7

Metal Transport in the *Rhizobium*-Legume Symbiosis

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Introduction

Iron, zinc, copper, molybdenum and some other transition metals are essential nutrients (Fraustro da Silva and Williams 2001). They are constitutive elements of around half of the proteins of a typical cell (Andreini et al. 2008), where they may act as structural elements, such as zinc in the zinc finger domain, but mostly as key elements in the active site of enzymes involved in almost every physiological process, from oxidative respiration to photosynthesis. These elements are growth-limiting nutrients for autotrophic organisms, since they form very stable complexes, resulting in low solubility and making their uptake difficult (Ruel and Bouis 1998, Fung et al. 2000, Grotz and Guerinot 2006). Moreover, living beings cannot accumulate high amounts of essential transition metals, since these elements can catalyze the production of free radicals in Fenton-style reactions or compete with other metals for the active site of metalloenzymes (Goldstein et al. 1993, Ranquet et al. 2007, Macomber and Imlay 2009). This is also the basis of the toxic effect of non-biogenic metals such as cadmium, lead or mercury.

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Consequently, a concerted set of systems must be in place to ensure high affinity metal uptake while simultaneously avoiding the noxious effects derived from metal accumulation. These systems include a number of metal transporter families, several types of organic molecules that can bind metals with high affinity, soluble proteins that shuttle metal in the cytosol (metallochaperones) or that simply bind excess metals (metallothioneins), and transcription factors that regulate the process (Wandersman and Delepelaire 2004, Waldron and Robinson 2009, Blindauer and Leszczyszyn 2010, Reyes-Caballero et al. 2011, Argüello et al. 2012). Overall, the mechanisms governing metal homeostasis are so efficient that the "free", hydrated, cytosolic metal concentration of a typical bacteria is in the pM-fM range (Outten and O'Halloran 2001), which is less than one free ion per cell. This has two important consequences for how metals are handled by the cell: one of them is the high metal affinity of all the elements involved in metal homeostasis; the other is that metal transport involves either the cotransport with a metal ligand (nicotianamine, glutathione, …) or the physical interaction between a metal delivery metallochaperone and the transporter itself.

In this chapter we focus on how metals are important for nitrogen fixation, and how metal uptake is carried out in the *Rhizobium*-legume system.

Metals and Symbiotic Nitrogen Fixation

Metal levels in the host legume are critical for the establishment and functionality of the symbiosis with rhizobia. For instance, the effect of low iron levels ranges from the inhibition of the nodulation to the loss of the capability to fix nitrogen (Tang et al. 1990, Tang et al. 1992, O'Hara 2001). This is due to the high number and relevance of metalloproteins involved in symbiotic nitrogen fixation.

Nitrogenase is one of the most abundant proteins in the symbiosome (around 10 percent) and arguably the most important enzyme of this symbiosis, since it catalyzes the conversion of N_2 into NH_4^+ (Miller et al. 1993). This enzyme is a multimeric protein that contains metallic cofactors, 2 FeMoCo, 2 P-clusters (Fe_8S_7), and one Fe_4S_4 cluster, totalling 34 iron and two molybdenum atoms. These metal cofactors direct the reducing electrons from a ferredoxin donor (also an iron-sulfur protein) through the nitrogenase Fe_4S_4 clusters to the P-clusters reaching the FeMoCo that would finally use them to break the triple bond in N_2 (Miller et al. 1993). This is an energetically inefficient process, in which reducing power is wasted in the production of H_2. To recover some of it, some rhizobia express the enzyme hydrogenase, a metalloprotein that carries a Ni-Fe cluster (O'Brian and Maier 1989, Palacios et al. 2005).

Leghemoglobin is also expressed at high levels in the nodule (around 20 percent of the total nodular protein) (Appleby 1984). This is a heme-carrying metalloprotein, whose iron confers the characteristic reddish color to functional nodules. Leghemoglobin is responsible of sequestering O_2, creating a microaerobic environment in which nitrogenase can function. In spite of the oxygen sensitivity of nitrogenase, the bacteroids use O_2-dependent respiration to obtain most of their energy. In order to be able to function in this microaerobic environment, the bacteroid expresses a high affinity cytochrome oxidase (*cbb*$_3$-type), whose synthesis requires a steady supply of copper (Preisig et al. 1996a, b).

Metalloproteins, such as catalase or superoxide dismutase (SOD), also play an important role in controlling reactive oxygen species (ROS) (Fridovich 1998). In the nodule, ROS are created by respiration in mitochondria and bacteroids, the direct reduction of O_2 by nitrogenase, hydrogenase and ferredoxin, and the autooxidation of leghemoglobin (Puppo et al. 1981, Dalton et al. 1991). Manganese and ferric-SOD are expressed in the nitrogen fixing areas of the nodule, where they seem to be controlling ROS (Rubio et al. 2004, 2007). The relative levels of Mn-SOD or of Fe-SOD appear to be controlled by iron availability in the nodule, in such a way that as the nodule senesces and heminic iron from the leghemoglobin is released, the relative levels of Fe-SOD increase, as a means to both protect against ROS, and to sequester free iron (Rubio et al. 2007). SODs transform superoxide anion to H_2O_2, which is still toxic. This peroxide is further detoxified by iron-containing catalases (Nicholls et al. 2001), which are critical for nodulation (Jamet et al. 2003, Hanyu et al. 2008). However, low levels of H_2O_2 are necessary for infection thread development and for nod signal transduction (Jamet et al. 2007, Cárdenas et al. 2008). Consequently, catalase overexpression negatively affects the symbiosis (Jamet et al. 2007). It has been hypothesized that a Cu, Zn-SOD is responsible for producing this H_2O_2 (Rubio et al. 2004, 2007).

Therefore, metals are essential nutrients for symbiotic nitrogen fixation, playing relevant roles from the transmission of the nod factor signal to the nitrogen fixation itself. Given that they are endosymbionts, and that many of these proteins are newly synthesized in the nodule, this means that for the metal to reach the symbiosome it has to cross several barriers. First, it has to be incorporated from the soil into the epidermal cells, where they symplastically reach the endodermis. Metals are transported to the vasculature and translocated to other parts of the plant, including the nodule. Then, it has to cross back the endodermis into the nodule cortex where the plant cells will uptake them to synthesize plant-derived nodular metalloproteins. Another part, the one required for bacteroid metalloproteins synthesis, has to cross not only the plasma membrane, but also the peribacteroid membrane and the rhizobial outer and inner

membranes. Given that biological membranes are impermeable to ions, metal transporters play an important role in delivering and controlling which and how metals reach the nodule.

Metal Transporters

Metal transport is as ancient as the first cell, and consequently many metal transporter families (CDF, ZIP or P_{IB}-ATPases) are conserved in all three domains of life. Attending to their substrate, we can classify them in two groups: those transporting metal ions and those transporting metal complexes. Similarly, the direction of transport seems to be conserved, and those families involved in loading the cytosol with metals (either from the cell surface or from an organelle lumen) do not normally export metal out of the cell and vice versa. These are the most common families of metal transporters:

Metal importers

- ZIP (Zrt1-Irt1 like Protein). They typically transport divalent metals (Fe^{2+}, Zn^{2+} and Mn^{2+}) (Eide 2004). They are mainly responsible for iron and zinc uptake in dicotyledonous plants (Vert et al. 2002).
- Nramp (Natural Resistance-Associated Macrophage Protein). Although first discovered in macrophages, these transporters are present in all domains of life (Forbes and Gros 2001, Nevo and Nelson 2006). Their role is complementary to ZIP transporters in many instances, and share similar substrates (Fe^{2+}, Mn^{2+}, Zn^{2+}) (Curie et al. 2000, Cailliatte et al. 2010).
- YSL (Yellow stripe-like). These transporters are present only in plants. Their substrate is a nicotianamine or related molecule complexed with metals (DiDonato et al. 2004). They are responsible for metal uptake from soil in monocotyledonous plants, as well as for long distance metal trafficking in both monocotyledonous and dicotyledonous plants (Curie et al. 2001, Jean et al. 2005).
- Ctr (Cu transporter). This is a homotrimeric protein present only in eukaryotes (Aller and Unger 2006, Dumay et al. 2006, De Feo et al. 2009). They transport Cu^{+} towards the cytosol, being the main copper uptake system in plants (Burkhead et al. 2009).
- MOT (Molybdenum Transporter). They are an extremely high affinity (nM k_M) system of molybdate import in eukaryotes (Tejada-Jiménez et al. 2007, Tomatsu et al. 2007, Tejada-Jiménez et al. 2011). In some cases, their function may be carried out by sulfate transporters (Fitzpatrick et al. 2008).

- ABC (ATP-Binding Cassette-type). These proteins transport a wide range of substrates, including mono- and oligosaccharides, organic and inorganic ions, amino acids, peptides, iron-siderophores, metals, polyamine cations, opines and vitamins (Davidson et al. 2008). The minimal structure of the ABC systems comprises two ATP binding domains for energy transduction, and two membrane-embedded domains forming the channel across the cytoplasmic membrane, which can be encoded in just one gene or by four of them (Rea 2007, Davidson et al. 2008). Most bacterial ABC uptake systems also contain a periplasmic solute-binding protein (SBP) that provides high-affinity binding to the corresponding substrate (Imperial et al. 1998). Extensive reviews on the mechanism and structure of ATP-driven transition metal transporters have been published recently (Cui and Davidson 2011, Klein and Lewinson 2011). While in bacteria they are mostly associated with metal import, in eukaryotes they are involved in metal detoxification (Rea 2007).
- TBDT (TonB-dependent transporter). They are the outer membrane proteins present only in bacteria. Most TBDT-like proteins are known to transport iron compounds (siderophores or heme) (Wandersman and Stojiljkovic 2000).
- HupE/UreJ. These bacterial transporters are involved in Ni^{2+} uptake (Brito et al. 2010).
- NiCoT (Ni^{2+}-Co^{2+} transporter). These transporters are involved in nanomolar Ni^{2+} and Co^{2+} uptake in bacteria, archaea and eukaryotes (Eitinger and Friedrich 1991, Eitinger et al. 2000).

Metal exporters

- P_{IB}-ATPases. These are members of the bigger P-type ATPase family that includes the Ca^{2+}, the Na^{+}/K^{+} and the H^{+} pumps (Palmgren and Nissen 2011). They are an ancient family of transporters present in all domains of life (Argüello 2003). They export Cu^{+}, Cu^{2+}, Zn^{2+}, Mn^{2+}, or Co^{2+}, either with detoxification purposes or to synthesize extracytoplasmic metalloproteins (González-Guerrero et al. 2010, Argüello et al. 2011, Raimunda et al. 2011).
- CDF (Cation Diffusion Facilitator). The homodimer of these transporters extrudes divalent cations (Zn^{2+}, Fe^{2+}, or Mn^{2+}) either to the cell exterior or to organelles to synthesize metalloproteins (Blaudez et al. 2003, Anton et al. 2004, Wei et al. 2004, Lu and Fu 2007).
- Ferroportin. These transporters have been found in animals and plants (McKie et al. 2000, Morrissey et al. 2009). They are involved in Fe^{2+} and Co^{2+} delivery to the vasculature and, consequently, in long distance metal transport.

- CCC1/VIT1. Members of this family have been found only in eukaryotes. They are involved in iron and manganese transport to the vacuole (Li et al. 2001, Kim et al. 2006).
- RND (Resistance-Nodulation-Division). These transporters are present only in Gram negative bacteria, since they form a protein complex that spans both cytoplasmic and outer membranes. The complex is formed by a trimeric inner membrane element and a trimeric outer membrane one that meets in the periplasm. This interaction is stabilized by a hexameric complex of a third protein (Long et al. 2010, Kim et al. 2011, Kulathila et al. 2011). They seem to be involved with metal detoxification from the periplasm (Anton et al. 1999, Nies 2003).

While not directly transporting metals, other transporters are also essential for metal homeostasis. Among them, probably the best characterized is the FRD3 citrate transporter in *Arabidopsis thaliana* (Rogers and Guerinot 2002, Durrett et al. 2007). FRD3 and its homologues are critical for iron transport across symplastically disconnected tissues (pericycle-xylem, embryo-seed envelope or pollen-anther tissue) (Roschzttardtz et al. 2011).

Plant Metal Uptake Transport and Symbiotic Nitrogen Fixation

As previously stated, bioavailable metal levels in soils are very low, especially in basic soils (Grotz et al. 1998). As a consequence, plants are often in a state close to metal deficiency, affecting crop production worldwide (Grotz et al. 1998, Ruel and Bouis 1998). This is particularly evident in the case of legumes since symbiotic nitrogen fixation requires relatively high amounts of metals. Consequently, as the nodulation process is being initiated, it also triggers the "metal deficiency response" (Terry et al. 1991). In dicotyledonous plants, this response involves an acidification of the surrounding soil to increase metal solubility, and the induction of metal reductases to reduce metals which are subsequently incorporated by epidermal metal transporters and also up-regulated (Puig et al. 2007, Andaluz et al. 2009, Bernal et al. 2012). Concomitant to this, and in order to ensure that the nodule needs are satisfied, the legume should down-regulate metal transporters directing metals to non-essential roles, as it seems to be the case of MtMTP1 (Chen et al. 2009).

To date, only three plant transporters have been associated with metal transfer to the nodule. The best characterized of them is *Lotus japonicus* SEN1, identified in a screen for *fix*$^-$ mutants (Hakoyama et al. 2011). SEN1 is a member of the CCC1/VIT1 family. It probably transports iron, since the nodules of *sen1* plants have lower iron concentrations. Histochemical and transcriptional data indicate that SEN1 is nodule-specific. All these

observations suggest that SEN1 is involved in iron delivery to the symbiosome. However, the authors of this work were not able to show the precise location of SEN1 in the cell, nor verify the metal transported. This was achieved in the other two identified nodule-specific metal transporters: the Nramp transporter GmDMT1 and the ZIP GmZIP1, both from *Glycine max* (Moreau et al. 2002, Kaiser et al. 2003). These transporters are located in the peribacteroid membrane. Yeast complementation assays indicate that they transport Fe^{2+} and Zn^{2+} respectively. Their precise direction of transport is not clear. The complementation data and biochemical analysis of homologous transporters indicate that they introduce metal towards the cytosol (Eide 2004, Nevo and Nelson 2006). However, in contradiction with this, an antibody raised against GmZIP1 inhibited Zn uptake in isolated symbiosomes (Moreau et al. 2002). Unfortunately, at that time it was not possible to study mutant lines of either gene, and consequently there is no data on the actual importance and role of each transporter in the symbiosis.

The nodule senesces a few weeks post infection. This is a genetically programmed process, often times coupled to flowering, in which the plant stops sending nutrients to the nodule and directs them instead to the seed (Fedorova et al. 2002, Puppo et al. 2005). It is estimated that around 50 percent of the nodular metal is transferred back to the seed (Burton et al. 1998), where it will be used for embryogenesis and germination (Sancenon et al. 2004, Kim et al. 2006, Roschzttardtz et al. 2011). Consequently, from a metal point of view, nodule senescence involves not only stopping metal delivery to the nodule but recycling the metal "stored" in the nodule. In this process, YSL transporters would play a role. As stated earlier, YSLs are involved in long-distance metal trafficking in which the metal substrate forms complexes with nicotianamine (DiDonato et al. 2004). Although no YSL transporter has as yet been identified in nodules, the presence of the nodule-specific senescence-induced nicotianamine synthase LjNAS2 indicates that this mechanism should be in place (Hakoyama et al. 2009). In this process of recycling metals, ferritins would also participate. Ferritins are plastidial iron storage proteins that form a shell-like structure that contains the iron in a quasi-crystalline form (Briat et al. 2010). In functional nodules, ferritins are highly expressed in the infected cells, but they are down-regulated as the nodule senesces or in the senescence areas of indeterminate nodules (Lucas et al. 1998). This is consistent with a role of ferritin in the protection against ROS (Briat et al. 2010). However, in *Lupinus* nodules, as the nodule senesces, ferritins are up-regulated in the nodular cortex, which might be responsible for protecting the rest of the plant against free radicals produced in the degradation of the nodule, as well as storing the iron before to its translocation to the vasculature via YSL transporters.

Metal Transport Mechanisms in the Bacterial Side

As already stated, the endosymbiotic state of rhizobia requires the uptake of high amounts of metals for the synthesis of metalloproteins. This has an additional layer of complexity, since these bacteria live surrounded by a plant-derived peribacteroid membrane immersed in the plant cytoplasm. Given the complexity of the symbiotic situation, most metal transport studies have been performed with free-living cultures, with further analysis of the effect that mutations in the different systems may have in symbiotic performance. We present here the main recent advances reported on rhizobial mechanisms for uptake of metals through outer and inner membranes. Metal efflux mechanisms, mainly involved in resistance against high levels of metals, have been covered in other reviews (Nies 2003, Macomber and Hausinger 2011, Raimunda et al. 2011).

Metallo-organic Complexes and Metal Uptake in Rhizobia

The extremely low solubility of iron under aerobic conditions is a problem that many organisms, including bacteria and plants, solve through the production of siderophores, organic compounds of low molecular weight (200–2000 Da) with high affinity for Fe^{3+} that allow the bacteria to scavenge the environment for this metal. Siderophores have a variety of chemical structures and form a family of at least 500 different compounds (Budzikiewicz 2010, Hider and Kong 2010). So far, the structures of three types of rhizobial siderophores have been characterized: cyclic trihydroxamates (vicibactin), α-hydroxycarboxylates (rhizobactin) and citrate-derivative hydroxamates (rhizobactin 1021 and schizokinen). Genes involved in the synthesis of these siderophores have been identified for different rhizobia (see O'Brian and Fabiano 2010 for a review).

Metallophores specific for other cations could be used for metal uptake. The analysis of a novel nickel uptake system through the outer membrane of *Helicobacter pylori* led to the hypothesis of the existence of a nickel-complexing compound (nickelophore) required to uptake this element when present at very low concentrations (Schauer et al. 2007). The genome of *Bradyrhizobium japonicum* encodes a similar outer membrane receptor for nickel (see below) suggesting that this metal could also be taken as a metallophore-complex in this bacterium. On the other hand, the existence of a chalkophore (the copper metallophore methanobactin) has been described in the alphaproteobacterium *Methylosinus trichosporium* and other methanotrophic bacteria (Balasubramanian et al. 2011), although the presence of such systems has not been studied in rhizobia.

In addition to bacterial metallophores, rhizobia can also use other complexes such as heme and citrate for uptake of iron or other metals.

Heme is a natural iron source for pathogenic bacteria, and it can be also used by rhizobia. Heme acquisition mediated by the *hmu* system has been described in different rhizobia, and the use of such a system for iron uptake from leghemoglobin in senescing nodules has been proposed (Balasubramanian et al. 2011). Citrate production and uptake of iron-citrate complexes have been described in *B. japonicum* free-living cells (Guerinot et al. 1990). Furthermore, Moreau et al. (1995) showed that isolated soybean bacteroids were able to transport iron citrate, and proposed this chemical species as the main supply for iron in symbiotic conditions. Besides this report , information on the "chemical landscape" of metal species available to the bacteroids within the nodules is scarce. For instance, while nickel is present as malate and citrate complexes in pea nodules (Cacho et al. 2010), other legumes show a different speciation for this metal (our unpublished results). This might affect the availability of nickel for the synthesis of Ni-enzymes such as hydrogenase, and might account for the marked host effect observed on expression of *R. leguminosarum* hydrogenase metalloenzyme (Brito et al. 2008).

Transporters for Metal Uptake through the Outer Membrane

Once the metallo-organic complexes are formed outside the cell, they are internalized through specific receptors located in the outer membrane (OM). Although transport of divalent cations through OM can proceed through general porins (Zeth and Thein 2010), the low availability of metal ions in the free form implies the requirement of receptors to import metallo-organic complexes. The OM does not maintain a proton gradient nor ATP synthesis, and the energy for transport through these receptors is collected from the cytoplasmic membrane by ExbB/ExbD membrane protein complexes and transduced to the OM by the periplasm-spanning inner-membrane protein TonB (Postle and Larsen 2007, Noinaj et al. 2010). For this reason these receptors have been designated as TonB-dependent transporters (TBDTs). The mechanism of uptake for ferric-siderophore complexes by a TBDT has been described in detail in the *E. coli* model system (Chakraborty et al. 2007). Most TBDT-like proteins are known to transport iron compounds (siderophores or heme) or cobalamine (Noinaj et al. 2010); recent reports, however, indicate that these kind of receptors are also involved in the uptake of nickel (Schauer et al. 2007) and zinc (Stork et al. 2010).

In the case of rhizobia, Fe^{3+}-siderophore complexes are recognized by different OM receptors depending on the siderophore. *R. leguminosarum* contains a receptor for vicibactin (FhuA) whose expression is induced in free-living cultures under low Fe conditions (Yeoman et al. 2000). Interestingly, *fhuA* expression is also induced in the meristematic zone of pea nodules, but not in mature bacteroids. Mutations in *fhuA*, however, do

not show significant effects on symbiotic performance (Yeoman et al. 2000). The TBDT for Fe-rhizobactin complex is encoded by *Sinorhizobium meliloti rhtA*. This gene is regulated by iron, and encodes a protein highly similar to enterobactin receptor LutA. *S. meliloti rhtA*$^{-}$ mutants have no significant defects in symbiotic performance (Lynch et al. 2001).

Genomic searches have unveiled the existence of other potential TonB-dependent receptors for which there is no experimental evidence. Analysis of the genome of 13 selected *Rhizobiaceae* strains revealed that the number of TBDT-like genes ranged from one in *Mesorhizobium loti* MAFF303099 to 14 in *Azorhizobium caulinodans* ORS571 (Lim 2010). The majority of the 54 rhizobial TBDTs identified in that study are predicted to be involved in the uptake of iron-siderophore (26) or heme (13). Also, *Mesorhizobium loti* TBDT-like encoded by *meso2063* is known to transport cobalamine, and *B. japonicum bll6948* might transport nickel. The latter assumption is based on the similarity of the *bll6948* gene product to a TBDT involved in nickel uptake in *Helicobacter pylori* (Schauer et al. 2007), and on the genomic context of *bll6948*, located between the gene encoding a nickel transporter of the NiCoT family (*hupN*) and a cluster of genes for the nickel-containing enzyme hydrogenase (Schauer et al. 2008). Although there are no direct studies on the relevance of this potential Ni-specific TBDT, data from transcriptomic analysis indicate that *bll6948* is induced, although at low level, in *B. japonicum* bacteroids (Chang et al. 2007).

Some rhizobia do not produce siderophores, but do synthesize OM receptors able to bind siderophore-metal complexes, allowing the uptake of iron chelates (Small et al. 2009). Such siderophores used by one organism but synthesized and secreted by other organisms are referred to as xenosiderophores. Examples of this type are ferrichrome and rhodotorulic acid, siderophores of fungal origin used by *B. japonicum* as a source of iron (Plessner et al. 1993). The use of xenosiderophores in rhizobia is extensively discussed in another review (O'Brian and Fabiano 2010). A recent report demonstrated that the expression of siderophore receptors from *Pseudomonas* in rhizobia isolated from pigeon pea resulted in better rhizosphere colonization and improved legume plant growth (Arif et al. 2012). This data suggests that iron availability is a major factor limiting the rhizobium-legume symbiosis, and that expression of heterologous siderophore receptors might constitute a potential tool to improve competitiveness and nitrogen fixation.

Although TBDTs appear to be the major OM metal receptors, other types of OM transporters might also participate in metal uptake. Recently, an outer membrane protein with a β-barrel structure (MnoP) has been described as essential for manganese uptake in *B. japonicum* (Hohle et al. 2011). This protein is similar to specific porins OmpA and OprB, and unrelated to the TBDTs described above, and might represent a distinct model for metal

uptake through the outer membrane. The fact that the expression of this protein is co-regulated with that of the inner membrane Mn^{2+} transporter MntH strongly suggests the existence of a two-step transport system for crossing both membranes in this bacterium (Hohle et al. 2011).

Mechanisms for Metal Uptake through Inner Membrane

Once in the periplasm, metal ions must cross the cytoplasmic membrane, the major permeability barrier for metal uptake. As described above, bacteria have developed a number of mechanisms to overcome this barrier.

ABC Transporters

ABC transporters are the prevalent transport systems in rhizobia (Young et al. 2006). *S. meliloti* contains 146 such systems, and similar numbers are present in other *Rhizobiaceae* (Galibert et al. 2001). The role of ABC transporters in the uptake of iron, manganese, cobalt, molybdenum and zinc has been demonstrated in free-living cultures of different rhizobia. Examples of iron-siderophore uptake systems include the *fhuBCD* system described in *Rhizobium leguminosarum* for uptake of vicibactin-Fe, and the *hmuTUV* system described in several rhizobia for heme-Fe complexes (O'Brian and Fabiano 2010).

A manganese-specific ABC importer (*sitABCD*) has been described in *S. meliloti*. Analysis of *S. meliloti* mutants affected in this operon indicates that this system is required for growth under manganese-limiting conditions. The same mutants display a normal symbiotic phenotype (Platero et al. 2003). A similar situation was found for the cobalt transport system (*cbtJKL*) recently described also in *S. meliloti* (Cheng et al. 2011). Expression of this system is controlled by a cobalamin-binding riboswitch located upstream of the operon. Mutants affected in this system are unable to grow on LB medium unless it is supplemented with cobalt. Interestingly, these mutants show a residual level of cobalt uptake, potentially due to an alternate, unspecific transport system. Such a system might be responsible for the normal symbiotic phenotype showed by the *cbt* mutants in symbiosis with alfalfa (Cheng et al. 2011).

The ModABC system mediates high affinity molybdate uptake in *B. japonicum* and probably also in other rhizobia, since it is encoded in most rhizobial genomes (http://genome.kazusa.or.jp/rhizobase/). *B. japonicum* mutants affected in the *mod* system lack nitrate reductase activity in free living cultures, and show reduced levels of nitrogen fixation in symbiosis with soybean. These deficiencies were corrected by addition of molybdenum to the plants (Delgado et al. 2006). Such complementation has a strong dependence on the presence of sulfate, suggesting that, in addition to being

mainly incorporated through the Mod system, molybdate is also taken up through sulfate transporters under symbiotic conditions, as it has been shown for a plant sulfate transporter (Fitzpatrick et al. 2008).

ABC transporters for zinc uptake homologous to the ZnuABC system described in *E. coli* and other bacteria (Hantke 2005) are present in the genome of most rhizobial strains. However, no specific work on zinc transport by endosymbiotic bacteria is available. The only mention of potential relevance of these transport systems in rhizobia is the induction of expression of *S. meliloti* Znu system in response to zinc limitation (Mauchline et al. 2006). Interestingly, Znu-deficient mutants of the phylogenetically relative animal pathogen *Brucella abortus* show a reduced intracellular survival and virulence (Kim et al. 2004).

There is not much information about the uptake of copper in rhizobia. Copper is required in bacteria mainly for proteins located either in the cytoplasmic membrane or in the periplasmic space. Based on that, some authors maintain that bacteria may not have a general requirement for cytoplasmic copper (Solioz et al. 2010). However, genes for two ATP-dependent systems (*nosDFYL* and *fixI*) have been linked to copper metabolism in rhizobia. The *nosDFYL* operon encodes an ABC uptake system. Since these genes are located in the nitrous oxide reductase gene clusters from *S. meliloti* and *B. japonicum* (Holloway et al. 1996, Velasco et al. 2004), their participation in the uptake of copper for the synthesis of this copper-metalloenzyme has been proposed (Holloway et al. 1996). Mutations in these genes do not affect symbiotic performance, suggesting that such a system is not critical for copper uptake under symbiotic conditions. On the other hand, *fixI* encodes a P-type Cu-ATPase known to participate in the synthesis of cbb_3-type heme-copper cytochrome *c* oxidase (cbb_3-Cox), an essential component of the symbiotic respiratory branch (Preisig et al. 1996a). These authors suggested the possibility that this ATPase imports Cu into the cytoplasm; however, this possibility has been contested by biochemical data that indicates that FixI is actually an efflux system (González-Guerrero et al. 2010). Then, FixI should participate in the transport of Cu from cytoplasm to periplasm for cbb_3-Cox biosynthesis. Copper uptake for cbb_3-Cox synthesis in rhizobia might instead be carried out by CcoA, an MFS transporter recently described in *Rhodobacter capsulatus* (see below).

Permease Metal Transporters

These are secondary transporters, dependent on proton motive force, composed by single proteins with multiple transmembrane domains in a monomeric or dimeric form. In the case of endosymbiotic bacteria, this type of transport system mediates uptake of nickel, cobalt and manganese ions.

Three main classes of secondary transport mechanisms for metal uptake have been described in rhizobia:

- *NiCoTs.* NiCoTs expression in *E. coli* identified ion preferences ranging from strict selectivity for nickel to a strong preference for cobalt through unbiased transport of both ions (Eitinger et al. 2005). *B. japonicum* HupN was the first member identified in rhizobia (Fu et al. 1994). The corresponding gene is located adjacent to a DNA region encoding the nickel-containing enzyme hydrogenase. Analysis of mutants affected in this gene revealed a significant Ni-dependent decrease in hydrogenase activity in free-living cells (Fu et al. 1994).
- *HupE/UreJ.* Their encoding genes are associated to gene clusters encoding NiFe hidrogenases in different rhizobia like *R. leguminosarum* and *A. caulinodans. R. leguminosarum hupE* encodes an integral membrane protein with six known transmembrane domains. Mutant analysis demonstrated the essentiality of this protein for the synthesis of *R. leguminosarum* NiFe hydrogenase both in free-living culture and in symbiosis (Brito et al. 2010). Genes encoding members of this group of transporters in other bacteria are sometimes preceded by sequences for cobalamin riboswitch RNA regulatory elements, suggesting that they might transport this cobalt compound rather than nickel (Schauer et al. 2008). So far, no evidence for a cobalt transporter of this type has been reported in rhizobia.
- *Nramp.* A relevant member of this group is MntH, a proton symporter acting as the main manganese uptake system in free-living cultures of *B. japonicum* (Hohle and O'Brian 2009). This transporter is not essential for the development of nodules nor for nitrogen fixation activity in symbiosis with soybean, suggesting that either the host provides enough manganese to make high-affinity transport unnecessary, or the bacterium has another mechanism for manganese acquisition that is not expressed in free-living cells (Hohle and O'Brian 2009). Interestingly, MntH plays a critical role in Mn^{2+} transport in the close phylogenetic relative *B. abortus,* where the presence of this manganese transporter is required for wild-type virulence in mouse (Anderson et al. 2009).

An additional type of secondary metal transporter might be relevant for rhizobia. A recent report described a gene (*ccoA*) required to maintain normal amounts of intracellular Cu and synthesis of cbb_3-Cox (Ekici et al. 2012) in the alpha-proteobacterium *Rhodobacter capsulatus,* strongly suggesting that the corresponding protein is a copper uptake transporter. CcoA is a member of the major facilitator superfamily (MFS) with 12 putative transmembrane helices split into two subdomains of six helices each, separated by a large cytoplasmic loop. Interestingly, this protein has homologs (*ca.* 40 percent identity) encoded in the genome of *Bradyrhizobium*, *Mesorhizobium* and

Sinorhizobium. Further studies are required to elucidate whether these homologs might function as copper transporters in rhizobia.

Global Analysis of Expression of Bacterial Metal Uptake Systems

The information on direct analysis of metal transport under symbiotic conditions is still scarce, due to the inherent difficulty of working with bacteroids. This information can be complemented by data arising form transcriptomic and proteomic analysis of genes expressed by rhizobia in the endosymbiotic state. A global study performed through transcriptomic analysis of symbiotic expression in *S. meliloti* revealed that genes involved in iron uptake were mostly repressed in the bacteroids (Becker et al. 2004). However, repression does not indicate that these genes are not expressed in the nodule bacteria. In fact, solute-binding proteins from ABC systems mediating uptake of iron, manganese and zinc were identified in the proteomic analysis of *S. meliloti* bacteroids isolated from alfalfa nodules (Djordjevic et al. 2003). In the case of *B. japonicum* soybean bacteroids, transcriptomic analysis (Pessi et al. 2007) revealed that components of the molybdenum uptake system ModABC were amongst the most strongly induced genes in bacteroids (over 120-fold as compared to free-living cells). This data is consistent with the relevant symbiotic role of this transporter deduced from the mutant analysis mentioned above (Delgado et al. 2006). Further proteomic analyses of *B. japonicum* bacteroids induced in soybean indicate the presence of proteins involved in iron transport, including the TonB-dependent ferrichrome receptors blr3904 and bll4920 (Delmotte et al. 2010). Finally, a recent report on the transcriptomics of *R. etli* bacteroids induced in *Phaseolus* indicates moderate induction of genes for inorganic ion transporters including *afuA3*, which encodes a solute binding protein from an ABC iron uptake system not expressed in free-living cells (Vercruysse et al. 2011).

Summary and Outlook

In the last two decades, our understanding of metal homeostasis processes in plants and bacteria has increased considerably, but it is still lagging in certain aspects such as the legume-rhizobium interaction. This is in spite of metals being essential nutrients for symbiotic nitrogen fixation and critical components of the key enzymes of this metabolic process. In this process of metal exchange between the symbionts, the peribacteroid membrane is critical. However, many transporters in this membrane remain to be discovered. With the development of genomic, transcriptomic, proteomic and metallomic tools we are filling this gap in knowledge. New elements involved in metal transfer to the symbiosome have been identified, as well

as the mechanisms involved in bacteroid metal uptake and use (summarized in Fig. 7.1). Interestingly, not many of the rhizobial transporters involved in metal uptake in free living conditions have a relevant role in symbiosis, probably reflecting a more favorable environment for metal uptake than in soils. Nevertheless, there are still many aspects to study, such as how the overall plant metal homeostasis mechanisms are affected by nodulation, what role rhizobial metal efflux transporters play in the invasion stage, which transporters are directing metals from the soil to the bacteroid, how metal speciation affects metal delivery, and how metals are directed to the corresponding apoproteins in the bacteroid cytosol.

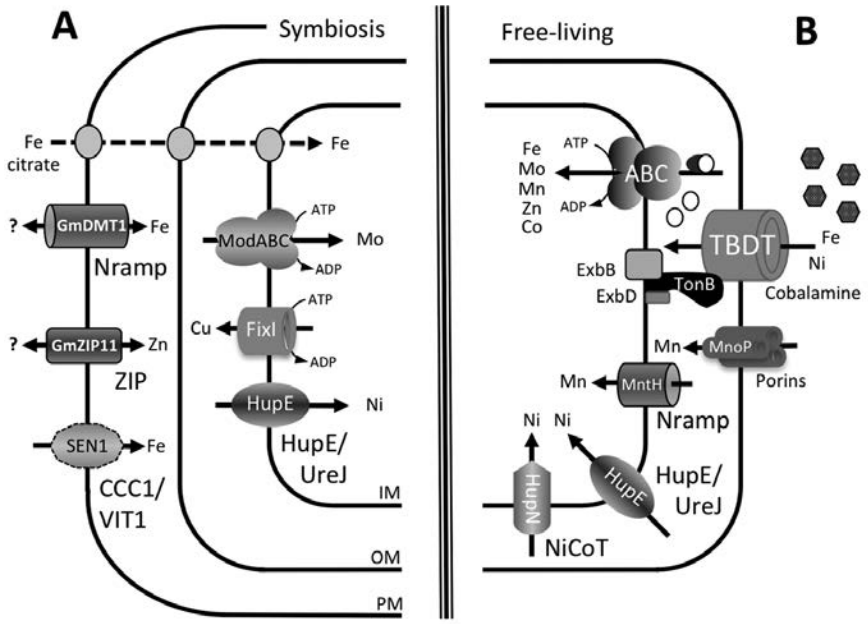

Figure 7.1 Metal uptake transporters described in *Rhizobium*-legume interactions. Transporters involved in metal uptake in symbiotic (A) and free-living (B) conditions are localized in the inner (IM), outer (OM) and plant membranes (PM). Siderophores are shown by hexagons whereas circles represent metal ions. Transport orientation for the plant Nramp and ZIP proteins remains elusive. The dotted arrow symbolizes an unknown mechanism for ferric citrate transfer across the plant and bacterial membranes. ABC: ATP-Binding Cassette transporters.

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