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The twin-arginine translocation (Tat) system is essential for *Rhizobium*–legume symbiosis

Stefania Meloni,[1] Luis Rey,[1] Stephan Sidler,[1]
Juan Imperial,[2] Tomás Ruiz-Argüeso[1] and
José M. Palacios[1]*
[1]*Laboratorio de Microbiología, Departamento de Biotecnología, Escuela Técnica Superior de Ingenieros Agrónomos, Universidad Politécnica de Madrid, and*
[2]*Consejo Superior de Investigaciones Científicas, Ciudad Universitaria s/n, 28040 Madrid, Spain.*

Summary

The Tat (twin-arginine translocation) system mediates export of periplasmic proteins in folded conformation. Proteins transported via Tat contain a characteristic twin-arginine motif in their signal peptide. Genetic determinants (*tatABC* genes) of the Tat system from *Rhizobium leguminosarum* bv. viciae were cloned and characterized, and a *tatBC* deletion mutant was constructed. The mutant lacked the ability for membrane targeting of hydrogenase, a known Tat substrate, and was impaired in hydrogenase activity. Interestingly, in the absence of a functional Tat system, only small, white nodules unable to fix nitrogen were induced in symbiosis with pea plants. Analysis of nodule structure and location of green fluorescent protein (GFP)-tagged bacteria within nodules indicated that the symbiotic process was blocked in the *tat* mutant at a stage previous to bacteria release into cortical cells. The *R. leguminosarum* Tat-deficient mutant lacked a functional cytochrome *bc*$_1$ complex. This was consistent with the fact that *R. leguminosarum* Rieske protein, a key component of the symbiosis-essential cytochrome *bc*$_1$ complex, contained a typical twin-arginine signal peptide. However, comparative analyses of nodule structure indicated that nodule development in the *tat* mutant was arrested at an earlier step than in a cytochrome *bc*$_1$ mutant. These data indicate that the Tat pathway is also critical for proteins relevant to the initial stages of the symbiotic process.

Accepted 10 February, 2003. *For correspondence. E-mail jpalacios@bit.etsia.upm.es; Tel. (+34) 91 336 5759; Fax (+34) 91 336 5757.

Introduction

Protein translocation across the bacterial membrane can proceed through a number of different routes. The best known pathway, designated general secretory (Sec) pathway, transports proteins through the cytoplasmic membrane by a threading mechanism in which proteins are maintained in extended conformation (Pugsley, 1993). However, some periplasmic proteins acquire partial or full conformation in the cytoplasm by binding cofactors (usually metal cofactors) in this cell compartment. Translocation of such proteins cannot proceed through the Sec pathway. A recently described Sec-independent pathway mediates the export of proteins in folded conformation (for reviews, see Berks *et al*., 2000; Robinson, 2000). This alternative pathway has been designated the twin-arginine translocation (Tat) system because of the characteristic twin-arginine motif (S/T-R-R-x-F-L-K) present in the signal peptide of the proteins translocated via this system. A similar pathway, called ΔpH to highlight its energy source, was identified previously in maize chloroplast, where it mediates the translocation of components of the photosynthetic apparatus (Voelker and Barkan, 1995; Settles *et al*., 1997). In bacteria, the proteins translocated via the Tat system include periplasmic proteins with cytoplasmic insertion of cofactors such as iron sulphur clusters, molybdopterin, polynuclear copper or FAD (Berks, 1996). Additionally, Tat-dependent translocation of non-cofactor-binding periplasmic proteins has been demonstrated (Stanley *et al*., 2000).

Most of the knowledge available on the Tat translocation pathway has been obtained with *Escherichia coli*. In this bacterium, four proteins are involved in the Tat translocon, namely TatA/TatE, TatB and TatC. TatA and TatE, encoded in different parts of the chromosome, are sequence-related proteins homologous to the Hcf106 component of the maize thylakoid ΔpH import pathway and are partially interchangeable *in vivo* (Sargent *et al*., 1998). Both proteins have a single transmembrane segment and an amphipathic helix probably located in the cytoplasm. TatB has a predicted structure similar to TatA, i.e. a single membrane-spanning domain followed by a potential amphipathic helical region, and is able to interact with TatC in a 1:1 ratio (Bolhuis *et al*., 2000). TatC is an integral membrane protein, initially predicted to have six transmembrane domains (Sargent *et al*., 1998). However, recent experimental evidence indicates that this protein has four transmembrane domains (Gouffi *et al*., 2002). A definitive model for the mechanism of Tat-dependent protein translocation has not yet been elucidated. The analysis of Tat complexes isolated from *E. coli* membranes indicated the presence of two types of large complexes: one complex consisting essentially of TatBC with small quantities of TatA, and the other complex containing TatA with small amounts of TatB (Bolhuis *et al*., 2001; de Leeuw *et al*., 2002). It has been demonstrated that the TatBC complex is able to bind proteins harbouring twin-arginine signal peptides (de Leeuw *et al*., 2002).

The analysis of sequenced genomes reveals that the Tat system is present in most bacteria, although it is absent in intracellular parasites and in bacteria with a solely fermentative metabolism (Berks *et al*., 2000). In *E. coli*, this system is essential only under certain growth conditions, particularly under those conditions involving the use of alternative electron acceptors such as dimethyl sulphoxide (DMSO) or trimethylamine oxide (TMAO) or the use of hydrogen as energy source (Sargent *et al*., 1998). In all these cases, periplasmic or membrane-bound proteins carrying the characteristic twin-arginine signal peptide have been identified as part of the corresponding redox complex. *E. coli* [NiFe] hydrogenase 2, a membrane-bound dimeric enzyme with a Tat-like signal peptide in the small subunit precursor, has been used as a model enzyme for the study of Tat-dependent translocation (Wu *et al*., 2000). For this enzyme, both subunits are required for Tat-dependent translocation to the periplasmic side of the membrane (Rodrigue *et al*., 1999).

Rhizobium leguminosarum bv. viciae is an alpha-proteobacterium able to establish a N_2-fixing symbiosis in root nodules of different legume plants such as peas and vetches. Development of the legume nodule results from a complex interaction between plant and bacteria in which both partners undergo dramatic changes (Schultze and Kondorosi, 1998). Differentiation of bacterial cells into bacteroids, the symbiotic form of the bacteria, involves significant changes affecting different structures in the cell (Oke and Long, 1999). The respiratory electron transport chain is modified for bacteroids to adapt to the microoxic conditions established within the nodule to allow nitrogenase activity. One of the key components of the *R. leguminosarum* respiratory chain is the cytochrome bc_1 complex, a branching point in the electron transfer pathway from the quinone pool to either the general aerobic oxidase aa_3 or the symbiosis-specific high-affinity terminal oxidase cbb_3 via *c*-type cytochromes (Delgado *et al*., 1998). Cytochrome bc_1 complex contains an Fe-S (Rieske) protein along with cytochromes of the *b* and *c* type (Berry *et al*., 2000). This complex is essential for symbiotic nitrogen fixation by *Bradyrhizobium japonicum* (Thony-Meyer *et al*., 1989) and *R. leguminosarum* (Wu *et al*., 1996).

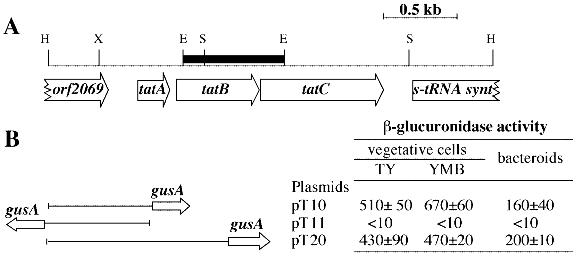
In this work, we analyse the *R. leguminosarum* bv. viciae Tat system and demonstrate that this system is essential for symbiotic nitrogen fixation, hydrogenase activity and cytochrome *c*-dependent respiration.

Results

Cloning and characterization of the tat *gene cluster from* R. leguminosarum *bv. viciae*

An internal fragment of the *tatC* gene from *R. leguminosarum* bv. viciae was obtained by polymerase chain reaction (PCR) amplification using the degenerate primers TAT1 (5′-CAYCTSAYTGARCTSCGYAM-3′) and TAT2 (5′-GARAASACRTCCGGCGGSGT-3′), designed from conserved regions of *tatC* genes from different bacteria, and genomic DNA from *R. leguminosarum* UPM791 as template. Sequencing of the 0.6 kb PCR product obtained showed that this fragment was in fact highly similar to *E. coli tatC* (data not shown). This DNA fragment was used as probe in a colony hybridization screening of a pLAFR1-based cosmid gene library of *R. leguminosarum* UPM791 (Leyva *et al*., 1987). Out of ≈2000 clones analysed, two colonies gave a positive signal. When Southern blots of *Eco*RI-digested DNA of the corresponding cosmids were hybridized to the probe cited above, two *Eco*RI fragments of ≈0.7 kb and 4 kb were identified in both cosmids. Fragments of the same size were identified with this probe in the *Eco*RI-digested genomic DNA from *R. leguminosarum* UPM791. After analysis with different restriction enzymes, an ≈2.9 kb *Hin*dIII fragment that contained this region of homology was subcloned in pBluescript and sequenced.

Nucleotide sequence determination of a region of 2700 bp DNA allowed the identification of three complete open reading frames (*orf1* to *orf3*) with high probability of encoding *R. leguminosarum* proteins. The deduced gene products from these ORFs showed significant similarity to Tat proteins from *E. coli* and other bacteria, so we designated them *R. leguminosarum tatA*, *tatB* and *tatC* genes respectively (Fig. 1). *R. leguminosarum tatA* encoded a protein of 63 amino acid residues showing 49.2% identity to *E. coli* TatA. Structure prediction of the deduced gene product indicated the presence of a single transmembrane domain in this protein (amino acid residues 4–21). The second gene in the cluster, *tatB*, encoded a gene product of 203 amino acid residues. This potential protein was 28% identical to *E. coli* TatB and also contained a single transmembrane domain (residues 4–17). The predicted gene product from *R. leguminosarum tatC* had 275 amino acid residues and showed 30% positional identity to *E. coli* TatC. Structural analysis predicted that TatC is an integral membrane protein with six potential transmem-



Plasmids	β-glucuronidase activity		
	vegetative cells		bacteroids
	TY	YMB	
pT10	510± 50	670±60	160±40
pT11	<10	<10	<10
pT20	430±90	470±20	200±10

Fig. 1. Genetic map and expression of *tat* genes from *R. leguminosarum*.
A. Physical map of the *tat* region. The solid horizontal bar corresponds to the deleted region in *tat* mutants SM61 and SP61. Horizontal arrows indicate the location and direction of transcription of the identified genes. s-tRNA synt, seryl-tRNA synthetase. Restriction site abbreviations: H, *Hin*dIII; X, *Xho*I; E, *Eco*RI; S, *Sal*I.
B. Analysis of *tat* gene expression. Horizontal lines indicate the DNA fragments cloned in front of the *gusA* reporter gene from plasmid pJP2. The table contains the values of GUS activity (Miller units) induced by each fusion in cells grown in either YMB or TY (Beringer, 1974) media and in pea bacteroids. Values correspond to the average of three independent assays ± SE.

brane helices and a cytoplasmic location for the protein N-terminus. Finally, 199 bp downstream from *tatC*, we identified the 5′ end of a potential ORF with high similarity to seryl-tRNA synthetases from different bacteria.

All three *tatABC* genes were closely linked: *tatB* and *tatC* overlapped by four nucleotides, whereas there was a 51 bp intergenic space between *tatA* and *tatB*. Upstream from *tatA*, we identified an inverted repeat sequence that may interrupt the transcription from an operon located upstream. Also, a palindromic sequence (aagcgc-N7-gcgctt) that may give raise to a stable RNA secondary structure was detected in the *tatA*–*tatB* intergenic region. This structure might affect transcription of downstream genes (*tatB* and *tatC*). In order to analyse the expression of *R. leguminosarum tat* genes, we constructed reporter gene fusion plasmids pT10 and pT20, containing *tatA* and *tatB* genes fused to β-glucuronidase respectively (see *Experimental procedures* and Fig. 1). The analysis of β-glucuronidase activity associated with these gene fusions in *R. leguminosarum* indicated that both pT10 or pT20 induced similar levels of GUS activity (≈ 450 Miller units) in vegetative cells, with no significant effect of growth media (TY or YMB) on the level of expression. When activity was measured in symbiotic cells, again similar levels of reporter activity were induced by both plasmids, although they were significantly lower (30–50%) than those in vegetative cells. Taken together, these data indicate constitutive expression of the Tat system and similar levels of transcription for *tatA* and *tatB* genes.

A functional Tat system is required for hydrogenase translocation and activity in R. leguminosarum *bv. viciae*

The analysis of the *R. leguminosarum* hydrogenase small subunit sequence (Hidalgo *et al*., 1990) revealed the presence of a Tat-type signal peptide, so we decided to test the effect of the Tat system first on hydrogenase activity and localization. To this end, an *R. leguminosarum* Tat-deficient mutant was constructed by substitution of a DNA fragment containing the 3′ end of *tatB* and the 5′ end of *tatC* by a spectinomycin resistance cassette (Fig. 1; for details, see *Experimental procedures*). In *R. leguminosarum* bv. viciae UPM791, hydrogenase activity is induced only in bacteroids (Brito *et al*., 1997). As the *tat* mutation affected the symbiotic interaction (see below), the effect of Tat on hydrogenase activity was determined using strain SP61, a Tat-deficient mutant derived from a modified *R. leguminosarum* strain (SPF25) that expresses hydrogenase activity in vegetative cells exposed to microoxic conditions (Brito *et al*., 2002). In order to increase the sensitivity of the assay, additional copies of the hydrogenase genes were added by introducing cosmid pALPF1. In this experiment, microaerobic cultures of the wild-type strain SPF25(pALPF1) exhibited high levels of uptake hydrogenase activity when using physiological (oxygen) or artificial (methylene blue) electron acceptors (Table 1). In contrast, no detectable levels of activity were found in the corresponding Tat-deficient mutant when oxygen was used as terminal electron acceptor, whereas low but significant levels of activity were detected in the mutant when we used methylene blue as electron acceptor. In order to analyse further the effect of the Tat system on the cellular distribution of hydrogenase, we determined hydrogenase activity in soluble and membrane fractions from SPF25(pALPF1) and its corresponding Tat-deficient derivative (Table 1). The hydrogenase activity was mainly associated with the membrane fraction in the wild-type strain, whereas no

Table 1. Effect of the *tat* mutation on hydrogenase activity[a] of *R. leguminosarum* whole cells and in soluble and membrane-bound fractions.

	Whole cells		Soluble		Membrane	
Strain	Oxygen	MB	Oxygen	MB	Oxygen	MB
SPF25(pALPF1)	18 400	27 900	240	3700	4900	7400
SP61(pALPF1)	<100	550	<100	<100	<100	<100

[a] Hydrogenase activity was expressed as nmol of H_2 taken up h^{-1} mg protein^{-1} using either oxygen or methylene blue (MB) as terminal electron acceptors. Values are the average of two replicates.

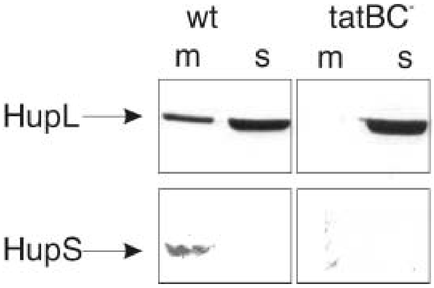


Fig. 2. Requirement of the Tat system for membrane targeting of hydrogenase subunits. Immunoblots of membrane (m) and soluble (s) fractions of *R. leguminosarum* microaerobic cultures resolved by SDS-PAGE (10% acrylamide gels) and revealed with anti-HupL and anti-HupS antisera. Bands corresponding to the mature forms of HupL and HupS are indicated by arrows. Strains: wt, strain SPF25(pALPF1); *tatBC*, SP61(pALPF1).

activity was found in any fraction from the Tat-deficient strain. It has to be noted that cell fractionation resulted in substantial losses of hydrogenase activity in the wild-type strain (Table 1), probably because of instability of the enzyme under aerobic conditions. A similar decrease in activity would lead to undetectable levels in the case of the Tat mutant.

We also analysed the potential effect of the *tat* mutation on membrane localization of the hydrogenase subunits by immunoblot assays using antisera raised against each subunit. In these experiments, we found, for each antiserum, a clear immunoreactive band in the membrane fraction of the wild-type strain (Fig. 2). By comparison with total extracts from a HupL processing-deficient *R. leguminosarum* HupD mutant, it was concluded that the band identified with the anti-HupL antiserum corresponded to the processed form of the large subunit (data not shown). Both HupL- and HupS-specific bands were absent in the membrane fraction of the *tatBC*-deficient mutant. In this mutant, the processed form of the hydrogenase large subunit could be detected in the soluble fraction, indicating that hydrogenase subunit synthesis was not affected by the *tat* mutation. In these experiments, hydrogenase small subunit could not be detected in either wild-type or mutant strain cytoplasms, probably because this subunit is highly unstable when not properly assembled in the membrane (Moshiri and Maier, 1988). We conclude from these results that the Tat system is essential for hydrogenase activity in *R. leguminosarum*, probably affecting translocation of the enzyme into the membrane.

A tatBC *mutation leads to a Fix-deficient phenotype in symbiosis with peas*

In order to assess the relevance of the *R. leguminosarum* Tat system for symbiosis, pea plants inoculated with *R. leguminosarum* strains UPM791 (wild type) and SM61 (*tatBC*) were grown on N-free solution under bacteriologically controlled conditions. After 3 weeks of growth, the plants inoculated with SM61 showed a chlorotic aspect, similar to that corresponding to the uninoculated controls. In contrast, the plants inoculated with UPM791 were green and healthy. Both strains induced nodulation in pea roots, but SM61 induced only small, round, white nodules lacking acetylene reduction activity, whereas strain UPM791 induced pink, normal nodules (Fig. 3A) expressing high levels of acetylene reduction activity (data not shown). After nodule surface sterilization, crushing and plating, colonies of normal appearance were obtained from plants inoculated with UPM791. In this experiment, no colonies could be recovered from extracts of SM61-induced nodules, suggesting that very low numbers of viable bacteria were present in these nodules. Microscopic examination of sections from these nodules revealed the presence of a very low number of infected cells and no infection threads (Fig. 3B). In contrast, nodules induced by wild-type strain UPM791 contained a high proportion of bacteroid-filled infected cells and abundant infection threads. These data suggest that the Tat-deficient mutant was able to elicit nodule formation, although the symbiotic process was arrested at an early stage.

In order to investigate further the potential involvement of Tat-dependent functions in the symbiotic process, we tagged our *R. leguminosarum* strains with green fluorescent protein (GFP). To do this, we used a constitutively expressed *gfp* gene carried by the plasmid pHC60 (Cheng and Walker, 1998). Roots and nodules from pea plants inoculated with the corresponding derivative strains were analysed by confocal microscopy. In both cases, infection threads were apparent in root hairs 11 days after inoculation, and nodule primordia were fluorescent, although the degree of fluorescence was significantly lower in plants inoculated with SM61 (data not shown). In contrast, analysis of sections from mature nodules demonstrated that those induced by SM61 exhibited virtually no fluorescence, whereas nodules induced by the wild-type strain were filled with fluorescent bacteria (Fig. 3B).

Finally, and in order to assess whether the observed Fix⁻ phenotype was a consequence of the deletion of *tat* genes, a complementation assay was carried out by introducing an intact copy of the *tat* gene cluster (pTAT) into strain SM61. This plasmid was able to revert fully the symbiotic phenotype of mutant SM61 (data not shown), indicating that the observed Fix deficiency of the mutant was indeed associated with the mutation in the *tat* genes.

Rhizobium leguminosarum *Tat mutant is deficient in cytochrome c-dependent respiration*

The Fix-deficient phenotype of the *R. leguminosarum tat* mutant prompted us to investigate potential Tat-dependent

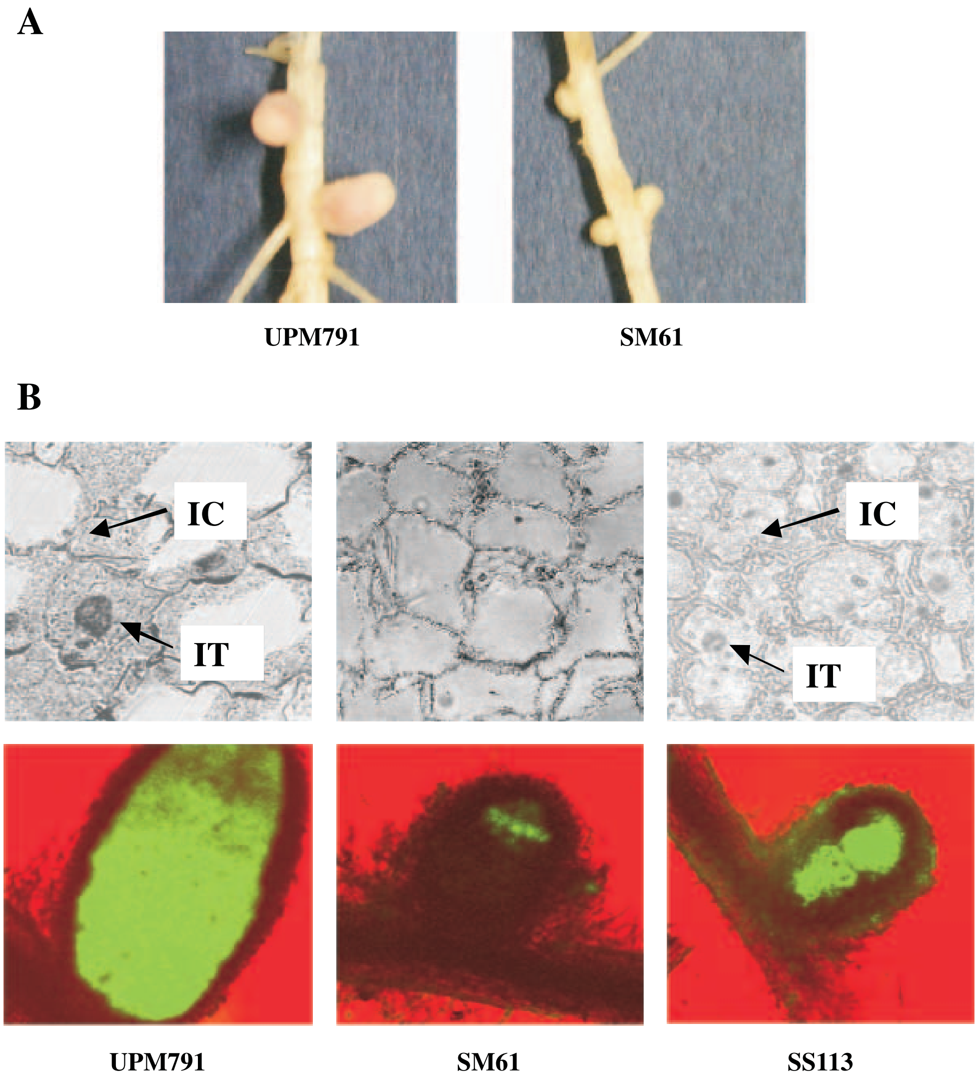


Fig. 3. Requirement of the Tat system for *Rhizobium*–pea symbiosis.
A. Pea nodules in roots 21 days after inoculation with strains UPM791 (wild type) and SM61 (*tatBC* mutant).
B. Analysis of nodule sections. Top, optical micrographs of nodule sections stained with toluidine blue. The presence of infected cells (IC) and infection threads (IT) is evident in strains UPM791 and SS113 (*fbcC* mutant) but not in SM61. Bottom, confocal images overlaying fluorescence plus transmission views of unfixed nodule sections. For this experiment, we used derivative strains carrying a constitutively expressed *gfp* gene in plasmid pHC60.

cellular traits essential for symbiosis. We had identified two extracytoplasmic proteins containing Tat-like signal peptides: hydrogenase small subunit (Hidalgo *et al*., 1990) and the Fe-S component (Rieske protein) of cytochrome *bc*$_1$ complex from *Bradyrhizobium japonicum* (Thony-Meyer *et al*., 1989). *R. leguminosarum* hydrogenase-deficient mutants have a normal Fix phenotype (Leyva *et al*., 1987). In contrast, a functional cytochrome *bc*$_1$ is known to be required for nitrogen fixation in the *B. japonicum*–soybean (Thony-Meyer *et al*., 1989) and *R. leguminosarum*–pea symbioses (Wu *et al*., 1996). A mutant strain (SS113) affected in the gene encoding the cytochrome *c*$_1$ component of the *bc*$_1$ complex from *R. leguminosarum* was obtained previously in our laboratory by insertion of a spectinomycin minitransposon (Sidler, 2002). From the analysis of this mutant, a partial sequence of the *R. leguminosarum* cytochrome *bc*$_1$ operon was obtained. The Rieske protein encoded in this operon contained a typical Tat-dependent signal peptide. A database search revealed the presence of Tat-dependent signal peptides in Rieske proteins from other rhizobia (Fig. 4). To test the effect of the *tat* mutation on cytochrome *bc*$_1$ functionality, we performed an assay for cytochrome *c* oxidase activity (Nadi assay, see *Experimental procedures*) in the wild-type strain UPM791 and in the mutants SM61 and SS113. In this experiment, the strain

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Rl  1  MLAVSEHETTSEGMGEPTRRDFLYLTTGMAGAVGAVAVAWP-IDQVRP
Sm  1     MSEHETSSETMGEPTRRDFLYLATGMAGVVGAGAAAWPFIDQMRP
At  1     MSEHVTNHDASGEPTRRDFLYLVTGMAGAVGAAAVAWPFIDQMRP
Ml  1          MSATDIHDPNRRDFLYVATGMAAVVGAGAVAWPFIDQMRP
Bj  1         MTTASSADHPTRRDFLFVATGAAAAVGGAAALWPFISQMNP
                       * *****   ** *  **  *  ** * *  *
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Fig. 4. Multiple alignment of Rieske protein N-terminal sequences from different Rhizobiaceae. Amino acid residues corresponding to the twin-arginine motif (S/TRRxFLK; Berks, 1996) are in bold. Asterisks indicate amino acid residues conserved in all the sequences. Sequences were aligned using the program CLUSTALW (Thompson *et al.*, 1994). Source for sequences: Sm, *Sinorhizobium meliloti* SMc00187; At, *Agrobacterium tumefaciens* NP_532912; Ml, *Mesorhizobium loti* mll2707; Bj, *Bradyrhizobium japonicum* p51130; and Rl, *Rhizobium leguminosarum*, AAN03339.

Table 2. Cytochrome *c*-dependent respiratory activities of membranes from different *R. leguminosarum* strains.

Strains	Genotype	Ubiquinol-cytochrome *c* reductase[a]	Oxidase[b]
UPM791	Wild type	115	+
SS113	*fbcC*	<5	–
SM61	*tatBC*	<5	–
SM61 (pTAT)	*tatBC*/*tatABC*	ND	+

a. μmol cytochrome *c* reduced min $^{-1}$ mg^{-1} protein.
b. Nadi test. +, blue colour formation in colonies within 20 s.
ND, not determined.

UPM791 gave a positive reaction, whereas the SM61 and SS113 mutants exhibited a negative reaction (Table 2). The functional impairment of the cytochrome bc_1 complex in the mutant strains was confirmed by determining the ubiquinol-cytochrome *c* oxidoreductase activity in membrane fractions. In this analysis, the wild-type strain UPM791 exhibited high levels of activity, whereas no detectable levels were observed in the mutants SM61 or SS113 (Table 2). Taken together, these results clearly indicate that cytochrome bc_1, and hence cytochrome *c*-dependent respiration, are not functional in vegetative cells of the mutant affected in the Tat system.

The deficiency in cytochrome bc_1 functionality could account for the observed symbiotic phenotype of the *tat* mutant. In order to verify this possibility, we analysed the structure of nodules induced by mutant strain SS113 (Fig. 3B). From this analysis, we concluded that, in spite of the bc_1 complex deficiency, SS113 was able to infect the whole central part of the nodule. In contrast, SM61 was virtually absent from it. These results indicate that the *tat* mutant is impaired in other traits, besides cytochrome bc_1 functionality, that are essential for earlier stages of *Rhizobium*–legume interaction.

Analysis of outer membrane integrity in the R. leguminosarum tat *mutant*

One possibility that might explain the symbiotic phenotype of the *tat* mutant could be a pleiotropic effect on the stability of the cell envelope. Pleiotropic defects in the cell envelope have been described in *E. coli tat* mutants. These mutants showed aberrant cell chain formation, probably caused by defects in cell septation, and also increased sensitivity to SDS (Stanley *et al.*, 2001). In order to test this possibility, we analysed cell morphology and SDS sensitivity in *R. leguminosarum* cultures grown in standard YMB medium to mid-log phase. No cell chain formation similar to that described in *E. coli* was detected in strain SM61 (data not shown), whereas SDS sensitivity was essentially unaltered in the same strain (Fig. 5A). Also, analysis of lipopolysaccharide (LPS) profiles in both wild-type and mutant strains showed no significant alterations associated with the Tat mutations (Fig. 5B). Taken together, these results indicate that the suppression of the Tat system does not result in major alterations in the *R.*

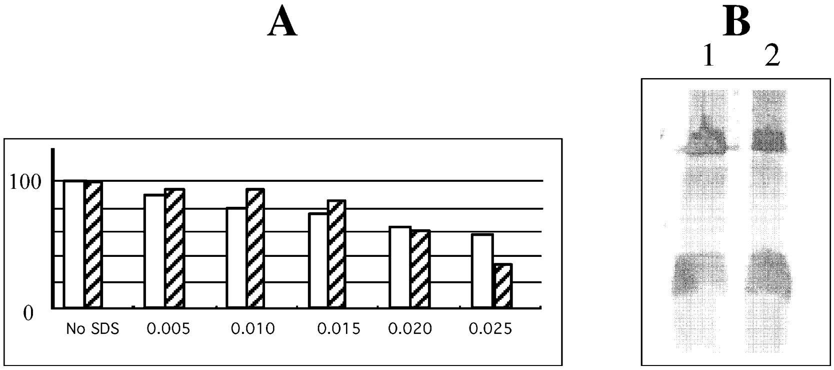

Fig. 5. Effect of Tat on *R. leguminosarum* outer membrane.
A. Analysis of SDS sensitivity. Cells were incubated overnight in YMB containing the indicated percentages of SDS. Growth was expressed in relative units, and a value of 100 was assigned to the final OD_{600} of the corresponding culture without SDS (UPM791, 0.430; SM61, 0.320). Values are the average of two replicates. Strains: UPM791, open bars; SM61, hatched bars.
B. Analysis of LPS fraction. Cell extracts were treated with proteinase K, resolved by SDS-PAGE (18% acrylamide) and silver stained. Lanes: 1, UPM791; 2, SM61.

leguminosarum outer membrane, at least in the conditions tested.

Discussion

In this study, we characterize the *tat* gene cluster from *R. leguminosarum* bv. viciae. Furthermore, we demonstrate for the first time that a functional Tat pathway is essential for biological nitrogen fixation by the *Rhizobium*–legume symbiosis. The Tat-dependent translocation system has mainly been described in *E. coli* (Berks *et al*., 2000). In this bacterium, the requirement for three proteins (TatA, TatB and TatC) has been demonstrated in a functional reconstituted system *in vitro* (Yahr and Wickner, 2001). A putative *tatABC* gene cluster was found in *R. leguminosarum*, and the high degree of positional identity of the predicted gene products with the corresponding *E. coli* components allowed a clear assignment to *tat* genes. The analysis of genome sequences in databases indicated that the region containing *tat* genes from *R. leguminosarum* is highly conserved in the chromosome of other *Rhizobiaceae* such as *Sinorhizobium meliloti* (Galibert *et al*., 2001), *Agrobacterium tumefaciens* (Wood *et al*., 2001) and *Mesorhizobium loti* (Kaneko *et al*., 2000). This conservation applies to the arrangement of *tat* genes, to the presence of significant intergenic space between *tatA* and *tatB* (31–90 nt) and also to the nature of the flanking genes (a gene homologous to *S. meliloti orf2069* upstream and seryl-tRNA synthetase downstream in all species studied). The only variation in gene synteny found in this region was an additional ORF (*orf2068*, between orf2069 and *tatA*) that was present only in *S. meliloti*. These data, and also the lack of *tat*-specific hybridization bands in hybridization experiments with plasmid DNA resolved by Eckhardt-type gels (data not shown), strongly suggest that *R. leguminosarum tat* genes are located in the chromosome. Although the *R. leguminosarum* genome is not available to perform the search for additional components of the Tat system, such as *E. coli* TatE (Sargent *et al*., 1998), the analysis of *S. meliloti* and *A. tumefaciens* genomes did not reveal the presence of TatE-related proteins other than TatA, suggesting that it might be absent in this group of bacteria.

The data obtained with *tat*–*gusA* fusions suggest a constitutive expression of *tat* genes in *R. leguminosarum* vegetative cells. Constitutive expression of *tat* genes has been documented previously in *E. coli*, and it was considered as indicative of the relevant role of the system for aerobic and anaerobic growth of this bacterium (Jack *et al*., 2001). Our results also indicate active expression of the Tat system in endosymbiotic conditions and, along with the data on the symbiotic phenotype of the Tat-deficient mutant, extend the relevance of the system to symbiotic life in rhizobia.

The mutation of the *R. leguminosarum tatBC* genes abolished membrane targeting of the HupS and HupL subunits. Export of the hydrogenase enzyme via Tat has been demonstrated in other bacteria such as *E. coli* (Wu *et al*., 2000), *Ralstonia eutropha* (Bernhard *et al*., 2000) and *Wollinella succinogenes* (Gross *et al*., 1999). In our experiments, the processed form of HupL was clearly detected in the mutant cytoplasm, indicating that the effect of Tat on hydrogenase activity was at the post-translational level as has been described previously for *E. coli* (Sargent *et al*., 1998). The lack of immunological detection of the small subunit, probably mislocated in the cytoplasm, was probably caused by the high instability of this subunit when not properly assembled in the membrane (Moshiri and Maier, 1988). The same reason could also account for the loss of activity in soluble extracts of the *tat* mutant. The effect of the *tat* mutation on the translocation of both enzyme subunits is consistent with the hitchhiker mechanism proposed for the Tat-dependent translocation of the *E. coli* hydrogenase 2 (Rodrigue *et al*., 1999).

There are no previous reports of the symbiotic relevance of protein export systems in *Rhizobiaceae*. Different periplasmic proteins are essential for the symbiotic process. This is the case for CycY, involved in cytochrome *c* synthesis (Fabianek *et al*., 1997). Also, periplasmic components of ABC transport systems, such as the Dct system for the uptake of dicarboxylates, are essential for bacteroid viability (Watson, 1990). However, their corresponding precursor proteins do not contain a Tat-type signal peptide. The first indication of symbiotic metabolic functions that might be defective in mutant SM61 came from an initial search for rhizobial proteins containing Tat-dependent signal peptides. Among others, this search identified the Fe-S component (Rieske protein) of cytochrome bc_1 complex (Fig. 4). It has been demonstrated that, in plant chloroplasts, the Rieske protein is translocated to the thylakoid lumen from the stroma by the phylogenetically related transport pathway designated ΔpH (Molik *et al*., 2001). On the other hand, it has been shown previously that bc_1 complex, as an obligate intermediate in electron transfer to the terminal oxidase cbb_3, is absolutely required for the *R. leguminosarum*–pea (Wu *et al*., 1996) and *B. japonicum*–soybean (Thony-Meyer *et al*., 1989) symbioses. In free-living conditions, the inactivation of genes for the bc_1 complex does not impair growth, probably because of the presence of one or several alternative oxidases. We propose that the *R. leguminosarum* Rieske protein is mislocated in the *tat* mutant SM61, thus leading to an impaired bc_1 complex. This conclusion is supported by two oxidase redox tests. First, ubiquinol-cytochrome *c* reductase activity was undetectable in membranes from SM61. Additionally, colonies of SM61 and SS113 failed to oxidize the Nadi reagent in a standard cytochrome oxidase assay. These data indicate that mutant SM61 has altered cytochrome *c*-dependent respiration, as is the case for the cytochrome c_1-deficient mutant SS113. This defect should lead to a Fix-deficient phenotype. Histological examination of nodules induced by strain SM61 revealed a noticeably aberrant morphology with no infected cells. This phenotype appears to be far more severe than that associated with the cytochrome bc_1-deficient mutant SS113. In the *B. japonicum*–soybean system, nodules induced by a cytochrome bc_1-deficient mutant also contained bacteroids (Thony-Meyer *et al*., 1989). We conclude from our results that the *tat* mutant strain interrupted the symbiotic process before bacteroid release in the cortex, and that the observed phenotype cannot be fully explained by the deficiency in cytochrome bc_1 complex. Thus, other Tat-dependent proteins are likely to be essential for symbiosis. These potential Tat substrates are likely to stay attached to the membrane or in the periplasm, where they will not be able to interact directly with host cells. These proteins could, instead, participate in the synthesis of other cellular components directly involved in this interaction, such as EPS or LPS, although we have not found an effect of Tat on outer membrane integrity in *R. leguminosarum*. However, it has to be noted that the analysis has been performed only in vegetative cells, as we cannot recover bacteroids from the mutant strain. It could be the case that some modifications in the cell envelope required for advanced stages in the infection process were Tat dependent. The analysis of the phenotype associated with the mutants altered in the different Tat substrates will hopefully clarify this point.

We have searched the *S. meliloti* genome for potential Tat substrates using TATFIND software (Rose *et al*., 2002). In this analysis, we have found 94 proteins harbouring potential Tat-like motifs in the N-terminus (Table 3; see also Table S1 in *Supplementary material*). No obvious candidates to explain the observed symbiotic phenotype were identified among these proteins. It is interesting to note the large number of periplasmic solute-binding proteins (22 proteins) likely to be dependent on Tat export. The presence of Tat-like signal sequences in solute-binding components of ABC transporters was reported previously in *Halobacteriaceae* (Bolhuis, 2002; Rose *et al*., 2002), although no experimental proof of their dependency on Tat export has been presented to date. A number of oxidoreductases, including Rieske protein (SMc00187) and periplasmic nitrate reductase NapA (SMa1236), were also identified. Most proteins contained predictable signal peptides of variable length (27–55 amino acid residues) and, for those proteins not showing such signal sequences, at least one transmembrane domain could be predicted, suggesting that they remain attached to the membrane. The genome of *R. leguminosarum* bv. viciae strain 3841 is currently being sequenced (http://www.sanger.ac.uk/Projects/R_leguminosarum) and, although no annotation is available yet, limited BLAST searches with *S. meliloti* potential Tat substrates suggest that, although the same types of proteins are probably dependent on Tat export in *R. leguminosarum*, some variations do occur. For instance, no sequences homologous to periplasmic nitrate reductase components were found in the *R. leguminosarum* database (data not shown).

The differences in the requirement for the Tat system under free-living versus symbiotic conditions suggest the existence of environmental conditions within the nodule requiring specific, Tat-dependent periplasmic or extracellular functions. Our data demonstrate the essentiality of the Tat system for the symbiotic interaction of *R. leguminosarum* with pea plants. Although the role of the Tat system in bacteria has been analysed mainly in free-living conditions, a recent report (Ochsner *et al*., 2002) demonstrated that, in *Pseudomonas aeruginosa*, the twin-arginine translocon is essential for the export of virulence determinants and, hence, for pathogenesis in a rat lung model. We have shown that this Sec-independent trans-

Table 3. Proteins containing Tat-type signal sequences in the *S. meliloti* genome.[a]

Predicted function	Protein designation
Periplasmic solute-binding proteins	SMc04135, SMc00265, SMc04251, SMc01628, SMc01642, SMc01647, SMc01966, SMc03196, SMc03124, SMa0392, SMa1651, SMa0466, SMa1860, SMa2075, SMb20231, SMb20284, SMb20383, SMb20538, SMb21215, SMb21345, SMb20660, SMb20671
Oxidoreductases	SMc02282, SMc01944, SMc04049, SMc03287, SMb21337, SMa0002, SMa1038, SMa1182, SMa1188, SMa1236, SMa1250, SMa1488, SMa1545, SMa2349, SMb20342,SMb20404, SMb21380, SMb21383, SMb21558
Hydrolases	SMc00047, SMc04449, SMc03243, SMb20216, SMb20221
Other functions	SMc04024, SMc00577, SMc00863, SMa0943, SMb20915, SMb20458
Unknown	SMc00187, SMc02243, SMc02385, SMc01184, SMc01459, SMc00738, SMc00817, SMc03964, SMc01779, SMc04137, SMc03063, SMc04218, SMc04167, SMc01724, SMc00116,SMc00814, SMc02177, SMc02316, SMc01769, SMa0025, SMa0308, SMa0380, SMa1507, SMa1793, SMa1811, SMa1819, SMa1927, SMa2129, SMa2299, SMa2361, SMb20127,SMb20320, SMb21329, SMb21544, SMb21551, SMb20881, SMb20910, SMb21492, SMb21516, SMb21521, SMb20703, SMc01281

a. A more detailed version of this table is available in *Supplementary material*.

location system is also essential for interaction with another eukaryotic host.

The relevance of the Tat system is highlighted by its involvement in at least two key biological processes: photosynthesis in plant chloroplasts (Settles *et al*., 1997) and nitrogen fixation in legume nodules (this work). In the case of *Rhizobium*, further investigation is required to identify additional targets for Tat that might result in the unveiling of new functions involved in the establishment of the symbiosis.

Experimental procedures

Chemicals

All enzymes were purchased from Roche and used according to the manufacturer's indications. Media constituents were from Oxoid. α-Naphthol and N,N dimethyl-*p*-phenylenediamine used for the 'Nadi' assay were from Sigma-Aldrich. All other chemicals were of reagent or electrophoresis grade.

Bacterial strains, plasmids, media and growth conditions

Strains and plasmids used in this work are listed in Table 4. *R. leguminosarum* strains were grown routinely at 28°C in YMB (Vincent, 1970). Plasmids were introduced into *R. leguminosarum* strains by conjugation using *E. coli* S17.1 as donor strain. For these matings, fresh cultures of donor and recipient strains were mixed on YMB plates and incubated overnight at 28°C. Transconjugants were selected on *Rhizobium* minimal medium (Rm; O'Gara and Shanmugam, 1976) plates supplemented with the corresponding antibiotics. For membrane preparations, cultures were grown on MM medium (Thorne and Williams, 1997). Antibiotic concentrations used were as follows (in $\mu g\ ml^{-1}$): ampicillin, 100; kanamycin, 50; spectinomycin, 100; and tetracycline, 5 for *R. leguminosarum* and 10 for *E. coli*. Analysis of sensitivity to SDS was carried out by overnight incubation of cultures inoculated to an $OD_{600} \approx 0.05$ with cells grown in YMB to stationary phase.

Recombinant DNA techniques

DNA manipulations including purification, restriction, ligation, agarose gel electrophoresis, PCR amplification, transformation into *E. coli* cells and Southern transfer into nylon membranes were carried out by standard methods (Sambrook and Russell, 2001). Labelling of DNA probes with digoxigenin (DIG), hybridization of DNA on nylon filters and chemiluminescent detection of hybridizing bands were performed using a DIG detection kit as specified by the manufacturer (Roche Molecular Biochemicals). Total DNA from *Rhizobium* strains was isolated as described previously (Leyva *et al*., 1987). Oligonucleotides used for PCR and sequencing reactions were ordered from Sigma.

Nucleotide sequence and accession number

Nucleotide sequence was determined with an ABI Prism

Table 4. Bacterial strains and plasmids.

Strains and plasmids	Description	Source or reference
Bacteria		
Rhizobium leguminosarum bv. viciae		
UPM791	128C53 wild type Str^r Nod$^+$ Fix$^+$ Hup$^+$	Ruiz-Argüeso *et al*. (1978)
SS113	UPM791 derivative, *fbcC*::mTn*5*SSoriR*gusA* Nod$^+$ Fix$^-$	This laboratory
SM61	UPM791 derivative, Δ*tatBC*::Spc, Nod$^+$ Fix$^-$	This work
SPF25	UPM791 with P*fixN*::*hupSL*	Brito *et al*. (2002)
SP61	SPF25 derivative, Δ*tatBC*::Spc, Nod$^+$ Fix$^-$	This work
Escherichia coli		
S17.1	*thi pro hsd*R$^-$ *hsd*M$^+$ *recA* RP4 2-Tc::Mu- Km::Tn*7* (Sp^r/Sm^r)	Simon *et al*. (1983)
Plasmids		
pBluescript (SK+, SK–)	Ap^r, lacZ′, T7Φ10 promoter, f1 ori	Stratagene
pSKΔEP	Derivative of pBluescript (SK$^-$), Δ*Eco*RI–*Pst*I restriction sites	This work
pSKX-H	Derivative of pSKΔEP containing a2.6 kb *Xho*I–*Hin*dIII fragment with *tatABC* from UPM791	This work
pSKTAT	Derivative of pSKX-H, Δ*Eco*RI–*Eco*RI::Spc, Spc^r	This work
pLAFR3	Cloning vector, *cos* IncP, Mob$^+$, Tra$^-$, Tc^r	Staskawicz *et al*. (1987)
pTAT	Derivative of pLAFR3, containing *tatABC* genes in a 2.9 kb *Hin*dIII fragment from UPM791	This work
pJP2	Promoterless *gusA* fusion vector; Tc^r	Prell *et al*. (2002)
pT10, pT11	pJP2 derivatives carrying fusions to the 5′ region of the *R. leguminosarum tatA* gene in direct and inverse orientations respectively	This work
pT20	pJP2 derivative, carrying a fusion to the 5′ region of the *R. leguminosarum tatB* gene	This work
pK18$_{mobsac}$	pK18 derivative, *sacB* Km^r	Schäfer *et al*. (1994)
pHC41	IncP, RK2 stabilization region, Tc^r	Cheng and Walker (1998)
pHC60	pHC41 derivative containing *gfp* gene constitutively expressed	Cheng and Walker (1998)
pAL618	Cosmid containing UPM791 *hup* cluster, Tc^r	Leyva *et al*. (1987)
pALPF1	pAL618 derivative containing P*fixN*::*hupSL*	Brito *et al*. (2002)

377 DNA sequencer (Perkin-Elmer) using a DNA sequencing kit (dRhodamine terminator cycle sequencing ready reaction; Perkin-Elmer). Nucleotide and deduced amino acid sequences were analysed using software from the GCG package (Genetics Computer Group, University of Wisconsin, USA), SEQUENCHER (Gene Codes Corporation), DNA STRIDER (C. Marck, Centre d'Études Nucleaires de Saclay, France) and BRUJENE (J. Vara, University Autónoma de Madrid, Spain). Protein structure was predicted using the HMMTOP server (http://www.enzim.hu/hmmtop). The 2700 bp sequence corresponding to the *tatABC* cluster and incomplete seryl-tRNA synthetase gene has been deposited in GenBank database under the accession number AF462068.

Generation of tat–gusA *fusions*

To investigate the expression levels of the *tat* genes, we constructed transcriptional fusions to the *gusA* gene in plasmid pJP2 (Prell *et al*., 2002). Two DNA regions were PCR amplified from plasmid pSKTAT. The DNA region cloned in plasmid pT10 spanned from the *Hin*dIII restriction site upstream of the *tat* region to *tatA* amino acid residue 37, and it was amplified using the Reverse primer and pTatA1 primer (5′-GGCGCGTCTTCGTCCGTCAT-3′). The DNA region corresponding to the insert in plasmid pT20 spanned from the same *Hin*dIII restriction site as in pT10 to *tatB* amino acid residue 120. This region was amplified using Reverse primer and pTatB1 primer (5′-CTACCAATGGCGGCGTTTCC-3′). Plasmid pT11 contained the same region as pT10 cloned in the opposite orientation and was used as a negative control.

Construction of tatBC *mutants*

The Ω spectinomycin/streptomycin resistance cassette from plasmid pHP45Ω (Prentki and Krisch, 1984) was used to replace a 0.7 kb *Eco*RI fragment containing part of the *tatB* and *tatC* genes (Fig. 1). This mutation in *tatBC* genes was mobilized and exchanged into the *R. leguminosarum* UPM791 genome by means of the suicide pK18*mobsac* vector (Schäfer *et al*., 1994). Potential *Rhizobium* recombinants were isolated as colonies Km^s, Spc^r. The fidelity of the double recombination event was confirmed by Southern blot using a DNA fragment internal to the *tatBC* region as probe (data not shown). The resulting strain was designated SM61. For analysis of hydrogenase phenotype, the same mutation was introduced in the SPF25 genome, thus generating mutant SP61.

Preparation of Rhizobium *cell fractions and assay for ubiquinol-cytochrome c oxidoreductase activity*

Rhizobium leguminosarum cultures (3 l) were grown in a fermenter (Microferm; New Brunswick), collected by centrifugation, resuspended in 36 ml of lysis buffer (10 mM Tris-HCl, pH 7.5), containing a protease inhibitor mixture (complete-Mini; Roche Molecular Biochemicals) and disrupted twice by high-pressure shearing at 100 MPa in a French Press cell (SLM Aminco). Lysates were cleared at 13 000 *g* for 30 min, and the supernatant was fractionated by ultracentrifugation at 100 000 *g* for 1 h. Finally, the pellet containing the membranes was resuspended in 12 ml of assay buffer (50 mM potassium phosphate, pH 7.0, 250 mM sucrose, 0.2 mM EDTA, 1 mM NaN_3, 2.5 mM KCN, 0.01% Tween 20). Ubiquinol-cytochrome *c* oxidoreductase activities of *R. leguminosarum* membranes were assayed as described for mitochondrial membranes (Nett *et al*., 2000). Membranes (≈ 5 mg of protein) were assayed at room temperature in 1 ml of assay buffer containing 50 μM horse cytochrome *c*. The reaction was started by adding 10 μl of 50 μM Ubiquinone50 (Coenzyme Q10; Sigma), and reduction of cytochrome *c* was monitored spectrophotometrically in a Shimadzu UV-2501-PC instrument set in dual wavelength mode (550 nm versus 539 nm). Activity values were calculated using an extinction coefficient of 21.5 mM^{-1} cm^{-1} for reduced minus oxidized cytochrome *c* (Ljungdahl *et al*., 1989). For analysis of the LPS fraction of *R. leguminosarum* vegetative cells, we used the procedure described by Lesse *et al*. (1990) modified as follows: 1 ml of YMB culture (OD_{600} ≈ 0.5) was centrifuged and resuspended in 60 μl of standard sample loading buffer. Cells were boiled for 10 min and treated with proteinase K (50 μg ml^{-1}) overnight at 37°C. Cell extracts were then boiled for 10 min and treated again with proteinase K for 3 h at 37°C. The resulting extract was resolved by SDS-PAGE (18% acrylamide gel) and stained using a commercial kit (Bio-Rad Silver Stain) according to the manufacturer's instructions.

Plant tests and nitrogenase activity

Pea (*Pisum sativum* L. cv. Frisson) plants were used as host for *R. leguminosarum* bv. viciae. Conditions for plant inoculation, growth on nitrogen-free nutrient solution under bacteriologically controlled conditions and bacteroid preparation have been described previously (Leyva *et al*., 1987). Nitrogenase activity was estimated by the acetylene reduction assay carried out under conditions described previously (Ruiz-Argüeso *et al*., 1978).

Hydrogenase activity and immunodetection

Hydrogenase activity was determined in whole cells and in cell fractions by an amperometric method as described previously (Ruiz-Argüeso *et al*., 1978). Cells were obtained from MM cultures grown in the fermentor under 1% oxygen tension. Cell membrane and soluble fractions were obtained as described above. Detection of hydrogenase subunits was carried out by standard immunoblot techniques on protein fractions resolved by SDS-PAGE using antisera raised against the *B. japonicum* hydrogenase large subunit and the *R. leguminosarum* hydrogenase small subunit (Brito *et al*., 1994).

Other enzyme assays

The β-glucuronidase activities of *Rhizobium* cell cultures and pea bacteroids were determined as described previously (Wilson *et al*., 1995). The protein content of cells and bacteroid suspensions was determined by the bicinchoninic acid method (Smith *et al*., 1985) after alkaline digestion in 1 N NaOH at 90°C for 10 min with BSA as standard. The Nadi assay for the detection of cytochrome *c*-dependent O_2 respiration was performed using a mixture of N,N,-dimethyl-*p*-phenylendiamine and α-naphthol on colonies grown on YMB plates as described previously (Marrs and Gest, 1973).

Preparation of nodule tissue for microscopy

Pea nodules, harvested at the indicated times after inoculation, were fixed in glutaraldehyde and processed for microscopy as described elsewhere (VandenBosch *et al*., 1989). Alternatively, unfixed nodule sections were obtained using a CO_2-freezing microtome. Optical microscopy was carried out with a Leica DRM microscope, and images were recorded with a Nikon Coolpix 900 digital camera. Confocal microscopy with GFP-tagged strains was carried out with a Leica TCS NT confocal microscope equipped with a krypton–argon laser.

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Supplementary material

The following material is available from
http://www.blackwellpublishing.com/products/journals/suppmat/mole/mole3510/mmi3510sm.htm
Table S1. Potential substrates for Tat export in Sinorhizobium meliloti.

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