A stomatin-like protein encoded by the *slp* gene of *Rhizobium etli* is required for nodulation competitiveness on the common bean

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***Rhizobium etli* strain TAL182 is a competitive strain for effective nodulation of beans. From this strain, a novel gene was isolated, *slp*, which is 669 bp in size and required for nodulation competition on the common bean. The *slp* knock-out mutant of TAL182 is defective in nodulation competition, shows reduced growth in the presence of 200 mM NaCl, KCl or LiCl and is complemented by the cloned *slp* gene. The deduced amino acid sequence of *slp* shows 66–72% similarity to stomatin proteins of *Homo sapiens*, *Mus musculus* and *Caenorhabditis elegans*. Expression of *slp* in *Escherichia coli* from a T7 promoter shows a 26 kDa protein which cross-reacts with human-stomatin-specific polyclonal antibody. Like the human stomatin protein, the *slp*-deduced protein, Slp, is very hydrophilic except for a single hydrophobic membrane-spanning domain. Among various bean-nodulating rhizobia, *slp* is present in *R. etli*, *Rhizobium leguminosarum* bv. *phaseoli* and *Rhizobium tropici* type A strains but is absent in *R. tropici* type B strains. It is also absent in *Bradyrhizobium* and several other *Rhizobium* spp.**

Keywords: nodulation competition, legume, symbiosis, nitrogen fixation, rhizobia

INTRODUCTION

Soil bacteria of the genera *Rhizobium*, *Bradyrhizobium*, *Azorhizobium* and *Sinorhizobium*, commonly known as rhizobia, form nitrogen-fixing symbiotic associations with members of the plant family Leguminosae. Rhizobial interaction with legumes requires continuous exchange of signals between the two partners. Rhizobial *nod* genes are induced in response to the inducer molecules flavonoids, present in the legume root exudate (for review, see Long, 1996). The products of the *nod* genes direct the synthesis of signal molecules, lipooligosaccharide Nod factors, which induce several physiological responses in the legumes, such as root hair curling, infection thread formation and localized cortical cell division resulting in nodule initiation. A second signal generated within the plant in response to the

rhizobial Nod factor may elicit further nodule morphogenesis (Hirsch, 1992). Rhizobia then invade through the infection thread where they continue to divide, nourished by the nutrients supplied by the plant, and finally reach the developing nodule. This invasion process involves the coordinated expression of many rhizobial and plant genes. While some of the *Rhizobium* genes involved in this process, such as *nodV* and *exo*, have been characterized (for review, see Leigh & Coplin, 1992), there are other, as yet unidentified, genes which may be involved in the regulation of growth of rhizobia through infection thread and nodule invasion. This class of genes may determine the capacity of a *Rhizobium* strain to compete for nodule occupancy.

Competition for nodule occupancy is an interesting biological phenomenon which is considered to be a limiting step for field legume inoculation. Inoculant strains often have to compete with indigenous rhizobia that may be unable to fix nitrogen in symbiosis. At present, little is known about the molecular basis of competitiveness and the genes that are involved in this process. Beattie & Handelsman (1993) described a

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strategy to identify nodulation competitiveness genes in *Rhizobium etli*. Recently, Mavingui *et al.* (1997) selected a competitive derivative of *Rhizobium* strain CFN299 by several cycles of selection for increased competitiveness for nodule formation among derivatives containing randomly amplified DNA regions. Previously, two *Sinorhizobium meliloti* genes, *nfe1* and *nfe2*, involved in nodulation efficiency and competitiveness were identified and characterized (Sanjuan & Olivares, 1991; Soto *et al.*, 1993). Another gene, *nfeC*, essential for nodulation competitiveness on soybean has been isolated and characterized from *Bradyrhizobium japonicum* (Chun & Stacey, 1994). Recently, we have shown that mimosine, a toxin produced by the tree legume *Leucaena*, provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains (Soedarjo & Borthakur, 1998).

R. etli TAL182 is a competitive strain for nodulation of the common bean and carries four indigenous plasmids with sizes ranging between 100 and 250 MDa. Previously, Borthakur & Gao (1996) isolated two overlapping cosmid clones containing genes for nodulation competition from the genomic library of *R. etli* strain TAL182 by functional complementation of *Rhizobium* sp. strain TAL1145, which nodulates bean but is non-competitive for nodule occupancy. By site-directed Tn5 insertion mutagenesis and subcloning, the genes for nodulation competition were localized within a 4.6 kb *Hind*III fragment common to both cosmids. Competition-defective mutants of TAL182 containing Tn5 insertions within the 4.6 kb *Hind*III fragment were identified. The competition defect of one of these mutants, RUH120, was complemented by plasmid pUHR207 containing the 4.6 kb *Hind*III fragment cloned in a broad-host-range plasmid vector. Thus it was shown that the 4.6 kb *Hind*III fragment in pUHR207 contained a gene or genes required for nodulation competition on beans. The objective of the present investigation was to characterize the genes for nodulation competition present in this DNA fragment. Interestingly, one of the genes in the 4.6 kb *Hind*III fragment in pUHR207 shows high sequence similarities with the human and murine stomatin genes at the amino acid level. Stomatin is an integral membrane protein in the red blood cells and is involved in the regulation of ion transport (Stewart, 1997).

METHODS

Bacterial strains and plasmids. *Rhizobium* strains and plasmids used in this study are listed with their sources and relevant properties in Table 1. *Rhizobium tropici* strains used in Southern hybridization were described previously by Martínez-Romero *et al.* (1991).

Bacterial media and growth conditions. Bacterial media, culture conditions and crosses were as described previously by George *et al.* (1994). For determining the growth rates, *Rhizobium* strains were grown in minimal medium (MM) which is based on the minimal medium described by Thorne & Williams (1997) and contains the following (l^{-1}): MOPS

(8.37 g, pH 7.2), KOH (1.2 g), $CaCl_2$ (1.12 g), $MgSO_4$ (0.48 g), NH_4Cl (1.07 g), K_2HPO_4 (0.21 g), KH_2PO_4 (0.05 g), mannitol (2.5 g), NaCl (5 mg), H_3BO_3 (1 mg), $ZnSO_4 \cdot 7H_2O$ (1 mg), $CuSO_4$ (0.5 mg), $MnCl_2 \cdot 4H_2O$ (0.5 mg), $NaMoO_4 \cdot 2H_2O$ (1 mg), EDTA (10 mg), thiamin (0.2 mg), pantothenate (0.01 mg) and biotin (0.2 mg). *Rhizobium* strains were grown by inoculating 50 ml MM in 250 ml screw-cap bottles with 0.5 ml *Rhizobium* culture. Each treatment was replicated three times. Cultures were grown at 28 °C with shaking, and growth was determined at 12 h intervals by measuring the OD_{600} in a Spectronic 401 spectrophotometer. *Escherichia coli* strains were grown in Luria-Bertani (LB) medium (Sambrook *et al.*, 1989). For determining osmotic stress tolerance, cultures were grown in MM medium to an OD_{600} of 0.5, at which point osmotic shock was applied by addition of a NaCl solution to a final concentration of 2.5 M and survival was monitored over a period of 3 h by plating for viable counting (Thorne & Williams, 1997).

Southern hybridization. Genomic DNA (approximately 3 µg) was digested with *Eco*RI, electrophoresed on a 1.0% agarose gel and transferred to a nylon membrane. For Southern hybridizations, DNA probes were labelled by random priming using a digoxigenin labelling and detection kit (Boehringer Mannheim). Digoxigenin-labelled probes were used for hybridization at 42 °C in a buffer containing 5 × SSC, 0.02% SDS, 2% blocking reagent and 50% formamide (1 × SSC is 0.15 M NaCl, 15 mM sodium citrate) for 12 h and washed at 60 °C with 2 × SSC, 0.1% SDS for 30 min. Chemiluminescent alkaline phosphatase substrate Lumi-Phos 530 (Boehringer Mannheim) was used to detect the signal by exposure to X-ray film.

DNA sequence analysis. The 4.6 kb *Hind*III fragment of plasmid pUHR207 was sequenced in both orientations. Another 300 bp region adjacent to the left end of the 4.6 kb *Hind*III fragment was sequenced from the 14.7 kb *Bam*HI fragment of pUHR200 subcloned in pUC18 (Fig. 1). Sequencing was done by a combination of subcloning and primer walking. The 4.6 kb *Hind*III fragment and its various *Sal*I, *Pst*I, *Eco*RI, *Pst*I-*Eco*RI and *Eco*RI-*Hind*III subfragments were cloned in pUC18 and sequenced using the pUC/m13 forward and reverse primers with an automated DNA sequencer (model 373A; Applied Biosystems) at the Biotechnology and Molecular Biology Instrumentation Facilities, University of Hawaii. The gaps in the sequence were filled in by sequences generated by using specific inner primers. The sequencing data were analysed by the GCG software and the Experimental BLAST Network Service (Altschul *et al.*, 1990). Protein sequence alignment was performed with the CLUSTAL W and Boxshade Server programs.

Insertional inactivation of *orf1* and the *slp* gene in TAL182.

To inactivate *orf1*, the 1.0 kb *Pst*I fragment of pUHR207 containing a middle segment of *orf1* (Fig. 1) was cloned in pBR322 and transferred to TAL182 by conjugation. As pBR322 cannot replicate in *Rhizobium*, it was expected that a single recombination between the cloned fragment and the corresponding homologous DNA in TAL182 would result in the interruption of *orf1* by the pBR322 sequences. Three tetracycline-resistant colonies were screened by Southern analysis and two of them were found to contain the insertion of pBR322 sequences in the expected position. One such colony in which *orf1* was found to be interrupted was selected as mutant RUH127. Similarly, to inactivate the *slp* gene, the 1.7 kb *Eco*RI-*Hind*III fragment of pUHR207 containing this gene (Fig. 1) was cloned in pUC18 and a 2.0 kb kanamycin-resistance cassette (Kan^R) from plasmid pSK101 (Shapira *et*

Table 1. Bacterial strains and plasmids

Strain/plasmid	Relevant characteristics	Reference/source
Strains		
<i>Rhizobium</i>		
TAL182	<i>R. etli</i> wild-type strain, Rif ^R	George <i>et al.</i> (1992)
TAL1145	<i>Leucaena</i> -nodulating <i>Rhizobium</i> sp., Rif ^R	George <i>et al.</i> (1994)
RUH120	Tn5-insertion mutant of TAL182, competition-defective, Rif ^R Kan ^R	Borthakur & Gao (1996)
RUH126	<i>slp</i> mutant of TAL182 with Kan ^R cassette inserted	This study
RUH127	<i>orf1</i> mutant of TAL182 with pBR322 inserted, Tet ^R	This study
<i>E. coli</i>		
BL21(DE3)pLysS	Contains a lambda prophage with an IPTG-inducible T7 RNA polymerase gene	Studier & Moffat (1986)
Plasmids		
pUHR68	Cosmid clone containing TAL182 DNA involved in nodulation competition	Borthakur & Gao (1996)
pUHR200	14.7 kb <i>Bam</i> HI fragment of pUHR68 cloned in the wide-host-range vector pTR102	Borthakur & Gao (1996)
pUHR207	4.6 kb <i>Hind</i> III fragment of pUHR68 cloned in the wide-host-range vector pTR102	Borthakur & Gao (1996)
pET21a(+)	T7 expression vector, Amp ^R	Novagen
pET21-669	<i>slp</i> gene cloned in pET21(+)	This study
pSK101	Kan ^R cassette from Tn5 cloned in pUC6	Shapira <i>et al.</i> (1983)

Fig. 1. Restriction maps of the 14.7 kb *Bam*HI fragment and the 4.6 kb *Hind*III fragment cloned in plasmids pUHR200 and pUHR207, respectively. The numbers in pUHR200 indicate sizes of DNA fragments in kb. The sequencing strategy is shown by horizontal arrows below pUHR207. The dimensions and directions of the three ORFs are indicated with open arrows below pUHR207. The positions of the Tn5 insertion in RUH120 (filled triangle) and the Kan^R cassette insertion in mutant RUH126 (open triangle) and the approximate position of the pBR322 insertion in RUH127 (open circle) are shown. B, *Bam*HI; E, *Eco*RI; H, *Hind*III; P, *Pst*I; S, *Sal*I.

al., 1983) was cloned into the *Sal*I cleavage site, located 92 bp downstream from the ATG start codon of the *slp* gene in the 1.7 kb fragment. The interrupted gene, *slp::Kan^R*, in the 3.7 kb *Eco*RI-*Hind*III fragment (1.7 kb fragment with the 2.0 kb Kan^R cassette) from this plasmid was cloned into a wide-host-range plasmid and transferred into TAL182. The *slp::Kan^R* allele was homogenized by double homologous recombination to obtain mutant RUH126.

PCR amplification of the *slp* gene. PCR-amplified fragments were used for cloning and generating probes for Southern hybridization. The PCR reactions were done using the GeneAmp PCR system 2400 (Perkin Elmer). Standard PCR reactions were done in a final volume of 50 µl which included 100 ng sample DNA, 200 µM each of dATP, dCTP, dGTP and

dTTP, 0.2 µM of each specific primer, 2.5 mM MgCl₂ and 1.5 U *Taq* polymerase (Promega). 'Hot start' at 94 °C for 5 min was used to avoid non-specific DNA amplification. Thermal cycling was done as follows for 25 cycles: denaturation for 30 s at 94 °C, primer annealing at 60 °C for 30 s and extension at 72 °C for 30 s. For Southern hybridization a 576 bp fragment of the *slp* gene was amplified using primers 5' GAG TCA AGG GAC CCG GCT TGAT 3' and 5' CTC CAT CGG GAA GGG GAA TAA 3'. For cloning the entire 669 bp *slp* gene with an added *Nde*I restriction site before the ATG start codon and a *Bam*HI site after the TGA stop codon, the following two primers were used: 5' GGC ATA TGA ACG CGG CGT CGT TCA CA 3' and 5' CAG GAT CCT CAC TTC GGC TTA ATG GCC ATT 3'.

Expression of *R. etli* Slp in *E. coli*. A PCR product containing the *R. etli* *slp* gene was cloned between the *Nde*I and the *Bam*HI sites of expression vector pET21a(+) (Novagen). The plasmid thus constructed was named pET21-669. Plasmid pET21-669 was used to transform the *E. coli* strain BL21(DE3)pLysS. This strain contains an inducible T7 RNA polymerase gene behind the *E. coli* *lacZ* promoter and the plasmid pLysS which encodes T7 lysozyme and represses the background levels of T7 RNA polymerase expression (Studier & Moffatt, 1986). The transformant was grown in LB broth to an optical density of 0.6 before the T7 polymerase was induced by adding IPTG to a final concentration of 1 mM with growth for additional 2–3 h. The cells from induced and uninduced cultures were then harvested and lysed and the total proteins were separated on a 12% polyacrylamide gel. The gel was stained with Coomassie Blue.

Western blot analysis. The proteins were transferred from the SDS gel to a nitrocellulose membrane (MSI) by using the Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad) under constant voltage of 75 V for 4 h at 4 °C. The efficiency of transfer was checked by staining the gel with Coomassie Blue. Blots were probed with a polyclonal antibody against human stomatin protein (Stewart *et al.*, 1992) used at a 1:1000 dilution followed by secondary antibody (goat anti-rabbit IgG conjugated with horseradish peroxidase, Bio-Rad; dilution 1:3000). Bound antigen was visualized by chemiluminescence using the ECL Western blotting detection reagents (Amersham Life Science).

Nodulation competition assay. *Phaseolus* beans (cv. Brazil 2) were grown in Leonard jar assemblies containing nitrogen-free nutrient solution as described previously (Borthakur & Gao, 1996). Eight replicates were used for each treatment with each Leonard jar containing two bean seedlings. One-week-old seedlings were inoculated with 10⁶ rhizobia diluted with sterile plant nutrient solution from a 2-d-old culture. Three inoculant ratios, 1:1, 1:9 and 9:1, between each mutant and TAL182 were used for competition studies. Plate counts of the rhizobia were made to verify the ratios of the mutants and TAL182 in the mixtures. A few plants were grown as uninoculated controls to check for cross-contamination. Plants were placed in a completely randomized block design. The uninoculated control plants appeared chlorotic and did not have root nodules, indicating that there was no cross-contamination of rhizobia between jar assemblies. Plants were harvested 4 weeks after inoculation for nodule typing.

RESULTS

Sequence analysis of the 4.6 kb *Hind*III fragment of pUHR207 and identification of three ORFs

The 4.6 kb *Hind*III fragment of plasmid pUHR207 that complemented the competition-defective mutant RUH120 was sequenced (Fig. 1). Sequence analysis showed three ORFs in this region. *orf1* is 2397 bp long and oriented in a right-to-left direction, extending beyond the 4.6 kb *Hind*III fragment by 282 bp at its 3' end to the adjacent fragment, whereas *orf2* and *orf3* are oriented left-to-right. The gap between *orf1* and *orf2* is 12 bp, while *orf2* and *orf3* are separated by 120 bp. *orf1* has 32% identity and 47% similarity with the *y4II* gene with unknown function from the symbiosis plasmid of *Rhizobium* sp. NGR234 (data not shown). *orf2* is 1404 bp, encodes a protein of 467 amino acids with a

calculated molecular mass of 48870 Da and has no significant homology with any known protein in the database.

orf3 encodes a stomatin-like protein

orf3 is 669 bp and its deduced amino acid sequence shows 66–72% similarity with the erythrocyte band 7 integral membrane protein or the stomatin protein of *Homo sapiens*, *Mus musculus* and *Caenorhabditis elegans* (Fig. 2). Because of this high homology of the deduced amino acid sequence with the stomatin protein, *orf3* was named *slp* (stomatin-like protein). Like the human stomatin protein, the deduced *slp* gene product, Slp, is very hydrophilic except for a single hydrophobic domain at the C-terminus. However, unlike the human stomatin protein, Slp does not have the N-terminal transit peptide and is smaller in size (Fig. 3). When the *slp* gene was expressed from a T7 promoter in *E. coli*, a 26 kDa protein was seen (Fig. 4a). The overexpressed Slp in *E. coli* cross-reacted with a polyclonal antibody specific for human stomatin protein (Fig. 4b), although the same antibody did not show detectable levels of cross-reaction with TAL182 proteins (data not shown).

Insertional inactivation analysis of *orf1*, *orf2* and the *slp* gene in TAL182

Mutants RUH127 and RUH126 were constructed by insertional inactivation of *orf1* and *orf3*, respectively, in TAL182. In the mutant RUH127, *orf1* was disrupted with the plasmid vector pBR322 through a single homologous recombination whereas in RUH126, the *slp* gene was interrupted by the Kan^R cassette through double homologous recombination (see Methods). Southern hybridization confirms that the mutant RUH127 contains a pBR322 insertion in *orf1* and the mutant RUH126 contains a Kan^R cassette insertion within the *slp* gene (data not shown). The competition-defective mutant RUH120 which was described previously (Borthakur & Gao, 1996) contains a Tn5 insertion in *orf2*. The precise location of the Tn5 insertion in RUH120 was also determined by sequencing. The Tn5 insertion was located 259 nucleotides downstream from the beginning of this *orf2* (Fig. 1). Mutants RUH120, RUH126 and RUH127 showed similar colony morphologies and growth rates in complete or minimal media.

The *slp* gene is required for nodulation competition on beans

orf1 mutant RUH127 and *slp* mutant RUH126 formed normal nitrogen-fixing nodules on beans which were indistinguishable in number, size, shape and colour from those formed by TAL182. These mutants were tested for nodulation competition with TAL182 by co-inoculating beans in paired combinations using different ratios of the two strains. The results of this experiment suggest that only RUH126, and not RUH127, is defective

Sto_Hs	1	M A E K R D T R D S E A Q R L P D S F K D S P S K G L G P C G W I L V A F S F L F T V I T F P I S I W M C I K I I K E Y
Sto_Mm	1	M S D K R Q S S H V Q S Q R I P E S F R E N S K T E L G A C G W I L V A V S F I F L I T F P I S I W C I K I V K E Y
Sto_Ce	1	-----M S Y V L I F L T F P V S V E M C I K -----E Y
Slp_Re	1	-----
Sto_Hs	61	E R A I I F R L G R I L Q G G A K G P G L F F I L P C T D S F I K V D M R T I S F D I P P Q E I L T K D S V T I S V D G
Sto_Mm	61	E R V I I F R L G R I L Q G G A K G P G L F F I L P C T D S F I K V D M R T I S F D I P P Q E V L T K D S V T I S V D G
Sto_Ce	22	Q R A V V F R L G R L V P - D V K G P G I F F I I P C I D T F L N I D L R V A S Y N V P S Q E I L S R D S V T V S V D A
Slp_Re	1	M N A A S F T L G R F T G - - V K G P G L I L L I P Y V Q Q M I R V D L R T R V L D V P S Q D V I S H D N V S V R V S A
Sto_Hs	121	V V Y Y R V Q N A T L A V A N T N A D S A T R L L A Q T T L R N V L G T K N L S O I L S D R E E I A H N M Q S T L D D
Sto_Mm	121	V V Y Y R V Q N A T L A V A N T N A D S A T R L L A Q T T L R N A L G T K N L S O I L S D R E E I A H N M Q S T L D D
Sto_Ce	81	V V Y F K V F D P I T S V V G V G N A T D S T K L L A Q T T L R T I L G T H T L S E I L S D R E K I S A D M K I S L D E
Slp_Re	59	V I Y F R V I D P E K S T I Q V E D F M M A T S Q L A Q T T L R S V L G K H D L D E M L A E R D R L N S D T Q E I L D A
Sto_Hs	181	A T D A W G I K V E R V E I K D V K L P V Q I Q R A M A A E A E A S R E A R A K V I A A E G M N A S R A L K E A S M V
Sto_Mm	181	A T D D W G I K V E R V E I K D V K L P V Q I Q R A M A A E A E A A R E A R A K V I A A E G M N A S R A L K E A S M V
Sto_Ce	141	A T E P W G I K V E R V E L R D V R L P S Q M Q R A M A A E A E A T R D A G A K I A A E G E T R A S A A L A E A A T I
Slp_Re	119	Q T D A W G I K V A T V E I K H V D I N E S M I R A I A R Q A E A E R E R R A K V I N A E G E Q C A A K L L E A E I
Sto_Hs	241	I T E S P A A I Q L R Y L Q T L T T I A A E K N S T I V F P L P I D M L O G I T G A K H S H L G -----
Sto_Mm	241	I T E S P A A I Q L R Y L Q T L T T I A A E K N S T I V F P L P V D M L O G I M G S N H -----
Sto_Ce	201	I S K S E G A M Q L R Y L H T L N A I S S E K T S T I T F P F P M E T L G G I S K V G S G G T S Q N F P V Q E M M N A A
Slp_Re	179	I A K Q P E A M Q L R Y L S T L N V I A S E K T S T I T F P F P M E L G N L M A I K P K -----

Fig. 2. Sequence comparison of deduced amino acid sequences from the *slp* gene of *R. etli* (Slp_Re) and the stomatin genes of *H. sapiens* (Sto_Hs) (Stewart *et al.*, 1992), *M. musculus* (Sto_Mm) (Gallagher *et al.*, 1996) and *C. elegans* (Sto_Ce) (Wilkinson-Sproat & Wohldman, 1994). Conserved residues are indicated by black areas, and similar residues are indicated by stippled areas.

Fig. 3. Comparison of the hydropathy plots of *R. etli* Slp with human stomatin protein (Stewart *et al.*, 1992) using the TopPred II computer program with a window of 21 amino acids (Claros & von Heijne, 1994).

in nodulation competitiveness on beans (Table 2). Plasmid pUHR207 containing the 4.6 kb cloned DNA complemented RUH126 for nodulation competitiveness (Table 2).

Growth of the *R. etli* *slp* mutant RUH126 is inhibited by monovalent salts

In humans, the stomatin gene is involved in the regulation of transport of sodium and potassium ions in

the red blood cells (Stewart, 1997). To determine if the *slp* gene is also involved in the transport of monovalent ions in *R. etli*, the wild-type strain TAL182, mutant RUH126 and the transconjugant of RUH126 containing pUHR207 were grown in minimal medium containing NaCl, KCl or LiCl. Compared to TAL182, the rate of growth and the final cell density of RUH126 were reduced in the presence of 200 mM NaCl, KCl or LiCl in the medium (Fig. 5). The growth of the transconjugant RUH126(pUHR207) in the presence of these salts was similar to that of TAL182, indicating that the *slp* gene in pUHR207 complemented the growth defect in RUH126 due to the presence of monovalent salts. These observations suggest that the *slp* gene may be involved in ion transport in *R. etli*. When osmotic stress was applied to TAL182, RUH126 and RUH126(pUHR207) for 15 min to 3 h, all three strains showed similar survival rates as judged from the colony counts from plating (data not shown). This indicates that the *slp* mutant does not have osmotic defects.

The *slp* gene is not present in all rhizobia

Southern analysis of genomic DNA of strains of various *Rhizobium*, *Bradyrhizobium* and *Sinorhizobium* species using the *slp* gene of TAL182 as the probe showed the absence of any hybridizing bands in *B. japonicum* USDA110, *Rhizobium* (*Aeschynomene*) strain Bta11, *Rhizobium leguminosarum* bv. *trifolii* WU290, *Rhizobium* sp. NGR234 and the *R. tropici* type B strains (Fig. 6). The *slp* probe hybridized with strains of *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *phaseoli*, *S. meliloti*, *R. etli*, *Rhizobium fredii* and *R.*

Fig. 4. (a) Expression of *R. etli* Slp in *E. coli*. Strain BL21(DE3)pLysS containing the cloned *slp* gene in plasmid pET21-669 was grown with IPTG for 2 and 3 h (lanes 1 and 2), or without IPTG for 3 h (lane 3). Extract from strain BL21(DE3)pLysS without additional plasmid grown for 3 h was loaded in lane 4 as a control. An additional protein band corresponding to 26 kDa in lanes 1 and 2 is indicated with an arrow. Size markers are in lane M. (b) Western blot analysis of overexpressed Slp in *E. coli* cross-reacting with human-stomatin-specific polyclonal antibody. The antibody cross-reacted with the overexpressed 26 kDa Slp (indicated with an arrow) and some other *E. coli* proteins in lane 1. The 26 kDa band was absent in the extract from the uninduced culture (lane 2). When the overexpressed 26 kDa Slp band was excised from a protein gel and reloaded on the protein gel, it again cross-reacted with the antibody (lane 3).

tropici type A strains. Among several bean-nodulating *Rhizobium* strains from the NifTAL collection (TAL strains), the *slp* gene did not hybridize with TAL490, TAL617 and *Rhizobium* sp. strain TAL1145.

DISCUSSION

The *slp* gene isolated in this study is required for nodulation competition on beans. The deduced amino acid sequence of the *slp* gene shows high homology with the human and murine stomatin peptides. Therefore, it was not surprising that the polyclonal antibody raised against human stomatin protein also cross-reacted with the overexpressed Slp in *E. coli*. This suggests that at

least some epitopes of the two proteins are similar. The Slp may be expressed at a very low level in TAL182 since the stomatin-specific antibody did not detect the presence of this protein in this strain in the Western blot analysis using a non-radioactive detection system. The antibody also cross-reacted with some *E. coli* proteins because it was produced by injecting rabbits with purified human stomatin protein expressed in *E. coli* (Stewart *et al.*, 1992). It is likely that during purification from *E. coli*, the stomatin protein was slightly contaminated with *E. coli* proteins. Human stomatin is an integral membrane protein involved in ion conductance across the membrane of red blood cells (Stewart *et al.*, 1992). It may be a part of the junctional complex of the membrane skeleton. In the absence of this protein, as in the case of a rare haemolytic anaemia, hereditary stomatocytosis, red blood cells leak sodium and potassium ions, suggesting that the protein has a role in the regulation of ion transport (Stewart, 1997). The *Rhizobium slp* gene also shows high homology with the *mec-2* gene of the soil nematode *Caenorhabditis elegans*, encoding a stomatin-like protein MEC-2 (Huang *et al.*, 1995). MEC-2 positively regulates the degenerin channel affecting the touch receptor axons of this nematode. MEC-2 links the mechanosensory degenerin channel and the microtubule cytoskeleton of the touch receptor neurons. The *mec-2* mutants are touch-insensitive. Thus, both stomatin and MEC-2 are membrane-associated proteins and interact with other proteins to regulate ion or mechanosensory channels. Slp, on the other hand, is a cytoplasmic protein with only one membrane-spanning domain at the C-terminus. It is different from both stomatin and MEC-2 in that it does not have an N-terminal hydrophobic membrane-spanning domain. The function of the missing N-terminal hydrophobic domain may be substituted by other membrane-associated proteins with which Slp interacts.

The striking sequence similarity between Slp and stomatin suggests that Slp has a similar function. This hypothesis is consistent with our finding that the *slp* mutant grows slower than the wild-type in medium containing 200 mM NaCl, KCl or LiCl. Stomatin is thought to inhibit membrane conductance whereas Slp may be involved in the transport of ions, which is coupled with nutrient uptake. Thus, the *slp* gene may provide a growth or competition advantage to the *Rhizobium* strain in the infection thread. The mutant RUH126 without Slp may have a disadvantage in ion exchange and nutrient uptake in the infection thread, resulting in the loss of capacity for competition for nodule invasion. However, Slp may not be a member of a primary ion channel system because the *slp* gene is not present in all rhizobia: a gene involved in a primary transport system is expected to be present in all rhizobia. Moreover, a mutation in a gene involved in a primary transport system should severely affect the growth of the mutant.

The *slp* gene is not essential for nodulation and nitrogen fixation because the mutant RUH126 forms nitrogen-fixing nodules on beans. Similarly, *R. tropici* type B

Table 2. Competitive nodule occupancies of *R. etli* strains on beans in paired inoculation experiments

Co-inoculated strains		Ratio	Nodule occupancy (%) [*] by:	
A	B	A:B	A	B
TAL182	RUH126	9:1	99.7 ± 1.0	0.3 ± 1.0
		1:1	94.8 ± 3.7	4.9 ± 3.7
		1:9	84.4 ± 6.4	15.6 ± 6.4
RUH126(pUHR207)	RUH126	9:1	95.4 ± 2.5	4.6 ± 2.5
		1:1	73.5 ± 6.8	26.5 ± 6.8
		1:9	30.0 ± 5.5	70.0 ± 5.5
TAL182	RUH127	9:1	85.0 ± 3.6	15.0 ± 3.6
		1:1	51.4 ± 10	48.6 ± 10
		1:9	12.1 ± 3.2	87.9 ± 3.2

^{*} Data are means ± SD from eight replicates.

Fig. 5. Growth of *R. etli* TAL182 (□), mutant RUH126 (○) and RUH126 containing pUHR207 (△) in MM (a), and in MM containing 200 mM NaCl (b), 200 mM KCl (c) or 200 mM LiCl (d).

strains and several other bean-nodulating strains such as TAL1145, TAL490 and TAL617 do not have the *slp* gene, and yet form effective nodules on beans. In these strains, the function of the *slp* gene may be substituted by another gene. In the absence of an *slp*-like gene or a substitute with similar function, the *R. tropici* type B strains may be less competitive for nodule occupancy on beans. In competition experiments involving *R. tropici* CIAT899 and *R. etli* TAL182 on beans, the latter was found to occupy most of the nodules (Borthakur & Gao, 1996). There is no homologue of *slp* in *Rhizobium* sp. strain TAL1145, which nodulates both tree legumes and

beans. This may be one of the reasons for the inability of this strain to occupy bean nodules when co-inoculated with TAL182. Besides *slp*, there might be other genes involved in nodulation competition in TAL182, because the capacity to occupy bean nodules in competition with TAL182 was only partially enhanced in transconjugants of TAL1145 containing the cosmid clone which contained *slp* (Borthakur & Gao, 1996).

The mutant RUH126 is similar to TAL182 except for the insertion of the Kan^R cassette into the *slp* gene. The strain was used to nodulate beans and was reisolated

Fig. 6. Southern hybridization of *Eco*RI-digested genomic DNA from various rhizobia with a 576 bp PCR-generated fragment from TAL182 containing *slp* sequences as the probe. The type A and type B strains of *R. tropici* are indicated. 1, TAL1145; 2, BR828; 3, BR833; 4, BR835; 5, BR836; 6, BR842; 7, BR846; 8, CFN299; 9, BR10043; 10, BR847; 11, BR850; 12, BR852; 13, BR857; 14, BR858; 15, BR859; 16, BR863; 17, BR864; 18, TAL182; 19, USDA110 (*B. japonicum*); 20, Bta1 (*Aeschynomene*); 21, F4B94 (*R. leguminosarum* bv. *viciae*); 22, NGR234; 23, CIAT899 (*R. tropici* type B); 24, NZP4013 (*S. meliloti*); 25, WU290 (*R. leguminosarum* bv. *trifolii*); 26, CE3 (*R. etli*); 27, USDA205 (*R. fredii*); 28, 8002 (*R. leguminosarum* bv. *phaseoli*); 29, TAL459; 30, TAL490; 31, TAL660; 32, TAL664; 33, TAL617; 34, TAL1140; 35, TAL1242; 36, TAL663.

from nodules before it was used in competition experiments. During the process it was cured of the plasmid pPH1J1, which was used to homogenize the Kan^R cassette insertion. The competition defect of this mutant is not due to the kanamycin cassette insertion per se but to the interruption in the *slp* gene, because it is complemented by the plasmid pUHR207 containing a cloned *slp* gene. Contrary to the competition-defective phenotype of RUH126, mutant RUH127, in which *orf1* was interrupted with the plasmid pBR322, was found to be not defective for nodulation competition on beans. In the competition experiments involving RUH126, the extent of double occupancies of nodules was not determined. It is possible that a few nodules occupied by the Kan^R mutant were also inhabited by TAL182. In an experiment involving mutant RUH127, which has a tetracycline-resistance marker, the extent of double occupancies was estimated to be $5 \pm 3\%$. Our competition experiments with beans were conducted in growth chambers under controlled conditions, which are different from those found in the field. However, in the present study we address competition as an aspect of nodule invasion which includes initial entry of rhizobia into the root cells through infection thread, followed by growth and proliferation through the infection thread and final release of rhizobia into the infected cells of the developing nodules. To avoid other aspects of interstrain competition in the rhizosphere favouring survival or death of any one strain before initial infection, we used an excess amount of rhizobia (at least 10^6 cells) for inoculating each Leonard jar assembly. Therefore, our results from the controlled growth room situation should indicate what does happen during nodule invasion in the natural soil environment.

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REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Beattie, G. A. & Handelsman, J. (1993). Evaluation of a strategy for identifying nodulation competitiveness genes in *Rhizobium leguminosarum* biovar *phaseoli*. *J Gen Microbiol* **139**, 529–538.
- Borthakur, D. & Gao, X. (1996). A 150-megadalton plasmid in *Rhizobium etli* strain TAL182 contains genes for nodulation competitiveness on *Phaseolus vulgaris* L. *Can J Microbiol* **42**, 903–910.
- Chun, J. Y. & Stacey, G. (1994). A *Bradyrhizobium japonicum* gene essential for nodulation competitiveness is differentially regulated from two promoters. *Mol Plant-Microbe Interact* **7**, 248–255.
- Claros, M. G. & von Heijne, G. (1994). TopPred II: an improved software for membrane protein structure predictions. *Comput Appl Biosci* **10**, 685–686.
- Gallagher, P. G., Turetsky, T. & Mentzer, W. C. (1996). Genomic organization and 5'-flanking DNA sequence of the murine stomatin gene (*Epb72*). *Genomics* **34**, 410–412.
- George, M. L. C., Robert, F. M. & Borthakur, D. (1992). Genetic analysis of *Rhizobium leguminosarum* bv. *phaseoli* mutants defective in nodulation and nodulation suppression. *Appl Environ Microbiol* **58**, 1050–1053.
- George, M. L. C., Young, J. W. P. & Borthakur, D. (1994). Genetic characterization of *Rhizobium* sp. strain TAL1145 that nodulates tree legumes. *Can J Microbiol* **40**, 208–215.
- Hirsch, A. M. (1992). Developmental biology of legume nodulation. *New Phytol* **122**, 211–237.
- Huang, M., Gu, G., Ferguson, E. L. & Chalfie, M. (1995). A stomatin-like protein necessary for mechanosensation in *C. elegans*. *Nature* **378**, 292–295.
- Leigh, J. A. & Coplin, D. L. (1992). Exopolysaccharides in plant-bacterial interactions. *Annu Rev Microbiol* **46**, 307–346.
- Long, S. R. (1996). *Rhizobium* symbiosis: Nod factors in perspective. *Plant Cell* **8**, 1885–1898.
- Martínez-Romero, E., Segovia, L., Mercante, F. M., Franco, A. A.,

- Graham, P. & Pardo, M. A. (1991). *Rhizobium tropici*, a novel species nodulating *Phaseolus vulgaris* L. beans and *Leucaena* sp. trees. *Int J Syst Bacteriol* **41**, 417–426.
- Mavingui, P., Flores, M., Romero, D., Martínez-Romero, E. & Palacios, R. (1997). Generation of *Rhizobium* strains with improved symbiotic properties by random DNA amplification (RDA). *Nature Biotechnol* **15**, 564–569.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanjuan, J. & Olivares, J. (1991). NifA-NtrA regulatory system activates transcription of *nfe*, a gene locus involved in nodulation competitiveness of *Rhizobium meliloti*. *Arch Microbiol* **155**, 543–548.
- Shapira, S. K., Chou, J., Richaud, F. V. & Casadaban, M. J. (1983). New versatile plasmid vectors for expression of hybrid proteins coded by a cloned gene fused to *lacZ* gene sequences encoding an enzymatically active carboxy-terminal portion of β -galactosidase. *Gene* **25**, 71–82.
- Soedarjo, M. & Borthakur, D. (1998). Mimosine, a toxin produced by the tree-legume *Leucaena* provides a nodulation competition advantage to mimosine-degrading *Rhizobium* strains. *Soil Biol Biochem* **30**, 1605–1613.
- Soto, M. J., Zorzano, A., Mercado-Blanco, J., Lepek, V., Olivares, J. & Toro, N. (1993). Nucleotide sequence and characterization of *Rhizobium meliloti* nodulation competitiveness genes *nfe*. *J Mol Biol* **229**, 570–576.
- Stewart, G. W. (1997). Stomatin. *Int J Biochem Cell Biol* **29**, 271–274.
- Stewart, G. W., Hepworth-Jones, B. E., Keen, J. N., Dash, B. C. J., Argent, A. C. & Casimir, C. M. (1992). Isolation of cDNA coding for an ubiquitous membrane protein deficient in high Na⁺, low K⁺ stomatocytic erythrocytes. *Blood* **79**, 1593–1601.
- Studier, F. W. & Moffatt, B. A. (1986). Use of bacteriophage T7 RNA polymerase to direct selective high level expression of cloned genes. *J Mol Biol* **189**, 113–130.
- Thorne, S. H. & Williams, H. D. (1997). Adaptation to nutrient starvation in *Rhizobium leguminosarum* bv. phaseoli: analysis of survival, stress resistance, and changes in macromolecular synthesis during entry to and exit from stationary phase. *J Bacteriol* **179**, 6894–6901.
- Wilkinson-Sproat, J. & Wohldman, P. (1994). 2.2 Mb of contiguous nucleotide sequence from chromosome III of *C. elegans*. *Nature* **368**, 32–38.

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