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The Fate of Intracellular Metal Ions in Microbes

Slade A. Loutet, Anson C. K. Chan, Marek J. Kobylarz, Meghan M. Verstraete, Stephanie Pfaffen, Bin Ye, Angel L. Arrieta, and Michael E. P. Murphy

Abstract

Metals are essential for all microorganisms as they are required as cofactors of enzymes that mediate metabolic processes that are indispensable for cellular energy production and growth. Some metals, such as zinc, are readily bound and serve as key structural elements of many macromolecules. Thus, to grow, microorganisms have an essential quota for several metals. The catalytic and other chemical properties of metals that microorganisms value create issues for metal management. Due to their high affinity for amino acids and their reactive nature, uptake, intracellular transport, and storage of metals are mediated by tightly regulated proteins. Protein chaperones function to supply some specific metals to sites of utilization and, in some cases, storage. In particular, iron is difficult to acquire and is stored as a mineral in protein nanocages. Other metals, when present in excess, induce the expression of export systems to maintain a defined intracellular concentration of readily exchangeable metal.

Introduction

Whether a microorganism is a pathogen or a commensal, dependency on metals is universal. Although metals and metalloids dominate the periodic table, the list of essential metals is short and likely varies between species. The mechanisms by which these metals came to have biological relevance were probably influenced by factors such as their chemical properties and bioavailability. The overall availability of each metal has changed over the course of geological history due to the onset of photosynthesis, which switched Earth from an anoxic to an oxic environment (Catling and Claire 2005). As a result, metals, such as iron (Fe), were sequestered and others, such as copper (Cu) and zinc (Zn), were solubilized. Defining the availability of a metal also depends on the

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subcellular localization within the cell, such as the bacterial periplasm versus cytoplasm, which have differing metal concentrations as well as oxidative versus reductive environments, respectively. As excessive metal concentrations can be highly toxic, tight control is maintained over the intracellular concentration of metals. Due to this supply limit, a given metalloprotein may not be bound to the metal for which it has the highest affinity but rather it must compete for it with all of the other metalloproteins present. This competition adds a layer of complexity that is difficult to monitor using current technologies.

The metabolic pathways requiring metals are diverse, with metals often found as key cofactors in both essential and nonessential processes (Figure 4.1), including the synthesis of biological molecules (amino acids, DNA, RNA, lipids, and carbohydrates) and energy production (tricarboxylic acid [TCA] cycle, electron transport chain, and photosynthesis). Our understanding

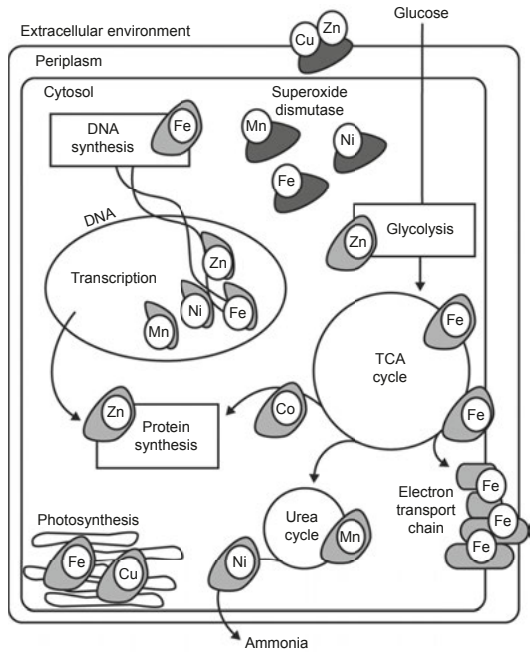

Figure 4.1 The major pathways in a bacterial cell that involve one or more metalloproteins (light gray). Examples of the metal ion(s) involved in each pathway are highlighted. Specific examples include ribonucleotide reductase (Fe, DNA synthesis), the Fur-like family (Zn/Ni/Fe/Mn, transcriptional regulation), threonyl-tRNA synthetase (Zn, protein synthesis), class II fructose-bisphosphate aldolase (Zn, glycolysis), aconitase and succinate dehydrogenase (Fe, TCA cycle), arginase (Mn, urea cycle), plastocyanin (Cu, photosynthesis), photosystems (Fe, photosynthesis), and cytochromes and succinate dehydrogenase (Fe, electron transport chain). This partial picture will continue to grow as studies continue to identify metalloproteins or reveal examples of metal promiscuity. In addition, proteins such as superoxide dismutase (dark gray) collectively utilize a variety of metals to perform the same biological function.

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of the fate of intracellular metals lies primarily within the detailed but often independent characterization of each protein that comprises the biochemical pathways within the cell.

In this chapter we aim to familiarize the reader with general principles concerning the function of metal ions in bacteria, the movement and storage of metals within bacterial cells, and the strategies of metal detoxification used by bacteria. We note that many gaps exist in the fundamental understanding of these processes in bacteria; in particular, little is known about these processes in relation to infection.

Forms and Concentration

Metals are trace elements in physiology. The total and readily exchangeable concentrations of specific metals are tightly regulated within the cell but vary greatly from metal to metal. Data on the content of specific metals per cell of many microorganisms are rarely available. The most comprehensive information in the literature is direct measurement of metal content by inductively coupled plasma mass spectrometry (ICP-MS) for *Escherichia coli*, some of which is summarized in Table 4.1. In general, available data stems from organisms that are easy to culture in a laboratory setting. Values obtained from a few species are often generalized without much supporting evidence. Large differences in metal content exist as the ratio of manganese (Mn) to iron can vary by over five orders of magnitude in bacteria (Lisher and Giedroc 2013).

Iron and zinc are the most abundant metals in *E. coli* (Table 4.1). Iron is necessary for almost all forms of life under physiological conditions, it exists primarily in one of two readily interconvertible oxidation states: the reduced Fe^{2+} ferrous form and the oxidized Fe^{3+} ferric form. Bacteria generally require

Table 4.1 Content in *Escherichia coli* of biological transition metals. Total elemental content reported by Nies (2007) from data by Outten and O'Halloran (2001).

Element	Ionic form	Content (μM)
Iron	Fe^{2+}/Fe^{3+}	180
Zinc	Zn^{2+}	270
Copper	Cu^{1+}/Cu^{2+}	18
Nickel	Ni^{2+}	<5
Cobalt	Co^{2+}	<0.5
Manganese	Mn^{2+}	1.7
Molybdenum	Mo^{6+}	8
Vanadium	V^{5+}	77
Tungsten	W^{6+}	no data

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approximately 10^{-7} to 10^{-5} M iron to achieve optimal growth. The total Fe content of *E. coli* is estimated to be ~200 μM of which 10–30 μM is accessible to a chelator, as determined by electron paramagnetic spectroscopy on whole cells (Yan et al. 2013). Zinc is present in larger quantity than iron (~300 μM) and occurs exclusively as divalent cation Zn^{2+}. The free Zn^{2+} concentration, as estimated by Zur and ZntR transcription assays in *E. coli*, is about 10^{-15} to 10^{-16} M (Outten and O'Halloran 2001); however, the readily exchangeable concentration is much higher (20 pM) and is more relevant to estimating the availability of this metal (Wang et al. 2011a).

Copper is both essential and toxic for many microorganisms. Approximately 20 μM of copper were measured, associated with *E. coli* cells. However, Cu-containing proteins are generally located outside the cytoplasm; thus no cytoplasmic metal is required. Because of the reducing cytoplasmic conditions, Cu^{1+} is likely the dominant oxidation state within the cell. Almost all Cu^{+} is bound by glutathione and other thiols. In yeast, the free Cu^{+} in the cytoplasm is virtually nonexistent, and in *E. coli* the concentration is estimated to be 10^{-21} M (Changela et al. 2003).

Nickel (Ni), typically used by microbes for anaerobic growth, is found at <5 μM in *E. coli* under aerobic conditions. Total Ni^{2+} in the cell may be increased under anaerobic growth when Ni-containing hydrogenase is expressed. The free concentration is estimated at 10^{-12} M based on the affinity of transcriptional regulator NikR in *E. coli* (Chivers and Sauer 2002). As detailed later, Ni- and Cu-trafficking systems have been discovered that explain the low exchangeable concentration of these metals in the cytoplasm.

For other metals, data are limited or nonexistent. For instance, Co^{2+} (total concentration of <0.5 μM in *E. coli*) remains the major free form of cobalt (Co) in microbes, but the cytoplasmic concentration has not yet been studied. Nevertheless, the free Co concentration is likely low due to its toxic competition with other biologically essential metal ions (Okamoto and Eltis 2011). Mn^{2+} (total concentration of ~2 μM in *E. coli*) is imported by microorganisms for use in Mn-specific enzymes, and can also be exchanged with iron in the metal-binding sites of some Fe proteins (Cotruvo and Stubbe 2012). The paucity of data on the overall metal content and speciation within many phyla of microorganisms is remarkable.

Functions

In biological systems, metals are observed to participate in various types of roles: they can act as signaling molecules, catalysts, or structural elements. Identification of the physiologically and functionally relevant metal in proteins is not straightforward. The favored metal bound to a metalloprotein can, in some cases, be estimated by the observed amino acid ligands and the geometry of the metal site, though mischaracterization can occur due to the

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promiscuity of metal-binding sites and the similar chemical properties of certain metals. Peptide deformylase is a prime example, emphasizing the need for careful identification of the native metal (Maret 2010). This enzyme was originally purified with bound Zn^{2+} and later extensively characterized with bound Ni^{2+}, a form that provided full activity. Subsequent studies, however, demonstrated that Fe^{2+} was bound *in vivo*. Moreover, Fe^{2+}-bound peptide deformylase is highly sensitive to inactivation by hydrogen peroxide (H_2O_2). During H_2O_2 stress, Mn^{2+} import is upregulated and can displace the bound Fe^{2+}. Mn^{2+}-bound peptide deformylase retains partial activity and is invulnerable to oxidation by H_2O_2 (Anjem and Imlay 2012). Species-based metal cofactor specificity has also been observed for proteins such as the superoxide dismutases (SODs), which can collectively utilize manganese, iron, binuclear copper–zinc, or nickel (Banci 2013). In yeast mitochondria, SOD can use either manganese or iron but selectively utilizes manganese, even though Fe concentrations are two orders of magnitude greater (Naranuntarat et al. 2009). Alternatively, isoenzymes utilizing different metals can functionally replace each other in a single organism. For example, NrdAB ribonucleotide reductase is an Fe enzyme essential for *E. coli* aerobic growth, but under periods of Fe restriction, the expression of Mn-dependent NrdEF is induced (Martin and Imlay 2011). Overall, this seemingly conflicting property of metal flexibility and selectivity attests to the finely tuned, but adaptable nature of metal homeostasis within an organism.

As a part of this adaptable nature, metals are involved in signal transduction pathways and are recognized by sensory proteins to maintain metal homeostasis. Changes in metal content can indicate a change in the environment, such as the transition experienced by a pathogen moving from an external environment to inside a host. In addition, host organisms actively sequester metals from bacterial commensals and pathogens alike. Therefore, bacteria must have the ability to sense the abundance of each metal and respond accordingly. Metal homeostasis is controlled by sensory proteins that bind specific metals and regulate uptake, export, or utilization of the metal. A classic example is the Fur-like family of related bacterial regulators—Fur, Zur, Mur, and Nur—which recognize and regulate Fe^{2+}, Zn^{2+}, Mn^{2+} and Ni^{2+} homeostasis, respectively (Fillat 2014). Metal complexes such as heme can also function as signaling molecules. One example is HrtR from the commensal *Lactococcus lactis*, which is a heme sensor that regulates a heme efflux system (Lechardeur et al. 2012).

In addition to their direct role in regulating metal acquisition, these sensory proteins can also modulate the expression of virulence and survival factors. For example, *Staphylococcus aureus* Fur was discovered to enhance the expression of several immunomodulatory proteins: protein A (SpA), staphylococcal immunoglobulin G-binding protein (Sbi), and extracellular fibrinogen-binding protein (Efb) (Torres et al. 2010).

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Deprivation of iron typically elicits a complex response which ultimately results in a metabolic shift to pathways that rely less on iron. In *S. aureus*, this "iron-sparing" response redirects its metabolism from the Fe-requiring tricarboxylic acid (TCA) cycle and respiratory chain to the Fe-independent glycolytic pathway (Friedman et al. 2006). Little is known of the potential metabolic shifts akin to "iron sparing," when bacteria find themselves in environments low in other metals. In some rare cases, select bacteria completely eliminate the need for a particular metal as a metabolic strategy. Notably, *Borrelia burgdorferi*—the only known bacteria that does not directly require iron or produce nucleotides *de novo*—is an obligate intracellular parasite that appears to utilize manganese in key proteins and depends on its (iron-requiring) host for essential nutrients (Posey and Gherardini 2000).

Transition metals (i.e., those elements in the d-block of the periodic table) play essential catalytic roles in microbial metabolic pathways. Of the transition metals, iron is commonly employed because its reduction potential is favorable for biochemical reactions. Iron is typically incorporated into enzymes as free ions, heme, or iron-sulfur (Fe-S) clusters, with the majority of Fe enzymes falling under the oxidoreductase class (Andreini et al. 2008). An essential free Fe-containing enzyme is the NrdAB ribonucleotide reductase, which produces deoxyribonucleotides from ribonucleotides for DNA synthesis and repair (Ando et al. 2011). The TCA cycle enzyme aconitase is a well-known Fe-S cluster enzyme responsible for the isomerization of citrate into isocitrate (Prodromou et al. 1992). Heme iron plays a major role in the electron transport chain as a cofactor in cytochromes, which mediate electron transfer that ultimately leads to the generation of ATP (Kamen and Horio 1970). Enzymes can also require more than one type of iron. For example, succinate dehydrogenase plays a role in both the TCA cycle and the electron transport chain, and contains three Fe-S clusters as well as a heme cofactor (Yankovskaya et al. 2003).

In biological molecules, cobalt is often bound in the center of a cyclic, four pyrrole ring structure (corrin). Derivatization of the corrin ring leads to a family of compounds known as the corrinoids, the most well-known of which is cobalamin (vitamin B12). Methionine synthase utilizes a cobalamin cofactor to transfer a methyl group to homocysteine, thus forming methionine (Drennan et al. 1994). The noncorrin Co enzyme methionine aminopeptidase is also involved in methionine metabolism and catalyzes the removal of the N-terminal methionine from newly synthesized peptides (Ben-Bassat et al. 1987). Methionine aminopeptidase is essential for the maturation, subcellular localization, and degradation of many proteins (Kobayashi and Shimizu 1999), and inactivation of this enzyme has been demonstrated to be lethal in organisms such as *E. coli* (Chang et al. 1989). Despite all of the work that has been done to characterize methionine aminopeptidase as a Co enzyme, some have suggested that iron is the true cofactor inside *E. coli* (Chai et al. 2008).

Zinc is an essential transition metal estimated to be used by 4–10% of proteins encoded by the genome of some organisms (Andreini et al. 2011). In

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fact, it is the second most abundant metal in characterized enzymes, behind magnesium and slightly ahead of iron (Andreini et al. 2008). The majority of prokaryotic Zn proteins are enzymatic in nature, although the Zn cofactor can participate as either a structural or catalytic component. In addition to acting as a catalytic Lewis acid, zinc is distinct from other common transition metals in that it lacks redox reactivity and is thus an excellent metal for stabilizing negative charges. Not surprisingly, examples of Zn proteins are found in all six major classes of enzymes, with the largest proportion being hydrolases such as alkaline phosphatase and thermolysin.

Nickel is an essential metal in some prokaryotes. For the gastric pathogen *Helicobacter pylori*, two Ni-containing enzymes contribute to its pathogenicity (Eaton et al. 1991; Olson and Maier 2002). Nickel-iron hydrogenase allows for the use of molecular H_2 as a substrate for respiration. Urease is an essential factor in stomach colonization; it neutralizes stomach acid through the production of ammonia.

No bacterial Cu-dependent proteins have been observed to be localized to the cytoplasm (Rensing and McDevitt 2013). Thus far, only ten types of Cu enzymes have been identified in prokaryotes, with cytochrome *c* oxidase present in most organisms. In *E. coli*, the major periplasmic Cu protein is CueO, a multicopper oxidase that is part of a Cu-resistance mechanism (Roberts et al. 2002). In cyanobacteria, copper is shuttled into the thylakoid, a specialized compartment with Cu-requiring photosynthetic and respiratory electron transport proteins (Tottey et al. 2005). All other characterized Cu proteins are localized either to the periplasm or to the cytoplasmic membrane with the active, Cu-binding site facing away from the cytoplasm.

In cases where the metal is structural, the selected metals tend to be redox inert and can act to neutralize repulsive negative charges. The most common metal in this capacity is Mg^{2+}, which plays a major role in stabilizing the structures of DNA and RNA backbone phosphate groups (Sreedhara and Cowan 2002) and assists in orienting the phosphate groups in nucleoside triphosphate (e.g., ATP, GTP, CTP, TTP) bound to enzymes (Cowan 2002). Ca^{2+} is another common structural metal, but it is primarily exploited by extracellular proteins, polysaccharides, and other cell wall components (Norris et al. 1991). A well-known structural feature containing a transition metal is the Zn finger motif, which mediates DNA- and RNA-binding as well as protein–protein interactions (Krishna et al. 2003). Zinc can also play a structural role in certain Fur proteins, such as those from *E. coli* and *H. pylori*, where it binds to a second, nonregulatory site (Jacquamet et al. 1998; Vitale et al. 2009).

Although the molecular details of metal uptake and efflux are relatively well studied and the list of characterized metal-binding proteins continues to grow, the distribution of such metals within a cell remains largely uncharacterized. Only recently have we begun to characterize the metalloproteome on a global scale with sufficiently high enough resolution to determine such detailed metal distribution information within a subcellular context. A recent

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study on the pathogen *Bacillus anthracis* revealed that cytoplasmic iron was primarily found in only four major pools: the electron transfer protein ferredoxin, the miniferritin Dps2, two co-eluted SODs (SodA1 and SodA2), and a single unidentifiable pool of metalloprotein(s) (Tu et al. 2012). Interestingly, manganese consisted of a single major pool, which was identified to be SodA1 as well. Through further investigation, Tu et al. revealed that SodA1 consists of a mixed population primarily bound to manganese *in vivo*, with the remaining population bound to iron. In contrast, SodA2 is exclusively bound to iron.

A key question facing metalloprotein research is the relative role of metals between commensal and pathogenic bacteria. Pathogens have been observed to carry a larger genetic toolbox of metal acquisition proteins and metalloenzymes required for pathogenicity and antibiotic resistance. For example, genes encoding receptors for the siderophores yersiniabactin (Ybt) and aerobactin are more common in extraintestinal pathogenic *E. coli* than in commensal strains (Lee et al. 2010). *S. aureus* produces two distinct SODs as compared to the less virulent coagulase-negative *Staphylococcus* spp., which generally have one (Valderas et al. 2002). In addition, many pathogenic Gram-positive bacteria possess FosB, a divalent metal-dependent thiol-S-transferase implicated in fosfomycin resistance (Roberts et al. 2013). The difference between a commensal and a related pathogen, however, may be at the level of expression control rather than gene content, as known genomic pathogenicity islands were identified in nearly 50% of randomly surveyed commensal fecal *E. coli* strains (Li et al. 2010a). A given organism may act as a commensal or a pathogen, depending on signals from its environment. Serotyping and genotyping studies have shown, in the same individual, that both asymptomatic intestinal *E. coli* and uropathogenic *E. coli* are clonally related (Grüneberg 1969; Yamamoto et al. 1997). Further confounding these issues are opportunistic pathogens, which take advantage of negative perturbations to the host defense systems.

Taken together, whether or not pathogens and commensals have different metal requirements remains an open question. They may have the same general metal requirements, since many of the roles played by metals discussed here are conserved among commensals and pathogens. They might also be distinguished by the possession and regulation of metal uptake and detoxification systems. Current techniques do not allow for detailed analyses of metal-requiring processes on a global and dynamic scale. Advances in high-resolution metallomics techniques, such as those discussed by Maret et al. (this volume), hold promise in providing great insight into metal functions and fluxes within a bacterium, and how these change under different environmental conditions. New data will also inform us on intra- and interspecies differences in metal usage. These data have the potential to lead novel tools for infection control, bioremediation, or bioengineering based on bacterial metal-dependent processes.

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Metallochaperones

Metallochaperones are a class of metalloproteins that bind metal ions and move them between uptake pathways, functional sites, storage systems, or detoxification pathways (Figure 4.2). They insert metal ions into other proteins via specific protein–protein interactions. Free metal ions must be quickly bound by the cognate metallochaperones to both facilitate transfer to the correct location and protect the cell from toxicity associated with free intracellular metal ions. The activity of metallochaperone proteins can be divided into three stages: (a) selective binding of the cognate metal ion, (b) identification of and interaction with the desired target metalloprotein, and (c) transfer of the metal ion to the target metalloproteins (Rosenzweig 2002).

In general, metal ions are bound to metallochaperones at exposed sites to facilitate protein-to-protein metal ion exchange. An example is the periplasmic protein CusF: a Cu^{1+} and Ag^{1+} chaperone for the CusCBA metal detoxification system (Mealman et al. 2012). CusF adopts a β-barrel fold and binds either Cu^{1+} or Ag^{1+} ions at a site at one end of the barrel (Xue et al. 2008). For a given metal, chaperones with different protein folds and binding pockets may exist. For example, Cu chaperones use at least four different protein folds and binding pockets (Robinson and Winge 2010). Purified metallochaperones are often poorly selective for binding of the correct metal ion *in vitro*. Ni^{2+} binding by UreE, the metallochaperone for urease assembly, can be outcompeted by Cu^{2+} and Zn^{2+} *in vitro* (Brayman and Hausinger 1996). In a bacterial cell, a number of factors likely contribute to the correct metal selection by metallochaperones (Tottey et al. 2005; Maret 2010). Bacteria maintain a limited metal pool through restriction of the readily exchangeable metal concentration by expressing storage systems and selective metal import and export systems. Compartmentalization within the bacterial cell can co-localize proteins in the presence of certain metals. Some metalloproteins undergo allosteric changes upon binding of only the correct metal ion. Finally, bacteria can alter their metabolism to exploit the most plentiful metals.

Once a metallochaperone has acquired its cognate metal ion, it must identify and interact with the correct target protein. These protein–protein interactions are driven by the factors that influence all protein–protein interactions, such as electrostatic or hydrophobic interactions between exposed surfaces (Reichmann et al. 2007). For metallochaperone–target protein interactions, other common features include a requirement for the correct metal ion and structural similarity between the metallochaperone and the target. The specificity of protein–protein interactions likely contributes to the target metalloproteins binding the correct metal despite energetic constraints. Finally, the metallochaperone must deliver its metal ion to the target protein. Metal transfer often involves ligand exchange reactions, where both proteins use the same ligands to coordinate the metal ion and, in a stepwise fashion, transfer the metal from the metallochaperone to the target. In some systems, such as the transfer

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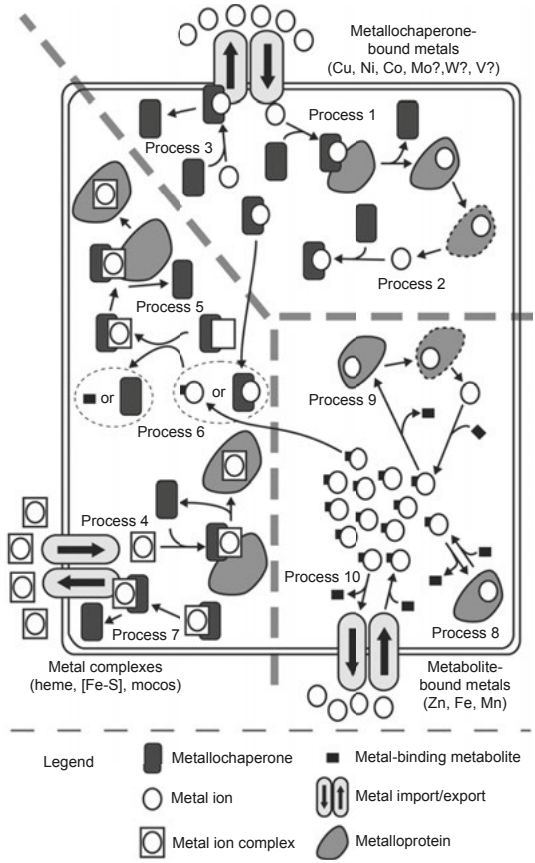

Figure 4.2 Metallochaperones are part of a complex set of metal pools that exist to minimize the concentration of free metal ions. The metalloprotein symbol represents proteins with a metal ion as a cofactor, with metal serving a structural role, or with metal being stored. Degraded metalloproteins are represented with dashed outlines. Most, but not all, of the processes illustrated have been experimentally demonstrated. For some metals (top), metallochaperones act to transport metal ions from import systems to target metalloproteins (Process 1). They can also recover metal ions after metalloprotein degradation (Process 2) and transport excess metal ions to export systems (Process 3). Metallochaperones perform similar functions for metal complexes (left). These complexes can be directly imported (Process 4) or they can be synthesized *de novo* (Process 5) with metals obtained from a metallochaperone protein pool or a metabolite-bound pool (Process 6). Metal complexes can also be exported from the cell (Process 7). Some metals (bottom right) are proposed to be bound by small metabolites to buffer the bacterium from free metal ions. The metal-bound metabolites are thought to be in equilibrium with some metal-bound proteins (Process 8). Metal bound to metabolites may bind tightly to metalloproteins and be released only upon protein degradation (Process 9). Theoretically, these metabolites would interact with import and export systems (Process 10). For illustrative purposes these interactions are all being shown as occurring within the bacterial cytoplasm; however, many of these processes also occur in the bacterial cell envelope.

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of Ni^{2+} from UreE to the urease enzyme complex (Farrugia et al. 2013), transfer of the metal ion to the target metalloprotein requires the assistance of accessory proteins. Accessory proteins can act as scaffolds to enhance protein–protein interactions; they can provide energy or other enzymatic functions. In the case of the urease complex (Farrugia et al. 2013), UreD acts as a scaffold between the UreABC enzyme and other proteins required for maturation of the enzyme. Ni^{2+} insertion into the urease complex requires GTP hydrolysis, a function provided by UreG. A third accessory protein, UreF, is suggested to enhance the interaction between the GTPase activity of UreG and Ni^{2+} insertion by UreE.

In cells, metal ions can also be found bound to nonprotein cofactors, such as iron bound to protoporphyrin IV (heme) or molybdenum (Mo) bound by a family of related cofactors (known as the Mocos). Metallochaperones exist for these types of complexes: CcmE is a periplasmic heme-binding protein that delivers heme to cytochrome *c* (Schulz et al. 1998), whereas specific chaperones deliver each of the Mocos to their appropriate targets (Neumann and Leimkühler 2011). Another well-known example of a nonprotein cofactor is the widely used Fe-S clusters (Johnson et al. 2005a). In bacteria, at least three different Fe-S biosynthetic clusters exist, with the IscU system being the most well characterized (Vickery and Cupp-Vickery 2007). Although precise mechanistic details are still being characterized, IscU is responsible for building the Fe-S cluster, which is then transferred to an acceptor protein, such as ferredoxin or aconitase, with the assistance of two accessory proteins (HscA and HscB) (Vickery and Cupp-Vickery 2007).

The state of knowledge with regards to metallochaperones for the metals varies. Numerous Cu and Ni chaperones have been described in the literature. Given the prevalence of Fe- and Zn-containing proteins, relatively little is known about metallochaperones for these elements. As proposed elsewhere (see Cavet et al., this volume), due to the large number of zinc, mononuclear iron, and Mn-containing proteins, we speculate that small molecule ligands (e.g., histidine and glutathione) rather than proteins might be used as chaperones for these elements. We believe that other metals used by bacteria in a smaller number of metalloproteins, including cobalt, vanadium, and tungsten, also likely use protein chaperones; however, little data concerning chaperones for these elements are available.

New avenues of metallochaperone research are likely to open up as emerging evidence from metalloproteome studies indicates that a wider variety of metals are incorporated into metalloproteins than previously thought. Metallochaperone research has mainly been confined to a subset of bacteria, particularly those causing disease. Environmental bacteria living in diverse ecosystems likely utilize additional sources of metals and contain novel metallochaperones. Finally, the proposed use of small molecule ligands as chaperones for zinc, manganese, and iron inside the bacterial cell awaits further study.

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Detoxification and Cytoplasmic Metal Export

Metals often get into microbial cells via influx pumps for essential elements and must be selectively handled by membrane transport mechanisms, as many are harmful to bacteria. Metals demonstrate toxicity due to their chemical affinity for thiol groups, which can alter enzyme specificity and disrupt cellular functions. Still, some metals (e.g., iron, cobalt, copper, and zinc) are essential micronutrients but can also be toxic at high levels (Bruins et al. 2000). The challenge for the bacterial cell is to maintain a level of homeostasis between metal uptake for nutrition and membrane transport out to avoid toxicity. Microorganisms have adapted by developing a variety of resistance mechanisms, including energy-dependent efflux, enzymatic transformations, sequestration by metal-binding proteins and siderophores, and precipitation of metal sulfides (Figure 4.3). These metabolic responses differ for each metal.

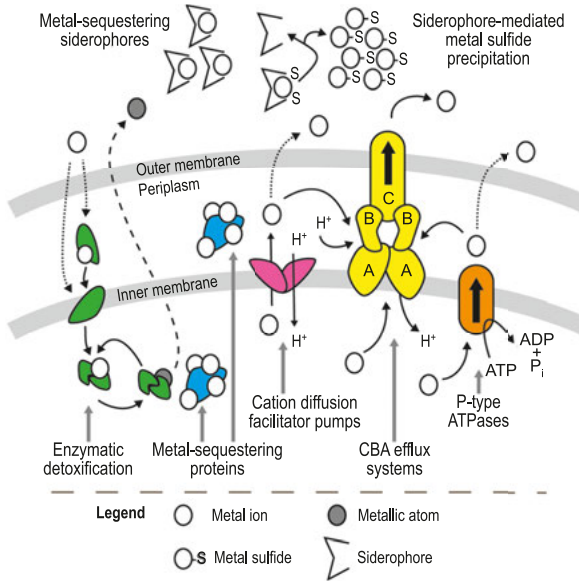

Figure 4.3 Examples of mechanisms of metal detoxification and resistance. Enzymatic detoxification (mercury reductase, green) alters a metal to a less toxic form. Mercury reduced to Hg^0 is volatile and diffuses out of the cell (dashed line). Periplasmic (SilE, blue) and cytoplasmic proteins (metallothionein, blue) mediate resistance by binding excess intracellular metals. Cation diffusion facilitator family pumps (CzcD, pink), CBA efflux transport systems (CzcCBA, yellow), and P-type ATPases (CadA, orange) are all energy-dependent efflux systems. Siderophores (e.g., pyoverdine) bind metals to lower the extracellular free metal ion concentration and can also participate in metal sulfide precipitation (e.g., pyridine-2,6-bis[thiocarboxylic acid]). For illustrative purposes a Gram-negative bacteria with an outer membrane is depicted: dotted lines illustrate the extracytoplasmic movement of metal ions in Gram-positive bacteria or movement of metal ions through the Gram-negative outer membrane that are poorly understood.

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The most frequent, widespread mechanism of metal resistance is energy-dependent efflux, generally without coincident covalent or redox chemistry. Efflux systems fall into a small number of families (Nies 2003; Silver and Phung 2005). These include P-type ATPases, which are single polypeptide determinants with an intermediate phosphorylated by ATP; cation diffusion facilitator pumps that are made up by a single polypeptide chemiosmotic pump; and export systems driven by resistance-nodulation-cell division (RND), which are comprised of three proteins: (a) an inner membrane RND protein, supported by (b) a membrane fusion protein (MFP) that provides a connection through the periplasmic space to (c) a protein belonging to the family of outer membrane factors (OMFs). These three proteins form a complex that can efflux its respective substrate from the cytoplasm, cytoplasmic membrane, or periplasm across the outer membrane to outside the cell. To differentiate this type of efflux mechanism from ATP-driven ABC transporters, the RND-driven export system is also referred to as a CBA efflux system.

Cadmium resistance, for example, is found widely in environmental and clinical isolates for which three types of cadmium efflux mechanisms have been discovered. The CzcD single membrane polypeptide chemiosmotic pump confers resistance by pumping out metal ions directly from the cytoplasm (Anton et al. 1999). The *czc* resistance determinant also encodes a CBA efflux system and contains the genes for the OMF CzcC, the MFP CzcB and the CzcA protein of the RND family (Nies et al. 1989). This efflux system may have the ability to pump metal ions out of both the cytoplasm and the periplasm (Nies 2003) as well as to confer some resistance to Co^{2+} and Zn^{2+} (Nies et al. 1989). CadA, a large single polypeptide P-type ATPase, can also contribute to Cd^{2+} resistance (Nucifora et al. 1989).

P-type ATPases and other transporters for metal efflux have also been found to be involved in extracytoplasmic protein metalation (Arguello et al. 2013). In *Rhodobacter capsulatus* the metalation of the periplasmic protein, cbb_3 cytochrome *c* oxidase (cbb_3-COX), requires transport of Cu^+ through a major facilitator superfamily transporter protein (Ekici et al. 2012). Gonzalez-Guerrero et al. (2010) described two nonredundant Cu^+-ATPases, CopA1 and CopA2, in *Pseudomonas aeruginosa*. CopA1 represents a classical Cu^+-ATPase for Cu^+ efflux, whereas CopA2 has comparably slower transport kinetics and higher affinity for Cu^+. Deletion of *copA2* results in a mutant with decreased cytochrome *c* oxidase activity, thus suggesting that the properties of CopA2 are suitable for participation in the assembly of cuproproteins like *ccb3*-COX.

Another metal resistance mechanism that has evolved for bacteria is the enzymatic detoxification of a metal to a less toxic form. Mercury resistance, encoded by the *mer* operon, is a broadly occurring toxic metal resistance system found in Gram-positive and Gram-negative bacteria from environmental, clinical, and industrial samples (Mathema et al. 2011). Bacteria have cleverly developed a way to bind extracellular Hg^{2+} and transport it into the cytoplasm

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using a series of cysteines on different proteins, termed a thiol "bucket brigade," to active sites on mercuric reductase, while ensuring that no toxic mercury is free within the cell to cause damage (Silver and Phung 2005). In Gram-negative bacteria, there is no outer membrane protein known to be involved in the Hg^{2+} transport system. Instead, a periplasmic MerP protein is thought to relay Hg^{2+} to the inner membrane protein MerT. Once Hg^{2+} is at the inner surface of the cytoplasmic membrane, it is likely transferred to mercuric reductase (MerA) by cysteine-pair to cysteine-pair exchange. Thereafter, Hg^{2+} is reduced to elemental mercury (Furukawa and Tonomura 1972; Schiering et al. 1991), which is volatile and able to diffuse out of the cell (Schelert et al. 2004).

Intracellular sequestration of toxic cations by metal-binding proteins serves as another method of metal resistance. Cd^{2+} and Zn^{2+} are commonly sequestered by cysteine-rich proteins, such as metallothionein (Shi et al. 1992). These small polythiol metal cation-binding proteins function to lower free metal ion concentrations within the cytoplasm and afford a level of resistance. Another example of binding by sequestration is the Sil Ag^{+} resistance system in which resistance is partly facilitated by a small periplasmic metal-binding protein, SilE (Gupta et al. 1999).

Metal resistance is also mediated through the secretion of siderophores, which are low molecular weight molecules primarily produced for ferric iron acquisition. Siderophores can serve as multifunctional metabolites that are able to bind other metals outside of the cell and protect against metal toxicity. An example of this environmental conditioning is the production of pyridine-2,6-bis(thiocarboxylic acid) (PDTC) siderophore by *Pseudomonas stutzeri* (Zawadzka et al. 2007). This siderophore can form poorly soluble complexes with Hg^{2+}, Cd^{2+}, Pb^{2+}, and As^{3+} and hydrolyze them to form insoluble metal sulfides. Precipitation of metals as metal sulfides using siderophores acts as an environmental detoxification mechanism by restricting the bioavailability of extracellular metals. Conversely, PDTC can also facilitate Zn utilization, as well as Fe uptake, in *Pseudomonas putida* (Leach et al. 2007). Another example of siderophores mediating bacterial tolerance to metals is the production of pyoverdine and pyochelin by *P. aeruginosa* (Braud et al. 2010). Despite their preference for iron, these siderophores are able to bind Al^{3+}, Co^{2+}, Cu^{2+}, Ni^{2+}, Pb^{2+}, and Zn^{2+} in the extracellular milieu, leading to increased *P. aeruginosa* resistance and decreased intracellular metal accumulation. Uropathogenic *E. coli* (UPEC) uses Ybt to chelate host Cu^{2+} and prevent its reduction to the more bactericidal form, Cu^{1+}, thus increasing the Cu resistance of UPEC (Chaturvedi et al. 2012). Importantly, the Cu^{2+}-Ybt complex was identified in samples from human patients with active urinary tract infections with UPEC. Though siderophores mediate metal detoxification outside of the cell, they are an intracellular crafted mechanism of resistance. The diversity seen in siderophore chemical composition, the upregulation of siderophore production in response to metals other than iron, the ability of siderophores to bind iron and other metals tightly, the fact that

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some bacteria make multiple siderophores, and the observation during infections that siderophores play roles in metal binding and detoxification all support the idea that siderophores have evolved to perform multiple physiological functions with regards to homeostasis of iron and other metals (Schalk et al. 2011).

Metal detoxification is a rapidly advancing field of research. *Campylobacter jejuni* has recently been shown to possess a membrane permease for the removal of roxarsone and nitarsone (organic arsenic molecules added to chicken and poultry feed to reduce bacterial infections and increase weight gain) from the cytoplasm (Shen et al. 2014). The permease was specific to these organic arsenic forms and played no role in arsenite or arsenate resistance. *Mycobacterium tuberculosis* produces an extracytoplasmic multicopper oxidase that participates in Cu resistance (Rowland and Niederweis 2013). Bacteria can also precipitate metals as inorganic sulfides, such as nickel sulfide and cobalt pentlandite (Co_9S_8) (Sitte et al. 2012). Many more detoxification strategies are being characterized as they are becoming more ubiquitous among bacterial populations in polluted environments and clinical isolates, where metal resistance systems are commonly co-selected for with antibiotic resistance genes due to genetic co-resistance and physiological cross-resistance mechanisms (Baker-Austin et al. 2006). There is growing concern that metal contamination is indirectly selecting for antibiotic resistance, and studies have shown that metal-contaminated environments contain an overabundance of antibiotic resistance genes (Knapp et al. 2011).

Storage

Iron in excess of that which is needed for cellular homeostasis is toxic and potentially lethal to the cell. Therefore, most organisms, including bacteria, have evolved a strategy that involves the use of proteins of the ferritin superfamily to protect cells from the harmful effects of excess iron (Andrews 2010). Ferritins are the major proteins that bind and store nonheme iron inside cells. The typical ferritin consists of 24 subunits, forming a hollow sphere that takes up soluble ferrous iron and oxidizes it at di-iron ferroxidase centers for storage as a ferric mineral within the central cavity. Mammalian ferritins are composed of two different subunits: the H-chain subunit contains the catalytic ferroxidase center, and the L-chain subunit contains mineral nucleation sites. In bacteria, two ferritin subtypes are found: the heme-containing bacterioferritin (BFR) and the bacterial ferritin. Both types are homopolymers in which the monomer contains the ferroxidase site and the mineral nucleation sites. Both BFR and bacterial ferritin play a role in Fe storage but may also have more specialized functions in Fe detoxification, depending on the organism (Le Brun et al. 2010). Although both could theoretically hold up to 4500 Fe atoms (Crichton and Declercq 2010), when isolated from non-overexpressing

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sources, BFR holds between 800 and 1600 Fe atoms whereas bacterial ferritin typically stores between 600 and 2300 Fe atoms (Lewin et al. 2005).

Outside the catalytic residues of the ferroxidase site, only a few residues are conserved between ferritin subfamilies (Andrews 2010), and amino acid sequence identities can be as low as 15%. Overall structure, however, is conserved among ferritins and BFR (Crichton and Declercq 2010). The ferroxidase center is located in the center of the monomer, a four helix bundle with a short fifth helix at the C-terminus, and consists of two Fe-binding sites: site A and site B. However, the ferroxidase centers of BFR and bacterial ferritins are distinct in their Fe-coordinating ligand arrangement. In bacterial ferritin, a third Fe-binding site, site C, is located near the ferroxidase center. In the *E. coli* ferritin A, this site is not essential for rapid Fe oxidation but has an effect on the Fe mineralization and Fe movement into the protein cavity (Bou-Abdallah et al. 2014). Residues coordinating to site C are highly conserved in bacterial ferritins, indicating the functional importance of this site (Le Brun et al. 2010).

Although ferritin is the most studied and best understood metal storage protein, little is known about how iron enters and exits ferritin. Iron entry and exit in ferritin is suggested to be through their threefold channels (Treffry et al. 1993). However, ferritins also have B-channels, which are located between two subunit dimers (Carrondo 2003). The roles of the channels in ferritin are not yet clear. Furthermore, the heme groups in BFR do not play a role in the Fe^{2+} uptake but may play an important role in Fe release (Yasmin et al. 2011).

A third Fe storage protein found in bacteria is the Dps (DNA-binding protein from starved cells) ferritin. Dps ferritin forms a 12mer and can store up to 500 Fe atoms (Crichton and Declercq 2010). However, the function of Dps ferritin lies more in protecting DNA against oxidative and mechanical stress as well as enzymatic degradation (Zeth 2012).

Other known metal storage proteins found in bacteria have a much lower storage capacity. For example, MoSto from *Azotobacter vinelandii* is a hexameric Mo storage protein that is functionally related to nitrogen fixation by supplying nitrogenase with molybdenum. It can store more than 100 Mo atoms per hexamer as a polyoxomolybdate cluster in an interior cavity (Kowalewski et al. 2012). Another example is *E. coli* SlyD, which can bind two to seven Ni ions in its unstructured C-terminal region. This protein is proposed to act as a reservoir for nickel, but also contributes to the insertion of nickel into the hydrogenase precursor protein by transferring Ni atoms to the auxiliary protein HypB and modulating its activities (Kaluarachchi et al. 2011).

Ferritin is the most studied and best understood metal storage protein and the only known storage protein with a high capacity of several hundred to thousands of metal ions. MoSto is the only other example that we have come across of a mass storage protein similar to ferritin. In contrast, other known storage proteins can bind few metal ions, and storage by these proteins is considered to be a secondary function.

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Concluding Remarks

Although much progress has been made on the fate of intracellular metal ions in bacteria, many key questions are still outstanding. We know that metal ions play diverse functional and structural roles in bacterial cells but that they can be toxic at high concentrations. Bacteria have evolved machinery to (a) take up metal ions, (b) transport and deliver metal ions to the appropriate locations in cells, and (c) sequester, store, and, if necessary, expel metal ions to maintain an appropriate concentration of metal ions. Of the transition metals, iron, copper, nickel, zinc, manganese, molybdenum, cobalt, vanadium, and tungsten have been shown to be used by bacteria. Bacteria are also exposed to and can detoxify many other toxic transition metals, such as chromium, cadmium, or mercury. This function makes bacteria potential agents for bioremediation.

Despite our advanced knowledge about many metalloproteins, we have yet to identify the complete metalloproteome, even in well-studied bacteria. New technologies should help to identify new metalloproteins and give us a greater sense of the metalloproteome of a given organism or a meta-metalloproteome from a complex, multispecies sample. A well-curated, online resource catalogue of known and predicted metalloproteins would be a welcome resource to metallo-microbiologists. One such platform was the Metalloprotein Database and Browser (Castagnetto et al. 2002), which provided a database of metal-binding sites from protein structures deposited in the Protein Data Bank; however, it is no longer available online. Other sites are available that provide some useful information. METAL-MACiE[1] provides detailed information on the roles played by metals in catalytic mechanisms of approximately 200 metalloenzymes (Andreini et al. 2009). BRENDA[2] provides data on enzyme metal-binding properties curated from publications (Schomburg et al. 2013). We envision a database that provides users lists of experimentally validated and predicted metalloproteins for a given organism of interest. Such a database is essential for the full development of metalloproteomics.

Current data are limited by the bacterial organisms that are commonly studied. We do not know the common trends (if any) in the use, storage, or detoxification of metals that differentiate pathogenic bacteria, human commensal bacteria, or environmental bacteria. As more environmental organisms are described, particularly those living in environments that are contaminated with metals, we will surely identify novel detoxification strategies, but it also seems possible that we will find bacteria using other transition metals functionally. More must also be done to assess the epidemiological risks of environmental metal pollution and how it functions in the maintenance and proliferation of antibiotic resistance.

1 http://www.ebi.ac.uk/thornton-srv/databases/Metal_MACiE/home.html

2 http://www.brenda-enzymes.org

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Finally, much work needs to be done to understand the concentrations and distributions of metals in the cell. ICP-MS has been used successfully in a number of organisms to establish total cellular content of metals (see Maret et al., this volume). As a next step, the distribution of the metals in different pools needs to be determined more accurately. How much of this metal is in readily exchangeable pools? How much is sequestered in storage systems, such as iron in ferritin or molybdenum in MoSto? How do these concentrations vary over time, stage of growth, and in the presence or absence of various nutrient sources? Are there differences between bacteria growing in suspension versus in a biofilm or in the presence or absence of other species of bacteria? In a biofilm, are particular metals sequestered in different regions of the biofilm? For metals with multiple uptake pathways in a given organism, do all uptake pathways feed into all metal pools, or do linkages exist between uptake pathways and cellular pools? The answers to these and similar questions are fundamental to our understanding of the use of metal ions by bacteria in the diverse ecosystems in which they live.

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