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Review

Bacterial zinc transporters and regulators

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Abstract

Zn^{2+} homeostasis in bacteria is achieved by export systems and uptake systems which are separately regulated by their own regulators. Three types of Zn^{2+} export systems that protect cells from high toxic concentrations of Zn^{2+} have been identified: RND multi-drug efflux transporters, P-type ATPases, and cation-diffusion facilitators. The RND type exporters for Zn^{2+} are only found in a few gram-negative bacteria; they allow a very efficient export across the cytoplasmic membrane and the outer membrane of the cell. P-type ATPases and cation-diffusion facilitators belong to protein families that are also found in eukaryotes. The exporters are regulated in bacteria by MerR-like repressor/activators or by ArsR-like repressors. For the high-affinity uptake of Zn^{2+}, several binding-protein-dependent ABC transporters belonging to one class have been identified in different bacteria. Zn^{2+} ABC transporters are regulated by Zur repressors, which belong to the Fur protein family of iron regulators. Little is known about low-affinity Zn^{2+} uptake under zinc-replete conditions. One known example is the phosphate uptake system Pit, which may cotransport Zn^{2+} in *Escherichia coli*. Similarly, the citrate–metal cotransporter CitM in *Bacillus subtilis* may help to supply Zn^{2+}.

Introduction

Zinc is, for most if not all bacteria, an essential trace element. Many bacterial enzymes contain zinc in the active center or in a structurally important site; the large group of DNA-binding proteins with zinc-finger motifs, however, are mainly found in eukaryotes and rarely in bacteria (Clarke & Berg 1998). In 1974, Bucheder & Broda showed in a careful study that the uptake of $^{65}Zn^{2+}$ in *Escherichia coli* is energy-dependent; in starved cells under anoxic conditions, uptake is stimulated by glucose and is more strongly stimulated by the addition of oxygen.

Only recently, with the availability of molecular genetic methods, have further details of bacterial Zn^{2+} transport and Zn^{2+} homeostasis been revealed. The first transporters identified were shown to confer resistance to high Zn^{2+} concentrations. In many cases, these transporters export, in addition to Zn^{2+}, also other toxic ions such as Cd^{2+}, Co^{2+}, and Pb^{2+}. In eukaryotes, resistance to high concentrations of Zn^{2+} is not only achieved by export, but also by binding to metallothionein. Among bacteria, this type of protein has to date only been observed in cyanobacteria (Robinson *et al.* 1998). Also, high-affinity zinc-uptake systems have been detected that help bacteria to live at extremely low Zn^{2+} concentrations, which are found, for example, in the serum of animals and humans. In addition, there are less well characterized low-affinity uptake systems that contribute to Zn^{2+} supply in a Zn^{2+}-rich, but non-toxic environment.

Figure 1 shows an overview of the different systems found in gram-negative bacteria grown under high, medium, or low Zn^{2+} concentrations. Examples from various bacteria are combined in this figure and will be addressed in the following paragraphs. What is noteworthy in the figure is the lack of uptake systems. Specific transporters active under Zn^{2+}-replete conditions have not been identified. Only a few unspecific systems are known to transport Zn^{2+}among

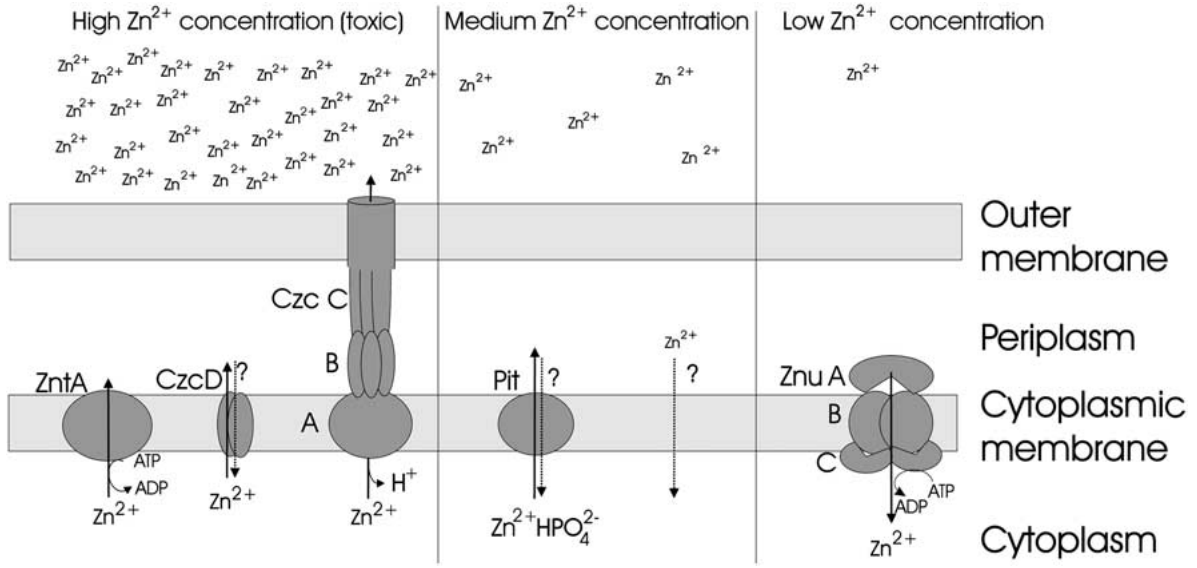

Fig. 1. Pathways for uptake and efflux of Zn^{2+} in gram-negative bacteria. Depending on the Zn^{2+} concentration in the medium, different types of Zn^{2+} transporters are synthesized. At limiting Zn^{2+} concentrations, binding-protein-dependent ABC transporters are induced (right). The Pit-like proteins might act as co-transporters to help satisfy the Zn^{2+} demands of the cell under Zn^{2+}-replete conditions (middle). Exporters of the CzcABC-like RND transporters seem to be very efficient in protecting the cells against toxic Zn^{2+} concentrations. Also, CzcD-like cation facilitators and P-type ATPases such as ZntA protect the cells against high Zn^{2+} concentrations (left). Obvious gaps in our knowledge are apparent: little is known about the passage of divalent ions across the outer membrane and about which transport systems supply the cells under Zn^{2+}-replete conditions.

other divalent cations. Although the outer membrane has many binding sites for divalent cations, little is known how Zn^{2+} passes through this membrane. Gram-positive bacteria, which do not have an outer membrane (Figure 1), have many binding sites for divalent cations in the teichoic acids, the polymeric ribitol phosphates bound to the thick peptidoglycan layer. These systems seem to act as cation exchangers on the surface of bacteria.

Zn^{2+} export systems (1): Cation diffusion facilitators

The highly Zn^{2+}-resistant gram-negative bacterium *Ralstonia metallidurans* (formerly Alcaligenes eutrophus) CH34, isolated from a decantation tank of a zinc factory, has a minimal inhibitory concentration for Zn^{2+} of 12 mM in a Tris-based medium. The *czcNICBADRS* gene cluster found on a large plasmid determines the high metal resistance by encoding two systems for the export of Zn^{2+}, Co^{2+}, and Cd^{2+} (Anton *et al.* 1999; Grosse *et al.* 1999). CzcD is a member of the cation diffusion facilitator family and exports these metals across the cytoplasmic membrane (Anton *et al.* 1999).

The *Staphylococcus aureus* Zn^{2+}-resistance determinant ZntA shows 38% identity to CzcD from *Ralstonia eutropha* (Xiong & Jayaswal 1998). A *zntA* mutant is sensitive to 0.5 mM Zn^{2+}compared to 5 mM for the parent strain. ZntR, a member of the ArsR family, regulates the expression of *zntA*. The nomenclature is very unfortunate since these ZntA and ZntR proteins are not related to the ZntA and ZntR proteins of *E. coli* and other bacteria, which are treated later in this review. Several other members of this protein family characterized in eukaryotes have important functions in the loading of vesicles with Zn^{2+} and in exporting Zn^{2+} from the cytoplasm (Palmiter *et al.* 1996); in bacteria sequence similarities indicate a wider distribution (Paulsen & Saier 1997). One homologue in *E. coli* encoded by *ybgR* is also active in Zn^{2+} transport (Patzer & Hantke, unpublished).

Zn^{2+} export systems (2): RND type exporters

The CzcD protein seems to be the first line of defense of *Ralstonia metallidurans* against high Zn^{2+} concentrations, since in a czcD mutant expression of the *czcCBA* genes is induced and the *czcD* mutant is slightly more sensitive to Zn^{2+} than the parent strain

(Anton *et al.* 1999). The proteins CzcA, CzcB, and CzcC form a sophisticated transport system that exports Zn^{2+}, Co^{2+}, and Cd^{2+} across two membranes, the cytoplasmic membrane and the outer membrane, thereby possibly also protecting the periplasmic space of *R. metallidurans* from the toxic metals. CzcA is a cation-proton antiporter located in the cytoplasmic membrane. CzcB seems to be a connector protein that is distantly related to AcrA (22% amino acid identity), a protein located in the periplasm as part of an acriflavin export pump. CzcC has a distant relationship to TolC and may connect CzcB to the outer membrane, forming a CzcABC protein complex (Rensing *et al.* 1997a). This arrangement allows extrusion of the toxic metals from the cytoplasm into the medium. The whole export system belongs to the widely distributed RND (resistance, nodulation, division) protein family (Tseng *et al.* 1999). The protein CzcA alone without CzcB and CzcC allows only a low level of metal ion resistance.

CzcS and CzcR belong to the large family of the two-component histidine sensor kinase regulators. Studies with *czcR* and *czcS* deletions, LacZ fusions and the analysis of *czcCBA* mRNA synthesis under inducing and non-inducing conditions has shown that CzcR/S regulate the *czc* genes (Grosse *et al.* 1999). The system was induced with 0.3 mM Zn^{2+} and less well with 0.3 mM Cd^{2+}, indicating a preference for Zn^{2+}.

Zn^{2+} export systems (3): P-type ATPases

P-type ATPases, named for the phosphorylated aspartate enzyme intermediate, form a large family of cation-transporting membrane proteins found in eukaryotes and bacteria. Two related subgroups transport Cu^{+} and Ag^{+}, and Zn^{2+}, Cd^{2+}, and Pb^{2+} (reviewed by Gatti *et al.* 2000).

A Cd^{2+}- and Zn^{2+}-resistance determinant encoded by the genes *cadA* and *cadC* has been found on the staphylococcal plasmid pI258. In a *Staphylococcus aureus* strain, the cloned genes raised the minimal inhibitory concentration of Cd^{2+} from 0.005 mM to 2.56 mM, while the minimal inhibitory concentration of Zn^{2+} was raised from 0.6 mM to 1.8 mM (Yoon & Silver 1991). These values illustrate the high toxicity of Cd^{2+} for the unprotected cell.

CadA is a P-type ATPase (Tsai *et al.* 1992), and CadC, important for full resistance, is a regulatory protein with similarities to ArsR proteins, which regulate arsenite/antimonite resistance ATPases (Shi *et al.* 1994; Rosenstein *et al.* 1994). The CadA/C transport system is also found in the gram-negative bacterium *Stenotrophomonas maltophila* and has 96% sequence identity to the staphylococcal CadA/C (Alonso *et al.* 2000). This high level of sequence identity of the two proteins indicates a recent horizontal gene transfer of *cadA* and *cadC* between a gram-positive and a gram-negative bacterium. In *Listeria monocytogenes*, the related CadA and CadC proteins (66% and 48% sequence identity to *S. aureus* CadA and CadC, respectively), are encoded by a transposon on a plasmid and confer only Cd^{2+} resistance and no Zn^{2+} resistance (Lebrun *et al.* 1994). The lack of Zn^{2+} resistance may be explained by the high intrinsic resistance of the *L. monocytogenes* and *B. subtilis* strains used (to 7 and 3.5 mM Zn^{2+}, respectively), which indicates the presence of additional Zn^{2+} exporters encoded on the chromosome. Also in *S. aureus*, the pI258 encoded CadA/C proteins increased the Zn^{2+} resistance level only by a factor of 3 which indicates that protection from Cd^{2+}may be the main function.

The *E. coli* ZntA protein, another member of the P-type ATPases, confers Cd^{2+} and Zn^{2+} resistance (Beard *et al.* 1997; Rensing *et al.* 1997b). The Zn^{2+} content of *E. coli* has been estimated as a nominal concentration of 0.6 mM if it were all free (Kung *et al.* 1976). In mammalian cells this value has been estimated at 0.2 mM (Palmiter & Findley 1995). The concentration of free Zn^{2+} is difficult to determine, the results depend on the methods used. *In vitro* studies suggest that thiolate-bound Cd^{2+} or Zn^{2+} are the best substrates for ZntA (Sharma *et al.* 2000). In the cell, most of these metals might be bound as thiolates of cysteine or glutathione. The main function of CadA- and ZntA-like proteins seems to be the extrusion of Cd^{2+} and Pb^{2+}, whereas Zn^{2+} export seems to be only an additional function.

In *Proteus mirabilis*, a mutation in a ZntA homologue leads to a defective swarming behavior with slower migration than the parent strain (Lai *et al.* 1998; Rensing *et al.* 1998). This unusual phenotype might arise through the disturbed Zn^{2+} homeostasis having an effect on swarm-cell differentiation.

In humans, two homologous P-type ATPases transport copper. Mutations in these proteins cause disorders of copper metabolism known as Wilson and Menkes diseases. To study the effects of Wilson disease mutations in *E. coli*, two mutants with site-directed mutations in ZntA were constructed, His475Gln and Glu470Ala, the human counterparts

of which cause Wilson disease. Both mutant proteins show a reduced metal-ion-stimulated ATPase activity (about 30–40% of the wild-type activity) and are phosphorylated much less efficiently than the wild-type proteins. These results suggest that the mutations affect major stages in the transport process of both P-type ATPases (Okkeri & Haltia 1999).

Members of the MerR/ZntR family or of the ArsR/SmtB family regulate Pb^{2+}, Cd^{2+}, and Zn^{2+} extrusion

The regulator of *zntA* in *E. coli*, ZntR, induces *zntA* transcription at 19 μM Cd^{2+} or 100 μM Zn^{2+} (Noll & Lutsenko 2000). Also Pb^{2+} is an efficient inducer of the system (Binet & Poole 2000). ZntR is a MerR-like regulator that binds as a repressor to the *zntA* promoter; in the presence of Cd^{2+} or Zn^{2+}, ZntR is converted into a transcriptional activator that changes the conformation of the promoter region and makes it a better substrate for the RNA polymerase (Outten *et al.* 1999).

MerR is a well-studied regulator of a Hg^{2+} detoxification system. Hg^{2+} is recognized by amino acid residue C82 from one monomer of the MerR dimer and by C117 and C126 from the second monomer of MerR. In a systematic study, mutated MerR was screened for other metal specificities. Mutations in 11 positions changed the responsiveness from Hg^{2+} to Hg^{2+} and Cd^{2+} (Caguiat *et al.* 1999), making the regulator less specific. Interestingly, in these mutants, the response to Zn^{2+} changed only slightly.

Other P-type ATPases like CadA are regulated by members of the ArsR/SmtB superfamily of repressors. The structure of SmtB has been solved. It is a dimeric repressor protein with a typical helix-turn-helix motif, the arrangement of the three core helices and the beta hairpin is similar to the HNF-3/forkhead, CAP and diphtheria toxin repressor proteins (Cook *et al.* 1998). However, the Zn^{2+} binding sites have not been characterized well. Both types of regulators, ArsR/SmtB and MerR/ZntR, are also found in connection with other metal transporters. Each of these regulators regulates very specifically only one system.

Binding-protein-dependent ABC transporters drive high-affinity metal uptake

High-affinity Zn^{2+} uptake systems in gram-negative and gram-positive bacteria have been characterized. One of the first described was in *E. coli* (Patzer & Hantke 1998). During the selection of recombinants with iron-regulated *lacZ* fusions using the transposing phage Mud1, fusions regulated by the availability of Zn^{2+} were obtained. Cells carrying these fusions grow as red colonies on MacConkey agar with Zn^{2+}-complexing chelators, indicating derepression of the *lacZ* fusion, while addition of $ZnCl_2$ leads to repression and growth of white colonies. On complex nutrient agar plates, growth of these mutants is inhibited by 5 mM EGTA or 0.4 mM EDTA. When $ZnCl_2$ is spotted on filter paper discs, a zone of growth around the disc is observed. Other metals, such as Ni^{2+}, Cu^{2+}, Mn^{2+}, and Fe^{2+}, do not stimulate growth. A much smaller zone of growth is observed with Co^{2+}, which might substitute for Zn^{2+} in some proteins and might lower the need for Zn^{2+}.

The Mud1 phage had inserted into one of three genes encoding a binding-protein-dependent ABC transporter. The gene *znuA* (zinc uptake) encodes a periplasmic binding protein, *znuB* encodes an integral membrane protein, and *znuC* encodes the ATPase component of the transporter. To prove the *in vivo* function of these genes, $^{65}Zn^{2+}$ uptake was measured in *znu* mutants and in the parent strain. In a HEPES-buffered medium, the uptake of $^{65}Zn^{2+}$ is the same for the mutants and the parent strain. Only when the cells were pre-grown in the presence of 5 mM EGTA did the mutant unexpectedly take up more $^{65}Zn^{2+}$ than the parent strain; this could be interpreted as the induction of another Zn^{2+} transporter in the mutant. Addition of 0.5 mM EGTA to the transport medium lowers the uptake by a factor of 10 in the induced parent strain, whereas no uptake is observed in *znu* mutants regardless of the growth conditions. Unfortunately the Zn^{2+} concentration in the uptake medium was not exactly defined. According to Zn^{2+} determinations, it was below 150 nM, and the free Zn^{2+} concentration was even lower through the addition of 0.5 mM EGTA (in the presence of 3 mM Mg^{2+}).

Sequence similarity searches revealed that ZnuA belongs to a large family of binding proteins that recognize either Zn^{2+}, Mn^{2+}, or $Fe^{?}$ as their substrate (Figure 2). Binding proteins of ABC transporters had been grouped into eight clusters (Tam & Saier 1993), but since these metal binding proteins had new characteristics, they were defined as the cluster 9 family of binding proteins by Dintilhac *et al.* (1997). These authors observed that competence of *adcABC* mutants of *Streptococcus pneumoniae* is Zn^{2+}-dependent. Mutants with mutations in the *adc*

Fig. 2. Sequence comparison of selected metal binding proteins (including the signal sequence, which may be the reason for the low similarity at the N-termini) of cluster 9 ABC permeases. The metal specificity of the binding proteins is not always clear; the existence of conflicting reports is indicated by ?. Identical residues are marked by an *, similar residues by ∧, and the positions involved in metal binding in the two known crystal structures of PsaA and TroA by #. Note the H-, D-, and E-rich domain in the Zn^{2+} binding proteins. Abbreviations of the organisms: HAEIN–*Haemophilus influenzae*; HAEDU–*Haemophilus ducreyi*; BACSU–*Bacillus subtilis*; STRPN–*Streptococcus pneumoniae*; STRGO–*Streptococcus gordonii*; YERPE–*Yersinia pestis*; SYNSP–*Synechocystis* sp.; TREPA–*Treponema pallidum*.

Fig. 2. Continued.

operon have a lower growth rate and are competence-defective during a stage after the interaction with the competence-stimulating peptide. Competence is restored after addition of Zn^{2+} to the chemically defined medium. The predicted AdcA sequence is similar to that of binding proteins of ABC transporters (22% identity to ZnuA) and has the characteristic N-terminal lipid anchor found in gram-positive bacteria. AdcB is similar to membrane components of ABC transporters (29% identity to ZnuB), and AdcC is an ATP binding protein (36% identity to ZnuC).

The PsaA protein (pneumococcal surface adhesin A) was identified as a second member of the cluster 9 proteins in *S. pneumoniae* (Dintilhac *et al.* 1997). The virulence and competence of *psaA* mutants are Mn^{2+}-dependent. Proteins closely related to PsaA have been detected in various caries-associated streptococci. The proteins FimA (fimbrial adhesin pre-

cursor) and ScaA (co-aggregation-mediating adhesin precursor) had been implicated in adhesion to fibrin monolayers, adhesion to saliva-coated hydroxyapatite, or aggregation with a *Streptomyces* strain, since mutants with mutations in the respective genes show a reduced adhesion and the proteins generated protective antibodies. A double function of these proteins as metal binding proteins and adhesins seems unlikely. Mutants with mutations in these transporters probably lose their fitness necessary for virulence and adhesion. Unfortunately, many homologues of these binding proteins are annotated in the databases as adhesins, which might be incorrect.

A crystal structure of the Mn^{2+}-specific binding protein PsaA from *S. pneumoniae* is available (Lawrence *et al.* 1998). The four amino acids H67, H139, E205, and D280 bind the metal. It has been noted by Lawrence *et al.* (1998) that in this structure, this site is occupied by Zn^{2+} in a tetrahedral coordination. Mn^{2+} is usually found in an octahedral geometry, which is difficult to reconcile with PsaA being a Mn^{2+} binding protein.

TroA is a metal binding protein in *Treponema pallidum* and also belongs to the cluster 9 family of ABC metal binding proteins. In the crystal structure, the four amino acids H68, H133, H199, and D279 bind Zn^{2+} (Lee *et al.* 1999). In Figure 2, it is obvious that these four amino acids are positioned in the places equivalent to those of the amino acids involved in binding in the PsaA structure. Lee *et al.* (1999) argue that the difference — E205 in PsaA and H199 in TroA — might be responsible for the substrate specificity of TroA for Zn^{2+} and PsaA for Mn^{2+} (Lee *et al.* 1999; Deka *et al.* 1999). However, the *troABC* genes encoding the metal ABC transporter are regulated by the DtxR homologue TroR, which only accepts Mn^{2+} and not Zn^{2+} (Posey *et al.* 1999), and the high similarity to binding proteins with Mn^{2+} specificity argue for a Mn^{2+} specificity of this transport system (Figure 2). Further research is needed to clarify this point.

A recent report on the closely related protein MtsA from *Streptococcus pyogenes* further challenges the situation. The binding protein MtsA shows more than 70% identity to PsaA, ScaA, and FimA, which have been shown to be manganese transporters. In contrast, recombinant MtsA does not show a specific interaction with Mn^{2+}, but does with Cu^{2+}, Zn^{2+}, and Fe^{3+} *in vitro*, and an *mtsA* mutant has a 50% lower iron content and a 30% lower Zn^{2+} content than the parent strain (Janulczyk *et al.* 1999). From these results, it has been postulated that the mtsABC genes encode a binding-protein-dependent transport system with a very broad metal specificity for Cu^{2+}, Fe^{3+}, and Zn^{2+}. Transport experiments with the mtsA mutant and the parent strain should verify the postulates.

The Yfe transport system (Figure 2) transports iron and manganese and also possesses an unusually low specificity (Bearden & Perry 1999). The cluster 9 family of binding-protein-dependent transport systems and the controversy surrounding their metal specificity has been extensively discussed by Claverys (2001).

Related Zn^{2+} binding-protein-dependent uptake systems in other bacteria have also been characterized. In *B. subtilis*, the *ycdHIyceA* genes are regulated by Zn^{2+} and the repressor Zur. Two lines of evidence suggest that the *ycdH* operon encodes a high-affinity Zn^{2+} transporter. First, a *ycdH* mutant is impaired in growth in low- Zn^{2+} medium. Second, mutation of *ycdH* alters the regulation of both the *yciC* (a gene regulated by Zn^{2+} and the repressor Zur) and *ycdH* operons such that much higher levels of exogenous Zn^{2+} are required for repression (Gaballa & Helmann 1998). In addition, the encoded proteins belong to the cluster 9 family of binding-protein-dependent ABC metal transporters.

The Zn^{2+} binding protein Pzp1 of *Hemophilus influenzae* was isolated because it is a homologue of the streptococcal FimA protein. Pzp1 was expected to be an adhesin (see above), but later studies indicated a periplasmic location. The recombinant protein purified from *E. coli* contains two Zn^{2+} ions per monomer. A pzp1 mutant was shown to be defective in growth. Suppression was achieved by addition of 100 μM Zn^{2+}, while no suppression was obtained with 100 μM Ca^{2+}, Mg^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+}, Mn^{2+}, or Fe^{3+} (Lu *et al.* 1997). Co^{2+} was not tested. A *znuA* mutant of *Haemophilus ducreyi* shows a strongly decreased virulence (Lewis *et al.* 1999).

The Pzp1 protein contains an extended region rich in histidine, aspartate, and glutamate residues, HHEHFHEDGDHDHDHK HEHKHDHKHD HDHDHDHKHE HKHDHEHHDH DHHEGLTTNW HVW, which may have a function in binding of Zn^{2+} or delivering Zn^{2+} to other proteins. It is interesting to note that only unambiguous Zn^{2+} binding proteins contain this H-, D-, and E-rich structure in front of the conserved HXW motif (position 171/173 of Pzp1 in Figure 2). H171 is in a Zn^{2+} binding position in the crystal structures of PsaA and TroA. Structural studies of Pzp1 in comparison to other ZnuA proteins with much smaller histidine-rich regions would be interesting.

Zur is a regulator of several high-affinity Zn^{2+} uptake systems

In order to identify the Zn^{2+}-dependent regulator of the znu genes in *E. coli*, constitutive mutants were isolated and tested for complementation by a gene bank of *E. coli*. A complementing gene, *yjbK* of the *E. coli* genome, was identified and named *zur* (for zinc uptake regulation). The Zur protein shows 27% sequence identity with the iron regulator Fur. High-affinity $^{65}Zn^{2+}$ transport of the constitutive zur mutant is tenfold higher than that of the uninduced parental strain (Patzer & Hantke 1998).

By inactivation of the *zur* gene, it has been demonstrated that Zur acts as a repressor and not as an activator. Some chromosomal mutant *zur* alleles have been sequenced to correlate the loss of Zur function with individual mutations. Wild-type and mutant Zur proteins have been purified to electrophoretic homogeneity. A considerable portion of the Zur mutant proteins, except ZurΔ46–91, accumulate in inclusion bodies. Wild-type Zur and ZurΔ46–91 form homo- and heterodimers. Dimerization is independent of metal ions since it also occurs in the presence of metal chelators (Patzer & Hantke 2000). Using an *in vivo* titration assay, the site affording Zur regulation was narrowed down to a 31-bp region in the promoter region of *znuA* and *znuCB*. This location was confirmed by DNase I footprinting assays. A single region comprising a nearly perfect palindrome is protected, which indicates direct binding of Zur. Zinc chelators completely inhibit DNA binding of Zur, and addition of Zn^{2+} in low concentrations enhances binding. Zur occupies its binding site only in the presence of Zn^{2+} or other divalent metal cations at low concentrations, as shown by DNase I footprinting. Zur protects a 29-nt approximate palindrome on each strand of the znu operator with a 3′ stagger of 4 nt (Patzer & Hantke 2000). This footprint resembles that of typical DNA binding dimers, such as classical helix-turn-helix proteins, e.g., the CI repressor from bacteriophage λ (Jordan & Pabo 1988). The observed 3′ stagger is indicative for coverage of the minor groove at the ends, but provides no information about the protein–DNA recognition contacts. Analysis of the mutant Zur proteins suggested an amino-terminal DNA contact domain around residue 65 and a carboxy-terminal dimerization and Zn^{2+}-binding domain. Footprinting experiments have indicated that although most of the mutant Zur proteins bind to the *znu* promoter *in vitro*, no protection is observed *in vivo* (Patzer & Hantke 2000).

Zur is active only in the reduced form. As a cytoplasmic protein, it has predominantly reduced thiols rather than oxidized disulfides due to the reducing conditions in the cytoplasm (Gilbert 1990). *In vitro*, the cysteine residues of Zur are easily oxidized to disulfides, as judged by the slower migration in SDS-polyacrylamide gels under reducing conditions compared to non-reducing conditions. Oxidized Zur does not bind DNA or considerable amounts of Zn^{2+}. This might indicate that Zn^{2+} is mainly bound by some of the nine cysteines found in Zur. In *E. coli* Fur, there are only four cysteines, all of which are conserved in Zur. At least two to three Zn^{2+} ions per dimer bind specifically to Zur (Patzer & Hantke 2000). It remains to be seen whether one of these Zn^{2+} ions is bound to the same site in Zur as was found in Fur at the conserved cysteines (positions 92 and 95; Jacquamet *et al.* 1998). These two cysteines are found in all Fur-like proteins except those from *Pseudomonas* and related organisms. The repressing activity of the Zur protein is Zn^{2+} specific since addition of Cd^{2+}, Hg^{2+}, Pb^{2+}, Mn^{2+}, Fe^{2+}, and Cu^{2+} led to a derepression of the Znu transport system *in vivo* (Patzer & Hantke 2000).

Zur, like Fur, seems to be widespread among bacteria, even in gram-positive bacteria and cyanobacteria, as indicated by sequence similarity searches. However, only in some cases has a functional Zn^{2+}-dependent regulator been demonstrated. Since small changes in the sequence might change the metal specificity, predictions always have to be examined. In *B. subtilis*, apart from Fur and PerR (regulator of the peroxide stress response) (Bsat *et al.* 1998), the third Fur-like homologue YqfV may act as Zur (Gaballa & Helmann 1998). The sequence similarity to *E. coli* Zur is not strong. A *zur* gene has also been found in *L. monocytogenes* (Dalet *et al.* 1999). Based on sequence similarity, the proposed Fur protein in *Staphylococcus epidermidis* (Heidrich *et al.* 1996) may be Zur rather than Fur. It is possible that other proteins designated as Fur homologues will turn out to be Zur proteins. In many of the partially sequenced genomes of various bacterial species, a Zur equivalent is found, e.g., in *Salmonella* strains, *Klebsiella pneumoniae, Yersinia pestis, Vibrio cholerae, Bordetella pertussis, Caulobacter crescentus, Pseudomonas aeruginosa*, and *Neisseria* strains. Since these organisms also possess a system homologous to Znu, it has been proposed that these proteins likewise are regulators of Zn^{2+} uptake. An alignment reveals two groups of Zur proteins — one is found in gram-negative bacteria, the second in gram-positive bacteria. Zur from

gram-positive bacteria is more similar than Zur from gram-negative bacteria to the *E. coli* Fur (Patzer & Hantke 2000). No evidence for autoregulation of Zur or for the influence of other regulators on Zur has been found. Zur has the very limited function of regulating Zn^{2+} uptake and metabolism in an environment poor in Zn^{2+}. To date, besides the *znuA* promoter, only two other Zur binding sites have been identified on the *E. coli* chromosome. No similarities to genes with known function have been found.

Low-affinity Zn^{2+} uptake systems

Little is known about low-affinity uptake systems for Zn^{2+} in *E. coli* or other bacteria. These systems are active at rich non-toxic concentrations of Zn^{2+} when *znuABC* is repressed by Zur. Cotransport of metals (van Veen *et al.* 1994) and Zn^{2+}(Beard *et al.* 2000) with phosphate via the Pit inorganic phosphate transport system has been observed. This system seems to be mainly responsible for the uptake of Zn^{2+}, but possibly also under certain conditions the efflux by metal exchange is catalyzed.

In *B. subtilis*, two systems have been found to be regulated by Zur and Zn^{2+}, the above-mentioned ABC transporter YcdHI, YceA, and the membrane protein YciC, which was assumed to be part of a low-affinity transport system for Zn^{2+} (Gaballa & Helmann 1998). YciC has similarity to nitrile hydratase activating proteins and may have a function in Zn^{2+} homeostasis and not in transport, but has not been studied further.

In *B. subtilis*, the metal–citrate uptake protein CitM has been shown to have a broad specificity for cotransport of Mg^{2+}, Ni^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}; however, Ca^{2+}, Ba^{2+}, and Sr^{2+} are recognized by the different but homologous transporter CitH (Krom *et al.* 2000). To which extent CitM contributes to the Zn^{2+} supply in the presence of citrate is not known.

ZraG and ZraH regulate the Zn^{2+} binding protein ZraP

In *E. coli* the periplasmic Zn^{2+} binding protein ZraP (former designation YjaI) had been postulated to be induced by the ZntR homologue PmtR from *Proteus mirabilis* (Noll *et al.* 1998). *E. coli* transformed with a PmtR-encoding plasmid grows better in the presence of 1 mM Zn^{2+} than the same strain transformed with only the vector. In addition, more ZraP is produced by the *pmtR*-containing *E. coli* strain. Recent studies by Leonhartsberger *et al.* (2001) indicate that the observed effects may be indirect. Their work has revealed that *zraP* is induced by the autoregulated two-component histidine kinase regulatory system ZraS/R encoded at a distance of 237 bp from *zraP*. ZraS, with the sequence signature of a sensor kinase, is found in the membrane fraction, and specific binding of the activator ZraR to the promoter region of *zraP* and *zraSR* has been demonstrated. Besides being a periplasmic Zn^{2+} binding protein, the function of ZraP is unknown. It might be a Zn^{2+} binding protein that protects periplasmic enzymes from high Zn^{2+} concentrations or it might be a sensor for the Zn^{2+} concentration in the periplasm which is reported to ZraS. ZraS then would act as a transmitter of the signal, and ZraR could induce an unknown protein in addition to ZraP and ZraS/R. The question remains whether other genes are regulated by ZraS/R.

Is EDDS a zincophore?

[S,S]Ethylenediamine disuccinic acid (EDDS) is produced by *Amycolatopsis orientalis* under Zn^{2+}-limiting conditions (Zwicker *et al.* 1998) and complexes Zn^{2+} and other divalent cations. EDDS is an isomer of the well-known chelator EDTA and is structurally related to certain siderophores (iron chelators). Siderophores are produced by bacteria and fungi under iron-limiting conditions. The siderophores bind Fe^{3+} specifically; these complexes are taken up by specific transport systems found in nearly every type of bacterium (Braun *et al.* 1998). Similarly, it is possible that *A. orientalis* uses EDDS as a zincophore to satisfy its Zn^{2+} demands. Experimental proof for this hypothesis is lacking. *E. coli* is unable to utilize Zn^{2+} bound to EDDS (Patzer & Hantke 1998).

Conclusions

Zinc transporters have been found in the following protein families: RND multi-drug efflux transporters, P-type ATPases, cation diffusion facilitators for export of the toxic metal Zn^{2+}, binding-protein-dependent ABC transporter, and phosphate or citrate metal cotransporters for the uptake of Zn^{2+} necessary for growth. It is remarkable that nearly every system is

regulated by its own regulator. In *E. coli*, three regulators — ZntR, ZraS/R, and Zur — are known to respond to Zn^{2+}. This is clearly different than the iron regulation where Fur (or DtxR in certain gram-positive bacteria) acts as a global regulator of many genes related to iron uptake and the oxidative stress response (Hantke & Braun 2000). Zn^{2+}-dependent regulators of export systems derepress or activate above 0.1 mM Zn^{2+}, while the Zur-like proteins derepress the uptake system below 10 μM Zn^{2+}.

It is astonishing that the reports on the metal specificity of different binding-protein-dependent ABC transporters found in pathogenic bacteria are so contradictory. In the crystal structures of PsaA and TroA, Zn^{2+} is found in the metal binding site, although *in vivo* observations and regulation indicate that Mn^{2+} is the main substrate (Claverys 2001). It is possible that these transporters are relatively unspecific because the host regulates its Me^{2+} concentrations within certain limits. This constant environment may allow an 'uncontrolled' uptake of different divalent cations by the pathogen. Since these systems have an important impact on the virulence of pathogens, this field will remain a subject of research and the results are eagerly awaited.

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