How do bacterial cells ensure that metalloproteins get the correct metal?

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Abstract | Protein metal-coordination sites are richly varied and exquisitely attuned to their inorganic partners, yet many metalloproteins still select the wrong metals when presented with mixtures of elements. Cells have evolved elaborate mechanisms to scavenge for sufficient metal atoms to meet their needs and to adjust their needs to match supply. Metal sensors, transporters and stores have often been discovered as metal-resistance determinants, but it is emerging that they perform a broader role in microbial physiology: they allow cells to overcome inadequate protein metal affinities to populate large numbers of metalloproteins with the right metals.

It has been estimated that one-quarter to one-third of all proteins require metals, although the exploitation of elements varies from cell to cell and has probably altered over the aeons to match geochemistry¹⁻³ (BOX 1). The proportions have been inferred from the numbers of homologues of known metalloproteins, and other deduced metalbinding motifs, encoded within sequenced genomes. A large experimental estimate was generated using native polyacrylamide-gel electrophoresis of extracts from ironrich Ferroplasma acidiphilum followed by the detection of metal in protein spots using inductively coupled plasma mass spectrometry⁴. The proportion of each proteome that requires iron, manganese, cobalt or zinc varies between the superkingdoms (Archaea, Bacteria and Eukaryotes), and within each kingdom the value tends to scale as a function of proteome size¹. Whatever the precise number of metalloproteins in any given cell, filling each one with the right metal is, literally, elemental to survival.

To understand the challenge associated with correctly populating each metalloprotein it is necessary to be familiar with the Irving–Williams series (sometimes also known as the natural order of stability for divalent transition metals)^{5,6}. Because proteins are flexible they offer imperfect steric selection between metals and this is especially true of nascent polypeptides. Under these conditions, affinities for metals have a tendency to follow a universal order of preference, which for essential divalent metals is the Irving–Williams series (Mg²⁺ and Ca²⁺ (weakest binding) < Mn²⁺ < Fe²⁺ < Co²⁺ < Ni²⁺ < Cu²⁺ > Zn²⁺).

Divalent (cupric) copper is highly competitive and is expected to bind tightly to metalloproteins, especially those containing sulphur and nitrogen ligands. Both monovalent (cuprous) copper, which is expected to predominate in a reducing cytosol, and trivalent (ferric) iron, which is expected to predominate in an oxidizing periplasm, are also highly competitive, as are several non-essential metals, such as cadmium, mercury and silver⁶ (BOX 2). How can a cell simultaneously contain some proteins that require copper or zinc and others that require uncompetitive metals, such as magnesium or manganese? In a most simplistic model in which proteins pick elements from a cytosol in which all divalent metals are present and abundant, all proteins would bind copper. Metals at the top of the stability series must be kept out of the binding sites for those lower down.

Research into the biology of metals in cells is at an interface between bioinorganic chemistry and cell biology. The roles of genes and proteins involved in metal homeostasis are commonly inferred using 'omics approaches: either predicted from genome sequences, recovered in screens for mutants with altered metal resistance, and/or detected as proteins or transcripts that change in abundance in response to a metal surplus or deficiency7-9. Atomic absorption spectrophotometry is used to quantify individual elements in mutant cells, extracts or individual proteins, whereas inductively coupled plasma mass spectrometry allows simultaneous detection of multiple elements and is highly sensitive. A range of biochemical and biophysical methods are used to visualize protein metal-binding sites and to uncover their physicochemical properties.

This Review summarizes mechanisms that bacterial and archaeal cells are presumed to use to restrict competitive metals to the correct proteins, together with the homeostatic proteins involved in these processes.

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Box 1 | Diversity of metalloproteins

Iron is prevalent in proteins that are involved in electron transfer and oxygen metabolism, in the form of haem or iron-sulphur clusters, and in a small subset of proteins (including ribonucleotide reductase and soluble methane monooxygenase) that directly bind one or two atoms of iron without any associated tetrapyrrole or inorganic sulphide⁶. In contrast to iron, zinc has a single oxidation state in solution and therefore is not a conduit for electrons, but rather is exploited to organize protein structure, as in zinc fingers, or (when coordinated to three rather than four protein ligands) to drive catalysis by acting as a Lewis acid. RNA and DNA polymerases require zinc, and zinc also associates with ribosomes, often representing a substantial proportion of the total cellular zinc quota. Unlike the other metals considered here, magnesium is a bulk element (found at millimolar concentrations) that is often weakly associated with proteins and is generally coordinated in the protein complex by ATP or ADP. Magnesium is required, for example, by the active sites of DNA polymerases, in ATPases and kinases, to polymerize actin, and in photosynthetic cyanobacteria, for chlorophyll. The remaining metals are required by fewer different proteins, and generally represent less than 30% of the total number of metalloproteins (less than 10% of all proteins). Nonetheless, some of these proteins are abundant. Cobalt is predominantly incorporated into vitamin B₁, in enzymes, whereas nickel is restricted to nine known enzymes, including urease, hydrogenase and some superoxide dismutases. Manganese is an obligatory cofactor in some other superoxide dismutases and has a special role in oxygen-evolving photosynthetic bacteria for the water-splitting enzyme. Molybdenum is incorporated into the molybdopterin cofactor, Moco, which is required in sulphite oxidases, nitrogenases and nitrate reductases. Finally, copper is used in the Cu, and Cu, sites of cytochrome oxidase and several periplasmic enzymes. There has been debate about whether copper needs to enter the bacterial cytosol⁸⁸. Copper is required for the cytoplasmic enzymes plastocyanin, in cyanobacteria, and particulate methane monooxygenase, in methanotrophs, but both of these proteins are localized within internal membrane structures. The multi-copper oxidase CueO is thought to acquire copper in the cytoplasm, and the cytoplasmic pathway for molybdopterin biosynthesis is known to require copper in other organisms and is also predicted to do so in bacteria and archaea^{89,90}. Proteins can require combinations of more than one metal; for example, iron plus nickel, haem-iron plus copper, and iron plus Moco⁶. Molybdenum can be replaced by vanadium in nitrogenases, and tungsten can be used in aldehyde oxidoreductase, formate dehydrogenase and acetyl hydratase, in a few selected organisms, mostly Archaea. The book by Fraústo da Silva and Williams⁶ that describes the biological chemistry of the elements is encouraged reading for those wanting greater insight into the wide-ranging uses of metals in proteins and the bioavailability of metals in different environments.

Metal availability over geological time (and in different habitats) has been influenced by the rise in dioxygen, coincident with the emergence of oxygenic photosynthesis. Under aerobic conditions, iron in its ferric form is sparingly soluble and small amounts are maintained in solution tightly bound to organic ligands (for example, in association with siderophores). By contrast, in anaerobic environments and at low pH, ferrous iron becomes soluble. Particulate sulphides of metals such as zinc and copper are oxidized under aerobic conditions and these metals therefore become soluble and more available as dioxygen increases. The ability of microorganisms to acquire metals from plants and animals is often restricted by tight metal associations with host organic ligands. In the oceans, different organic complexes of cobalt, zinc, iron and copper influence the bioavailability of elements to different species and control community structure. Nickel is an example of a metal that is used by many microorganisms, but not by humans.

Some of the observations are pertinent to yeast and other microbial eukaryotes, but these microorganisms are not the main focus of this article. The mechanisms are generic, but a smaller complement of homeostatic proteins is currently known in archaea compared with either Gram-positive or Gram-negative bacteria. The fidelity of the homeostatic proteins themselves is at the top of a hierarchy of metal selectivity, as their specificity is now proposed to influence metal occupancy of other proteins¹⁰. Finally, we consider how these proteins discern metals. We note that few studies have explicitly set out to test the mechanisms by which cells overcome the limitations of coordination chemistry to correctly populate metalloproteins, a challenge that perhaps requires a systems-based approach.

Metal selection by folding location

To directly explore how a cell overcomes the challenge imposed by the Irving–Williams series, the most abundant copper and manganese proteins were recently identified in the periplasm of a cyanobacterium¹¹. This organism was chosen because it has a high demand for both copper and manganese, which are required for proteins that are involved in photosynthesis. Copper and manganese were chosen because they lie at opposite extremes of the stability series and therefore selectivity should be especially demanding. Unexpectedly, the identified proteins, MncA for manganese and CucA for copper, were both cupins (β -barrels) with identical sets of metal ligands. Consistent with the Irving-Williams series, a 10,000-fold molar excess of manganese was needed relative to copper to fill the MncA barrel with manganese when folding occurred in vitro. How can this be achieved in vivo when an equivalent site in CucA must fill with copper? The answer is that MncA is a Tat (twin-arginine translocase) substrate that folds in the cytoplasm to acquire manganese, whereas CucA is a Sec (general secretory pathway) substrate that folds in the periplasm: the localization of folding within the cell permits different ligands to bind (FIG. 1). Once folded, the manganese site of MncA is buried and the metal becomes trapped inside the protein with a negligible off rate, and therefore MncA can subsequently coexist with CucA because its metal is now impervious to replacement by more competitive elements. This exemplifies how a cell can overcome the inherent metal-binding preferences of proteins and highlights the importance of compartmentalizing metals to maintain a cytoplasm in which copper is either absent or tightly buffered and bound.

Lewis acid

A chemical that can accept a pair of electrons from a Lewis base.

Box 2 | Non-essential metals in proteins

Mercury and cadmium are both highly thiophilic and are therefore liable to out-compete zinc, especially for protein-binding sites that contain cysteine and thus inactivate zinc-requiring proteins⁶. Resistance determinants for these metals have been isolated from plasmids, and transposable elements of organisms that have been recovered from environments that are subject to anthropogenic metal pollution. Several gene families, the chromosomal relatives of which are associated with the management of essential metals, were first discovered as resistance determinants for non-essential metals; for example, MerR (mercury sensing; first detected DNA-under-winding transcriptional activator⁹¹) and CadC (cadmium exporting; first detected P₁-type ATPase²⁴). Cadmium can replace zinc in carbonic anhydrase of the diatom Thalassiosira weissfloqii, and it is plausible that an enzyme might have evolved to function with cadmium in prokaryotes from cadmium-polluted, zinc-deficient habitats⁹². Silver and gold are non-essential metals from the same triad in the periodic table as copper and can inactivate copper proteins. Silver resistance determinants, such as the sil operon, become problematic if silver is extensively used for its antimicrobial properties (for example, on catheters)93. Remarkably, resistance determinants for oxyanions of the toxic metalloid arsenic are widely distributed throughout prokaryotic genomes⁹⁴.

MncA required at least as large an excess of manganese relative to zinc to acquire manganese *in vitro*, which also revealed the importance of tight control over the number of atoms of zinc in the cytoplasm¹¹.

Relative affinities for metals

Cells restrict the numbers of metal atoms within the cytoplasm such that metals do not compete with other metals for a limited pool of proteins, but rather each protein competes with other proteins for a limited pool of metal¹⁰. Metal occupancy of metalloproteins thus becomes, in part, a function of the relative metal affinities of the different proteins rather than their absolute metal affinities (BOX 3). But how does the cell discern the different metals and control their effective intracellular concentrations?

Metal transporters. A balance between the actions of metal-specific importers and exporters can dictate how many atoms of each metal accumulate¹². In Gramnegative bacteria, metals probably diffuse across the outer membrane through porins, such as outer-membrane protein F (OmpF)13. Energy-coupled importers are also active for inorganic elements (notably iron) that are acquired in complex with, for example, siderophores, which are too large for porins. These siderophore complexes exploit β-barrel outer-membrane pores, such as FhuA (ferric hydroxamate uptake) and FecA (ferric citrate uptake), which are energized by TonB, a protein that straddles the periplasm¹⁴. At the cytoplasmic membrane, the metal transporters, some for import and others for export, include ABC (ATP-binding cassette)type ATPases¹⁵, P₁-type ATPases¹⁶, RND (resistance and nodulation) proteins¹⁷, cation diffusion facilitator (CDF) proteins¹⁸, NiCoT (Ni and Co transporter) proteins^{19,20}, CorA (Co resistance)²¹, NRAMP (natural resistance associated with macrophage protein)²² and ZIP (Zrt/ Irt-like protein)-family transporters²³.

ABC-type ATPases selectively import manganese, zinc, nickel or iron across the cytoplasmic membranes of Gram-negative and Gram-positive bacteria, aided by substrate-binding proteins that are located within the

periplasm in Gram-negative bacteria and are sometimes associated with the plasma membrane¹⁵. P,-type ATPases are mostly metal exporters, but a few are metal importers. P₁-type ATPases are widespread and have also been described in archaea. They generally possess one (or more) cytosolic metal-binding domain that contains a pair of metal-coordinating cysteine residues within a ferredoxin-like fold^{24,25}. In some cases, these domains interact with specific cytosolic copper-binding proteins known as metallochaperones. An interaction with a metallochaperone occurs in the handful of P,-type ATPases that are thought to be associated with copper import, and in these cases the metal-binding domain is presumed to pass metal to the metallochaperone²⁶. For the exporters, the vector for metal transfer is the reverse: the metal is transferred to the ATPase metal-binding domain. A metallochaperone has also been shown to directly donate copper to the transmembrane metal-binding sites of a P₁-type ATPase, thereby bypassing the cytosolic metal-binding domains²⁷. Intriguingly, a ferredoxin-like fold was also recently determined for the soluble domain of a CDF zinc exporter called ferrous-iron efflux pump F (FieF; also known as YiiP)28. CDF proteins drive the efflux of surplus transition metals from the cytoplasm, probably through proton antiport, but the importance of the common fold in their soluble domains remains to be discovered¹². RND proteins mediate the efflux of metals, such as cobalt, zinc, nickel or copper, across the outer membranes of Gram-negative bacteria. Each genome contains some complement of these assorted classes of metal transporters, as illustrated for Escherichia coli in FIG. 2 and reported elsewhere²⁹. As the divalent species (plus monovalent copper) of the respective metals become more competitive, on moving up the Irving-Williams series, it is anticipated that the number of atoms must match the number of correct binding sites with increasing precision to avoid the displacement of metals further down the series. In *E. coli*, this correlates with an increasing emphasis on the exporters of surplus metal ions relative to importers (FIG. 2).

Metal-responsive transcription. Transcription of the genes that encode the metal transporters is commonly under the control of metal sensors. Seven families of soluble metal sensors have been identified³⁰⁻³⁵ (FIG. 3). In addition, there are multiple two-component metal-sensing histidinekinases and response regulators³⁶, some individual sensors, such as the molybdate regulator ModE37,38, and IscR, which detects iron-sulphur cluster status. Our understanding of metal-responsive transcriptional control is thus exceptionally well advanced in prokaryotes compared with eukaryotes, for which only a relatively small number of metal-sensing transcriptional regulators are known, mostly in yeast. Representatives of several of the families of metal sensors have also been described in archaea. Although some of the metal sensors regulate only a single metal transporter, others control regulons that have been discovered from transcription profiling in mutants, together with bioinformatics searches of genomes for known DNA-binding sites³⁹⁻⁴¹. Different representatives of

Thiophilic

A strong binder to thiol sulphurs.

Porin

A β -barrel protein that allows the diffusion of molecules (up to ~ 1.5 kDa) across the outer membrane.

Antiport

Coupled transport of two molecules in opposing directions.



Figure 1 | Localization-dependent ligand binding and trapping through folding. The most abundant copper and manganese proteins in the periplasm of the cyanobacterium Synechocystis sp. PCC 6803 are both cupins, CucA and MncA, respectively, that bind their respective metals using identical sets of ligands. Both CucA and MncA bind copper in preference to manganese in vitro, but MncA is a Tat (twin arginine translocase that exports folded proteins) substrate, which allows MncA to fold in the cytoplasm where it entraps manganese. Once the metal is bound inside the folded protein, there is a negligible off rate. By maintaining a cytoplasm in which competitive metals are tightly bound and buffered, it becomes possible for proteins to acquire uncompetitive metals, such as manganese. CucA is a Sec (general secretory pathway) substrate that folds in the periplasm after export and acquires the more competitive metal copper. RR indicates the twin arginine motif in MncA. Figure is modified, with permission, from Nature REF. 11[®] Macmillan Publishers Ltd. All rights reserved.

each of the sensor families can regulate gene expression in response to different metals (TABLE 1), and one of the ongoing challenges is to determine which metal (or metals) each homologue detects within its respective organism. The residues that form the sensory metal-binding sites have been defined in some of these proteins by detecting loss of perception in site-directed mutants coupled with in vitro assays of metal binding and by solving structures. For example, eight distinct metal-sensing motifs (and one non-metal-sensing motif) have been identified in ArsR (arsenical resistance operon repressor)-SmtB proteins⁴². These motifs correlate with the perception of different elements, and have been designated α 3, α 3N, α 5, α 5C, α 3N α 5, α 5-3, α 4C and α 3C based on the locations of the sensory residues within known or predicted protein folds⁴². Remarkably, the iron-sensing sites of the first identified and probably most widely known metal regulator, Fur (ferric uptake regulator), remain poorly defined, partly owing to the complications caused by the additional presence of structural zinc sites. We note that Fe-Fur most commonly functions by repression but can also directly activate transcription on some promoters⁴³. Fe-Fur also acts indirectly in the regulation of some genes by modulating the expression of RyhB small RNA (sRNA)44. The RyhB anti-sense sRNA anneals to target mRNAs to repress translation. Finally, expression of the magnesium transporter MgtE in *Bacillus subtilis* is regulated by a riboswitch, the M box⁴⁵. When the magnesium concentration is sufficient, MgtE binds to this ribonucleotide sequence, thereby exposing a terminator structure that halts transcription in the leader region.

Each sequenced genome generally contains representatives of several of these sensor families, as illustrated for E. coli in FIG. 3. Although the roles of regulators in sensing specific metals are commonly inferred from protein-sequence similarity, this is often a poor predictor of the metals detected. For example, ArsR-SmtB proteins have been separated into eight clades based on overall sequence similarity, but these clades do not correlate with the eight sensory motifs⁴². Multiple sensory motifs are found in most clades and most of the motifs occur in several clades. The implication is that there has been extensive convergent and/or concerted evolution, and the outcome is that microbiologists are being misled into assuming roles for sensors in the detection of the wrong metals (for example, a sensor could be presumed to detect iron based on sequence similarity, but could in fact be a manganese sensor). Bioinformatics searches using metal-sensing motifs, if known, provide more accurate predictions of which metals are detected.

The metal affinities of metal sensors are particularly important. These values are presumed to define the boundaries between metal sufficiency and metal excess or deficiency. Accordingly, the manganese sensor MntR has a 10⁻⁵ molar manganese affinity whereas the MgtE riboswitch of *B. subtilis* has a 10⁻³ molar magnesium affinity; these values match the estimated ~10 micromolar and ~ 1 millimolar cytoplasmic concentrations of the respective metals⁴⁵⁻⁴⁷. The *E. coli* nickel sensor NikR has two binding sites, which have nickel affinities of 10⁻¹² molar and 10⁻⁷ molar. Occupancy of both metal sites gives a tighter DNA affinity than occupancy of only the high-affinity metal site. This suggests that two levels of gene regulation depend on the metal concentration³⁰. The two zinc sensors of *E. coli*, <u>Zur</u> and <u>ZntR</u>, have zinc affinities of 10⁻¹⁵ molar⁴⁸. At concentrations greater than femtomolar Zn-Zur represses genes that encode an ABC type ATPase for zinc import, whereas ZntR activates expression of a P₁-type ATPase for zinc export. This implies that all cytoplasmic zinc is bound and buffered to this low value and any surplus is expelled. In this way, zinc can be withheld from weaker metal-binding sites. The copper affinity of the E. coli copper sensor CueR, which regulates a copper-exporting P₁-type ATPase and the exported multi-copper oxidase CueO, was estimated to be 10⁻²¹ molar (zeptomolar)⁴⁹. Again, this implies that all cytoplasmic copper is bound and buffered to an extremely low value. By restricting the effective concentration of the competitive metals at the top of the Irving-Williams series, weaker metal-binding sites remain available to less-competitive inorganic ions.

Controlling the number of protein ligands. Metal sensors modulate the number of ligands for some metals in addition to controlling accumulation of the metals. For example, surplus atoms of iron are stored in an oxidized

Box 3 | Troublesome metal affinities

Our knowledge of the relative metal affinities of metalloproteins is not ideal. Polyhistidine-tagged metalloproteins have sometimes been erroneously used to study metal-binding properties of a protein in vitro. It is not always easy to measure the metal affinity of a protein with any degree of accuracy. For example, published K₄ values (association constant) vary by up to ten orders of magnitude for some known cuprous, cupric or cobalt protein interactions^{95–99}. Pitfalls arise from assays that are performed without any free metal remaining in solution during titrations, invariably owing to the lack of any effective competition with a metal buffer. Reported values of 10⁻⁶ molar are in fact often only minimal values that are dictated by the lowest protein concentration at which metal binding can be accurately monitored. When competing chromogenic (colour producing) ligands are used, the ability of the small molecule to form adducts with the protein is often not appreciated. It is also common for the conditions of the assay to be imperfect owing to unnoticed competition from buffer solutions (dithiothreitol, Tris or even overlooked EDTA), protonation of ligands owing to use of an unsuitable pH and, commonly, oxidation of Cys ligands because anaerobic conditions were not used. To better understand how cells operate as systems to restrict metals to the correct proteins, there is a need for more widespread, and more precise, enumeration of protein metal affinities both for the metals the proteins bind in vivo and, perhaps especially, the ones they do not.

> form within ferritins (TABLE 1). The E. coli ferritin genes, bfr and ftnA, are controlled by Fe-Fur-regulated expression of RyhB50. Some cyanobacteria and Pseudomonas species express metallothioneins^{51,52}, under the control of zinc sensors, such as SmtB53, to tightly bind and sequester surplus atoms of zinc. Such small genes encoding poorly conserved metallothioneins (proteins with low sequence complexity) might be hidden within other bacterial and archaeal genomes. A previously cryptic copper metallothionein gene has recently been discovered in several species of pathogenic Mycobacteria⁵⁴. By producing a sequestration protein, the metals are withheld from other proteins that must be occupied by less-competitive ions. Transcription profiles have also shown that E. coli switches metabolism to minimize the number of ironrequiring proteins that are expressed when iron is less abundant55. Conversely, metabolism can also be switched to exploit a metal that has become plentiful. This is exemplified by the replacement of iron-requiring, soluble methane monooxygenase with the copper-requiring particulate enzyme in methanotrophic bacteria^{56,57} and the substitution of haem-iron-containing cytochrome c_c with copper-containing plastocyanin for photosynthetic electron transport in cyanobacteria⁵⁸, in both cases in response to elevated copper levels.

> *A note about protein folding*. An association with a metal cofactor enables folding in many enzymes; for example, manganese–MncA¹¹. Provided folding discerns the right metal, it becomes possible for only the correct metal to be entrapped, with a negligible off rate. According to this scenario, excess competitive metals have the potential to interfere with folding by reducing the folding rate.

Metal-insertion by delivery proteins

Unexpectedly, when the responses of the nickel sensor NikR were tested against changes in exogenous nickel, NikR was found to be unaffected by deletion of the NikA–E importer⁵⁹. This seems peculiar given that hydrogenase, one of the rare examples of a nickelrequiring enzyme, is completely inactive in *E. coli* without NikA–E because it cannot acquire nickel. The implication is that NikA–E nickel supply is 'hard-wired' to hydrogenase whereas NikR is supplied by an alternative route⁵⁹. It seems that the nickel ions are not simply released into the cytoplasm, but are trafficked to the correct protein destination.

Genetic analyses in microorganisms have uncovered classes of proteins that are essential for metal-cofactor insertion into specific metalloproteins during biosynthesis. Some of these pathways lead first to the synthesis of organic metal cofactors, such as molybdopterin and tetrapyrroles (for example, iron in haem, cobalt in cobalamin, magnesium in chlorophyll and nickel in F_{430}), but others pass metals directly to metalloproteins with no intermediate. These pathways transfer metals to their correct destinations through sequential ligand-exchange reactions. Under these conditions, the specificity of protein-protein interactions determines which apoprotein receives the metal. Conformational change in the holoprotein following metal insertion means that off rates can be negligible and thus the metal becomes kinetically trapped inside the correct enzyme.

Nickel metallochaperones. Hydrogenases liberate hydrogen gas to dissipate the surplus-reducing potential under anaerobic conditions. These enzymes are currently receiving attention owing to the potential to exploit hydrogen as an alternative bioenergy source, especially if expressed in phototrophic microorganisms and therefore coupled to photosynthesis60. The large subunit of hydrogenase contains a unique nickel-iron active site that is generated by an elaborate metal-insertion and protein-processing pathway^{61,62} (FIG. 4a). The gene names vary between organisms and some organisms possess multiple enzymes. The small subunit of hydrogenase also requires extensive processing and must acquire multiple iron-sulphur clusters. The large subunit binds to a chaperone, HypC (hydrogenase accessory protein), that is thought to modify the protein to gain access to its active site and to introduce iron. Prior to this step, HypC itself must undergo a series of interactions with HypD to acquire iron and then with HypE and HypF to acquire one carbon monoxide and two cyanide ligands. Nickel is inserted into the large subunit by a dedicated metallochaperone that is composed of two proteins, HypA and HypB, in a step that consumes GTP. In E. coli, but not in all hydrogenase-containing bacteria, HypB also interacts with a peptidyl-prolyl isomerase (SlyD) with a carboxy-terminal region that is rich in metal-binding histidine and cysteine residues⁶². SlyD influences cellular nickel accumulation and hydrogenase metallocentre assembly, and is thought to act as a nickel reservoir. The HypAB or HypAB-SlyD complex obtains nickel from the NikA-E importer, and the implication is that this occurs directly, through ligand exchange, rather than indirectly, through nickel release to the cytosol⁵⁹. After nickel insertion into the large subunit, the HypC chaperone dissociates and the hydrogenase undergoes C-terminal proteolytic processing before the mature protein binds to the hydrogenase small subunit.

Metallothionein

A small, cysteine-rich metal-binding protein.

Apoprotein A protein without a metal cofactor.

Holoprotein

A mature protein with a metal cofactor.



Figure 2 | **The complement of metal transporters in Escherichia coli.** The number of atoms of each metal in a cell is a function of net influx and efflux, which is mediated by specific metal importers and exporters. Each organism has a different complement of transporters with different types of transport proteins that handle the various metals in different species. CDF, cation diffusion facilitator; Cir, colicin I receptor; CusA–D, copper-sensitive operon; EfeU, elemental ferrous iron uptake; Feo, ferrous iron-uptake system; Fep, ferric enterobactin-transporting protein; Fiu, ferric iron uptake; NRAMP, natural resistance associated with macrophage protein; RND, resistance and nodulation; ZIP, Zrt/Irt-like protein; Znu, zinc uptake; ZupT, zinc-uptake transporter.

Urease hydrolyses urea to carbamate and ammonia, which can serve as a nitrogen source to allow bacteria to grow on urea precursors. Urease is most notably essential for virulence of Helicobacter pylori in gastroduodenal infections, and is therefore associated with pyloric ulcers63. Urease (which is composed of UreA-C) contains pairs of nickel atoms that are deeply buried within the enzyme, where they are inaccessible to competing ligands. Nickel is delivered to an apo-urease complex by a metallochaperone, UreE. Consistent with the nickel demand of the urease trimer, the UreE dimer in Klebsiella aerogenes binds six nickel atoms, whereas in Sporosarcina pasteurii the UreE dimer binds only one nickel atom^{64,65}. A UreA-C apoprotein complex first associates with the accessory proteins UreD, UreF and UreG, before being carbamylated. UreE then inserts nickel in a GTP-dependent step (FIG. 4b), and the accessory proteins dissociate to release the active holoenzyme with its nickel atoms.

Iron-sulphur cluster biogenesis and insertion. It has been estimated that approximately 5% of all *E. coli* proteins require iron-sulphur inorganic clusters⁶⁶. The clusters are assembled on a protein scaffold using sulphur donated by enzymes that liberate the element from cysteine^{66,67}. Iron can be delivered by homologues of the eukaryotic protein frataxin, or <u>CyaY</u> in *E. coli*, although CyaY is not essential under normal

growth conditions (FIG. 4c). Three operons are involved in cluster biosynthesis, *nif* (nitrogen fixation), *isc* (iron-sulphur cluster) and *suf* (sulphur assimilation), and some components have analogous roles but are adapted to adverse conditions, such as oxidative stress. Additional proteins subsequently assist in transferring the clusters to the correct holoenzymes. A key finding in relation to the theme of this Review is that cobalt toxicity in *E. coli* results from the inactivation of pathways that depend on iron-sulphur cluster proteins⁶⁸. This is because cobalt (a metal further up the Irving–Williams series) replaces iron in the clusters during their vulnerable assembly phase. Once the clusters are inside the holoprotein they become protected from cobalt⁶⁸.

Copper metallochaperones. Copper homeostasis in *Enterococcus hirae* involves two P_1 -type ATPases, <u>CopA</u> and <u>CopB</u>, a DNA-binding repressor, <u>CopY</u>, and <u>CopZ</u>, a small soluble protein that shares the same ferredoxin fold as the cytosolic metal-binding domains of the ATPases²⁶. CopZ is a copper metallochaperone that interacts with the CopA copper importer and the CopY regulator (FIG. 4d). Donation of copper from CopZ to CopY is consistent with impaired copper detection by the sensor in CopZ mutants, and has been confirmed by *in vitro* assays of metal transfer between the purified proteins^{69,70}. Many prokaryotes contain similar



Figure 3 | **The complement of metal sensors in** *Escherichia coli*. The number of atoms of each metal in a cell is under the control of metal sensors that detect excess or deficiency of specific metals and regulate the expression of metal transporters. These sensors can also modulate the number of ligands for each metal by controlling the production of metal-sequestering proteins and switching metabolism to adjust the demands of metalloenzymes. The mode of action of each regulator is indicated by arrows away from the DNA (metal derepression), towards the DNA (metal corepression) or twisting of the DNA (metal-dependent activation by DNA under-winding), as well as two-component phosphotransfer (Pi). Metal specificities are indicated and the colours reflect the regulator families: MerR (mercury sensing; blue), ArsR–SmtB (light pink), Fur (ferric uptake regulator; red), two-component regulators (light blue), DtxR (diphtheria toxin repressor; dark green), CsoR–RcnR (orange), NikR (grey) and ModE (light pink). RyhB is a regulatory RNA that is under the control of Fur. Different organisms contain different complements of sensors, and the types of sensors that detect each metal vary between species. *K* represents the affinity for the sensed metal.

open reading frames to CopZ, although some of the homologues are periplasmic proteins that are involved in mercury resistance⁷¹. In B. subtilis, CopZ donates copper to a copper exporter, and in Synechocystis sp. PCC 6803 a related copper metallochaperone, Atx1 (antioxidant 1; a yeast homologue), interacts with two copper-transporting P₁-type ATPases to supply copper to plastocyanin^{72,73}. Nuclear magnetic resonance (NMR) solution structures of the B. subtilis and cyanobacterial copper chaperones in complex with the transporters have revealed structural changes during docking that trigger metal release^{74,75}. This release involves the displacement of a histidine imidazole ligand away from the copper coordination sphere in Atx1 (REF. 75). Tyrosinases contain a pair of copper atoms at the active site. A second protein, the tyrosinase cofactor MelC1, is required for the correct expression

of tyrosinase⁷⁶. Genetic and biochemical studies have established that MelC1 forms a heterodimer with apotyrosinase and serves as a metallochaperone for the incorporation of copper into the active enzyme.

Metal discrimination by homeostatic proteins

How do the homeostatic proteins themselves break free from the constraints of metal affinity? For the correct metal to be passed to a nascent polypeptide by an accessory protein, such as a metallochaperone, the accessory protein must somehow acquire the correct metal in the first place. Similarly, metal sensors must discern the elements if they are to control the number of available metal atoms and the number of bona fide protein ligands.

Elaborate features of the primary and secondary coordination spheres of the homeostatic proteins impose biases in favour of the correct metal, and redox

Table 1 Families	s of cyto	solic metal sensors and stores	
F 1	M / 1		

Founder	Metal	Additional representatives	Metals
Sensors*			
Fur	Fe	Zur, Mur and Nur	Zn, Mn and Ni
DtxR	Fe	IdeR, MntR, ScaR and SirR	Fe, Mn, Zn and Cd
NikR	Ni	None	None
MerR	Hg	ZntR, CueR, PbrR and CoaR	Zn, Cu, Pb and Co
ArsR–SmtB	As–Zn	CadC, CmtR, CzrA, NmtR and KmtR	Cd, Cd, Co, Ni and Ni
CsoR–RcnR	Cu–Ni	None	None
СорҮ	Cu	None	None
Stores [‡]			
Ferritins	Fe	Bfr, FtnA and Dps	None
Metallothioneins	Zn	SmtA and BmtA	None

*Different prokaryotes contain different complements of each of the seven families of sensors. Representatives of all of the families have been documented in Gram-positive and Gram-negative bacteria, but only a subset have been described to date in archaea. Fur, DtxR and NikR family proteins are co-repressors that bind to DNA after binding metal. At a few promoters, Fe-Fur directly functions as an activator. MerR family proteins are activators that after binding metal under-wind a suboptimally spaced promoter region to more optimally align RNA-polymerase recognition sequences. ArsR-SmtB, CsoR-RcnR and CopY are derepressors that have impaired binding to DNA after binding metal, and thus metal association alleviates repression. Two-component regulators that detect metals are not listed. [‡]Metal-regulated stores for surplus atoms. Unlike more standard ferritins, such as FtnA, Bfr (bacterioferritin) contains haem. Dps may protect DNA from reactive oxygen species rather than storing iron. ArsR, arsenical regulator; BmtA, bacterial metallothionein; CadC, cadmium resistance regulator: CmtR. cadmium Mycobacterium tuberculosis regulator: CoaR. cobalt-transporting ATPase regulator; CopY, copper P-type ATPase regulator; CsoR, copper-sensitive operon regulator; CueR, copper efflux regulator; CzrA, cobalt and zinc resistance regulator; Dps, DNA protection during starvation; DtxR, diphtheria toxin regulator; FtnA, ferritin A; Fur, ferric-uptake regulator; IdeR, iron-dependent regulator; KmtR, nickel M. tuberculosis regulator; MerR, mercury responsive regulator; MntR, manganese transport regulator; Mur, manganese-uptake regulator; NikR, nickel-responsive regulator; NmtR, nickel M. tuberculosis regulator; Nur, nickel-uptake regulator; PbrR, lead-responsive regulator; RcnR, regulation of cobalt and nickel repressor; ScaR, Streptococci coaggregation-mediating adhesion regulator; SirR, Staphylococcal iron-regulator repressor; Smt, Synechococcus metallothionein regulator; ZntŘ, zinc-transport regulator; Zur, zinc-uptake regulator.

d–*d* transition

An electronic transition between *d*-orbitals in a metal atom.

Winged helix-turn-helix structure

A structure found in many DNA-binding proteins with wings formed by a pair of β -strands.

Tetrahedral geometry

Four atoms are arranged around a central atom, thereby defining the vertices of a tetrahedron.

Octahedral geometry

Six atoms are symmetrically arranged around a central atom, thereby defining the vertices of an octahedron. changes can introduce additional selectivity as the metal is passed from favoured ligands for one oxidation state to the favoured ligands for another, a paradigm for high-affinity iron uptake in yeast. Metal selection thus becomes the sum of multiple partitioning events. Here we outline the potential mechanisms of metal selection — most notably allostery and access — which are not the products of absolute metal affinity. An implication is that proteins of metal homeostasis that exploit factors other than absolute metal affinity enable the entire cell to escape from limitations imposed by universal orders of metal preference.

Metal-specific allosteric change. A DNA-binding transcriptional repressor, NmtR, that detects surplus nickel and cobalt in *Mycobacterium tuberculosis* was found to have a tighter affinity for zinc, which it does not detect *in vivo*, than for either of the metals which it does detect⁷⁷. Site-directed mutagenesis inferred that the metal-sensing site of NmtR is composed of six metal-binding residues⁷⁷. The related protein SmtB has the opposite metal specificities — it detects zinc but does not detect nickel^{51,77} — and in SmtB only four binding residues were identified from the same type of analysis⁷⁸. NmtR and SmtB are predicted to

have similar winged helix-turn-helix structures, and their metal-sensing residues are located in the same regions of the respective folds (at a dimeric interface formed between anti-parallel C-terminal α -helices). When metals bind to proteins they often alter the spectral properties of the molecule, and this is especially true of cobalt. Depending on the coordination geometry and the types of ligands, the cobalt-protein complex either absorbs strongly or weakly at diagnostic wavelengths. Cobalt-NmtR absorbs weakly in the region of 500–700 nm (owing to *d*-*d* transitions), whereas cobalt-SmtB absorbs strongly. This implies that cobalt in SmtB binds to four residues in a tetrahedral geometry, whereas cobalt in NmtR binds to six residues in an octahedral geometry⁷⁷. Zinc-protein complexes do not provide such features in ultravioletvisible spectra, but zinc-binding sites can be explored using X-ray absorption spectroscopy, and this method established that zinc binds to NmtR using only four of the six metal-sensing residues⁷⁹. Although zinc binds to NmtR tightly, it does so in a different manner to cobalt and nickel. Zinc is thus ineffective at triggering the allosteric mechanism and therefore fails to alleviate repression in vivo. Nickel and cobalt are sensed because they bind in their preferred octahedral geometry, which is necessary to cause the conformational change in the sensor that impairs its binding to DNA77,79.

MntR⁸⁰, Fur⁸¹ and NikR⁸² have all been shown to bind metals with an order of affinity that is similar to the Irving-Williams series, and yet they sense different metals: manganese, iron and nickel, respectively. Metals such as nickel, zinc and cobalt, all of which bind to MntR more tightly than manganese, do not induce the correct allosteric change⁸⁰. The structure of zinc-NikR is similar to that of apo-NikR but is unlike nickel-NikR, because nickel, but not zinc, binds in square-planar geometry to correctly position an α-helix to induce DNA binding⁸². Selectivity at the level of an allosteric change can also mediate specificity in other classes of protein involved in metal homeostasis. For example, the metal transporter AztA (Anabaena zinc-transporting ATPase) is more selective for zinc, and against cadmium, than an equivalent transporter, ZntA, from E. coli^{83,84}. The main difference is that AztA has dual cytosolic metal-binding domains and cadmium reconfigures these domains in a different way to zinc; the cadmium form of AztA is non-productive for transport⁸³.

Specificity mediated by protein contact. As described above, access to metals in enzymes can be mediated by specific interactions with metal-delivery proteins, such as metallochaperones, and this can also apply to the homeostatic proteins. For example, the copper sensor CopY in *E. hirae* acquires copper from the CopZ metallochaperone^{69,70} (FIG. 4d). In theory, failure of other metal sensors in this organism to form heterodimers with CopZ would prevent them from gaining access to copper. Direct protein contact between the nickel importer and cytosolic Nik delivery proteins is a likely explanation for why Nik-imported



Figure 4 | **Pathways of metal supply.** Protein interactions mediated by metallochaperones and associated cofactors impose a kinetic component to metal specificity for hydrogenase (**a**), urease (**b**), iron–sulphur-cluster biogenesis and subsequent enzyme insertion (**c**). Protein interactions also mediate copper trafficking in *Enterococcus hirae*, in which the copper chaperone interacts with the metal-binding domain (MBD) of the P₁-type ATPase designated CopA, which, unlike *Escherichia coli* CopA, is an importer, and copper sensor CopY (**d**). Ls and Ss represent the large and small subunits of hydrogenase, respectively. Csd, cysteine desulphurylase.

nickel is destined only for hydrogenase. This implies that the metal acquired by the delivery proteins is a function of their interaction with the importer rather than of their metal-binding preference⁵⁹. The soluble metal-binding domain of the zinc exporter ZiaA (zinc-transporting ATPase) from *Synechocystis* sp. PCC 6803 binds Cu⁺ more tightly than zinc and has a similar fold to the soluble metal-binding domain of the copper exporter <u>PacS</u> (P-type ATPase for copper and silver)⁸⁵. However, the copper-delivering metallochaperone Atx1 only interacts with PacS, not ZiaA, and so this specificity in the protein–protein interaction prevents ZiaA from gaining access to copper⁸⁵.

Relative metal affinities of sensor proteins. The diphtheria toxin repressor (DtxR) of <u>Corynebacterium</u> <u>diphtheriae</u> regulates iron homeostasis and diphtheria toxin production in response to iron, but when expressed in *B. subtilis* DtxR also responds to manganese with similar sensitivity to iron⁸⁶. The related sensor of *B. subtilis*, MntR, only senses manganese. If two residues from the DtxR metal-sensing site are replaced with their counterparts from MntR, DtxR loses the ability to respond to iron in the heterologous host, except in a *fur* mutant, in which iron levels become abnormally high⁸⁶. Similarly, when NmtR was transferred from a mycobacterial cell to a cyanobacterial cell it retained the ability to sense cobalt but lost the ability to sense nickel77. Unlike the mycobacterial cell, the cyanobacterial cell does not accumulate sufficient nickel to trigger the sensor and therefore this difference in metal specificity is dictated by differences in access to nickel in the cytoplasm of the two organisms. Presumably, the set points of metal sensors (the effective concentration of metal that triggers the switch) are differently attuned in different species, and one intriguing prediction is that the metal specificity of sensors is determined by their relative metal affinities. For example, iron-sensing sites would be expected to bind zinc more tightly than ferrous iron, but, provided the zinc sensor binds zinc even more tightly, the zinc sensor would respond to surplus zinc first, thereby inhibiting further uptake, promoting efflux and preventing the iron-sensing sites from gaining access to zinc. By ranking the affinities of the different sensors for each metal, it should be possible to identify those metals for which the cellular system is based on relative metal affinity. Crucially, the affinities of each regulator for all of the metals that it fails to sense, in addition to those it does detect (FIG. 3), need to be determined, and remarkably, this has not yet been done for a full complement of metal sensors of any cell. Perhaps the challenge associated with measuring metal affinity (BOX 3) is one of the reasons why.

Prospects

The observation that cells must precisely integrate supply and demand for different metals to manage metal–protein interactions has pervasive implications, and here are two examples. Synthetic biology aims to rewire metabolic pathways to introduce non-natural traits of value to biotechnology. Because metals drive catalysis, it is anticipated that the success of such ventures will often depend on the coincident engineering of the supply of metal cofactors. Indeed, it is plausible that the metal affinities of enzymes will require adjustment such that they become optimally poised to acquire the correct element in the context of the heterologous cellular milieu. Calprotectin limits the proliferation of *Staphylococcus aureus* in abscesses by chelating manganese and zinc to cause a disadvantageous change in bacterial gene expression, and an intriguing possibility is that some virulent strains might have evolved resistance to such metal limitation. Do the demands of the Irving– Williams series introduce a vulnerability to bacteria and create an opportunity for therapeutics⁸⁷?

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Acknowledgements

The authors have been supported by Biotechnology and Biological Sciences Research Council (BBSRC) Plant and Microbial Sciences (PMS) grant BB/E001688/1.

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