Functional characterization of the *Bradyrhizobium japonicum* modA and modB genes involved in molybdenum transport

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A modABC gene cluster that encodes an ABC-type, high-affinity molybdate transporter from *Bradyrhizobium japonicum* has been isolated and characterized. *B. japonicum* modA and modB mutant strains were unable to grow aerobically or anaerobically with nitrate as nitrogen source or as respiratory substrate, respectively, and lacked nitrate reductase activity. The nitrogen-fixing ability of the mod mutants in symbiotic association with soybean plants grown in a Mo-deficient mineral solution was severely impaired. Addition of molybdate to the bacterial growth medium or to the plant mineral solution fully restored the wild-type phenotype. Because the amount of molybdate required for suppression of the mutant phenotype either under free-living or under symbiotic conditions was dependent on sulphate concentration, it is likely that a sulphate transporter is also involved in Mo uptake in *B. japonicum*. The promoter region of the modABC genes has been characterized by primer extension. Reverse transcription and expression of a transcriptional fusion, P<sub>modA</sub>·<sub>lacZ</sub>, was detected only in a *B. japonicum* modA mutant grown in a medium without molybdate supplementation. These findings indicate that transcription of the *B. japonicum* modABC genes is repressed by molybdate.

INTRODUCTION

Molybdenum (Mo) is an essential element for bacteria, since it serves as a cofactor for a number of enzymes involved in the metabolism of carbon, nitrogen and sulphur. Before synthesis of molybdoenzymes, uptake of molybdate (the more stable form of Mo), molybdate activation to an appropriate form, and incorporation of Mo into the organic part of the Mo-cofactors, are required (Pau & Lawson, 2002). The molybdate uptake system has best been characterized in *Escherichia coli*, in which incorporation of molybdate by the cell is mediated by a high-affinity ABC-type transport system encoded by the modABC genes. ModA binds molybdate in the periplasm, ModB is the transmembrane component of the permease, and ModC provides the energizer function on the cytoplasmic side of the membrane (for reviews see Grunden & Shanmugam, 1997; Self et al., 2001). Similar systems for molybdate transport have been characterized in *Rhodobacter capsulatus* (Wang et al., 1993), *Azotobacter vinelandii* (Luque et al., 1993; Mouncey et al., 1995, 1996), *Staphylococcus carnosus* (Neubauer et al., 1999) and *Anabaena variabilis* (Thiel et al., 2002; Zahalak et al., 2004). Immediately following the modC gene, another ORF, designated modD, has been found in *E. coli* and *R. capsulatus*, the deletion of which has no phenotypic effect (Maupin-Furlow et al., 1995; Wang et al., 1993). Expression of the modABCD operon of *E. coli* is tightly regulated and requires molybdate starvation. This regulation is achieved by a repressor protein, ModE, the product of the *modE* gene located in the modEF operon, which is transcribed divergently from *modABCD* (Grunden et al., 1996). ModE has a helix–turn–helix region in the N-terminal segment and is a member of the LysR family of transcriptional regulators. The ModE–molybdate complex is the active form of the protein and binds to target DNA as a dimer (Grunden et al., 1996, 1999; Anderson et al., 1997). Although ModF has similarity to ModC, a mutation or deletion within the *modF* gene has no effect on molybdate uptake (Grunden et al., 1996).

In addition to the ModABC transporter, uptake of molybdate in *E. coli* can be carried out by a low-affinity sulphate-transport system encoded by the cysPTWA and sbp genes. The product of *sbp* binds either sulphate or molybdate, and CysP recruits thiolsulphate, while CysT and CysW are the permeases for sulphate/molybdate transport into the cell, and CysA has ATPase activity (Sirko et al., 1990; Kertesz, 2001). Since a double modA cysA mutant is still able to take
up molybdate, a third transport system is involved in molybdate uptake in *E. coli* (Rosentel *et al.*, 1995). Physiological experiments suggest that molybdate transport through this third transport system is competitively inhibited by selenite (Lee *et al.*, 1990).

*Bradyrhizobium japonicum* is a Gram-negative soil bacterium which can exist either as a free-living organism or as a nitrogen-fixing root-nodule symbiont of its soybean host plant. Within the nodules, bacteria differentiate into bacteroids, which reduce atmospheric dinitrogen (*N₂*) into ammonia (*NH₄⁺*), a reaction catalysed by the molybdoenzyme nitrogenase (Lawson & Smith, 2002). In addition to assimilating nitrate (*NO₃⁻*) to *NH₄⁺* (Bergersen, 1977), *B. japonicum* is also capable of denitrification, that is, the reduction of *NO₃⁻* or nitrite (*NO₂⁻*) via nitric oxide (NO) and nitrous oxide (*N₂O*) to *N₂*, when cultured under oxygen-limiting conditions (Bedmar *et al.*, 2005). The first step of denitrification, the anaerobic reduction of nitrate to nitrite, is carried out by the periplasmic Mo-containing nitrate reductase (Delgado *et al.*, 2003).

Mo uptake has been shown to occur in *B. japonicum* free-living cells and bacteroids (Graham & Maier, 1987; Maier & Graham, 1988). Despite the importance of Mo in *N₂* fixation, there has been very little work on the mechanisms involved in uptake of molybdate in rhizobia (Johnston *et al.*, 2001). The recently sequenced *B. japonicum* USDA110 genome (Kaneko *et al.*, 2002; see http://www.kazusa.jp/rhizobase/) revealed the existence of three putative sets of *mod* genes: one corresponds to ORFs *blr8160, bhr8161* and *blr8162*, a second to *bhr6951, bhr6952* and *bhr6953*; while further copies of *modB* (*blr1719*) and *modC* (*bl1780*) are present within the symbiosis island of the *B. japonicum* genome (Göttfert *et al.*, 2001). In this paper, we report the phenotypic analysis of mutant strains carrying insertions in the *modA* and *modB* *B. japonicum* genes, which correspond to ORFs *blr8160* and *bhr8161*, respectively. Regulatory studies indicate that expression of the *B. japonicum mod* genes described in this work is repressed by Mo.

**METHODS**

**Bacterial strains and growth conditions.** *B. japonicum* USDA110 (US Department of Agriculture, Beltsville, MD) was used in this study. Yeast extract/mannitol (YEM) medium (Vincent, 1974) was used for routine cultures of *B. japonicum*. Anaerobic cultures were kept in YEM medium supplemented with 10 mM KNO₃ (YEMN) in completely filled, rubber-stoppered serum bottles. Aerobic cultures were kept in Bergersen’s minimum medium (Bergersen, 1977), in which glutamate was substituted by 10 mM KNO₃ (BMN). YEM, YEMN and BMN medium were prepared with MilliQ water and high-quality chemical products. When required, Na₂MoO₄·xH₂O and K₂SO₄ were added to the medium. Antibiotics were added to *B. japonicum* cultures at the following concentrations (μg ml⁻¹): spectinomycin, 200; streptomycin, 200; kanamycin, 200; 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 for the production of double-stranded plasmid DNA sequencing templates, and *E. coli* S17-1 (Simon *et al.*, 1983) served as the donor in conjugal plasmid transfer. The antibiotics used were (μg ml⁻¹): ampicillin, 200; streptomycin, 20; spectinomycin, 20; kanamycin, 25; tetracycline, 10.

Transposon mutagenesis and isolation of chlorate-resistant mutants. *B. japonicum* USDA110 mutants were isolated following random mutagenesis with Tn5-mob by use of suicide plasmid pSUP2021 (Simon *et al.*, 1983). Kanamycin-resistant transconjugants were replica-plated onto YEMN containing 15 mM KClO₃. Plates were placed into anaerobic jars fitted with vents (GasPak 150; BBL Microbiology Systems) and the atmosphere inside the jars was made microaerobic by evacuating and refilling with O₂/Ar (2:98, v/v) three times. Plates were incubated at 28 °C for 1 week and then air-exposed until the appearance of colonies. Physical verification of Tn5 insertions was carried out by DNA hybridizations performed with digoxigenin–dUTP-labelled probes (Roche). The chemiluminescence method was applied to detect hybridization bands. One of the chlorate-resistant mutants obtained, *B. japonicum* 0507, was used for further study.

DNA manipulation and sequencing. Chromosomal and plasmid DNA isolations, restriction enzyme digestions, agarose gel electrophoresis, ligations and *E. coli* transformations were performed according to standard protocols (Sambrook *et al.*, 1989). A 5-9 kb BamH1 fragment containing chromosomal DNA from the region flanking the Tn5 insertion in *B. japonicum* 0507 was cloned into plasmid pBluescript K+ (pBS, Stratagene) to obtain plasmid pCHL7B. Then a 2 kb Hpal/Sac1 fragment from pCHL7B was used as probe to screen a *B. japonicum* USDA110 cosmid library. The cosmID CBG20-23 was thus identified containing *B. japonicum* cloned DNA, and a 4-5 kb Pst1 and an 8-8 kb EcoRI fragment showing homology with the probe were subcloned in plasmid pBS to yield plasmids pBG0502 and pBG0504, respectively (Fig. 1). Finally, a 1-8 kb Sal1 and a 2-7 kb Sal1/PstI fragment from pBG0502 were ligated to pBS to produce plasmids pBG0505 and pBG0509, respectively. DNA from pBG0505, pBG0509 and pBG0504 was sequenced on both strands by using pBS-specific primers and the Sanger dideoxy chain-termination method. The sequencing reactions were analysed in a DNA sequencer (model 373 Strecht and dye primers from Applied Biosystems). To fill gaps, specific synthetic oligonucleotides complementary to the internal sequences were used as primers.

Construction of a modA mutant. The *modA* gene was mutated by performing gene-directed mutagenesis by marker exchange. A 0-9 kb EcoRV fragment from pBG0502 harbouring the entire *B. japonicum* USDA110 *modA* gene was subcloned into pBS to obtain plasmid pBG0510 (Fig. 1). Then, the 2 kb Smal fragment (5′Spce3′Sm) interposon of pPH451 (Pretkni & Kirsch, 1984) was inserted into the NrdI site of the 0-9 kb EcoRV fragment from pBG0510, resulting in plasmid pBG0511 (data not shown). Finally, a 2-9 kb Sal1/PstI fragment from pBG0511 was subcloned into plasmid pK18mobsac (Schäfer *et al.*, 1994) to obtain plasmid pBG0512 (data not shown), which was transferred via conjugation into *B. japonicum* USDA110 using *E. coli* S17-1 as donor. Potential double recombinants were selected by growth on agar plates containing 10 % sucrose. Mutant strains resistant to spectinomycin/streptomycin but sensitive to kanamycin were checked by Southern hybridization experiments (data not shown) for correct replacement of the wild-type fragment by the Ω interposon. The mutant derivative *B. japonicum* 0512 was obtained and was used in this study.

Genetic complementation of modA and modB mutant strains. The recombinant plasmid pBG0523 containing the complete *modABC* region was obtained by cloning a 2-7 kb Apal–Xbal fragment from pBG0509 (Fig. 1) into the Apal/XbaI-digested pBR1MC3-3 (Kovach *et al.*, 1994) (data not shown). Using *E. coli* S17-1 as donor, pBG0523 was transferred by conjugation into *B. japonicum* 0507 and
0512, resulting in strains 0507-0523 and 0512-0523, respectively. The correct genomic structure of the transconjugants was confirmed by Southern blot analysis of genomic DNA preparations.

**Transcript analysis.** Transcripts of mod genes were analysed by primer extension. RNA was isolated from *B. japonicum* USDA110 and its mutant derivative 0512. Cells were grown aerobically in YEM medium supplemented or not supplemented with 0·5 μM molybdate. One hundred millilitres of cells was collected into chilled tubes, pelleted and subjected to RNA isolation as described elsewhere (Nienaber et al., 2000). Primer extension was performed with primer 5′-CGAACACGTTGATTTTGTG-3′, which is complementary to positions 73–94 downstream of the putative ATG start codon of modA. Sixty picomoles of the primer was labelled with 10 U T4 polynucleotide kinase and 80 μCi (3·0 MBq) [γ-32P]ATP in a total volume of 17 μl. About 10⁸ c.p.m. labelled primer was hybridized to 20 μg total RNA, overnight at 30 °C. Primer extension was carried out with avian myeloblastosis virus reverse transcriptase (Roche). Electrophoresis of cDNA products was done in a urea/polyacrylamide sequencing gel to separate the reaction products, and dry gels were exposed to X-ray film and visualized.

**Construction of a PmodA–lacZ fusion.** To construct a transcriptional fusion of the mod promoter region to the reporter gene lacZ (PmodA–lacZ fusion), the 877 bp SalI/NcoI fragment from pBG0509 containing the mod promoter region was subcloned into the EcoRI site of pMP220 (Spanik et al., 1987), yielding plasmid pBG0513 (Fig. 1). To monitor modA expression, pBG0513 was used to transform *E. coli* S17-1, and then transferred via conjugation into *B. japonicum* strains USDA110 and 0512.

**Plant growth conditions.** *Glycine max* L. Merr., cv. Williams seeds were surface-sterilized with 96% ethanol (v/v) for 30 s, immersed in H₂O₂ (15%, v/v) for 8 min, washed five times in sterile water and germinated in darkness at 28 °C for 48 h. Selected seedlings were planted in sterile Leonard jars and placed in controlled environmental chambers under conditions previously described (Delgado et al., 1989). Plants were inoculated at sowing with 1 ml cell suspension (approx. 10⁷ cells per seed) of a single strain of *B. japonicum*. Plants were grown for 42 days in an N-free nutrient solution (Rigaud & Puppo, 1975), prepared by using MilliQ water and high-quality chemical products and supplemented or not supplemented with 0·8 μM molybdate. The sulphate concentration in the mineral solution used for plant growth was 3·5 or 10 mM.

**Plant tests.** 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 leghaemoglobin content of soybean nodules was determined by fluorimetry, as described previously (Delgado et al., 1993).

**Analytical methods.** For determination of β-galactosidase activity, cells were grown in YEM medium, supplemented or not supplemented with 0·5 μM molybdate, until the OD₆₀₀ of the culture was higher than 0·4. β-Galactosidase activities were determined with permeabilized cells from at least three independently grown cultures, as described by Miller (1972). All media and materials used for incubations were sterilized at 120 kPa and 110 °C for 30 min before use. Methyl viologen-dependent nitrate reductase activity was analysed as

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**Fig. 1.** Organization of the *B. japonicum* USDA110 modABC genes. Arrows indicate the location and orientation of the deduced ORFs. The insertions containing the streptomycin/spectinomycin resistance gene (Ω, Sm/Spc) and the Tn5 in the modA and modB genes to obtain mutant strains 0512 and 0507, respectively, are marked. A detail of the construct used to generate pBG0513 which carries a transcriptional fusion between the modA promoter region and lacZ is also shown. E, EcoRI; Ev, EcoRV; Nc, NcoI; Nr, NruI; P, PstI; S, SalI.
described by Delgado et al. (2003). Nitrite was estimated after diazotization by adding the sulphanilamide/naphthylethylene diamine dihydrochloride reagent (Nicholas & Nason, 1957). Protein concentration was estimated by using the Bio-Rad assay, with BSA as standard.

RESULTS AND DISCUSSION

Isolation and characterization of a B. japonicum chlorate-resistant mutant

Because chlorate is reduced to cytotoxic hypochlorite by respiratory nitrate reductases, resistance to chlorate has been widely used to obtain nitrate-reductase-deficient mutants. However, most chlorate-resistant cells show mutations affecting molybdenum uptake or metabolism (Stewart, 1988). For isolation of chlorate-resistant mutants, Tn5 was used to mutagenize wild-type B. japonicum USDA110 cells. Out of 6000 Kmr transconjugants from four independent matings, twelve mutants were obtained that were able to grow on YEMN plates containing 15 mM chlorate. Sequence analysis of the cloned DNA from the mutant strain B. japonicum 0507 (Fig. 1) revealed that Tn5 was inserted in an ORF that showed homology with modB genes from other bacteria (data not shown).

modABC genes

The modA, modB and modC genes isolated in this work are 186, 333 and 642 bases in length, respectively, and encode proteins of 260 (23.3 kDa), 231 (24.5 kDa) and 220 (24.3 kDa) amino acid residues, respectively. The deduced primary sequence of ModA and ModB shows 64 and 61% identity to their homologues in Agrobacterium tumefaciens, respectively (Wood et al., 2001). The deduced primary sequence of ModC has 53% identity with the translated sequence of the modC gene from Mesorhizobium loti (Kaneko et al., 2000). Interestingly, at the C-terminal end, ModC is 130–160 amino acid residues shorter than ModC from other bacteria.

The modABC gene products described here show only a 25, 34 and 46% identity, respectively, with the translated sequences of ORFs brl6951, brl6952 and brl6953 of the B. japonicum USDA110 genome (Kaneko et al., 2002; see http://www.kazusa.jp/rhizobase/). The products of the other two ORFs, brl1719 and brl1780, show a 26 and 40% identity with the products of the modB and modC genes described in this work. Families of paralogue genes are not uncommon in B. japonicum, as documented in the genome database for rhizobia (http://www.kazusa.jp/rhizobase/).

Transcription analysis of the mod genes

Primer extension experiments were performed to analyse mod transcripts in cells of B. japonicum strain USDA110 and modA mutant strain 0512 grown under different conditions (Fig. 2a). No transcript was detected when RNA from the wild-type strain was used, regardless of the presence or absence of 0.5 mM molybdate in the growth medium (Fig. 2a, lanes 1 and 2, respectively). Whereas no transcript was detected with RNA from the B. japonicum modA mutant grown in a medium supplemented with molybdate,
a transcriptional start site that initiates at a T, 34 nt upstream of the putative translational start codon, was detected when cells were grown under Mo-limiting conditions (Fig. 2a, lanes 3 and 4, respectively).

Inspection of the DNA sequence (Fig. 2b) revealed a purine-rich Shine–Dalgarno-like sequence (GGAG) 12 bases upstream of the putative translational start codon of modA. Comparison with the modA promoter regions from other bacteria showed that very few bases were conserved (data not shown). This observation, which has also been reported by Anderson et al. (1997), explains the difficulty in identifying a putative ModE-binding site by sequence comparison.

Expression of the modABC genes was studied using a transcriptional PmodA–lacZ fusion, which was transferred by conjugation into B. japonicum strain USDA110 and the modA mutant strain 0512. After growth in a medium supplemented or not supplemented with 0.5 μM molybdate, cells of strain USDA110 with the PmodA–lacZ fusion had very low levels of β-galactosidase activity (Table 1). Similarly, when cells of the modA mutant containing PmodA–lacZ were grown in the presence of molybdate, activity values were negligible (Table 1). In the absence of molybdate, however, levels of activity were 13-fold higher than those found in the presence of molybdate (Table 1). Both β-galactosidase activity (Table 1) and primer extension (Fig. 2a) experiments demonstrated that transcription of mod genes is repressed by molybdate. The lack of expression in cells of the parental strain grown in a medium not supplemented with molybdate could be explained if traces of molybdate in the growth medium were transported into the cells by the high-affinity ModABC transporter. Because of the mutation in the modA gene, traces of molybdate in the medium were not taken up by the mutant cells, and the expression of the mod genes was not repressed. In E. coli, expression of the mod operon also requires molybdate starvation (Rech et al., 1995; Rosentel et al., 1995). The repressor protein ModE binds molybdate to form a complex which, in turn, binds to the promoter region to inhibit transcription of mod genes (Grunden et al., 1996, 1999; Anderson et al., 1997; McNicholas et al., 1998). A ModE-like protein has not been annotated in the genome sequence of B. japonicum (http://www.kazusa.jp/rhizobase/). The presence of ModE-like proteins in bacteria is not common. Out of 20 prokaryotes containing mod genes, only five have been found to show ModE homologues (Self et al., 2001).

**Effects of mod mutations on nitrate reductase activity**

Synthesis of Mo-cofactors is crucial for aerobic and anaerobic growth when nitrate is the only nitrogen source or the only respiratory substrate, respectively. Under the latter conditions, the assimilatory and respiratory Mo-containing nitrate reductases have to be synthesized to support nitrate reduction (Richardson et al., 2001). In order to assess the function of the mod genes with respect to the activity of the molybdopterin-requiring nitrate reductase enzyme, in addition to the modB::Tn5 mutation, the modA gene was also mutated by marker exchange mutagenesis (Fig. 1). In contrast to B. japonicum USDA110, cells of the modA mutant strain 0512 (Fig. 3a) or the modB chlorate-resistant 0507 strain (data not shown) were unable to grow anaerobically in YEMN medium. After incubation of the cells under these conditions, levels of nitrate reductase in cells of the modA and modB mutant strains were very low compared to those detected in cells of strain USDA110 (Table 2). Complementation of B. japonicum 0512 or 0507 with pBG0523 containing the wild-type modABC genes restored both nitrate reductase activity (Table 2) and the ability of the cells to grow in YEMN medium (Fig. 3a). Addition of molybdate to the medium at concentrations ≥0.35 μM fully restored the ability of the modA mutant to respire nitrate (Fig. 3b). Although the results presented here refer to cells of B. japonicum grown under anaerobic conditions, similar results were obtained for aerobically grown cells in BMN medium (data not shown). The ability of molybdate concentrations ≥0.35 μM to support the growth and expression of nitrate reductase of the mod mutants under anaerobic conditions indicates that another system is involved in molybdate transport. It has previously been observed in E. coli that molybdate can be taken up by the sulphate-transport system (Rosentel et al., 1995). To investigate the role of sulphate on molybdate transport,

<table>
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<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth conditions</th>
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<tr>
<td></td>
<td></td>
<td>YEM</td>
</tr>
<tr>
<td>110-0513</td>
<td>PmodA–lacZ wild-type</td>
<td>9.54 (3.0)</td>
</tr>
<tr>
<td>0512-0513</td>
<td>PmodA–lacZ modA</td>
<td>113.62 (10)</td>
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*Table 1. Expression of β-galactosidase from a PmodA–lacZ fusion in B. japonicum parental and modA mutant background*

Cells were grown aerobically in YEM medium supplemented or not supplemented with molybdate. Values, in Miller units, are mean and standard error of the mean (in parentheses) for at least three cultures which were assayed in duplicate.
the mod mutants were tested for the ability to grow under anaerobic conditions in a medium containing different sulphate concentrations. The results presented in Fig. 3(c) show that the amount of molybdate required for anaerobic growth of strain 0512 in a medium supplemented with 1 mM sulphate was 0.5 μM (Fig. 3c). This value is higher than the 0.35 μM molybdate needed for growth of the modA mutant in a medium containing 0.5 mM sulphate (Fig. 3b, c). These findings suggest that the addition of sulphate, which causes repression of the sulphate-transport system (Kredich, 1987; Ohta et al., 1971), increased the molybdate requirement for anaerobic growth of the mod mutants. Conversely, at 5 μM sulphate, addition of 0.25 μM molybdate to the medium was sufficient for cell growth (data not shown). The chlorate-resistant mutant B. japonicum 0507 showed growth characteristics similar to those found in B. japonicum 0512 (data not shown). These results indicate that in B. japonicum, molybdate is transported by the sulphate-transport system in the absence of a functional high-affinity molybdate-transport system.

**Effects of mod mutations on nitrogenase activity**

Under all conditions examined in this study, the number of nodules and the nodule dry weight from soybeans inoculated with either the wild-type B. japonicum USDA110 or its modA mutant derivative were not significantly different (data not shown). Similarly, no differences in plant dry weight (PDW) and N content were observed among plants nodulated with any of the strains used for inoculation after growth in Mo-containing mineral solution, regardless of the sulphate concentration in the solution (Table 3). Decreases of 17 and 20% in PDW and N content, respectively, were

**Table 2. Nitrate reductase activity in B. japonicum parental and mod mutant strains**

Since the mod mutants did not grow anaerobically with nitrate, cells were grown aerobically in YEM medium, collected by centrifugation and incubated anaerobically in YEMN supplemented or not supplemented with molybdate. Data, in nmol NO\textsubscript{2}/C\textsubscript{0} \textsubscript{2} produced (mg protein\textsuperscript{-1} min\textsuperscript{-1}), are mean and standard error of the mean (in parentheses) for at least three cultures which were assayed in duplicate.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Growth conditions</th>
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<tr>
<td></td>
<td></td>
<td>YEMN</td>
</tr>
<tr>
<td>USDA110</td>
<td>Wild-type</td>
<td>50.39 (3.0)</td>
</tr>
<tr>
<td>0512</td>
<td>modA</td>
<td>0.38 (0.3)</td>
</tr>
<tr>
<td>0507</td>
<td>modB</td>
<td>0.50 (0.3)</td>
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<tr>
<td>0512-0523</td>
<td>modA with pBG0523</td>
<td>47.32 (3.5)</td>
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<tr>
<td>0507-0523</td>
<td>modB with pBG0523</td>
<td>49.45 (4.0)</td>
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**Fig. 3.** (a) Nitrate-dependent anaerobic growth of wild-type B. japonicum USDA110 (■), modA mutant derivative 0512 (●), and modA mutant complemented with plasmid pBG0523 (▲). (b, c) Nitrate-dependent anaerobic growth of modA mutant derivative 0512 in a medium containing either 0.5 mM sulphate (b) or 1 mM sulphate (c) and supplemented with 0.25 (○), 0.30 (□), 0.35 (▲), 0.45 (■) or 0.5 μM (●) molybdate. Cells were grown anaerobically in YEM medium supplemented with 10 mM KNO\textsubscript{3}. Growth of the cells under anaerobic conditions was measured by monitoring OD\textsubscript{600}.
Table 3. Plant dry weight and plant nitrogen content of soybeans nodulated by *B. japonicum* wild-type and mutant derivative 0512

The units of plant dry weight (PDW) are grams per plant; the units of plant nitrogen content (N) are milligrams per plant. Plants were grown for 42 days with an N-free nutrient solution supplemented or not supplemented with 0-8 µM molybdate. The growth medium contained either 3-5 or 10 mM sulphate. Values in individual columns followed by the same letter are not significantly different at \( P \leq 0.05 \) (\( n = 12 \)).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of ( \text{SO}_4^{2-} ) (mM)</th>
<th>Without molybdate</th>
<th>With 0.8 µM molybdate</th>
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<tbody>
<tr>
<td></td>
<td>PDW</td>
<td>N</td>
<td>PDW</td>
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<tr>
<td>USDA110</td>
<td>3.5</td>
<td>2.4 (a)</td>
<td>62.6 (a)</td>
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<td>3.5</td>
<td>10</td>
<td>2.4 (a)</td>
<td>60.5 (a)</td>
</tr>
<tr>
<td>0512</td>
<td>3.5</td>
<td>1.7 (b)</td>
<td>41.4 (b)</td>
</tr>
<tr>
<td>10</td>
<td>1.06</td>
<td>30.8 (c)</td>
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observed in soybeans inoculated with the parental strain after growth of the plants in mineral solution without molybdate containing 3-5 or 10 mM sulphate (Table 3). Decreases of about 37 and 46% in PDW and N content, respectively, were found in plants nodulated by the mutant strain and supplied with a mineral solution without molybdate and with 3-5 mM sulphate (Table 3). Under Mo-deficient conditions, decreases of 63 and 59% in PDW and N content, respectively, were found in plants inoculated with the mutant strain that were supplied with 10 mM sulphate (Table 3).

In a similar manner to the observations for PDW and plant N content, the acetylene-reduction rates and leghaemoglobin content of plant nodules formed by the *modA* mutant grown with 3-5 mM sulphate were reduced by about 40 and 20%, respectively, by molybdate deficiency, compared with the values observed in Mo-supplemented plants (Table 4). As observed in Table 3, addition of 10 mM sulphate to the Mo-deficient nutrient solution decreased the nitrogenase activity and leghaemoglobin content by about 60 and 39%, respectively, compared with the values observed in plants grown in a Mo-containing medium (Table 4). Similar results were obtained when the *modB* 0507 mutant strain was used (data not shown).

Mo transport has been studied in free-living nitrogen fixers, such as *Klebsiella pneumoniae* (Imperial *et al.*, 1985), *A. vinelandii* (Luque *et al.*, 1993; Mouncey *et al.*, 1995, 1996) and *R. capsulatus* (Wang *et al.*, 1993). Although *modABC* homologues have been found in the genome sequence of symbiotic nitrogen fixers, such as *Sinorhizobium meliloti* (Galibert *et al.*, 2001), *M. loti* (Kaneko *et al.*, 2000) and *B. japonicum* (Kaneko *et al.*, 2002), whether this is the only transporter for rhizobia and whether it operates in bacteroids remains to be seen. In this paper, we describe for the first time a symbiotic phenotype of rhizobia strains affected in the high-affinity Mo transporter ModABC. Taken together, the results presented in Tables 3 and 4 clearly indicate that the *mod* genes described in this work are required for a fully effective symbiosis under Mo-limiting conditions. As for free-living cells, the increase in sulphate concentration in the rooting medium of the plants inoculated with the *mod* mutants reduced the symbiotic

Table 4. Acetylene reduction activity (ARA) and leghaemoglobin (Lb) content in nodules of soybeans nodulated by *B. japonicum* wild-type and mutant derivative 0512

The units of ARA were micromoles C2H2 reduced per gram nodule dry weight per hour; the units of Lb content were milligrams per gram nodule fresh weight. Plants were grown for 42 days with a nutrient solution supplemented or not supplemented with 0-8 µM molybdate. The growth medium contained either 3-5 or 10 mM sulphate. Values in individual columns followed by the same letter are not significantly different at \( P \leq 0.05 \) (\( n = 12 \)).

<table>
<thead>
<tr>
<th>Strain</th>
<th>Concentration of ( \text{SO}_4^{2-} ) (mM)</th>
<th>Without molybdate</th>
<th>With 0.8 µM molybdate</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ARA</td>
<td>Lb</td>
<td>ARA</td>
</tr>
<tr>
<td>USDA110</td>
<td>3.5</td>
<td>331 (a)</td>
<td>9-35 (a)</td>
</tr>
<tr>
<td>3.5</td>
<td>10</td>
<td>335 (a)</td>
<td>9-15 (a)</td>
</tr>
<tr>
<td>0512</td>
<td>3.5</td>
<td>236 (b)</td>
<td>6-74 (b)</td>
</tr>
<tr>
<td>10</td>
<td>154 (c)</td>
<td>4-95 (c)</td>
<td>383 (a)</td>
</tr>
</tbody>
</table>
properties of the plants. Again, it is possible that repression of the sulphate transporter resulted in inhibition of molybdate uptake by bacteroids, which lends support to the suggestion that *B. japonicum* possesses at least two independent systems for molybdate incorporation within each free-living or symbiotic cell.

ACKNOWLEDGEMENTS

This work was supported by grants BMC2002-04126-C03-02 and FIT-050 000-2001-30 from Dirección General de Investigación to E.J.B. The support of the Junta de Andalucía (PAI/CVI-275) is also acknowledged. A.T.-A. was the recipient of a fellowship from Agencia Española de Cooperación Internacional (AECI). We thank Wilson Teran Perez for his help with the primer extension experiments.

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