Zn(II) metabolism in prokaryotes

Dayle K. Blencowe, Andrew P. Morby *

Cardiff School of Biosciences (2), Cardiff University, Museum Avenue, P. O. Box 911, Cardiff CF10 3US, UK

Received 7 October 2002; received in revised form 29 January 2003; accepted 29 January 2003

First published online 26 April 2003

Abstract

It is difficult to over-state the importance of Zn(II) in biology. It is a ubiquitous essential metal ion and plays a role in catalysis, protein structure and perhaps as a signal molecule, in organisms from all three kingdoms. Of necessity, organisms have evolved to optimise the intracellular availability of Zn(II) despite the extracellular milieu. To this end, prokaryotes contain a range of Zn(II) import, Zn(II) export and/or binding proteins, some of which utilise either ATP or the chemiosmotic potential to drive the movement of Zn(II) across the cytosolic membrane, together with proteins that facilitate the diffusion of this ion across either the outer or inner membranes of prokaryotes. This review seeks to give an overview of the systems currently classified as altering Zn(II) availability in prokaryotes.

© 2003 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Metal ion; Zinc; Ion transport; Metallo-chaperone

Contents

1. Zinc in biology .......................................................... 292
   1.1. Prokaryotic Zn(II) metabolism ........................................ 292
2. Primary mechanisms of Zn(II) import ..................................... 292
   2.1. ZnuABC: a high-affinity Zn(II) uptake system ....................... 292
3. Secondary mechanisms of Zn(II) import ................................... 294
   3.1. Nramp and Mramp: broad-spectrum cation transporters .......... 294
4. Protein-specific alterations in prokaryotic Zn(II) sensitivity .............. 294
   4.1. ZupT: a Zn(II) import system in E. coli .......................... 294
5. Broad-spectrum metal ion importers ..................................... 294
   5.1. Mg(II) uptake systems that also import a variety of other metal ions ...... 294
6. Metallo-chaperones ................................................... 295
   6.1. Cyanobacterial metallothioneins .................................... 295
   6.2. PZP1: a periplasmic Zn(II) metallo-chaperone in Haemophilus .......... 296
   6.3. Potential E. coli metallo-chaperones .................................. 296
7. Primary mechanisms of Zn(II) export ................................... 296
   7.1. Cd(II) resistance in S. aureus ........................................ 296
   7.2. The zia operon of Synechocystis PCC 6803 .......................... 299
   7.3. The zntA gene of E. coli .............................................. 300
8. Secondary mechanisms of Zn(II) export .................................. 302
9. Putative Zn(II) export systems ......................................... 302
   9.1. A chromosomal Zn(II)/Co(II) resistance determinant in S. aureus .......... 302
   9.2. The zntB gene of E. coli and zntB from Salmonella enterica .......... 302
10. Genomic approaches to Zn(II)-metabolism ................................ 303
11. Concluding remarks .................................................. 303
Acknowledgements ....................................................... 303
References .................................................................. 303

* Corresponding author. Tel.: +44 (2920) 874128; Fax: +44 (2920) 874116. E-mail address: morby@cardiff.ac.uk (A.P. Morby).
1. Zinc in biology

Zinc, and indeed other transition metal ions, present an interesting chemical paradox to living cells. Whilst excess Zn(II) can have significant toxicity and can act as a potent disrupter of biological systems, it is also an essential (micro-)nutrient that plays important roles in numerous physiological processes [1–3], as it serves as a cofactor in members of all six major functional classes of enzymes [4] and is especially important in the maintenance of protein structure [5–10].

Zinc occurs naturally as the divalent cation Zn(II) (Zn$^{2+}$) and has no redox activity under physiological conditions [11]. With the exception of copper, Zn(II) is the strongest intracellular Lewis acid and is predominantly co-ordinated to proteins via either the sulfur ‘thiol’ moieties of cysteine residues or the amino ‘imidazole’ ligands of histidine residues [12–16]. Although Zn(II) forms strong or labile co-ordinate bonds to these moieties, Zn(II) complexes tend to be thermodynamically stable [17].

1.1. Prokaryotic Zn(II) metabolism

Prokaryotic organisms are single cell entities that have immediate contact with their proximal environment and are separated from it by a set of cellular membranes. Since prokaryotic organisms are devoid of sub-cellular compartments, the major mechanisms that maintain cellular Zn(II) concentrations (Fig. 1) are limited to the highly regulated processes of Zn(II) import, metal ion sequestration by metallo-chaperones and Zn(II) export across the cytoplasmic membrane.

The import and export of Zn(II) is accomplished by a mixture of unique bacterial transport proteins, and proteins that belong to ubiquitously distributed protein superfamilies that contain members identified in all three kingdoms. Uptake systems for Zn(II), as well as other essential metal ions, have to differentiate between ions that are structurally very similar. Hence, most cells have two types of import systems: those that have high substrate specificity, which can be coupled to an energy source, and those that are non-specific and are usually driven by the diffusion gradient across the cytoplasmic membrane. Export systems for Zn(II) tend to have evolved to transfer metal ions of the same group across the cytoplasmic membrane, with ions being grouped on the basis of electronic structure and chemical similarity, e.g. Zn(II)/Cd(II)/Pb(II) or Ag(I)/Cu(I).

Membrane transport mechanisms can be further categorised on the basis of thermodynamics into primary and secondary systems, depending upon the energy source used for active transport. Primary mechanisms utilise chemical energy to transfer metal ions across the cytoplasmic membrane, whereas secondary active transport mechanisms use the energy stored in electrochemical gradients for the same purpose [18].

The regulation of most of these systems is controlled at the level of transcription and is mediated by metal-responsive regulators, which modulate expression of genes in response to respective substrate concentrations. Hence, the cellular level of loosely bound, labile Zn(II) is sustained at close to required levels in conditions of either metal ion limitation or excess.

2. Primary mechanisms of Zn(II) import

2.1. ZnuABC: a high-affinity Zn(II) uptake system

ABC transporters are comprised of one, but more often two, transmembrane proteins that form a pore in the membrane allowing the transport of a specific substrate from one side of the membrane to the other [19–21]. On the cytosolic membrane face of this pore are one or two ATP-binding regions that act to provide the energy for substrate transport from ATP hydrolysis. The Znu proteins of Escherichia coli belong to a recently defined sub-family of uniquely bacterial divalent metal ion ABC transport systems that are dependent upon an extra-cytoplasmic metal-binding protein [22].

The znu structural genes are transcribed in a divergent pattern on the chromosome from a spacer region that lacks the presence of an obvious promoter, with znuA being upstream and transcribed on the complementary strand to znuBC. Located next to znuA, and also transcribed from the complementary strand, is yebA, which encodes a hypothetical Zn(II)-dependent protease [23] of unknown function. YebA has significant similarity to the Zn(II)-containing staphylococcal enzyme, lysostaphin, which is an enzyme that degrades the cross-links in peptidoglycan and elastin [24].

ZnuA has primary sequence similarity to the periplasmic binding protein of the Mn(II) transport system of Synechocystis sp. [25] and has a cleavable periplasmic leader sequence [23]. This indicates that ZnuA is located outside the cytoplasmic membrane and has the potential to bind metal ions. ZnuB is a hydrophobic protein that has similarity to the membrane component of other ABC transport systems, whilst ZnuC has strong sequence similarities to the ATPase subunit of the ATP transporters [23]. Evidence obtained by Patzer and colleagues suggests ZnuABC constitutes a high-affinity Zn(II) uptake system in E. coli that is functionally dependent on both ZnuA and ATP.

This system is regulated by Zur, which is a member of the Fur family of bacterial metal-responsive regulators [23,26] and can sense sub-femtomolar concentrations of cytosolic Zn(II) [27]. In its native state Zur forms a dimer [28], which in the presence of excess Zn(II) binds to a regulatory sequence of dyad symmetry that is located within the central znu operator region and prevents the binding and activity of RNA polymerase. This Zur dimer
is only active in the reduced form [28] and has two distinctive metal-binding sites [17]. The first binds Zn(II) very tightly and is functionally analogous to the Zn(II)-binding site in Fur, whereas Zn(II) is more readily exchanged in the second site, which is similar to the metal-sensing site in the Zn(II) metallo-regulatory protein SmtB from Synechococcus [17,29–31].

Zur proteins seem to be widespread among Gram-negative, Gram-positive and cyanobacteria ([28], and references therein). In addition, a number of homologous, ABC-type, Znu high-affinity Zn(II) permease systems have also been identified in other bacteria. These include a system from Listeria monocytogenes [32], two from Haemophilus sp., [33–35] two from Streptococcus sp., [22,36,37] as well as systems in Treponema pallidum [38] and Neisseria gonorrhoeae [39].

A common Zn(II)-responsive metallo-regulatory protein (also designated Zur) is thought to regulate two coding regions that have been identified in Bacillus subtilis [40] and are thought to have a number of parallels with the ZRT Zn(II) transport systems that regulate Zn(II) uptake in the yeast Saccharomyces cerevisiae [41–47].
3. Secondary mechanisms of Zn(II) import

Secondary mechanisms of metal ion active transport, either uniporters or antiporters, catalyse the import or export of metal cations. The natural resistance-associated macrophage protein (Nramp) super-family [48] are a ubiquitous group of membrane proteins that are found in archaea, bacteria and eukaryotes and are associated with resistance to infection by intracellular pathogens [48–50]. Evidence suggests that members of the Nramp super-family use the electrochemical proton gradient as the major driving force for the broad-specificity import of divergent metal cations [49–57].

3.1. Nramp and Mnt: broad-spectrum cation transporters

Comparative genomic analyses have identified several Nramp homologues in micro-organisms such as Mycobacterium sp. (known as Nramp), Salmonella typhimurium and E. coli [58–61]. Functional characterisation of the E. coli Nramp homologue, designated MntH, confirms that, like its eukaryotic homologues, MntH is a proton-dependent divergent cation importer, which is able to facilitate the intracellular accumulation of several divergent metal ions, with substrate preference of Mn(II) > Cd(II) > Co(II) > Fe(II) > Zn(II) and to a lesser extent Ni(II) > Cu(II) [62]. MntH is regulated by the external availability of metal ions and by the dual action of the iron-dependent and manganese-dependent repressor proteins Fur and MntR [60,63]. It is possible that MntH represents the single Mn(II) active transport system that was identified by Bhattacharyya [64] and Silver et al. [65] in 1970.

4. Protein-specific alterations in prokaryotic Zn(II) sensitivity

In addition to the primary and secondary mechanisms of Zn(II) import in prokaryotes, proteins have been identified that confer Zn(II) sensitivity when expressed at high levels, which are indicative of further Zn(II) import systems. These proteins include a recently characterised bacterial member of the ZIP (ZRT, IRT-like protein) superfamily of metal ion transporters, previously reported only in eukaryotes [66–71].

4.1. ZupT: a Zn(II) import system in E. coli

Similar to the ZIP super-family members found in humans and yeast [46,72–77], ZupT from E. coli is involved in the uptake of Zn(II) across the cellular membrane and into the cell cytosol [68]. ZupT appears to have a lower affinity for Zn(II) than the ZnuABC system and may also be a broad-range metal ion transport protein, potentially mediating the import of cations such as Cd(II) and possible Cu(I) into E. coli cells [68]. This theory is consistent with the previously reported metal ion specificities of the Arabidopsis thaliana ZIP proteins [70].

In addition to these cation-specific metal ion transporters, Zn(II) may also be able to enter prokaryotic cells via a variety of other mechanisms, which have the ability to act as broad-spectrum metal ion importers.

5. Broad-spectrum metal ion importers

The outer membranes of many bacteria, including E. coli, contain a group of proteins known as porins [78–80]. These proteins form cylindrical, fluid-filled channels in the outer membrane of cells that allow selective hydrophilic solutes of up to 600 Da, including divergent metal cations such as Zn(II), to diffuse across into the periplasmic space. Zn(II) may then be imported into the cytosol of cells such as E. coli as a neutral metal phosphate via the constitutively expressed inorganic phosphate uptake system (Pit) [81], which also transports a diverse array of other metal cations into the cell [82–85]. Zn(II) can also be imported into cells as an alternative substrate of many broad-spectrum Mg(II) uptake systems [86–88].

5.1. Mg(II) uptake systems that also import a variety of other metal ions

Mg(II) is the most abundant divalent cation in biological systems [89], and has the largest hydrated radius of all divergent cations of biological interest [90]. In E. coli there are two systems for Mg(II) uptake. System I is a high-affinity, fast and non-specific metal transport system (MIT), designated CorA. CorA was identified as a Mg(II) uptake protein [87,91,92] and is ubiquitous throughout a variety of Gram-negative and Gram-positive bacteria [88,90,93,94] as well as in the yeast S. cerevisiae [95]. This system is constitutively expressed [90,96], and mediates the influx of Mg(II) as well as Ni(II), Co(II) and perhaps Zn(II) into the cytoplasm of E. coli cells [88]. System II is a specific Mg(II) transport system [96,97] that is regulated in response to Mg(II) concentration [88,96]. System II is analogous to the Mgt system [97] and, like CorA, has homologues in many other organisms. Probably the best characterised system is from S. typhimurium [98–104], which includes MgtA, a P-type ATPase that may transport Zn(II) better than Mg(II) ([11] and references therein). There is over 88% similarity between the MgtA proteins from S. typhimurium and E. coli [105], which also catalyses ATP-dependent Mg(II) uptake [106]. Hence, there is the possibility that MgtA from E. coli also transports Zn(II) and may provide the energy-dependent Zn(II) uptake phenotype observed by Bucheder and Broda [107].

In addition to these Mg(II) transport proteins, two other proteins have also been shown to confer Zn(II) ion...
uptake into prokaryotic cells. MgtE from *Bacillus firmus* OF4 is thought to be a Mg(II) and Co(II) uptake protein of diverse specificity that extends to incorporate Zn(II) and/or copper, serving as an internal transport mechanism that deliver these divalent cations to apo-metalloproteins [127,131–139].

In contrast to their eukaryotic relatives [140–147], bacterial MTs have not previously been widely reported. In *Pseudomonas putida*, there have been reports of three MT-like proteins [148–150], whereas Cd(II)-binding components have been isolated from *E. coli* [151].

Most recently bacterial metallothioneins (BmtA) (some corresponding to those initially reported in *P. putida*) have been isolated and characterised from a range of strains including *Anabaena PCC7120, Pseudomonas aeruginosa*, *P. putida* and *E. coli*, all of which bind Zn(II). It is of interest to note that some contain the GATA-type one-Zn(II)-finger structure similar to that seen in SmtA, the best studied prokaryotic MT that has been identified in a range of cyanobacterial strains [152,153].

6.1. Cyanobacterial metallothioneins

SmtA is a small, non-essential, cysteine-rich protein that is found in cyanobacteria and has a comparatively high affinity for Zn(II) in preference to Cd(II), Cu(II) and Hg(II) [152,154–159]. SmtA differs from eukaryotic MT as it contains histidine residues [160].

The abundance of SmtA increases following the exposure of cyanobacterial cells to elevated concentrations of Zn(II) and Cd(II), but not Cu(II) [161], which is the result of an increased level of *smtA* transcripts [157]. The chromosomal *smtA* locus along with *smtB*, the gene encoding its trans-acting metal-dependent repressor [29], is divergently transcribed from a central operator–promoter region [158].

SmtB is member of the ArsR family of metallo-regulatory proteins [162,163] and represses the expression of *smtA* by binding to an imperfect inverted repeat that is situated between the sites of transcriptional and translational initiation of *smtA* [30,157]. It may also bind to the two other regions of the operator–promoter region in a multimeric form at higher concentrations [29,30]. This regulation is then sensitive to fluctuations in metal ion concentrations, with the potential tetrahedral binding of Zn(II) by an SmtB homodimer complex causing dissociation of SmtB from its principal promoter complex or either of its two other potential regulator binding sites [31,164].

Although the suggestion that MT can form a putative cytosolic Zn(II)-translocating metallo-chaperone is only tentative, periplasmic proteins have been identified in a number of bacterial species that could be classified as Zn(II)-translocating metallo-chaperones. Principally these include the periplasmic ZnuA protein orthologues of the bacterial ABC metal permeases as well as PZP1, a Zn(II)-binding periplasmic metallo-chaperone from *Haemophilus influenzae*. 

Chaperones, as the name implies, act to escort or usher interactive entities through the potentially hostile environments in which they find themselves, whilst facilitating appropriate partnerships. The primary function of chaperones is to protect, not only shielding the entity from the environment, but more often than not protecting the environment from the more active nature of its inhabitant, thus preventing unfavourable interactions between the two. A family of soluble metal-binding proteins, known as metallo-chaperones [110], are thought to facilitate the intracellular trafficking of metal ions. In addition to the characterisation of the relatively new copper metallo-chaperones of eukaryotes [41,47,111–116] and potential bacterial chaperones [117–119], probably the best known biological molecule that sequesters metal ions and mediates metal-dependent gene expression is metallothionein (MT) [120–129]. However, even after extensive research, the biological function of MT remains unclear.

Although MT is induced in many organisms as a consequence of exposure to toxic levels of metal ions, where it is thought MT acts as a protective ‘sponge’, this is likely to be a property of MT rather than an evolutionary function [128]. Evidence indicates that MT is not essential for metalloprotein synthesis [122,126,130]. However, it is possible that MTs might function as cytosolic metallo-chaperones of the more physiologically important metal ions such as Zn(II) and/or copper.
6.2. PZP1: a periplasmic Zn(II) metallo-chaperone in *Haemophilus*

With the completion of the *H. influenzae* genome sequence [165] came the identification and partial characterisation of a putative adhesin B protein, periplasmic zinc-binding protein- (PZP1) [33,34].

This 37-kDa periplasmic protein shows significant similarity (49.2% identity/59.4% similarity) to ZnuA from *E. coli* and is thought to be involved in the uptake of Zn(II) into *H. influenzae* [23,33]. Since a null mutation made in the *pzp1* coding sequence confers a Zn(II)-dependent growth phenotype on cells grown aerobically, it is likely that PZP1 is part of the major Zn(II) uptake system in this pathogenic prokaryote. Sequence analysis indicates that PZP1 has similarity to another putative adhesin B protein that contains an ATP-binding cassette [165] and it has been speculated that PZP1 and this uncharacterised protein may interact, possibly with other protein(s), to form a Zn(II) import system [33], similar to the Znu system from *E. coli* [23].

6.3. Potential *E. coli* metallo-chaperones

Other proteins in *E. coli* that have the potential to form Zn(II)-binding metallo-chaperones include an unidentifed 20-kDa periplasmic spy protein that is only produced when cells are incubated with millimolar concentrations of Zn(II) [166,167]. YdaE and ZraP (formerly YjaI). YdaE has significant primary structural similarity with SmtA from *Synechococcus* and has recently been shown to interact with Zn(II) [153].

ZraP is a 20.4-kDa membrane-associated protein that undergoes a specific Zn(II)-induced cleavage to release a 12-kDa carboxy-terminal Zn(II)-binding region into the periplasm, which is involved in acquisition of tolerance to high Zn(II) concentrations. The expression of zraP is regulated by ZraS and ZraR (formerly HydH and HydG, respectively), which may form part of a Zn(II)-responsive phosphorylation cascade [166,168].

It is interesting to note the recent report detailing the release of Zn(II) ions bound to SmtA expressed in *E. coli* due to nitric oxide exposure [169]. Further studies of such phenomena certainly may well prove illuminating in terms of the global cellular function of Zn(II).

The identification of Zn(II) chaperones represents a beginning of the dissection of pathways that carry Zn(II) within the cell and target it to specific cellular sites. Such studies hold a great deal of promise for understanding the cellular consequences of Zn(II) metabolism.

7. Primary mechanisms of Zn(II) export

Similar to the systems for Zn(II) import into prokaryotic cells, the systems for Zn(II) export are also made up of proteins that can be separated into protein families and can be grouped on the basis of thermodynamics. However, unlike the systems that import Zn(II) into the cell cytosol, the proteins that export Zn(II) across the cytoplasmic membrane into the periplasm often have more precise substrate specificities.

Like the primary mechanisms of Zn(II) import, the proteins involved with the export of Zn(II) can also utilise the energy derived from ATP hydrolysis to translocate metal cations across the cytoplasmic membrane. Perhaps the best studied super-family of these proteins are the P-type ATPases. One sub-group of this super-family involved in transition metal ion export are the ‘soft metal ion-translocating’ P-type ATPases [13,170–174], which can be further sub-divided into two classes [13]: the Cu(I)/Ag(I)-translocating ATPases and the Zn(II)/Cd(II)/Pb(II)-translocating proteins (Fig. 2).

Representative members of these sub-family groups of P-type ATPases, which appear to be present in all three kingdoms, include the Cd(II)/Zn(II)/Pb(II) export protein CadA, from *Staphylococcus aureus* [175,176] and the Cu(I)/Ag(I)-transporting CopA and CopB from *Enterooccus hirae* [177–180], as well as proteins associated with both Wilson’s and Menkes’ diseases [181–186]. More recently, members that transport Ag(I), Co(II) or Pb(II) have also been identified [187–190].

These integral membrane ATPases form an important class of ion transport proteins that serve to maintain suitable cellular ionic conditions [173] and are characterised by the transient formation of a covalent phosphorylated intermediate during the catalytic cycle [170,171,191,192], as well as consensus domains for both ATP binding and hydrolysis [173]. The energy released by the removal of the γ-phosphate from ATP is coupled to the translocation of ions across biological membranes. In addition, these soft metal ion-translocating proteins, which have also been designated the ‘CPx-type ATPases’ [173] or ‘P1 heavy metal-transporting P-type ATPases’ [174], exhibit several novel features. These include unique number and topology of the membrane-spanning segments located in the carboxy-terminal region of the protein [193,194] as well as distinctive, highly polar, amino-terminal regions that contain one to six repeats of a conserved metal-binding domain. These domains are 70–100 amino acids in length and most commonly contain a motif sequence with similarity to the ‘heavy metal-associated (HMA) motif’, GMTCxxC (where x is equivalent to any amino acid) [181,195] (Fig. 3). One of the first Zn(II)/Cd(II)- and Pb(II)-translocating members of this sub-family of proteins was identified in Cd(II)-resistant strains of *S. aureus*.

7.1. Cd(II) resistance in *S. aureus*

Initial studies showed that genes encoding Cd(II) resistance were carried on several ‘penicillinase’ plasmids [196], the best characterised example of which is pL258 of *S. aureus*.
This bacterium is a common human pathogen that is associated with a number of diseases. Its plasmid-encoded Cd(II) resistance operon consists of two overlapping open reading frames [175]. The first, cadC, encodes a 122-amino acid, soluble protein and the second, cadA, encodes a 727-amino acid, integral membrane protein. CadA from \emph{S. aureus} is a soft metal ion-translocating P-type ATPase [13,174,197], and confers resistance to Cd(II), Zn(II) and Pb(II) [175,198–201]. The topology of CadA, encoded on plasmid pI258, has been determined [202] and conforms to that predicted for the soft metal ion-transporting P-type ATPase sub-family [173,174,193,203,204] (Fig. 4).

CadA catalyses the ATP-dependent efflux of Cd(II) from the cell cytosol in an electro-neutral exchange that transfers one Cd(II) ion out of the cell whilst accumulating two protons [175,176,205]. Unsurprisingly, the cadmium resistance operon is primarily induced by Cd(II) [200]. Whilst Zn(II) appears to be a rather poor inducer of the system [200], the range of cation concentrations tested during induction profiling was limited to a maximum of 100 \( \mu \)M, far below the toxic level for Zn(II).

CadC is an extrachromosomally encoded metallo-regulatory repressor protein of the ArsR super-family of metallo-regulatory proteins [162,206–208] and negatively regulates expression of the \emph{cad} operon in response to metal ions. The binding of thiophilic divalent cations, including Cd(II), Zn(II) and Pb(II), to this \emph{trans}-acting repressor protein allosterically regulates the DNA-binding activity of CadC to the \emph{cad} operator region, CadC binding to the proposed region as a high-affinity DNA-CadC dimer in the absence of metal ions and dissociating in the presence of the inducers [199,200,209–214]. The presence of \emph{cadC} in the operon is essential for its ability to confer maximal resistance to metal ions [199,200]. When produced, CadC chelates metal ions using at least three cysteine residues, which are conserved in all CadC homo-

---

**Fig. 2.** Organisation of the P-type ATPase super-family highlighting the phylogenetic relationship of the primary structures of the soft metal ion-transporting sub-group. The five sub-groups of the P-type ATPase super-family are spatially arranged. The dendrogram representing the sub-group of soft metal ion-transporting ATPases (highlighted in box) has been reproduced from Rensing [13] with GenBank accession numbers for each representative member given therein. Briefly: Zn(II)/Pb(II)/Cd(II) pumps include ZntA \emph{E. coli} (P37617), CadA from \emph{B. firmus} (AAA22858) and that encoded by the \emph{S. aureus} plasmid pI258 (AAB59154). Cu(I)/Ag(I) pumps include MNK, human Menkes’s disease-related protein (Q04656), WND, human Wilson’s disease-related protein (U03464) and CopA from \emph{E. coli} (AAB02268). The sub-group of soft metal ion-transporting P-type ATPases is growing rapidly, so only representative members are shown. The calculated matching percentages are indicated at each branch point.
Bacterial resistance to Cd(II) is usually based on the efflux of this divalent metal ion and Cd(II) resistance determinants are widespread [175,215-218]. Although the founder members of the growing group of Cd(II) resistance determinants were first identified in S. aureus [198,219,220], plasmid-borne Cd(II)-resistant determinants have been more recently identified in S. aureus [226,227], P. putida [228], and Halobacterium salinarum [229]. As well as the metal-binding and putative metal-binding domains of a series of other proteins including the Cu(I) chaperones Atx1, Hah1 and CopZ [112,178,327], the block shading indicates the presence of conserved or highly similar residues across all the protein regions shown. The positions of secondary structure elements are represented at the top of the figure along with the positions of the metal-binding pockets from MerP, CopZ and Zn(II)-transporting ATPases ZntA and CadA [175,215,238], as well as the domains from the Hg(II) chaperone MerP [119,195,328,329]. If a sequence is preceded by ~, this indicates the presence of amino-terminal extensions.

logues, and potentially a single carboxylic acid group, which is conserved as either an aspartate or a glutamate in all identified members of the ArsR family [210].

Although these proteins were initially characterised because of the ability to confer Cd(II) tolerance, in many cases, Zn(II) resistance determinants were genetically indistinguishable from those conferring resistance to Cd(II). Other members of the soft metal ion-transporting P-type ATPases family, such as ZiaA from Synechocystis PCC 6803 and ZntA from E. coli, were primarily identified be-

Fig. 4. Membrane topology of CadA, a Cd(II) and Zn(II) efflux protein (adapted from [202]).
7.2. The zia operon of Synechocystis PCC 6803

The zia system was identified in the fully sequenced genome of the cyanobacterium Synechocystis PCC 6803 [234] and comprises two open reading frames, ziaA and ziaR, which encode a Zn(II) transport protein and its cognate regulator and are divergently transcribed from a central promoter region [235]. ZiaA is a soft metal ion-translocating P-type ATPase that has sequence similarity to CadA. Whilst the expression of ziaA, under the control of its upstream regulatory sequences, confers both an increased Zn(II) tolerance and a reduction in Zn(II) accumulation by cells, its disruption leads to Zn(II) hypersen-

![Fig. 5. Alignment of the primary structure of ZntA with its CadA homologue from B. firmus and that encoded by the S. aureus plasmid pI258. Identical residues are boxed in grey with similar residues coloured in blue. Some of the characteristic features of soft metal-transporting P-type ATPases are coloured in red. Other potential metal-binding residues are highlighted in green. The protein sequence for ZntA is taken from [238], that for B. firmus from [175] and that for S. aureus plasmid pI258 from [215].]
sitivity and a reduced periplasmic compartmentalisation for this ion [235]. Hence, ZiaA mediates the efflux of Zn(II) from the cell cytosol to the periplasmic space of cyanobacterial cells.

ZiaR belongs to the ArsR super-family of metal-responsive repressor regulators [162] and binds to the zia operator–promoter region in the absence of Zn(II), forming a Zn(II)-responsive repressor of ziaA transcription [235]. The perception of Zn(II) by ZiaR is conferred by the histidine residue at position 116 and either or both cysteine residues located at positions 71 and 73 of the primary structure [235]. It has been suggested that ziaR and the upstream open reading frame, sll0793, which is predicted to encode a membrane-bound protein, are co-transcribed as there is only an 11-bp spacer region between them. This implies that sll0793 may also be involved in mediation of Zn(II) by the zia system. However, as yet, there is no evidence to support this.

A more extensive review of this subject by Cavet et al. appears in this issue [160].

In addition to these systems, E. coli also contains two open reading frames that encode proteins belonging to the soft metal ion-translocating P-type ATPase family. The first has now been designated copA and encodes a Cu(I) and possibly Ag(I) export protein [179,236], the second is zntA [237–239].

7.3. The zntA gene of E. coli

The zntA gene from E. coli encodes a member of the P-type ATPase super-family [240] and displays sequence similarity to a range of metal-translocating P-type ATPases from a diverse array of organisms [13,177,179,183,185,235,241–248]. Most notably these include strong primary structural similarity to CadA (Fig. 5). zntA_Ecol encodes a 732-amino acid residue, approx. 77-kDa integral cytoplasmic membrane protein, which has all the hallmarks of a soft metal ion-translocating P-type ATPase (Fig. 6). These features include a cysteine-rich hydrophilic amino-terminal region that contains both a single metal-binding motif (GMDCAC) and a unique triplet thiol cluster located within a CCCDGC motif. ZntA_Ecol also has a number of potential membrane-spanning segments within its carboxy-terminal region that are interspersed with two additional cytosolic regions. Contained within the larger, 280-amino acid aspartyl kinase domain, is the hallmark phosphorylation motif, Asp-Lys-Thr-Gly (DKTG). Located within the smaller 145-amino acid phosphatase domain, which is situated further to the amino-terminus, is a Thr-Gly-Glu-Ser (TGES) motif that is thought to be involved in the hydrolysis of the phospho-intermediate (Fig. 6).

ZntA_Ecol is a Zn(II)-dependent Zn(II) and Cd(II) export protein that catalyses the energy-dependent efflux of Zn(II) and Cd(II) from the cell cytosol and has the ability to confer Zn(II)/Cd(II) and Pb(II) tolerance to E. coli cells [201,237,238,249], with the expression of zntA_Ecol resulting in a reduction in the amount of 65Zn(II) associated with E. coli cells [249] and interruption at the 5' end of zntA_Ecol causing increased cellular Zn(II) association in Zn(II)-supplemented medium [239]. These studies also suggest that ZntA_Ecol is the primary mechanism of modulating Zn(II) concentration in the E. coli system as cells were unable to adapt to the altered levels of Zn(II) [249] and everted vesicles prepared from E. coli ΔzntA exhibited no accumulation of 65Zn(II) [238].

In addition, the amino-terminal region of ZntA_Ecol confers both Zn(II) and Cd(II) tolerance to E. coli cells and forms a metal-binding domain that sequesters 65Zn(II) during in vitro binding assays [249]. The function(s) of this sequestration event remains to be demonstrated. Moreover, the true functional roles of the putative amino-terminal metal-binding domains of soft metal ion-translocating ATPases are unknown.

It is estimated that the Michaelis constant (K_M) of Zn(II) transport by ZntA_Ecol is in the range of approximately 10 μM, which is thought to be similar to the labile intracellular Zn(II) concentration and therefore enables E. coli to respond to minor changes in cytosolic content [238]. These results are consistent with data obtained by Okkeri and co-workers, who have shown that ATPase activities of ZntA_Ecol reached a maximal turnover rate when stimulated by 20 μM Zn(II) [250]. In addition, this
group has shown that there are two major Zn(II)-induced, vanadate-sensitive membrane proteins that are phosphorylated by γ32P-labelled ATP [250]. These correspond to the predicted monomer and dimer sizes of ZntA\textsubscript{Ecol}, which is consistent with the archetypal dimerisation of P-type ATPases [171,194].

The metal ion-stimulated ATPase activity of purified, reduced ZntA\textsubscript{Ecol} has shown that the ‘hard’ metal cations Ca\textsuperscript{2+}, Na\textsuperscript{+} and Ba\textsuperscript{2+} or the ‘soft’ metals Co(II), Ni(II), Cr(III), Cu(I and II), Sb\textsubscript{2}O\textsubscript{7} and AsO\textsubscript{2} did not stimulate any ATPase activity, whereas both Pb(II) and Zn(II) stimulated activity between pH 5.5 and pH 8 (the total range investigated) [251]. These results are similar to those obtained by Okkeri and co-workers who also demonstrated that ATPase activity was stimulated by Pb(II) [250]. However, Sharma and co-workers [251] place the order of activity as Pb(II) > Zn(II) > Cd(II) at pH 6.0, Okkeri has an order of Zn(II) > Pb(II) > Cd(II).

Thiolate complexes of Cd(II) and Hg(II) were also able to stimulate the ATPase activity of ZntA\textsubscript{Ecol} at neutral pH [251]. It is interesting to note that during these investigations an oxidised form of ZntA\textsubscript{Ecol} was not active, suggesting that the ability of the protein to translocate metal ions might depend on its cysteine residues, and that these residues may be easily oxidised.

Metal ion specificity studies have shown that transition metal ions can have effects on either ZntA\textsubscript{Ecol}\textsubscript{-}P hydrolysis or the phosphorylation state of ZntA\textsubscript{Ecol} [250]. This is borne out by the differing effects that substrate ions have on the transition metal ion transport cycle of this P-type ATPase. Whilst Zn(II) stimulates both ZntA\textsubscript{Ecol}\textsubscript{-}P hydrolysis and ZntA\textsubscript{Ecol} phosphorylation, Cd(II) is more effective at promoting ZntA\textsubscript{Ecol} phosphorylation and is less effective at stimulating ZntA\textsubscript{Ecol}\textsubscript{-}P hydrolysis and metal ion transfer [250]. Whilst this demonstrates that these two metal ions affect different parts of the reaction cycle, it has been suggested that ZntA\textsubscript{Ecol} has at least two functionally different metal-binding sites with slightly different specificities [250]: one that is involved in the hydrolysis of the ZntA\textsubscript{Ecol}\textsubscript{-}P intermediate and could be synonymous with the binding site for the ion to be translocated, and another which is involved in the catalysis of phosphoryl transfer from ATP to ZntA\textsubscript{Ecol}.

At present the locations of metal-binding sites in ZntA\textsubscript{Ecol} and indeed other soft metal ion-translocating P-type ATPases are being mapped. However, the function of these sites in the metal ion translocation pathway remains unclear. Whilst the conserved metal-binding sites within the amino-terminal regions of ZntA\textsubscript{Ecol} and its homologues clearly bind metal ions [195,249,252,253], these regions appear not to be essential for enzyme phosphorylation [250] or metal ion translocation [230,254,255]. Additionally, the unique CP\textsubscript{x} motif (where x can be cysteine, histidine or serine) may also have the ability to transiently sequester metal ions during the metal ion transport cycle. This signature motif is located within the sixth putative membrane-spanning segment of ZntA\textsubscript{Ecol}, as well as many if not all soft metal ion P-type ATPases, and is thought to be situated in the transmembrane ion channel or the ion translocation domain [173].

Of particular functional interest is the E\textsubscript{X}\textsubscript{1}HP cluster (a motif located 34–43 residues distal from the aspartate residue in the –DKTG– phosphorylation motif that contains a conserved histidine-proline doublet sequence characteristic of soft metal ion-translocating P-type ATPases) [250]. This motif is involved in the metal ion transport cycle of ZntA\textsubscript{Ecol} and possibly of other soft metal ion-translocating P-type ATPases as mutations (Glu\textsubscript{70}Ala and His\textsubscript{27}Gln) made within this cluster reduce metal ion stimulation of ATPase activity of ZntA [250], implying that these residues are involved in the binding and/or sensing of metal ions. Furthermore, these mutations correspond to those found within the Cu(I)-transporting P-type ATPases associated with Wilson’s and Menkes’ disease states, where they lead to reduced Cu(I) transport, either reducing the uptake of Cu(I) from the gut and other tissues (in Menkes’ disease) or conferring a lack of Cu(I) export in the liver resulting in excess Cu(I) in the brain and liver (in Wilson’s disease) [182–186,256–258].

7.3.1. Transcriptional control of zntA\textsubscript{Ecol} expression

The expression of zntA\textsubscript{Ecol} is under the control of ZntR\textsubscript{Ecol}, a trans-acting activator that is essential for the Zn(II)-induced transcription of zntA\textsubscript{Ecol} [259–261]. ZntR\textsubscript{Ecol} exhibits 34% identity with the MerR metallo-regulatory protein, which is required for the Hg(II)-induced expression of the plasmid- and transposon-encoded mercury detoxification systems [262–270]. Similar to MerR, ZntR\textsubscript{Ecol} acts as a dimeric metallo-regulatory protein and tightly binds to its cognate promoter, P\textsubscript{zntA} (located upstream of the zntA start codon), regardless of the presence of a bound metal ion [259,271].

The P\textsubscript{zntA} promoter is reminiscent of the archetypical mer promoters from Tn501 and Tn21 transposon-encoded mercuric ion resistance mechanisms [272,273]. The separation of the −10 and −35 regions of the γ\textsuperscript{70} promoter sequence of P\textsubscript{zntA} is 20 bp. This promoter structure not only extends the spacer region between the elements from the consensus 17 ± 1 bp [274], but also defines a rotational separation around the DNA helix, which together makes P\textsubscript{zntA} a poor substrate for the binding of the γ\textsuperscript{70} subunit of RNA polymerase. Located within the spacer region of P\textsubscript{zntA} is a perfect 11–11-bp inverted repeat, which is slightly offset towards the −35 element [259] and is the binding site for the ZntR\textsubscript{Ecol} dimer [259,271].

ZntR\textsubscript{Ecol} regulates transcription from P\textsubscript{zntA} by a metal-induced DNA distortion mechanism that is thought to be a widespread attribute of the MerR family members [264,270,271,275–281]. Similar to the Hg(II) MerR activation of mer promoters [10], ZntR\textsubscript{Ecol}-dependent expression from P\textsubscript{zntA} exhibits a sigmoidal response to changes in the Zn(II) concentration, with induction occurring from 100
μM to a maximum 75-fold increase at 1.1 mM Zn(II) [259]. Furthermore, ZntR_EaSl has a femtomolar sensitivity to Zn(II) ions [282] and can bind up to two Zn(II) ions per monomer [271], which makes it one of the most sensitive regulatory proteins characterised to date.

8. Secondary mechanisms of Zn(II) export

Probably the best characterised system of Zn(II) transfer that employs a secondary mechanism is encoded by the czc determinant from R. metallidurans (formerly Alcaligenes eutrophus or Ralstonia eutropha) [283–289] strain CH34, which contains at least eight determinants that encode resistance to metal ions [189,286,287,289–291] that are found on either the bacterial chromosome or one of two large, endogenous mega-plasmids, pMOL28 (180 kb) [292] and pMOL30 (238 kb) [284,286]. Of these R. metallidurans CH34 has three ‘classical’ plasmid-encoded metal resistance systems of diverse specificity. These include: chr, carried on pMOL28 and encoding resistance to chromate (CrO$_4^{2−}$) [293–295]; ccr, which is also found on pMOL28 where it confers resistance to Co(II) and Ni(II) [293,296–301]; and cze, which mediates resistance to Co(II), Zn(II) and Cd(II) [290,294,302,303] and is encoded on the larger pMOL30 plasmid [284].

Both the ccr and cze systems are very closely related, with equivalent tri-component, chemiosmotic efflux systems that are functionally very similar [303–306]. These two systems have become the first members of a new bacterial family of resistance, modulation and cell division (RND)-driven transporters [307,308] that channel cations across both inner and outer membranes and the periplasmic space, with the cze determinant becoming the archetypal system for all new members of this family of ‘trans-envelope transporters’ [309,310] are compared.

Many ‘ccz-type’ systems have now been isolated; these include many from the Ralstonia spp. of β-Proteobacteria [286,287,289,311], possible determinants from S. aureus [312,313] and one involved in mammalian host cell cytopathicity with Legionella pneumophila [314]. In addition, similar systems have been identified in P. aeruginosa strains isolated from metal-polluted environmental sources and within the clinical isolate P. aeruginosa type (PAO1) [233].

These RND-driven transport systems are reviewed in detail by Nies [315], elsewhere in this issue.

9. Putative Zn(II) export systems

9.1. A chromosomal Zn(II)/Co(II) resistance determinant in S. aureus

Staphylococcal strains that lack the plasmid-encoded heavy metal resistance determinants identified by Novick [198] still show resistance to some heavy metal ions. In the recent past Xiong and Jayaswal [313] have identified a chromosomal determinant that confers resistance to both Zn(II) and Co(II). Isolated from a genomic library, the region of DNA containing this determinant comprised two consecutive open reading frames, here designated zntASaur and zntRSaur, which encode proteins that show significant similarity to the cation diffusion facilitator (CDF) superfamily of proteins [46,66,304,316], and the ArsR family of regulators, respectively. The genomic organisation suggests that zntASaur and zntRSaur are co-transcribed in an operon, which encodes a Zn(II)/Co(II) export pump and its cognate regulatory protein [313,317].

The ZntASaur protein is predicted to have six transmembrane segments ending with a hydrophilic C-terminal region. Interestingly, ZntASaur contains two histidine-rich regions, one located near the amino-terminus and the other at the carboxy-terminus, which have the potential to coordinate metal ion binding.

A genomic knockout of zntASaur conferred a Zn(II)- and Co(II)-sensitive phenotype to the host strain, which was complemented by the re-introduction of a trans copy of the intact operon into the strain [313]. The sensitive strain also accumulated more cytosolic Zn(II). When present in multiple copies, the zntASaur operon conferred a Zn(II)/Co(II)-tolerant phenotype with multiple copies of zntASaur leading to a decreased intracellular Zn(II) content [313]. Together these data suggest that the zntASaur gene product is involved in the export of Zn(II) ions from S. aureus cells. Since sequence analysis revealed the lack of any characteristic ATP-binding sites in the ZntASaur primary structure, this indicates that transport of metal ions is powered by an alternative energy source or mechanism [313].

9.2. The zitB gene of E. coli and zntB from Salmonella enterica

Other proteins that have been associated with variations in Zn(II) tolerance of E. coli cells include ZitB (formerly YbgR), another member of the CDF family of proteins that is thought to mediate a specific Zn(II)-induced Zn(II) efflux [318]. Analysis of ZitB has shown that a number of residues are essential for function (His$_{53}$, His$_{159}$, Asp$_{163}$ and Asp$_{186}$) and that neither the N- nor C-terminal histidine-rich regions were essential for function [319].

More recently a novel Zn(II) export system has been identified in Salmonella enterica serovar Typhimurium [320]. ZntB is homologous to the CorA family of cation transporters that, surprisingly, is unable to function as a Mg(II) uptake system. Instead this protein appears to function as a novel Zn(II) efflux pathway in enteric bacteria that confers tolerance to both Zn(II) and Cd(II) [320].
10. Genomic approaches to Zn(II)-metabolism

Transcriptional profiles have been established for *E. coli* cells adapted to grow in media containing substantially elevated levels of a range of transition metal ions, including Zn(II) [321]. The data suggest that a number of gene products, previously unconnected with Zn(II), appear to play a role in the generation of tolerance. Expression of the transposition-associated protein InsA7 can confer a slight growth advantage on *E. coli* when challenged by Zn(II) or Cd(II). In silico analysis of the primary sequence of InsA7 highlighted the presence of two CxxC motifs that are associated with protein–cation interactions. These studies also identified a range of other genes whose products have either been shown to bind metal ions or are predicted to do so.

A proteomic approach to defining Zn(II)-binding proteins in *E. coli* [322] has identified 10 proteins not previously known to interact with Zn(II). Whilst the techniques used to identify Zn(II)-binding proteins may shed light on the distribution of Zn(II) in the cell, it must be borne in mind that chemical and structural integrity may be compromised in proteins that have undergone the lengthy extraction and separation processes inherent in proteomic studies and this may destroy valid metal-binding sites or create conditional ion-binding sites.

11. Concluding remarks

Zn(II) is involved in a wide variety of cellular processes and the maintenance of cellular Zn(II) status is essential for survival. Therefore, an improved understanding of Zn(II) acquisition, assimilation and metabolism is of great significance. Investigations carried out in prokaryotic organisms have characterised many mechanisms for ‘channelling’ Zn(II) ions, which include mechanisms for Zn(II) import, export and potential *intra*cellular trafficking by Zn(II) metallo-chaperones. Each metal ion has one or more low-affinity uptake systems, which tend to have broad substrate specificity and import metal ions when they are freely available. Additionally, when the supply of metal ions is limited, high-affinity systems have also been found. These systems are selective for their target metal ions and are tightly regulated according to metal ion requirements. The regulation of these systems by metal-responsive transcriptional regulators modulates expression of the genes encoding metal ion binding and/or transport proteins. Hence, metal ion homeostasis is maintained in conditions of either metal ion limitation or excess.

As access to ‘genomic’ technologies increases and the range of bacterial genome sequences proliferates it is strikingly apparent that the number of gene products involved in, or affected by, Zn(II) metabolism is far greater than first expected and this, perhaps, reflects the importance of metal ions in biology.

The future lies in the understanding of Zn(II) (metal ion) availability in the cell and the pathways by which these magical crumbs of structure and catalysis are sensed and ultimately targeted to appropriate cellular sites. It is our opinion that such endeavours will prove fruitful in understanding many fundamental processes in prokaryotes and some more complex but less important life forms!

Acknowledgements

The work presented here was aided by the generous donation of unpublished information by Dr Chris Rensing (University of Tucson, AZ, USA). We are particularly indebted to Professor Nigel Brown (University of Birmingham, Birmingham, UK) and Professor John Kay for critical reading of this work prior to publication.

References


ily SMF by NRAMP2, a member of the mammalian natural resistance-associated macrophage protein family. J. Biol. Chem. 272, 28933–28938.


Tao, T., Snavely, M.D., Farr, S.G. and Maguire, M.E. (1995) Magnesium transport in Salmonella typhimurium: mgtA encodes a P-type ATPase and is regulated by \( ^{25}\text{Mg}^{2+} \) in a manner similar to that of the mgtB P-type ATPase. J. Bacteriol. 177, 2646–2662.


Yamaguchi, Y., Heiny, M.E. and Gitlin, J.D. (1993) Isolation and


