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Is Metal Uptake Related to Ecological Niche in the Vertebrate Host?

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Abstract

There is a complex interplay between the vertebrate host and the microbes that inhabit its mucosal surfaces in their competition for essential metal ions. Mucosal surfaces in the host constitute an array of diverse ecological niches that vary substantially in the availability of metal ions from the external environment and from the host. The microbes that inhabit different mucosal surfaces vary in the degree to which they are uniquely adapted to, and are restricted to, the host mucosal environment. This chapter reviews current understanding of metal ion homeostasis in the host, the mechanisms of metal ion acquisition in microbes, and the degree to which the specific mucosal niche impacts the repertoire of metal ion acquisition mechanisms that the microbes possess.

Introduction

Our aim in this chapter is to provide a synopsis of the mechanisms of essential metal ion acquisition in microbes that inhabit the vertebrate host, highlighting how different mucosal environments may influence the repertoire of acquisition systems that are present. An appreciation of the relative exposure of microbes to metal ions from the environment or derived from the host and their available metal ion acquisition systems is an essential foundation for understanding the impact of metals ions on infection.

Metal Ion Homeostasis in Vertebrates

Metal ions are essential to biological systems, as they participate in discrete chemistries that are not possible by organic molecules alone. Iron (Fe), zinc

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(Zn), copper (Cu), and manganese (Mn) are essential to both bacterial and mammalian cells alike. However, the human body also requires molybdenum (Mo), selenium (Se), chromium (Cr), cobalt (Co) and other metals for select biochemistries. While the homeostasis (acquisition, distribution, storage, and elimination) of iron has received a great deal of attention, the homeostasis of some of the other aforementioned metals has not been as well defined.

The Functional Role of Essential Metal Ions: A Brief Glance at Metallobiochemistry

A comprehensive review of the functional role of metals in biological systems is well beyond the scope of this chapter. In the sections below, discussion focuses on some of the well-studied biological roles of essential metals.

Redox-Active Metals: Iron, Copper, Molybdenum, Cobalt, Manganese, and Chromium

Current understanding of Fe and Cu homeostasis stems from their vital and diverse functions in cellular chemistry. Indeed, iron is an essential player in ATP production, cellular division, DNA repair, and antioxidant defense, and is a required moiety for distributing oxygen in the human body. The ability of iron to undergo redox chemistry (i.e., $Fe^{2+} \leftrightarrow Fe^{3+}$) makes it ideal for participating in electron donor/receiving reactions in the electron transport chain and other important redox reactions. Copper also lends itself to electron transfer in the electron transport chain, as a critical cofactor in cytochrome *c* oxidase. The ability of copper to be oxidized (Cu^{2+}) or reduced (Cu^{+}) gives it utility in antioxidant defense in the enzyme Cu/Zn-superoxide dismutase (Cu/Zn-SOD) (Turski and Thiele 2009). The necessity of redox-active metals in attenuating reactive oxygen species is also evidenced by some cells expressing an Fe-dependent SOD.

The human body requires cobalt in its corrin form as cobalamin (vitamin B12). The importance of bacteria to human health is demonstrated by our reliance on them for the synthesis of cobalamin. The versatility of the cofactor cobalamin gives it utility in a range of essential cellular pathways: fatty acid and amino acid metabolism, DNA synthesis, and energy production. Within these pathways, cobalamin participates in small molecule transfer. Some classic examples of this are methyl group transfer (methyl transferases) and hydrogen atom handling (ribonucleotide reductases) (Banerjee and Ragsdale 2003).

In their free ionic form, molybdenum and chromium are typically found as oxyanions in biological systems: MoO_4^{2-}, and CrO_4^{2-}, respectively. However, human cells have found ways to exploit them for biological functions. Cofactor manipulation of molybdenum allows for the shuttling of electrons between molybdenum's IV, V, and VI oxidation state (Schwarz et al. 2009). This gives enzymes such as xanthine oxidase the capacity to participate in two electron

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transfer reactions. Currently, the debate on Cr^{3+} necessity to human health and nutrition is still ongoing. Vincent (2010) proposed that Cr^{3+} is involved in glucose metabolism, through the interaction with various aspects of the insulin-signaling pathway. To date, however, there has been no apparent mechanism that explains this phenomenon in detail.

As a micronutrient, manganese finds one of its most important utilities in Mn-dependent SOD in the mitochondria, and is therefore essential for antioxidant defense. The proposed mechanism of action for manganese in SOD is the formation of a Mn^{3+} intermediate that oxidizes superoxide (Sheng et al. 2012).

Nonredox-Active Metals: Zinc and Selenium

Though not redox active, Zn^{2+} finds use as a catalytic, structural, and regulatory cofactor in the cell (Maret 2013). One of the best-studied catalytic functions of zinc is the activation of water in carbonic anhydrase. Water activation by zinc in carbonic anhydrase allows for the formation of bicarbonate (HCO_3^-) from carbon dioxide (CO_2), an essential function for maintaining the pH of the blood. Zinc is also required for maintaining the structure of the Zn-finger family of transcription factors. Additionally, zinc has also been demonstrated to participate in cell signaling as a secondary messenger, thus inducing the expression of certain genes (Yamasaki et al. 2007).

Selenium is required to biosynthesize the noncoded amino acid selenocysteine. One of the better-documented functions of selenocysteine is its importance in glutathione peroxidase, an enzyme that can detoxify hydrogen peroxide (H_2O_2). Other notable enzymes that require selenocysteine to operate are deiodinases (removal of iodine from thyroid hormones) and formate dehydrogenases (detoxification of formate in the liver) (Rayman 2000).

Acquisition and Transport of Essential Metal Ions

Iron plays a critical role in biological redox reactions but its innate insolubility and the potential for free iron to mediate the generation of toxic by-products in the presence of oxygen has prompted the development of complex systems for maintaining its solubility through ligating to various organic acids and proteins. Thus mammals have developed effective systems for handling iron and regulating the level of available iron (De Domenico et al. 2008). Although less studied, there are likely complex systems for handling other essential metals. One critical component of metal ion homeostasis is the mechanism for uptake by gut epithelial cells, and studies have revealed several pathways (Figure 2.1). However, due to the varying forms of metal ions in dietary sources and the complexities introduced by the interplay with the gut microbiota, it remains to be seen whether there are additional metal ion uptake pathways and to what extent they contribute under varying dietary conditions and microbiota compositions.

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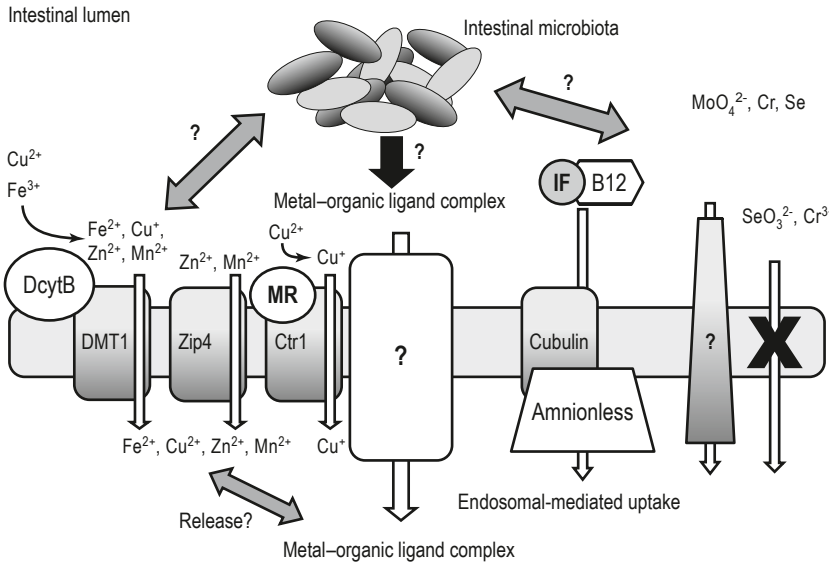

Figure 2.1 Essential metal uptake into intestinal epithelium cells from the gut lumen. The divalent metals Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} can be transported by divalent metal transporter 1 (DMT1) (Vashchenko and MacGillivray 2013). Fe^{3+} must be reduced to Fe^{2+} by the duodenal cytochrome B (DcytB) prior to internalization by DMT1 (Kim et al. 2008; Lichten and Cousins 2009; Vashchenko and MacGillivray 2013). Zip4 and Ctr1 mediate the uptake of Zn^{2+} or Mn^{2+} and Cu^+, respectively (Kim et al. 2008; Lichten and Cousins 2009; Vashchenko and MacGillivray 2013). Reduction of Cu^{2+} to Cu^+ by a metaloreductase, possibly DyctB, is required for import by Ctr1. Vitamin B12 (cobalamin) requires its own transporter complex—cubulin-amnionless—which internalizes a complex of B12 and intrinsic factor (IF) by an endosomal-dependent pathway (Kozyraki and Cases 2013). The exact transport mechanism of MoO_4^{2-}, Cr (III and VI), and Se remain a point of discovery and controversy (Lichten and Cousins 2009; Zeng et al. 2011; Mendel and Kruse 2012).

Essential Metal Uptake by the Gut Epithelia

The divalent metal transporter 1 (DMT1) is capable of transporting a number of the essential metal ions (Fe^{3+}, Cu^{2+}, and possibly Zn^{2+} and Mn^{2+}) into the gut epithelial cell. This process, however, generally requires the activity of the duodenal cytochrome B (DcytB), which reduces dietary Fe^{3+} to Fe^{2+} or Cu^{2+} to Cu^+ for transport by DMT1 into the intestinal epithelial cell (Vashchenko and MacGillivray 2013). Copper can also be transported by a high affinity Cu transporter, Ctr1, a process that also requires coupling to a metalloreductase (Kim et al. 2008). The Zip4 family of transporters on the apical surface of the intestinal epithelia specifically transports zinc, but may also transport manganese (Lichten and Cousins 2009).

Microorganisms also have strict requirements for iron, copper, zinc, and manganese and have mechanisms of acquisition that involve modifying the

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form of metal present in food sources. Current models for metal ion acquisition, however, do not generally account for how eukaryotic cells address the impact of the gut microbiota and compete for various forms of metal ions that may be present. The serendipitous discovery of siderocalin, a secreted protein that binds iron complexed to bacterial siderophores (Flo et al. 2004), provides an example of additional complexities in the competition between the microbiota and host in the competition for metal ions. An obvious question is whether there are additional surface proteins and systems in the gut epithelial that are devoted to acquisition of metal ion complexes that exist due to the presence of the gut microbiome.

Cobalamin (vitamin B12) is the form of cobalt that is transported by gut epithelial cells and there is a complex system for optimizing its utilization. Cobalamin is bound by the salivary gland-derived glycoprotein, haptocorrin (transcobalamin), in the upper gastrointestinal tract to protect the sensitive vitamin from acidic breakdown (Kozyraki and Cases 2013). Cobalamin is then passed from haptocorrin to the parietal cell-produced glycoprotein—intrinsic factor (IF)—in the duodenum following the degradation of haptocorrin. The cobalamin-IF complex is then transported from the lumen by an endosomal-dependent pathway into the enterocyte by the cubilin-amnionless complex (Kozyraki and Cases 2013).

The uptake of molybdenum, chromium, and selenium is poorly characterized. Molybdenum is likely absorbed as MoO_4^{2-} by an active transport process, which may be impeded by the presence of competing anions such as sulphate (Mendel and Kruse 2012). Uptake of chromate through anion transporters is considered a source of toxic exposure. The entry of dietary Cr(III) into the human body may require coordination by an organic ligand (Lichten and Cousins 2009), and Se absorption is enhanced in its amino acid—selenocysteine and selenomethionine—form (Zeng et al. 2011).

The Distribution of Essential Metals throughout the Body

Bioaccessible dietary iron taken up by the enterocyte is transported to the basal membrane where it is loaded onto transferrin (Tf), a bi-lobed glycoprotein capable of binding two Fe^{3+} with high affinity (Figure 2.2). Ferrous iron (Fe^{2+}) is exported from the enterocyte through ferroportin and converted to Fe^{3+} by the ferroxidase hephaestin, for loading iron onto Tf (Vashchenko and MacGillivray 2013). Transferrin transports iron to cells that require iron for growth, particularly erythrocyte precursors which require iron for the oxygen transport protein hemoglobin. Iron is efficiently recycled in the body through (a) direct engulfment of senescent erythrocytes and other cells, (b) macrophages and hepatocytes, or (c) recapture of released hemoglobin or heme by the serum glycoproteins haptoglobin and hemopexin, and taken up by hepatocytes. The recycled iron from macrophages and hepatocytes is then loaded onto Tf through ferroportin and a ferroxidase, as in the enterocyte. The regulation of

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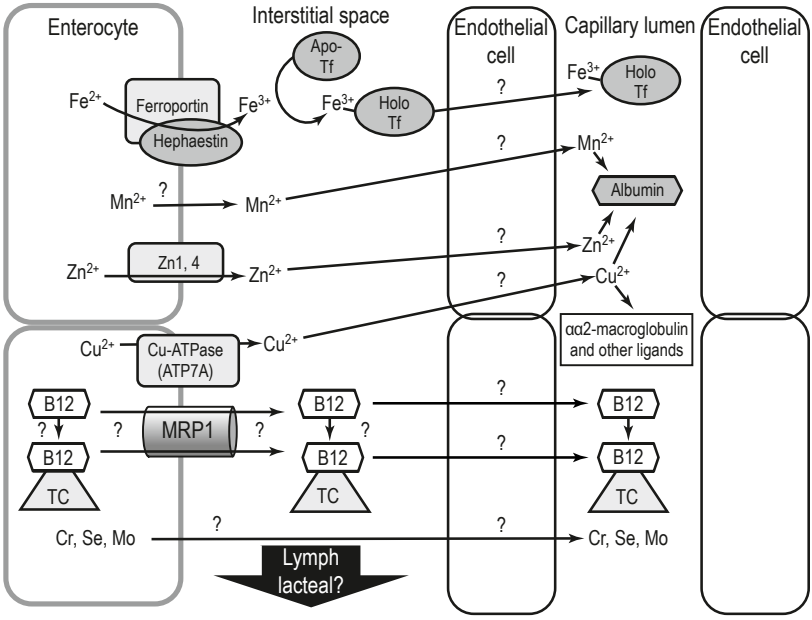

Figure 2.2 Essential metal transport from the enterocyte to the systemic circulation. Iron is transported from the enterocyte to the interstitial space via export through ferroportin and oxidation of Fe^{2+} to Fe^{3+} by hephaestin. This provides the form of iron that is bound by apo-transferrin (apo-Tf) (Vashchenko and MacGillivray 2013). Zn^{2+} and Cu^{2+} are both exported from the enterocyte by specific transporters: Znt1, 4 and ATP7A (Cu-ATPase), respectively (Kim et al. 2008; Lichten and Cousins 2009). Cobalamin (B12) is exported through MRP1, the multidrug protein, and binds to transcobalamin (TC) for distribution in the blood (Kozyraki and Cases 2013). As indicated by the numerous question marks, there is considerable uncertainty as to how the metal ions released from the basal membrane of the enterocyte are transported to the lumen of the blood vessels and bound to proteins thought to be responsible for their distribution throughout the body.

Fe homeostasis by hepcidin takes advantage of the critical role that ferroportin plays in export of iron from cells, and influences the stability of ferroportin (Zhang and Enns 2009).

Cobalamin is likely exported from the enterocyte through the multidrug resistance protein 1 (MRP1) transporter following release from the IF cubulin-amnionless complex via lysosomal acidification (Kozyraki and Cases 2013; Cole 2014). From the enterocyte, cobalamin is delivered through the systemic circulatory system by binding to transcobalamin (Kozyraki and Cases 2013). Further studies are required to establish whether cobalamin binds to transcobalamin before exiting the enterocyte, while in the interstitial space, or upon reaching systemic circulation.

Copper is exported from the enterocyte by a Cu-ATPase (ATP7A) (Kim et al. 2008) but the ligands that it binds for transport throughout the body are not

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firmly established, although albumin and α_2-macroglobulin are likely candidates (Collins et al. 2010). It has also been suggested that the serum Fe oxidase, ceruloplasmin, may play an integral role in Cu transport through the systemic circulation, since 90% of serum copper is bound by ceruloplasmin. However studies in mice lacking ceruloplasmin demonstrated no apparent defect in Cu absorption or distribution (Kim et al. 2008).

Export of zinc from the enterocyte relies on the Zn transporters Znt1 and Znt4 (Lichten and Cousins 2009), whereas Mn export may rely on ferroportin (Madejczyk and Ballatori 2012). Albumin has been implicated as the molecule responsible for carrying both zinc and manganese in the blood (Michalke et al. 2007; Lu et al. 2008).

Although there is information on the pathways for exporting metal ions from the enterocyte, many questions remain: Where does the apo-Tf that binds the Fe^{3+} at the basal membrane of the enterocyte originate (Figure 2.2)? Is apo-Tf produced locally by enterocytes or by leukocytes, or is it transported from the lumen of capillaries? Are the proteins involved in binding other metal ions also present at the basal surface of the enterocyte, and, if so, what is their origin? How is the Fe-loaded Tf transported to the lumen of blood vessels for distribution throughout the body? How are other metals ions, or metal ions complexed to proteins, transported into the lumen of the blood vessels? Are metal ions or metal ion complexes transported through the lymphatics along with cell fluids, just as chylomicrons are? Clearly, current gaps in our understanding of how metal ions enter the systemic circulation remain a field for future discovery in metal ion homeostasis (Figure 2.2).

Mechanisms and Pathways for Metal Ion Acquisition in Microbes

Sources of Metal Ions

The descriptions of metal ion homeostasis in the preceding section focused on the acquisition, distribution, and storage of metal ions within the body. As such, they provide insight on the forms of metal ions that would be available in the extracellular milieu during invasive infection (Hood and Skaar 2012) but do not necessarily provide information regarding what is available on mucosal surfaces.

Due to our understanding of Fe homeostasis, we have a good grasp of the sources of iron available to invading microbes within the mammalian host (Hood and Skaar 2012). The predominant form of extracellular iron within the body is in the form of Tf, which is normally partially saturated (~30%), resulting in rapid sequestration of any available Fe^{3+} by Tf. The structurally related glycoprotein, lactoferrin (Lf), is normally at nearly 1,000-fold lower levels than that of Tf. However, since it is released from neutrophil granules at sites of infection and inflammation, the local concentrations can be substantial. The

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efficient recycling of hemoglobin and heme by haptoglobin and hemopexin not only limit the levels of free hemoglobin and heme, the levels of hemoglobin-haptoglobin and heme-hemopexin complexes are also kept very low, except under conditions of extensive hemolysis.

Although the study of homeostasis of other essential metal ions is less extensive than for iron, there are common features and considerations. The majority of the zinc, manganese, and copper in plasma is bound to albumin or other serum proteins, and calprotectin-releasing cells (e.g., neutrophils) can release calprotectin for Zn and Mn chelation (Hood and Skaar 2012). Just as there is some uncertainty of how metal ions from the diet are transported into serum (Figure 2.2), it is not readily apparent whether or how the components of serum involved in metal ion coordination are transported into the interstitial fluids, except when there is damage to blood vessels during infection or inflammation.

The production of Lf by glandular epithelial cells (Figure 2.3) has led to the general assumption that Lf would be more readily available on mucosal surfaces than Tf. This assumption has been shown, however, to be incorrect for the male genitourinary tract in humans (Anderson et al. 2003). Still, the levels of Lf increase upon gonococcal challenge, are likely due to the concomitant inflammatory response. It is important to consider that Lf is secreted in its apo form and does not in itself represent a means of delivering host iron to the mucosal surface. Lactoferrin will, however, readily complex any free Fe^{3+} that is available. Similarly, the secretion of members of the S100 family of vertebrate proteins would result in chelation of other essential metal ions but would not provide a supply of host-derived metal ions (Hood and Skaar 2012). Aside from the process of bleeding, there currently are no well-described mechanisms by which host metal ion sources would be available on the mucosal surfaces where most microbes reside. Metal ions could certainly be transported to mucosal surfaces by the secretions from glandular cells, which are significant components on mucosal surfaces, but it may not be a well-controlled process. The prevalent forms of the metal ions on the mucosal surface are not well known.

When considering the availability of essential metal ions on various mucosal surfaces, one not only has to consider the degree to which external sources (food, dust particles) are available and the degree to which host sources may be available, but also the degree to which the microbes themselves influence the available sources. Many microbes produce and secrete small Fe-binding chelators, termed siderophores, which are capable of binding and sequestering iron in diverse environments. The selective capture by surface proteins enables the microbe to compete effectively for iron when the supply is limited. Comparable systems for the specific capture of copper, zinc, or manganese have not been identified, but the demonstration that some siderophores may have comparable affinities of iron, copper, and zinc (Brandel et al. 2012) suggests that under some conditions siderophores could provide a means for other essential metal ions to piggyback on siderophore-mediated systems. For

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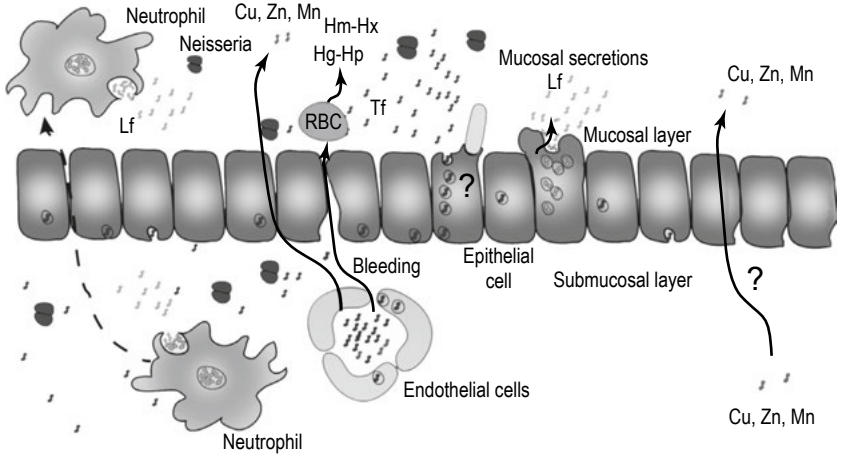

Figure 2.3 Host-derived essential metal ion sources on mucosal surfaces. Potential routes for movement of host-derived metals to the mucosal surfaces consist of migration of host cells (neutrophil), bleeding, and mucosal secretions. The recent observation that *Helicobacter pylori* is capable of hijacking the Tf recycling system and bringing Tf-bound iron to the epithelial surface (Tan et al. 2011) begs the question of whether microbes on mucosal surfaces have a variety of undiscovered mechanisms for bringing essential metal ions to the mucosal surface. Not to be ignored is that some bacteria can be transcytozed across the epithelial cell layer and may reside in the submucosal space.

example, the Fe-binding catecholate siderophore yersiniabactin (Ybt) released by *Escherichia coli* also has the capacity to bind to copper. This Cu chelation capacity of Ybt contributes to higher Cu resistance in urinary tract infection isolates (Chaturvedi et al. 2012).

Transport across the Cytoplasmic Membrane

Unlike the eukaryotic host, microbes (yeast, bacteria) do not transport complexes of metal ions with proteins into the cell. Thus the final stages of transport of metal ions across the cytoplasmic membrane are either with the free metal ion or as a metal ion complexed with small molecules. This process occurs irrespective of what the physiological source of metal ion is. The removal or capture of the metal ion from the host protein is a necessary prior step for protein-metal ion complexes, and the mechanisms vary considerably between Gram-negative and Gram-positive bacteria (Figures 2.4 and 2.5).

A common mechanism for the transport of essential metal ions across the cytoplasmic membrane is through the action of ATP-binding cassette (ABC) transporters (Rees et al. 2009). In addition to the cytoplasmic membrane complex, which consists of a cytoplasmic ATPase bound intimately to an inner membrane transporter, a specific binding protein component is normally

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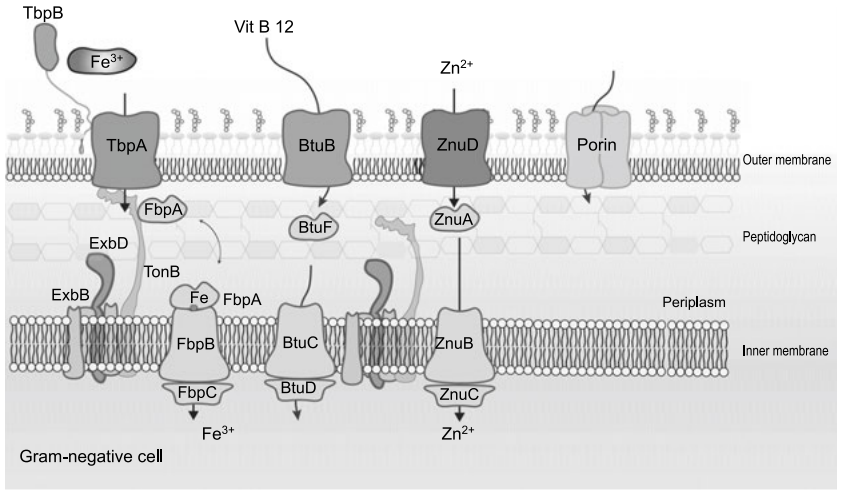

Figure 2.4 Metal ion transport in Gram-negative bacteria. In this figure, only transport systems in which transport across the inner membrane is mediated by an ABC transport system (FbpABC, ZnuABC, BtuFCD) have been included. These systems typically have a periplasmic binding protein (FbpA, ZnuA, BtuF) that delivers the metal ion or metal ion complex to an inner membrane complex consisting of an inner membrane transporter (FbpB, ZnuB, BtuC) and a cytoplasmic ATPase component (FbpC, ZnuC, BtuD). For completeness, a porin in the outer membrane (right-hand side) has been included, although it is an unlikely mode for transport of essential metal ions across the outer membrane. The ZnuD outer membrane transporter and the ZnuABC transport pathway represent the simplest system in which the outer membrane receptor binds Zn and transports it across the outer membrane. The BtuB outer membrane receptor and BtuFCD transport system for transporting vitamin B12 (cobalamin) is a representative for heme and siderophore receptors in that the metal ion complex is directly bound and transported into the cell. The most complex system represented by the TbpAB, FbpABC system involves removal of metal ion from the protein carrier at the cell surface and transport of the metal ion into the cell. This process may involve a lipoprotein component that facilitates the transport process by delivery of the metal-containing protein to the integral outer membrane receptor protein.

present as it not only confers specificity on the transport process but also is effective at capturing substrate for the transport process. In Gram-negative bacteria the binding protein is soluble and contained within the periplasmic space (Figure 2.4), whereas in Gram-positive bacteria the proteins are lipoproteins anchored to the cytoplasmic membrane (Figure 2.5).

A variety of ABC transport systems for essential metal ions have been identified and characterized in bacteria, including FbpABC for Fe^{3+}, ZnuABC for zinc, MntABC for manganese, and NosVFD for copper. Careful biochemical and physiological studies, however, may be needed to define the substrate preference of some transport systems clearly, particularly for the Mn transporters. The structural features of the periplasmic binding proteins and related lipoproteins that directly bind metal ions vary from the venus fly-trap structure of

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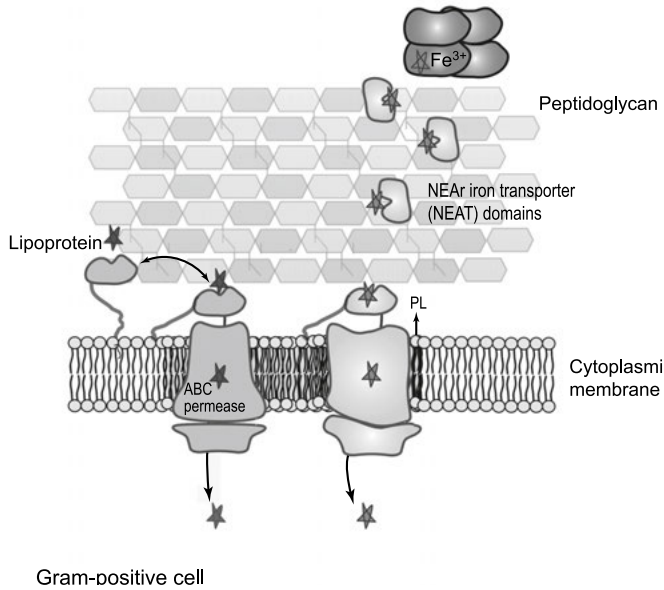

Figure 2.5 Metal ion transport in Gram-positive bacteria. Metal ions or metal ion complexes (outlined as stars) can be captured directly by a tethered lipidated binding protein and transported across the inner membrane by the inner membrane permease-ATPase complex of the ABC transport system. A series of membrane-anchored proteins (proteins with near iron transporter, NEAT, domains) are required for the removal of heme from physiological heme sources such as hemoglobin or hemoglobin-haptoglobin complexes. After shuttling the heme between a series of NEAT domain proteins, the heme is transferred to the lipid-anchored binding protein and transported across the inner membrane by the ABC transporter complex.

the cluster 1 type (FbpA-Fe), which involves considerable domain movement upon metal ion binding, to the more rigid cluster 8/9 type (TroA-Zn) proteins (Krewulak and Vogel 2008). Thus, the processes of metal ion removal and transport into the cell may vary. ABC transporters are also responsible for the transport of iron-siderophore complexes, heme iron, and cobalamin (B12) into the cell. In addition, because the periplasmic binding proteins and lipoproteins all belong to the cluster 8 type, it is likely that the mechanism of removal and transport is similar to that proposed for the BtuFDC complex based on the atomic resolution structural information (Rees et al. 2009).

In addition to the ABC transporters illustrated in Figures 2.4 and 2.5, there are a variety of other essential metal ion transport systems in the cytoplasmic membrane, such as the FeoAB (Fe^{2+}), MntH (Mn), and ZupT (Zn) transporters, the latter being homologs of the eukaryotic NRAMP and ZIP transporter families. These transporters tend to have relatively broad substrate specificities *in vitro*, and determining whether their physiological role relates to a specific metal ion in the mammalian host can be elusive. Similarly it is unclear whether

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ABC transport systems or these types of transporters play more important roles in different ecological niches or under different conditions.

Transport across the Gram-Negative Outer Membrane

The Gram-negative outer membrane provides a semipermeable barrier containing substrate-specific porins and nonspecific porins that allow diffusion of small molecules of the appropriate size and charge characteristics to pass readily through their central channels along a concentration gradient (Figure 2.4). However, essential metal ions and metal ion complexes are generally in low concentrations in the external milieu; they move against a concentration gradient or are not suitable for passing through the standard porin proteins. Thus specific channels are required to facilitate the transport of metal ions and metal ion complexes across the outer membrane and serve to bind and capture the metal ion or metal ion complexes.

Most Gram-negative bacteria have outer membrane receptor complexes that mediate the transport of specific metal ions or metal ion complexes across the outer membrane belonging to the TonB-dependent class of outer membrane proteins. TonB-dependent receptors interact with the cytoplasmic membrane-anchored TonB complex that is required for providing energy for the transport across the outer membrane (Shultis et al. 2006). The simplest TonB-dependent systems are represented by ZnuD (a Zn-specific transporter), siderophore receptors, heme receptor proteins, and BtuB as they appear simply to bind the metal ion (Zn) or metal ion complex (iron-siderophore, heme, or cobalamin) and mediate its transport across the outer membrane in a TonB-dependent manner (Figure 2.4). The apparent independence of ZnuD-mediated Zn transport from TonB (Stork et al. 2010) is an interesting phenomenon, but whether it applies to acquisition of zinc from physiological sources in the host is still an open question. To some extent this is reminiscent of the inability to demonstrate a dependence for utilization of iron in Tf on the surface lipoprotein TbpB with TbpB-deficient strains until tested in the host (Baltes et al. 2002).

Additional complexities arise when metal ions or metal ion complexes are bound to proteins such as Tf, Lf, hemoglobin, hemoglobin-haptoglobin, or heme-hemopexin, and their removal is intrinsic to the overall transport process. The simplest are the single component receptors such as the TbpA2 receptor or heme/hemoglobin receptors (HmbR). The process for heme acquisition may be facilitated by production and secretion of protein hemophores to extract heme from hemoglobin and other heme sources, analogous to siderophores (Wandersman and Delepelaire 2004). The most complex are receptor transporter complexes consisting of a TonB-dependent integral membrane protein and a surface lipoprotein, which facilitates the efficiency of the transport process such as the TbpA-TbpB Tf receptors (Morgenthau et al. 2013) and the HpuA-HpuB hemoglobin-haptoglobin receptors (Rohde and Dyer 2004). The role of the accessory surface lipoprotein can be difficult to demonstrate, and

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the importance in the transport process may only become evident in the host (Baltes et al. 2002).

Structural studies with TonB-dependent transporters have led to insights into the process by which these proteins utilize energy transduced by the inner membrane TonB-Exb-ExbD complex (Figure 2.4) to mediate the transport of iron-siderophore complexes, nickel complexes, or vitamin B12 across the outer membrane (Noinaj et al. 2010). In addition, ongoing structural and functional studies are beginning to provide insights into how the surface receptor complexes are able to extract iron from Tf and mediate its transport across the outer membrane. Although there is some preliminary evidence, it is not well established whether the process of transport across the outer membrane is effectively coupled to the subsequent transport of ligand by the periplasm to the cytoplasm ABC transport system.

Transport in Gram-Positive Bacteria

Although Gram-positive bacteria do not have to contend with the potential barrier that the Gram-negative outer membrane represents, they still have to deal with relatively low concentrations of essential metal ions that are frequently bound to host proteins. The high binding affinity of the lipoprotein component of ABC transporters may be sufficient for capturing some metal ions and metal ion complexes from the external milieu, but the competition with host proteins has required additional components for acquiring some metal ion or metal ion complexes (Figure 2.5). Thus production of siderophores provides one mechanism for acquiring iron from host Tf, and many bacteria produce lipoproteins capable of binding complexes of iron and siderophores produced by other bacteria (Beasley et al. 2011) (Figure 2.5). More complex systems involving a series of heme-binding proteins anchored to the cell wall are involved in acquiring heme iron from various physiological sources of heme. The cell wall-anchored proteins shuttle heme and deliver it to a lipid-anchored binding protein that transports heme iron into the cell through an ABC transport pathway (Tiedemann et al. 2012) (Figure 2.5).

Metal Ion Acquisition on Mucosal Surfaces

Metal Ion Acquisition in the Gastrointestinal Tract

Mucosal surfaces of the gastrointestinal tract represent a diverse set of ecological niches to which the colonizing microbes adapt, ranging from aerobic environments present in the oral cavity to the acidic environment of the stomach to the nearly anaerobic environment of the colon. The microbial communities that are established throughout the gastrointestinal tract are continuously

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exposed to microbes from the external environment, and this can impact the composition of the microbial flora and the development of disease.

Although the microbes may be well adapted to the local environment, many of the microbes in the lower gastrointestinal tract (stomach and intestine) are acquired through the fecal-oral route. Thus they must also adapt to survival outside of the human or vertebrate host. In addition, the microbes are exposed to varying food sources and may thus require diverse capabilities and strategies for metal ion acquisition. As a consequence, versatile mechanisms of metal ion acquisition, such as the siderophore-mediated Fe acquisition mechanism, are often found in pathogens and inhabitants of the gastrointestinal tract. The secretion of siderocalin (lipocalin2) by the host has been shown to limit proliferation of enteric pathogens that produce siderophores capable of being bound by siderocalin (Flo et al. 2004). The production of siderophores that are not bound by siderocalin is a strategy pathogens have used to overcome this growth limitation.

Although microbes colonizing the gastrointestinal tract are expected primarily to access food sources of metal ions, there are unique niches where access host Fe sources may be an advantage. For example, it has recently been demonstrated that *H. pylori* is able to modulate the Tf recycling pathway in host epithelial cells with CagA and VacA, resulting in transport of submucosal Tf to the epithelial surface (Tan et al. 2011). Clearly this is just one example of unique adaptations to specific niches within the diverse microbial surfaces of the gastrointestinal tract that are likely to be revealed with further study.

Metal Ion Acquisition in the Respiratory and Genitourinary Tracts

Unlike the gastrointestinal tract, where there is a large intake of material containing metal ions from the environment, the microbes that inhabit the mucosal surfaces of the genitourinary tract (particularly the male genitourinary tract) and upper respiratory tract—excluding the oral cavity and oropharynx—may rely primarily on host sources of metal ions (Figure 2.3). Since many of the microbes and pathogens that reside in this niche are acquired primarily from other hosts through the close contact and exchange of material from the host (e.g., respiratory droplets, direct exchange during sexual contact) they are highly adapted to the only niche they inhabit: the host's upper respiratory or genitourinary tract. This is reflected in a relatively small genome size, many host-specific interactions, and, commonly, natural transformation systems that facilitate antigenic variation of surface components.

Since metal ions on the upper respiratory and genitourinary tract mucosal surfaces may be primarily obtained from endogenous sources (Figure 2.3), it is perhaps not surprising that some bacteria which reside in this niche have developed mechanisms of acquiring metal ions directly from host proteins. Many of the host-specific receptors that bind to host Fe-containing complexes (Tf, Lf, hemoglobin-haptoglobin, heme-hemopexin) have only been found in bacteria

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that reside in the genitourinary and upper respiratory tracts (see Tf, Lf, Hg-Hp, Hm-Hx in Figure 2.3). It is also important to note that some bacteria are capable of attaching to host surface proteins to mediate transcytosis across epithelial cells, and thus may be able reside in the submucosal space (Sadarangani et al. 2011) where the host Fe-binding complexes would be readily available. This raises the question of whether the ability to use host proteins directly for Fe acquisition is restricted to bacteria that are capable of accessing a different niche. Genomic comparisons of pathogenic and commensal *Neisseria* species (Marri et al. 2010) suggest that some commensals have a different, and to some extent, mutually exclusive mechanism for Fe acquisition, which may be a reflection of different niches. Thus our current appreciation of the mechanisms of metal ion acquisition by bacteria which reside on the genitourinary and upper respiratory mucosal surfaces may be biased by the natural focus on disease-causing bacteria.

There has been a fairly extensive focus on the study of Fe acquisition mechanisms of the Gram-negative pathogenic bacteria which reside exclusively in the genitourinary and upper respiratory tracts that directly acquire iron from host proteins and do not produce siderophores. The lack of siderophore production is also observed in the important Gram-positive pathogen, *Streptococcus pneumoniae,* leading to an impression that reliance primarily on host-derived sources of metal ions by inhabitants of the genitourinary and upper respiratory tracts may lead to less reliance on siderophore-mediated mechanisms. This may reflect the inhabiting of a particular subniche or just a bias in the microbes being studied. A more important question regarding these mucosal surfaces is: What is the source of essential metal ions, and how do the inhabitants access them? The novel finding that *H. pylori* is capable of bringing Tf-Fe to the epithelial system by hijacking the recycling pathway begs the question of whether the inhabitants of the genitourinary and upper respiratory tracts have a diverse array of similar mechanisms for ensuring a sufficient supply of essential metal ions.

Conclusions

It is readily apparent that there are many unanswered questions regarding metal ion homeostasis and the interplay between microorganisms that colonize and infect the vertebrate host. Many of the questions will be addressed through ongoing studies that are being pursued, but some questions may require new initiatives and collaborations. The following are a limited set of questions that we feel warrant attention:

- How do microbiota modulate the bioavailability of metal ions from dietary sources? What are the primary forms of metal ions that the enterocyte is exposed to as a consequence of microbiota actions?

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- How are metal ions released from the enterocyte transported to the lumen of the blood vessels? What is the source of apo-Tf that accepts ferric ion from the enterocyte, and how does the resulting Fe-loaded Tf enter the lumen of the blood vessels?
- How are metal ions in serum delivered to the tissues? Are essential metal ions from the host a primary source for microbes that inhabit the upper respiratory and genitourinary tracts? If so, what are the primary mechanisms for delivery of metal ions onto the mucosal surfaces?

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