake of Cd^{2+}. Only in cyanobacteria have metallothionein-like proteins been characterized (Smt). Efflux is carried out in gram-positive bacteria by P-type ATPases; in gram-negative bacteria it takes the form of RND-driven transenvelope transport, and possibly also carried out by CDF transporters. **F** For mercury, the resistance determinants encode the transport systems. MerT interacts with a periplasmic mercury-binding protein, MerP. Transport by MerC may be in addition to that by MerT or may substitute for MerT transport, depending on the respective resistance determinant. Inside the cell, Hg^{2+} is reduced to metallic mercury, which diffuses out of the cell and its environment

membrane (Suwalsky et al. 1998). Every person in the world may have a contact with copper in coins daily; however, one has to digest about 275 coins for a lethal effect (Yelin et al. 1987), as has been shown in the case of a mentally disturbed individual. This person died from copper intoxication following a massive ingestion of coins (Yelin et al. 1987).

Besides copper/zinc superoxide dismutases, the most important function of copper is in the cytochrome *c* oxidase and related enzymes, which are oxygen-dependent terminal oxidases in the respiratory chain of many organisms. Two copper centres exist in the cytochrome *c* oxidase (Iwata et al. 1995) and they have different roles in the catalytic cycle. The Cu_A center is responsible for the uptake of electrons from the soluble cytochrome *c* and for delivery to the haem aa_3/Cu_B complex, which finally reduces molecular oxygen to water (Ostermeier and Michel 1997); the resulting energy is used to pump protons across the cytoplasmic membrane (Michel et al. 1998).

Plasmid-encoded copper resistance in *E. coli* strongly interacts with chromosomally encoded functions (Fong et al. 1995; Gupta et al. 1995, 1997; Rogers et al. 1991), and the actual mechanism may depend on the growth phase (Brown et al. 1995). Although the copper resistance determinants were shown to be homologous in *E. coli* and *Pseudomonas* species, the phenotype of the two copper-resistant bacteria is different. While *E. coli* remains colourless, resistant *Pseudomonas* strains turn blue on high-copper-containing media because copper is accumulated in the periplasm and outer membrane (Cooksey 1993, 1994) (Fig. 1B). The periplasmic CopA protein shows conservation of several predicted copper-binding sites. In addition, the CopC and CopD proteins seem to catalyse copper uptake into the cytoplasm. Related copper-resistance determinants were found in various *Pseudomonas* (Lin and Olson 1995; Vargas et al. 1995) strains and in *Xanthomonas campestris* (Lee et al. 1994).

In the gram-positive bacterium *Enterococcus hirae*, copper metabolism seems to be much clearer than in gram-negative bacteria (Fig. 1B). *E. hirae* contains a *cop* operon with two structural genes, both encoding a P-type ATPase. While CopA is probably responsible for copper uptake and copper nutrition, CopB (35% identical to CopA) is responsible for copper efflux and detoxification (Odermatt et al. 1992, 1993). Both proteins seem to transport silver as well as copper (Odermatt et al. 1994). Obviously, monovalent cations are being transported (Solioz and Odermatt 1995).

Copper-transporting P-type ATPases have been found in a variety of organisms, in cyanobacteria (Kanamaru et al. 1995; Phung et al. 1994) and in eukaryotes; however, in *S. cerevisiae*, the copper P-type ATPase does not transport copper across the cytoplasmic membrane (Fig. 2A). For uptake into the yeast cell, Cu^{2+} is first reduced by the iron/copper-specific reductases FRE1p, FRE2p and FRE7p to Cu^{+} (Georgatsou et al. 1997; Hassett and Kosman 1995; Martins et al.

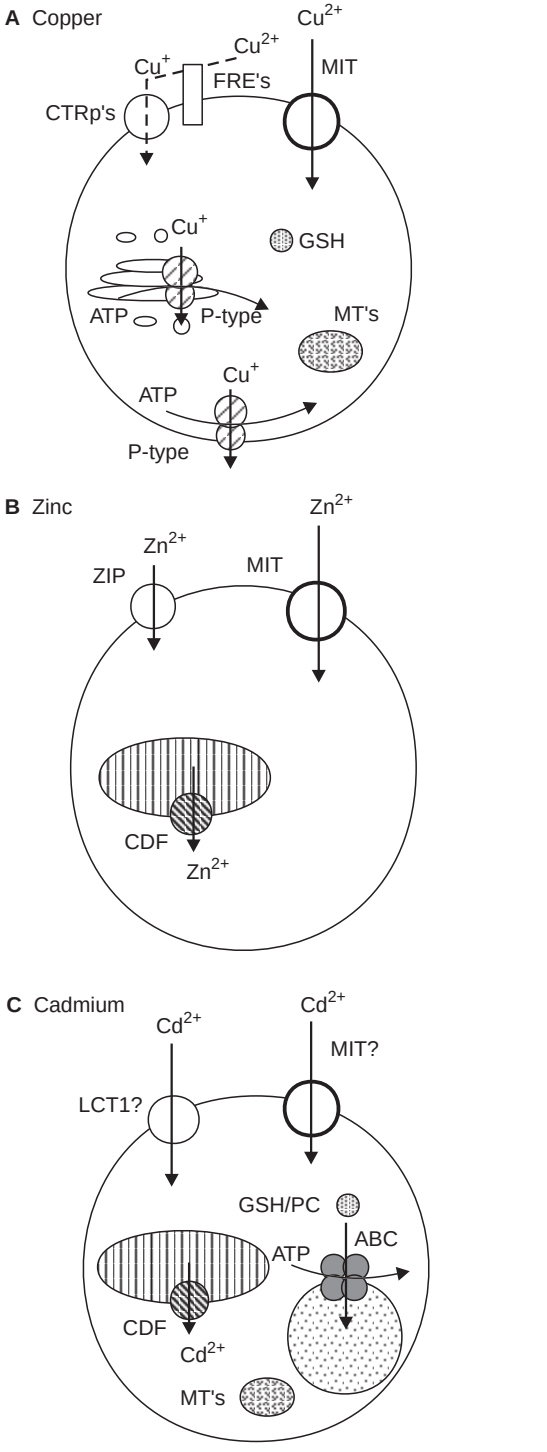

Fig. 2A–C Protein families involved in heavy-metal metabolism in yeast. **A** In *Saccharomyces cerevisiae*, a MIT system takes up Cu^{2+}, while CTRp systems transport Cu^{+}, which has been previously reduced by FREp systems. Copper is bound to glutathione (*GSH*) and metallothioneins (*MT's*). P-type ATPases transport copper into the trans-Golgi system, and may detoxify copper by efflux in mammalian cells. **B** Zinc is taken up by ZIP and MIT transporters, and CDF proteins may detoxify the mitochondrion. **C** MIT- and possibly LCT1-like transporters take up the toxic heavy-metal cation. It is complexed by metallothioneins (*MT*) and glutathione/phytochelatin (*GSH*/*PC*), and the resulting bisglutathionato complexes (or PC complexes) are sequestered into the vacuole by ABC transporters. CDF proteins may protect the mitochondrion

1998), which is transported into the cell by the CTR1p transporter (Dancis et al. 1994a, b; Hassett and Kosman 1995). CTR1p is a novel protein with two related putative copper transporters (CTR2p, CTR3p) in yeast (Paulsen et al. 1998) and a homologue in man (Zhou and Gitschier 1997). In addition, Cu^{2+} is accumulated by the CorA-related transporters ALR1p and ALR2p (Dancis et al. 1994a, b; 1994; Hassett and Kosman 1995).

Inside the yeast cell, copper may be bound by various compounds, and a copper-bisglutathionato complex is likely to be formed. The metallothioneins of yeast, CUP1p and CRS5p (Presta and Stillman 1997), probably store copper. For synthesis of cytochrome *c* oxidase, copper is delivered into the mitochondria by COX1p (Amaravadi et al. 1997; Beers et al. 1997; Glerum et al. 1996). ATX1p, CCSp and the copper P-type ATPase CCC2p are required for copper insertion into proteins of the trans-Golgi network (Casareno et al. 1998; Lin and Culotta 1995; Lin et al. 1997; Yuan et al. 1997, 1995).

The progress of understanding copper homoeostasis in yeast also sheds some light on copper homoeostasis in general (Askwith and Kaplan 1998). *E. coli* also harbours a P-type ATPase, probably required for copper homoeostasis (AtcU, gb 1786691), besides the plasmid-mediated copper-resistance determinant, which is homologous to the *Pseudomonas* system. P-type ATPases also seem to control copper flow in *H. pylori* (Ge et al. 1995) and *Listeria monocytogenes* (Francis and Thomas 1997), two pathogens. In man, defects in the function or expression of copper-transporting P-type ATPases are responsible for two hereditary diseases, Menke's and Wilson's. As in yeast, the two proteins reside in the trans-Golgi network at low copper concentrations, but appear in the cytoplasm and cytoplasmic membrane at higher concentrations (Dierick et al. 1997; Francis et al. 1998; LaFontaine et al. 1998; Vulpe and Packman 1995). Obviously, although alternative splicing of the Menke's gene and protein isoforms seems to exist, the protein itself is reversibly transported, and this transport may be regulated by copper (Petris et al. 1996). In addition to the copper P-type ATPase in man, more genes for homologous P-type ATPases have been identified in mouse, rat, and *Caenorhabditis elegans* (Koizumi et al. 1998; Schilsky et al. 1998; Yoshimizu et al. 1998). Thus, the copper-dependent transport of the P-type ATPase may occur in all eukaryotes.

No life without zinc (Zn)

Zinc occurs exclusively as the divalent cation Zn^{2+}. With its completely filled d orbitals, the zinc cation is not able to undergo redox changes under biological conditions. It is used to complex polypeptide chains, for example, when redox reactions are not desired, and, as a Lewis base, mainly to activate water (Coleman 1998). Zinc is a component in such a variety of enzymes and DNA-binding proteins, such as zinc-finger proteins, which also exist in bacteria (Chou et al. 1998), that life seems not to be possible without this redox-inactive former of tight complexes.

The toxicity of zinc to *E. coli* is similar to the toxicity of copper, nickel and cobalt (Table 2). Zinc toxicity in man may be based on zinc-induced copper deficiency (Fosmire 1990). Zinc is less toxic than copper, in a mentally disturbed human, 461 zinc-containing coins were required for a lethal effect (Bennett et al. 1997). Zinc may be complexed by various cellular components (Daniels et al. 1998; DiazCruz et al. 1998; Jiang et al. 1998; Palmiter 1998), and is transported by members of a variety of protein families (Fig. 1C). Unspecific and fast uptake of Zn^{2+} is mediated by Mg^{2+} transport systems, as shown in *Ralstonia* sp. CH34 (Nies and Silver 1989a). Three transporter groups contribute to the observed zinc transport by those systems: the CorA MIT transporter transports zinc in *S. cerevisiae* (MacDiarmid and Gardner 1998), and CorA has been shown to be present in archaea and many bacteria (Smith et al. 1998; Smith and Maguire 1995), but magnesium transport by CorA was not inhibited by Zn^{2+} (Snavely et al. 1989a, b). A second type of potential chemiosmotically driven transporter forms the MgtE family (Smith et al. 1995), which also seems to transport zinc. This protein is present in *Providencia stuartii* and a few other gram-negative and gram-positive bacteria; however, it is not as broadly distributed as CorA (Townsend et al. 1995).

The third magnesium/zinc transporter is MgtA from *S. typhimurium*, a P-type ATPase that may transport zinc better than magnesium (Snavely et al. 1989a, b; Townsend et al. 1995). MgtA is regulated by magnesium starvation (Tao et al. 1998, 1995), and zinc may interfere with this process, which is at least partially dependent on the PhoPQ two-component regulatory system. However, the MgtA P-type ATPase is not the inducible high-specificity uptake system for zinc. A periplasmic zinc-binding protein was found in *Haemophilus influenzae* to be important for zinc uptake (Lu et al. 1997), and ABC transporters (or the evidence for such transporters) were found in *Streptococcus pneumoniae*, *Streptococcus gordonii*, and *E. coli* (Dintilhac et al. 1997; Kolenbrander et al. 1998; Patzer and Hantke 1998). The *E. coli* transporter responds to zinc deficiency and is regulated by Zur, which is homologous to the Fur main iron regulator in bacteria (Patzer and Hantke 1998).

In addition to transport by the CorA-related ALR1p and ALR2p proteins, uptake of zinc into *S. cerevisiae* is mediated by ZRT1p high-affinity and ZRT2p low-affinity transporters of the ZIP family (Paulsen et al. 1998; Zhao and Eide 1996a, b). The related proteins ZIP1, ZIP2, ZIP3 and may be even ZIP4 have now also been found in *Arabidopsis thaliana* (Grotz et al. 1998). Since the ZIP family seems to be present in plants, protozoa, fungi, invertebrates and vertebrates (Fox and Guerinot 1998), uptake of zinc should follow the same pattern in all eukaryotes.

Two systems are used for zinc detoxification in bacteria, P-type efflux ATPases and RND-driven trans-

porters (Fig. 1C). In *E. coli* (Beard et al. 1997; Rensing et al. 1997b) and in the cyanobacterium *Synechocystis* (Thelwell et al. 1998), the ZntA or the ZiaA P-type ATPase respectively may be responsible for zinc efflux. Moreover, P-type ATPases mediating cadmium resistance also bring about zinc efflux in most cases.

While the P-type ATPases transport zinc only across the cytoplasmic membrane, the RND systems (Table 1) are thought to transport across the complete cell wall of gram-negative bacteria, outer membrane included, a process named "transenvelope transport" (Nikaido 1996, Paulsen et al. 1996; Saier et al. 1994). The first RND system cloned was the cobalt/zinc/cadmium resistance (Czc) system from *Ralstonia* sp. CH34 (Mergeay et al. 1985; Nies et al. 1987). Resistance mediated by Czc is based on energy-dependent metal ion efflux (Nies and Silver 1989b). The Czc determinant contains three structural genes coding for subunits of the membrane-bound efflux complex $CzcCB_2A$ (Nies et al. 1990; Rensing et al. 1997c). The driving force for the export of the heavy-metal cations is not ATP, but the proton-motive force (Nies 1995). As shown with the reconstituted, purified CzcA protein, the proton gradient itself, and not the charge gradient, is required to drive zinc transport (Goldberg et al. in preparation).

In Czc as well as in other transenvelope transporters, one component transports the substrates across the cytoplasmic membrane; this transporter may be a RND, an ABC (Table 1) or a MFS (major facilitator superfamily) protein or protein complex. In the Czc system, this transporter is CzcA. CzcB, a membrane fusion protein (MFP), contains a cytoplasmic anchor, a hydrophobic α-helix at its amino terminus, a coil-to-coil structure that might span the periplasmic space and a carboxy terminus that may contain a hydrophobic β-barrel and inserts this protein into the outer membrane. The third subunit, CzcC, may be an integral outer-membrane protein or may contact an integral outer-membrane protein. Together, all three components could transport Co^{2+}, Zn^{2+} and Cd^{2+} across cytoplasmic membrane, periplasm and outer membrane (Rensing et al. 1997c).

A component of the Czc regulatory system, CzcD, is the patriarch of yet another family of proteins, CDF (Table 1), which mostly contains zinc transporters (Nies and Silver 1995; Paulsen and Saier 1997). CDF proteins have been found in many bacteria. In *S. aureus* (Fig. 2B), the CDF transporter ZntA mediates resistance to zinc and cobalt (Xiong and Jayaswal 1998). *S. cerevisiae* contains at least two members of the CDF family, ZRC1p and COT1p. ZRC1p mediates zinc and cadmium resistance (Kamizomo et al. 1989) and is involved in regulation of the glutathione level (Kobayashi et al. 1996). COT1p may substitute ZRC1p, although it is mainly a cobalt transporter, (Conklin et al. 1994, 1992). Since COT1p transports its substrate across a mitochondrial membrane, both proteins could be involved in heavy-metal metabolism of the yeast mitochondrion. By heterologous expression in *Ralstonia* sp. CH34, it has been shown that CzcD as well as the yeast transporters are energy-dependent efflux systems (Anton et al. in preparation). Thus, ZRC1p and COT1p might function in the efflux of surplus cations from the mitochondrion.

Four CDF proteins have been found in mammals, ZnT1, 2, 3, and 4. ZnT2 and ZnT3 are closely related and transport zinc into vesicles, ZnT2 into lysosomes (Palmiter et al. 1996a) and ZnT3 into synaptic vesicles (Palmiter et al. 1996b). ZnT1 detoxifies zinc by efflux across the cytoplasmic membrane (Palmiter and Findley 1995). The recently identified ZnT4 has a different function because it may be responsible for zinc secretion into milk (Huang and Gitschier 1997).

Arsenic (As), a well-known toxin

Arsenic is a heavy metalloid and acts sometimes as a metal, sometimes not. Mainly it occurs as As(V) in AsO_4^{3-}, arsenate, and as As(III) in AsO_2^-, arsenite. Arsenate is structurally highly related to PO_4^{3-}, thus, its main toxicity results from its interference with the metabolism of the major bioelement phosphorus. In rural Germany, it was used in historical times to speed up the inheritance process by disposing of the old owner of the house, farm and land; it was therefore called "inheritance powder". Because of its toxicity, arsenic has no function as a trace element; however, bacteria may use it as electron acceptor for anaerobic respiration (Laverman et al. 1995). Aerobic bacteria like *Alcaligenes faecalis* are able to oxidize arsenite again; thus, a geomicrobial redox cycle of arsenic exists, similar to the iron and sulfur cycles.

After arsenate has been taken up by phosphate transport systems (Fig. 1D), there is a problem with its detoxification: the structural similarity makes it difficult to export arsenate effectively because of the high phosphate concentration in the cell (Nies and Silver 1995). Thus, arsenate detoxification has to involve an initial step to differentiate it from phosphate. This step is the reduction of arsenate to arsenite (Ji et al. 1994; Ji and Silver 1992). For the resistance determinant in *E. coli*, arsenate reduction by the ArsC protein is coupled to glutathione (Oden et al. 1994) via glutaredoxin (Gladysheva et al. 1994; Liu and Rosen 1997). For ArsC from *S. aureus*, the electron donor is thioredoxin (Ji et al. 1994).

Arsenite then leaves the bacterial cell. Since anion export from bacterial cells is always driven by the chemiosmotic gradient, simple arsenite efflux systems are composed of just one efflux protein, the ArsB product (Wu et al. 1992). Examples are the plasmid-encoded system from *S. xylosus* (Rosenstein et al. 1992) and the chromosomally encoded system in *E. coli* (Diorio et al. 1995). In addition to the efflux only mediated by ArsB, arsenite transporters exist that are composed of an ArsB pore plus an ArsA ATPase. The best studied example is the plasmid-encoded arsenical resistance of *E. coli* (Chen

et al. 1986). The ArsB protein in these systems is able to function alone (Kuroda et al. 1997), therefore arsenite efflux carried out by the $ArsA_2B$ complex is driven chemiosmotically and by ATP (Dey and Rosen 1995). ArsA acts as a dimer with four ATP-binding sites, and related proteins have been found in bacteria, archaea, fungi, plants and animals (Li et al. 1996; Li and Rosen 1998; Zhou and Rosen 1997). Arsenite transporters related to ArsB have been found in *S. cerevisiae* (Rosenstein et al. 1992; Wysocki et al. 1997) and also in man (KurdiHaidar et al. 1998a, b).

In the pathogenic protozoon *Leishmania*, a P-glycoprotein-related ABC transporter is responsible for arsenite resistance (Papadopoulou et al. 1994). Cells of these organisms are able to gain resistance to arsenite and antimonium by efflux (Dey et al. 1994). As(III) is most rapidly detoxified as an As(III)-glutathione conjugate (Dey et al. 1996) or trypathione conjugate (Mukhopadhyay et al. 1996); however, the glutathione conjugate transporter is different from the P-glycoprotein-related protein, which seems to export non-conjugated arsenite (Legare et al. 1997; Papadopoulou et al. 1996).

Molybdate is the biologically most important heavy metal oxyanion

Molybdenum occurs mostly as Mo(VI) in molybdate. Molybdenum is an important trace element, since it is able to perform oxyanion catalysis without being as toxic as chromate. Although molybdate may also be transported by sulfate uptake systems, the main import into bacterial cells is catalysed by an inducible ABC transporter (Grunden and Shanmugam 1997). For most enzymes, molybdate is bound to a specific molybdate cofactor (Romão et al. 1995; Schindelin et al. 1996), a pterin mono- or dinucleotide. In nitrogenase, however, the enzyme able to assimilate molecular nitrogen, the specific iron/molybdenum cofactor does not involve a pterin, and Mo is bound to homocitrate, sulfur and a histidine residue (Bolin et al. 1993; Chan et al. 1993).

Silver (Ag), a precious metal with medical use

Silver is isoelectronic to copper; however, while the standard electrochemical potential of the Cu^{2+}/Cu^{+} pair is −268 mV, the potential of the Ag^{2+}/Ag^{+} pair is 1.56 V at pH 7. Thus, the main ionic forms of the two elements are Cu^{2+} but Ag^{+}. The monovalent silver cation forms a tight complex with sulfur, the solubility product of Ag_2S being 6.62×10^{-50}, but only 1.28×10^{-36} for CuS, which makes silver very toxic (Table 2). Because of its toxicity, silver is no trace element, but it has been used a long time as an antimicrobial agent in medicine (Slawson et al. 1992) and in coins. In most countries, the eyes of newborn children are treated with a drop of silver nitrate to prevent infections with *Neisseria* strains. Consequently, silver-resistant bacteria have been evolved, but only recently have any molecular studies been performed. The copper-effluxing ATPase CopB from *E. hirae* was found to transport Ag^{+} as well as Cu^{+} (Solioz and Odermatt 1995), the K_m of both substrates being identical. Silver resistance in *E. coli* was recently explained (Gupta et al. 1999). Resistance is catalysed by a RND-type transporter with remarkable similarity to the Czc system from *Ralstonia* sp. strain CH34. Thus, silver resistance may be based on RND-driven transenvelope efflux in gram-negative bacteria, efflux by P-type ATPases in gram-positive organisms, and additional complexation by intracellular compounds.

Cadmium (Cd), the best-known toxic heavy metal

The solubility product of CdS is 1.4×10^{-29} but 2.91×10^{-25} for ZnS (Weast 1984). Thus, cadmium is more toxic (Ragan and Mast 1990) than zinc (Table 2). Although a tremendous amount of work has been done, especially on cadmium toxicity in microorganisms, no defined mechanisms of action have been highlighted. The effects may be summed up under the general headings "thiol-binding and protein denaturation", "interaction with calcium metabolism and membrane damage" and "interaction with zinc metabolism", or loss of a protective function. Only in rare cases has an important single mechanism been found. Mutation of *dsbA*, encoding a product required for disulfide formation, leads to cadmium sensitivity in *E. coli* (Rensing et al. 1997a). Thus, DsbA is a target for cadmium in the periplasm of gram-negative bacteria. The influence of the additional proteins induced under cadmium stress in *E. coli* is not understood (Ferianc et al. 1998).

On the molecular level, cadmium uptake is barely understood (Fig. 1E). In *Ralstonia* sp. CH34 (Nies and Silver 1989a), and maybe also in *S. cerevisiae* (Liu et al. 1997), cadmium is accumulated by the magnesium system(s). In other bacteria, cadmium enters the cell by some manganese uptake system (Burke and Pfister 1986; Laddaga et al. 1985; Tynecka and Malm 1995). In plants, cadmium is taken up by the calcium uptake system (Clemens et al. 1998).

Resistance to cadmium in bacteria is based on cadmium efflux. Cyanobacteria, however, contain metallothioneins (Olafson et al. 1979). Amplification of the *smt* metallothionein locus increases cadmium resistance (Gupta et al. 1992) and deletion of it decreases resistance (Gupta et al. 1993; Turner et al. 1993, 1995). The metallothionein gene, *smtA*, is controlled by the SmtB repressor (Huckle et al. 1993; Morby et al. 1993; Turner et al. 1996), which also regulates a zinc-transporting P-type ATPase (Thelwell et al. 1998). Since cyanobacteria contain a variety of RNA- and P-type transport systems, transport may also be important for cadmium resistance in these bacteria.

In gram-negative bacteria, cadmium seems to be detoxified by RND-driven systems like Czc, which is

mainly a zinc exporter (Nies 1995; Nies and Silver 1989b) and Ncc, which is mainly a nickel exporter (Schmidt and Schlegel 1994). In gram-positive bacteria, the first example of a cadmium-exporting P-type ATPase was the CadA pump from *S. aureus* (Nucifora et al. 1989; Silver et al. 1989). This protein was the first member of a subfamily of heavy-metal P-type ATPases, and all the copper, lead and zinc transporters found later are related to this protein. Cadmium resistance in other gram-positive bacteria was also found to be mediated by CadA-like proteins (Liu et al. 1997).

In *S. cerevisiae* (Fig. 2C), cadmium is bound by glutathione, and the resulting cadmium-bisglutathionato complex is transported by the YCF1p transporter, an ABC transporter, into the vacuole (Li et al. 1997, 1996). This may be a general principle in all eukaryotes. The multidrug-resistance-associated protein from man may complement a *YCF1* mutation with respect to cadmium resistance (Tommasini et al. 1996). If phytochelatins are formed from the glutathione, the resulting cadmium-phytochelatin complexes are transported (Inouhe et al. 1996; Wu et al. 1995) by the HMT1p ABC transporter instead (Ortiz et al. 1992, 1995), and a similar transporter has also been found in *A. thaliana* (Tommasini et al. 1996). Although transport by CDF transporters like ZRC1 and binding by metallothioneins may also be involved in cadmium metabolism in all eukaryotes, the main detoxification seems to be mediated by transport of glutathione/phytochelatin complexes by ABC transporters into the vacuoles.

Antimonite, a rare toxin

Antimonite is isolelectronic to arsenite and has been mentioned above in the section on arsenical compounds. Antimonite enters the *E. coli* cell by the glycerol facilitator, GlpF (Sanders et al. 1997). It is detoxified by all systems giving resistance to arsenite by efflux (Rosenstein et al. 1992; Sanders et al. 1997). Since antimonite also serves as an inducer of these resistance systems, biosensors for antimonite and arsenite have been developed (Ramanathan et al. 1997; Scott et al. 1997; Tauriainen et al. 1997).

Tungsten (W), the beneficial exception

Tungsten is by far the heaviest element with any beneficial function. In sea water tungsten is present at 1% of the concentration of molybdenum (Weast 1984) but, in some anaerobic environments, WS may be more readily available than MoS. Thus, all tungsten-containing enzymes have been found in bacteria and archaea, mostly those with an anaerobic metabolism. The first tungsten-containing enzyme found was a reversible formate dehydrogenase (Andreesen and Ljungdahl 1973) and more groups of proteins followed (Kletzin 1997). Some methanogenic bacteria contain tungsten- and molybdenum-containing enzymes for the same purpose. Interestingly, the tungsten enzymes are expressed constitutively while their Mo counterparts were induced only in the presence of molybdate (Hochheimer et al. 1998). Like molybdenum, tungsten may be used as constituent of a tungsten cofactor; however, tungsten-containing nitrogenases have not been reported so far.

Mercury (Hg), the heavy metal with the strongest toxicity

The affinity of Hg^{2+} to thiol groups is even stronger than the affinity of cadmium to sulfide; the solubility product of HgS is 6.38×10^{-53} (Weast 1984). Consequently, it is the most toxic of all the elements tested in *E. coli* (Table 2). Mercury has been used in amalgam for tooth fillings for decades; however, recent results question the use of this element (Lorscheider et al. 1995).

Because of its high toxicity, mercury has no beneficial function. However, since bacteria are very likely to be confronted with toxic Hg^{2+} concentrations, mercury resistance determinants, *mer*, are very widespread (Silver 1996; Silver and Phung 1996). Resistance to mercury (Fig. 1F) is based on its unique peculiarities: its redox potential [its electrochemical potential of Hg(II)/Hg(0) at pH 7 is +430 mV] and the vapour pressure/melting/boiling point of metallic mercury, which is extraordinarily low for a metal [melting point −39 °C, boiling point 357 °C (Weast 1984)]. Thus, living cells are able to reduce Hg^{2+} to the metal, which does not remain inside the cell with the potential of becoming oxidized again, but leaves the cell by passive diffusion (Silver 1996; Silver and Phung 1996). Once outside, however, metallic mercury may be oxidized again by other bacteria (Smith et al. 1998).

To prevent toxic effects of Hg^{2+} on periplasmic proteins in gram-negative bacteria, Hg^{2+} is transported into the cell via specific uptake systems (Fig. 1F). In gram-negative bacteria, it is bound by the periplasmic Hg^{2+}-binding protein MerP as the first step of detoxification (Qian et al. 1998). MerP probably delivers the toxic cation to the mercury transporter MerT for transport into the cytoplasm (Hobman and Brown 1996). Alternatively, or in addition to MerTP, another uptake route exists which involves the MerC protein (Hamlett et al. 1992; Sahlman et al. 1997). Once inside the cell, Hg^{2+} is reduced with NADPH to Hg(0) by the MerA protein, which is related to glutathione reductase and other proteins (Schiering et al. 1991).

Organomercurials, which are more toxic than Hg^{2+}, may also be detoxified if the *mer* resistance determinant encodes a MerB organomercurial lyase in addition to the other Mer proteins (Silver 1996; Silver and Phung 1996). After cleavage by MerB, the resulting Hg^{2+} is reduced by MerA. The high toxicity of organomercurials and other methylated and alkylated heavy-metal compounds makes it very unlikely that these kinds of chemical modification of heavy metals are metal-resistance

mechanisms. Methylation has been observed for arsenic, mercury, tin, lead, selenium and tellurium (Fatoki 1997).

Because of its high toxicity, and the unique combination of reducibility and the ability of the product to volatolise, mercury is an ideal candidate for bioremediation. To increase the ability of the natural bacterial soil community to remediate Hg, the bacterial MerA reductase was first actively expressed in yeast (Rensing et al. 1992), then in plants (Rugh et al. 1998a, 1996), even in useful plants (Rugh et al. 1998b). Together with a *mer*-operator-based *lux* biosensor (Selifonova et al. 1993), this is the first step towards a real sensing and remediation of a heavy-metal contamination.

Lead (Pb) is not as bad as its reputation

Lead is no transition element, but belongs to the element group IVa, C, Si, Ge, Sn, Pb. In sea water, it is even more rare than mercury (Weast 1984). Owing to its low solubility (lead phosphate especially is insoluble, with a solubility product of 10^{-54}) its biologically available concentration is low. Thus, lead is not extraordinarily toxic for microorganisms (Table 2).

Lead has been used in large amounts for 2500 years (Hong et al. 1994), recently as a fuel additive, although the toxicity of lead for animals and man has been well known for a long time (Johnson 1998). Lead acts on the central nervous system, on blood pressure and on reproduction (Goyer 1993). In rural Albania, repair of a broken mill stone with lead and the resulting contamination of the flour recently led to the death of two people (Panariti and Berxholi 1998).

Lead-tolerant bacteria have been isolated (Trajanovska et al. 1997), and precipitation of lead phosphate within the cells of these bacteria has been reported (Levinson and Mahler 1998; Levinson et al. 1996). In *Ralstonia* sp. CH34 it has been shown that resistance to lead is mediated by a P-type ATPase (Borremans and van der Lelie, unpublished observation). Moreover, the CadA P-type ATPase is also able to transport Pb^{2+} (Rensing et al. 1998). Thus, lead resistance may also be based predominantly on metal ion efflux.

Uranium, the radioactive exception

Uranium, the natural element with the highest atomic number, is an actinide and mainly occurs as U(VI) in UO_2^{2-}. In this form, its toxicity to bacteria is low (Table 2); however, the deliberate ingestion of 15 g (!) uranium acetate led to acute renal failure in man (Pavlakis et al. 1996). Various ionic forms are possible, and it may be used as a substrate for anaerobic respiration (Lovley et al. 1991). No other beneficial actions of this radioactive element are known. As with many heavy metals, biotechnologically inspired investigations speculate on bioremediation of uranium by binding to bacteria, e.g. to *Citrobacter* (Jeong et al. 1997; Yong and Macaskie 1998), *E. coli* (Basnakova et al. 1998) or *Pseudomonas aeruginosa* (Hu and Reeves 1997).

Biotechnological use of heavy-metal resistance: an opinion

Biotechnology aims to create value by transforming a cheap substance into an expensive product. There are three areas for using heavy-metal resistance in biotechnology: first, adding metal resistance to a microorganism may facilitate a biotechnological process, which may or may not be linked to heavy metals. Second, heavy-metal-resistant bacteria may be used for any kind of bio-mining of expensive metals, directly on ores or by recovering metals from effluents of industrial processes. Third, heavy-metal-resistant bacteria may be used for bioremeditation of metal-contaminated environments.

How metal resistance can be added to a microorganism of biotechnological use depends on the amount of control one has over the process, which itself depends on the increase of value the process creates. In a highly controlled fermentor reaction, the insertion of a heavy-metal-resistance determinant into the chromosome of a particular bacterium is easily brought about by molecular genetics, if the toxic effect of a heavy metal has to be diminished. On the other hand, a sewage plant with limited control over the cleaning process probably does not allow the use of a highly modified organism. However, in these cases, heavy-metal-resistant natural bacteria may be established in the sewage plant, or plasmids with a broad host range of replication and metal-resistance expression could easily be introduced into the bacterial community. The presence of heavy metals will cause the plasmids to be stably maintained in the bacterial population. In all cases, determinants for efflux systems should be used, since detoxification by efflux is more economical for bacteria than binding, except in the case of mercury.

For biomining of ores, either the bacteria must be able to solubilize the respective metal directly, e.g. by reduction or oxidation, or the biotechnological transformation of another element, metal or not, is used in an indirect process. A few metals may be reduced or oxidized by bacteria, e.g. copper and iron. The indirect interaction with other elements is limited to sulfur, carbon, some metals, and the effect of the organic acids excreted by the bacteria. For recycling of metals in an industrial effluent, the value of the metal obtained must be higher than the value of the bacteria used. In most cases, the high costs of growing bacteria and the low specificity of the bacterial accumulation process make such a cleaning procedure unattractive.

Bacterial bioremediation has many problems. Although the binding of metals to bacteria has been described for many years, the commercial use of this procedure is slow. It is probably too expensive to grow bacteria and use them to bind metals; simple ion exchangers are cheaper and do the same job. There are a

few exceptions. Owing to their high metabolic power, bacteria have long been known for their essential function in the global cycle of elements. The sulfur circle may be used to remediate metals. First, acidophilic, aerobic chemolithoautotrophs like *Thiobacillus* solubilize heavy metals by producing sulfuric acid and maybe some complexing agents. In a second step, anaerobic sulfur-respiring bacteria produce H_2S, which precipitates the heavy-metal cation again. The metal sulfides may finally be used in chemical processes to purify the metal. However, this process must pay its way, by preventing expensive waste products and/or by the value of the metal obtained. Secondly, bacteria may be able to bind metals from extremely diluted solutions, a procedure that is only interesting if the metal is expensive or very toxic and has to be removed. Phytoremediation may be a third exception; however, the section on chromate shows the problems involved in getting plants to transport chromium into shoots or leaves. Much work has to be done to generate plants that grow faster than the natural accumulators and that might be used with the existing agricultural techniques.

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