Rhizobium tropici nodulation factor sulfation is limited by the quantity of activated form of sulfate

Rémy Poupot[a], Esperanza Martinez-Romero[b], Fabienne Maillet[c], Jean-Claude Prome[a,*]

[a]*Laboratoire de Pharmacologie et Toxicologie Fondamentales, CNRS, 205 route de Narbonne, 31077 Toulouse Cedex, France*
[b]*Departamento de Genética Molecular, Centro de Investigacion sobre Fijacion de Nitrogeno Universidad Nacional Autonoma de Mexico, Ap. P 565-A Cuernavaca, Morelos, Mexico*
[c]*Laboratoire de Biologie Moléculaire des Relations Plantes-Microorganismes, CNRS-INRA, BP 27, 31326 Castanet-Tolosan Cedex, France*

Received 6 April 1995; revised version recived 8 June 1995

Abstract ***Rhizobium tropici*** **is a broad host-range symbiont of** ***Phaseolus vulgaris*****. This bacterium produces a mixture of sulfated and non-sulfated *N*-methylated pentameric nodulation (Nod) factors. To understand the genetic bases of the partial sulfation of *R. tropici* Nod factors, which might be involved in the broad host-range of this species, we introduced in *R. tropici* CFN299 the recombinant plasmid pGMI515 carrying a set of nodulation (*nod*) genes of *R. meliloti*, including those involved in the sulfation of *R. meliloti* Nod factors. The CFN299 (pGMI515) transconjugant produced only sulfated Nod factors, but approximately half of them were no more *N*-methylated. Mutations in *R. meliloti nodH* gene did not decrease the Nod factor sulfation whereas inactivation of the *nodPQ* genes restored the production of a mixture of sulfated and non-sulfated molecules. These results suggest that the limiting step in *R. tropici* Nod factor sulfation is the production of activated sulfate donors. Mutations in the *R. meliloti nodFEG* and *nodH* genes did not change the *N*-methylation pattern, whereas mutations in *nodPQ* increased the degree of *N*-methylation, suggesting a metabolic link between sulfation and methylation of *R. tropici* Nod factors.**

Key words: Host-specific Nod gene; Nod factor; *Rhizobium tropici*; Sulfation

1. Introduction

Symbiotic bacteria of the genera *Azorhizobium*, *Bradyrhizobium* and *Rhizobium* specifically trigger root- or stem-nodule organogenesis on leguminous host plants. In these newly developed organs, infecting bacteria, under a bacteroid differenciated form, reduce atmospheric nitrogen in ammonia [1].

Recognition between the symbiotic organisms and bacterial infection of the host plant, resulting in nodule organogenesis, are based on a chemical dialogue between prokaryotic and eukaryotic partners [2]. Legume roots secrete and exude flavonoids which regulate expression of the nodulation (*nod* or *nol*) genes of the bacterium [3] that are involved in the biosynthesis and excretion of lipo-oligosaccharide molecules called nodulation (Nod) factors [2]. The common *nodABC* operon is responsible for the synthesis of the lipo-oligochitin backbone, and specific nodulation genes encode a variety of enzymes that are able to modify this basic core, making Nod factors plant-specific [4,5].

R. meliloti synthetizes sulfated nodulation factors [6,7], and the presence of the sulfate group is necessary for NodRm factors to induce nodule organogenesis on alfalfa, leguminous host of *R. meliloti* [8]. The sulfation pathway of NodRm factors has been elucidated [9]: three specific nodulation genes of the bacterium are implicated in the sulfation process. The *nodP* and *nodQ* genes (termed *nodPQ*) are involved in the synthesis of ATP-derived activated forms of sulfate, APS and PAPS [10,11]. PAPS is hypothesized to be the substrate of a sulfotransferase, encoded by *nodH* [9], which introduces a sulfate group in O-6 position of the reducing *N*-acetyl-D-glucosamine residue of NodRm factors [9,12].

In *R. meliloti* two copies of the specific *nodPQ* operon are present. One is borne on the pSymA megaplasmid, the other one on the second megaplasmid, pSymB [13]. Both loci are functional and are able to ensure the synthesis of activated forms of sulfate in the sulfation pathway of NodRm factors. Inactivation of a single copy of *nodPQ* leads to a partial sulfation of the NodRm factors, provoking an extension of the bacterial host range [9]. The authors suggested that in *nodPQ* mutants, a limiting step of sulfation is the decrease in APS and PAPS synthesis.

Previously, we have shown that the broad host range tropical bacterium *R. tropici* [14] (strain CFN299) produces partially sulfated nodulation factors [15]. This ability to produce a mixture of sulfated and non-sulfated Nod factors is believed to play a role in the broad host-range of this species. Only the sulfated forms of the NodRt factors induce nodule organogenesis in *Phaseolus vulgaris* (common bean) [16]. Genetically, *R. tropici* is not as well known as *R. meliloti*, *R. leguminosarum* biovar *viciae* or *B. japonicum*. In *R. tropici*, *nod* genes involved in the sulfation of Nod factors have not been identified so far.

Herein, we addressed the question of the sulfation pathway step that may be limiting in sulfation of NodRt factors. We introduced various combinations of the *R. meliloti nod* genes involved in the sulfation process into *R. tropici*. The pGMI515 plasmid, harboring a set of specific nodulation genes from the symbiotic plasmid of *R. meliloti* [17], was transferred in *R. tropici*. Our results indicate that in *R. tropici* limiting amount of activated form of sulfate is responsible for the partial sulfation of NodRt factors. On the contrary, the sulfotransferase activity of *R. tropici* is sufficient to enable a complete sulfation of NodRt factors. Moreover, we have found that the complete sulfation of NodRt factors interferes with the *N*-methylation

*Corresponding author. Fax: (33) 6117-5994.

Abbreviations: APS, adenosine 5′-phosphosulfate; HPLC, high pressure liquid chromatography; PAPS, 3′-phosphoadenosine 5′-phosphosulfate.

SSDI 0014-5793(95)00737-7

pathway of Nod factors, resulting in a severe default of *N*-methylation.

2. Materials and methods

2.1. Bacterial strains and culture conditions

All strains used in this study were derived from the wild-type strain *R. tropici* CFN299. pGMI 515 is a derivative of the self-transmissible RP_4 plasmid carrying around 26 kb from the symbiotic megaplasmid of *R. meliloti* [17]. It harbors host-specific *nod* genes (*nodE*, *nodF*, *nodG*, *nodH* and *nodPQ*) and two regulatory loci ($nodD_3$ and *syrM*) (Fig. 1).

The pGMI515 derivatives, carrying Tn5 insertions in the various *nod* genes of interest, are detailed in Table 1. These plasmids were conjugatively transferred into *R. tropici* from *E. coli* GMI3540 donors, the plasmid transfer being selected by tetracyclin (10 μg/ml) and the auxotrophic *E. coli* donors being counter-selected by growth on a minimum selective medium as previously described [9].

For Nod factor production, all the *R. tropioci* derivatives used in this study were grown in liquid minimal medium, in the same conditions as previously described [15]. Induction of *R. tropici* nodulation genes was achieved using 1.5 mM naringenin (4′,5,7-trihydroxyflavanone; Sigma). The *R. meliloti* nodulation genes, borne on the pGMI515 plasmid, did not need flavonoid induction insofar as they are constitutively expressed due to the presence in multicopy of the regulatory genes $nodD_3$ and *syrM*, cloned in the same plasmid [19].

All the *R. tropici* strains (the wild-type strain and derivatives) showed the same growth kinetics.

2.2. Purification of Nod factors

Four liters of cultures were centrifuged, sterilized by filtration (Millipore HA 0.45 μm), and the filtrate was neutralized with ammonia and extracted twice, first with 1 liter, then with 500 ml of butanol. The butanol extract, after vacuum evaporation, was washed in a water/ethyl acetate partition. Nod factors remained in the aqueous phase.

This crude extract was purified by HPLC on a semi-preparative C_{18} reversed-phase column (250 × 7.5 mm; Spherisorb ODS2, 5 mm; ColoChrom), using a water/acetonitrile gradient [15]. Each of the Nod factor containing fractions were further purified by HPLC on an analytical C_{18} reversed-phase column (250 × 4.6 mm, Spherisorb ODS1, 5 μm, ColoChrom), as described for NodRt-V (Me,S) factors [15]. Detection was done by UV absorbance at 220 nm.

2.3. Constituent and structural analyses

The structure of Nod factors purified from the different strains was achieved using the procedures described in [15]: molecules were hydrolyzed with 3 N HCl; sugars were derivatized as alditol acetates and fatty acid as methylester; 2-butyl glycosides were prepared to determine the series of the sugars; permethylation studies were done according to Ciucanu and Kerek and the partially methylated residues were analyzed as described [15].

2.4. Analytical methods

Gas chromatography analyses were done on a Girdel 30 gas chromatograph equipped with an OV1 bound capillary column (0.32 mm × 30 m, Spiral France).

Mass spectrometry studies were recorded on an AutoSpec Instrument (VG Analytical, Manchester, UK) fitted with a cesium bombardment source. The matrix was a (1:1 v/v) mixture of *m*-nitrobenzyl alcohol/glycerol, spiked either with 1% trichloracetic acid in water or with a solution of sodium iodide (1 mg/ml).

Gas chromatography coupled to mass spectrometry experiments were performed on a Hewlett-Packard 5989A mass spectrometer in electronic impact ionization mode.

NMR spectra were recorded on a Brücker AC-200 spectrometer (Karlsruhe, Germany), using 1 mg of sample dissolved in 0.5 ml of methanol (CD_3OD 99.95% from Euriso Top).

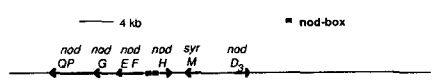

Fig. 1. *R. meliloti nod* genes and regulatory loci on the pGMI515 plasmid.

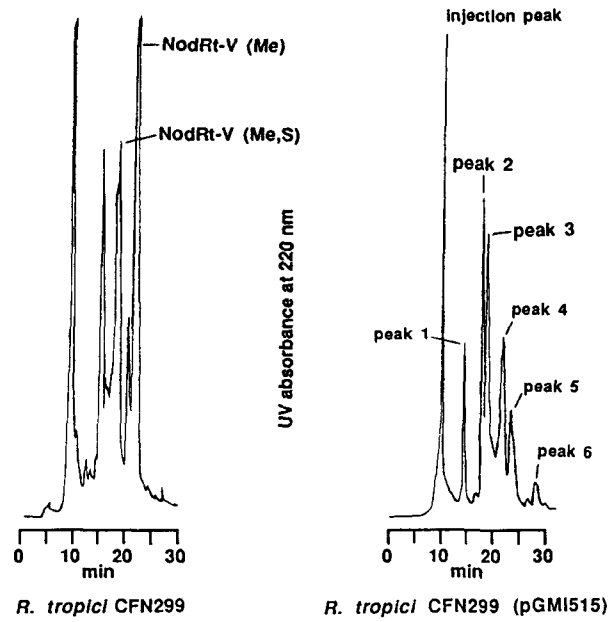

Fig. 2. Comparative semi-preparative C_{18}-HPLC chromatograms of the butanol extracts of the culture media of the wild type *Rhizobium tropici* CFN299 and the transformed strain *Rhizobium tropici* CFN299 pGMI515.

3. Results

3.1. R. tropici CFN299 (pGMI515) produced only sulfated Nod factors

After naringenin induction of nodulation genes, four liters of sterile culture medium from *R. tropici* CFN299 (pGMI515), carrying *R. meliloti nod* genes (Fig. 1), were extracted with butanol, followed by ethyl acetate washing, as already described [15]. Compounds of the washed aqueous phase were then purified by semi-preparative C_{18} reversed-phase HPLC. The chromatographic profile of the purified extracts was quite different from the *R. tropici* CFN299's one (Fig. 2). These differences were confirmed by fast atom bombardment-mass spectrometry analysis of the HPLC fractionation.

Only two peaks of the HPLC chromatogram contained Nod factors, detected by their classical fragmentation pattern: (a) peak three contained the sulfated NodRt-V (Me,S) factor identified by its $(M + H)^+$ ion at *m/z* 1350, whose structure had been elucidated as already described [15]; (b) peak two revealed the presence of a new NodRt factor identified by its $(M + H)^+$ ion at *m/z* 1336 (Fig. 3). This ion showed a first loss of 80 mass units at *m/z* 1256, corresponding to a sulfate group. This assignment was confirmed by cationization with the sodium ion, giving a $(M\text{-}H + 2Na)^+$ ion at *m/z* 1380. The spectrum in Fig. 3 showed clear fragmentations of the *N*-acetylglucosamine oligomeric core with successive losses of 221, then 203 mass units, ended at *m/z* 426, corresponding to the acylated glucosaminyl extremity of the molecule.

This difference of 14 mass units in all fragments of the mass spectrum, compared to the NodRt-V (Me,S) factor, suggested that the molecules had no *N*-methyl group at their non-reducing end. This hypothesis was confirmed by structural characterization of the molecules contained in peak two.

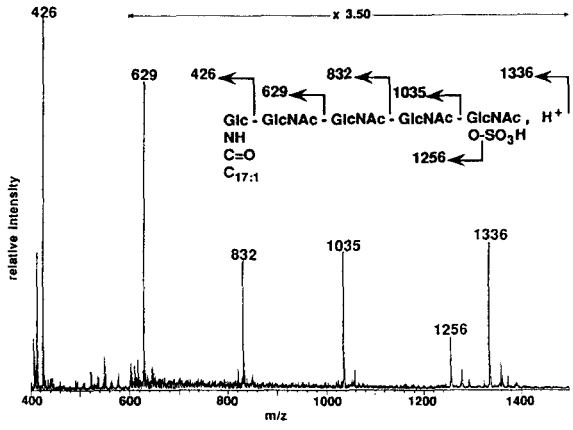

Fig. 3. Positive ion FAB mass spectrum of the NodRt-V (S) factor, with the corresponding fragmentations.

Complete hydrolysis of the molecules liberated only D-glucosamine and *cis*-vaccenic acid, detected in capillary gas chromatography as alditol acetate and methylester derivatives, respectively. Permethylation studies confirmed (1→4) glycosidic linkages between saccharidic residues [15]. ^{1}H NMR spectrum enabled the location of the sulfate group on O-6 position and showed no singlet at δ 3.00 ppm corresponding to the *N*-methyl group signal could be observed [15].

Thus, *R. tropici* CFN299 (pGMI515) produced two types of Nod factors after naringenin induction of its nodulation genes. The first one corresponded to the described NodRt-V (Me,S) factor [15], the second one to a new non-methylated signal called NodRt-V (S) (Fig. 4). Non-sulfated factors were not detected.

Four liters of culture filtrate from *R. tropici* CFN299 (pGMI515) yielded 4 mg of Nod factors, a similar amount than the wild-type strain *R. tropici* CFN299. Each type of Nod factors represented approximately half of the total production.

3.2. *Role of* R. meliloti *nod genes in the sulfation and methylation of* R. tropici *Nod factors*

R. tropici CFN299 carrying pGMI515 *nodH*$^-$ (= pGMI869) exhibited the same Nod factor production than the *R. tropici* CFN299 (pGMI515) strain. Thus, inactivation of the *R. meliloti* sulfotransferase gene does not change the degree of Nod factor sulfation.

Table 1
Bacteria and plasmids used in this study

Strain or plasmids	Relevant characteristics or genotypes	References
Strain		
CFN299	Nal^r	[14]
Plasmids		
pGM1515	*R. meliloti* hsn insert in RP$_4$ Tc^r, Ap^r	[17]
pGM1436	pGM1515 *nodQ* 115::Tn$_5$	[18]
pGM1869	pGM1515 *nodH* 2313::Tn$_5$	this work
pGM1435	pGM1515 *nodE* 2309::Tn$_5$	[18]
pGM1433	pGM1515 *nodF* 2407::Tn$_5$	[18]
pGM1870	pGM1515 *nodG* 957::Tn$_5$	[18]

In contrast, *R. tropici* CFN299 carrying pGMI515 *nodPQ*$^-$ (= pGMI436) mutant plasmid had a modified production of Nod factors. The main difference was the presence of non-sulfated molecules corresponding to the described NodRt-V (Me) factor [15], for about a half of the production, besides NodRt-V (Me,S) and NodRt-V (S) factors, with a quarter of the total production for each.

As it is known that *nodFE* genes of *R. meliloti* intervene in the biosynthesis of specific fatty acids transferred on the N-atom at the non-reducing end of NodRm factors [20], three other constructions have been studied: CFN299 transconjugants with *nodE*$^-$ or *nodFE*$^-$ or *nodG*$^-$ mutations in pGMI515 (= pGMI435, pGMI433 and pGMI870, respectively). These bacteria showed the same Nod factor production as CFN299 (pGMI515). Thus, the *nodPQ* genes of *R. meliloti* seem responsible for both the increased sulfation and decreased methylation of Nod factors.

4. Discussion

4.1. *Limiting step in NodRt factor sulfation*

The introduction of pGMI515 in *R. tropici* CFN299 caused the bacteria to produce an altered profile of Nod factors. While the original wild-type *R. tropici* strain produced a mixture of sulfated and non-sulfated molecules [15], the transconjugant produced only sulfated molecules. The total amount of secreted Nod factors was comparable to the level observed with the wild-type strain.

The analysis of Nod factors produced by this transconjugant strain, in comparison to other strain constructions harboring derivatives of pGMI515 with mutations in different host-specific nodulation genes (i.e. *nodH* and *nodPQ*), led us to conclude that the limiting step in the sulfation process of NodRt factors in *R. tropici* is the production of activated forms of sulfate (APS or PAPS), rather than a default of sulfotransferase activity.

R. meliloti has two copies of *nodPQ* [13], and single mutants in *nodPQ* produce both sulfated and non-sulfated molecules [9]. In this respect, *R. tropici* CFN299 resembles a *R. meliloti* *nodPQ*$^-$ mutant. Accordingly, only one copy of *nodPQ* had been found in *R. tropici* (unpublished results), suggesting that a gene dosage effect of *nodPQ* could be responsible for the production of a mixture of sulfated and non-sulfated Nod factors in *R. tropici*.

Another hypothesis could be that *nodD*$_3$ and *syrM* regulatory genes of *R. meliloti* are involved in the observed phenomena.

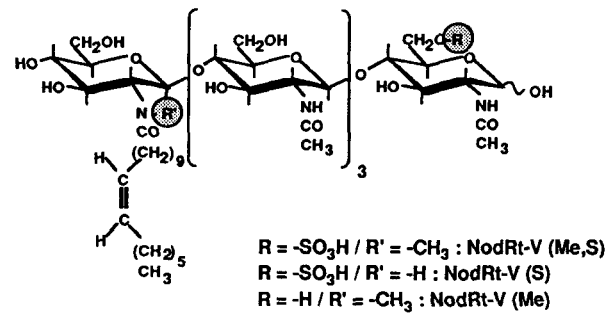

Fig. 4. Structural features of NodRt factors in this study: NodRt-V (Me,S); NodRt-V (S) and NodRt-V (Me).

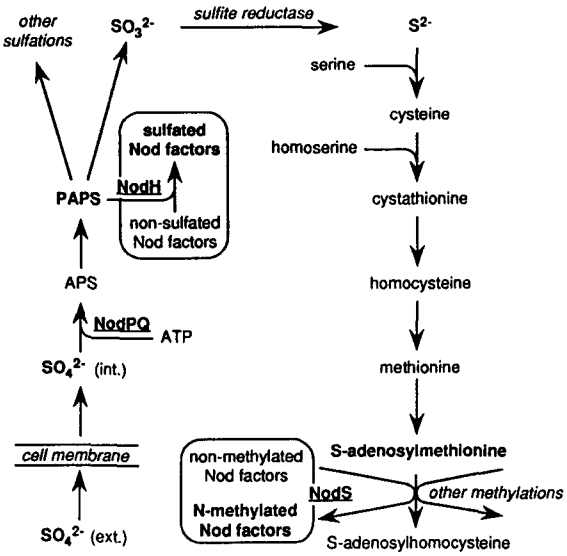

Fig. 5. Sulfated compounds involved in sulfation and *N*-methylation pathways in Nod factors biosynthesis, from [9] and [24].

When present, they could activate a higher expression of the endogenous *nodH* homolog of *R. tropici*. Against this putative involvement, primarily, it was recently shown that these regulatory genes are specifically responsible for the transcription control of the *nodFE* operon of *R. meliloti* [25]. Secondly, this could not explain why a mutation in *nodPQ* did not restore the wild type methylation pattern (see section 4.2 below).

R. strain NGR234 is the other bacteria producing both sulfated and non-sulfated Nod factors [21]. In this case, NodNGR234 factors bear a sulfate group or an acetate group, mutually exclusive, on the 2-*O*-methyl-L-fucose residue at the non-reducing end of the molecules. No homology between *R.* strain NGR234's nodulation loci and *nodH* or *nodPQ* genes of *R. meliloti* has been described yet, and there are no biochemical data about the duality in sulfation/acetylation of NodNGR234 factors.

4.2. *Metabolic link between Nod factor sulfation and methylation*

An unexpected alteration in the Nod factors produced by the transconjugant *R. tropici* CFN299 (pGMI515) was the decrease in the rate of *N*-methylation.

In *R. meliloti*, host-specific nodulation genes *nodE* and *nodF* [20], together with the common nodulation gene *nodA* [12,22], are responsible for the biosynthesis and the transfer of the C_{16} unsaturated fatty acids on the N-atom of the terminal non-reducing D-glucosamine residue of NodRm factors. The *nodFE* genes have been introduced in *R. tropici* CFN299 with pGMI515. Thus, one hypothesis was that these genes could interfere with the *N*-methylation of NodRt factors at the non-reducing end of the molecules. We have shown, however, that mutations in *nodFE* or in *nodG* genes did not modify the structure of Nod factors in the transconjugant strains. Thus, the *nodE*, *nodF* and *nodG* genes were not responsible for the decrease in Nod factor *N*-methylation observed in *Rhizobium tropici* CFN299 (pGMI515). The *nodH* gene was also found not to influence *N*-methylation. Only the *nodPQ* genes were shown to decrease the *N*-methylation level.

In *Azorhizobium caulinodans*, it has been shown that the *N*-methylation of NodARc factors [23] is under the control of the host-specific nodulation gene *nodS* [24]. The NodS protein is a methyltransferase, which specifically *N*-methylates NodARc factors, utilizing *S*-adenosylmethionine as methyl group donor. Thus, this *N*-methylation pathway requires sulfated intermediates.

If the same methylation process is operating in *R. tropici*, it is likely that the sulfated intermediate pool is not sufficient to ensure both a complete *O*-sulfation and *N*-methylation of Nod factors (Fig. 5). A competition should appear between the activation of oxidized (for *O*-sulfation) and reduced (for *N*-methylation) forms of sulfate in the sulfur metabolism of the bacterium. This could be the reason of the decrease in *N*-methylation observed in NodRt factors when they are fully sulfated. Nevertheless, this cannot explain why, in *Rhizobium tropici* CFN299 (pGMI515 *nodPQ*$^-$), sulfated Nod factors are only partly *N*-methylated whereas the total quantity of sulfated Nod factors is not higher than in *Rhizobium tropici* CFN299. Connections between sulfation and *N*-methylation pathways in Nod factor biosynthesis are now being investigated in the transconjugant strains of *R. tropici*.

Acknowledgements: We are grateful to J. Dénarié for his critical review of the manuscript. This work was financially supported by VLIR/ABOS grant from Belgium, and by CNRS, Université Paul Sabatier, Région Midi-Pyrénées and E.E.C. (Biotech and Science programs).

References

[1] Long, S.R. (1989) Cell 56, 203–214.
[2] Dénarié, J. and Cullimore, J. (1993) Cell 74, 951–954.
[3] Peters, N.K., Frost, J.W. and Long, S.R. (1986) Science 233, 977–980.
[4] Fisher, R.F. and Long, S.R. (1992) Nature 357, 655–660.
[5] Göttfert, M. (1993) FEMS Microbiol. Rev. 104, 39–64.
[6] Lerouge, P., Roche, P., Faucher, C., Maillet, F., Truchet, G., Promé, J.-C. and Dénarié, J. (1990) Nature 344, 781–784.
[7] Roche, P., Lerouge, P., Pontus, C. and Promé, J.-C. (1991) J. Biol. Chem. 266, 10933–10940.
[8] Truchet, G., Roche, P., Lerouge, P., Vasse, J., Camut, S., de Billy, F., Promé, J.-C. and Dénarié, J. (1991) Nature 351, 670–673.
[9] Roche, P., Debellé, F., Maillet, F., Lerouge, P., Faucher, C., Truchet, G., Dénarié, J. and Promé, J.-C. (1991) Cell 67, 1131–1143.
[10] Schwedock, J. and Long, S.R. (1990) Nature 348, 644–647.
[11] Schwedock, J.S., Liu, C., Leyh, T.S. and Long, S.R. (1994) J. Bacteriol. 176, 7055–7064.
[12] Atkinson, E.M., Palcic, M.M., Hindsgaul, O. and Long, S.R. (1994) Proc. Natl. Acad. Sci. USA 91, 8418–8422.
[13] Schwedock, J. and Long, S.R. (1989) Mol. Plant-Microbe Interact. 2, 181–194.
[14] Martinez-Romero, E., Segovia, L., Mercante, F.M., Franco, A.A., Graham, P. and Pardo, M.A. (1991) Int. J. Syst. Bacteriol. 41, 417–426.
[15] Poupot, R., Martinez-Romero, E. and Promé, J.-C. (1993) Biochemistry 32, 10430–10435.
[16] Martinez, E., Poupot, R., Promé, J.-C., Pardo, M.A., Segovia, L., Truchet, G. and Dénarié, J. (1993) in: New Horizons in Nitrogen Fixation (Palacios, R., Mora, J. and Newton, W.E. eds.) pp. 171–175, Kluwer Academic, Dordrecht/Boston/London.
[17] Truchet, G., Debellé, F., Vasse, J., Terzaghi, B., Garnerone, A.-M., Rosenberg, C., Batut, J., Maillet, F. and Dénarié, J. (1985) J. Bacteriol. 164, 1200–1210.
[18] Faucher, C., Camut, S., Dénarié, J. and Truchet, G. (1989) Mol. Plant-Microbe Interact. 2, 291–300.
[19] Maillet, F., Debellé, F. and Dénarié, J. (1990) Mol. Microbiol. 4, 1975–1984.

[20] Demont, N., Debellé, F., Aurelle, H., Dénarié, J. and Promé, J.-C. (1993) J. Biol. Chem. 268, 20134–20142.
[21] Price, N.P.J., Relic, B., Talmont, F., Lewin, A., Promé, D., Pueppke, S.G., Maillet, F., Dénarié, J., Promé, J.-C. and Broughton, W.J. (1992) Mol. Microbiol. 6, 3575–3584.
[22] Röhrig, H., Schmidt, J., Wieneke, U., Kondorosi, E., Barlier, I., Schell, J. and John, M. (1994) Proc. Natl. Acad. Sci. USA 91, 3122–3126.
[23] Mergaert, P., Van Montagu, M., Promé, J.-C. and Holsters, M. (1993) Proc. Natl. Acad. Sci. USA 90, 1551–1555.
[24] Geelen, D., Mergaert, P., Geremia, R.A., Goormachtig, S., van Montagu, M. and Holsters, M. (1993) Mol. Microbiol. 9, 145–154.
[25] Demont, N., Ardourel, M., Maillet, F., Promé, D., Ferro, M., Promé, J.-C. and Dénarié, J. (1994) EMBO J. 13, 2139–2149.