Role of XinT2 in *Sinorhizobium meliloti*

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ABSTRACT

P_{IB}-ATPases are transmembrane enzymes which couple the efflux of cytosolic metals to the hydrolysis of ATP. To study the roles of a putative Ni-transporting ATPase (XinT2), we analyzed the Nickel content of the symbiotic bacteria *Sinorhizobium meliloti*. Our lab has previously shown that the *xinT2::mTn5* mutant strain exhibits sensitivity to nickel. In this work we challenged *S. meliloti* wild type and mutant cells with sub lethal concentrations of Ni and Fe and observed that deletion of XinT2 lead to cytosolic accumulation of both metals.

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1. Introduction

1.1 Overview

Heavy metals play a myriad of biological roles in enzymes including proteinases, dehydrogenases and peptidases when present in the desired concentrations. These metals are essential in physiological process including respiration, photosynthesis, and nitrogen metabolism and are required for synthesis of carbohydrates, proteins, phosphates, auxins, RNA and ribosomes (1). These same vital elements can have extremely detrimental effects, binding irreversibly with proteins and producing harmful free radicals (2). This delicate balance has given rise to highly specific mechanisms for maintaining metal homeostasis within the cell. The P_{1B}-type ATPase are among these mechanism. Although much research has been done on similar metal transporter such as Cu^{+} and Zn^{2+} ATPases, fewer aspects are documented concerning related heavy metal ATPases (3). These P_{1B}-type ATPases have been identified in all kingdoms of life and are the prevalent P-type ATPase in archaea and bacteria, including Rhizobacteria residing in the nodules of legumes (4). The role of a novel Ni^{+} P_{1B}-ATPase in *Sinorhizobium meliloti* is the focus of this study.

1.2 Significance of Nickel in Legumes and their *Rhizobium*

Nickel has recently been identified as an essential element in plant survival when present in low concentrations (0.05-10 mg/kg dry weight) (5). Ni deficiencies cause development inhibition and reduction of crop yields due to disruption of photosynthesis by leaf chlorosis. Nickel scarcity upsets nitrogen metabolism, a process facilitated by rhizobacteria in legumes (6).

More common however, is excess nickel in plants as anthropogenic factors have influenced soil and air quality (7). Burning of fossil fuels, organic fertilizers and manures, vehicle emissions, and household and industrial waste all contribute to this pollution (8). A number of factors determine the rate of Ni uptake including Ni^{+2} concentrations, plant metabolism, acidity of the soil, organic matter composition, and the presents of other metals. Specifically, Zn^{2+} and Cu^{2+} are the main Ni^{2+} competitors due to their similar physicochemical characteristics (9).

Nickel accumulation in plants leads to adverse effects including growth inhibition, reduction of crop yield, disruption of photosynthesis, leaf chlorosis, necrosis, and wilting (figure 1). Accumulation in soil dramatically affects microbial concentration and their activities, resulting in decreased soil fertility (10). Depleted nutrient pools in soil as well as direct effects of toxicants have shown to have detrimental effects on legumes such as green gram (*Vigna radiate*) and chickpeas (*Cicer arietinum*) (1). Critical toxic levels range from 10 mg/kg dry weight for Ni^{2+} sensitive plants, 50 mg/kg dry weight for moderately tolerant plants, and 1000 mg/kg dry weight for nickel hyper accumulators (9).

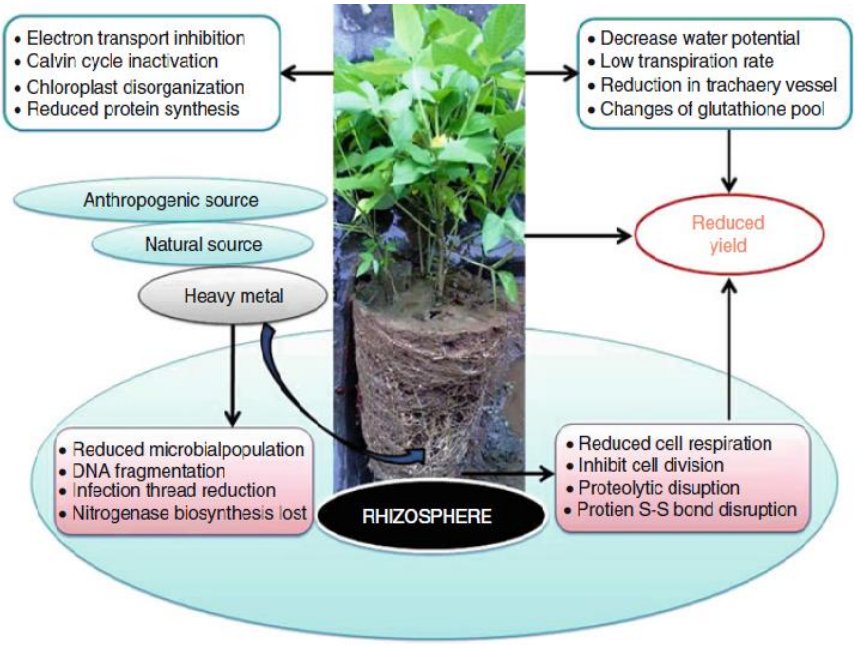

Figure 1. Toxicity of heavy metal to various metabolic stages of plants including Rhizobium–legume symbiosis (11)

1.3 Role of Iron in Plants and *Rhizobacteria*

Leghemoglobin plays an important role in the roots of legumes as an oxygen carrier and heme-proteins, much like hemoglobin found in animals. These proteins are red in color and share similar structural and chemical characteristics (12). On major deviation in leghemoglobin is that its affinity for oxygen is roughly ten times higher than human hemoglobin. Production of leghemoglobin is dependent on the presence of rhizobacteria in the roots of these legumes. This protein is thought to be a shared product of the plant and the bacterium. The iron-rich heme is produced by the rhizobacteria, while the plant manufactures the apoprotein (12).

Oxygen transport is particularly important in nodules as the nitrogenase responsible for N_2 fixation is oxygen sensitive. Leghemoglobin ensures optimal conditions for fixation by buffering the concentration of free oxygen in the cytoplasm (12). The amount of oxygen must be in a range that allows bacterial respiration but does not inhibit the function of the nitrogenase.

1.4 *Sinorhizobium meliloti*

The gram negative bacterium *S. meliloti* inhabits the roots of legumes filling a critical void in the in the plant's biological processes. Legumes cannot extract and fix N_2 from the soil as other plants do, and rather develop an organ known as a nodule. These nodules house *S. meliloti*, forming a symbiotic relationship and granting the plants the ability to construct amino acids from N_2 in the air. In return, the bacteria is provided with energy from the plant's photosynthesis and carbon from malate, obtained through the oxidation of glucose (12).

This symbiotic relationship is initiated as the legume excretes signaling flavonoids. The bacteria respond with the secretion of Nod (nodulation) factors, signaling the curling of root hairs, subsequently trapping the rhizobacteria (Figure 2). The bacteria cause an infection thread, granting them passage into the root cortex where cell division will commence. Then, rhizobacteria differentiates into N_2 fixing bacteroides in the newly formed nodules (13).

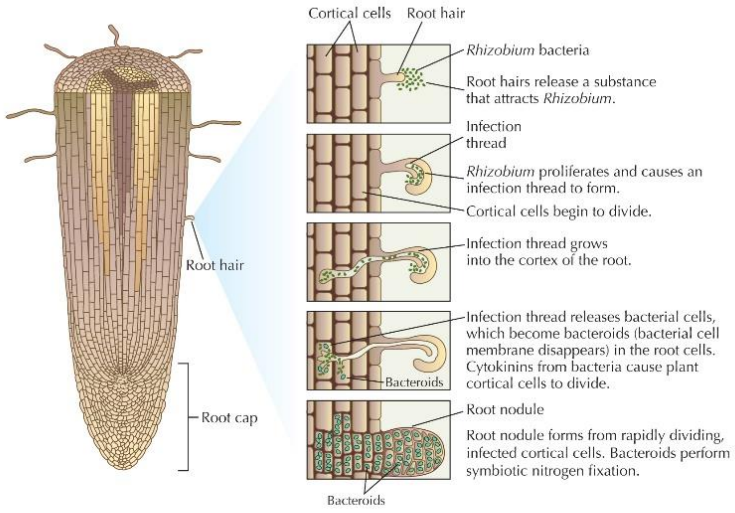

FIGURE 6.26. Symbiosis of *Rhizobium* bacteria with legumes.

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Figure 2. Symbiosis of rhizobacteria in legumes. The bacteria are attracted to the root hairs of the legume where infection takes place. Once the infection reaches the root cortex, the rapidly dividing bacteriodes form the nodule (13)

Once this relationship has been initiated, atmospheric nitrogen can be converted by the bacteria for use by the legume. The energy intensive reduction of nitrogen gas to ammonia requires 16 ATP molecules and a complex set of enzymes. These nitrogenases break nitrogen bonds and subsequently bond hydrogen, as shown in the reaction below (14). The oxygen sensitive reaction requires rhizobium to have the highest rate of respiration of any organism to maintain low cytosolic oxygen levels.

$$N_2 + 3H_2 \xrightarrow{\text{energy}} 2NH_3$$

In addition to the direct effects rhizobium have on the legumes they inhabit, these bacteria can also transfer symbiotically formed nitrogen to non-legumes, facilitating overall growth of neighboring and crop rotated plants (15). Rhizobium also provide soil with growth-promoting substances such as phytohormones; auxins, cytokinins and abscisic acids; lumichrome, riboflavin, LCOs (lipochitin oligosaccharides) and vitamins. Furthermore, these bacteria have been utilized for their ability to synthesize siderophores, solubilized inorganic phosphorus, and even infect roots of other non-legume plants including rice, wheat, and corn (15).

1.5 Structure and Function of P-type ATPases

P-type ATPases are membrane proteins responsible for the transport of ions by ATP hydrolyzation. Figure 3 depicts a phylogenic analysis of multiple P-type ATPases of different species, suggesting that sequence homology between enzymes also indicates functional similarities (10). P-type ATPases are divided into sub-categories based on the ions they

transport. P_{IA}-ATPases are bacterial K+ ion transporters, while P_{IB} transport "soft" transition metal ions such as those targeted in this study. P_{II}-ATPases are Ca2+ pumps (types A and B) as well as the Na+/K+ and H+/K+ pumps in animals (type C.) P_{III}-ATPases transports H+ to control membrane potential. P_{IV}-ATPases are lipid flipases which maintain membrane symmetry. The ion specificity of P_V-ATPases have not yet been defined (16).

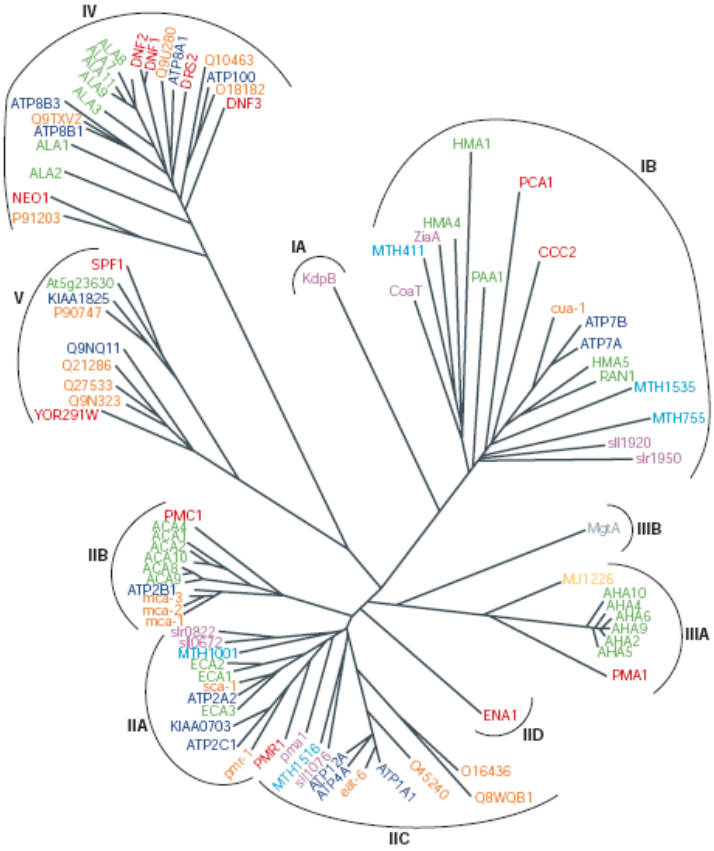

Figure 3: Phylogenic tree of the P-type ATPase families. The subfamilies cluster according to substrate specificity, even across multiple species. Gene products are color coded by producing species: green – *Arabidopsis thaliana*; orange – *Caenorhabditis elegans*; grey – *Escherichia coli*; dark blue – *Homo sapiens*; light blue – *Methanobacterium thermoautotrophicum*; yellow – *Methanococcus jannaschii*; purple – *Synechocystis* PCC6803; and red – *Saccharomyces cerevisiae (17)*

The structure of P-type ATPases are highly homologous, all sharing four major domains (Figure 4). The transmembrane (TM) domains consists of the 6-10 helices containing specific

residues responsible for substrate affinity. The phosphorylation (P) domain is both the largest and most highly conserved cytoplasmic domain, containing the signature DKTGT sequence. In this sequence, D is the residue to be reversely phosphorylated during ion transport. The nucleotide binding (N) domain is bound to the P domain via a hinge of antiparallel peptide strands (18). Crystallographic data shows that only the adenosine of the ATP is pocketed by the N domain's conserved Phe. Finally, the actuator (A) domain lies on a separate transmembrane helix is responsible for protecting the phosphoryl group from unwanted hydrolysis (19).

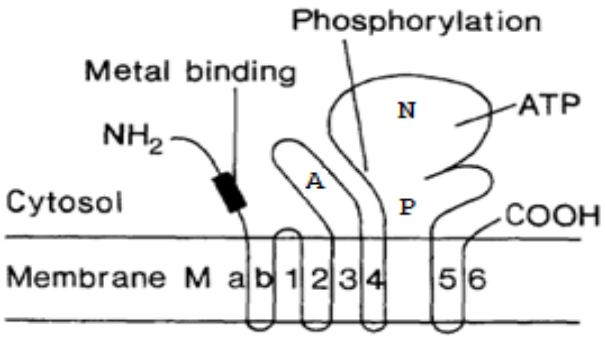

Figure 4. Structure model of Bacterial P_{IB}-ATPase. Actuator (A), Phosphorylation (P), and Nucleotide binding (N) domains are labeled (19).

P-type ATPases are membrane transporters defined by the E1/E2 Albers-Post catalytic cycle (Figure 4). The cycle begins when cytoplasmic ATP binds to the ATPase and a metal ion binds at the transmembrane binding site. Phosphorylation of the enzyme at an Asp group generates a conformational change forming an enzyme substrate complex (19). The metal is then released from the enzyme and out of the cells cytoplasm. The enzyme returns to its original conformation by releasing a phosphate group, allowing further binding of ATP and metal ions (20).

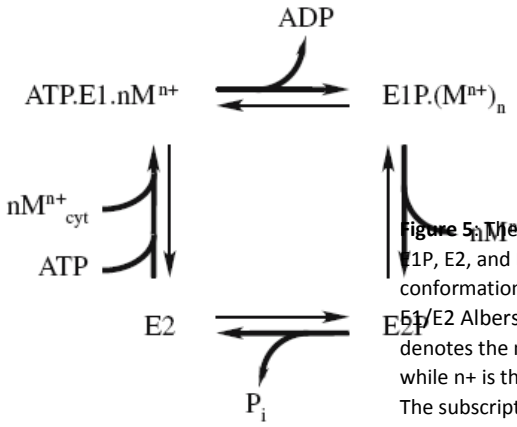

Figure 5. The E1/E2 catalytic cycle. E1, E1P, E2, and E2P are the primary conformations of the enzyme in the E1/E2 Albers-Post catalytic cycle. M_n denotes the metal being transported while n+ is the valence of the metal. The subscript of M_{n+} represents the location of the metal with respect to the membrane. n denotes that the stoichiometry of transfer is unknown. (19)

1.7 Ion Specificity

Mechanisms for ion selectivity are still an area of much debate, especially for PIB-type ATPases. Proposed mechanisms for metal transporters of this study are derived from better characterized P (II)-type ATPases (Ca-ATPase and Na/ K ATPases) (21). Three transmembrane segments have been identified to have a role in ion binding and transport of a given substrate. The equivalent transmembrane sequences of P_{1B} -type ATPases (H6, H7, and H8) are proposed to play a similar role in transition metal ion transport (22). Further research proved conserved amino acid sequences in H7 and H8 predict metal selectivity of all five PIB-type ATPases. Diverse side chains (thiol, hydroxyl, carbonyl, amide, imidazolium) which participate in metal coordination during transport, strongly suggesting a role in substrate specificity (23). Presence or absence of amino-terminal metal-binding domains further suggest particular metal specificity for each subgroup (21).

1.6 Objectives

The aim of this project is to analyze the effect of XinT2 deletion on the cytosolic content of transition metals like Ni, Fe, Cu, Co, Mn of S. Meliloti. AAS will be utilized to quantify the presence of these metals within *S. meliloti* cells. Furthermore, we hope to study the effect of increasing iron concentration in the growth of *S. meliloti* cells lacking XinT2. We believe XinT2 is a PIB-type ATPase required for controlling cytosolic nickel levels in these rhizobacteria. Elevated levels of transition metals within mutant compared to wild type cells will confirm the function of the XinT2 gene.

2. Materials and methods

2.1 Bacterial culture

S. meliloti (WT2011) strain was generously provided by Dr. Jacques Batut (University of Toulouse, France). *S. meliloti* transposon mutant *xinT2::mTn5* strain was obtained from Dr. Anke Becker (Center for Biotechnology, University of Bielefed, Germany. *S. meliloti* strains (wild type and *xinT2::mTn5*) were grown in 5 g/l tryptone, 3 g/l yeast extract and 3mM $CaCl_2$ (TY media). Selection of strains was achieved with *Streptomycin* (200 μM) for the wild type strain and *Neomycin* (100 μM) for *xinT2::mTn5* mutant strain.

2.2 Whole Cell Metal Content Determinations by Atomic Absorption Spectroscopy

Five ml cultures of both wild type and *xinT2::mTn5* mutant cells were grown in an incubator overnight at 30º C. The cultures were stressed with sub lethal concentrations of nickel and iron (1 mM $NiCl_2$, 0.5 mM $FeCl_2$). 1mM $FeCl_2$ cultures resulted in precipitation of metal ions, which would skew AAS results. Cells grown in the absence of metals were used as control. After incubation, cells were washed three times in 5 ml of washing buffer containing 50 mM HEPES pH 7.5 and 500 mM NaCl (bottles and beakers were rinsed with deionized H_2O + 5% HNO_3 (trace metal grade) for buffer preparations). The samples were resuspended in 100 µl deionized H_2O and a 10 µl aliquot was used for protein content determination (Bradford, 1976). The remaining 90 µl of each sample were acid digested with 200 µl (HNO3, trace metal grade) for 1 h at 80°C and left overnight at 20°C. Digestions were concluded after addition of 60 µl of 30% H_2O_2 and dilution to 1 ml with water. Metal content in digested samples was measured by atomic absorption spectroscopy (Aanalyst 300; Perkin-Elmer, Foster City, CA, USA). Five independent biological replicates of each sample where used for metal determinations. The standards used were: Ni 12, 24, 48 ppm; Fe- 8, 50, 100 ppm; Cu- 8, 16, 32ppm; Co- 8, 16, 64ppm; Mn- 8, 16, 32ppm

2.2 *S. meliloti* Growth under Fe Stress

S. meliloti wild type and *xinT2::mTn5* mutant strains where grown in a *Rhizobium* defined medium (RDM), a minimal media containing; 6g/L KNO_3, 1g/L $CaCl_2$ $2H_2O$, 2.5g/L $MgSO_4.7H_2O$ (RDMA), 10g/L K_2HPO_4, 10g/L KH_2PO_4 (RDMB), .25 mg/ml Biotin stock (4ml), 10 mg/ml thiamine stock (1ml), and 5 g/l sucrose. Sinorhizobium strains RDM liquid cultures

were inoculated at 0.2 OD_{600} from overnight cultures and supplemented with increasing concentration of $FeCl_3$ (0, 25uM, 50uM, 100uM, 250uM, 500uM, 1mM, 2mM). Cells were grown for either 24 or 48 h and OD_{600} was measured.

3. Results

3.1 Atomic Absorption Spectroscopy

In order to elucidate the participation of XinT2 in nickel exportation, *S. meliloti* cells were cultured overnight with sub lethal concentrations of Ni and the cytosolic concentration of Ni was analyzed for the wild type and the mutant *xinT2::mTn5* strains. Supporting our hypothesis, *xinT2::mTn5* strains accumulated four times as much nickel (960.88 $\pm$ 115.15 nmol/mg to 249.66 $\pm$ 31.19 nmol/mg) than the wild type when under 1 mM nickel stress (figure 6). These results suggests XinT2 codes for the primary nickel transporter in *S. meliloti*, playing a major role in heavy metal detoxification. Wild type nickel levels of the non-treated and iron stressed samples were higher than their mutant counterpart (WT vs. mutant in nmol/mg protein; non treated- 12.55 $\pm$ 2.12 vs. 4.96 $\pm$ 2.73, Fe stressed- 9.28 $\pm$ 3.75 vs. 6.61 $\pm$ 1.13). These results further strengthen the original hypothesis, proving the mutant cells' inability to transport nickel into the cytosol to reach optimal concentrations.

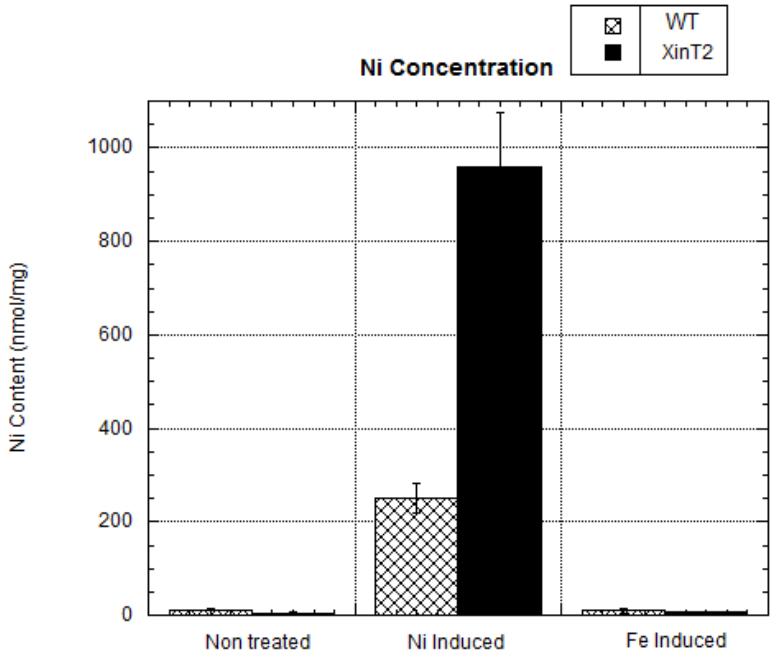

Figure 6. Ni concentration of wild type and *xinT2::mTn5* cells (nmol/mg protein) under metal stress

Interestingly, when the media was supplemented with 0.5 mM $FeCl_3$ a significant increase in the total Fe level of the *xinT2::mTn5* mutant was detected. Figure 7 shows the XinT2 mutant accumulated 3.5 fold more Fe than the wild type (152.25 $\pm$ 32.18 nmol/mg wild type vs. 524.66 $\pm$ 85.87 nmol/mg protein *xint2::mTn5* strain). In accordance with these results, we also see a deficiency in Fe concentrations for non-treated and Ni stressed cultures. The cellular factors leading to these results are not completely understood. P-type ATPases have extreme substrate specificity, as discussed above, making it hard to conclude XinT2 is responsible for transport of both metals. Taken together however, these results suggest a role of XinT2 controlling Ni^{+} (and perhaps Fe^{2+}) levels.

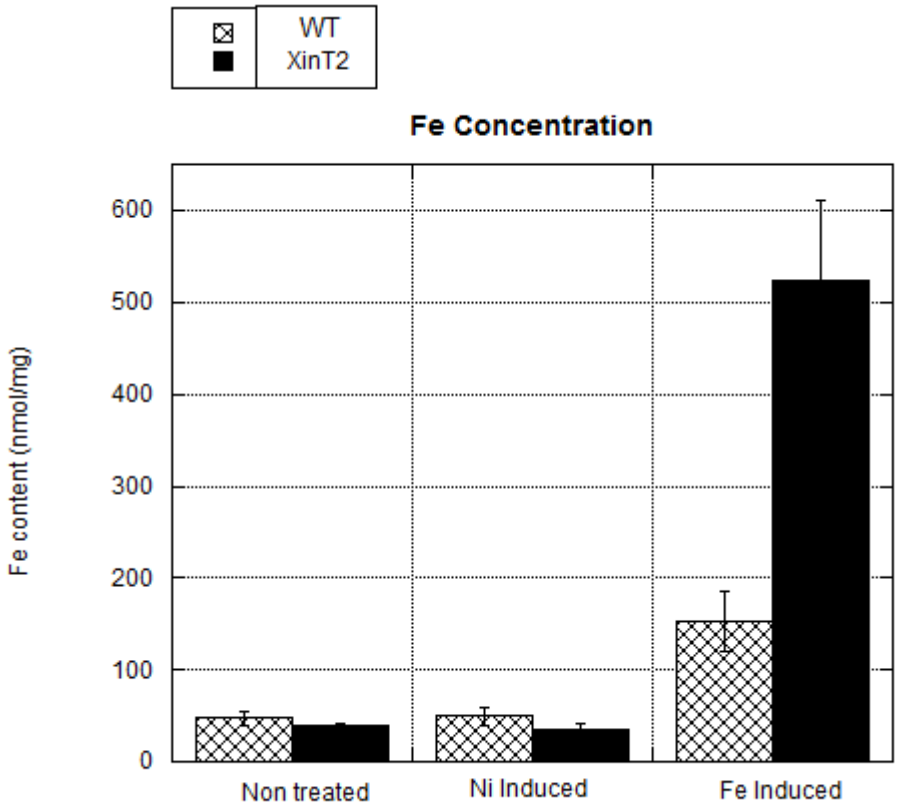

Figure 7. Iron concentration of wild type and *xinT2::mTn5* cells under metal stress (nmol/mg protein)

Table 1. Control metals (Cu, Co, Mn) under non-treated, nickel induced, and iron induced conditions

Metal	Control		1 mM Ni in TY media		0.5 mM Fe in TY media	
	WT	XinT2	WT	XinT2	WT	XinT2
Cu	4.36 ± 0.94	2.12 ± 0.96	0.68 ± 0.16	5.54 ± 1.00	3.45 ± 1.31	6.03 ± 2.00
Co	0.49 ± 0.20	0.27 ± 0.18	0.12 ± 0.034	0.29 ± 0.15	0.52 ± 0.09	3.31 ± 0.12
Mn	4.05 ± 1.33	2.19 ± 0.73	1.76 ± 0.21	1.94 ± 0.66	2.83 ± 0.66	2.79 ± 1.2

Control metals, cobalt and manganese, suggest a high selectivity for nickel by the transporter. We can conclude that there are specific transporters other than XinT2 responsible for transportation of these transition metals. Copper seems to be the only metal of the controls that deviates from this trend. Most strikingly under nickel stress, cytosolic copper accumulation proved eight times higher in *xinT2::mTn5* than wild type strains (5.54 ± 1.00 vs. 0.68 ± 0.16 nmol/ mg protein). These results advocate that there are multiple mechanisms for copper transport in *S. meliloti*, and that Xint2 may play a role in transporting not only nickel, but also iron and copper.

3.2 Iron improves *xinT2::mTn5* mutant growth.

Since an increase in cytosolic Fe content was observed, the role of XinT2 maintaining cytoplasmic Fe levels was evaluated by measuring the growth rate in the presence of increasing Fe concentrations. *S. meliloti* cells were grown in defined RDM media lacking Fe, and increasing concentrations of this metal were added as indicated in figure 7. An OD_{600} was measured after 24 and 48 hours. Figure 7 shows that *xinT2::mTn5* is not sensitive to Fe^{2+}, moreover, both the wild type and the mutant strain exhibited better growth when cultured with higher iron concentrations. For instance, addition of 0.5 mM Fe to the culture media led to an increase in the OD compared to the non-treated control (figure 8). However, no differences where observed between the wild type and the *xinT2::mTn5* mutant strain (figures 8 and 9).

In order to determine the Fe concentration that is toxic for S. meliloti *xinT2::mTn5* mutant strain, the cells were incubated with up to 2 mM Fe. However, higher concentrations of resulted in precipitation of the metal, therefore, we were unable to determine the Fe minimal inhibitory concentration (MIC).

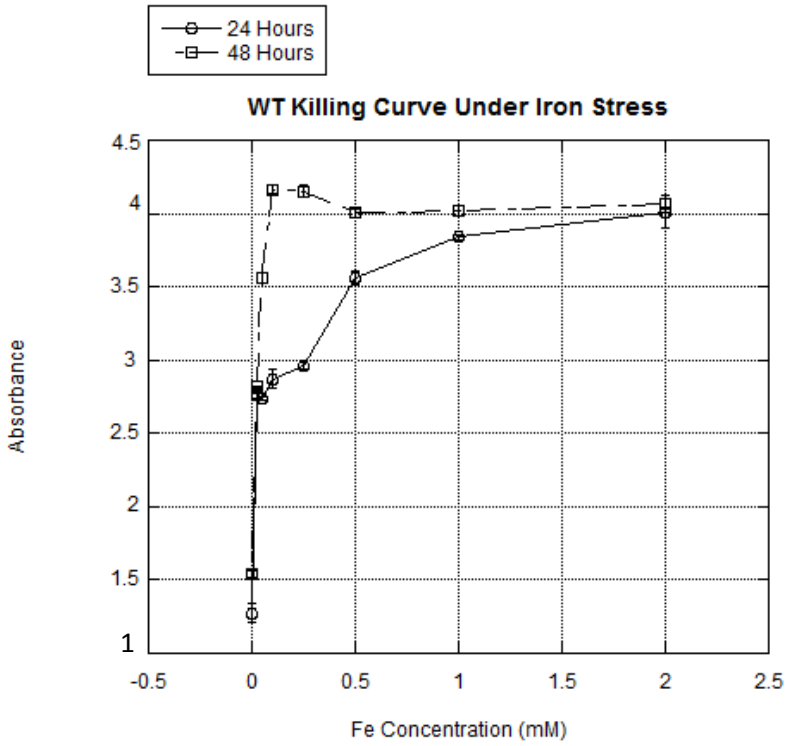

Figure 8. Growth of wild type *S. meliloti* cells under iron stress

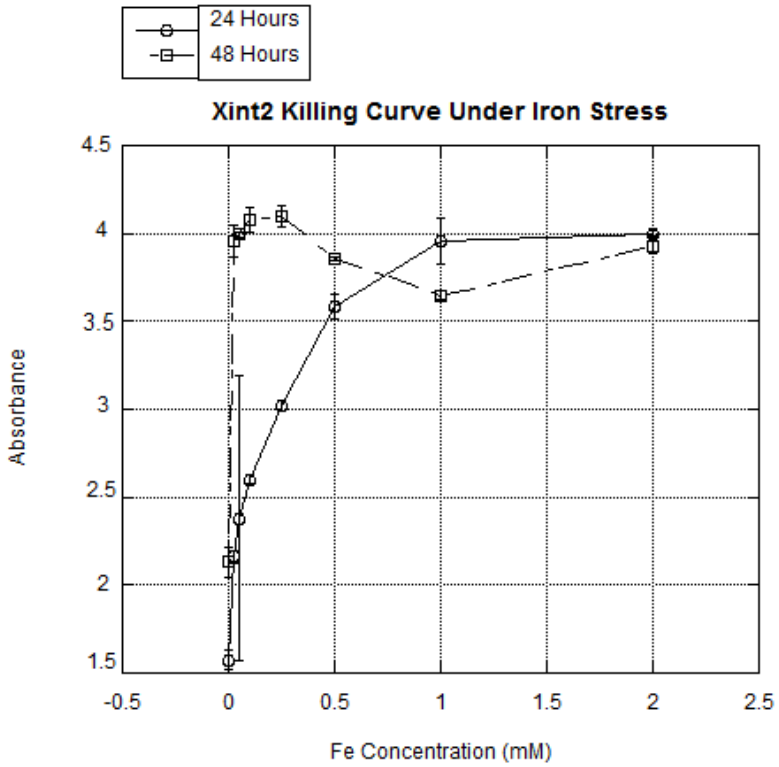

Figure 9. Growth of *xinT2::mTn5* cells under iron stress

4. Conclusion

The SmA1163 gene codes for a putative P_{IB}-type ATPase located between the 637388 and 639787 base pairs of the *S. meliloti* 1021 chromosome. The 2011mTn5STM mutation is a transposon insertion, causing a deletion and subsequently, the loss of efficient nickel transportation through the cell membrane. Nickel accumulation to critical toxic levels results in eventual death of the rhizobacteria and the legume with which it shares a symbiotic relationship (24). Studying these adverse effects and their prevention could lead to greater crop yields in the agriculture industry.

Through a series of experiments we were able to determine the role that Xint2 plays in heavy metal transport. This was accomplished by comparing the function of *xinT2::mTn5* with its wild type counterpart under heavy metal stress. We observed that *S. meliloti* cells under nickel stress were unable to export the metal ions when the SmA116 gene was knocked out.

The use of AAS provided conclusive evidence that the *xinT2::mTn5* mutant lacked the ability to efficiently rid the cell of excess nickel, which when greatly accumulated can be toxic for the cells. The data was quite striking as the strain lacking this transporter contained nearly four times as much nickel within its cytosol when compared to the wild type strain. In accordance with these results, *xinT2::mTn5* mutants were unable to supply the cell with sufficient nickel levels under control conditions.

Iron accumulation was also a target of study as it had been proposed that the Xint2 mutant may also affect iron accumulation. When under $FeCl_2$ stress, mutant strains accumulated 3.5 times more iron than the control. These results, along with insufficient iron concentrations under control conditions, led to a similar conclusion as found with nickel. It seems XinT2 is responsible for transporting metal substrates other than just nickel, including iron and perhaps copper.

The results suggested by the copper AAS demonstrated to be particularly surprising, as a Cu^+ ATPase in *S. meliloti* has already been well defined (gonz) (25). The data points to the conclusion that there are multiple methods of copper transport in these particular cells. These outcomes do not necessarily conform to previous research regarding the extreme specificity of P-type ATPases (21).

An attempt to create a killing curve of *S. Meliloti* strains under increasing concentrations of iron proved unsuccessful, as the cells survived and even thrived under iron stress. This can be attributed to the absence of iron in the RDM media. Under these conditions, raising the concentration only brought the media closer to optimal conditions for cell growth, well below critical toxic levels. Similar research has shown iron stress up to 25 mM to have improved symbiotic parameters, such as biomass production and nodulation, before detrimental effects were observed (10). Attempts to raise the iron content resulted in precipitation and iron accumulation outside of the *S. meliloti* cells. Compared to similar rhizobacteria, *S. meliloti* is known to have an increased tolerance to heavy metal stress (10). With the inability to significantly raise iron concentrations without precipitation, we were unable to reach levels to cause cell death. This precipitation may have also skewed OD results, giving higher absorbance readings at higher iron concentrations.

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