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 ndv 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
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 cosmids 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 HindIII fragment common to both cosmids. Competition-defective mutants of TAL182 containing Tn5 insertions within the 4-6 kb HindIII fragment were identified. The competition defect of one of these mutants, RUH120, was complemented by plasmid pUHR207 containing the 4-6 kb HindIII fragment cloned in a broad-host-range plasmid vector. Thus it was shown that the 4-6 kb HindIII 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 HindIII 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 Martinez-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), CaCl2 (1.12 g), MgSO4 (0.48 g), NH4Cl (1.07 g), K2HPO4 (0.21 g), KH2PO4 (0.05 g), mannitol (2.5 g), NaCl (5 mg), H3BO3 (1 mg), ZnSO4·7H2O (1 mg), CuSO4·5H2O (0.5 mg), MnCl2·4H2O (0.5 mg), NaMoO4·2H2O (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-cup 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 OD600 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 OD600 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 EcoRI, electrophoresed on a 1% 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 × SS, 0.02% SDS, 2% blocking reagent and 50% formamide (1 × SS 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 HindIII fragment of plasmid pUHR207 was sequenced in both orientations. Another 300 bp region adjacent to the left end of the 4-6 kb HindIII fragment was sequenced from the 147 kb BamHI fragment of pUHR200 subcloned in pUC18 (Fig. 1). Sequencing was done by a combination of subcloning and primer walking. The 4-6 kb HindIII fragment and its various SalI, PstI, EcoRI, PstI–EcoRI and EcoRI–HindIII 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 Biotecnology and Molecular Biology Instrumentation Facilites, 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 sip gene in TAL182.**

To inactivate orf1, the 1.0 kb PstI 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 sip gene, the 1.7 kb EcoRI–HindIII fragment of pUHR207 containing this gene (Fig. 1) was cloned in pUC18 and a 2.0 kb kanamycin-resistance cassette (KanR) from plasmid pSK101 (Shapira et
**Table 1. Bacterial strains and plasmids**

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>Relevant characteristics</th>
<th>Reference/source</th>
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<tr>
<td><strong>Strains</strong></td>
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<tr>
<td><em>Rhizobium</em></td>
<td>R. etli wild-type strain, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>George <em>et al.</em> (1992)</td>
</tr>
<tr>
<td>TAL182</td>
<td><em>Leucaena</em>-nodulating <em>Rhizobium</em> sp., Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>George <em>et al.</em> (1994)</td>
</tr>
<tr>
<td>TAL1145</td>
<td>Tn5-insertion mutant of TAL182, competition-defective, Rif&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Borthakur &amp; Gao (1996)</td>
</tr>
<tr>
<td>RUH120</td>
<td><em>slp</em> mutant of TAL182 with Kan&lt;sup&gt;R&lt;/sup&gt; cassette inserted</td>
<td>This study</td>
</tr>
<tr>
<td>RUH126</td>
<td><em>orf1</em> mutant of TAL182 with pBR322 inserted, Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td><strong>E. coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BL21(DE3)pLysS</td>
<td>Contains a lambda prophage with an IPTG-inducible T7 RNA polymerase gene</td>
<td>Studier &amp; Moffat (1986)</td>
</tr>
<tr>
<td>pUHR68</td>
<td>Cosmid clone containing TAL182 DNA involved in nodulation competition</td>
<td>Borthakur &amp; Gao (1996)</td>
</tr>
<tr>
<td>pUHR200</td>
<td>147 kb BamHI fragment of pUHR68 cloned in the wide-host-range vector pTR102</td>
<td>Borthakur &amp; Gao (1996)</td>
</tr>
<tr>
<td>pUHR207</td>
<td>46 kb HindIII fragment of pUHR68 cloned in the wide-host-range vector pTR102</td>
<td>Borthakur &amp; Gao (1996)</td>
</tr>
<tr>
<td>pET21a(+)</td>
<td>T7 expression vector, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Novagen</td>
</tr>
<tr>
<td>pET21-669</td>
<td><em>slp</em> gene cloned in pET21(+)</td>
<td>This study</td>
</tr>
<tr>
<td>pSK101</td>
<td>Kan&lt;sup&gt;R&lt;/sup&gt; cassette from Tn5 cloned in pUC6</td>
<td>Shapira <em>et al.</em> (1983)</td>
</tr>
</tbody>
</table>

**Fig. 1.** Restriction maps of the 147 kb BamHI fragment and the 46 kb HindIII 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<sup>R</sup> cassette insertion in mutant RUH126 (open triangle) and the approximate position of the pBR322 insertion in RUH127 (open circle) are shown. B, BamHI; E, EcoRI; H, HindIII; P, PstI; S, SalI.

*al., 1983*) was cloned into the SalI 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<sup>R</sup>* in the 3.7 kb *EcoRI–HindIII* fragment (1.7 kb fragment with the 2.0 kb Kan<sup>R</sup> cassette) from this plasmid was cloned into a wide-host-range plasmid and transferred into TAL182. The *slp::Kan<sup>R</sup>* allele was homogenotized 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 MgCl<sub>2</sub> 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' CAG GAT CCT CAC TTC GGC TTA 3'. For cloning the entire 669 bp *slp* gene with an added NdeI restriction site before the ATG start codon and a BamHI site after the TGA stop codon, the following two primers were used: 5' GGC ATA TGA ACG CCG CTT GCC CAG ATT 3' and 5' CAG GAT CCT CAC TTC GGC TTA ATG GCC ATT 3'.

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Expression of *R. etli* *slp* in *E. coli*. A PCR product containing the *R. etli* *slp* gene was cloned between the *NdeI* and the *BamHI* 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 inoculated with 10^6 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 *HindIII* fragment of pUHR207 and identification of three ORFs**

The 4.6 kb *HindIII* 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 *HindIII* 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 y4lll 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
Rhizobium slp gene encoding a stomatin-like protein

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 Bta1, 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. vicieae, R. leguminosarum bv. phaseoli, S. meliloti, R. etli, Rhizobium fredii and R.
**DISCUSSION**

The *slp* gene isolated in this study is required for nodulation competition on beans. 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. 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

<table>
<thead>
<tr>
<th>Co-inoculated strains</th>
<th>Ratio A:B</th>
<th>Nodule occupancy (%) by:</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>A</td>
<td>B</td>
</tr>
<tr>
<td>TAL182</td>
<td>9:1</td>
<td>997 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>948 ± 37</td>
</tr>
<tr>
<td></td>
<td>1:9</td>
<td>844 ± 64</td>
</tr>
<tr>
<td>RUH126(pUHR207)</td>
<td>9:1</td>
<td>954 ± 2.5</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>735 ± 68</td>
</tr>
<tr>
<td></td>
<td>1:9</td>
<td>300 ± 5.5</td>
</tr>
<tr>
<td>TAL182</td>
<td>9:1</td>
<td>850 ± 36</td>
</tr>
<tr>
<td></td>
<td>1:1</td>
<td>514 ± 10</td>
</tr>
<tr>
<td></td>
<td>1:9</td>
<td>121 ± 3.2</td>
</tr>
</tbody>
</table>

*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 cosm id clone which contained *slp* (Borthakur & Gao, 1996).

The mutant RUH126 is similar to TAL182 except for the insertion of the KanR cassette into the *slp* gene. The strain was used to nodulate beans and was reisolated.
from nodules before it was used in competition experiments. During the process it was cured of the plasmid pH13J, 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 deletion of the epb72 gene, because it is regulated from two promoters.

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