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

María J. Delgado, Alvaro Tresierra-Ayala, Chouhra Talbi and Eulogio J. Bedmar

Correspondence

María J. Delgado
mdelgado@eez.csic.es

Departamento de Microbiología del Suelo y Sistemas Simbióticos, Estación Experimental del Zaidín, CSIC, PO Box 419, 18080 Granada, Spain

Received 15 July 2005
Revised 14 October 2005
Accepted 16 October 2005

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_{modA} -*lacZ*, 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-Furrow *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 thiosulphate, 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

Abbreviation: PDW, plant dry weight.

The GenBank/EMBL/DDBJ accession number for the *B. japonicum* *mod* gene sequence reported in this paper is AF446208, and this corresponds to ORFs *blr8160*, *blr8161* and *blr8162* from the recently sequenced *B. japonicum* USDA110 genome (see <http://www.kazusa.jp/rhizobase/>).

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 (N_2) into ammonia (NH_4^+), a reaction catalysed by the molybdoenzyme nitrogenase (Lawson & Smith, 2002). In addition to assimilating nitrate (NO_3^-) to NH_4^+ (Bergersen, 1977), *B. japonicum* is also capable of denitrification, that is, the reduction of NO_3^- or nitrite (NO_2^-) via nitric oxide (NO) and nitrous oxide (N_2O) to N_2 , 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 N_2 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 *blr160*, *blr161* and *blr162*; a second to *blr6951*, *blr6952* and *blr6953*; while further copies of *modB* (*blr1719*) and *modC* (*blr1780*) 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 *blr160* and *blr161*, 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 KNO_3 (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 KNO_3 (BMN). YEM, YEMN and BMN medium were prepared with MilliQ water and high-quality chemical products. When required, $Na_2MoO_4 \cdot xH_2O$ and K_2SO_4 were added to the medium. Antibiotics were added to *B. japonicum* cultures at the following concentrations ($\mu g\ ml^{-1}$): 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* DH5 α (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 conjugative plasmid transfer. The antibiotics used were ($\mu g\ ml^{-1}$): 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 $KClO_3$. 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 O_2/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 *Bam*HI fragment containing chromosomal DNA from the region flanking the Tn5 insertion in *B. japonicum* 0507 was cloned into plasmid pBluescript KS+ (pBS, Stratagene) to obtain plasmid pCHL7B. Then a 2 kb *Hpa*I/*Sac*I fragment from pCHL7B was used as probe to screen a *B. japonicum* USDA110 cosmid library. The cosmid cGBj20-23 was thus identified containing *B. japonicum* cloned DNA, and a 4.5 kb *Pst*I and an 8.8 kb *Eco*RI 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 *Sal*I and a 2.7 kb *Sal*I/*Pst*I 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 *Eco*RV 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 *Sma*I fragment (Ω Spc/Sm interposon) of pHP45 Ω (Prentki & Kirsch, 1984) was inserted into the *Nru*I site of the 0.9 kb *Eco*RV fragment from pBG0510, resulting in plasmid pBG0511 (data not shown). Finally, a 2.9 kb *Sal*I/*Pst*I 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 *Apa*I-*Xba*I fragment from pBG0509 (Fig. 1) into the *Apa*I/*Xba*I-digested pBBR1MCS-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

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*.

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 μ M 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'-CGAACACGGTGATGGTCTTGTC-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 μ Ci (3.0 MBq) [γ - 32 P]ATP in a total volume of 17 μ l. About 10^5 c.p.m. labelled primer was hybridized to 20 μ g 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 P_{modA} -*lacZ* fusion. To construct a transcriptional fusion of the *mod* promoter region to the reporter gene *lacZ* (P_{modA} -*lacZ* fusion), the 877 bp *SalI*/*NcoI* fragment from pBG0509 containing the *mod* promoter region was subcloned into the *EcoRI* site of pMP220 (Spaink *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. 10^8 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 μ M 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 μ M 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

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 Km^r 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).

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 *blr6951*, *blr6952* and *blr6953* of the *B. japonicum* USDA110 genome (Kaneko *et al.*, 2002; see <http://www.kazusa.jp/rhizobase/>). The products of the other two ORFs, *blr1719* and *bll1780*, 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/>).

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 µM 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,

Fig. 2. (a) Mapping of the transcription start site of *B. japonicum modA* by primer extension. The sequence ladder shown was generated with pBG0509 DNA and the same *modA* primer used for the transcript mapping. The transcriptional start site is marked with an arrow. For RNA isolation, cells of the wild-type strain USDA110 (lanes 1 and 2) and the *modA* mutant strain 0512 (lanes 3 and 4) were cultured aerobically in YEM medium supplemented (lanes 1 and 3) or not supplemented (lanes 2 and 4) with 0.5 μ M molybdate. (b) The *modA* 5' region. The nucleotide at which transcription initiates is shown in bold and marked +1 above. A potential Shine–Dalgarno (RBS) sequence is underlined. The putative translation start codon is shown in bold. The N-terminal amino acid sequence of the ModA protein is also shown.

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 P_{modA} –*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 μ M molybdate, cells of strain USDA110 with the P_{modA} –*lacZ* fusion had very low levels of β -galactosidase activity (Table 1). Similarly, when cells of the *modA* mutant containing P_{modA} –*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 μ M 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 μ M 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 1. Expression of β -galactosidase from a P_{modA} –*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.

Strain	Relevant genotype	Growth conditions	
		YEM	YEM + 0.5 μ M molybdate
110-0513	P_{modA} – <i>lacZ</i> wild-type	9.54 (3.0)	8.43 (4.0)
0512-0513	P_{modA} – <i>lacZ</i> <i>modA</i>	113.62 (10)	9.34 (4.0)

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 μ M (●) molybdate. Cells were grown anaerobically in YEM medium supplemented with 10 mM KNO_3 . Growth of the cells under anaerobic conditions was measured by monitoring OD_{600} .

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 μ M (Fig. 3c). This value is higher than the 0.35 μ M 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 μ M sulphate, addition of 0.25 μ M 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_2^- produced (mg protein) $^{-1}$ min $^{-1}$, are mean and standard error of the mean (in parentheses) for at least three cultures which were assayed in duplicate.

Strain	Relevant genotype	Growth conditions	
		YEMN	YEMN + 0.5 μ M molybdate
USDA110	Wild-type	50.39 (3.0)	51.68 (4.0)
0512	<i>modA</i>	0.38 (0.3)	49.60 (4.5)
0507	<i>modB</i>	0.50 (0.3)	50.80 (3.5)
0512-0523	<i>modA</i> with pBG0523	47.32 (3.5)	48.50 (4.0)
0507-0523	<i>modB</i> with pBG0523	49.45 (4.0)	50.25 (4.5)

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 $P \leq 0.05$ ($n \leq 12$).

Strain	Concentration of SO_4^{2+} (mM)	Without molybdate		With 0.8 µM molybdate	
		PDW	N	PDW	N
USDA110	3.5	2.4 (a)	62.6 (a)	2.9 (a)	78.5 (a)
	10	2.4 (a)	60.5 (a)	3.1 (a)	79.1 (a)
0512	3.5	1.7 (b)	41.4 (b)	2.7 (a)	76.9 (a)
	10	1.06 (c)	30.8 (c)	2.9 (a)	75.4 (a)

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 C_2H_2 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 $P \leq 0.05$ ($n \leq 12$).

Strain	Concentration of SO_4^{2+} (mM)	Without molybdate		With 0.8 µM molybdate	
		ARA	Lb	ARA	Lb
USDA110	3.5	331 (a)	9.35 (a)	400 (a)	9.00 (a)
	10	335 (a)	9.15 (a)	392 (a)	8.83 (a)
0512	3.5	236 (b)	6.74 (b)	393 (a)	8.40 (a)
	10	154 (c)	4.95 (c)	383 (a)	8.05 (a)

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.

REFERENCES

- Anderson, L., Palmer, T., Price, N., Bornemann, S., Boxer, D. & Pau, R. (1997). Characterisation of the molybdenum-responsive ModE regulatory protein and its binding to the promoter region of the *modABCD* (molybdenum transport) operon of *Escherichia coli*. *Eur J Biochem* **246**, 119–126.
- Bedmar, E. J., Robles, E. F. & Delgado, M. J. (2005). The complete denitrification pathway of symbiotic N-fixing bacteria *Bradyrhizobium japonicum*. *Biochem Soc Trans* **1**, 11–16.
- Bergersen, F. J. (1977). A treatise on dinitrogen fixation. In *Biology*, section III, pp. 519–556. Edited by R. W. Hardy & W. Silver. New York: Wiley.
- Delgado, M. J., Olivares, J. & Bedmar, E. J. (1989). Nitrate reductase activity of free-living and symbiotic uptake hydrogenase-positive and uptake hydrogenase-negative strains of *Bradyrhizobium japonicum*. *Arch Microbiol* **151**, 166–170.
- Delgado, M. J., Garrido, J. M., Ligerio, F. & Lluch, C. (1993). Nitrogen fixation and carbon metabolism by nodules and bacteroids of pea plants under sodium chloride stress. *Physiol Plant* **89**, 824–829.
- Delgado, M. J., Bonnard, N., Tresierra-Ayala, A., Bedmar, E. J. & Müller, P. (2003). The *Bradyrhizobium japonicum* *napEDABC* genes encoding the periplasmic nitrate reductase are essential for nitrate respiration. *Microbiology* **149**, 3395–3403.
- Galibert, F., Finan, T. M., Long, S. R. & 53 other authors (2001). The composite genome of the legume symbiont *Sinorhizobium meliloti*. *Science* **293**, 668–672.
- Göttfert, M., Röthlisberger, S., Kundig, C., Beck, C., Marty, R. & Hennecke, H. (2001). Potential symbiotic-specific genes uncovered by sequencing a 410-kilobase DNA region of the *Bradyrhizobium japonicum* chromosome. *J Bacteriol* **183**, 1405–1412.
- Graham, L. & Maier, R. J. (1987). Variability in molybdenum uptake activity in *Bradyrhizobium japonicum* strains. *J Bacteriol* **169**, 2555–2560.
- Grunden, A. M. & Shanmugam, K. T. (1997). Molybdate transport and regulation in bacteria. *Arch Microbiol* **168**, 345–354.
- Grunden, A. M., Ray, R. M., Rosentel, J. K., Healy, F. G. & Shanmugam, K. T. (1996). Repression of the *Escherichia coli* *modABCD* (molybdate transport) operon by ModE. *J Bacteriol* **178**, 735–744.
- Grunden, A. M., Self, W. T., Villain, M., Blalock, J. E. & Shanmugam, K. T. (1999). An analysis of the binding of repressor protein ModE to *modABCD* (molybdate transport) operator/promotor DNA of *Escherichia coli*. *J Biol Chem* **274**, 24308–24315.
- Imperial, J., Ugalde, R. A., Shah, V. K. & Brill, W. J. (1985). Mol[−] mutants of *Klebsiella pneumoniae* requiring high levels of molybdate for nitrogenase activity. *J Bacteriol* **163**, 1285–1287.
- Johnston, A. W. B., Yeoman, K. H. & Wexler, M. (2001). Metals and the rhizobial-legume symbiosis-uptake, utilization and signalling. *Adv Microb Physiol* **45**, 112–156.
- Kaneko, T., Nakamura, Y., Sato, S. & 21 other authors (2000). Complete genome structure of the nitrogen-fixing symbiotic bacterium *Mesorhizobium loti*. *DNA Res* **7**, 331–338.
- Kaneko, T., Nakamura, Y., Sato, S. & 17 other authors (2002). Complete genomic sequence of nitrogen-fixing symbiotic bacterium *Bradyrhizobium japonicum* USDA110. *DNA Res* **9**, 189–197.
- Kertesz, M. A. (2001). Bacterial transporters for sulfate and organo-sulfur compounds. *Res Microbiol* **152**, 279–290.
- Kovach, M. E., Phillips, R. W., Elzer, P. H., Roop, R. M., II & Peterson, K. M. (1994). pBBR1MCS: a broad-host-range cloning vector. *Biotechniques* **16**, 800–802.
- Kredich, N. M. (1987). Biosynthesis of cysteine. In *Escherichia coli and Salmonella typhimurium: Cellular and Molecular Biology*, pp. 419–428. Edited by F. C. Neidhardt, J. L. Ingraham, K. B. Low, B. Magasanik, M. Schaechter & H. E. Umbarger. Washington, DC: American Society for Microbiology.
- Lawson, D. M. & Smith, B. E. (2002). Molybdenum nitrogenases: a crystallographic and mechanistic view. *Met Ions Biol Syst* **39**, 75–119.
- Lee, J. H., Wendt, J. C. & Shanmugam, K. T. (1990). Identification of a new gene, *molR*, essential for utilization of molybdate by *Escherichia coli*. *J Bacteriol* **172**, 2079–2087.
- Luque, F., Mitchenall, L. A., Chapman, M., Christine, R. & Pau, R. N. (1993). Characterization of genes involved in molybdenum transport in *Azotobacter vinelandii*. *Mol Microbiol* **7**, 447–459.
- Maier, R. J. & Graham, L. (1988). Molybdate transport by *Bradyrhizobium japonicum* bacteroids. *J Bacteriol* **170**, 5613–5619.
- Maupin-Furlow, J. A., Rosentel, J. K., Lee, J. H., Deppenmeier, U., Gunsalus, R. P. & Shanmugam, K. T. (1995). Genetic analysis of the *modABCD* (molybdate transport) operon of *Escherichia coli*. *J Bacteriol* **177**, 4851–4856.
- McNicholas, P. M., Chiang, R. C. & Gunsalus, R. P. (1998). Anaerobic regulation of the *Escherichia coli* *dmsABC* operon requires the molybdate responsive regulator, ModE. *Mol Microbiol* **27**, 197–208.
- Mesa, M., Alché, J. D., Bedmar, E. J. & Delgado, M. J. (2004). Expression of *nir*, *nor* and *nos* denitrification genes from *Bradyrhizobium japonicum* in soybean root nodules. *Physiol Plant* **119**, 1–7.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Mouncey, N. J., Mitchenall, L. A. & Pau, R. N. (1995). Mutational analysis of genes of the *mod* locus involved in molybdenum transport, homeostasis, and processing in *Azotobacter vinelandii*. *J Bacteriol* **177**, 5294–5302.
- Mouncey, N. J., Mitchenall, L. A. & Pau, R. N. (1996). The *modE* gene product mediates molybdenum-dependent expression of genes for the high-affinity molybdate transporter and *modG* in *Azotobacter vinelandii*. *Microbiology* **142**, 1997–2004.
- Neubauer, H., Pantel, I., Lindgren, P. & Götz, F. (1999). Characterization of the molybdate transport system ModABC of *Staphylococcus carnosus*. *Arch Microbiol* **172**, 109–115.
- Nicholas, D. J. D. & Nason, A. (1957). Determination of nitrate and nitrite. In *Methods in Enzymology*, vol. 3, pp. 981–984. Edited by S. P. Colowick & N. O. Kaplan. New York: Academic Press.
- Nienaber, A., Huber, A., Göttfert, M., Hennecke, H. & Fischer, H. M. (2000). Three new NifA-regulated genes in the *Bradyrhizobium*

japonicum symbiotic gene region discovered by competitive DNA–RNA hybridization. *J Bacteriol* **182**, 1472–1480.

Ohta, N., Galsworthy, P. R. & Pardee, A. B. (1971). Genetics of sulfate transport by *Salmonella typhimurium*. *J Bacteriol* **105**, 1053–1062.

Pau, R. N. & Lawson, D. M. (2002). Transport, homeostasis, regulation, and binding of molybdate and tungstate to proteins. *Met Ions Biol Syst* **39**, 31–74.

Prentki, P. & Kirsch, H. M. (1984). *In vitro* insertional mutagenesis with a selectable DNA fragment. *Gene* **29**, 303–312.

Rech, S., Deppenmeier, U. & Gunsalus, R. P. (1995). Regulation of the molybdate transport operon, *modABCD*, of *Escherichia coli* in response to molybdate availability. *J Bacteriol* **177**, 1023–1029.

Richardson, D. J., Berks, B. C., Russell, D. A., Spiro, S. & Taylor, C. J. (2001). Functional, biochemical and genetic diversity of prokaryotic nitrate reductases. *Cell Mol Life Sci* **58**, 165–178.

Rigaud, J. & Puppo, A. (1975). Indole-3-acetic acid catabolism by soybean bacteroids. *J Gen Microbiol* **88**, 223–228.

Rosentel, J. K., Healy, F., Maupin-Furlow, J. A., Lee, J. H. & Shanmugam, K. T. (1995). Molybdate and regulation of *mod* (molybdate transport), *fdhF*, and *hyc* (formate hydrogenlyase) operons in *Escherichia coli*. *J Bacteriol* **177**, 4857–4864.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* **145**, 69–73.

Self, W. T., Grunden, A. M., Hasona, A. & Shanmugam, K. T. (2001). Molybdate transport. *Res Microbiol* **152**, 311–321.

Simon, R., Priefer, U. & Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in Gram negative bacteria. *Biotechnology* **1**, 784–791.

Sirko, A., Hryniewicz, M., Hulanicka, D. & Böck, A. (1990). Sulfate and thiosulfate transport in *Escherichia coli* K-12: nucleotide sequence and expression of the *cysTWAM* gene cluster. *J Bacteriol* **172**, 3351–3357.

Spaink, H. P., Okker, J. H., Wijffelman, C. A., Pees, E. & Lugtenberg, B. J. J. (1987). Promoters in the nodulation region of *Rhizobium leguminosarum* Sym plasmid pRL1J1. *Plant Mol Biol* **9**, 27–39.

Stewart, V. (1988). Nitrate respiration in relation to facultative metabolism in enteric bacteria. *Microbiol Rev* **52**, 190–232.

Thiel, T., Pratte, B. & Zabalak, M. (2002). Transport of molybdate in the cyanobacterium *Anabaena variabilis* ATCC 29413. *Arch Microbiol* **179**, 50–56.

Vincent, J. M. (1974). Root-nodule symbioses with *Rhizobium*. In *The Biology of Nitrogen Fixation*, pp. 265–341. Edited by A. Quispel. New York: American Elsevier Publishing Company.

Wang, G., Angermüller, S. & Klipp, W. (1993). Characterization of *Rhodobacter capsulatus* genes encoding a molybdenum transport system and putative molybdenum-pterin-binding proteins. *J Bacteriol* **175**, 3031–3042.

Wood, D. W., Setubal, J. C., Kaul, R. & 48 other authors (2001). The genome of the natural genetic engineer *Agrobacterium tumefaciens* C58. *Science* **294**, 2317–2323.

Zabalak, M., Pratte, B., Werth, K. J. & Thiel, T. (2004). Molybdate transport and its effect on nitrogen utilization in the cyanobacterium *Anabaena variabilis* ATCC 29419. *Mol Microbiol* **51**, 539–549.