

# Multiplicity of Sulfate and Molybdate Transporters and Their Role in Nitrogen Fixation in *Rhizobium leguminosarum* bv. *viciae* Rlv3841

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*Rhizobium leguminosarum* Rlv3841 contains at least three sulfate transporters, i.e., SulABCD, SulP1 and SulP2, and a single molybdate transporter, ModABC. SulABCD is a high-affinity transporter whose mutation prevented growth on a limiting sulfate concentration, while SulP1 and SulP2 appear to be low-affinity sulfate transporters. ModABC is the sole high-affinity molybdate transport system and is essential for growth with NO<sub>3</sub><sup>−</sup> as a nitrogen source on limiting levels of molybdate (<0.25 μM). However, at 2.5 μM molybdate, a quadruple mutant with all four transporters inactivated, had the longest lag phase on NO<sub>3</sub><sup>−</sup>, suggesting these systems all make some contribution to molybdate transport. Growth of Rlv3841 on limiting levels of sulfate increased *sulB*, *sulP1*, *modB*, and *sulP2* expression 313.3-, 114.7-, 6.2-, and 4.0-fold, respectively, while molybdate starvation increased only *modB* expression (three- to 7.5-fold). When grown in high-sulfate but not low-sulfate medium, pea plants inoculated with LMB695 (*modB*) reduced acetylene at only 14% of the wild-type rate, and this was not further reduced in the quadruple mutant. Overall, while *modB* is crucial to nitrogen fixation at limiting molybdate levels in the presence of sulfate, there is an unidentified molybdate transporter also capable of sulfate transport.

Sulfur (S) is an essential element for living organisms and is usually available to bacteria, fungi, and plants as sulfate (SO<sub>4</sub><sup>2−</sup>) (Kertesz and Mirleau 2004). Sulfate is a strong acid that is completely ionized at physiological pH, necessitating its uptake by active transport systems. Sulfate transport in bacteria is catalyzed by ABC-type transporters from the SulT family as well as MFS transporters from the SulP family (Kertesz 2001).

SulT is the main sulfate and thiosulfate transporter and has been studied in detail in *Salmonella typhimurium* and *Escherichia coli*. This transport system consists of a complex of five types of subunits encoded by *sbp* (sulfate-binding protein gene), *cysP*, *cysU*, *cysW*, and *cysA* (Kertesz 2001; Sirko et al. 1995). Sulfate assimilation is initiated by the periplasmic SBP or thiosulfate binding protein (CysP), which interacts with the membrane permease components CysT and CysW. CysA comprises a nucleotide-binding (ATP-binding) and a regulatory domain that changes conformation upon binding of ATP

(Hryniewicz et al. 1990; Scheffel et al. 2005). In *S. typhimurium* and *E. coli*, SulT subunits are encoded by the *cysPTWA* operon, with *sbp* located elsewhere on the genome (Kertesz 2001). Single *cysP* and *sbp* mutants in *E. coli* are able to utilize both sulfate and thiosulfate as a sole S source, demonstrating that the two proteins overlap in their solute range. By contrast, a mutant in *cysT* was a cysteine auxotroph, as was the *cysP* and *sbp* double mutant (Sirko et al. 1995). Genes encoding these components are tightly regulated at two levels by transcriptional regulators of the LysR family. However, CysB appears to play the main role in activation of genes encoding the sulfate uptake complex upon S starvation (Kredich 1996; Piłsyk and Paszewski 2009).

Many genera lack SulT but, instead, contain a sulfate permease from the SulP superfamily, consisting of hundreds of sequenced members, although few have been functionally characterized (Saier et al. 2006). They are found in prokaryotes, including *Yersinia enterocolitica*, *Bacillus subtilis* (two copies), and *Pseudomonas aeruginosa*, and are present in a number of incomplete genome sequences (Kertesz 2001; Piłsyk and Paszewski 2009). Several of the latter contain two or more SulP-type permeases (Kertesz 2001). *P. aeruginosa* contains both SulT and SulP transporters, with SulT expressed under low-sulfate conditions (Quadroni et al. 1999). Due to their low substrate specificity, sulfate permeases may also transport other tetra-oxyanions like molybdate (Kertesz 2001).

Molybdenum (Mo), in its readily soluble form of molybdate (MoO<sub>4</sub><sup>2−</sup>), is essential for bacterial growth (Smith et al. 1997). It is incorporated into apoenzymes such as Moco, a Mo cofactor comprising a mononuclear Mo atom coordinated to the S atoms of a pterin called molybdopterin (Hille 2002). In *E. coli*, high-affinity molybdate transport is mediated by an ABC-type ModABC transport system. ModA is a periplasmic molybdate-binding protein, ModB is a transmembrane permease, while ModC is an ATPase that energizes transport on the cytoplasmic side of the membrane (Grunden and Shanmugam 1997). Similar systems for molybdate transport have been characterized in *Rhodobacter capsulatus*, *Azotobacter vinelandii*, *Anabaena variabilis*, and *Bradyrhizobium japonicum* (Delgado et al. 2006; Zahalak et al. 2004). The *modABC* operon of *E. coli* is negatively regulated by molybdate via a regulatory protein, ModE (Anderson et al. 2000). In strains deficient in molybdate transport or mutated in *modE*, there is little transcription of *modABC* (Anderson et al. 2000). However *E. coli*, as well as using ModABC, transports molybdate via SulT (Kertesz 2001; Sirko et al. 1990).

*Rhizobium leguminosarum* bv. *viciae* is a gram-negative soil bacterium that induces formation of nitrogen (N)-fixing nodules

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with several hosts, including pea (*Pisum sativum*), lentil (*Lens culinaris*), and *Vicia* spp. Within nodules, rhizobia differentiate into bacteroids that reduce atmospheric di-nitrogen ( $N_2$ ) to ammonia through the Mo-iron nitrogenase complex, thus providing the plant with a source of fixed N (Terpolilli et al. 2012). In turn, the plant must provide carbon, phosphorous, N, S, and Mo. Considering the special function played by the nitrogenase complex in *Rhizobium* species, uptake of molybdate and sulfate are particularly important and their similar structural characteristics mean they can be transported into the cell by the same type of carriers. Moreover, repression of sulfate transport may result in inhibition of molybdate uptake by bacteroids, but the mechanism is unknown (Delgado et al. 2006). Despite the importance of S and Mo in N fixation, there has been very little work on the mechanisms involved in their uptake in rhizobia. We investigated the roles of *R. leguminosarum* sulfate and molybdate transporters in free-living bacteria and during N-fixing symbiosis on *Pisum sativum* by analyzing the phenotypes of mutant strains, each lacking one to four of the putative sulfate and molybdate transporter systems identified in the Rlv3841 genome.

## RESULTS

### Molybdate and sulfate transport systems.

Bioinformatic analysis of Rlv3841 revealed the presence of a single putative molybdate transporter (ModABC) and three putative sulfate transporters (Fig. 1). ModABC is an ABC transporter composed of a SBP encoded by *modA* (RL4685), a permease encoded by *modB* (RL4686), and an ATP-binding protein encoded by *modC* (RL4687).

One of the three putative sulfate transporters in Rlv3841 is an ABC transporter of the SulT family. Located on plasmid pRL11 (Young et al. 2006), *sulA* (pRL110374) encodes a SBP, *sulB* (pRL110373) and *sulC* (pRL110372) encode permease components, and *sulD* (pRL110371) encodes an ATP-binding protein (Fig. 1). The remaining sulfate transporters are two chromosomal

SulP-type permeases, SulP1 (RL2866) and SulP2 (RL2944). To characterize the role of these transporters and interaction between sulfate and molybdate transport, single and multiple mutants were made in all these transporters by combining the mutated Mod and Sul transport systems (each marked with a different antibiotic resistance gene) by means of mutagenesis and transduction between Rlv3841 and mutant strains (Table 1).

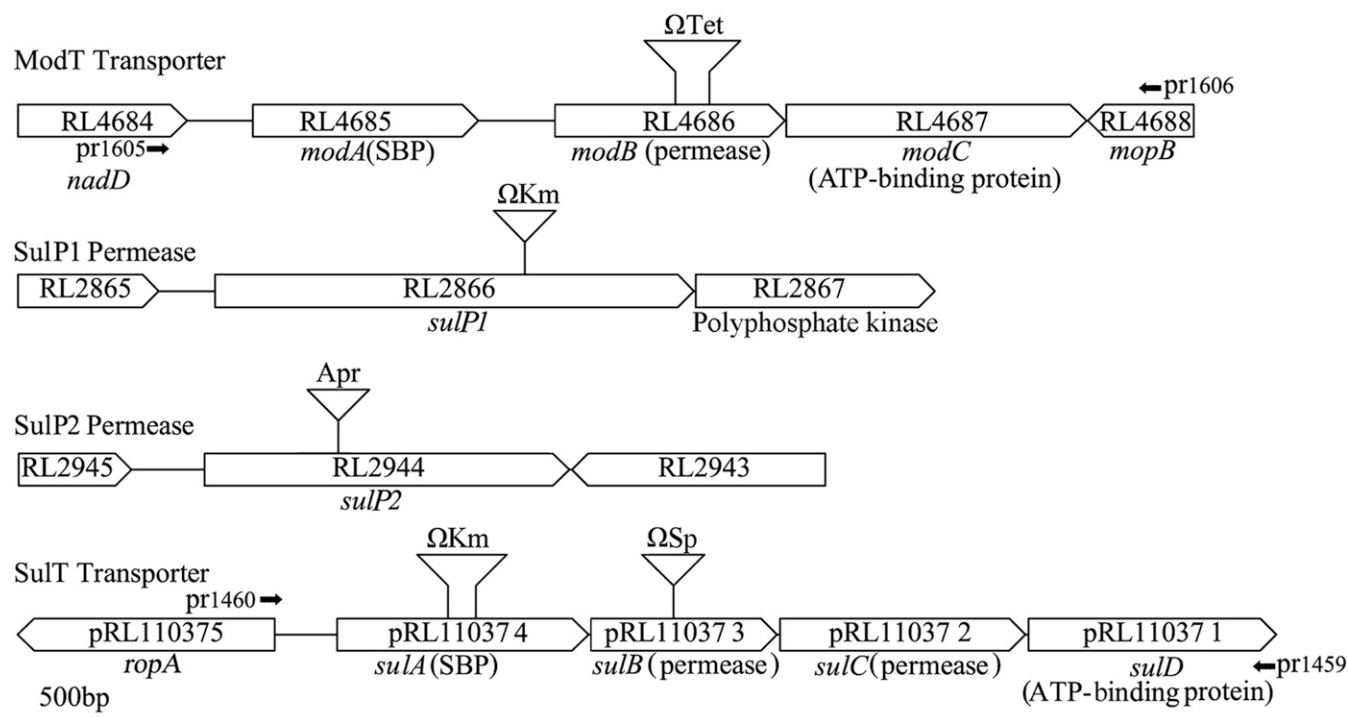
### Growth of *sul* mutants on sulfate.

To investigate the role of each of the three Sul transport systems identified bioinformatically, growth of strains mutated in each system was compared with that of wild-type Rlv3841. A mutant in the Sul system, by inactivation of either *sulA* (pRL110374) or *sulB* (pRL110373) (Fig. 1), gave strains LMB595 and LMB584, respectively (Table 1), which were unable to grow in liquid acid minimal salt (AMS) medium supplemented with 20  $\mu$ M sodium sulfate as the sole S source (Figs. 2 and 3A). Ability to grow in this media was restored to these Sul mutants either by complementation with pLMB731 (*sulABCD* cloned in a multicopy plasmid), giving strains LMB631 and LMB633 (Fig. 2), or by addition of 2 mM sodium sulfate to the growth medium (Fig. 3B). Mutants in either *sulP1* or *sulP2* (strains LMB700 and LMB694) grew at the same rate as wild-type Rlv3841 with 20  $\mu$ M sulfate as sole S source (Fig. 3A). This shows that SulABCD is the main high-affinity sulfate transport system in Rlv3841.

Single *sulB*, *sulP1*, and *sulP2* mutants grew at the same rate as wild type on 2 mM sulfate (Fig. 3B). However, the triple mutant LMB708 (*sulB sulP1 sulP2*) had slightly reduced growth compared with the wild-type strain (Fig. 3B). This is consistent with SulP1 and SulP2 being transporters with a lower affinity for sulfate but suggests there are one or more additional unidentified low-affinity S transporters in Rlv3841, as this triple mutant is still able to grow.

### Growth of *sul* and *mod* mutants on molybdate.

Comparison of growth of transport-system mutants was used to discover their role in molybdate transport in Rlv3841. In



**Fig. 1.** Organization of genes encoding a molybdate transporters (ModABC) and sulfate (Sul) transporters in Rlv3841. Arrows indicate location and orientation of open reading frames. The solid black arrows indicate the positions of primers used to generate the polymerase chain reaction products cloned to complement mutations in *modABC* (pr1605/pr1606 product cloned in pLMB801) and *sulABCD* (pr1459/pr1460 product cloned in pLMB731). Positions of insertion of genes encoding resistance to antibiotics (Apr = apramycin, ΩKm = kanamycin, ΩSp = spectinomycin, ΩTet = tetracycline) into *mod* and *sul* genes to obtain mutants are shown.

contrast to Rlv3841, strain LMB695 (*modB*) failed to grow aerobically in AMS glucose nitrate ( $\text{Glc}/\text{NO}_3^-$ ) media, nitrate acting as the sole N source, without the addition of molybdate (Fig. 4A). This media was used because nitrate reductase, required for growth of rhizobia on nitrate as the sole N source, is a molybdoenzyme (Ferroni et al. 2011). Therefore, growth of rhizobia in this medium is dependent on sufficient Mo being available to the bacterial cell. By complementing LMB695 (*modB*) with plasmid pLMB801 (containing the whole of *modABC*), strain LMB741 (LMB695[pLMB801]) was able to grow in AMS  $\text{Glc}/\text{NO}_3^-$  medium, using nitrate as the sole N source without added molybdate (Fig. 4A). Addition of molybdate to AMS  $\text{Glc}/\text{NO}_3^-$  medium at concentrations  $\geq 0.25 \mu\text{M}$  supported slow growth of LMB695 (*modB*) with a very long lag phase

(Fig. 4B, C, and D). Moreover, at a molybdate concentration of  $2.5 \mu\text{M}$ , the quadruple mutant in which all four Mod and Sul transport systems are inactivated (Table 1, LMB709) had a longer lag phase and slightly reduced growth rate compared with the other mutant strains (Fig. 4D), indicating a role for Sul transporters in molybdate uptake.

To further investigate whether sulfate can block molybdate transport, Rlv3841 and LMB695 (*modB*) were grown in S- and Mo-free AMS  $\text{Glc}/\text{NO}_3^-$  medium supplemented with  $0.5 \mu\text{M}$  sodium molybdate and sodium sulfate at either  $50 \mu\text{M}$  (low) or  $2 \text{ mM}$  (high concentration) (Fig. 5). A concentration of  $0.5 \mu\text{M}$  sodium molybdate was chosen because it is sufficiently high to enable growth on  $\text{NO}_3^-$  as the sole N source when the high-affinity ModABC is inactivated in LMB695 (*modB*). There were

**Table 1.** Strains, vectors, and plasmids

Names	Description <sup>a</sup>	Reference	Names	Description <sup>a</sup>	Reference
<b>Strains</b>					
Rlv3841	<i>Rhizobium leguminosarum</i> bv. <i>viciae</i> , Str <sup>r</sup>	Johnston and Beringer 1975	pRK2013	Helper plasmid for mobilization; Kan/Neo <sup>r</sup>	Figurski and Helinski 1979
LMB584	Rlv3841 <i>sulB</i> :: $\Omega\text{Sp}$ , Spc <sup>r</sup> Str <sup>r</sup>	This study	pRK415-1	IncP broad host-range cloning vector, Tet <sup>r</sup>	Keen et al. 1988
LMB595	Rlv3841 $\Delta\text{sulA}$ :: $\Omega\text{Km}$ , Kan/Neo <sup>r</sup> Str <sup>r</sup>	This study	<b>Plasmids</b>		
LMB631	LMB584[pLMB731], Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB670	pr1382/pr1383 PCR product of <i>sulA</i> cloned in pJET1.2/blunt; Amp <sup>r</sup>	This study
LMB633	LMB595[pLMB731], Kan/Neo <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB671	pr1388/pr1389 PCR product of <i>sulB</i> cloned in pJET1.2; Amp <sup>r</sup>	This study
LMB694	Rlv3841 <i>sulP2</i> ::Apr, Apr <sup>r</sup> Str <sup>r</sup>	This study	pLMB674	$\Omega\text{Sp}$ cassette cloned into unique <i>FspAI</i> site in pLMB671; Amp <sup>r</sup> Spc <sup>r</sup>	This study
LMB695	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB675	<i>BglIII</i> fragment from pLMB674 cloned into <i>BamHI</i> site of pJQ200SK; Gm <sup>r</sup> Spc <sup>r</sup>	This study
LMB696	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulB</i> :: $\Omega\text{Sp}$ , Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB680	Inverse PCR with pr1384/pr1385 of pLMB670, digested with <i>EcoRI</i> and religated; Amp <sup>r</sup>	This study
LMB697	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulB</i> :: $\Omega\text{Sp}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB681	$\Omega\text{Km}$ cassette cloned into <i>EcoRI</i> site in pLMB680; Amp <sup>r</sup> Kan/Neo <sup>r</sup>	This study
LMB698	Rlv3841 <i>sulB</i> :: $\Omega\text{Sp}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup>	This study	pLMB682	<i>XhoI/XbaI</i> fragment from pLMB681 cloned in pJQ200SK; Gm <sup>r</sup> Kan/Neo <sup>r</sup>	This study
LMB699	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulB</i> :: $\Omega\text{Sp}$ , <i>sulP1</i> :: $\Omega\text{Km}$ , Kan/Neo <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB731	pr1459/pr1460 PCR product (containing <i>sulABCD</i> ) cloned in pRK415-1 <i>XbaI</i> site; Tet <sup>r</sup>	This study
LMB700	Rlv3841 <i>sulP1</i> :: $\Omega\text{Km}$ , Kan/Neo <sup>r</sup> Str <sup>r</sup>	This study	pLMB734	pr1463/pr1464 PCR product containing <i>sulP1</i> cloned in pJET1.2; Amp <sup>r</sup>	This study
LMB701	Rlv3841 <i>sulP1</i> :: $\Omega\text{Km}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Kan/Neo <sup>r</sup> Str <sup>r</sup>	This study	pLMB751	pr1467/pr1468 PCR product containing <i>sulP2</i> cloned in pJET1.2; Amp <sup>r</sup>	This study
LMB702	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB752	<i>XmnI/SmaI</i> containing apramycin resistance from pIJ773 cloned in pLMB751; Apr <sup>r</sup> Amp <sup>r</sup>	This study
LMB705	Rlv3841 <i>sulP1</i> :: $\Omega\text{Km}$ , <i>sulB</i> :: $\Omega\text{Sp}$ , Kan/Neo <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup>	This study	pLMB754	pr1473/pr1474 PCR product containing <i>modB</i> into pJET1.2; Amp <sup>r</sup>	This study
LMB706	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulP1</i> :: $\Omega\text{Km}$ , Str <sup>r</sup> Tet <sup>r</sup> Kan/Neo <sup>r</sup>	This study	pLMB755	$\Omega\text{Tet}$ cassette cloned into <i>EcoRI</i> site of pLMB754; Amp <sup>r</sup> Tet <sup>r</sup>	This study
LMB707	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulP1</i> :: $\Omega\text{Km}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Kan/Neo <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB756	<i>BglIII</i> fragment from pLMB755 cloned into <i>BamHI</i> site of pJQ200SK; Gm <sup>r</sup> Tet <sup>r</sup>	This study
LMB708	Rlv3841 <i>sulB</i> :: $\Omega\text{Sp}$ , <i>sulP1</i> :: $\Omega\text{Km}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Kan/Neo <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup>	This study	pLMB778	<i>NotI</i> fragment of pLMB752 cloned into <i>NotI</i> -digested pJQ200Sp, Apr <sup>r</sup> Spc <sup>r</sup>	This study
LMB709	Rlv3841 <i>modB</i> :: $\Omega\text{Tet}$ , <i>sulB</i> :: $\Omega\text{Sp}$ , <i>sulP1</i> :: $\Omega\text{Km}$ , <i>sulP2</i> ::Apr, Apr <sup>r</sup> Kan/Neo <sup>r</sup> Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB784	<i>EcoRI</i> end-filled $\Omega\text{Km}$ cassette cloned into pLMB734 <i>SmaI</i> site; Amp <sup>r</sup> Kan/Neo <sup>r</sup>	This study
LMB741	LMB695[pLMB801], Spc <sup>r</sup> Str <sup>r</sup> Tet <sup>r</sup>	This study	pLMB785	<i>XhoI</i> fragment from pLMB784 cloned into <i>XhoI</i> site of pJQ200SK; Gm <sup>r</sup> Kan/Neo <sup>r</sup>	This study
<b>Vectors</b>			pLMB801	pr1605/pr1606 PCR product (containing the <i>modABC</i> operon) cloned in pJP2Spc <i>XbaI/KpnI</i> ; Spc <sup>r</sup>	This study
pIJ773	aac(3)IV (AprR) oriT, Apr <sup>r</sup>	Gust et al. 2003			
pJET1.2	Vector for cloning polymerase chain reaction (PCR) products, Amp <sup>r</sup>	Thermo Fisher Inc.			
pJP2Spc	Stable broad host-range cloning vector, Spc <sup>r</sup>	P. Poole, unpublished			
pJQ200SK	pACYC-derivative, P15A origin of replication, Gm <sup>r</sup>	Quandt and Hynes 1993			
pJQ200Sp	pJQ200SK-derivative, P15A origin of replication, Gm <sup>r</sup> Spc <sup>r</sup>	P. Poole, unpublished			
pHP45 $\Omega\text{Sp}$	pBR322-derivative carrying $\Omega\text{Sp}$ , pHP45 replicon, Spc <sup>r</sup>	Fellay et al. 1987			
pHP45 $\Omega\text{Km}$	pBR322-derivative carrying $\Omega\text{Km}$ , pHP45 replicon, Kan/Neo <sup>r</sup>	Fellay et al. 1987			
pHP45 $\Omega\text{Tet}$	pBR322-derivative carrying $\Omega\text{Tet}$ , pHP45 replicon, Tet <sup>r</sup>	Fellay et al. 1987			

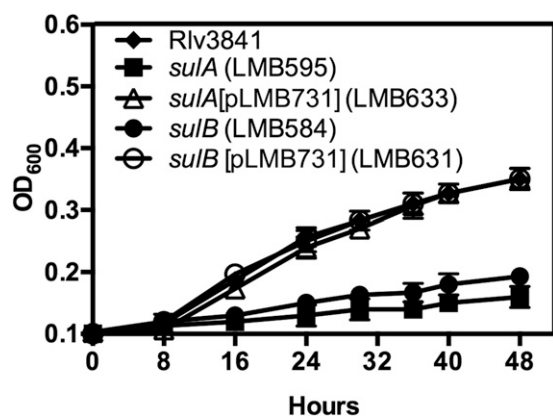
<sup>a</sup> Str, Spc, Kan, Neo, Tet, Apr, Amp, and Gm = streptomycin, spectinomycin, kanamycin, neomycin, tetracycline, apramycin, ampicillin, and gentamicin, respectively; <sup>r</sup> indicates resistance.

no differences observed between LMB695 (*modB*) and Rlv3841 when grown in medium supplemented with the low concentration of 50  $\mu\text{M}$  sulfate. This confirms that, even with ModABC inactivated, 0.5  $\mu\text{M}$  molybdate is sufficient to allow growth on  $\text{NO}_3^-$ . However, growth of LMB695 (*modB*) showed a long delay in the presence of 2 mM sulfate (Fig. 5). This is consistent with the sulfate transporters also transporting molybdate.

### Transcriptional analysis of sulfate and molybdate transport genes.

Studies on regulation of genes coding for the sulfate and molybdate transport systems were carried out using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) to quantify the levels of mRNA for specific genes under different conditions. Wild-type Rlv3841 was grown overnight in AMS Glc/ $\text{NH}_4^+$  with 1  $\mu\text{M}$  sodium molybdate, supplemented with sodium sulfate at either 100  $\mu\text{M}$  or 2 mM. Growth in the lower concentration of sulfate increased *sulB*, *sulP1*, *modB*, and *sulP2* expression  $313.3 \pm 7.2$ -,  $1,14.7 \pm 69.8$ -,  $6.2 \pm 2.8$ -, and  $4.0 \pm 2.3$ -fold, respectively. Thus these molybdate and sulfate transporters are negatively regulated by sulfate. The high-affinity sulfate transporter SulABCD (shown by expression of *sulB*) and the low-affinity SulP1 system were particularly highly induced when the level of sulfate was low. Similar regulation by sulfate concentration occurs in *E. coli* and *P. aeruginosa* (Hummerjohann et al. 1998; Tralau et al. 2007; van der Ploeg et al. 1996).

Further qRT-PCR was performed to examine levels of gene expression in LMB584 (*sulB*), LMB694 (*sulP2*), LMB700 (*sulP1*), and LMB701 (*sulP1 sulP2*) compared with Rlv3841, grown overnight in AMS Glc/ $\text{NH}_4^+$  with 2 mM sulfate (Table 2). Expression of *sulP1* and *sulP2* in LMB584 (*sulB*) were increased 4.8- and 8.7-fold compared with levels in Rlv3841 (Table 2). However, as there was no significant difference in expression of either *sulP2* in LMB700 (*sulP1*) or of *sulP1* in LMB694 (*sulP2*) compared with Rlv3841 (Table 2), it suggests that SulABCD may be involved in monitoring sulfate levels in the cell. Nevertheless, in the double mutant LMB701 (*sulP1 sulP2*), the relative expression of *sulB*, part of Sul, decreased 3.6-fold, indicating that SulABCD is negatively regulated by deletion of both *sulP1* and *sulP2* genes (Table 2). This suggests a complex interregulation of the sulfur transport systems. However, in each of these mutants, *modB* expression showed no significant up- or downregulation, indicating that these mutations in Sul transport systems do not affect *modB* transcription.

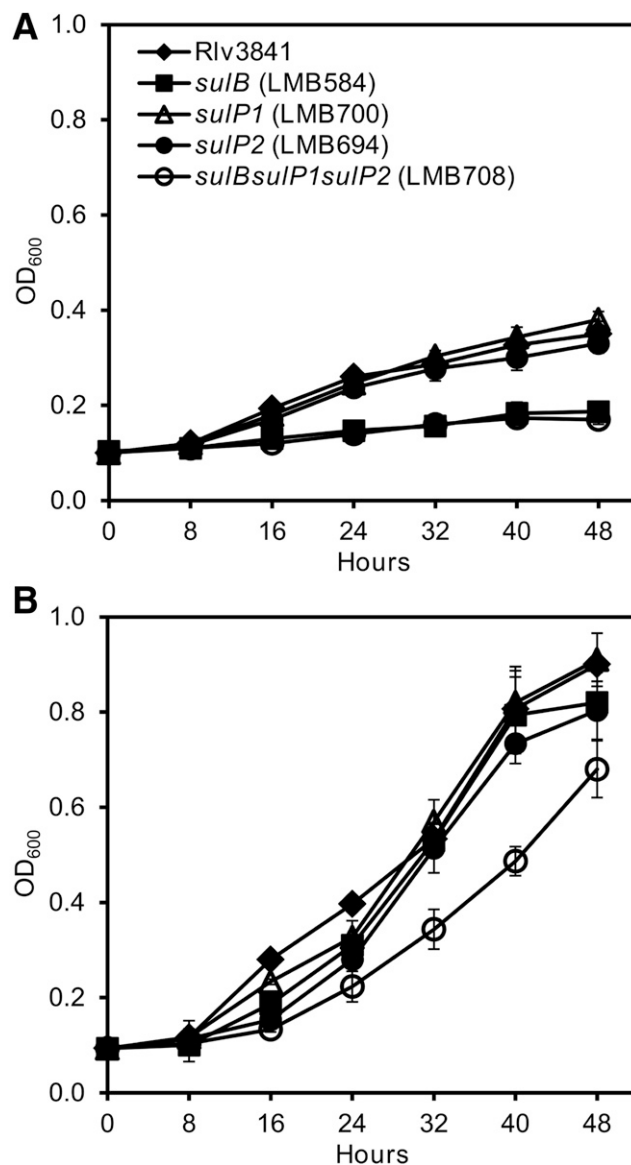


**Fig. 2.** Growth of Rlv3841, sulfate (*sul*) mutants, and complemented strains in 20  $\mu\text{M}$   $\text{Na}_2\text{SO}_4$  as sole sulfate source added to S- and molybdenum (Mo)-free acid minimal salt Glc/ $\text{NH}_4^+$  with 1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ . Wild-type Rlv3841 was compared with mutants  $\Delta$ *sulA* (LMB595) and *sulB* (LMB584) and complemented mutants  $\Delta$ *sulA*[pLMB731] (LMB633) and *sulB*[pLMB731] (LMB631). Data are from three biological samples  $\pm$  standard deviation.

To analyze gene expression levels under different molybdate concentrations, analysis by qRT-PCR was performed on RNA from wild-type Rlv3841 grown in AMS Glc/ $\text{NO}_3^-$  with 2 mM sodium sulfate, in the presence or absence of 10  $\mu\text{M}$  sodium molybdate. With no added molybdate, *modB* expression in early log phase and late log phase increased 3.0- and 7.5-fold, respectively (Table 3), suggesting *modB* is negatively regulated by molybdate. Expression of *sulB*, *sulP1*, and *sulP2* were not significantly altered (Table 3), indicating that these sulfate transporter systems are not regulated by molybdate.

### Role of Sul and Mod transporters in N fixation.

In order to assess N-fixing capacity of Mod and Sul transport system mutants, *Pisum sativum* seedlings were inoculated with *R. leguminosarum* strains and, after 3 to 4 weeks, acetylene reduction activity was measured (Table 4). When pea plants were grown without addition of Mo and S in the plant-growth



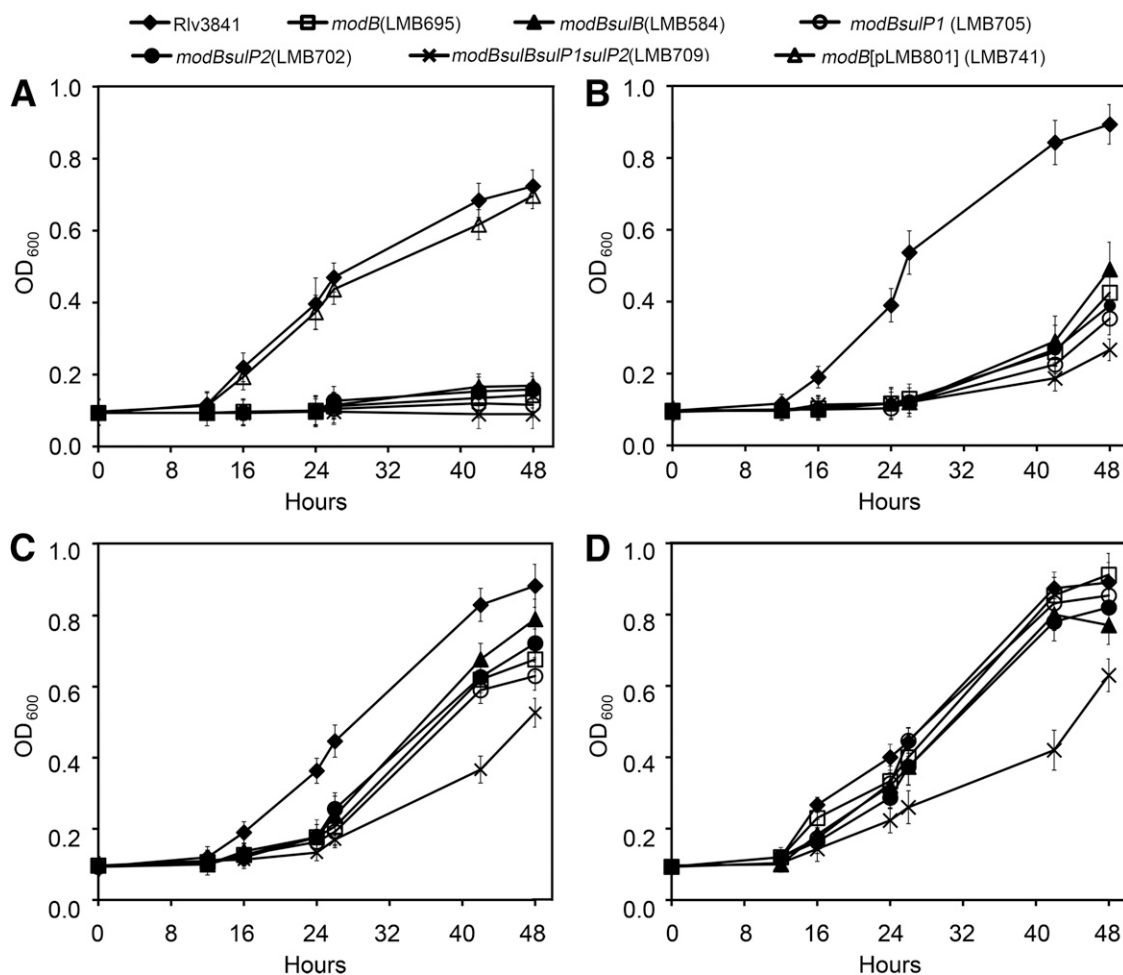
**Fig. 3.** Growth of Rlv3841 and Sul mutants in **A**, 20  $\mu\text{M}$   $\text{Na}_2\text{SO}_4$  (low sulfate) and **B**, 2 mM  $\text{Na}_2\text{SO}_4$  (high sulfate) media. Sulfate was added to sulfate- and molybdenum-free acid minimal salt Glc/ $\text{NH}_4^+$  supplemented with 1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ . Wild-type Rlv3841 was compared with mutants *sulB* (LMB584), *sulP1* (LMB700), *sulP2* (LMB694), and *sulB sulP1 sulP2* (LMB708). Data are from three biological samples  $\pm$  standard error of the mean.

media, acetylene reduction activity was not significantly different in any of the mutant strains from that of Rlv3841 (Table 4). Plants were not discernably different in green color and nodules appeared pink and of similar size in all cases (data not shown). Thus, in the absence of added sulfate and molybdate even the quadruple mutant (LMB709) with all four transporter systems inactivated was not affected in its ability to fix N, as measured by acetylene reduction. These results suggest there must be one or more other systems able to transport trace levels of either sulfate, molybdate, or both in Rlv3841 bacteroids, enabling reduction of acetylene at wild-type levels in pea nodules.

Acetylene reduction activity of plants infected with the mutants was assessed with pea plants grown in the presence of 10 mM sodium sulfate but with no added molybdate (Table 4). The logic was that sulfate at 10 mM would block access of molybdate to sulfate transporters, forcing uptake of molybdate to be by systems specific for this ion. Acetylene reduction by plants inoculated with the single *modB* mutant (LMB695) was only 14.4% of that of pea plants inoculated with wild-type Rlv3841, while a triple *sul* mutant (LMB708) was not affected in acetylene reduction (Table 4), indicating the decrease is due to mutation of *modB* alone. There were also many more small pale nodules compared with the wild type in the *modB* mutant (data not shown). The Mo-dependent phenotypes of the *modB* mutant LMB695 was only apparent in media with a high sulfate

concentration. The addition of 1  $\mu$ M sodium molybdate to pea plants was sufficient to restore acetylene reduction in LMB695 to the wild-type level, even in the presence of 10 mM sodium sulfate (Table 4). Paradoxically, when *modB* was combined with the three combinations of double *sul* mutations, there was no decrease in the rate of acetylene reduction. However, when all three characterized *sul* mutations were combined with *modB* in a quadruple mutant (LMB709), the rate of acetylene reduction was again reduced to 17.3% of wild type. Thus, a strain mutated in *modB* alone (LMB695) or one mutated in *modB* combined with all three characterized *sulB*, *sulP1*, *sulP2* (LMB709) systems had reduced acetylene reduction but not strains with *modB* mutated in combination with partial knockouts in S transport. Since this was unexpected, the experiment was repeated with a similar result (Table 4, final column). This is a complex phenotype that suggests mutating one or more Sul transporters changes either the expression, activity, or both of the remaining Sul transporters, enabling cells to scavenge enough Mo from the medium, even in the absence of ModB. This complex regulatory effect is only overcome once all three characterized Sul transporters are mutated. Presumably because the single *modB* mutation has no effect on the S transport systems, this gives a simple fix reduced phenotype.

Overall, these data reveal the presence of at least one unidentified molybdate transporter, which must also be capable of

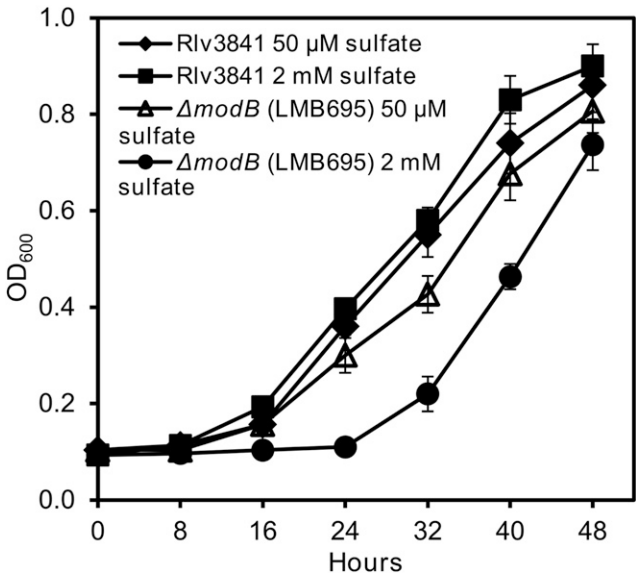


**Fig. 4.** Growth of Rlv3841, ModABC, and ModABC/Sul mutants at different concentrations of molybdate. Strains were grown in sulfate- and molybdenum-free acid minimal salt Glc/NO<sub>3</sub><sup>-</sup> supplemented with 2 mM Na<sub>2</sub>SO<sub>4</sub> and **A**, no added Mo, **B**, 0.25  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, **C**, 1  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, and **D**, 2.5  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>. Wild-type Rlv3841 was compared with mutants *modB* (LMB695), *modB* *sulB* (LMB696), *modB* *sulP1* (LMB706), *modB* *sulP2* (LMB702), and *modB* *sulB* *sulP1* *sulP2* (LMB709) and complemented ModABC mutant *modB*[pLMB801] (LMB741). Data are from three biological samples  $\pm$  standard error of the mean.

sulfate transport. This is apparent because in S- and Mo-free plant-growth medium, the quadruple mutant, in which one or more of these unidentified systems will still be active, reduces acetylene at the same rate as wild-type Rlv3841. However, in the presence of 10 mM sulfate, during which this system is inhibited, *modB* mutants show a large decrease in ability to reduce acetylene. We assume this system transports sulfate, although, formally, the data shows its ability to transport molybdate is inhibited by sulfate. The multiplicity of overlapping sulfate and molybdate transporters explains why bacterial phenotypes lacking the ability to fix N have not been seen for transporters of these ions that are essential for N fixation.

## DISCUSSION

The presence of multiple transport systems for uptake of sulfate and molybdate is likely to mean that the requirement for these anions within the cell is crucially important to members of genus *Rhizobium*. We have seen a similar scenario in the numerous transport systems not only present in the genome of



**Fig. 5.** Growth of Rlv3841 and a ModABC mutant in 50  $\mu\text{M}$   $\text{Na}_2\text{SO}_4$  (low sulfate) and 2 mM  $\text{Na}_2\text{SO}_4$  (high sulfate) media. Sulfate was added to sulfate- and molybdenum (Mo)-free acid minimal salt Glc/ $\text{NO}_3^-$  supplemented with 0.5  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ . Growth of wild-type Rlv3841 was compared with that of *modB* (LMB695). Data are from three biological samples  $\pm$  standard error of the mean.

this organism but also activated in different environments, e.g., the rhizosphere (Ramachandran et al. 2011), and in bacteroids (Karunakaran et al. 2009). This leads to robustness, because an organism doesn't rely totally on a single method of uptake of an important substrate and also confers ability to 'fine tune' transport depending upon the environment. However, this multiplicity of transport systems means that it is difficult to get clear knock-out phenotypes and makes it hard to study even transport systems crucial to the bacteria.

Characterization of mutant strains shows that ABC transporter SulABCD is the main high-affinity system responsible for transport of sulfate in Rlv3841, with SulP1 and SulP2 acting as lower-affinity systems. The strong induction of expression (>300-fold) of this Sul system from Rlv3841 in low-sulfate media, measured by qRT-PCR of *sulB*, is similar to that of the Sul system encoded by SMb21130-SMb21133 in *Sinorhizobium meliloti*. The expression of SMb21133 (encoding the SBP) was examined with a GFP transcriptional fusion and was shown to be induced by sulfate-limitation (Mauchline et al. 2006).

Mutation of ModABC was sufficient to prevent growth on nitrate without the addition of molybdate to the growth medium, indicating that, in wild-type Rlv3841, ModABC is able to scavenge Mo from the medium (Mo is notoriously difficult to totally remove from growth media) (Fig. 4). This growth effect can be seen because nitrate reductase is a molybdoenzyme required for growth of *Rhizobium* species on nitrate (Ferroni et al. 2011). The long lag phase in growth of ModABC mutant at concentrations of molybdate (0.25 to 2.5  $\mu\text{M}$ ) (Fig. 4) can be explained by the observation that mutations in metal transporters often cause long lag phases rather than outright loss of growth in bacterial strains. This is presumably due to slow accumulation of the metal by transporters other than ModABC, enabling a critical intracellular concentration for growth to be reached after a lag. The fact that strain LMB709, the quadruple mutant (mutated in all four Sul and ModABC transport systems), has a longer lag and slower growth at 2.5  $\mu\text{M}$  molybdate (Fig. 4D) is consistent with SulABCD, SulP1, and SulP2 being able to carry out molybdate uptake to some extent. We did not isolate all combinations of Sul transporter mutations to determine their individual contribution to molybdate transport, as the overall effect is modest and they may all contribute to some extent. Furthermore, there must be least one additional way for molybdate to enter the cell to enable the slow growth observed in the quadruple mutant. The idea that the Sul transporters play some part in molybdate uptake in Rlv3841 is substantiated by the observation that growth of *modB* mutant (LMB695) is inhibited by a high concentration of sulfate (Fig. 5), which could be due to sulfate inhibiting molybdate uptake by one or more of the Sul transport systems. A similar situation in which

**Table 2.** Expression of genes involved in uptake of sulfate measured by quantitative reverse transcription-polymerase chain reaction analysis

Strain <sup>y</sup>	Gene expression <sup>z</sup>			
	<i>modB</i>	<i>sulB</i>	<i>sulP1</i>	<i>sulP2</i>
Experiment A				
LMB584 ( <i>sulB</i> )	0.61 $\pm$ 0.21	0.003 $\pm$ 0.00	*4.78 $\pm$ 1.48	*8.69 $\pm$ 3.11
LMB694 ( <i>sulP2</i> )	0.75 $\pm$ 0.10	0.980 $\pm$ 0.42	0.96 $\pm$ 0.05	nd
LMB700 ( <i>sulP1</i> )	0.86 $\pm$ 0.35	0.640 $\pm$ 0.22	nd	0.68 $\pm$ 0.26
LMB701 ( <i>sulP1 sulP2</i> )	0.84 $\pm$ 0.31	*0.280 $\pm$ 0.09	nd	nd
Experiment B				
Rlv3841 (wild-type)	*6.18 $\pm$ 2.79	*313.290 $\pm$ 7.18	*114.71 $\pm$ 69.80	*3.96 $\pm$ 2.34

<sup>y</sup> In experiment A, relative expression of genes in mutant strains were compared with Rlv3841. Bacteria were grown overnight in acid minimal salt (AMS) Glc/ $\text{NH}_4^+$  supplemented with 2 mM  $\text{Na}_2\text{SO}_4$  and 1  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ . In experiment B, relative expression of genes was compared between Rlv3841 grown overnight in AMS Glc/ $\text{NH}_4^+$  supplemented with  $\text{Na}_2\text{SO}_4$  at either 100  $\mu\text{M}$  (low sulfate) or 2 mM (high sulfate).

<sup>z</sup> In experiment A, asterisks (\*) indicate gene expression was at least twofold different from Rlv3841. In experiment B, asterisks (\*) indicate expression was at least twofold greater when grown under low compared with high sulfate ( $P \leq 0.05$ ). Values are given  $\pm$  standard error of the mean. nd = not determined.

molybdate can be also taken up by the sulfate transport system has been observed in both *E. coli* (Rosentel et al. 1995) and *Bradyrhizobium japonicum* (Delgado et al. 2006).

The reduction of N to ammonia by bacteroid nitrogenase is dependent on Mo (in the form of molybdate), making molybdate acquisition by bacteroids essential for a functional symbiosis. This is illustrated, in this work, when pea plants were grown in media with a high concentration of sulfate, and bacteroids with a mutation in *modB* reduced acetylene at only 14.3% of the wild-type rate (Table 4). This highlights the overlap between sulfate and molybdate transport because, in low-sulfate medium, *modB* mutants fixed N at wild-type rates, showing they could acquire sufficient molybdate, presumably by a system that also transports sulfate. There is also likely to be complex regulation of S transporters, since a partial inactivation of S transport in a *modB* background restored acetylene reduction to wild-type rates. This suggests mutation of one or two of the *sul* transporters causes a compensation by one or more remaining transporters, resulting in an increased steady state accumulation of molybdate. The effect can be very subtle, because only trace amounts of molybdate need to be obtained by bacteroids. However, the effect appears real, as it could be repeated, and once all three characterized *sul* transporters were mutated in a *modB* background, acetylene reduction was once again severely inhibited (Table 4). Finally, the effects on growth

and acetylene reduction in the quadruple mutant (*modB sulB sulP1 sulP2*) demonstrates that there must be yet another system (or systems) that is able to transport both molybdate and sulfate. This system cannot be capable of high-affinity molybdate uptake, because mutation of *modB* alone is capable of preventing growth on  $\text{NO}_3^-$  as a sole N source on Mo-free media. From these data, we conclude that molybdate transport is essential for N fixation in low molybdate growth conditions, particularly when sulfate is abundant.

## MATERIALS AND METHODS

### Bacterial growth and media.

The strains and plasmids used in this study are listed in Table 1. *Rhizobium* strains were grown at 28°C in either tryptone yeast extract (Beringer 1974) or AMS (Poole et al. 1994) with D-glucose (10 mM) as a carbon source and  $\text{NH}_4\text{Cl}$  (10 mM) or  $\text{NaNO}_3$  (10 mM) as a nitrogen source (referred to as AMS Glc/ $\text{NH}_4^+$  and AMS Glc/ $\text{NO}_3^-$ , respectively). For growth and qRT-PCR experiments, cells were grown in S- and Mo-free medium based on AMS, in which all sulfate salts have been replaced with chlorides, and phosphate buffer (10 mM) was used instead of MOPS. When required,  $\text{Na}_2\text{SO}_4$  and  $\text{Na}_2\text{MoO}_4$  were added to the concentration stated. Antibiotics were used at the following concentrations ( $\mu\text{g/ml}$ ): ampicillin, 50; apramycin, 50; gentamicin, 20; kanamycin, 20; neomycin, 80; spectinomycin, 100; streptomycin, 500; and tetracycline, 5. Strains were grown at 28°C with shaking (200 rpm) for liquid media. To monitor culture growth, optical density at 600 nm ( $\text{OD}_{600}$ ) measured on three independent cultures.

### Construction of strains with mutations in genes encoding sulfate and molybdate transporters.

In order to produce a series of stable mutants in the sulfate and molybdate transporters, which could be combined to give strains with two, three, and four mutated transporters, genes were interrupted by insertion of an antibiotic resistance gene. In each case, the mutation of a gene in *Rhizobium* species was checked by PCR, with mapping primers designed to regions up- and downstream of the position of antibiotic resistance insertion. Construction of the strains mutated in a single gene is initially described (Fig. 1), followed by the methods by which the mutations were combined.

To mutate *sulA*, primers pr1382 and pr1383 were used to PCR-amplify *sulA* (pRL110374) from Rlv3841 genomic DNA, as described previously (Karunakaran et al. 2009), and the 3.0-kb product was cloned into linearized vector pJET1.2/blunt (Thermo Fisher Scientific, Hemel Hempstead, U.K.), according to the manufacturer's instructions, giving plasmid pLMB670. A

**Table 3.** Expression of genes involved in molybdate and sulfate uptake<sup>x</sup>

Gene	Average fold change in expression <sup>y</sup>		Bacteroids <sup>z</sup>
	Early log phase	Late log phase	
<i>modA</i>	Nd	Nd	**2.85
<i>modB</i>	*3.04 ± 1.21	*7.51 ± 4.66	1.78
<i>sulB</i>	1.74 ± 1.44	1.12 ± 0.22	0.25
<i>sulP1</i>	0.76 ± 0.47	1.23 ± 0.39	1.80
<i>sulP2</i>	0.67 ± 0.49	1.10 ± 0.16	0.83

<sup>x</sup> Measured by quantitative reverse transcription-polymerase chain reaction analysis of Rlv3841 grown in molybdate-free and molybdate containing media and in pea bacteroids. In the microarray experiments, all media were high in sulfate and molybdenum. Bacteria were grown in sulfate and molybdenum-free acid minimal salt Glc/ $\text{NO}_3^-$  supplemented with 2 mM  $\text{Na}_2\text{SO}_4$  and containing either no added molybdate or 10  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ .

<sup>y</sup> As compared with molybdate-containing media. Values are given ± standard error of the mean. One asterisk (\*) indicates gene expression was at least twofold higher in media with no added molybdate compared with that supplemented with 10  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , with *P* value ≤ 0.05. Nd = not determined.

<sup>z</sup> From microarray data on 21-day pea bacteroids (Karunakaran et al. 2009). Two asterisks (\*\*) indicate expression was at least twofold higher in bacteroids than in free-living Rlv3841 (Karunakaran et al. 2009), with *P* value < 0.05.

**Table 4.** Symbiotic behavior of *Rhizobium leguminosarum mod* and *sul* mutants measured by acetylene reduction

Strain	Genotype	Micromoles acetylene per plant per hour <sup>y</sup>			
		Mo-free S-free	Mo-free 10 mM $\text{Na}_2\text{SO}_4$ <sup>z</sup>	1 $\mu\text{M}$ $\text{Na}_2\text{MoO}_4$ 10 mM $\text{Na}_2\text{SO}_4$	Mo-free 10 mM $\text{Na}_2\text{SO}_4$ <sup>z</sup>
Rlv3841	Wild-type	2.33 ± 0.03a	3.69 ± 0.59a	3.52 ± 0.44a	4.15 ± 0.17a
LMB584	<i>sulB</i>	2.48 ± 0.05a	3.40 ± 0.15a	3.33 ± 0.28a	3.62 ± 0.15a
LMB695	<i>modB</i>	2.50 ± 0.22a	0.57 ± 0.12b	3.08 ± 0.15a	1.69 ± 0.23b
LMB696	<i>modB sulB</i>	2.22 ± 0.12a	3.56 ± 0.41a	2.69 ± 0.32a	3.76 ± 0.10a
LMB699	<i>modB sulB sulP1</i>	2.19 ± 0.31a	3.63 ± 0.42a	2.85 ± 0.28a	3.64 ± 0.32a
LMB697	<i>modB sulB sulP2</i>	2.03 ± 0.12a	3.03 ± 0.50a	3.33 ± 0.30a	3.55 ± 0.48a
LMB707	<i>modB sulP1 sulP2</i>	2.38 ± 0.29a	3.18 ± 0.15a	2.85 ± 0.28a	3.60 ± 0.09a
LMB708	<i>sulB sulP1 sulP2</i>	2.34 ± 0.28a	3.64 ± 0.40a	3.25 ± 0.20a	3.70 ± 0.36a
LMB709	<i>modB sulB sulP1 sulP2</i>	2.01 ± 0.29a	0.64 ± 0.66b	2.76 ± 0.13a	2.15 ± 0.17b

<sup>y</sup> Data are averages of five independent plants ± standard error of the mean. Mo = molybdenum; S = sulfur. Letters a and b indicate whether acetylene reduction rates were significantly different from wild-type grown under the same conditions, with *P* value < 0.01.

<sup>z</sup> These two experiments are independent repeats. Uninoculated control points were not nodulated and did not reduce acetylene.



282-bp deletion was introduced into *sulA* by inverse PCR with primers pr1384 and pr1385, using pLMB670 as template. This PCR product was cut with *EcoRI* and was religated to give plasmid pLMB680. The kanamycin-resistance cassette from *EcoRI*-digested pHP45 $\Omega$ Km (Fellay et al. 1987) was cloned into the unique *EcoRI* site of pLMB680, producing pLMB681. The *XbaI/XhoI* fragment from pLMB681 was cloned into pJQ200SK to create pLMB682. Plasmid pLMB682 was conjugated into strain Rlv3841, and a kanamycin-resistant  $\Delta$ *sulA* mutant (LMB595) was isolated by selecting for recombination, using the *sac* mutagenesis strategy, as previously described (Kumar et al. 2005). The insertion was mapped by PCR, using primers pr1386 and pr1387 together with pOTfarforward.

To mutate *sulB*, primers pr1388 and pr1389 were used to PCR-amplify the *sulB* (pRL110373) region from Rlv3841 genomic DNA. The 2.8-kb product was cloned into pJET1.2/blunt, giving plasmid pLMB671. The spectinomycin-resistance cassette from pHP45 $\Omega$ Sp (Fellay et al. 1987) was cloned into the unique *FspAI* site of pLMB671, which is within the *sulB* gene, to produce pLMB674. The *BglII* fragment from pLMB674 was cloned into *BamHI*-digested pJQ200SK to create pLMB675. Plasmid pLMB675 was conjugated into strain Rlv3841, to produce a spectinomycin-resistant *sulB* mutant (LMB584), as previously described (Kumar et al. 2005). This insertion was mapped by PCR, using primers pr1390 and pr1391 together with pOTfarforward.

To mutate *modB*, primers pr1471 and pr1472 were used to PCR-amplify *modB* (RL4686) from Rlv3841 genomic DNA, and the 2.7-kb PCR product was cloned into pJET1.2/blunt, giving plasmid pLMB754. The tetracycline-resistance cassette from pHP45 $\Omega$ Tet (Fellay et al. 1987) was cloned by digesting pLMB754 with *EcoRI* (there are two sites approximately 70 bp apart that lie within *modB*) and ligating, to produce pLMB755. The *BglII* fragment from pLMB755 was cloned into *BamHI*-digested pJQ200SK to create pLMB756. Plasmid pLMB756 was conjugated into strain Rlv3841 and LMB584 (*sulB*) to produce tetracycline-resistant *modB* mutants LMB695 (*modB*) and double mutant LMB696 (*modB sulB*), as previously described (Kumar et al. 2005). This insertion was mapped by PCR, using primers pr1473 and pr1474 together with pOTfarforward.

To mutate *sulP1*, primers pr1463 and pr1464 were used to PCR-amplify *sulP1* (RL2866) from Rlv3841 genomic DNA, and the 3.7-kb product was cloned into pJET1.2/blunt, giving plasmid pLMB734. Plasmid pHP45 $\Omega$ Km (Fellay et al. 1987) was digested with *EcoRI*, and the ends were filled to clone the kanamycin-resistance cassette into the unique *SmaI* site (within *sulP1*) of pLMB734 to produce pLMB784. The *XhoI* fragment from pLMB784 was cloned into *XhoI*-digested pJQ200SK to form pLMB785. Plasmid pLMB785 was conjugated into strains Rlv3841, LMB584 (*sulB*), and LMB696 (*modB sulB*) to produce kanamycin-resistant *sulP1* mutants LMB700 (*sulP1*), double mutant LMB705 (*sulB sulP1*), and triple mutant LMB699 (*modB sulB sulP1*), respectively, as previously described (Kumar et al. 2005). This insertion was mapped by PCR, using primers pr1465 and pr1466 together with pOTfarforward.

To mutate *sulP2*, primers pr1467 and pr1468 were used to PCR-amplify *sulP2* (RL2944) from Rlv3841 genomic DNA, and the 3.5-kb product was cloned into pJET1.2/blunt, giving plasmid pLMB751. The 0.9-kb *XmnI/SmaI* fragment encoding apramycin resistance from pIJ773 (Gust et al. 2003) was cloned into the unique *AfeI* site of pLMB751, which is within the *sulP2* gene, to produce pLMB752. The *NotI* fragment from pLMB752 was cloned into pJQ200Sp to form pLMB778. Plasmid pLMB778 was conjugated into strain Rlv3841 to produce apramycin-resistant *sulP2* mutant LMB694, as previously described (Kumar et al. 2005). This insertion was mapped by PCR, using primers pr1504 and pr1505 together with primers Apr-for and Apr-rev.

Following generation of strains with a single mutation, these mutations were then combined to construct double, triple, and quadruple mutants, as described below. To introduce the apramycin resistance-marked *sulP2::Apr* mutation into strains to make further mutants, the general transducing phage RL38 was used to lyse strain LMB694 (*sulP2::Apr*). Using transduction (Buchanan-Wollaston, 1979) of apramycin resistance to LMB695 (*modB*), strain LMB702 (*modB sulP2*) was generated. In a similar way, transduction of double mutant LMB696 (*modB sulB*) was used to generate LMB697 (*modB sulB sulP2*) and transduction of triple mutant LMB699 (*modB sulB sulP1*), to generate LMB709 (*modB sulB sulP1 sulP2*) (Table 1).

In order to introduce the spectinomycin resistance-marked *sulB:: $\Omega$ Spc* mutation into strains to make further mutants, the general transducing phage RL38 was used to lyse strain LMB584 (*sulB:: $\Omega$ Spc*). Transduction of spectinomycin resistance to LMB694 (*sulP2*) was used to generate strain LMB698 (*sulB sulP2*). In a similar way, transduction of LMB700 (*sulP1*) was used to generate LMB705 (*sulB sulP1*) and transduction of double mutant LMB701 (*sulP1 sulP2*) was used to generate triple mutant LMB708 (*sulB sulP1 sulP2*).

To introduce the tetracycline-marked *modB:: $\Omega$ Tet* mutation into strains, the general transducing phage RL38 was used to lyse strain LMB695 (*modB:: $\Omega$ Tet*). Selecting for tetracycline-resistant transductants of LMB700 (*sulP2*) generated double mutant LMB706 (*modB sulP1*), and transduction of LMB701 (*sulP1 sulP2*) generated triple mutant LMB707 (*modB sulP1 sulP2*) (Table 1).

#### Complementation of ModABC and Sul mutants.

To complement the ModABC mutant, primers pr1605 and pr1606 were used to amplify the whole *modABC* operon from Rlv3841 (Fig. 1). The 3.5-kb PCR product was digested with *XbaI/KpnI* and was cloned into *XbaI/KpnI*-digested pJP2Spc, resulting in plasmid pLMB801. Plasmid pLMB801 was conjugated into LMB695 (*modB*) strain using pRK2013 as helper plasmid. Using selection for spectinomycin resistance, complemented strain LMB741 was isolated according to the method previously described (Figurski and Helinski 1979).

To complement Sul mutants, primers pr1459/pr1460 were used to amplify *sulABCD* genes from Rlv3841 (Fig. 1). The 4.2-kb PCR product was digested with *XbaI* and was then cloned into *XbaI*-digested pRK415-1 (a broad-host range plasmid, stably maintained in *Rhizobium* spp.), resulting in plasmid pLMB731. Plasmid pLMB731 was conjugated into LMB584 (*sulB*) to make complemented strain LMB631 and was conjugated into LMB595 ( $\Delta$ *sulA*) to make complemented strain LMB633 by selection for tetracycline resistance.

#### RNA isolation and qRT-PCR analysis.

Transcripts of *mod* and *sul* genes were analyzed using qRT-PCR. RNA was prepared from Rlv3841 and its mutant derivatives by extracting RNA from three independent cultures. Bacterial cells were grown aerobically in S- and Mo-free AMS Glc/NO<sub>3</sub><sup>-</sup> or AMS Glc/NH<sub>4</sub><sup>+</sup>, supplemented, if required, with either sulfate (100  $\mu$ M or 2 mM Na<sub>2</sub>SO<sub>4</sub>), molybdate (1  $\mu$ M or 10  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>), or both. Cultures were grown overnight or were harvested at early (OD<sub>600</sub> = 0.3) or late (OD<sub>600</sub> = 0.7) log phase.

Bacterial cultures (12 ml) were added directly to 24 ml pf RNAlater (Ambion, Hemel Hempstead, U.K.) and were harvested by centrifugation at 4°C (Karunakaran et al. 2009). RNA was treated twice with DNase (TURBO DNA-free; Ambion). cDNA was synthesized using SuperScript II reverse transcriptase and random hexamers (Invitrogen, Hemel Hempstead, U.K.). Quantification of cDNA was carried out using the SensiMix SYBR No-ROX kit (Bioline, London), and real-time amplification of the PCR products was performed on samples in



triplicate, using the CFX real-time PCR system (Bio-Rad, Hemel Hempstead, U.K.), as described by Mulley et al. (2011). Primers for *modB*, *sulB*, *sulP1*, *sulP2*, and *gyrB1* qRT-PCR are detailed in Supplementary Table S1. PCR consisted of an initial incubation step for 3 min at 95°C, followed by 35 cycles for 5 s at 95°C, 10 s at 62°C, and 5 s at 72°C. Housekeeping gene *gyrB1* (RL0012) was used as a calibrator gene, and results were analyzed as previously described (Prell et al. 2009). Statistical analysis of data sets was performed using REST (Pfaffl et al. 2002).

### Plant experiments and acetylene reduction.

Seeds of *Pisum sativum* cv. Avola were surface-sterilized, were placed in 1-liter pots containing sterile vermiculite, and were watered with sterile S-, Mo-, and N-free rooting solution (1 mM CaCl<sub>2</sub>·2H<sub>2</sub>O, 100 μM KCl, 800 μM MgCl<sub>2</sub>·7H<sub>2</sub>O, 10 μM Fe EDTA, 35 μM H<sub>3</sub>BO<sub>3</sub>, 9 μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 0.8 μM ZnCl<sub>2</sub>, 0.3 μM CuCl<sub>2</sub>·5H<sub>2</sub>O, 7.2 mM KH<sub>2</sub>PO<sub>4</sub>, and 7.2 mM Na<sub>2</sub>HPO<sub>4</sub>). When required, 10 mM Na<sub>2</sub>SO<sub>4</sub> and 1 μM Na<sub>2</sub>MoO<sub>4</sub> were added to the rooting solution. Inoculated plants were incubated in a controlled-environment chamber and were harvested at 3 to 4 weeks. Acetylene reduction was determined for five independent plants in each case, as previously described (Allaway et al. 2000).

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