

## Identification and cloning of nodulation genes from the stem-nodulating bacterium ORS571

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**Summary.** After random Tn5 mutagenesis of the stem-nodulating *Sesbania rostrata* symbiont strain ORS571, Nif<sup>-</sup>, Fix<sup>-</sup> and Nod<sup>-</sup> mutants were isolated. The Nif<sup>-</sup> mutants had lost both free-living and symbiotic N<sub>2</sub> fixation capacity. The Fix<sup>-</sup> mutants normally fixed N<sub>2</sub> in the free-living state but induced ineffective nodules on *S. rostrata*. They were defective in functions exclusively required for symbiotic N<sub>2</sub> fixation. A further analysis of the Nod<sup>-</sup> mutants allowed the identification of two *nod* loci. A Tn5 insertion in *nod* locus 1 completely abolished both root and stem nodulation capacity. Root hair curling, which is an initial event in *S. rostrata* root nodulation, was no longer observed. A 400 bp region showing weak homology to the *nodC* gene of *Rhizobium meliloti* was located 1.5 kb away from this *nod* Tn5 insertion. A Tn5 insertion in *nod* locus 2 caused the loss of stem and root nodulation capacity but root hair curling still occurred. The physical maps of a 20.5 kb DNA region of *nod* locus 1 and of a 40 kb DNA region of *nod* locus 2 showed no overlaps. The two *nod* loci are not closely linked to *nif* locus 1, containing the structural genes for the nitrogenase complex (Elmerich et al. 1982).

**Key words:** Nitrogen fixation – Stem nodulation – Tn5 mutagenesis – *nod* genes – *nodC* homology

### Introduction

Gram-negative soil bacteria of the genera *Rhizobium* and *Bradyrhizobium* symbiotically associate with leguminous plants and induce the differentiation of new plant organs, the nodules. Inside the nodules the bacteria reduce molecular dinitrogen into ammonium which is made available to the plant host as a nitrogen source. With few exceptions the nodules are formed on the roots of the host plants. Some legume species, belonging to the genera *Neptunia*, *Aeschynomene* and *Sesbania*, however also bear aerial nodules on their stem (for a review, see Dreyfus et al. 1984). A very profuse stem nodulation is induced on the annual tropical legume *Sesbania rostrata* by a particular bacterial strain ORS571 (Dreyfus and Dommergues 1981). This plant/bacterium interaction presents some very interesting features.

Strain ORS571 induces effective nodules on both roots and stems of *S. rostrata*. The stem nodulation occurs at

predetermined sites, namely root primordia, that are evenly distributed on vertical lines all along the stem (Duhoux and Dreyfus 1982). The dormant root primordia can differentiate either into nodules, when infected with strain ORS571, or into roots when immersed in water. Apart from this particular interaction with its plant host, strain ORS571 is unique among symbiotic N<sub>2</sub>-fixing bacteria because it can also fix N<sub>2</sub> in the free-living state and grow at the expense of N<sub>2</sub> as sole nitrogen source (Dreyfus et al. 1983; Gebhardt et al. 1984). Although it has a generation time similar to that of the fast-growing rhizobia, strain ORS571 is more closely related to the slow-growing bradyrhizobia (Jarvis et al. 1986). Recent results from an elaborate taxonomic study indicate that strain ORS571 actually represents a new genus (Dreyfus et al., in preparation), more closely related to the genus *Xanthobacter* than to the genus *Bradyrhizobium*.

To date the molecular genetic analysis of strain ORS571 has been focused on the study of genes essential for nitrogen fixation (*nif*). Three *nif* loci have been identified after random mutagenesis and direct screening for Nif<sup>-</sup> mutants on agar plates (Donald et al. 1986). One of these loci, *nif* locus 1, contains genes homologous to the *nifHDK* and the *nifE* genes of *Klebsiella pneumoniae* (Elmerich et al. 1982; Norel et al. 1985), and to the *nifA* regulatory gene of *Rhizobium meliloti* (Donald et al. 1986). We are interested in the study of the ORS571 nodulation genes, and in particular in genes specifically required for either root or stem nodulation. Indeed, the early interaction between ORS571 and *S. rostrata* is different for root versus stem infection. Root nodulation occurs, as in most legumes, via root hair curling and subsequent infection thread formation (Olsson and Rolfe 1985). Stem nodulation in contrast occurs via a mechanism related to the “crack entry” observed in a few tropical legumes, such as *Arachis hypogaea* (Chandler 1978). Stem nodulation starts by direct intercellular infection and proliferation of ORS571 in the intercellular space of the basal cells of the root primordia. Simultaneously, cortical cells are induced to dedifferentiate and the bacteria penetrate in the resulting meristematic cells via infection threads (Tsien et al. 1983; Duhoux 1984). The identification of genes essential for these two modes of infection may contribute to a better understanding of the mechanisms underlying infection via root hair curling or crack entry. Furthermore, given its different taxonomic position it will be informative to compare the ORS571 *nod* genes with those of the *Rhizobiaceae*.

**Table 1.** Bacterial strains and plasmids

Strain	Relevant characteristics	Source or reference
ORS571	Stem-nodulating strain of <i>Sesbania rostrata</i> ; new genus, new species	Dreyfus and Dommergues (1981); Dreyfus et al., in preparation
ORS571-1	Tn5 Nod <sup>-1</sup>	This work
ORS571-2	Tn5 Nod <sup>-2</sup>	This work
ORS571-14	Tn5 Nif <sup>-14</sup>	This work
<i>Rhizobium meliloti</i>		
1021	Str <sup>r</sup>	Meade et al. (1982)
<i>Escherichia coli</i>		
HB101	F <sup>-</sup> pro leu thi lacY Str <sup>r</sup> r <sup>-</sup> m <sup>-</sup> EndoI <sup>-</sup> RecA <sup>-</sup>	Boyer and Roulland-Dussoix (1969)
BHB2600	SupE <sup>+</sup> SupF <sup>+</sup> r <sub>k</sub> <sup>-</sup> m <sub>k</sub> <sup>+</sup> met <sup>-</sup>	Hohn (1979)
SM10	RP4-2-Tc::Mu integrated ( <i>tra</i> Km <sup>r</sup> ) <i>thi thr leu</i> SuIII <i>lac recA</i>	Simon et al. (1983)
Plasmid		
pBR325	Ap <sup>r</sup> Tc <sup>r</sup> Cm <sup>r</sup>	Bolivar (1978)
pHC79	Ap <sup>r</sup> Tc <sup>r</sup> λ cos	Hohn and Collins (1980)
pSUP2021	pBR325 mob::Tn5 Ap <sup>r</sup> Tc <sup>r</sup> Cm <sup>r</sup> Km <sup>r</sup>	Simon et al. (1983)
pLAFR1	Wide host range cosmid Tc <sup>r</sup>	Friedman et al. (1982)
pRK2013	rep ColeE1 Km <sup>r</sup>	Diitta et al. (1980)
pRS2	pBR322 containing <i>nifHDK</i> genes of ORS571	Elmerich et al. (1982)
pEK12	pBR322 containing <i>nodABC</i> of Rm41	Schmidt et al. (1984)
pJS209	pIN-II-A2 containing the <i>nodC</i> gene of Rm41	John et al. (1985)
pJS204	pIN-II-A2 containing the <i>nodA</i> gene and a portion of the <i>nodB</i> gene of Rm41	Schmidt et al. (1984)
pGMI149	pRK290 containing <i>nod</i> genes of Rm1021	Truchet et al. (1985)
pRG70	pLAFR1 containing <i>nod</i> locus 1 of ORS571	This work
pRG71	pHC79 with partial <i>SalI</i> insert from <i>nod</i> locus 1, containing Tn5 Nod <sup>-1</sup>	This work
pRG72	pBR325 with 18.5 kb <i>EcoRI</i> fragment, containing Tn5 Nod <sup>-1</sup>	This work
pRG701	pBR325 containing 12.7 kb <i>EcoRI</i> fragment of pRG70	This work
pRG7011	3 kb <i>EcoRI-BamHI</i> fragment of pRG701 in pBR325 <i>EcoRI-BamHI</i>	This work
pRG20	pLAFR1 containing <i>nod</i> locus 2 of ORS571	This work
pRG21	<i>SalI</i> fragment of <i>nod</i> locus 2 in pHC79, contains part (Km <sup>r</sup> ) of Tn5 Nod <sup>-2</sup>	This work

**Table 1.** (continued)

Plasmid	Relevant characteristics	Source or reference
pRG30		This work
pRG32		This work
pRG33		This work
pRG34		This work
pRG35		This work
	pLAFR1 clones overlapping <i>nif</i> locus 1	
pRG31	Partial <i>SalI</i> insert of <i>nif</i> locus 1 in pHC79; contains Tn5 Nif <sup>-14</sup>	This work
pRG801	7.35 kb <i>EcoRI</i> fragment subcloned in pBR325; contains homology to pRG701 insert	This work

In order to identify ORS571 nodulation genes we carried out a random Tn5 mutagenesis and screened the mutant bacteria for defective nodulation. In this paper we describe the isolation of two independent mutants which have lost all nodulation capacity. We report on the cloning and characterization of these *nod* loci and on the comparison by DNA hybridization to the common *nod* genes of the fast-growing rhizobia (Kondorosi et al. 1984).

### Materials and methods

**Bacterial strains and plasmids.** The bacterial strains and plasmids used in this study are listed in Table 1.

ORS571 cultures were grown at 37° C in LSR medium containing per litre: 5 g D-L sodium lactate; 5 g disodium succinate·6H<sub>2</sub>O; 1 g yeast extract; 1 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>; 0.1 g NaCl; 0.2 g MgSO<sub>4</sub>·7H<sub>2</sub>O; 1.67 g K<sub>2</sub>HPO<sub>4</sub>; 0.87 g KH<sub>2</sub>PO<sub>4</sub>; pH 6.8.

*R. meliloti* was grown at 28° C in TY medium (Beringer 1974); *Escherichia coli* cultures were grown in Luria broth medium (Miller 1972) at 37° C.

**Tn5 mutagenesis.** Late exponential growth phase cultures of *E. coli* SM10(pSUP2021) and strain ORS571 were mixed in equal proportions. One hundred microlitres of the mixture was plated on solid LSR medium and incubated overnight at 37° C. The cells were resuspended and plated on selective LSR medium with 100 µg/ml streptomycin and 100 µg/ml kanamycin. Sm<sup>r</sup> Km<sup>r</sup> ORS571 colonies were purified on the same medium. In order to distinguish between pSUP2021 cointegration and Tn5 transposition, the Sm<sup>r</sup> Km<sup>r</sup> colonies were replica-plated on LSR plates with 12.5 µg/ml chloramphenicol and on LSR plates with 7.5 µg/ml tetracycline. We never saw expression of the tetracycline resistance gene of pSUP2021 in *E. coli*. However, in ORS571 the gene was expressed and caused resistance to 7.5 µg/ml tetracycline. The carbenicillin resistance encoded by the pSUP2021 plasmid could not be used as a marker for cointegration because strain ORS571 itself is highly resistant to this antibiotic.

**Plate assay for auxotrophy and for free-living N<sub>2</sub> fixation.** ORS571 colonies were replica-plated onto minimal LSO medium plates (Elmerich et al. 1982) with or without 1 g/l (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> added as a nitrogen source. One percent agar-

ose (Sigma) was used to solidify these media.  $\text{NH}_4^+$ -containing plates were incubated in the air; plates without  $\text{NH}_4^+$  were incubated in a growth chamber at 37° C under a continuous flow atmosphere consisting of  $\text{N}_2:\text{O}_2$  (97:3 v/v). Auxotrophic mutants did not grow on either type of plates.  $\text{Nif}^-$  mutants grew perfectly well on the  $\text{NH}_4^+$  medium but showed very poor growth under  $\text{N}_2$  fixation conditions.

**Plant tests.** Root nodulation tests and reisolation of bacteria from surface-sterilized nodules were performed as described by Vincent (1970).

Nitrogenase activity in root nodules was assayed by measuring the reduction of acetylene to ethylene by gas chromatographic analysis as described by Hardy et al. (1968). Stem nodulation was assayed on *S. rostrata* plants by applying a drop of an exponentially growing ORS571 culture along the rows of well-developed root primordia.

**Root hair curling.** Light microscopic analysis of root hair curling was carried out as described by Vincent (1970).

**Triparental matings.** In order to mobilize pLAFR1 clones from *E. coli* to ORS571 or vice versa, triparental matings were carried out as described by Ditta et al. (1980) using pRK2013 as a helper plasmid. pLAFR1-containing ORS571 transconjugants were selected by plating the mating mixture on LSR agar plates with 500 µg/ml carbenicillin and 10 µg/ml tetracycline. To mobilize pLAFR1 clones back to *E. coli*, a rifampicin-resistant derivative of HB101 was used as the recipient strain and transconjugants were selected on LB plates with 100 µg/ml rifampicin and 10 µg/ml tetracycline.

**DNA preparation.** Total DNA of strain ORS571 and of *R. meliloti* 1021 was prepared according to Dhaese et al. (1979). In order to obtain clear lysates, bacteria were washed once in 0.4 vol. cold TES (50 mM Tris, 50 mM NaCl, 5 mM EDTA, pH 8.0), and once in 0.4 vol. cold TE (20 mM Tris, 20 mM EDTA, pH 8.0). The cells were resuspended in 0.2 vol. 50 mM glucose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, and treated with 20 mg/ml lysozyme at 37° C for 30 min. Pronase (preheated at 37° C) was added (500 µg/ml final concentration), and the cells were incubated for 30 min. Lysis was completed by adding SDS (2.5% final concentration) and incubating at 37° C.

For small-scale plasmid preparation the method of Birnboim and Doly (1979) was used. For large-scale plasmid isolation the scaled-up version of the same method was used followed by purification of the plasmid DNA by cesium chloride-ethidium bromide gradient centrifugation (Maniatis et al. 1982).

**DNA biochemistry and recombinant DNA techniques.** The standard techniques described by Maniatis et al. (1982) were used for digestion of DNA with restriction endonucleases (purchased from Boehringer, Mannheim, FRG), gel electrophoresis, Southern blotting, dephosphorylation of DNA, purification of DNA from agarose gels, ligation, and transformation of *E. coli*. For cloning of ORS571-1, ORS571-2, and ORS571-14 DNA in the cosmid vector pHC79, total ORS571 DNA was partially digested with *SaII* to yield a good representation of restriction fragments in the 20–30 kb size range. Ligation to *SaII*-cut pHC79, in vitro packaging, and transfection to *E. coli* HB101 were

as described by Maniatis et al. (1982). After transfection, *E. coli* HB101 colonies resistant to 25 µg/ml kanamycin and to 100 µg/ml carbenicillin were selected, in order to isolate ORS571 DNA segments mutated by Tn5 transposition. A pLAFR1 gene library of ORS571 DNA partially digested with *EcoRI* was provided by Dr. F. De Bruijn (Max-Planck-Institut für Züchtungsforschung, Köln, FRG), and introduced into *E. coli* BHB2600 by transfection and selection for *E. coli* colonies resistant to 10 µg/ml tetracycline.

**DNA hybridization.** DNA hybridization was carried out in a non-fat dry milk based reagent as a substitute for Denhardt's hybridization mix, according to Johnson et al. (1984). Filters were preincubated in 3 × SSC (0.45 M NaCl, 0.045 M sodium citrate·2H<sub>2</sub>O), 0.25% w/v non-fat dry milk (Gloria) for 2 h at 60° C. Hybridization was carried out overnight at 68° C in 3 × SSC, 0.25% w/v dry milk, 0.5% SDS, 1 mM Na<sub>2</sub>-EDTA. <sup>32</sup>P-labelled DNA probes were prepared by nick translation as described by Rigby et al. (1977). The blots were washed 6 times for 15 min at 68° C in 3 × SSC, 0.5% SDS, and a final time in 3 × SSC.

For low-stringency hybridization the same conditions were used but the hybridization and the washes were carried out at 60° C. Colony hybridization was carried out according to Maas (1983). The blots were exposed to X-ray films at –70° C with an intensifying screen.

## Results

### Random transposon mutagenesis of strain ORS571

For the mutagenesis of strain ORS571 we used the pSUP2021 vector (Simon et al. 1983) which carries a Tn5 transposon on an efficiently mobilizable pBR325 derivative. The limited host range plasmid pBR325 cannot be maintained as a separate replicon in ORS571. The Tn5-encoded resistance to streptomycin, which is not expressed in *E. coli* (Putnoky et al. 1983), is expressed in ORS571. Thus, after mating between *E. coli* SM10(pSUP2021) and ORS571, streptomycin was used both to eliminate the *E. coli* donor and to select, in combination with kanamycin, for Tn5-harboured ORS571 derivatives.  $\text{Sm}^r \text{Km}^r$  ORS571 transconjugants appeared at a frequency of about  $10^{-7}$ . Two thousand of these colonies were purified and screened for the presence of pSUP2021 vector-encoded markers, for auxotrophy and for free-living nitrogen fixation. They were all tested for nodulation capacity and symbiotic  $\text{N}_2$  fixation by inoculating the roots of sterile *S. rostrata* plantlets individually grown in test-tubes.

Approximately half of the  $\text{Sm}^r \text{Km}^r$  ORS571 colonies also expressed the vector-encoded resistance to chloramphenicol and/or tetracycline, indicating a cointegration of pSUP2021 in the ORS571 genome. The other half of the  $\text{Sm}^r \text{Km}^r$  colonies were sensitive to both chloramphenicol and tetracycline, and may have arisen by transposition of Tn5.

Auxotrophic mutants were found with a frequency of 1%. Some were deficient in nodulation or in symbiotic  $\text{N}_2$  fixation. These were not studied further. Among the non-auxotrophic ORS571 derivatives three major types of symbiotically defective mutants were found. A first group of four  $\text{Nif}^-$  mutants no longer fixed dinitrogen, either in the free-living or in the symbiotic state. They were easily recognized by their defective growth on solid medium when at-

mospheric N<sub>2</sub> was the nitrogen source. In root nodules, induced by these mutants, no acetylene reduction was detected. The nodulated *S. rostrata* plants showed a nitrogen-starved phenotype.

In a second group of seven Fix<sup>-</sup> mutants, the free-living N<sub>2</sub> fixation was not affected at all, but ineffective root nodules were formed, without measurable acetylene reduction and again the nodulated plants looked nitrogen-starved. These Fix<sup>-</sup> mutants are defective in functions exclusively required for symbiotic N<sub>2</sub> fixation. Finally, we found two mutants, strains ORS571-1 and ORS571-2, which did not nodulate *S. rostrata* roots. They were not affected in their free-living N<sub>2</sub> fixation capacity. These Nod<sup>-</sup> mutants formed the basis of our present study.

#### Features of Nod<sup>-</sup> mutants

The Nod<sup>-</sup> mutants showed a slightly different phenotype when inoculated on roots of *S. rostrata*. Strain ORS571-1 caused no noticeable response, whereas strain ORS571-2 reproducibly induced a faint swelling at the junction of the lateral roots to the main root, precisely at the position where root nodules are normally induced by the wild-type strain ORS571. No effect was observed with either mutant upon inoculation of *S. rostrata* stems. Light microscopic examination (data not shown) of the root hairs present near the origin of the lateral roots, revealed normal root hair curling in the presence of the Nod<sup>-</sup> mutant 2 and a beginning of infection thread formation. No root hair curling was observed in the presence of the Nod<sup>-</sup> mutant 1. An elaborate morphological study of the effect caused by these and other symbiotic ORS571 mutants on root hair curling and infection thread formation will be described separately (Dreyfus et al., in preparation).

Both Nod<sup>-</sup> mutants were sensitive to chloramphenicol and to tetracycline. Total DNA, digested with *Eco*RI, did not hybridize to nick-translated pBR325 DNA. With <sup>32</sup>P-labelled DNA of the Tn5-containing vector pSUP2021 as a probe, a single 18.5 kb DNA fragment was detected in Southern hybridization of total *Eco*RI-digested ORS571-1 DNA, corresponding to Tn5 transposition in a wild-type *Eco*RI fragment of about 12.7 kb. With strain ORS571-2 a 12 kb *Eco*RI fragment hybridized to the pSUP2021 probe, indicating transposition of Tn5 in an approximately 6 kb wild-type *Eco*RI fragment. By selecting for the Tn5-encoded resistance to kanamycin, the mutated DNA regions of strains ORS571-1 and ORS571-2 were cloned in *E. coli* plasmid vectors, for instance as partial *Sal*I inserts in the cosmid vector pHC79 (clone pRG71 in Fig. 1, clone pRG21 in Fig. 2) or as a single *Eco*RI fragment in pBR325 (clone pRG72 in Fig. 1). From a comparison of the physical map of these Tn5-containing clones with the map of clones complementing the Nod<sup>-</sup> mutations (see below) it was concluded that the Nod<sup>-</sup> phenotype of strains ORS571-1 and ORS571-2 was caused by the insertion of a Tn5 transposon (Figs. 1 and 2).

#### Cloning of nodulation genes

A pLAFR1 gene library of ORS571 DNA, constructed by Dr. F. De Bruijn (MPI, Köln), was used to select a clone which complemented the Nod<sup>-</sup> mutant ORS571-2. The pLAFR1 library was mobilized en masse from *E. coli* BHB2600 into ORS571-2 in a triparental mating with

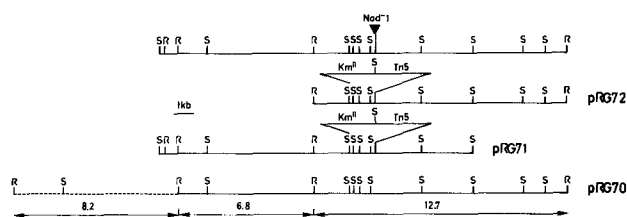


Fig. 1. Physical map of *nod* locus 1 of strain ORS571. The *Eco*RI (R) and *Sal*I (S) restriction map of a 20.5 kb DNA region surrounding the Nod<sup>-</sup>1 Tn5 insertion is presented together with the map of the inserts of clones pRG70, pRG71, and pRG72. pRG71 and pRG72 contain part of the mutated region of strain ORS571-1, cloned in the vectors pHC79 and pBR325, respectively. The 8.2 kb *Eco*RI fragment of the complementing pLAFR1 clone pRG70, indicated in dotted lines, was probably artificially joined to the rest of pRG70 during the cloning (see text)

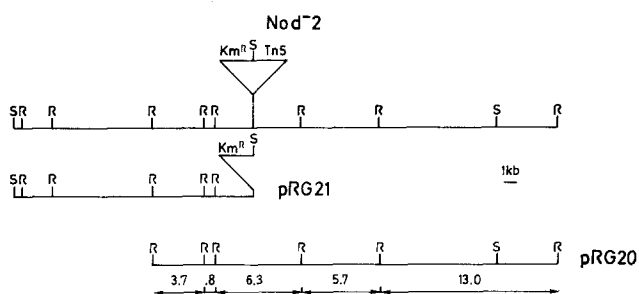


Fig. 2. Physical map of *nod* locus 2 of strain ORS571. A 40 kb DNA region surrounding the Nod<sup>-</sup>2 Tn5 insertion was characterized. The *Sal*I (S) and *Eco*RI (R) restriction maps of the inserts of clones pRG21 and pRG20 are presented. pRG21 contains part of the mutated region of strain ORS571-2. The pLAFR1 clone pRG20 complements the Nod<sup>-</sup> phenotype of ORS571-2

*E. coli* (pRK2013) as the source of a helper plasmid. About 3,000 Tc<sup>r</sup> ORS571-2 transconjugants were pooled and mass inoculated on *S. rostrata* roots. A few nodules appeared. Bacteria were reisolated from the surface-sterilized nodules and the pLAFR1 clone they contained was mobilized back to *E. coli* HB101 Rif<sup>r</sup> in a triparental mating, in order to facilitate its analysis. Thus, a clone, pRG20, was identified which contains five *Eco*RI fragments of 0.8, 3.7, 5.7, 6.3 and 13 kb, respectively (Fig. 2). Southern hybridization of <sup>32</sup>P-labelled pRG20 DNA to *Eco*RI-digested total DNA of the wild-type strain and of the strain ORS571-2 showed that the 6.3 kb fragment of pRG20 corresponds to the Tn5-containing *Eco*RI fragment in the Nod<sup>-</sup> mutant (data not shown). When pRG20 was introduced into strain ORS571-2, all Tc<sup>r</sup> transconjugants tested had regained the wild-type nodulation capacity. They induced normal N<sub>2</sub>-fixing nodules on both roots and stems of *S. rostrata*. No effect on the Nod<sup>-</sup> phenotype of strain ORS571-1 was observed upon introduction of pRG20.

A similar in vivo complementation experiment with the pLAFR1 gene bank in strain ORS571-1 did not yield any nodules. Therefore, we used the Tn5-harboring 18.5 kb *Eco*RI fragment of ORS571-1, cloned in pBR325 (clone pRG72 in Fig. 1) as a hybridization probe to select the corresponding wild-type sequence from the pLAFR1 gene library by colony hybridization. Of 384 colonies 1 hybridized very strongly, while several others showed varying degrees of hybridization. The latter clones contained one or

another of a series of repeated sequences present at several positions in the ORS571 genome (Goethals et al., unpublished results). The one, strongly hybridizing clone, pRG70, contained three *EcoRI* fragments of 6.8, 8.2 and 12.7 kb, respectively, that of 12.7 kb being the wild-type fragment homologous to the Tn5-harboured hybridization probe. When pRG70 was mobilized into ORS571-1, the nodulation capacity was restored. No effect of pRG70 was noticeable in Nod<sup>-</sup> strain ORS571-2. For an as yet unknown reason the mobilization frequency of clone pRG70 into several recipient strains was much lower than usual. When, for instance, pRG20 was mobilized into strain ORS571-1 or ORS571-2, Tc<sup>r</sup> transconjugants appeared with a frequency of  $5 \times 10^{-1}$  whereas the frequency of mobilization of pRG70 into the same recipient strains was 1,000 times lower. This feature might explain why pRG70 could not be selected by in vivo complementation of the nodulation deficiency after mobilization en masse of the pLAFR1 library into strain ORS571-1.

#### Physical characterization of the two *nod* loci

The *SalI* and *EcoRI* restriction maps of the two ORS571 DNA regions identified by Nod<sup>-</sup> Tn5 insertions are presented in Figs. 1 and 2. They are based on restriction analysis of Tn5-containing clones, isolated from the mutant strains, and of the corresponding complementing pLAFR1 clones.

A 20 kb DNA region (*nod* locus 1) surrounding the Nod<sup>-</sup> mutation of ORS571-1 was characterized. From a comparison of the overlapping regions of the partial *EcoRI* clone pRG70 and the partial *SalI* clone, pRG71, it followed that pRG71 contains an authentic piece of ORS571-1 DNA, and that the 6.8 kb and 12.7 kb *EcoRI* fragments of pRG70 are authentic adjacent fragments of the wild-type genome. The additional 8.2 kb *EcoRI* fragment of pRG70, however, most probably represents a cloning artefact. Indeed, the leftmost 2.4 kb *SalI* fragment of pRG71 is not present as such in pRG70 (Fig. 1). Moreover, Southern hybridization of total ORS571 DNA and pRG70 DNA digested with *SalI*, *HindIII*, or *BamHI* to <sup>32</sup>P-labelled DNA of the 6.8 kb *EcoRI* fragment of pRG70 clearly demonstrated that the 8.2 kb and 6.8 kb *EcoRI* fragments are not present as neighbouring fragments in the ORS571 genome as they are in clone pRG70 (data not shown). When *EcoRI*-digested total ORS571 DNA was hybridized against a pRG70 probe, three *EcoRI* fragments identical in size to those in pRG70 were detected. Therefore, we have no evidence for potential genomic rearrangements as the cause of the observed discrepancies; the most likely explanation is an artificial ligation of two independent *EcoRI* sites during the construction of the cosmid library.

A 40 kb DNA region from *nod* locus 2, corresponding to the Nod<sup>-2</sup> mutation was characterized. The clone pRG20 contains an authentic piece of ORS571 DNA derived from this region. Southern hybridization of *HindIII*, *BamHI*, and *SalI* digests of total ORS571 DNA and of pRG20 DNA to a <sup>32</sup>P-labelled pRG20 probe revealed fragments in the genomic digests identical in size to internal fragments of the pRG20 partial *EcoRI* insert (data not shown).

From a comparison of the physical map of *nod* loci 1 and 2 in Figs. 1 and 2 it follows that there are no restriction fragments common to these two loci. In addition, no cross-

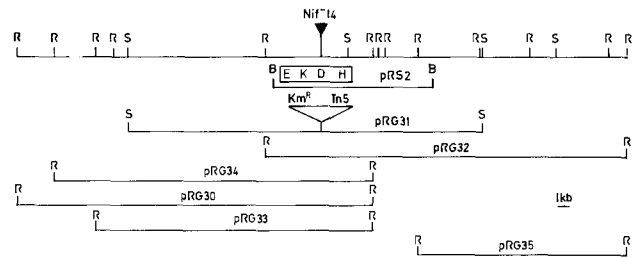


Fig. 3. Physical map of the *EcoRI* (R) and *SalI* (S) sites of a 50 kb DNA region of strain ORS571 containing *nif* locus 1. The partial *SalI* clone pRG31 contains the Nif<sup>-</sup> Tn5 insertion of strain ORS571-14. The clones pRG32, pRG34, pRG30, pRG33, and pRG35 contain partial *EcoRI* fragments in the vector pLAFR1. pRS2 contains ORS571 genes homologous to the *nifHDK* and the *nifE* genes of *Klebsiella pneumoniae* (Elmerich et al. 1982; Norel et al. 1985) in a 13 kb *BamHI* (B) fragment

hybridization was observed between the clones pRG20 and pRG70. The two Nod<sup>-</sup> mutations are separated from each other by at least 27 kb.

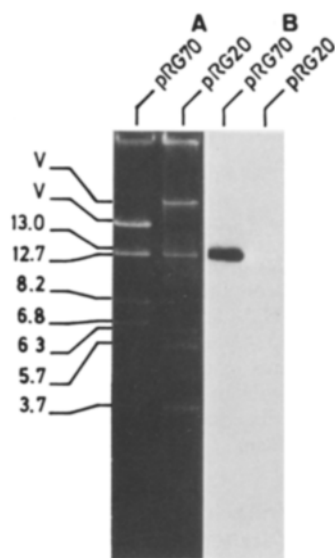
#### Location of the two *nod* loci relative to *nif* locus 1

The *nif* locus 1 of strain ORS571 contains the structural genes for the nitrogenase complex. The clone pRS2 was initially isolated by Elmerich et al. (1982) on the basis of homology to the cloned *nifHDK* genes of *K. pneumoniae*. pRS2 consists of a 13 kb *BamHI* fragment of ORS571 cloned in the vector pBR322. We used several approaches to isolate large clones derived from *nif* locus 1. From one of our randomly isolated Nif<sup>-</sup> mutants, strain ORS571-14, a partial *SalI* fragment was cloned in the cosmid vector pHC79 by selecting for the Tn5-encoded kanamycin resistance (clone pRG31). Restriction analysis of clone pRG31 in comparison with clone pRS2 (Fig. 3) showed that the Nif<sup>-</sup> 14 Tn5 insertion was located in the *nifD* gene, as mapped by Elmerich et al. (1982). The clone pRG30 (Fig. 3) was isolated by mobilization en masse of the pLAFR1 gene library into ORS571-14 and selecting for Nif<sup>+</sup> transconjugants. The clones pRG32, pRG33, pRG34, and pRG35 were selected from the pLAFR1 gene bank by colony hybridization using the 13 kb insert of pRS2 as a hybridization probe. This set of clones allowed the construction of a physical map of a 50 kb DNA region containing *nif* locus 1 (Fig. 3).

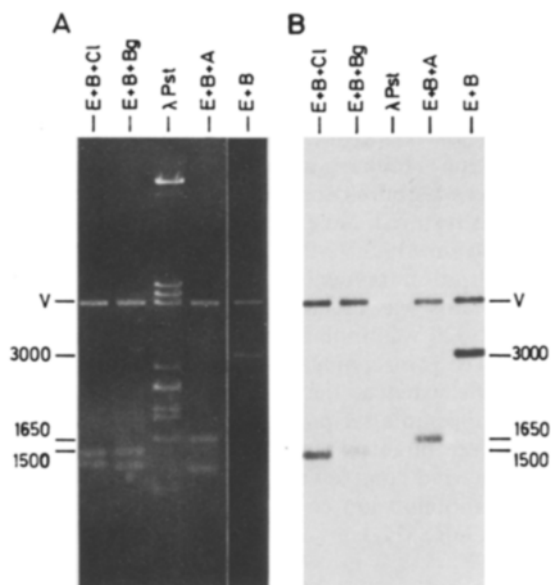
Comparison of the *EcoRI* and *SalI* restriction maps of *nif* locus 1 and *nod* loci 1 and 2 (Figs. 1–3) shows that there are no overlaps between these three regions, since there are no common restriction fragments. The Nod<sup>-1</sup> mutation is separated from the *nifHDK* genes by at least 35 kb, the Nod<sup>-2</sup> mutation by at least 42 kb. Up to now we have not obtained any evidence for the presence of large plasmids in strain ORS571. The symbiotic genes of strain ORS571 seem to be spread over a number of possibly widely separated loci on the chromosome.

#### Homology between *nod* locus 1 and the common *nod* genes of *R. meliloti*

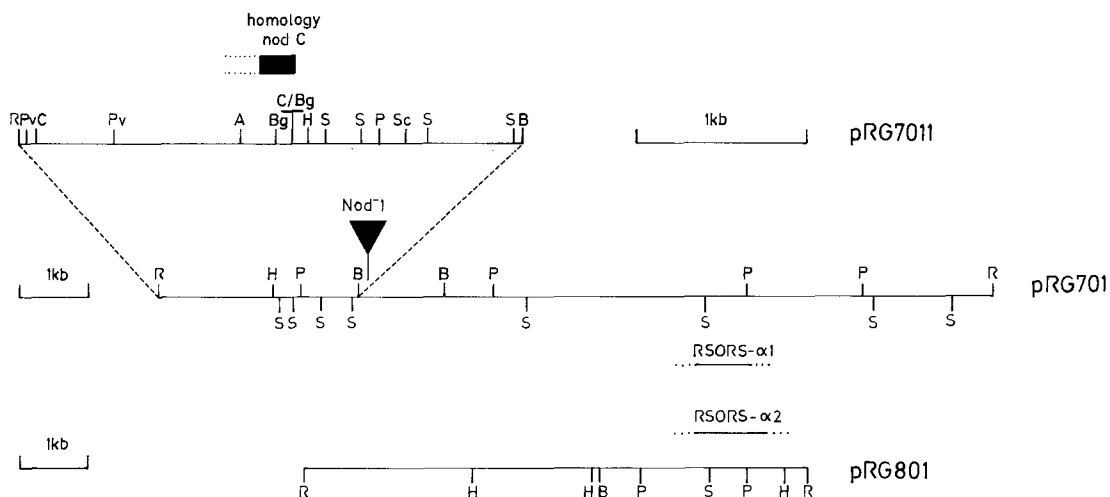
In order to investigate whether the *nod* loci of strain ORS571 are related to the common *nod* genes of *R. meliloti*, Southern-blotted *EcoRI* digests of the clones pRG20 and pRG70 were hybridized to the <sup>32</sup>P-labelled 3.5 kb *EcoRI*-



**Fig. 4A, B.** Homology of the 12.7 kb *Eco*RI fragment from *nod* locus 1 to the common *nod* genes of *Rhizobium meliloti*. **A** *Eco*RI digests of the pLAFR1 clones pRG70 and pRG20. The vector band (v) in pRG20 is larger than pLAFR1 and may consist of a cointegrate between pLAFR1 and the helper plasmid pRK2013. **B** Hybridization to the *Eco*RI-*Bam*HI insert of pEK12 containing the *nodABC* genes of *R. meliloti* 41 (Schmidt et al. 1984). The hybridization was carried out under low stringency at 60° C



**Fig. 5A, B.** Hybridization of pRG7011 digests to the *nodC* gene of *Rhizobium meliloti*. Several restriction enzyme digests of pRG7011 (**A**) were Southern-blotted and hybridized (**B**) at 60° C to the purified 1.8 kb *Eco*RI fragment from pJS209 (John et al. 1985). Contaminating pIN-II-A2 vector sequences in the probe are responsible for revealing the pBR325 band (V). E, *Eco*RI; B, *Bam*HI; Cl, *Cla*I; Bg, *Bg*III; A, *Apa*I



**Fig. 6.** Location of common *nod* homology and a copy of RSORS $\alpha$  in *nod* locus 1 of ORS571. A restriction map of the 12.7 kb *Eco*RI insert of pRG701 is presented with a more detailed extension of the 3 kb *Eco*RI-*Bam*HI fragment in which the homology to a *nodC* probe from *Rhizobium meliloti* was mapped (black box). A 7.35 kb *Eco*RI fragment from ORS571, cloned in pBR325 (pRG801), contains a copy of a repeated sequence RSORS $\alpha$ -2 that is also present in pRG701 (RSORS $\alpha$ -1). A, *Apa*I; B, *Bam*HI; Bg, *Bg*III; C, *Cla*I; H, *Hind*III; P, *Pst*I; Pv, *Pvu*II; R, *Eco*RI; S, *Sal*I; Sc, *Sac*I

*Bam*HI insert of the clone pEK12 (Schmidt et al. 1984) which contains the *nodABC* genes of *R. meliloti*. Under low stringency hybridization conditions, at 60° C in 3  $\times$  SSC, the probe hybridized only to the 12.7 kb *Eco*RI fragment of pRG70 (Fig. 4). A unique weakly hybridizing band of similar size was revealed in *Eco*RI-digested total ORS571 DNA (data not shown). No homology between pEK12 and pRG70 was detected when hybridization was carried out at 65° C. Subsequently, the homology to the *nodABC* probe was narrowed down to a 3 kb *Eco*RI-*Bam*HI subfragment. A detailed restriction map of this fragment, subcloned in

*Eco*RI-*Bam*HI-cut pBR325 (pRG7011; Fig. 6) was constructed and several digests were Southern-blotted and hybridized to two different probes. First, we used the *nodC*-containing 1.8 kb *Eco*RI subfragment from pJS209 (John et al. 1985). From Figs. 5 and 6 it can be seen that the rightward 1.65 kb *Apa*I-*Bam*HI fragment and the leftward 1.5 kb *Cla*I fragment from the pRG7011 insert contain *nodC* homologous sequences. The *nodC* homology is located in the overlap between these fragments. When pRG7011 was digested with *Eco*RI+*Bg*III+*Bam*HI, almost all hybridization signals disappeared. We concluded

that a 0.1 kb *Bgl*II fragment (which had run off the 0.8% agarose gel) contains the strongest homology. Preliminary sequence data confirmed this conclusion. Secondly, we used the *Eco*RI insert of pJS204 (Schmidt et al. 1984). This fragment contains *nodA* and part of *nodB* from *R. meliloti*. With this probe no conclusive hybridization results were obtained. Any potential sequence homology to *nod* locus 1 must be extremely weak, much weaker than with the *nodC* probe.

The  $\text{Nod}^{-1}$  mutation, which is located approximately 1.5 kb to the right of the *nodC* homology (see Fig. 6) was not complemented by pGMI149, a broad host range clone containing all *nod* genes from *R. meliloti* 1021 (Truchet et al. 1985).

## Discussion

As an initial approach in searching for nodulation genes in strain ORS571 we carried out a random Tn5 mutagenesis. Using the Tn5-carrying vector pSUP2021 from Simon et al. (1983) in ORS571, we obtained both true Tn5 transposition and vector cointegration in approximately equal proportions. Vector cointegration mediated by the entire Tn5 transposon or by its IS50-R element is a well-documented phenomenon in *E. coli*, where it is dependent on the *recA* product (Hirschel et al. 1982a, b). After the mutagenesis we screened not only for nodulation-defective ORS571 derivatives but also for mutants affected in the free-living and/or symbiotic  $\text{N}_2$  fixation capacity.  $\text{Nod}^{-}$ ,  $\text{Nif}^{-}$ , and  $\text{Fix}^{-}$  ORS571 mutants were isolated. The  $\text{Nif}^{-}$  mutants had lost both free-living and symbiotic  $\text{N}_2$  fixation capacity. One of them was located in *nif* locus 1, a region of the ORS571 genome that contains genes homologous to the *nifHDK* genes of *K. pneumoniae* (Elmerich et al. 1982; Donald et al. 1986). The map position of the other  $\text{Nif}^{-}$  mutants has not been determined.

The  $\text{Fix}^{-}$  ORS571 mutants fixed  $\text{N}_2$  normally in the free-living state but induced ineffective nodules on *S. rostrata*. Although we have not yet further characterized them, we want to point out the particular interest of this type of mutants and the great advantage of strain ORS571 for making the distinction between mutations affecting the  $\text{N}_2$  fixation process *sensu stricto* and mutations affecting other functions required exclusively for the symbiotic association. The latter functions may be involved in bacteroid differentiation, interaction with the plant host, or regulation of symbiotic  $\text{N}_2$  fixation.

In the present paper we concentrated on the characterization of two *nod* loci from strain ORS571, identified by two independent Tn5 insertions which caused the loss of both root and stem nodulation capacity. With strain ORS571-1, mutated in *nod* locus 1, no noticeable response of either stems or roots of *S. rostrata* was observed. The strain no longer induced the curling of the root hairs present near the origins of lateral roots on the host plant. A pLAFR1 clone, pRG70, was isolated which complements the  $\text{Nod}^{-1}$  mutation, and a physical map of a 20.5 kb DNA region surrounding the  $\text{Nod}^{-1}$  mutation was constructed. Subsequently, we investigated whether *nod* locus 1 shows any homology to the common *nodABC* genes of *R. meliloti* (Kondorosi et al. 1984). This set of three contiguous genes, together with an adjacent regulatory gene *nodD* (Mulligan and Long 1985) is structurally and functionally conserved in the very related fast-growing *Rhizobium* species *R. meli-*

*loti*, *R. leguminosarum*, *R. trifolii*, and *R. phaseoli* (Kondorosi et al. 1984; Török et al. 1984; Rossen et al. 1984; Djordjevic et al. 1985; Lamb and Hennecke 1986). Also, a more widely divergent fast-growing *Rhizobium* species, the wide host range strain MPIK3030, has been shown to contain a gene homologous to *nodC* of *R. meliloti* (Bachem et al. 1985). The presence of common *nod* genes has been demonstrated in several species of the slow-growing bradyrhizobia (Noti et al. 1985; Russell et al. 1985; Marvel et al. 1985; Lamb and Hennecke 1986). In strain ORS571, which is taxonomically sufficiently divergent from rhizobia and bradyrhizobia to be included in a new genus (Dreyfus et al., in preparation), we observed a weak homology to the *nodC* gene of *R. meliloti*. Under low stringency DNA hybridization conditions, a *nodC*-specific probe hybridized to a 400 bp sequence some 1.5 kb away from the  $\text{Nod}^{-1}$  mutation in *nod* locus 1. The hybridization was strongest to the rightmost 200 bp of this sequence. Homology to the *nodAB* genes of *R. meliloti* could not convincingly be demonstrated and if it exists at all in ORS571, it must be extremely weak and barely detectable. The  $\text{Nod}^{-1}$  mutation was not complemented by clone pGMI149 which contains the common *nod* genes of *R. meliloti* 2011 (Truchet et al. 1985). However, preliminary results from an extensive site-specific mutagenesis of *nod* locus 1 indicate that Tn5 insertions in the region of *nodC* homology affect the nodulation capacity of strain ORS571 and can be complemented by pGMI149 although not to the full wild-type  $\text{Nod}^{+}$  phenotype. The 12.7 kb *Eco*RI fragment of *nod* locus 1, in which the *nodC* homology and the  $\text{Nod}^{-1}$  Tn5 insertion are located, also contains a copy of two unrelated sequences that are repeated several times in the genome of ORS571. These sequences are not present however in *nod* locus 2 or in *nif* locus 1 (Goethals et al., unpublished results). Further characterization is needed in order to determine their nature and to investigate any possible role in symbiosis.

The second *nod* locus of strain ORS571 did not show any homology to the common *nod* genes and may contain genes comparable to the host-specific nodulation genes (*hsn*) of *R. meliloti* (Kondorosi et al. 1984). Strain ORS571-2, mutated in *nod* locus 2, induced a slight swelling at the junction of the lateral roots with the main root of *S. rostrata* and root hair curling still occurred. The physical map of a 40 kb DNA region containing *nod* locus 2 showed no overlap with *nod* locus 1. Neither of the two *nod* loci is closely linked to *nif* locus 1 of which we determined a physical map stretching 25 kb to the left and to the right of the *nifHDK* genes. As we never obtained evidence for the presence of large plasmids in strain ORS571, we assume that *nod* locus 1, *nod* locus 2, and *nif* locus 1 of ORS571 are located on the chromosome, an organization which may reflect the closer relatedness of strain ORS571 to the bradyrhizobia than to the fast-growing rhizobia.

At present, we are carrying out an extensive site-specific mutagenesis of the *nod* loci of strain ORS571 and are comparing the effect of mutations on the interaction of ORS571 with stems and roots of *S. rostrata*.

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