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Transition metal transporters in rhizobia: Tuning the inorganic micronutrient requirements to different living styles

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A group of bacteria known as rhizobia are key players in symbiotic nitrogen fixation (SNF) in partnership with legumes. After a molecular exchange, the bacteria end surrounded by a plant membrane forming symbiosomes, organelle-like structures, where they differentiate to bacteroids and fix nitrogen. This symbiotic process is highly dependent on dynamic nutrient exchanges between the partners. Among these are transition metals (TM) participating as inorganic and organic cofactors of fundamental enzymes. While the understanding of how plant transporters facilitate TMs to the very near environment of the bacteroid is expanding, our knowledge on how bacteroid transporters integrate to TM homeostasis mechanisms in the plant host is still limited. This is significantly relevant considering the low solubility and scarcity of TMs in soils, and the *in crescendo* gradient of TM bioavailability rhizobia faces during the infection and bacteroid differentiation processes. In the present work, we review the main metal transporter families found in rhizobia, their role in free-living conditions and, when known, in symbiosis. We focus on discussing those transporters which could play a significant role in TM-dependent biochemical and physiological processes in the bacteroid, thus paving the way towards an optimized SNF.

1. Introduction

One of the main entries of nitrogen (N) in its biogeochemical cycle is the Biological Nitrogen Fixation (BNF), where inert molecular nitrogen (N_2) is reduced to ammonia (NH_3). This is achieved by a varied group of water- or soil-dwelling archaea and bacteria microorganisms harbouring the enzyme nitrogenase, generally known as diazotrophs[1]. Numerous diazotrophs fix nitrogen in free-living conditions, having the machinery to assemble the nitrogenase enzyme, to energize the process, and to protect the oxygen-sensitive nitrogenase. Other diazotrophs fix nitrogen only when interacting with other organisms, in so called symbiotic nitrogen fixation (SNF). Here, the partners share energy production and protection of the nitrogenase duties, since nitrogenase machinery is encoded only in prokaryotes. SNF performed between a group of bacteria called rhizobia and legume plants has been extensively studied because of its agricultural interest as an environmentally-friendly alternative to the use of fertilizers. As a result of the interaction, the plant develops new organs, the nodules, where they provide an optimal environment to maintain obligate aerobe rhizobia metabolism and the microaerobic environment to protect nitrogenase.

Diazotrophy is a biochemical process requiring numerous transition metals (TM) (cobalt, copper, iron, manganese, molybdenum, nickel and zinc), as has been found in several free-living and symbiotic diazotrophs[2-4]. At the same time, it is well known that most TMs at high intracellular levels induce cytotoxicity, via the production of free radicals (Fenton-like reactions), or outcompeting the cognate metal in metalloproteins[5].

TM transporters are key players in mechanisms leading to the TM homeostasis and metalloprotein assembly required for diazotrophy in SNF. Several families participating in the efflux/influx of TM in rhizobia have been identified. Emphasizing the complexity of the rhizobia-legume symbiotic process, with a few exceptions, many of these transporters seem dispensable for SNF. Although some were indeed proposed as key players in SNF, they represent strain-specific transporters involved in iron and manganese homeostasis (see 4.1.2 and 5.3.2, respectively), or in the metalation of a fundamental cuproprotein that sustains the process energetically (see 5.2.1). Importantly, this reveals the lack of systematic and formal analyses of the information gathered in recent years from the different rhizobia-legume model organisms. It also highlights the necessity to integrate data from the –omics and high resolution imaging fields with those existing in classical physiological, biochemical, and genetic studies, in order to identify TM transporters as potential candidates in the rhizobia-legume symbiotic interphase.

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2. What happens to the bacteria during rhizobia-legume symbiosis?

Rhizobia are heterotrophic organisms with a versatile metabolism. When living in soil, they mostly use plant root exudates and reduced organic compounds released by biomass degradation as C and N sources (reviewed in[6]). Upon nitrogen starvation, rhizobia and legumes establish a molecular dialogue, which marks the formation of the nodule and the infection of the plant by the rhizobia. Briefly, the process starts with the perception of root-secreted flavonoids by the rhizobia. This induces their motility towards the root, in addition to the synthesis and secretion of Nod factors to the soil. Nod factors specifically interact with plant cell membrane kinase receptors, triggering a fundamental Ca^{2+}-dependent signalling pathway[7, 8]. From this point on, there are events occurring in parallel in both symbionts:

-On the plant side: A shepherd hook structure formed at the tip of a root hair will trap rhizobia attached to the root surface. There, a transcellular channel-like structure will be formed, known as infection threads (IT)[9, 10, 11]. In the inner root, asymmetric divisions of certain root tissues result in the protrusion of a cell mass, which driven by a detailed developmental program, results in the organogenesis of the nodule[12, 13]. Depending on what cellular type is divided and the developmental program initiated, the nodules may or may not present a permanent meristem, among other differentiating features. Based on those criteria, nodules are classified as determinate, spherical, such as those found in *Glycine max* (soybean), *Phaseolus vulgaris* (bean) or *Lotus japonicum*; or indeterminate cylindrical nodules, such as those of *Medicago truncatula*, *Pisum sativum* (pea), and *Vicia faba* (broad bean). The absence of a meristem determines that the rhizobia inside these nodules are synchronized (evolve temporally through the different phases of the symbiotic process: infection>fixation>senescence). Indeterminate nodules, due to the presence of a meristem, develop a spatial zonation coinciding with the different phases of the symbiotic process: meristem (zone I), infection zone (zone II), nitrogen fixing zone (zone III), and the senescent zone (zone IV)[12, 14].

-On the rhizobia side: bacteria attached to the root hairs and enclosed in the shepherd hook structure progress through the IT mainly by cell division. Once at the end of the IT, the rhizobia will directly interact with plant membrane receptors in infection droplets (anatomical structures where there is no plant cell wall), and are endocyted [10, 11]. Once inside the plant cell, rhizobia will multiply and segregate, forming an organelle-like structure called symbiosome, a plant-derived membrane surrounding the rhizobia. Depending on the symbiotic plant partners, each symbiosome contains one or several bacteria. Afterwards, rhizobia will carry out several endoreduplication cycles, followed or not by a differentiation program. The terminal differentiation is controlled by the plant through different signals, including the host Nodule Cysteine-Rich peptides (NCR), among other factors[15, 16]. The ability to fix nitrogen seems independent of the differentiation program and it is performed in the endocyted and endoreduplicated bacteria, now called bacteroids.

It is generally assumed that once endocyted, bacteroids become dependent on the nutrients provided by the plant. I.e. the plant infected cell supplies reduced carbon to the bacteroids as C_4-dicarboxilic acids (malate, succinate and fumarate), which are then taken up by DctA in the bacteroid, a member of the MFS family required for malate uptake[17]. Bacteroids also readjust their metabolism to meet the metabolic demand imposed by nitrogen fixation (16 ATP molecules per mol of N_2 reduced). For that, they obtain energy using a strictly respiratory metabolism once they are within the nodule microaerobic environment generated by the plant leghemoglobins[18]. Comparative studies using transcriptomics, proteomics and metabolic modelling, showed that among the main alterations during symbiosis, is the shut-off of the glycolytic pathway[19, 20]. This apparent limitation in TCA cycle could be explained by low free O2 tension in the nodule and overcome by the induction of the high O2 affinity *cbb3*-cytochrome c oxidase (*cbb3*-COX) and several enzymes of the cycle[6] (Table S1). Indeed, a metabolic modelling study shows significant increase rates in several reactions of the TCA cycle simulated at the bacteroid stage[21].

Nitrogen assimilation through amino acid and protein anabolism is expected in free-living diazotrophic rhizobia. However, in the bacteroids, the biochemical pathways enabling N autotrophy are shut down. Therefore, bacteroids become organelle-like auxotrophs for branched aminoacids. This effect was observed in *R. leguminosarum,* where two broad-specificity amino acid transporters from the ABC-family, Aap and Bra, are required for effective SNF [22 6] .

All the above indicated the capacity of rhizobia to thrive in the heterogeneous menu of C and N present in the soil *versus* the nodule. This is achieved due to the integration of transport systems tuning the fluxes upon metabolic demands. So the immediate questions are: What are the metabolic requirements of TM in rhizobia during symbiosis? Do rhizobia tune the TM fluxes to fulfil this demand?

3. TM requirement for rhizobia during symbiosis

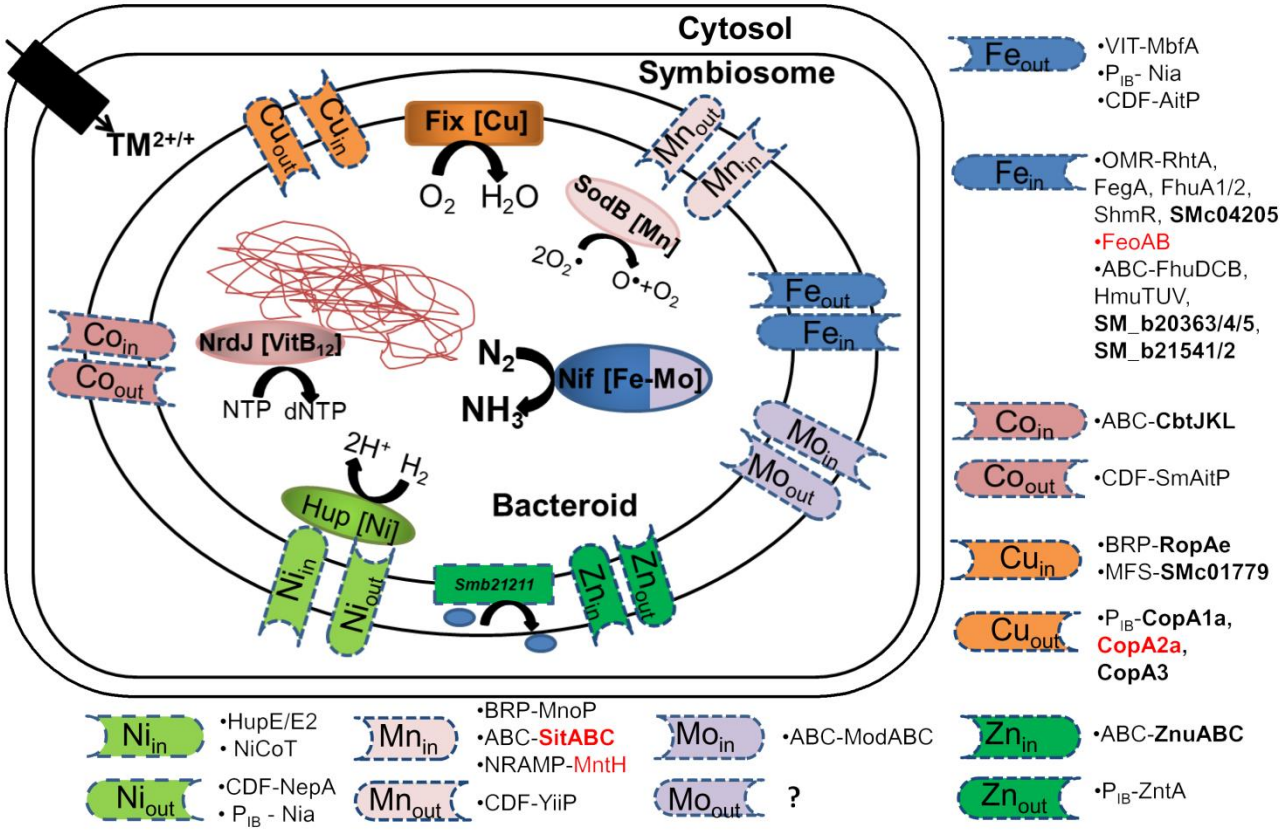

Figure 1. **SNF is highly dependent on TMs.** Bacteroids in the symbiosome rely on TMs for several processes, as depicted. Feeding TMs at this stage requires a battery of TM transporters (importers and exporters) ultimately involved in rhizobia homeostasis and bacterial physiology. The functional roles of the transporters mostly depend on transport specificity, transport direction and temporal expression during infection. Specificity and transport directions are indicated with the element symbol of the ion transported and a subscript (TM_{in} = importers; TM_{out}=exporters). Systems located at the outer membrane are Outer Membrane Receptors (OMR) and Brucella-Rhizobium Porin (BRP). Systems at the inner membrane are Cation diffusion facilitator (CDF), ATP Binding Cassette-type (ABC), P-type-$_{IB}$-ATPases (P_{IB}), Nodulation-Resistance-Membrane Protein (NRAMP), HupE-UreJ and NicoT. The systems have been assigned through *in vitro* phenotypic analysis and to a lesser extent by biochemical characterization of transport (see text). Transporters induced in nodules (Table S1) are marked in bold and those required for optimal SNF in red letters. Nif (nitrogenase), NrdJ (vitamin B12-dependent ribonucleotide reductase), Fix (*cbb*$_3$-cytochrome c oxidase), SodB (superoxide dismutase B), Hup (hydrogenase), SMb21211 (putative lipid A phosphetanolamine transferase) are metalloproteins depending on Fe/Mo, Co, Cu, Mn, Ni and Zn ions (with TM as co-factors or co-enzymes), respectively, induced in the bacteroids. Blue circle= putative Lipid A modification by SMb_21211; ?= unidentified transporter.

During their lifecycle, rhizobia face different challenges to satisfy the TM quota and to avoid toxicity, based on the different availability of TM in the soil or the nodule, and on their own requirement of TM in those environments (as their metabolism and metalloproteome change).

When rhizobia-legume symbiosis is established, nodulation triggers metal uptake mechanisms in the plant to ensure an optimal metal supply for SNF. Also, nodules become the most important sink for nutrients with around one third of plant Fe (and a large proportion of their Zn, Cu, and Mo) relocated there[23]. For instance, the Fe and Zn nodule distribution observed with synchrotron X-Ray Fluorescence (S-XRF) and fluorescent probes showed that, in *M. truncatula* nodules, TM reach the nodule through the peripheral vascular bundles, and are released at the apical part of the nodule. There, they first accumulate in the apoplast, while in zone III most of the metals are inside rhizobia-infected cells and bacteroids [24, 25]. This data implied the existence of a set of transporters in transition zone II/III that incorporate TM in the rhizobia-infected cells: MtNramp1 for iron[26]; MtZIP6 for zinc [25]; MtCOPT1 for copper[27], and MtMOT1.3 for Mo (as molybdate)[28]. From there, MtSEN1, a VIT1/CCC1 family member, could be responsible for iron transport in the symbiosome membrane[29], although direct evidences of its subcellular localization and transported substrate are still required. Delivery of copper and zinc could be mediated by yet unidentified P_{IB}-ATPases, CDF proteins or PLAC8 family members[4, 30].

As mentioned above, diazotrophy is a highly TM-dependent process. Undoubtedly, such a high requirement reflects in the synthesis of very abundant metalloproteins during the symbiotic process (Fig. 1; Table S1). Among the proteins present in the bacteroid are: the FeMo-nitrogenase, the most abundant protein in the bacteroid, which contains up to 32 atoms of iron and 1 of molybdenum; terminal *cbb3*-COX, copper proteins are critical for the oxygen consumption and energy production; cytosolic superoxide dismutase B (SodB), involved in reactive oxygen species (ROS) detoxification, requires Mn as cofactor; Co (as vitamin B12) seems required during the infection process since vitamin B12-dependent enzymes, such as the Class II ribonucleotide reductase (NrdJ), become upregulated there; Zn is required for symbiosis[25, 31],

but the Zn^{2+}-dependent proteins (both on the plant and the bacterial side) remain unknown.

Therefore, considering the different availability of TM in the nodule compared to the soil, and the increased demand of TM in the bacteroid, we expect a variable set of TM transporters in the bacteroid compared to the free-living bacteria, dedicated to achieving TM homeostasis and metalloprotein synthesis in such a challenging environment.

4. Iron: The way in

Iron is the most abundant TM in the symbiosome[32], so in this section we will examine current knowledge on the different mechanisms that rhizobia use to internalize this fundamental micronutrient from the soil as free-living bacteria. We will also focus on the bacteroid Fe acquisition in the symbiosome (as endosymbionts), as this remains unknown for several rhizobia members.

4.1 Scavenging iron

Iron in soils is mostly present as low-soluble oxides and hydroxides[33]. To solubilize it, bacteria synthesize and secrete siderophores, low molecular weight organic compounds with high affinity for TM, especially Fe^{3+} [34] . Based on the chemical moiety that allows the interaction with Fe, there are three main types of siderophores: catecholates, hydroxamates and carboxylates[35, 36]. In rhizobia, most synthesized siderophores are of the hydroxamate type, including cyclic trihydroxamates (vicibactin), α-hydroxycarboxylates (rhizobactin) and mixed-type (rhizobactin 1021 and schizokinen)[37, 38 39, 40] . Genes involved in the synthesis of these siderophores have been identified in different rhizobia, including *Sinorhizobium meliloti* and *Rhizobium leguminosarum*[41, 42] (reviewed in [43]), which are then capable of sequestering and internalizing Fe^{3+} by means of endogenously produced siderophores. Other rhizobia, such as *Bradyrhizobium japonicum*, although unable to synthesize siderophores, recognize and scavenge those produced by others, therein called xenosiderophores[44, 45 46]. Independent of the source, Fe^{3+}-siderophore complexes are internalized into the cytosol by energy-dependent transport systems located at the outer and the inner membrane (see below). It is also interesting to note that *B. japonicum* quickly acquired the ability to incorporate artificial Fe-ligand complexes, such as the iron-fertilizer Fe-EDDHA by mutating some of these systems[47], which opens up the possibility that rhizobia may be using other plant siderophores, such as flavins and coumarins. Mutants in siderophore synthesis showed no symbiotic phenotype[41, 42], although some reports suggest that the altered nutritional status of the bacteria due to the lack of siderophores may influence nitrogen fixation activity [48-50] .

4.1.1 Outer membrane (OM) receptors/porins

A fundamental prerequisite in Gram-negative bacteria for TM uptake is their entry into the periplasmic space. In rhizobia, this is achieved by two families of the Outer Membrane Pore-forming Protein (OMPP) superfamily I, the OM receptor and the Brucella-Rhizobium Porin (BRP) families[51]. Both families are integrated with other transport systems at the inner membrane. The OM receptor members present binding sites for organic molecules, while OM porin members behave as channels [52]. The first case is particularly important for Fe uptake: once Fe^{3+} is solubilized, the acquisition of siderophores, heme and other Fe-compounds is mediated by outer membrane OM receptors of the TonB-dependent transporter (TBDT) family[53]. TBDTs usually present a TonB interacting domain, which allows their interaction with the TonB/ExbB/ExbD complex, responsible for energizing the process of uptake via the OM receptor[54 55, 56].

Native siderophores, such as rhizobactin 1021 or vicibactin, are acquired by the pair, OM receptor/inner membrane transporter RhtA/RhtX[41, 57] and FhuA/FhuCDB[58, 59], while *B. japonicum* imports the xenosiderophores, ferrichrome and rhodotorulic acid, via the OM receptors, FegA, FhuA1 and FhuA2 (FoxA)[43, 45, 46, 60]. Pre-infection TM nutrition is a relevant factor for the rhizobium-legume symbiosis since the expression of siderophore receptors from *Pseudomonas* in rhizobia isolated from pigeon pea resulted in better rhizosphere colonization and improved legume plant growth[61].

Heme was recognized in early works as an Fe source for rhizobia[62], and more recently intracellular heme-degrading oxygenases and monoxygenases (*hmuO*, *hmuQ*, *hmuD*) involved in tetrapyrrole degradation and Fe^{2+} released have been identified[63-65]. Heme is imported through the OM receptor ShmR and then the HmuTUV ABC-type transport system (section 4.1.3), which has been described in different rhizobia [66 60].

The oligotrophy and capacity of rhizobia to thrive in diverse niches is exemplified in the Fe natural and artificial sources which they can use. Rhizobia can also use citrate-Fe complexes[44], probably through a homolog of the FecA system in *E. coli*[67], which is potentially relevant since isolated soybean bacteroids were able to transport Fe and Fe-citrate[68-70]. *S. meliloti* encodes a putative FecAR system (SMc02177-SMc02178) with expression in the nodule but no visible roles in nitrogen fixation[71]. Another ortholog system is formed by FecARI orthologs (SMc04203-SMc04205), with increased expression in the fixing zone (Table S1).

TBDTs are essential for acquiring siderophores and heme in free-living conditions, but their role in the endosymbiont remains unclear. One of the most abundant proteins in root cells infected with rhizobia is leghemoglobin[18]. Unexpectedly rhizobia mutants of heme transporters and TonB/ExbB/ExbD energizing system showed no defect in symbiosis or nitrogen fixation[72] . As mentioned, *B. japonicum fegAB* operon is involved in ferrichrome uptake in free-living conditions, and the mutant produces white, non-fixing nodules *in planta* [73]. The lack of availability of this fungal source of siderophore in the nodule precludes postulating FegA as an Fe entry. However, proteomic studies of nodules, symbiosome and/or peribacteroid membranes from different symbiosis partners have reported the existence of TBDT in the peribacteroid membrane (reviewed in[74]).

4.1.2 FeoAB and the missing path for Fe^{2+} entry

Pathogenic facultative-anaerobic bacteria present an Fe^{2+} transport system at the inner membrane required to establish infections, the FeoAB system[75]. In *B. japonicum,* the membrane protein FeoB is required for Fe^{3+} uptake in aerobic free-living cells and during symbiosis[76]. Under free-living conditions, transcription of the operon was downregulated by high Fe^{3+} content in the media. The observation that FeoB is required for growth *in vitro* at a broad range of Fe^{3+} concentrations indicates that even the low expression level under replete Fe condition sustains transport activity and the intracellular Fe quota. This is an unexpected finding since the role of high-affinity importers, such as FeoAB, would be necessary only at low substrate concentrations. While the functional characterization of FeoAB in *B. japonicum* has improved our understanding of Fe homeostasis in bacteroids, this Fe-import pathway seems to be specific for *Bradyrhizobium* strains since other rhizobia genres, such as *Rhizobium* or *Ensifer*/*Sinorhizobium* lack FeoAB homologs [77].

Although FeoAB is not ubiquitous in rhizobia, an interesting question emerges since iron is most probably present in bulk soils in its ferric form (Fe^{3+}). Several reports showed that rhizobia present some sort of ferric reductase activity prior to iron acquisition[50, 76 47]. To date, one inner membrane, *frcB* reductase has been described[78]. However this gene is not responsible for Fe^{3+}-siderophore complex dissociation, since a mutant is still able to internalize iron-siderophore complexes[47]. As mentioned above, Fe^{2+} is the preferred source of iron in bacteroids according to biochemical assays[68, 69]. From the microaerobic environment and the presence of antioxidant compounds in the symbiosome and the nodule, we can infer that bacteroids do not require the presence of a periplasmic ferric reductase at this stage.

4.1.3 ABC (ATP-Binding Cassette-type) transporters

The ABC transporter family is highly distributed and conserved in nature. In Gram-negative bacteria, they participate in the import of a myriad of substrates across the cytosolic membrane[79]. Most members of the family are constituted by monomeric or dimeric membrane-embedded permease subunits which interact with monomeric or dimeric cytosolic soluble subunits. This interaction promotes the energy transduction of ATP hydrolysis from the soluble domains to the permease allowing substrate transport across the membrane. ABC-transporter participation in TM homeostasis is highly specific, due to a third sub-unit found in the periplasm that may function as a TM or TM-adduct chaperone aiding trans-envelope transport from the OM receptor to the inner membrane component of the ABC-transport system for subsequent internalization[80, 81].

Siderophore ABC-transporters. It has been proposed that the canonical ABC-transporter FhuDCB participates in the import of the cyclic trihydroxamate siderophore vicibactin in *R. leguminosarum*[58]. Consistent with a function in iron uptake, the operon genes were up-regulated in Fe-depleted media, and mutants induced overproduction of the siderophore with reduced vicibactin uptake rates. Importantly, N_2 fixation in pea nodules infected with those mutants was unaffected. Even more striking is the fact that model rhizobia like *B. japonicum* USDA110 and *S. meliloti* 2011, lack an *fhuDCB* operon and thus an important but dispensable mechanism for Fe nutrition in the bulk soil or the rhizosphere. As noted earlier, *B. japonicum* is able to import xenosiderophores which would then gain cytoplasmic access through the less selective HmuUV permease (see below)[60] or the FeoAB pathway, which involves previous Fe^{3+} release and reduction[47].

Heme ABC-transporters. Although heme utilization as an Fe source was long thought to be a trait in evolution of bacterial pathogens it is now recognized that rhizobia import heme for degradation and Fe utilization[49, 65, 82] . HmuTUV is the ABC-transporter involved in tetrapyrrole uptake [66, 83]. The periplasmic chaperone HmuT transports heme from ShmR to the permease HmuU. ATP hydrolysis in the cytosolic subunit HmuV drives heme import. Studies on *S. meliloti* 2011 probed the participation of HmuUV in the uptake of ferric-hydroxamates[60]. Here a fourth periplasmic component, FhuP, binds and transports the siderophore between OM receptors FhuA1/2 to the HmuU permease. This is an important iron uptake mechanism for organisms lacking FhuDCB and the capacity to synthesize native siderophores, like *B. japonicum* USDA110. These genes are regulated by Fe responsive transcription factor RirA[77] (see Section 4.4). This assures the integration of different transport systems in Fe import and maximizes the ion uptake rates when cells sense scarcity of the micronutrient or an increase in the turnover rate of labile Fe-S clusters.

4.1.4 Putative Fe importers for nodulation

In rhizobia like *S. meliloti* lacking FeoAB systems, the entrance pathway for Fe into bacteroids is still uncharacterized. As none of the previously described Fe-import systems seem to participate in Fe uptake at the infection stage, the system must be regulated by specific nodule environmental signals; i.e., low O2 tension, specific Fe-chelate, plant nodule specific peptides, etc. A bioinformatics analysis of genes coding for putative membrane and metal-binding proteins induced in the nodule[84, 85] or over represented at the N_2 fixation zone[86] is shown in Table S1. Interestingly, several transcripts of operons coding for Fe transport systems at the outer and inner membranes were overrepresented in zone III or nearby. Further investigation of these candidates will clarify their roles in Fe import into the bacteroids. Considering the total high level content of Fe in infected cells, the basal activity of a repressed system could be sufficient to sustain the Fe metal quota for cell growth, and likely nitrogenase assembly, as observed *in vitro* for *B. japonicum* FeoAB[76], thus hiding potential candidates in transcriptomic analysis.

4.2 Intracellular handling of iron

To prevent the harmful effects that free TM can exert in the cytosol, all living organisms present three strategies: i) chelation by small organic ligands; ii) storage in dedicated proteins such as bacterioferritins (iron) or metallothioneins (copper, zinc); or iii) delivery to the apoproteins required at that specific moment leading to functional holoprotein synthesis[87] .

In nature, there are a myriad of small organic ligands for iron, chemically diverse but containing oxygen, nitrogen or sulfur-donor atoms. Among these are organic acids, whose affinity is higher at acidic pH; redox molecules such as ascorbic acid; siderophores during their synthesis and before their secretion; and, last but not least, some free amino acids[88, 89]. The importance of these compounds in cytosolic iron homeostasis is not clear and requires further research in bacteria.

Among the storage proteins, rhizobia present in their genomes at least a ferritin-like gene, and those of *B. japonicum* and *S. meliloti* have already been characterized[76, 90]. Bacterioferritin (Bfr) is a multimeric protein (24 subunits form a cage) in which up to 4000 iron atoms can be stored. This protein is ubiquitous in bacteria and plays an important role in both iron storage and iron/redox detoxification[91, 92]. Although still speculative, Fe-storage molecules might represent a dynamic pool with a specific role in the integration of Fe fluxes across cytoplasmic membrane to the Fe-protein synthesis demand[93]. Furthermore, when influx rates exceed the requirement for holo-protein assembly, Fe^{2+} accumulation and toxicity could be avoided via Fe-storage. If Fe-storage capacity is surpassed, efflux rates should speed up (see section 4.3). Sankari & O'Brian (2016) showed that Bfr is involved in iron detoxification and oxidative stress protection in *B. japonicum*[94]. A recent report also demonstrates that *S. meliloti* Bfr seems to prevent iron toxicity, oxidative stress, and to work as an iron storage that can be remobilized when the nutrient is scarce[90]. However, when *M. sativa* is inoculated with the *bfr* mutant, there is no difference in nitrogenase activity, suggesting that it is not essential during symbiosis[90].

The second family of storage proteins are metallothioneins, described in several bacteria, including several free-living diazotrophs. These are small proteins enriched in cysteine residues involved in copper and zinc binding; their small size and heterogeneous sequence makes automatic annotation and homology searches difficult (reviewed in[95-97]). To date there are no reports in the literature characterizing any native rhizobial metallothionein.

The metalation of apoproteins to obtain the functional holoprotein (at cellular scale the metalloproteome) is the final purpose of metal acquisition. However we are still at an early stage in this field both experimentally and computationally[98-101]. Nitrogen fixation is itself an interesting model to study Fe cytosolic fluxes and protein metalation due to its high metal requirements. As an example, among the pathways to assemble iron-sulfur clusters (reviewed in[102]) model rhizobia *R. leguminosarum* and *S. meliloti* encode a complete Suf operon[103, 104] although potential NifUS homologs are also present in their genomes. Further biochemical evidence is needed to clarify how the metalation of proteins is occurring in both free-living condition and endosymbiosis.

4.3 Preventing iron toxicity and more

Iron is needed by all organisms and it requires specialized transport systems. The high content levels in soils (3 orders of magnitude above those of Co and Ni for example), the possibility of becoming soluble at acidic pH plus the capacity to participate in redox reactions make Fe potentially toxic. New evidence supports the roles of different transport systems in Fe^{2+} efflux in bacteria[105]. In this section, we will analyze current knowledge of Fe export systems in rhizobia and in the soil-borne plant pathogen *Agrobacterium tumefaciens*. We include recently described Fe-transporting members of the Cation Diffusion Facilitator (CDF) family in non-enteric bacteria[106], as many orthologs in rhizobia might be under the regulation of Fe-related transcriptional factor RirA.

4.3.1 Vacuolar Iron Transporter (VIT) family

Members of this family were first found and characterized in yeast[107, 108] and plants[109] with the main function of Fe^{2+} and Mn^{2+} transport into vacuoles to avoid potential cytotoxic effects resulting from accumulation. Their main structural characteristic is the presence of soluble and membrane-embedded, N- and C- domains, respectively. Some members have an extended N-terminal with the presence of a ferritin/erythrin(Er)-like domain capable of binding and oxidizing Fe^{2+} [110]. In the plant-pathogen bacteria, *A. tumefaciens* this VIT-membrane-bound ferritin (MbfA) was shown to participate in H2O2 resistance[111] and Fe homeostasis as an Fe^{2+} exporter required to avoid Fe^{2+} accumulation and toxicity in low pH conditions[112]. In *B. japonicum*, experiments *in vitro* determined that MbfA is involved in Fe^{2+} detoxification[76]. Recent transcriptomic studies have shown that, in *M. truncatula* 28 dpi-nodules, *mbfA* is upregulated, specifically at the beginning of the N_2 fixation zone[86] (Table S1). In this zone, Fe localization has been shown to be mainly intracellular which correlates with the high Fe demand for leghemoglobin synthesis and nitrogenase assembly in the plant cell and the bacteroid, respectively[24, 113]. In the case of *S. meliloti*, an Irr operator-promoter region (section 4.4) is present upstream of the gene, indicating that Irr could possibly regulate its transcription. However, the mutant *mbfA* does not show Fe-sensitivity (Mihelj P. & Raimunda D. communication). *In vivo* studies will be required to discern the role of MbfA in rhizobia Fe^{2+} homeostasis after bacteroid differentiation.

4.3.2 P_{IB}-ATPases

P_{IB}-ATPases are active primary transporters that couple ATP hydrolysis to transition metal efflux. The transport mechanism is described by an Albers-Post E1/E2 catalytic cycle [114]. Members of the family shows a core structure formed by 6 TMD, and three cytosolic domains named A (actuator), P (phosphorylation) and N (nucleotide binding)[115, 116]. Mechanistically, the binding of the TM to an intracellular exposed transport binding site allows phosphorylation by ATP of the P-domain, which drives conformational changes with release of the substrate in extra-cytoplasmic media[117, 118]. Invariant residues in TMD4-6 allowed the definition of 5 sub-groups with unique transport specificities [119]. P_{IB4}-ATPases were initially characterized in Co^{2+} transport but new evidence suggests a main role as Fe^{2+} transporters[105, 120, 121]. This has been shown for saprophytic and pathogenic organisms. For example, in *Bacillus subtilis,* PfeT is required for Fe^{2+} resistance

and the PfeT-like CtpD is required for virulence in *Mycobacterium tuberculosis*[122]. For both cases, but particularly for *M. tuberculosis* pathogenesis, the P_{IB4}-ATPase mediated Fe^{2+} efflux is part of a detoxification mechanism avoiding the TM over-accumulation[123]. Analysis of *R. leguminosarum*, *B. japonicum*, *S. meliloti*, *S. etli* and *A. tumefaciens* genomes shows that only the latter, a plant pathogen, contains a PfeT-like ortholog. Interestingly, all these organisms share an ortholog of the recently proposed Fe-transporting CDF member (see next section) in *Pseudomonas aeruginosa*, AitP[106]. However, many reference bacterial genomes lack both transporters, pointing to the existence of a yet undescribed Fe^{2+}-export system. P_{IB5}-ATPases are the least studied sub-group, with two members characterized in different *S. meliloti* strains (Rm1021 and CCNWSX0020). Importantly the phenotypic and biochemical analysis showed that in Rm1021, the P_{IB5}-ATPases Nia is able to transport Ni^{2+} and Fe^{2+} [124] and that, at the C-terminal, the protein presents the Hemerythrin-like (Hr) domain, which coordinates Fe^{2+} in a diiron center. As suggested for the homologous Hr domain in the P_{IB5}-ATPase member of *Acidothermus cellulolyticus*[125] it is likely that Nia-Hr senses O2 regulating the Fe^{2+} transport activity. Considering the high-Fe^{2+}, the microaerobic environment of the nodule, and the molecular characterization of Nia, it is tempting to hypothesize a role for Nia at the nodule stage regulating Fe^{2+} levels in the bacteroid. Further *in vivo* studies will provide a clearer scenario for the function of Nia Fe^{2+}-mediated transport in the rhizobia-legume interphase.

4.3.3 Roles of CDF in Fe^{2+} homeostasis.

Recent phenotypic characterization of a CDF member in *P. aeruginosa*, AitP, demonstrated that it plays a role in Fe^{2+} and Co^{2+} homeostasis[106]. In this organism, AitP has an apparent role in protecting against Fe^{2+} induced redox stress, probably via Fe^{2+} export. Supporting a putative role in Fe^{2+} homeostasis of AitP-like CDF members, a previous work identified the AitP homolog in *S. meliloti* Rm1021, SMc04167, as part of a RirA-modulated regulon[126]. Further studies are required to determine with certainty a role of this CDF sub-group in Fe^{2+} homeostasis in rhizobia.

4.4 The regulation of iron homeostasis in Rhizobia

In a very simplistic view, metal homeostasis is defined as the dynamic equilibrium reached for steady intracellular metal concentration resulting from the coordinated expression of metal scavenging molecules, importers, exporters, small ligands, storage proteins and structural/functional metalloproteins. To coordinate the expression and presence of this complex network of proteins, cells require a dedicated machinery including metal sensors, intermediaries (signal-transducers) and effectors (transcription factors, siRNA, etc.) that regulate the expression, synthesis and/or turnover of the above mentioned enzymes and transporters.

In most proteobacteria, iron homeostasis is controlled by the iron sensor Fur (ferric uptake regulator). Fur was first described in *E. coli* and characterized as a metalloprotein able to bind Fe and acting as a negative repressor. In rhizobia, Fur is not an Fe regulator, but rather recognizes Mn, and therefore these isoforms are called Mur[72, 127-131], although their consensus sequences do not differ significantly from those found in true Fur isoforms[77]. Another regulator of Fe homeostasis in proteobacteria is RhyB, a small antisense RNA. RhyB is activated by Fur, and binds its target genes mediated by the RNA chaperone Hfq (probably in an antisense manner) (reviewed in[132]). This causes either destabilization of the mRNA, repression of translation, direct activation of translation or dis-coordinate regulation of the operon. Among its target genes are: *fumA*, *acnB*, *sodB*, *fur*, *shiA*, *iscSUA*[133].

Instead of Fur, rhizobia present two transcriptional regulators denominated Irr[134, 135] and RirA[136, 137], which do not perceive Fe directly but through heme and Fe-S cluster, respectively [138-140]. Irr was first described in *B. japonicum* as a repressor during Fe sufficiency[134, 135, 141]. Irr is also present in other model rhizobia, such as *R. leguminosarum*[72, 142]. Functional analysis of these Irr genes showed some differences between the *B. japonicum* and the *R. leguminosarum*/*S. meliloti* Irr protein[140]. In the absence of Fe, Irr is active, since the apo-form of Irr recognizes the iron control element (ICE) TTTAGAA-N_3-TTCTAAA [77, 143] and activates or represses gene expression, depending on the target gene. Among the genes positively regulated by Irr in *B. japonicum* are *hmuR*, *hmuT*, and *tonB-exbBD* while among the negatively regulated genes are blr7895 and *bfr* (bll6680)[143-145]. Under high iron conditions Irr interacts with a ferrochetalase, which catalyzes the last step in heme synthesis (the assembly of Fe in the protoporphyrin ring). These nascent heme molecules bind at certain sites of the Irr and target it for degradation[139, 144, 146]. In *B. japonicum* there seems to exist a control of Irr by itself and also by the Fur regulator[147], although it is now considered a Mur version[140] while in *R. leguminosarum*/*S. meliloti*, Irr proteins act only as repressors of gene expression as it is deduced from the transcriptomic analysis of *irr* mutants. Finally in this case, the protein is stable even under replete iron conditions[148, 149].

RirA (rhizobial iron regulator) is a metalloprotein described in *R. leguminosarum* acting as a repressor in high iron conditions[136, 137]. RirA belongs to the Rrf2 family of putative regulators and does not bind free iron but Fe-S clusters, which act as a proxy of the nutritional status of the cell. RirA is found in several rhizobia but not in *B. japonicum* [77, 140]. When there is enough iron for the synthesis of Fe-S clusters, RirA is metallated with a [4Fe-4S] cluster and binds to the consensus sequence iron-responsive operator (IRO) motif (TGA-N_9-TCA)[77], preventing transcription of numerous genes involved in iron uptake, among them rhizobactin synthesis operon (*rhbABCDEF*), putative siderophore type ABC transporter genes (SMa1746, SMb21432 and SMc01659) and heme uptake (*hmuPSTUV*)[126]. Under low iron, the [4Fe-4S] cluster decays to an [2Fe-2S] cluster with lower affinity for DNA, and if Fe starving persists it decays to the apo-RirA form with no affinity for DNA [150].

To exert a more precise control of Fe homeostasis, Irr and RirA are interconnected in close relatives such as *A. tumefaciens* [151]. RirA and Irr control each other expression, and both act

oppositely in some shared genes through binding to their specific regulatory sequences[77, 152], as is the case for the *S. meliloti bfr* gene[90].

Other aspects of Fe homeostasis like secretion of siderophores seem to be controlled by other factors. For example, the extra-cytoplasmic sigma factor of RNA polymerase, RpoI, controls siderophore production in *R. leguminosarum*[153, 154]. In *S. meliloti* the iron-sulfur cluster assembly pathway is regulated by an RpoH-type sigma factor SufT[104], involved in stress response.

5 Homeostasis of other essential metals

Other TMs are essential for SNF[155 30]. We will briefly summarize here those required in co-enzymes, or as cofactors of key enzymes that sustain this process. We will also focus on the putative importers, exporters and storage molecules participating in TM homeostasis considering the different needs of the rhizobia upon transition from the free-living to the endosymbiotic lifestyle as bacteroids.

5.1 Cobalt and nickel

Some of the enzymes participating in metabolic pathways leading to SNF depend on Co^{2+} or Ni^{2+}. Thus it is expected that rhizobia possess mechanisms to capture and acquire these ions during infection and bacteroid differentiation in nodules. In indeterminate nodules rhizobia cells go through endoreduplication of their chromosome[156 157](Fig. 1). This would require a high demand of dNTPs, which anabolism depends broadly on the vitamin B12-dependent (Class II) ribonucleotide reductases (RNR)[158]. *S. meliloti* synthesizes the co-enzyme vitamin B12 *de novo* via an oxygen-dependent pathway[159]. Importantly, the upregulation of putative Co^{2+} importers, vitamin B12-dependent RNR and enzymes participating in vitamin B12 synthesis was observed in zones of bacteroid differentiation and N_2 fixation in *M. truncatula* nodules[86] (Table S1). Instead, Ni^{2+} participates as a cofactor in the [NiFe]-hydrogenase expressed in many, but not all, rhizobia-legume symbioses in the microaerobic environment of nodules to oxidize H_2, a byproduct of nitrogenase activity.

5.1.1 Co^{2+} ABC-transporters

Similar to Fe, Co is found in holo-proteins as a coenzyme when coordinated in a tetrapyrole structure (corrinoid enzymes), or as a metal cofactor (non-corrinoid enzymes). Although TonB-dependent ABC-transport systems specific for cobalamins, like BtuBFCD present in *E. coli* and *Salmonella*[160, 161], have been predicted in rhizobial genomes, the evidence indicates that the specific substrate transported is non-coordinated Co^{2+}. In *S. meliloti,* the operon *cbtJKL* was found to be negatively regulated by the presence of cobalamin in the growing media[162]. This regulation is likely mediated by a vitamin B12 riboswitch that lies at the 5'-UTR of the operon inducing early translation or transcription termination upon the interaction. This unique regulation was first described as a conserved bacterial regulatory mechanism in the vitamin B12 synthesis pathway[163]. Importantly, cells lacking CbtJKL transporter showed significantly reduced Co^{2+} uptake rates and deficient growth which was rescued by Co^{2+} addition to the media. Thus, *cbtJKL* would be required for vitamin B12 synthesis, although redundancy with other low affinity Co^{2+}-importers was predicted, especially at the nodulation stage, since shoot weights of plants infected with *cbtJKL* mutants were similar to those infected with the wild-type parental strain and low transcription levels in nodules were detected. Noticeably, transcription of the system in the nodule reaches maximal levels in bacteroid differentiation and N_2 fixation zones[86] (Table S1).

5.1.2 HupE - Specific Ni^{2+} permeases

Specific strains of *B. japonicum* and *R. leguminosarum* encode NiFe-hydrogenases in their genomes[164] . These enzymes catalyze the oxidation of H_2 produced during SNF in nodules and their expression is concomitant with that of nitrogenase. Therefore, provision of Ni^{2+} to the bacteroid must be assured in order to eliminate this potential toxic byproduct and to maximize efficiency of the process [165 164]. In pea nodules nickel is present as malate and citrate complexes[166]. This might affect the availability of Ni for the synthesis of NiFe-hydrogenase, and might account for the marked host effect observed on expression of *R. leguminosarum* NiFe-hydrogenase[167]. In *R. leguminosarum,* two homologous membrane proteins, HupE and HupE2, allow Ni^{2+} uptake to support hydrogenase metalation[168]. Structural and functional characterization showed that HupE is highly specific for Ni^{2+} and that the transport mechanism is not driven by the proton motive force[169]. These permeases are functionally equivalent to the unrelated NiCoT family member HupN from *B. japonicum*[170], which was also shown to support hydrogenase synthesis exclusively.

5.1.3 Roles of CDF in Co^{2+} and Ni^{2+} homeostasis

Although Co and Ni soil abundance is low compared to Fe, it is expected that rhizobia face increased bioavailability of these TM, and concomitant potential cytotoxic effects, in free-living conditions. This could be explained by the high stability constants of Fe^{3+} adducts in the aerobic soil environment[87]. In enterobacteria, Co^{2+} accumulation leads to Fe-S cluster disassembly, and enzymes with water-accessible centers were proved to suffer inactivation [171]. One of the main targets is the Fe-S aconitase in the TCA cycle [172]. As with Co^{2+}, Ni^{2+} accumulation is toxic and thus export systems are necessary to keep intracellular levels within safe limits. Like *P. aeruginosa* AitP, rhizobia orthologs have been linked to Co^{2+} and Ni^{2+} homeostasis, participating in the efflux of these TM. In *R. leguminosarum* and *S. etli*, DmeF and CepA mutants showed increased sensitivity to Co^{2+} and both gene transcripts were induced by Co^{2+} *in vitro*[173 174]. In *R. leguminosarum*, the DmeF mutant was also Ni^{2+}-sensitive and its transcription was upregulated by the presence of the ion. Similar phenotypes were observed in *A. tumefaciens* DmeF mutant strains (AtDmeF), with intracellular accumulation of both ions[175]. Importantly, in this organism it was shown that transcriptional

regulation is mediated by DmeR, a member of the RcnR transcriptional repressor factor family[175, 176]. A CepA paralog in *S. etli*, NepA was characterized as a Ni^{2+} exporter[173]. Although AitP-like CDFs have been suggested to have roles in Fe^{2+} homeostasis[105, 106], there is still a lack of evidence to support their participation in Fe^{2+} transport in rhizobia. In this line, the *S. meliloti* SMc04167/AitP-like mutant shows increased Co^{2+} sensitivity and when the gene is deleted in the *mbfA* mutant background Fe^{2+} sensitivity is observed (Mihelj P. *et al.*, unpublished results). Regarding the roles of CDFs in SNF, host plants infected with RlDemF and AtDmeF mutants showed similar phenotypes as the parental wild-type strains[174, 175]. Whereas the nodule has been demonstrated to participate in TM recycling, as a sink organ, the total low levels of non-essential metals in plants like Co^{2+} [177], could hinder any roles for these CDFs at these stages. Moreover, even when these ions could be accumulated in the cell plant organelles, Co^{2+} and Ni^{2+} toxicity in the bacteroid could be unnoticed or require higher levels of intracellular accumulation as bacteroids become TCA cycle-independent at this stage (see Section 2), with no apparent aconitase Fe-S cluster attack by Co^{2+}/Ni^{2+}. On the other hand, Co^{2+} and Ni^{2+} accumulation in bacteroids with no targets to damage might be beneficial in N_2 fixation or bacteroid differentiation processes which rely on biochemical pathways with key enzymes requiring these ions (Figure 1). Although existing evidence suggests no significant roles for Co^{2+}/Ni^{2+}- CDFs, we postulate that accumulation during infection of Co^{2+} and Ni^{2+} (and possibly Fe^{2+}, see section 4.3.3) in AitP-like mutants could translate into a beneficial trait for the symbiotic interaction and ultimately, to increased plant growth.

5.2 Copper

Copper is an essential element in bacteria as it is a cofactor of *cbb3*-COX and extracellular enzymes involved in ROS detoxification, free radical formation, etc.[178]. However, similar to Fe^{2+}, intracellular free Cu^{+} produces cytotoxicity and ROS and RNS (Reactive Nitrogen Species) via Fenton reactions. This fact plus the lack of identification of cytosolic Cu^{+} pools, together with the compartmentalization of cuproenzyme assembly -mainly outside the cytosolic compartment- led in the past two decades to the characterization of the major Cu^{+} export systems in bacteria[178, 179]. Recent evidence gathered from other bacterial organisms points to the existence of intracellular Cu^{+} storage[180] and suggests that Cu transporters likely participate in the cofactor assembly to apo-enzymes[181]. This is supported by the major role of bacteroid cuproproteins in SNF, and the synchronized increased transcription observed during bacteroid differentiation of both transporters and the apo-proteins (Table S1). Thus, it is reasonable to propose the existence of Cu importers and cytosolic Cu storage with roles in assisting the cuproenzyme synthesis process.

5.2.1 Roles of P_{IB1}-ATPases in Cu^{+} homeostasis, in metalloprotein assembly and bacteroids differentiation

Early genetic studies on *S. meliloti* identified the presence of a Cu^{+}-transporting P_{IB1}-ATPase coding gene, *fixI*, in the operon containing the structural and accessory proteins for the heme-copper *cbb3*-COX[182]. This oxidase was shown to be the last component of the electron transport chain in bacteroids and due to its high O2 affinity is essential for bacteroid respiration at the microaerobic (20 nM free O2) environment at the nodules[183] . Deletion of *fixI* in rhizobia causes a significant decrease of SNF (fix-)[182, 184]. This was proven to be consequence of the participation of *fixI* in *cbb3*-COX Cu^{+} assembly. Later, it was described that deletion of an ortholog gene in *S. meliloti*, *actP*, led to increased Cu^{+} sensitivity and decreased tolerance to acidity[185]. Phylogenetic analysis of sequenced genomes and biochemical studies of the *actP* and *fixI* paralogs members in *P. aeruginosa* defined two sub-groups of Cu^{+}-ATPases, CopA1/ActP-like and CopA2/FixI-like [186, 187]. Importantly, these studies demonstrated that all Cu^{+}-ATPases export Cu^{+} to the periplasm, although with different biochemical properties that adapt to their functional role in the cell. In *P. aeruginosa,* CopA1 has high transport capacity (high V_{max}) and low Cu^{+} affinity (high $K_{1/2}$), while CopA2 shows low capacity (low V_{max}) and high Cu^{+} affinity (low $K_{1/2}$). At the transcriptional level CopA1 is Cu^{+} inducible while CopA2 only responds to oxidative stressors. Noticeably, inactivation of both genes leads to opposite effects regarding *cbb3*-COX. As expected, deletion of CopA2 inhibits the activity, but deletion of CopA1 showed an increase in enzyme activity, an observation that later was described by Ekici *et al.* (2014)[181].

Recent work by Patel *et al.* (2014) showed that the five P_{IB1}-ATPases in *S. meliloti* (CopA1a, CopA1b, FixI1, FixI2 and CopA3) play non-redundant roles and importantly, propose new functions for CopA1b, FixI2 and CopA3[188]. While FixI2 and CopA1b show similar biochemical characteristics *vs.* the paralogues CopA1a and FixI1, their different participation during infection would result from different kinetics of their expression. The roles of CopA3 are less clear. It is part of an operon with two other genes, and similarly to FixI1 is under the regulation of FixK. The fact that it is induced by microanaerobiosis and nitrosative stress, like FixI1, suggests a role in cupro-protein metalation at the nodule stage[188]. Supporting this data, maximal expression of CopA3 within the nodule was seen at the N_2 fixation zone[86] (Table S1).

Studies of the P_{IB}-ATPases of *S. meliloti* CCNWSX0020 isolated from *Medicago lupulina* growing in gold-mine tailings show that it lacks CopA1a, the archetypical Cu^{+}-transporting ATPase[189]. The bacteria genome only has a CopA1b, which apparently participates together with the Zn^{2+}-transporting P_{IB2}-ATPase, ZntA, in Zn^{2+} homeostasis. This homolog is, equally to *S. meliloti* 2011 CopA1b, induced by Cu^{+} and unexpectedly capable to complement an *E. coli* Zn^{2+}-sensitive strain[189]. The data suggest that the strain has alternative ways to reach Cu^{+}-homeostasis and raise the question on what is the determinant for Cu^{+} resistance in this organism during free-living stage.

5.2.2 Putative $Cu^{2+/+}$ importers

In *Rhodobacter capsulatus,* a member of the Major Facilitator Superfamily (MFS), CcoA, was characterized as a Cu^+ importer involved in the assembly of *cbb3*-COX. Cells lacking the importer showed decreased *ccb3*-COX assembly and activity [190]. Interestingly, the phenotype was reverted by spontaneous mutations in CopA1-like gene[181]. This reflects that, as observed previously in *P. aeruginosa*[186], lack of Cu^+ export capacity might correlate with increased Cu^+ storage available for cuproprotein synthesis [186]. Thus, truncation of a specific pathway can result in lack of metalloprotein synthesis, while the opposite might be encountered when a general efflux path is inhibited, in conditions where the metal is present at sub-lethal concentrations.

Recently, a member of the Brucella-Rhizobium Porin (BRP) family in *Rhizobium etli* CFN42, *ropAe,* was characterized as responsible for Cu entry into the periplasm[191]. The mutation of the gene in a *copA* mutant background alleviates the Cu^+ sensitive phenotype. Whether the *ropAe* mutation affects *cbb3*-COX assembly and nodulation negatively requires to be tested. Importantly the regulation of *ropAe* is induced by lack of Cu^+ and, although the mechanism of this regulation is still unknown, the homolog gene in *S. meliloti* is maximally expressed at the fixation zone in *M. truncatula* (Table S1).

5.3 Manganese

Mn^{2+} is a cofactor for intracellular enzymes involved in central metabolism pathways and oxidative stress protection. In *B. japonicum* pyruvate kinase requires Mn^{2+} as cofactor instead of Mg^{2+} [192], and Mn^{2+}-dependent SODs have been characterized in rhizobia[193, 194]. In *S. meliloti*, the symbiotic characterization of the Mn^{2+}-SOD mutant showed that the enzyme is required to overcome the rise of reactive oxidative species (ROS) in ITs at initial stages of plant root colonization and to avoid early bacteroid senescence[195, 196]. Although contrary evidence exists regarding SODs participation in SNF the increased expression of Mn^{2+}-dependent enzymes, including Mn^{2+}-SOD, at the N_2 fixation and bacteroid differentiating zone (Table S1) is apparently well-orchestrated to that of importing Mn^{2+} transport systems.

5.3.1 Outer Membrane Porins

The Brucella-Rhizobium Porin (BRP) family member MnoP is responsible for specific Mn^{2+} uptake in *B. japonicum* and is under the negative regulation of Mur, the rhizobial Mn^{2+}-responsive transcription factor. Although a homolog protein is missing in *S. meliloti*, the new evidence points that SNF in galegoid plants is dependent on Mn^{2+}-transporting systems at the cytosolic membrane of the bacteroid[197]. Further studies will discern whether the Mn^{2+} entry to the periplasm involves a specific porin at the outer membrane or outer membrane receptors able to recognize and transport Mn^{2+}-loaded metallophores.

5.3.2 Mn^{2+} ABC-transporters and NRAMP

Early studies in *S. meliloti* identified a putative Fe-transporting ABC-system next to the *fur* gene involved in Mn^{2+} uptake[198]. The transcriptomic analysis of a deletion mutant of *fur* in *S. meliloti* identified the operon *sitABCD* as the most up-regulated genes[127]. Fur was described as a global Fe dependent repressor and it was expected that genes involved in siderophore synthesis were increased in its absence as observed in enteric and non-enteric bacteria. However, repressed siderophore synthesis was observed in the *fur* mutant and the growth of a *sitA* mutant was confirmed to be dependent on Mn^{2+} content in the media. The same study concluded that Mn^{2+} drives the inhibition of the *sitABCD* operon via Fur[127], and proposed the name Mur (manganese uptake regulator) in rhizobia for *fur* orthologs[77, 128]. Pointing to the role of *sitABCD* in bacteroids, SNF was attenuated in nodules infected with the *sitA* mutant. Later, these results were confirmed and functional characterization of *sitABCD* was extended, showing that this ABC-transporter is involved in superoxide resistance[199]. Although the assembly and activity of the Mn^{2+}-dependent SodB was shown to be disrupted in the *sitA* mutant, a *sodB* mutant was comparable to WT in nodulation fitness and SNF activity. Thus it was concluded that the role of SitABCD in nodulation might be related to the metalation of a yet unidentified Mn^{2+}-dependent protein or to the limitation of intracellular Mn^{2+} as high free-Mn^{2+} levels have been linked to increased capacity to handle oxidative stress in other bacterial species becoming resistant to ROS[200, 201].

In *B. japonicum* the NRAMP family member, MntH, was found to be regulated by Mur[77] and involved in Mn^{2+} uptake[202]. Although this strain lacks *sitABCD* the mutation of this system does not affect the symbiosis with the phaseoloid plant clade soybean. The recent proposition that nodulation in phaseoloid plants does not depend on Mn^{2+} transport systems in the infecting rhizobia, supports this data. Hood *et al.* (2017) proved that the *R. leguminosarum* double mutant *mntH sitABCD* failed to induce nodulation only in galegoid plants, meaning that the Mn^{2+} requirement, and thus Mn^{2+} import systems, are not ubiquitous in all rhizobia-legume symbioses but rather specific on those established between rhizobia and galegoid clade legumes[197].

Highlighting the unique requirements for Mn^{2+} in bacteroids of indeterminate nodules, *R. leguminosarum sitABCD* and *mntH* show maximal expression at the N_2 fixation zone[197] as do their paralogs in *S. meliloti*[86, 127]. Interestingly, only *sitABCD* is under the regulation of Mur in *S. meliloti* and whether MntH participates in any aspect of the bacteroid physiology is unknown. Finally, the characterization of the players in the outer membrane bacteroid for Mn^{2+} uptake and the Mur-independent regulatory mechanisms leading to MntH expression will aid in unveiling how Mn^{2+} homeostasis in the bacteroid translates to optimal SNF in indeterminate nodules.

5.3.3 Roles of CDFs in Mn^{2+} homeostasis

Current studies have shown that the mutation of CDF family members SmYiiP and EmfA in *S. meliloti* and *S. etli*, respectively, led to an increase in Mn^{2+} sensitivity and in the first case, this phenotype correlated to increased intracellular accumulation of the ion[203, 204]. Resistance to oxidative stress

was not observed in the SmYiiP mutant but nodulation assays indicated that its infection capacity was affected[203]. While the mechanism by which Mn^{2+} regulates this process is unknown earlier studies on *R. leguminosarum*, showed that cells deprived of Mn^{2+} had an increased rate of attachment to pea roots and a deeper penetration in the cortex zone[205]. More recently a study in *B. japonicum* provided evidences showing that Mn^{2+} interferes in Mg^{2+}-dependent enzyme activities[206]. While the participation of SmYiiP and EmfA in Mn^{2+} homeostasis in free-living bacteria is accepted, further biochemical work is required to reveal the metabolic pathways affecting SNF in legumes.

5.4 Molybdenum

With the only exception of nitrogenase, Mo-dependent enzymes rely on the organic moiety molybdopterin, the coenzyme named Mo cofactor (Moco)[207]. As opposed to heme and corin rings, all organisms requiring Moco posseses the biosynthetic pathway and thus an ABC-importer assuring the coenzyme internalization or a salvage pathway, is not expected.

5.4.1 $MoO4^{2-}$ ABC-transporters

Initial characterization of Mo importers in *E. coli* provided evidence that Mo is uptaken as molybdate ($MoO4^{2-}$) by the ABC-transport system ModABC[208]. In *B. japonicum* a *modABC* mutant showed decreased activity of the Moco-dependent nitrate reductase and lower SNF in absence of $MoO4^{2-}$ in the nutrient solution[209]. The regulation of these importers depends on a LysR-type transcription factor, ModE with an N-terminal DNA binding domain, and a $MoO4^{2-}$ binding/sensing C-terminal domain[210]. In all cases, repression of the operon occurs in the presence of $MoO4^{2-}$. However, shortened ModE forms are present in approximately one third of sequenced organisms[211] including *S. meliloti* and *A. tumefaciens*. A common theme in these versions is the lack of the $MoO4^{2-}$ binding domain[212] raising the question of the mechanism leading to *modABC* repression still observed in these organisms. An interesting finding in the *S. meliloti-M. truncatula* pair is the increased expression of enzymes involved in Moco synthesis and the Moco dependent xanthine dehydrogenase in bacteroids of the N_2 fixation zone (Table S1). The latter is required for NH_3 incorporation into ureids in the cytosol of phaseoloid plant cells[213]. Two questions arise from these observations; why is xanthine dehydrogenase overexpressed in the nodule when it is known that NH_3 is fixed in the plant cytosol cell and secondly, why is it expressed in a plant host that delivers N_2 mainly as amides instead of ureids.

The fact that the phenotype of *B. japonicum modB* mutant was rescued by $MoO4^{2-}$ supplementation in the bacteria or the plant media, and that $SO4^{2-}$ presence accentuated the need of $MoO4^{2-}$ led to propose $SO4^{2-}$ importers as alternative routes for Mo acquisition[209]. However, in *R. leguminosarum* a mutant lacking *modB*, the concomitant mutation of the three possible $SO4^{2-}$ importers did not result in further decreased levels of nitrogenase activity[214]. PerO is a low affinity transporter of $MoO4^{2-}$ in other diazotrophs, however the mutants of the two isoforms present in *S. meliloti* show a higher tolerance to high tungstate (100 mM) but not to high $MoO4^{2-}$ (300mM)[215]. It would be interesting to study the role of PerO in a more realistic growth condition and during symbiosis.

5.5 Zinc

Zn^{2+} is required for gene transcription, ribosomal protein synthesis, and as a structural cofactor in enzymes that handle redox-stress[216, 217, 218]. Highlighting the significance of Zn^{2+} in symbiotic nitrogen fixation, the plant transporter MtZIP6 has been described as an important player in Zn^{2+} transport towards the infected cells[25].

5.5.1 Zn^{2+} ABC-transporters

Bacteria possess two main zinc ABC importers: ZnuABC and TroABC[219]. ZnuABC participation in Zn^{2+} import was first recognized in *E. coli*[220]. Later studies in *S. meliloti* demonstrated that a similar system is involved in high-affinity Zn^{2+} uptake[221]. In *S. meliloti znuABC* mutant cells became EDTA-sensitive and the individual genes of the operon could complement the respective mutant in *E. coli*. The system seems under the transcriptional regulation of Zur (Zinc Uptake Regulator), since a Zur-box is present between *znuA* and *znuBC*. Thus divergent transcription of the operon is promoted by the regulator dissociation at low Zn^{2+} levels. According to transcriptomic data, ZnuABC system is expressed in nitrogen fixing zone in *M. truncatula-S. meliloti* symbiosis[86] (Table S1). Its requirement in symbiosis was not tested with these symbiotic partners, but soybean plants inoculated with a *znuA S. fredii* mutant show a decreased shoot biomass and a higher number of nodules[31].

TroABC, has been proposed in *A. tumefaciens* on the basis that a Zur-box is present next to the operon, its genes are up-regulated at low Zn^{2+} levels and in the *zur* mutant, and a double mutant *znuA-troC* decreased growth is observed under Zn^{2+} deficiency in the growing media[222]. In *S. meliloti* the gene with higher similarity to TroA is SitA (33% similarity, 94 % coverage). However, a Mur-box is found flanking the *sitABCD*[77]. This is a clear example on how the differential regulation in transcription of orthologous periplasmic components of ABC-transport systems integrates a response to achieve different TM transport needs. In this line, TroA members have been pointed as unspecific for TM binding *in vitro* and thus the physiological substrate difficult to predict based on these data and the sequence analysis[219].

5.5.2 Roles of P_{IB2}-ATPases in Zn^{2+} homeostasis

The Zn^{2+} accumulation leads to cytotoxicity and it is known that exporting systems are the main components of the homeostatic machinery in rhizobia. Early genomic studies in *S. meliloti* identified ZntA as the major player in Zn^{2+}-homeostasis [223]. As expected transcription of these P_{IB2}-type ATPases is induced when cells are exposed to high Zn^{2+} levels by an still undescribed mechanism. Recently it has been shown that *S. meliloti* CCNWSX0020 *zntA* mutant cells accumulate Zn^{2+}[189]. All these data point to ZntA as the archetypical Zn^{2+}-ATPase in charge of the metal efflux to achieve Zn^{2+} homeostasis in free-

living conditions. Further work is required to evaluate its participation during infection or nodulation.

5.6 Transporters with an unclear role in TM homeostasis

5.6.1 HME-RND (Heavy Metal Efflux - Resistance-Nodulation-Division)

RND family proteins participate in a wide range of substrate efflux in bateria. This is mainly achieved by active transport from the periplasm or cytosol to the extracellular milieu[224]. Members of the family export specifically amphiphilic organic compounds, including antibiotics. A sub-group of TM transporting RNDs has been described in the homeostasis of these ions[225, 226]. In general RNDs function as tripartite complex named CBA, where RNDs are A, a periplasmic soluble protein is B and an outer membrane porin is C. However different architectures have been described in delta-proteobacteria[179]. More recently the roles of RND-like proteins and RND homologs present in non-conventional operons were proposed to be involved in *S. meliloti* CCNWSX00200[227] and *M. amorphaceae*[228] TM homeostasis. Although nodulation was affected when the RND-like protein was absent in the first case, it is not obvious how it participates in the infection process and the substrate involved, a TM or an organic signal molecule.

6 Perspectives

In the last two decades, our understanding of TM homeostasis in plants and free-living bacteria has increased considerably. However, the knowledge on nutritional aspects still lags behind in the rhizobia-legume symbiosis[155, 229]. This is in spite of TM exchange between symbionts being critical, as they are essential cofactors in the enzymes required for SNF. Specifically, the space-temporal changes in permeability changes associated to TM transport at the peribacteroid membrane, of plant origin, and at the bacterial outer and inner membranes remain unresolved.

The particular organization of the nodule makes a challenge to study the interchange of nutrients, and specifically of TM between the symbionts. Among the reasons for such difficulty are: i) the complex signaling cascade and the numerous developmental steps required for a successful interaction ii) rhizobia do not fix nitrogen in free living conditions (is an obligate symbiotic diazotroph) so the study of the TM requirement for N_2 fixation has to be done within the nodule; iii) the qualitative and quantitative nutritional requirements of the two organisms are different, because the TMs considered essential are not the same in both organisms and because the concentrations in which are suboptimal/optimal/toxic also differ (reviewed for plants in[230]).

By far, iron acquisition by bacteroids has received more attention than that of other transition element ions. Interestingly, not many of the rhizobial transporters involved in iron uptake in free living conditions have a relevant role in symbiosis[66, 83, 60] and to date only one gene involved in bacteroid iron uptake has been characterized, *B. japonicum* FeoAB[76, 231]. This could reflect a more favorable environment, due to a higher concentration of iron in the symbiosomal space[32], or due to a higher bioavailability for metal uptake (acidic pH or presence of soluble iron-complexes)[44, 68, 69, 232] than in soils. Alternatively, the lack of symbiotic phenotype could be a consequence of functional redundancy between uptake transport systems rising during symbiosis.

Importers for other TMs have been determined (summarized in Fig. 1), except for the copper transporter at the inner membrane. Briefly, *B. japonicum* ModABC for molybdate uptake[209, 214], *S. fredii* ZnuA for zinc [221], *S. meliloti* CbtJKL for cobalt[162], *R. leguminosarum* HupE and HupE2 for Ni^{2+} uptake [168, 169], and SitABCD and/or MntH for manganese[127, 197, 202]. From the mentioned transporters, it emerges the question if we can extend the essentiality of these systems to all the rhizobia-legume associations. ModABC seems the universal system of Mo acquisition in both free-living and symbiotic diazotrophs[233]. However Mn requirement in symbiosis was controversial for years, since opposed phenotypes for mutants in the SitABCD system in *S. meliloti* and MntH in *B. japonicum* were found[127, 202]. Recently, Hood *et al.* (2017) studied the Mn requirement with a *R. leguminosarum* strain able to nodulate both galegoid and phaseoloid legumes. Specifically, they showed a high requirement of Mn in the bacteroids developed in galegoid legumes, since the mutants in SitABC and MntH transporters present a phenotype on these ones but not in phaseoloid legumes [197]. Interestingly, *R. leguminosarum* requires PstSCAB system for Pi acquisition when interacting with the phaseoloid legume *Sophora flavescens* but not with the galegoid legume *P. sativum*[234]; whereas MgtE is required for *R. leguminosarum* magnesium uptake in the symbiosis with the galegoid legumes *P. sativum* and *Vicia hirsuta* but not with *V. faba*, also a galegoid legume [235]. Somehow, nutrient requirements during the endosymbiotic stage show similitudes with the terminal bacteroid differentiation fate, which is influenced by both the host (the presence of NCR peptides in the genomes of different galegoid and phaseoloid legumes, and the nodule developmental program) and by the rhizobia (presence of peptide transporters and cell envelope)[15, 16, 236, 237, 238]. Whether terminal bacteroid differentiation influences transition metal homeostasis requires further examination, but interestingly addition of NCR peptides *in vitro* induces the expression of several metal transporters in *S. meliloti*[239, 240].

Additionally, the cassette of transporters present in a specific strain would influence the options to acquire and detoxify transition metals. For example, the iron FeoAB system seems present exclusively in *Bradyrhizobium* species[43, 77]. The rest of rhizobia would require a yet uncharacterized strategy to take up iron from the peribacteroid space. Currently there are 107 genomes in the RhizoDB (http://xbase.warwick.ac.uk/rhizodb/)[241], so the comparison of the 'transportomes' in different strains able to effectively nodulate the same host, or the 'transportome' of strains able to nodulate different ones, combined with a metaanalysis of the numerous transcriptomes available for the different rhizobia-legume associations would provide an interesting

framework to identify potential candidates for transition metal acquisition in SNF.

Once transition metals are inside the bacteria they have to be targeted to metallate certain apoproteins. This is one of the most challenging topics in the biology of metals, and will be interesting to study rhizobial metalloproteomes (in free-living bacteria and bacteroids) with the bottom-up and top-down mass spectrometry approaches recently developed[99, 242], plus tailor-made biochemical and genetic approaches. Significant advances have been made in the characterization of the intracellular machinery required for the assembly of the FeMo-nitrogenase[243]. At the cell membrane, the repertoire of transport systems and associated TM chaperones involved in the copper acquisition for the *cbb3*-COX assembly has been expanded in other bacteria[190 244]. Counterintuitively, the Cu assembly to *cbb3*-COX depends directly on a Cu^{+}-exporting P_{IB}-ATPase[186, 187] and new evidence supports that it is indirectly dependent on the net rate of Cu flux resulting from individual importing and exporting systems[181]. In this sense it will be interesting to evaluate whether tuning of these TM transport systems derive in SNF improvement, especially in those agricultural areas showing low TM bioavailability.

In a more speculative fashion, we need to revisit two seemingly contraposed assumptions in rhizobia-legume symbiosis. First, that rhizobia depend on the nutrients delivered by the plant in the symbiosome, where they would be limiting[30, 245 4], and second, that rhizobia performance *in planta* is independent of the growth conditions *ex planta*. According to the iron distribution in *M. truncatula* nodules [24], rhizobia progressing through the ITs would be directly exposed to the iron delivered to the apoplast in the apical zone of the nodule. Additionally, the concentration of iron described in soybean symbiosomes (0.5-2.5 mM) would result toxic in free living conditions. These results point to rhizobial metal efflux transporters as important players to: i) prevent iron toxicity during the infection, and ii) modulate the higher iron levels required during the synthesis of the nitrogenase enzyme. The iron content in the cells previous to plant infection seems relevant on the nodulation competitiveness[49, 61] . However, this seems independent of the storage of iron, since a Bfr mutant does not show a phenotype in symbiosis [90].

In summary, future research in TM homeostasis in the rhizobia-legume interphase will allow: i) to identify the plant transporters which deliver TM ions to the symbiosome, and to quantify the amount of metal diverted to this compartment; ii) to characterize the speciation of TM in the peribacteroid space, which can be modulated by both the plant and the rhizobia; iii) to identify the transporters responsible for metal uptake into the bacteroid at both the outer and inner membrane; iv) to determine the role of rhizobial metal efflux transporters during the infection stage (inside the nodule but out of the cell) and in the bacteroid (es endosymbiont); v) to unravel how metals are directed to the corresponding apoproteins in the bacteroid cytosol, and vi) the regulation of TM homeostasis in the bacteroid. All these questions and hypotheses will require the exploitation of -omic approaches (genomic, transcriptomic, (metallo)proteomic and metabolomic); the refinement of metal quantification techniques (Inductively Coupled Plasma-Mass Spectrometry, ICP-MS, and Total reflection X-Ray Fluorescence, TXRF), metal 'imaging' techniques (Laser Ablation-ICP-MS (LA-ICP-MS), synchrotron X-Ray Fluorescence (S-XRF) or Nanoscale Secondary Ion Mass Spectrometry (NanoSIMS); and last but not least, the proper application of tailor-made biochemical and genetic approaches, including the development of bacterial reporters (nutrient sensors) and genetic screenings performed *in planta*.

Conflicts of interest

There are no conflicts to declare.

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