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Research Note

Identification and Characterization of a *Rhizobium leguminosarum* bv. *phaseoli* Gene that Is Important for Nodulation Competitiveness and Shows Structural Homology to a *Rhizobium fredii* Host-Inducible Gene

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DNA sequence analysis of a 1.4-kb *Sal*I–*Hin*dIII segment located approximately 2 kb upstream of the *Rhizobium leguminosarum* bv. *phaseoli syrM* gene revealed the presence of an open reading frame (ORF3) encoding a putative 295-amino acid polypeptide with a molecular mass of 33,401 Da. ORF3 is homologous to a *R. fredii* host-inducible gene. The proteins encoded by *R. l.* bv. *phaseoli* ORF3 and by the *R. fredii* host-inducible gene share 37% sequence identity. In contrast to the *R. fredii* host-inducible gene, expression of ORF3 is not induced in the presence of *Phaseolus vulgaris* root exudates or by specific flavonoids, able to induce nodulation genes in *R. l.* bv. *phaseoli*. A *R. l.* bv. *phaseoli* ORF3 mutant was constructed by site-directed deletion/replacement mutagenesis. This mutant strain is not affected in symbiotic nitrogen fixation but exhibits a delay in nodulation on *Phaseolus vulgaris*. Moreover, this mutant was shown to be defective in competition for nodulation.

The formation of nitrogen-fixing nodules in the *Rhizobium*–legume symbiosis is a multi-step process involving recognition and invasion of plant root hairs by the bacteria, release of bacteria in the host cells, and the differentiation into bacteroids able to reduce atmospheric nitrogen (Michiels and Vanderleyden 1994). Bacterial genes involved in different stages of the *Rhizobium leguminosarum* bv. *phaseoli*–legume symbiosis including nodulation genes (Davis and Johnston 1990; Vàzquez et al. 1991), nitrogen fixation genes (Quinto et al. 1985; Michiels and Vanderleyden 1993; Michiels et al. 1994), and genes involved in the production of surface components (Diebold and Noel 1989) have been identified. In this study, we report the characterization of a *R. l.* bv. *phaseoli* gene that is involved in competition for nodulation and that is homologous to a *R. fredii* host-inducible gene.

Escherichia coli and *R. l.* bv. *phaseoli* strain CNPAF512 cells were grown as described (Michiels et al. 1994). We have previously identified a *R. l.* bv. *phaseoli* DNA region containing three open reading frames (ORF): ORF1, *syrM,* and the *nolN* homolog ORF2 (Michiels et al. 1993; Fig. 1). DNA sequence analysis of a 1.4-kb *Sal*I–*Hin*dIII segment located approximately 1.1-kb upstream of ORF1 indicated the presence of one ORF (designated as ORF3) reading in the rightward direction (Figs. 1 and 2). The size of ORF3 is 885 bp. Several putative ATG initiation codons are located at the 5′ end of ORF3 but only the one at nucleotide 473 was preceded by a potential ribosome-binding site (underlined in Fig. 2) with strong homology to the consensus ribosome binding-site of *E. coli* (Shine and Dalgarno 1974). We tentatively assigned this codon as the ORF3 initiation codon. ORF3 encodes a 295-amino acid polypeptide with a calculated molecular mass of 33,401 Da. This polypeptide was compared with protein sequences from the Swiss-Prot database. Significant homology was found only with the deduced amino acid sequence of a *Rhizobium fredii* host-inducible gene (Sadowsky et al. 1988). Since no name has yet been assigned to this *R. fredii* host-inducible gene, we will refer to it as *R. fredii* ORF1. Amino acid sequence alignment indicates that the gene products of ORF3 and *R. fredii* ORF1 contain 37% identical residues and 8% conservative substitutions (Fig. 3). In *R. fredii,* a second host-inducible gene is located downstream from *R. fredii* ORF1 (Sadowsky et al. 1988). No such a gene was detected downstream from *R. l.* bv. *phaseoli* ORF3 (results not shown). Regions homologous to *R. fredii* ORF1 were reported in *Bradyrhizobium* strains but were not detected in *R. l.* bv. *trifolii*, *R. l.* bv. *viciae* or, contrarily to our results, in *R. l.* bv. *phaseoli* (Sadowsky et al. 1988).

R. fredii ORF1 as well as a second ORF located downstream from *R. fredii* ORF1 have been shown to be inducible in the presence of soybean root exudates (Olson et al. 1985). Analysis of the inducing substance indicated that it has properties of 4′-7-dihydroxyisoflavone, daidzein (Sadowsky et al. 1988). In addition, *R. fredii* ORF1 was also induced by several commercially available isoflavones and flavones (Sadowsky et al. 1988). These compounds are also involved in the activation of several nodulation (*nod*) genes in rhizobia (reviewed by Long 1989; Göttfert 1993). The inducible *nod*

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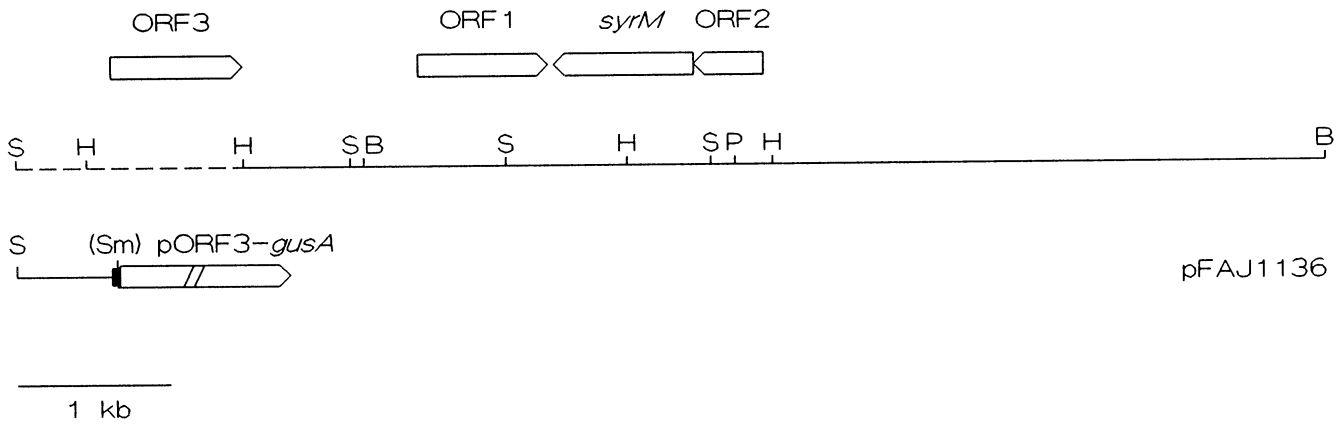

Fig. 1. Physical map of the ORF3 region of *Rhizobium leguminosarum* bv. *phaseoli*. The region that was sequenced is indicated as a dashed line. The structure of the *gusA* fusion is shown below the physical map. The blackened box represents the ORF3 coding region fused to *gusA*. The *gusA* gene is not drawn to scale. Restriction sites within parentheses were lost during cloning procedures. Restriction sites are: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; S, *Sal*I; Sm, *Sma*I. The *Sal*I restriction site at the left of the DNA segment that was sequenced originates from the polylinker of the original λ phage that was isolated from the λEMBL3 gene bank of CNPAF512.

genes are generally preceded by a promoter that contains a highly conserved DNA region, the so-called *nod* box. No such a promoter sequence was detected in front of *R. fredii* ORF1 or the ORF located downstream (Sadowsky et al. 1988). Instead, the 5′ regions of these genes contained a 17-bp conserved DNA region with 82% consensus. To test whether expression of the *R. l.* bv. *phaseoli* ORF3 was inducible by *Phaseolus vulgaris* root exudates or flavonoids, we constructed a transcriptional pORF3–*gusA* fusion. A 0.5-kb *Sal*I–*Sma*I fragment containing the promoter region and the 5′ end of ORF3 was first cloned into the *Sma*I site of pUC18, after blunting of the *Sal*I-digested DNA end with the Klenow polymerase fragment of *E. coli* polymerase. In this construct, the 5′ end of ORF3 was located near the pUC18 *Kpn*I restriction site. Next, the plasmid was digested with *Kpn*I and ligated to the 3.8-kb *Bam*HI fragment of pWM6 (Metcalf et al. 1993) containing a *uidA–aph* cassette, after blunting of the DNA ends of both fragments with T4 and Klenow DNA polymerases, respectively. From the construct carrying the *uidA–aph* cassette in the correct orientation, the pORF3–*uidA* (or pORF3–*gusA*) fusion was recloned as a 4.3-kb *Bam*HI–*Eco*RI fragment into the broad host range plasmid pLAFR3 (Staskawicz et al. 1987). The resulting plasmid was named pFAJ1136. pFAJ1136 was transferred into *R. l.* bv. *phaseoli* CNPAF512, and expression of the plasmid was monitored in the presence of *P. vulgaris* root exudates (*P. vulgaris* cv. Carioca and *P. vulgaris* cv. Ouro negro) or several commercially available flavonoids (Table 1). As a control plasmid we used pGUS32Km containing the *Rhizobium* sp. BR816 nodABC promoter fused to the *gusA* reporter gene (van Rhijn et al. 1994). From Table 1, it can be seen that expression of pGUS32Km was clearly induced in *R. l.* bv. *phaseoli* CNPAF512 in the presence of *P. vulgaris* root exudates as well as the flavonoids naringenin, genistein, kaempferol, and apigenin. No such an induction was observed in the *R. l.* bv. *phaseoli* wild-type strain CNPAF512 or in the ORF3 mutant strain Rp1001 (see further), containing pFAJ1136.

To determine the role of ORF3 during symbiosis, a *R. l.* bv. *phaseoli* ORF3 mutant was constructed (Fig. 4). A 2-kb *Sal*I fragment containing the complete ORF3 sequence was cloned

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   1  GTCGACCCAGATCTGGGTCGACCTGCAGGTCAACGGATCAGGAGGAGACGAGGAGCGGCT
  61  GGAGGCGGTAAGACGAACTATAACGTGGATGGACGCTGGCGATGTTAATGCAACTACAAT
 121  CACTGCGGCTGGCCAAGCCGCCGGCACTGTTCCGCCAGAGCTCCAGAAGCTCGCGGACGA
 181  CAACCGGCTGTGAGCAGCTTCCCGAGCGGGTCCGGACGACGCTGGCAGAACCAGTGATCG
 241  CATCGGGCTATATGGGCCCGCAGTCGTCTTTCCGCCCAGTTCGCGGAAGTTTAGTCCCCT
 301  CTAGGCGAGACCCAGGAGCCGCGGCAGCAACCGCGTACCCCCCCCGCTTCATCGAAGCTT
 361  AGCAGTCACGTATCCGCTTTATAGGCCGGACATGTGAGCGCACCGTTCCCACATGTGAAG
 421  CGACTGAAAAGGATGCGCTTCATCACACCTTTCACTCTCAGGAGAGACCGCCATGGATAA
                                                      M  D  N
 481  CACAGAACGATCGCTGCGCTACAACATTTCCGAGGAAGATGATCGCACCCGGGCCATGGC
        T  E  R  S  L  R  Y  N  I  S  E  E  D  D  R  T  R  A  M  A
 541  GGGGTGGATCGAGAATATGACCGGGGCACAAACACTTACGGGCATGCCGTCCCGATCAAC
        G  W  I  E  N  M  T  G  A  Q  T  L  T  G  M  P  S  R  S  T
 601  GTCAGATCCCCATGCTCGTTCGCCTTCGTCGCCAACGCGGCCATACTCGCTCTCTTCCGA
        S  D  P  H  A  R  S  P  S  S  P  T  R  P  Y  S  L  S  S  E
 661  ACCTCCAATTAAGGTGCTCGACAGATCGACATTTGCCAGAAAAGTGAGGGAATTTTACGG
        P  P  I  K  V  L  D  R  S  T  F  A  R  K  V  R  E  F  Y  G
 721  TGATGATATCAAGCACATTTCCGTCAATCCACAAGAGTACTCGCCCTTCGTGTCCTCTAA
        D  D  I  K  H  I  S  V  N  P  Q  E  Y  S  P  F  V  S  S  K
 781  AGCCCTGCGTACGGCTACCATCGCTCGACTCTACGGCGGCACCGACAATGATACGCCCAG
        A  L  R  T  A  T  I  A  R  L  Y  G  G  T  D  N  D  T  P  S
 841  CGCGCGATATTTCAGCTATGATCTGGGAGAACGAAGCGTTGGGCTTTTAAGAACAGAGGA
        A  R  Y  F  S  Y  D  L  G  E  R  S  V  G  L  L  R  T  E  D
 901  TGGATTTAGCATCGAGAACGAGCCGTGGCGGGAACTTTTTCCCGGTCGGAAAAGAATCAC
        G  F  S  I  E  N  E  P  W  R  E  L  F  P  G  R  K  R  I  T
 961  TTCGATTGTAGACCTTCGCGTTACTCATCCGCTTGTCGAGAACGCAGGCGATATTCTGCT
        S  I  V  D  L  R  V  T  H  P  L  V  E  N  A  G  D  I  L  L
1021  CGAACATCCAACTTCAACTCGACGGCGAACAGCCGTTAATCATGTCACGTCCCGTTCGCC
        E  H  P  T  S  T  R  R  R  T  A  V  N  H  V  T  S  R  S  P
1081  AGAGATGGGAGCCCGTCTAGAGCAGATGGGTTTCGTTGACGTGGGTCAAAATCGCTGGGT
        E  M  G  A  R  L  E  Q  M  G  F  V  D  V  G  Q  N  R  W  V
1141  GCTCGACCCTACGCAACATCCGGACAAGTGGACAAAGAATAGCAAGGGTGGATGGCAGCG
        L  D  P  T  Q  H  P  D  K  W  T  K  N  S  K  G  G  W  Q  R
1201  CGCAGGCAAACCTTGGAGTTATCTCTCGAAAACCGTGGACTATCTTAGCGACGATGAAGC
        A  G  K  P  W  S  Y  L  S  K  T  V  D  Y  L  S  D  D  E  A
1261  AGGTGTCGAGGGGCATTCGAGTGAAGCCAGTGTCGAGACAGATTCATCCGATGATGACCC
        G  V  E  G  H  S  S  E  A  S  V  E  T  D  S  S  D  D  D  P
1321  CTCTTGGTACTTCGAACACCTCAATTTGAACCGCGAATAAAAGCTT
        S  W  Y  F  E  H  L  N  L  N  R  E  -
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Fig. 2. Nucleotide sequence of a 1,366-bp *Sal*I-*Hin*dIII fragment containing *Rhizobium leguminosarum* bv. *phaseoli* ORF3. Appropriate subclones of this fragment were constructed in pUC19. Double-stranded DNA was sequenced according to the dideoxynucleotide chain-termination method as described by Sanger et al. (1977). DNA sequences were run on an automated sequencer (A.L.F., Pharmacia-LKB). Sequences were analyzed using the software package of PCGENE (IntelliGenetics Inc., Mountain View, California). The predicted amino acid sequence of ORF3 is shown below the DNA sequence.

into *Sal*I digested pSUP202-1 (*Hin*dIII site of pSUP202 [Simon et al. 1986] filled in with Klenow polymerase, Michiels et al. 1991). This plasmid was digested with *Xba*I and ligated to the 2.3-kb *Bam*HI–*Hin*dIII fragment originating from pGV910 (Van den Eede et al. 1992) and conferring resistance to spectinomycin (30 μg ml^{-1}, Spc) and streptomycin (100 μg ml^{-1}, Str), after blunting of the DNA ends of both fragments with the Klenow polymerase fragment of *E. coli* polymerase. To construct a deletion derivative, this plasmid, pFAJ1115, was digested with *Bam*HI and *Hin*dIII, blunt-ended with Klenow and religated, yielding pFAJ1116. The

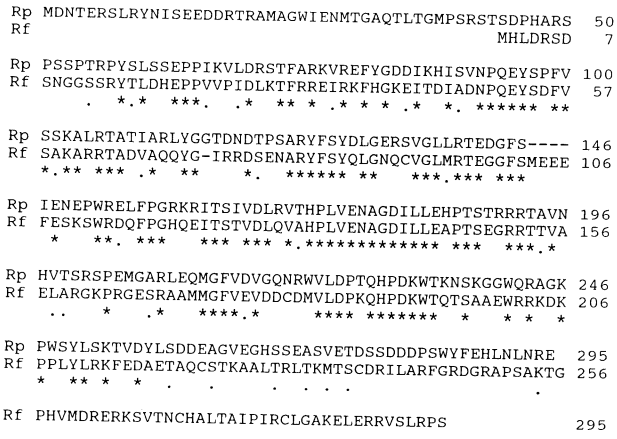

Fig. 3. Amino acid alignment of the *Rhizobium leguminosarum* bv. *phaseoli* ORF3 (Rp) and the *R. fredii* ORF1 (Rf) encoded proteins. The sequence alignment was carried out with the program PALIGN. Identical amino acids are indicated by asterisks and positions with similar residues (S-T-A; L-V-I-M; K-R; D-E; Q-N; F-Y-W) are marked with dots.

selected plasmid carried the Str/Spc resistance gene in the correct orientation (see Fig. 4) and contained a deletion of the carboxy-terminal 0.7-kb *Xba*I–*Hin*dIII fragment of ORF3. The entire insert of plasmid pFAJ1116 was then removed as a 4-kb *Sal*I–*Cla*I fragment and ligated into *Eco*RI-digested pSUP102 (Simon et al. 1986), after blunting of the DNA ends of both fragments. This plasmid was called pFAJ1117. Finally, the 3.8-kb *Bam*HI fragment of pMH1701 (Hynes et al. 1989) containing the *nptI-sacB-sacR* genes was blunt ended and ligated into pFAJ1117, which had been linearized with *Eco*RI and blunt-ended with Klenow, yielding pFAJ1127. The *sacB* gene confers sucrose sensitivity on gram-negative bacteria. Next, pFAJ1127 was recombined into ORF3 of the *R. l.* bv. *phaseoli* strain CNPAF512. Transconjugants were isolated on TY medium containing nalidixic acid (30 μg ml^{-1}, Nal), Spc, and 5% sucrose. Double recombinants were isolated as sucrose-resistant tetracycline-sensitive transconjugants. These transconjugants were further analyzed by Southern blot hybridization. One of the isolated double recombinants was named Rp1001.

The constructed mutant strain (Rp1001) was tested for nodulation (Table 2, Fig. 5), nitrogen fixation (Table 2), and competition for nodulation (Table 3). Plants inoculated either with Rp1001 or the wild-type strain were nodulated equally as judged from plant nodule numbers and plant nodule dry weight, indicating that ORF3 is not essential for nodulation. Similarly, the inactivation of ORF3 did not result in a reduction of the symbiotic nitrogen fixation activity when tested 13 days after inoculation (Table 2).

The mutant strain was also tested for its competitive capacities for nodule formation against the wild type. From Table 3 it can be seen that in mixed inoculation experiments Rp1001 is always outcompeted by the wild-type strain. At a 1:1 ratio of viable cells, the wild-type strain occupied 93% or more of the nodules formed. Since growth was only scored on medium containing Nal or Nal and Str, we were unable to distinguish between single (mutant alone) or double (mutant and wild-type) infections. Therefore the percentage of the nodules occupied solely by Rp1001 is most likely considerably lower than the values indicated in Table 3. Data obtained

Table 1. Expression of p*nodABC-gusA* (pGUS32Km) and pORF3-*gusA* (pFAJ1136) fusions in *Rhizobium leguminosarum* bv. *phaseoli* CNPAF512 and Rp1001 grown in the presence of root exudates or flavonoids

Root exudate (RE)[b] or flavonoids[c]	β-Glucuronidase activity (units)[a]		
	CNPAF512 (pGUS32Km)	CNPAF512 (pFAJ1136)	Rp1001 (pFAJ1136)
RE *P. vulgaris* 'Carioca'	177	15	17
RE *P. vulgaris* 'Ouro negro'	81	14	20
Naringenin	348	12	16
Quercetin	34	14	16
Kaempferol	154	14	15
Apigenin	84	13	16
Genistein	135	11	15
No inducer	36	15	17

[a] The expression tests were carried out as described by van Rhijn et al. (1994). Data are the means of three replicates. Units were calculated as defined by Miller (1972).
[b] Root exudates were prepared according to van Rhijn et al. (1994).
[c] Flavonoids were used at a concentration of 500 nM.

at a low Rp1001/wild-type CNPAF512 ratio in the inoculum mixture were unreliable because of a low background nodulation activity of Nal^r Str^r contaminating strains.

To further analyze the strongly diminished competitiveness of the ORF3 mutant, we examined several bacterial phenotypes that have been previously implicated in nodulation competitiveness. Upon examination of bacterial motility and bacterial proliferation on the roots of *P. vulgaris* plants, no differences between the wild-type and the ORF3 mutant strain were observed (data not shown). However, both strains differed with respect to their speed of nodulation (Fig. 5). Rp1001 exhibits a delay in nodulation of approximately 2 days as compared to the wild-type strain. The average number of nodules per plant of the mutant strain 6 or 7 days after inoculation differed significantly from that of the wild-type strain (Student's *t* analysis at $t = 0.05$). Similarly, at day 7 the average plant nodule dry weight of plants inoculated with Rp1001 was at least 3 times lower than the controls. From day 8 postinoculation, no differences in average nodule number or nodule dry weight were observed between both strains.

The competitive properties of a strain are determined by various bacterial characteristics including the host range of the bacteria, bacterial motility, the production of bacteriocins, the speed of nodulation and cell surface characteristics (reviewed by Triplett 1990). In *R. l.* bv. *phaseoli*, at least some of the genetic information affecting the competitiveness is present on megaplasmids other than the symbiotic plasmid (Brom et al. 1992). Although the exact biological function of *R. fredii* ORF1 is unknown, inactivation of this gene strongly reduces the competitive capacity of the bacteria (Sadowsky et al. 1988). In paired competition studies with the *R. fredii* wild-type strain, the *R. fredii* ORF1 mutant strain occupied approximately 1% of the nodules formed. However, the *R. fredii* mutant strain (3F1) used in these competition studies contained, in addition to a MudI(Kan, *lac*) insertion in *R. fredii* ORF1, a deletion covering at least a *nif* and a *nod* region (Olson et al. 1985), making it difficult to correlate the MudI(Kan, *lac*) insertion and the observed phenotype. In *R. l.* bv. *phaseoli*, the ORF3 gene is clearly important for nodulation competitiveness since the corresponding mutant was

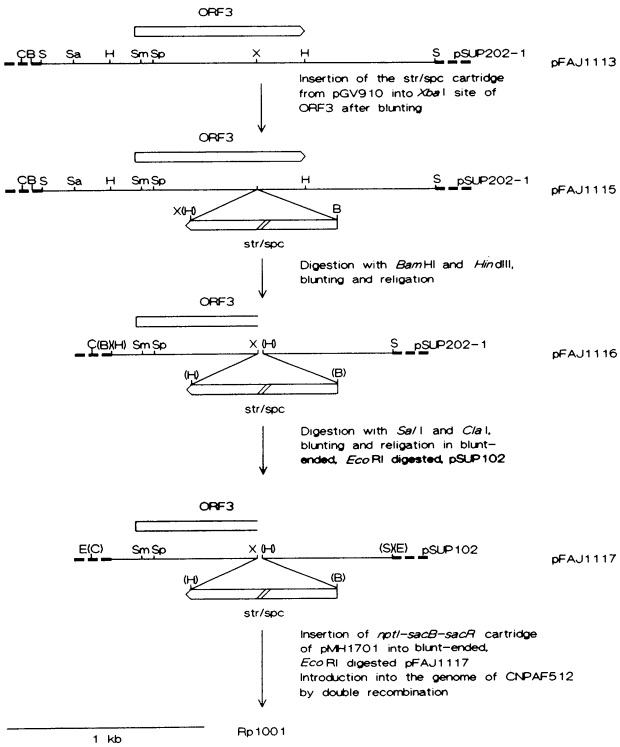

Fig. 4. Construction of the *Rhizobium leguminosarum* bv. *phaseoli* ORF3 mutant Rp1001 (see also text). *R. l.* bv. *phaseoli* DNA is represented by thin lines, plasmid DNA is represented by a thick line. Plasmid DNA and the str/spc cartridge are not drawn to scale. Plasmid numbers are given in the right margin. Restriction sites are: B, *Bam*HI; C, *Cla*I; H, *Hin*dIII; S, *Sal*I; Sa, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; X, *Xba*I. Restriction sites within parentheses were lost during cloning procedures.

Table 2. Phenotypical characterization of the *Rhizobium leguminosarum* bv. *phaseoli* wild-type strain CNPAF512 and the ORF3 mutant strain Rp1001 during symbiosis[a]

	Strain	
	CNPAF512	**Rp1001**
Nodule number (number/plant)	148 (31)	168 (51)
Nodule dry weight (g/plant)	0.028 (0.006)	0.032 (0.006)
ARA (μmol of C_2H_2 $plant^{-1}$ h^{-1})	7.33 (0.96)	7.03(0.80)

[a] All plant experiments were conducted in a randomized complete block design. Standard deviations are given within parentheses. *Phaseolus vulgaris* 'Carioca' seeds were surface sterilized by immersion in 95% ethanol for 30 s followed by an acidified solution of 0.2% $HgCl_2$ (wt/vol) for 1.5 min and finally were washed with 10 changes of sterile distilled H_2O. The seeds were germinated in the dark for 3 days on 1.0% water agar plates. Individual seedlings were transferred aseptically to 250-ml cylindrical flasks containing 150 ml of Jensen medium in 1.2% (wt/vol) agar slants and 60 ml of distilled water. Seedlings were inoculated with approximately 1×10^8 cells and grown in the growth chamber at 25°C with a 12-h photoperiod. Plant nodule number, nodule dry weight and acetylene reduction activity (ARA) were determined 13 days after inoculation. Values are the means of seven different plants. All uninoculated controls were devoid of nodules.

severely outcompeted by the wild-type strain in competition tests.

In contrast to *R. fredii* ORF1, the expression of *R. l.* bv. *phaseoli* ORF3 is not induced in the presence of the root exudates of its host, *Phaseolus vulgaris*. This observation is in accordance with the fact that no *nod* box-like sequence nor

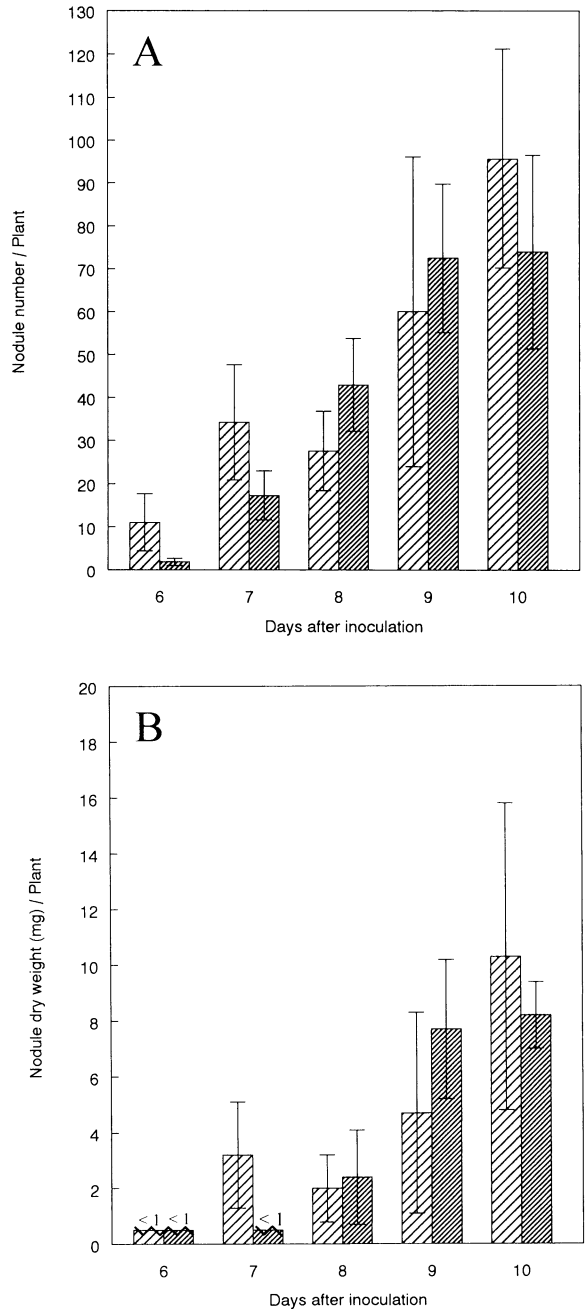

Fig. 5. Nodulation kinetics of the *Rhizobium leguminosarum* bv. *phaseoli* wild-type strain CNPAF512 and the ORF3 mutant Rp1001 on *Phaseolus vulgaris* cv. Carioca. The mean plant nodule number (**A**) and the average nodule dry weight (mg) per plant (**B**) are indicated as a function of time. Plants were grown as described in the footnote of Table 3 and harvested daily from the 6th day until the 10th day after inoculation. Data are the result of six independent tests. Vertical bars denote the standard deviations. Symbols: broad stripes, *R. leguminosarum* biovar *phaseoli* wild-type strain CNPAF512; narrow stripes, Rp1001.

sequences homologous to the *R. fredii* ORF1 5′ region were detected in the ORF3 promoter region. Recently, several bacterial genes (*nfe*, nodule formation efficiency) involved in competition for nodulation were characterized in *R. meliloti* and *Bradyrhizobium japonicum* (Soto et al. 1993; Chun and Stacey 1994). Inactivation of these genes also causes a delay in nodule formation. In *R. meliloti*, expression of the *nfe*

Table 3. Competition for plant nodulation between the *R. leguminosarum* bv. *phaseoli* CNPAF512 wild-type and the ORF3 mutant strain Rp1001.

Strains in the inoculation mixture (%)		Nodules occupied by Rp1001 (%)
CNPAF512	Rp1001	
100	0	3 (7)
57	43	26 (12)
50	50	7 (6)
13	87	54 (8)
0	100	97 (5)

[a] Competition experiments were carried out on plants grown in 250-ml cylindrical pots filled with a 1:2 (vol/vol) mixture of quartz sand and vermiculite. Pots were watered with a 1:2 strength N-free nutrient medium (1× medium contains K_2SO_4 435 mg l^{-1}, $MgSO_4.7H_2O$ 616 mg l^{-1}, $CaSO_4$ 272 mg l^{-1}, $CaHPO_4$ 360 mg l^{-1}, Fe-citrate 0.005 mg l^{-1}, H_3BO_4 2.86 mg l^{-1}, $MnCl_2.4H_2O$ 1.81 mg l^{-1}, $ZnSO_4.7H_2O$ 0.22 mg l^{-1}, $CuSO_4.5H_2O$ 0.08 mg l^{-1}, $H_2MoO_4.H_2O$ 0.02 mg l^{-1} and $CaCl_2$ 0.002 mg l^{-1}). Tests were replicated five times. The seedlings were inoculated with approximately 10^8 cells of parental and mutant strains in different ratio's of viable cells. The percentages of viable cells of the *R. leguminosarum* biovar *phaseoli* wild-type strain CNPAF512 and the ORF3 mutant strain Rp1001 in the inocula are indicated in the two columns at the left of the table. Percentages in the right colomn refer to the nodules containing Nal^r Str^r bacteria. Standard deviations are given within parentheses. The *R. leguminoasrum* biovar *phaseoli* wild-type strain CNPAF512 is naturally resistant against Nal (30 μg ml^{-1}), but sensitive to Str (100 μg ml^{-1}). Plants were analyzed 2 weeks after inoculation. Twenty nodules from each plant were collected randomly on the plant root and surface sterilized (Vincent 1970). The treated nodules were crushed on selective TY media containing either Nal (30 μg ml^{-1}) or Nal and Str (100 μg ml^{-1}). Plates were incubated at 30°C and growth was scored after 2 days.

genes was shown to be regulated by *nifA* and *rpoN* genes. The regulation of expression of the *nfeC* gene in *B. japonicum* is more complex and is controlled by two independently regulated promoters. One of these promoters is probably RpoN-dependent and is activated only in bacteroids. The second promoter is similar to the *E. coli* –35/–10 type of promoters and is expressed under aerobiosis. In contrast to the *nfe* genes in *R. meliloti* and *B. japonicum, R. l.* bv. *phaseoli* ORF3 is not preceded by a typical –24/–12, RpoN-dependent, type of promoter and is therefore probably not regulated by RpoN. Possibly the *R. l.* bv. *phaseoli* ORF3 is expressed only under specific conditions. Further analysis should clarify the mechanisms and signals that control the expression of ORF3.

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