MOLYBDATE TRANSPORT IN THE *Bradyrhizobium japonicum* - *Glycine max* L. SYMBIOSIS

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ABSTRACT

For the molybdoenzymes synthesis as the nitrogenase, the molybdenum, in its most stable form, the molybdate, must be transported inside the cell. In *Bradyrhizobium japonicum*, the *modABC* genes code for a high-affinity ABC-type molybdate transporter. This work allowed to study the effect of inoculation of soybean plants with strains affected in the molybdate transport. *modA* and *modB* mutants, unable to grow in culture media under molybdate-deficient conditions, were used in our experiments. When soybean plants were inoculated with one of these strains and grown in a molybdate-deficient mineral solution did not affect the nodulation, but the nitrogen-fixing ability of the *mod* mutants was severely impaired. Addition of molybdate to the nutrient mineral solution used for plant growth fully restaured the wild-type phenotype, and the amount of molybdate required for supression of the mutant phenotype was dependent on sulfate concentration. Molybdate concentration required for the functioning of the mutant strains was greater when the medium was supplemented with high amounts of sulfate. Our results suggest the existence in *B. japonicum*, at least, three independent molybdate transport systems, a high-affinity transporter encoded by the *modABC* genes, a low affinity system corresponding to a sulfate transporter, and a third transporter that would be functional in the presence of high sulfate concentrations.

Keywords: soybean, Bradyrhizobium, mod genes, molybdate transport, nitrogenase.

INTRODUCTION

Soybean seed (*Glycine max* L., *Fabaceae* family) is one of the most nutritious legumes and of higher energy value, it contains about 37% of high-quality proteins, which is almost twice the meat proteins, four times the egg proteins and twelve times the milk proteins. It has 18% of unsaturated fat; vitamins A, E, F and B, and is one of the richest sources of lecithin, essential substance for the cells because it dissolves the bad cholesterol and helps the assimilation of vitamins (Arnau, 2009).

Soybean plant establishes symbiotic association with *Bradyrhizobium japonicum*, a bacterium that, like other species of the *Rhizobiales* order, is characterized by its ability to infect the

roots of legumes and form nodules, specialized organs within which, as bacteroids, made the biological fixation of atmospheric nitrogen (N_2), or the reduction of nitrogen in ammonium (NH_4^+), a reaction catalyzed by nitrogenase (Lawson and Smith, 2002). This association enables the legumes to grow in soils of low fertility, with deficient in N combined, where other plants can not grow.

Molybdenum is an essential element for soil microorganisms, since it serves as a cofactor for different enzymes involved in the metabolism of nitrogen, carbon and sulfur. Before the synthesis of molybdoenzymes, uptake of molybdate (the more stable form of molybdenum, which in this study it will be called Mo), its activation to an appropriate form, and its incorporation into the organic fraction of the molybdenum-cofactors, are required (Pau and Lawson, 2002). In *Escherichia coli*, the incorporation of Mo is mediated by a high-affinity ABC-type transport system, encoded by the *modABC* genes. ModA protein binds Mo in the periplasm, ModB is a transmembrane component of the permease, and ModC provides the energizer function on the cytoplasmic side of the membrane (Grunden and Shanmugam, 1997; Self *et al*., 2001). Moreover, in this bacterium, uptake of Mo can be carried out by sulfate-transporter, a low-affinity system for Mo, which is encoded by the *cysPTWA* and *sbp* genes. The *sbp* product binds either sulfate or Mo, CysP recruits thiosulfate, CysT and CysW are permeases for sulfate/Mo transport, and CysA has ATPase activity (Sirko *et al*., 1990; Kertesz, 2001).

On the other hand, it has shown that cysteine inhibits the transport and use of sulfate in some bacteria (Stewart and MacGregor, 1982; Trudinger and Loughlin, 1982; Ugalde *et al*., 1985) and that the cysteine addition to the culture medium inhibited the mutation effects of *modABC* genes in *K. pneumoniae* strains (Ugalde *et al*., 1985). Because a mutant affected in both transport systems (Mo and sulfate) was still able to incorporate Mo, in presence of high concentrations of this element, Rosentel *et al*. (1995) suggested that in *E. coli*, a third transport system is involved in Mo uptake.

In *B. japonicum*, molybdenum is part of important proteins, including the nitrogenase, an molybdoenzyme that reduces atmospheric dinitrogen (N_2) into ammonia (NH_4^+) (Lawson and Smith, 2002). This bacterium is also capable of denitrification, that is, the reduction of nitrate (NO_3^-) to nitrite (NO_2^-), via nitric oxide (NO) and nitrous oxide (N_2O) to N_2, when the cells are cultured under oxygen-limiting conditions (Bedmar *et al*., 2005). The first reaction of denitrification, is carried out by the periplasmic Mo-containing nitrate reductase (Delgado *et al*., 2003).

Despite the important role they play in nature, especially in the biogeochemical cycle of N, are unknown mechanisms involved in the transport of Mo in the rhizobia. *modABC* genes of *B. japonicum* have been described and some mutants (*modA*$^-$ and *modB*$^-$) have been obtained and characterized mostly under free living conditions (Delgado *et al*., 2006),. For this reason, the aim of this work was to study the effect of inoculation of soybean plants with strains affected in the Mo transport.

MATERIALS AND METHODS

Bacterial strains and growth conditions

The wild-type strain *B. japonicum* USDA110 (U. S. Department of Agriculture, Beltsville, MD) was used in this study. Yeast extract/mannitol (YEM)

medium (Vincent, 1974) was used for routine cultures of this bacterium. Anaerobic cultures were kept in YEM medium supplemented with 10 mM KNO_3 (YEMN) in completely filled, rubber-stoppered serum bottles. Sometimes, the cultures were kept in Bergersen's minimum medium (Bergersen, 1977), in which glutamate was substituted by 10 mM KNO_3 (BN). All these media were prepared with MilliQ water and high-quality chemical products. When required, $Na_2MoO_4.2H_2O$ and K_2SO_4 were added to the medium.

The selective capacity of culture media was obtained adding some antibiotics in the following concentrations (μg mL^{-1}): spectinomycin, 200; streptomycin, 200; kanamycin, 200 and tetracycline, 100. *E. coli* strains were cultured in Luria-Bertani (LB) medium (Miller, 1972) at 37 °C. *E. coli* DH5α (Stratagene) was used as host in standard cloning procedures, and *E. coli* S17-1 (Simon *et al*., 1983) was used as the donor in conjugative plasmid transfer, The antibiotics used were (μg mL^{-1}): ampicillin, 200; streptomycin, 20; spectinomycin, 20; kanamycin, 25 and tetracycline, 10.

Stewart (1988) described most chlorate-resistant cells show mutations affecting molybdenum uptake or its metabolism. On the other hand, he determined that the mutation of *E. coli* strains chlorate-resistant can be suppressed increasing the Mo concentration in the culture medium. Then, chlorate-resistant mutants of wild-type *B. japonicum* USDA110 were isolated following random mutagenesis Tn*5-mob* using of suicide plasmid pSUP2021 (Simon *et al*., 1983). So, they were obtained kanamycin-resistant transconjugants, these were replicated onto YEMN medium containing 15 mM $KClO_3$. One of the chlorate-resistant mutants obtained (*B. japonicum* 0507), unable to grow in aerobic or anaerobic conditions using nitrate as unique nitrogen source or respiratory substrate, respectively, was selected. Sequence analysis of the cloned DNA from this mutant strain revealed that, the transposon was inserted in an ORF that showed homology with *modB* genes from other bacteria (mainly *Sinorhizobium meliloti*). Chromosomal and plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations, *E. coli* transformations and sequencing, were performed according to standard protocols (Sambrook, Fritsch and Maniatis, 1989). So, the *modABC* genes of *B. japonicum* USDA110, were identified (Delgado *et al*., 2006).

Performing gene-directed mutagenesis by inserting of Ω Spc/Sm interposon of pHP45Ω, the *modA* gene was mutated (Delgado *et al*., 2006). This mutant was designated as *B. japonicum* 0512.

Plant growth conditions

Soybean (*Glycine max* L. Merr., cv. Williams) seeds were surface-sterilized with 96% ethanol (v/v) for 30 s, immersed in H_2O_2 (15%, v/v) for 8 min, then washed five times thoroughly in sterile water and germinated in darkness at 28°C, during 2 or 3 days. Selected seedlings were planted in sterile Leonard jar assemblies filled with vermiculite.

For determination the effect that mutations in *modA* and *modB* genes could have on nitrogen fixation in the symbiosis *B. japonicum*-soybean, plants (two per jar) were inoculated at sowing with 1mL cell suspension (approx. 10^8 cells per seed) of a single bacterial strain (USDA110, 0507 and 0512 of *B. japonicum*). Plants were grown for 42 days, in a nitrogen-free nutrient solution

(Rigaud and Puppo, 1975), prepared by using MilliQ water and high-quality chemical products, so that the plant growth depended only on the N_2 fixation. Depending on the experiment, the solution was supplemented or not supplemented with 0.2, 0.4, 0.8 and 1.2 µM of Mo. The sulfate concentration in the mineral solution used for plant growth was 3.5 or 10 mM. For determination the effect of cysteine on the requirements of Mo, a mineral solution with 3.5 mM of sulfate, supplemented or not supplemented with 0.4 µM Mo and 1.6 mM cysteine.

Plants were grown under controlled light conditions (500 µε m^{-2} x s, 400-700 nm of wavelength), temperature (25ºC/17ºC, day/night), relative humidity (65%), The photoperiod light/dark was 16 h/8 h (day/night).

Analytical determinations

The ability to fix N_2 was determined by acetylene reduction to ethylene: the acetylene-dependent ethylene production was assayed by gas chromatography on detached root systems excised at the cotyledonary node, essentially as described by Mesa *et al*. (2004). Plant and nodule dry weight, and tissue N (Kjeldahl analysis) were assayed on plant samples that had been heated at 60°C for 48 h. The leghemoglobin content of soybean nodules was determined by fluorimetry, as described previously (Delgado *et al*., 1993). The soluble protein concentration in extract cytosolic of nodules was estimated by using the Bio-Rad assay, with Bovine Serum Albumin (Sigma), as standard.

In the statistical analysis for comparisons of the average value of measurements of all parameters studied, the Statgraphics Plus Program (Fisher test), was used.

RESULTS AND DISCUSSION

Effect of soybean plants inoculation with *mod* mutants on nitrogenase activity

In *B. japonicum* has been shown the presence of *modABC* genes encoding a high-affinity transporter for Mo, so, consequently, *mod* mutants were unable to grow when these were cultured in minimal medium (BN), without addition of Mo (Delgado *et al*., 2006).

Although our results showed no significant differences between the number of nodules formed by each of the strains (71 ± 4) or between their dry weight (0.184 ± 0.006 g), the values of nitrogenase activity, dry weight and nitrogen content of plants inoculated with the wild-type strain were significantly higher than ($p \leq 0.05$) those inoculated with the mutant strains 0507 and 0512 when soybean plants were grown in the presence of concentrations of Mo ≤ 0.4 µM. However, these differences were not observed when Mo concentrations were ≥ 0.8 µM (Table 1).

These results have relation with those reported by Delgado *et al*. (2006), they indicated that the 0512 mutant strain regains the ability to grow under free-living conditions when was grown on minimal medium (BN) supplemented with Mo concentrations ≥ 0.35 µM. On the other hand, the concentration of leghemoglobin and protein in the cytosol of the nodules of plants grown without addition of Mo and inoculated with the mutant strains 0507 and 0512 were about 61% and 25% respectively, lower than ($p \leq 0.05$) the values determined in nodules of plants inoculated with the wild-type strain (Table 2). These differences were not observed when the nutrient solution was supplemented with 0.8 µM

Mo (Table 2). Since that nitrogenase is a molybdoenzyme, it seems logical the existence of differences in the symbiotic parameters of the plants, due to the difficulty of the mutant strains 0507 and 0512, either for the synthesis or activity of this enzyme.

Table 1. Acetylene reduction activity (ARA), plant dry weight (PDW) and nitrogen content [N] of soybean plants inoculated with *B. japonicum* wild-type and their mutants 0507 and 0512 and, cultured in presence of different Mo concentrations.

Mo concentration (μM)	*B. japonicum* strain								
	USDA110			0507			0512		
	ARA	PDW	[N]	ARA	PDW	[N]	ARA	PDW	[N]
0.0	266**a**	2.4**a**	76**a**	101**b**	1.5**b**	40**b**	100**b**	1.7**b**	41**b**
0.2	414**a**	3.0**a**	76**a**	237**b**	2.0**b**	46**b**	248**b**	2.1**b**	48**b**
0.4	395**a**	2.9**a**	77**a**	258**b**	2.2**b**	47**b**	260**b**	2.3**b**	50**b**
0.8	400**a**	2.9**a**	79**a**	397**a**	2.9**a**	78**a**	393**a**	2.7**a**	77**a**
1.2	395**a**	3.0**a**	78**a**	388**a**	2.9**a**	79**a**	380**a**	2.8**a**	78**a**

The units of ARA were μmol ethylene reduced g^{-1} nodule dry weight h^{-1}; the units of PDW are g $plant^{-1}$ and the units of [N] are mg $plant^{-1}$. Values in individual columns followed by the same letter are not significantly different at $p \leq 0.05$, (n=12).

Table 2. Leghemoglobin (Lb) and protein contents in nodules of soybean plants inoculated with *B. japonicum* wild-type and their mutants 0507 and 0512 and, cultured in presence of different Mo concentrations.

B. japonicum strain	Mo concentration (μM)			
	0		0.8	
	Lb	Protein	Lb	Protein
USDA110	5.69 **a**	23.44 **a**	6.52 **a**	24.48 **a**
0507	2.19 **b**	16.89 **b**	6.61 **a**	24.32 **a**
0512	2.23 **b**	17.46 **b**	6.81 **a**	25.01 **a**

The units of Lb and protein content were mg g^{-1} nodule fresh weight. Values in individual columns followed by the same letter are not significantly different at $p \leq 0.05$, (n=12).

Effect of sulfate on Mo transport in the *B. japonicum*-soybean symbiosis

Because the defects in the efficiency of N_2 fixation produced in the mutant strains disappeared when it was added Mo (≥ 0.8 μM) to the mineral solution of Rigaud and Puppo (1975), it is evident that *B. japonicum* possesses the system codified by *mod* genes and another transporter

system of Mo. In *Clostridium pasteurianum* (Elliot and Mortenson, 1975) and *Escherichia coli* (Lopez *et al*., 1993), has been shown that sulfate competitively inhibits the Mo transport and that in *E. coli*, the Mo can be incorporated into the cell through the sulfate transport system (Rosentel *et al*., 1995). Moreover, in *B. japonicum* has been observed that the addition of sulfate suppresses its transport (Ohta *et al*., 1971; Kredich, 1987), and it was necessary an increase in the Mo concentration for anaerobic growth of the mutant strain 0512, under free-living conditions (Delgado *et al*., 2006).

To investigate the role of sulfate on Mo transport in the *B. japonicum*-soybean symbiosis, a mineral solution supplemented or not supplemented with 0.8 µM Mo, was used and, in each case, the solution was supplemented with 3.5 or 10 mM sulfate. Independently of the strain or the treatment used, there were no significant differences in the number (73 ± 4) and the dry weight of nodules (0.180 ± 0.007 g) formed by each strain. Similarly, there were not differences in the dry weight and the N content between plants nodulated with any of the strains, regardless of the sulfate concentration used, when soybean plants grew up with a solution was containing 0.8 µM Mo (Table 3). By contrast, in plants inoculated with the wild-type strain and grown without the addition of Mo, the dry weight and the nitrogen content decreased about 17% and 20%, respectively, when the mineral solution was containing 3.5 mM or 10 mM sulfate (Table 3). When plants were inoculated with the mutant strains and grown without the addition of Mo but supplemented with 3.5 mM sulfate, the decrease in dry weight and the nitrogen content was approximately 37% and 46%, respectively (Table 3).

Table 3. Plant dry weight (PDW) and nitrogen content [N] of soybean plants inoculated with *B. japonicum* wild-type and their mutants 0507 and 0512 and, cultured in presence of different Mo and sulfate concentrations.

***B. japonicum* strain**	**Sulphate concentration (mM)**	**Mo concentration (µM)**			
		0		**0.8**	
		PDW	[N]	PDW	[N]
USDA110	3.5	2.5 **a**	63 **a**	3.0 **a**	79 **a**
	10	2.4 **a**	61 **a**	3.1 **a**	80 **a**
0507	3.5	1.8 **b**	40 **b**	2.8 **a**	78 **a**
	10	1.0 **c**	30 **c**	2.7 **a**	79 **a**
0512	3.5	1.8 **b**	41 **b**	2.8 **a**	77 **a**
	10	1.1 **c**	31 **c**	2.9 **a**	77 **a**

The units of PDW are g plant^{-1} and the units of [N] are mg plant^{-1}. Values followed by the same letter are not significantly different at $p \leq 0.05$, (n=12).

Up to 63% and 59% decrease in dry weight and nitrogen content was detected when plants were inoculated with the mutant strains and grown in the absence of Mo and presence of 10 mM sulfate (Table 3). Similarly, the values of acetylene reduction to ethylene and leghemoglobin (Lb) in plant nodules formed by the 0507 or 0512 mutants, grown in absence of Mo and in presence of 3.5 mM sulfate, were reduced by nearly 40% 20%, respectively, compared with plants supplemented with Mo (Table 4). The addition of 10 mM sulfate to the Mo-deficient nutrient solution, decreased the nitrogenase activity and Lb content around 60% and 39%, respectively, compared with the values observed in plants grown in a Mo-containing solution (Table 4).

Table 4. Acetylene reduction activity (ARA) and leghemoglobin (Lb) content in nodules of soybean plants inoculated with *B. japonicum* wild-type and their mutants 0507 and 0512 and, cultured in presence of different Mo and sulfate concentrations.

***B. japonicum* strain**	**Sulphate concentration (mM)**	**Mo concentration (μM)**			
		0		**0.8**	
		ARA	Lb	ARA	Lb
USDA110	3.5	336 **a**	9.5 **a**	398 **a**	9.2 **a**
	10	339 **a**	9.2 **a**	390 **a**	9.0 **a**
0507	3.5	226 **b**	8.9 **b**	395 **a**	8.9 **a**
	10	149 **c**	4.9 **c**	396 **a**	9.1 **a**
0512	3.5	232 **b**	6.7 **b**	289 **a**	9.2 **a**
	10	151 **c**	5.0 **c**	290 **a**	8.9 **a**

The units of ARA were μmol ethylene reduced g^{-1} nodule dry weight h^{-1} and the units of Lb content were mg g^{-1} nodule fresh weight. Values in individual columns followed by the same letter are not significantly different at $p \leq 0.05$, (n=12).

Effect of the cysteine on the requirements of Mo

As already indicated, mutant strains do not grow in minimal medium (BN), supplemented with Mo concentrations <0.35 μM (Delgado *et al*., 2006) and values of nitrogenase activity, dry weight and nitrogen content of plants inoculated with wild-type strain were significantly higher ($p \leq 0.05$) to that of those inoculated with the mutant strains when soybean was grown in the presence of concentrations ≤ 0.4 μM Mo (Table 1). The use of a nutrient solution of soybean plants with 0.4 μM Mo, 3.5 mM sulfate and 1.6 mM cysteine, restored the wild-type phenotype of the 0507 and 0512 mutant strains because there was no difference between the values of activity nitrogenase, dry weight or N content of plants inoculated with the wild-type strain and the values of plants inoculated with the mutant strains (Table 5).

These results have relation with those reported by Ugalde *et al*. (1985), who described that the addition of cysteine to the culture medium suppresses the effects

Table 5. Acetylene reduction activity (ARA), plant dry weight (PDW) and nitrogen content [N] of soybean plants inoculated with wild-type *B. japonicum* and their 0507 and 0512 mutants and, cultured in presence of Mo (0.4 µM), MgSO4 (3.5 mM) and absence or presence of cysteine (1.6 mM).

Cysteíne (1.6 mM)	*B. japonicum* strain								
	USDA110			0507			0512		
	ARA	PDW	[N]	ARA	PDW	[N]	ARA	PDW	[N]
Absent	266**a**	2.4**a**	76**a**	101**b**	1.5**b**	40**b**	100**b**	1.7**b**	41**b**
Present	324**a**	2.7**a**	78**a**	337**b**	2.9**b**	79**b**	335**b**	2.8**b**	78**b**

The units of ARA were µmol ethylene reduced g^{-1} nodule dry weight h^{-1}; the units of PDW are g $plant^{-1}$ and the units of [N] are mg $plant^{-1}$. Values in individual columns followed by the same letter are not significantly different at $p \leq 0.05$, (n=12).

of the mutation of *modABC* genes in *Klebsiella pneumoniae* strains.

As it has been observed in *B. japonicum*, the addition of sulfate causes the suppression of its transport (Ohta *et al*., 1971; Kredich, 1987), as in *S. typhimurium* (Dreyffus and Pardee, 1966) and *E. coli* (Rosentel *et al*., 1995), if the cysteine inhibits the sulfate transport, and mutant strains grew in the presence of cysteine and the symbiotic parameters were similar to those of the wild-type strain, it is possible the existence of a third transport system of Mo in *B. japonicum*, that would be functional in the presence of high sulfate concentrations.

CONCLUSIONS

The mutation in *modA* or *modB* genes of *Bradyrhizobium japonicum* did not affect its ability to form nodules on soybean plants, but decreased the ability to fix atmospheric nitrogen.

modA and *modB* genes of *Bradyrhizobium japonicum* are required for a fully effective *B. japonicum*-soybean symbiosis under Mo-limiting conditions.

Bradyrhizobium japonicum possesses, at least, three Mo transport systems, a high-affinity ABC-type transport system encoded by the *modABC* genes, the second, probably, a sulfate transporter that allows the incorporation of Mo with lower efficiency, and a third, which would only act under excess sulfate conditions.

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