Studies on *Rhizobium meliloti* Plasmids and on Their Role in the Control of Nodule Formation and Nitrogen Fixation: The pSym Megaplasmids and the Other Large Plasmids

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Introduction

At the moment the *Rhizobium*-legume symbiosis is the most efficient agricultural system for the biological fixation of atmospheric nitrogen. Lucerne is, with soyabean, the crop which provides the largest amount of fixed nitrogen throughout the world (Hanson 1972). Strains of the symbiotic partner of lucerne, *Rhizobium meliloti,* are fast-growing rhizobia and constitute, on the basis of symbiotic properties, numerical taxonomy, nucleic acid hybridization and genetic studies, a homogenous cluster clearly different from the *R. leguminosarum* grouping of strains of *R. leguminosarum, R. phaseoli* and *R. trifolii* (Gibbins and Gregory 1972, Kondorosi et al. 1980).

Large plasmids of molecular weight greater than 90 million have been described in all the strains of fast-growing rhizobia investigated so far (for a review see Dénarié et al. 1981). In the *R. leguminosarum* grouping, plasmids of various sizes in the range of M.W. 120–550 million have been shown to carry genes controlling such symbiotic properties as host-specificity and nodule formation (Beynon et al. 1980, Hooykaas et al. 1981) and nitrogen fixation (Nuti et al. 1979, Prakash et al. 1981, Krol et al. 1982). No size class could be established among these plasmids, and no correlation was found between the presence of symbiotic genes and the size of the plasmids (Krol et al. 1982).

In this paper we review the results from our laboratory which show that in *R. meliloti* two size classes of large plasmids exist: (1) megaplasmids (M.W. > 450 million) which are present in all the strains and carry *nif* and *nod* genes and (2) plasmids in the M.W. range of 90 million to about 250 million which do not carry nitrogenase genes, share limited DNA homology and seem not to be essential for effective nodulation of *Medicago sativa.*

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3 This paper is dedicated to the memory of our friend Jean-Simon Julliot who died in an accident on February 21st,1982

Molecular Genetics of the Bacteria-Plant Interaction
(ed. by A. Pühler)

Indigenous Plasmids of *R. meliloti:* Two Size Classes

To ensure maximum genetic diversity among the *R. meliloti* strains examined, thirty isolates were selected from various geographical origins (Table 1) and from the three nodulation groupings proposed by Brockwell and Hely (1966) according to their nodulation properties on various *Medicago*, *Melilotus* and *Trigonella* species.

Table 1. List of *R. meliloti* strains [a] and their plasmid content

Geographical origin	Code number
South Africa	Rf22 (3) [b]
North America	12 (2); V7 (2); S14 (2) Balsac (3); 102F51 [c] (2) 102F28 (4); S26 [c] (1) S33 [c] (2); 54032 (2) II (3); 3DoA20a (2)
South America	U45 [c] (2); B294 (2) U54 (2)
Oceania	1322 (2); RCR2011 [c] (= SU47) (1) CC169 (2); CC2003 (2) CC2013 (1); CC2090 (2) CC2093 (2)
Europe	L5-30 (2); 41 (2) 311 (3); A145 (1) Sa10 (2); Lb1 (2) Ls2a (3); Ve8 (3)

[a] The strains have been described in Casse et al. (1979) and Rosenberg et al. (1981)

[b] The number of ccc DNA bands observed after agarose gel electrophoresis according to Rosenberg et al. (1981) is given in parentheses

[c] Used in commercial inoculants

Using an alkaline denaturation procedure for DNA extraction it was possible to detect the presence of large plasmids in the M.W. size range from 90 million to around 250 million in all the strains except four: RCR2011, CC2013, S26 and A145 (Casse et al. 1979, Jouanin et al. 1981, Rosenberg et al. 1981). The number of such pRme plasmids varied from 1 to 3 according to the strain (Table 1). It is worth noting that only two strains carried a plasmid of molecular weight lower than 85 million, and no very small plasmids (M.W. $<$ 10 million) suitable as cloning vehicles could be detected.

Slight modifications by Banfalvi et al. (1981) and Rosenberg et al. (1981, 1982) of the analytical 'in gel' lysis technique of Eckhardt (1978) allowed the detection, in addition to the pRme plasmids already described, of plasmids of very high molecular weight greater than 450 million. One such plasmid was present in every strain (Rosenberg et al. 1981, 1982). We suggested calling them *megaplasmids* to distinguish them from the other pRme plasmids.

The Megaplasmids

Localization of Nitrogenase Structural Genes

A direct physical approach to the location of *Rhizobium nif* genes was made possible by the finding that the DNA sequences of the nitrogenase structural genes were highly conserved among various nitrogen-fixing bacteria (Nuti et al. 1979, Ruvkun and Ausubel 1980). Recombinant plasmids carrying *nif* genes isolated from *Klebsiella pneumoniae* could be used as probes to show that at least the nitrogenase structural genes *nif* D and H are located on plasmids in strains of the *R. leguminosarum* cluster (Nuti et al. 1979, Krol et al. 1982, Prakash et al. 1981).

A recombinant plasmid pRmR2 has been constructed which contains at least part of *R. meliloti* 2011 *nif* D and H genes (Ruvkun and Ausubel 1980, 1981). We used this hybrid plasmid as a probe to investigate the physical location of *nif* genes in eighteen different *R. meliloti* strains: A145, S26, L530, 41, V7, U45, 102F51, 12, 1322, Ls2a, Balsac, Ve8, S14, 102F28, Rf22, RCR2011, CC2013 and CC2020. Lysates obtained by the 'lysis in gel' procedure (Rosenberg et al. 1982), were submitted to electrophoresis, blotted and hybridized with a pRmR2 probe (Fig. 1). The ccc DNA band corresponding to the megaplasmid of each of the eighteen strains hybridized strongly with the *nif* DH probe.

On the contrary no hybridization could be seen with the ccc DNA bands of the pRme plasmids of smaller M.W. (90–250 million), except in one strain V7 in which the pRmV7*a* of M.W. around 200 million hybridized with pRmR2 (Rosenberg et al. 1981). On the other hand this plasmid has been shown not to hybridize with *K. pneumoniae nif* DH (Prakash et al. 1981); it is thus likely that the hybridization observed at the level of the pRmeV7*a* was due to a DNA sequence which is present in addition to the *nif* DH genes in pRmR2.

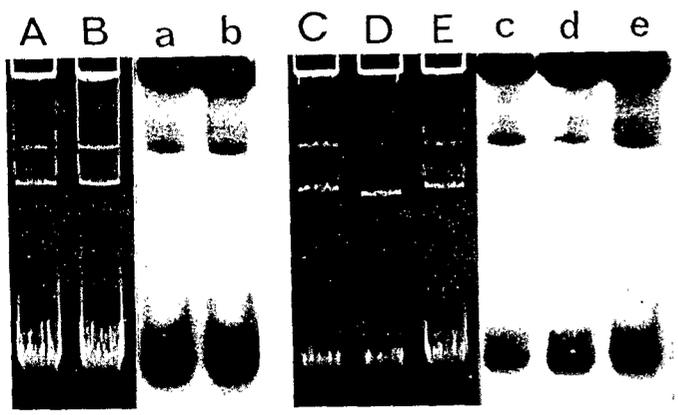

Fig. 1. Agarose gel electrophoresis of lysates from *R. meliloti* strains, *A* = 1322; *B* = 12; *C* = 102F51; *D* = L5-30; *E* = U45. *Small letters* indicate the autoradiograms of the corresponding Southern blots after hybridization with pRmR2

Therefore *nif* genes D and H are located, in all the strains investigated, on the megaplasmid and not on smaller plasmids.

Localization of *nod* Genes

Independent spontaneous Nod$^-$ derivatives of L5-30 have been fortuitously isolated after storage in the cold. They were derivatives of L5-30 since they had the same pattern of sensitivity to a set of specific bacteriophages as the wild-type. All still possessed two plasmids: the megaplasmid and the pRmeL5-30*a* (90 million). No deletion of the smaller plasmid could be detected by restriction enzyme analysis. On the contrary, in all the Nod$^-$ mutants studied, hybridization with the *K. pneumoniae nif* DH was no longer observed (Rosenberg et al. 1981). These *nif nod* deletions were not large enough to be detected by a difference in electrophoretic mobility of the covalently closed circular forms of the megaplasmid. The same results were obtained with Nod$^-$ derivatives of strains Lb1 and Sa10.

Similarly Banfalvi et al. (1981) have observed a simultaneous loss of *nod* and *nif* genes in derivatives of strain 41 which carried a deletion of the megaplasmid. In this book Ausubel et al., Dusha et al., and Long demonstrate that *nod* genes are also located on the megaplasmid in strain RCR2011.

Therefore in the five strains investigated so far (L5-30, 41, Sa10, Lb1 and RCR2011) *nod* genes are located on the megaplasmid.

The fact that spontaneous deletions of the megaplasmid, selected on the Nod$^-$ phenotype, result in a loss of the nitrogenase structural genes indicate that these *nif* genes are located only on the megaplasmid and not on the chromosome.

The pRme Plasmids

In the *R. leguminosarum* cluster, some plasmids of molecular weight in the range 90–250 million have been shown to share a considerable degree of homology (Prakash et al. 1981, Prakash and Schilperoort 1982), and to control symbiotic properties see Dénarié et al. 1981). We wanted to know if it was also the case in *R. meliloti* for the pRme plasmids of this size class (= pRme plasmids).

DNA Homology Studies

The study of sequence conservation of pRme plasmids was done with strains of various geographical origin harboring only one pRme plasmid: L5-30 (Europe; M.W. of pRme: 91 million), 41 (Europe, 150 million), 102F51 (North America, 100 million), 12 (North America, 107 million), V7 (North America, 197 million), 54 (South America, 153 million) and 1322 (New Zealand, 121 million). Covalently closed circular DNA was isolated by an alkaline denaturation procedure (Jouanin et al. 1981). DNAs were treated with various restriction endonucleases. The restriction patterns were markedly dissimilar. No band common to all the plasmids studied could be

detected whatever the endonuclease used, either giving a great number of fragments such as *Eco*RI, *Hin*dIII or *Bam*HI or a low number such as *Sma*I, *Kpn*I or *Xba*I.

Southern DNA/DNA hybridization experiments were performed for more precise estimation of sequence homologies. Purified pRme molecules, radioactively labelled by nick-translation, were hybridized against Southern blots of restriction fragments of the plasmids from other strains. In all experiments hybridization could be detected suggesting that a certain level of sequence homology exists among all the pRme plasmids studied.

The number of hybridizing bands and the intensity of hybridization were different according to the probe and the plasmid blotted (Fig. 2). There were always only a limited number of restriction fragments which hybridized demonstrating that plasmids share only a limited extent of homology. Experiments on thermal denaturation revealed a high stability of the hybrids suggesting a low sequence divergence within the short hybridizing sequences. No obvious correlation could be found between the extent of pRme homology and the geographical origin of the strains.

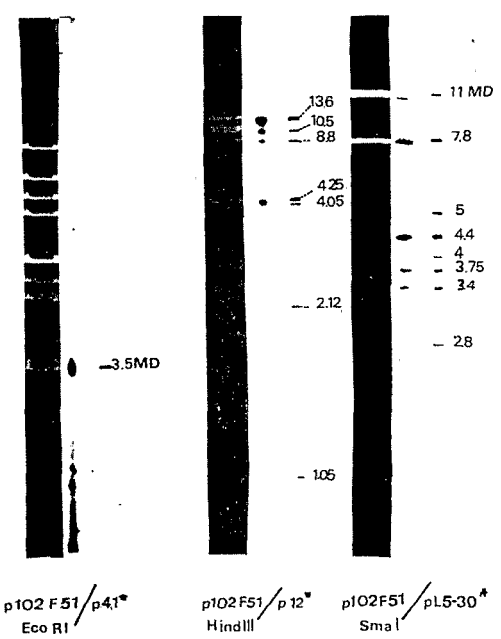

Fig. 2. Restriction endonuclease digests were fractionated on agarose gels, transferred to nitrocellulose sheets and hybridized against ^{32}P-labelled plasmid DNA as probe (Jouanin et al. 1981). *Left track* restriction pattern of plasmid to be blotted; *right track* autoradiogram after hybridization of the plasmid probe with the digested DNA of left track

In order to study the organization of homologous sequences between pRme, we constructed the physical map of one of these plasmids, pRme41 (150 million), isolated from the well studied strain 41 (Banfalvi et al. 1981). This map was constructed by the technique of Depicker et al. (1980): fragments resulting from incomplete digestion of pRme41 by *Hin*dIII were cloned in pBR322 (De Lajudie 1981). Overlapping fragments were used to build the map. Figure 3 summarizes the results concerning the location of pRme41 fragments homologous with pRme from strains L5-30,

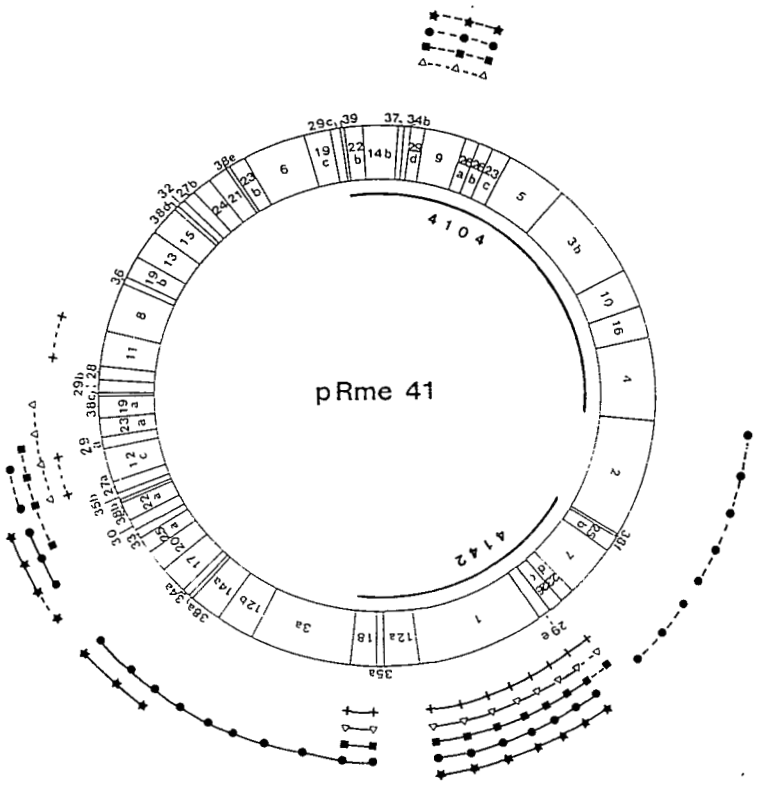

Fig. 3. *Hin*dIII map of pRme41 plasmid DNA and the regions of homology with *Rhizobium* pRme and *Agrobacterium* Ti plasmid DNA. Regions of weak homology are represented by *interrupted lines* (– – –); +++ pRmeL5-30; △ △ △ pRme102F51; ■ ■ ■ pRme1322; ● ● ● pTiC58; * * * pTiB6S3; *lines* and *numbers* inside the physical map refer to the pGMI4104 and pGMI4142 episome used in the genetical experiment

102F51 and 1322, Ti plasmids from *Agrobacterium tumefaciens* C58 (nopaline type) and B6S3 (octopine type). pRme probes hybridized mainly with fragments clustered within three regions of the map, the strongest hybridization being observed within the region cloned in the pGMI4142 episome. Surprisingly the extent of homology of pRme41 DNA was much greater with Ti plasmids than with the other pRme. Both C58 and B6S3 Ti plasmids hybridized strongly with the 4142 region, as did the other pRme plasmids, but also with regions extending further (clockwise).

The use of cloned fragments of pRme41 as probes allowed us to show that the regions of pRme41 from fragment *Hin*dIII 12b to 17 and from 33 to 35b respectively are homologous to the conserved regions C and B of Ti plasmids, regions which are involved in replication and transfer functions (De Greve et al. 1981, Engler et al. 1981). The '4142' region of pRme41 strongly hybridized with regions of Ti plasmids which as far as we know have not yet been genetically characterized. No homology could be detected between pRme41 and T-DNA. The pRme plasmids were not very

different from the Nif-plasmids of the *R. leguminosarum* group with respect to their homology with Ti plasmids (Prakash and Schilperoort 1982). In other words the pRme plasmids of *R. meliloti* do not seem to share more homology among themselves than they do with other plasmids of *Rhizobiaceae.*

Genetic Studies

Genetic studies were performed on the plasmid pRme41. The strategy used was to clone the pRme41 plasmid into the broad host-range plasmid RP4 and to use some of the resulting RP4-prime episomes to insert specifically transposons into pRme41 and to mobilize it.

Partial *Hin*dIII digests of pRme41 were cloned into the Km gene (*Hin*dIII site) of RP4. Some of the RP4-primes are illustrated in Fig. 3. The pGMI4104 episome was introduced into an *E. coli* strain carrying transposon Tn5 (Km^R) in the chromosome. pGMI4104::Tn5 hybrids were selected by transfer into another *E. coli* strain, and introduced into a strain 41 recipient by mating. Recombination between the homologous region of pRme41 and the pGMI4104::Tn5 led to the insertion of Tn5 into pRme41 (Fig. 4). Elimination of the RP4-prime in *Rhizobium* was achieved by selecting clones resistant to two phages (PRRI and GU5) specific for RP4-carrying bacteria. Strain 41 (pRme41::Tn5) was fully effective.

Tagging of pRme41 by Tn5 allowed the study of its transfer. It was self-transmissible to another strain of *R. meliloti,* strain L5-30, at a low frequency ($\cong 10^{-7}$). The two plasmids pRme L5-30 and pRme41::Tn5 were compatible. Transfer of pRme41::Tn5 into *A. tumefaciens* C58 could not be detected. pGMI4142, a RP4-

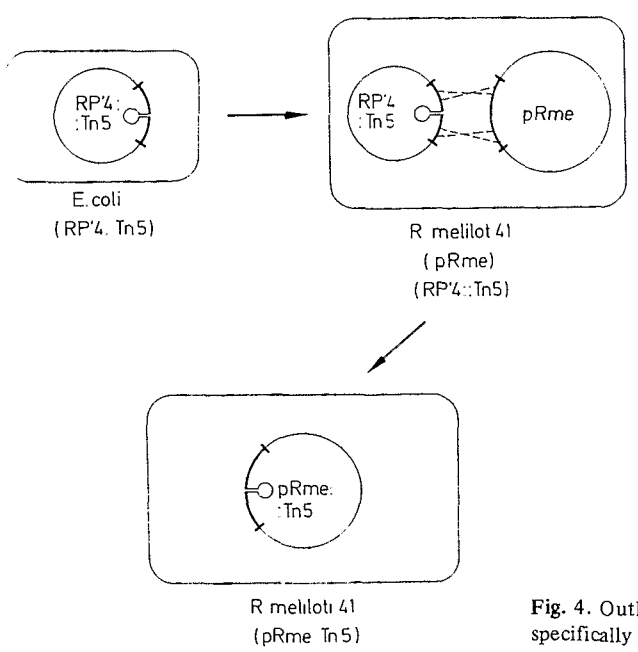

Fig. 4. Outline of strategy used to insert specifically Tn5 into pRme41 plasmid

prime having no common pRme41 *Hin*dIII fragment with pGMI4104 (see Fig. 3), was used to mobilize pRme41::Tn5 by DNA homology (Julliot and Boistard 1979). A strain 41 (pRme41::Tn5) (pGMI4142) could transfer pRme41::Tn5 into *R. trifolii* LPR5001 and *A. tumefaciens* C58 at a frequency of around 5×10^{-7} (Fig. 5). The *R. trifolii* and *A. tumefaciens* pRme41::Tn5^{+} transconjugants were unable to form nodules on *M. sativa* (Rosenberg et al., in preparation). pRme41::Tn5 was compatible with pTiC58 but not with the cryptic large plasmid pAtC58 (Rosenberg et al., in preparation). Strain 41 (pRme::Tn5) behaves, as does the wild-type 41, like an opine-utilizing strain of *A. tumefaciens:* mutants able to use octopine as a sole carbon and nitrogen source can be isolated. When pRme41::Tn5 was introduced into C58C1, a nopaline *A. tumefaciens* strain cured of its Ti plasmid, the ability to catabolize opines was not restored.

Attempts to cure strain 41 of the pRme41::Tn5 plasmid by various treatments (heat, phosphorus starvation, lauryl sulphate, acridine orange, etc.) were unsuccessful: no Km^{S} derivative could be detected. Insertion of bacteriophage Mu into RP4 is known to prevent replication of the resulting RP4::Mu in *Rhizobium* (Boucher et al. 1977) and *Agrobacterium* (Van Vliet et al. 1978). Surprisingly, insertion of Mu into the RP4-prime pGMI4142 (carrying a fragment of pRme41) did not significantly decrease its transfer frequency from *E. coli* to *R. meliloti* 41. Nevertheless, the presence of pGMI4142::Mu led to some instability of pRme41::Tn5. A Km^{S} derivative, cured at the same time of pRme41::Tn5 and pGMI4142::Mu, could be isolated; it was called 41-Cal (Fig. 5). The fact that the strain was actually cured of the pRme41 was checked by preparing a radioactive probe of purified pRme41 and hybridizing with the *Hin*dIII digested total DNA of strains 41 and 41-Cal; no hybridizing band could be detected with 41-Cal. Strain 41-Cal gave rise to octopine-utilizing clones as

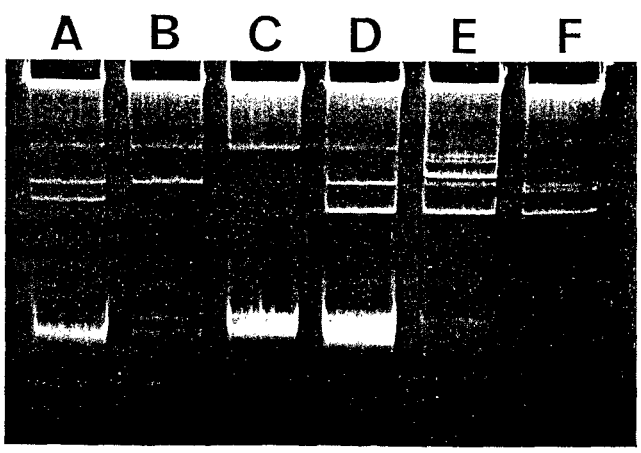

Fig. 5. Agarose gel electrophoresis of lysates from strains *A* = *R. meliloti* L530 (pRme41::Tn5); *B* = *R. meliloti* 41 (pRme41::Tn5); *C* = *R. meliloti* 41-Cal; *D* = *R. meliloti* 41 (pRme41::Tn5) (pGMI4142); *E* = *R. trifolii* LPR5001 (pRme41::Tn5) (pGMI4142); *F* = *A. tumefaciens* C58 (pRme41::Tn5) (pGMI4142)

effectively as the wild-type showing that opine catabolism is not controlled by the pRme41 plasmid. The cured strain 41-Cal nodulated ten varieties of *Medicago sativa* and fixed nitrogen as effectively as the wild-type 41 (Rosenberg et al., in preparation). These results indicate that pRme41 does not carry genes essential for nodulation and nitrogen fixation on *M. sativa*.

Conclusions

Studies on the size of plasmids and their role in the control of symbiotic properties for strains of the *R. leguminosarum* cluster present a very diverse picture. No essential plasmid size class has clearly emerged. *nif* DH were shown to be located on plasmids of molecular weight from 130 to 550 million in *R. leguminosarum* (Nuti et al. 1979, Prakash et al. 1981, Krol et al. 1982). Plasmids in the molecular weight range 100–250 million were found to carry genes controlling nodulation and host-specificity in *R. leguminosarum, R. phaseoli* (Beynon et al. 1980) and *R. trifolii* (Hooykaas et al. 1981).

On the contrary in *R. meliloti* a general picture has emerged. There are clearly two size classes of large plasmids.

1. Megaplasmids for which we propose the term pSym (for control of symbiotic properties): (a) their molecular weight is greater than 450 million; (b) they are present in all effective strains; (c) they carry nitrogenase structural genes and, in all the cases studied so far, *nod* genes.

2. Plasmids which we propose to call pRme (for nomenclature see Casse et al. 1979): (a) their molecular weight varies from 90 million to about 250 million; (b) their number varies with the strain and they may be absent; (c) they do not carry nitrogenase structural genes.

An important question to answer is: what functions are carried on these large cryptic pRme plasmids (each carrying more than 100 genes)? Possibly these include: (1) functions related to the infectious process on legumes, for instance host-specificity for hosts other than the ones tested, or non-obligatory functions such as hydrogen-uptake, etc.; (2) life in the rhizosphere and (3) life in the soil.

Genes essential for the symbiotic relationship with *M. sativa*, such as *nod, fix* and *nif* are located on huge plasmids probably carrying more than 400 genes. The fact that *nif* genes have not been observed in effective strains of *R. meliloti* on a plasmid of smaller size indicates that no large segment of the pSym can be deleted without altering bacterial viability or symbiotic effectiveness and suggest that *Rhizobium* genes required for an effective symbiosis with *M. sativa* are numerous and clustered on a single replicon independent of the chromosome. The genetic study of these huge molecules, that is the inventory of genes controlling the sequential stages of the symbiotic process, the study of the organization of these genes on the megaplasmid, of the sequential expression of these genes during the formation of nodules and the establishment of nitrogen-fixation, require other genetic tools. In the following chapter we describe a strategy for the genetic study of the *R. meliloti* pSym megaplasmid.

Acknowledgements. This work was supported by the "Biological Nitrogen Fixation" grant from Société Nationale Elf Aquitaine and Entreprise Minière et Chimique and by an INSERM grant (ATP Recombinaisons Génétiques no. 79-104). Part of the work was performed at the Laboratoire de Biologie Moléculaire Végétale (Laboratoire associé CNRS no. 40) of Université Paris-Sud, Orsay. The authors would like to thank Dr. Bill Broughton for reviewing the manuscript.

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