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Current Review

Plasmids in Rhizobia: The Role of Nonsymbiotic Plasmids

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Rhizobium, Bradyrhizobium, and *Azorhizobium* genera, belonging to the Rhizobiaceae family, are able to develop a root-nodule symbiosis with legume plants. The importance of this association lies in the high level of fixed molecular dinitrogen (N_2), the effectiveness of the process, and the economic, social and ecological interest of the plants involved. Symbiotic nitrogen fixation takes place in specialized structures (the nodules) originated in the legume roots as a consequence of the microsymbiont infection. In the nodules, bacteria are transformed into bacteroids, which are able to use photosynthate as energy, electron, and carbon sources to carry out the transformation of N_2 to ammonia. After this process, ammonia is incorporated to the plant metabolism. The whole process involves a complex interaction between the two partners (bacteria and plants).

A general feature in species of the genus *Rhizobium* is the existence of a large amount of extrachromosomal DNA. These plasmids vary in number (1 to 10) and size, but usually they are of high molecular mass (100 to 300 Mda), reaching as occurs in *R. meliloti* with the so-called megaplasmids, a molecular mass > 1,000 Mda. They can constitute a large percentage of the cell genome, e.g., up to 45% of the *R. etli* genome (Martínez-Romero and Palacios 1990; Martínez-Romero 1994). In some rhizobial species, most of the essential genes required in the symbiotic process are located in plasmids, which have been named traditionally symbiotic plasmids or pSyms. However, most of the rhizobia harbored plasmids are not essential for the establishment of a complete symbiotic state, these are called non-pSym, cryptic or simply large plasmids. pSyms as well as non-pSym plasmids are stably maintained through successive generations. This suggests the presence of an accurate mechanism that ensures an equal partitioning among daughter cells. On the other hand, a role of plasmids in rhizobial survival can not be ruled out, despite the metabolic burden that their maintenance implies.

Information about the involvement of plasmid genes in symbiotic or saprophytic performance is increasing quickly. This paper is an overview of the current knowledge on genetic and physiological traits harbored by plasmids in rhizobia, mainly those not devoted directly to development of the symbiosis. We also discuss the stability of these plasmids, and the possible exploitation of characteristics such as high stability and broad host range transmissibility in the construction of new vectors.

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PLASMIDS IN RHIZOBIA: THE PSYM PLASMIDS

The development of techniques for plasmid detection in gels (Eckhardt 1978) and their further modifications (Plazinski et al. 1985 and references therein) made possible the detection of large plasmids besides those already detected by the alkaline lysis procedure (Casse et al. 1979; Jouanin et al. 1981). Thus, the most upper band frequently detected in plasmid profiles of *R. meliloti* strains actually corresponds to two megaplasmids (Banfalvi et al. 1985) with extremely large molecular sizes (up to 1,700 kb) (Burkhardt and Burkhardt 1984; Burkhardt et al. 1987; Charles and Finan 1991; Margolin and Long 1993). It is in the megaplasmids of *R. meliloti* strains where symbiotic genes are located. The molecular size of plasmids carrying symbiotic genes for other *Rhizobium* species is highly variable. pSyms as well as other plasmids have been found in different *Rhizobium* species: *R. leguminosarum* bv. *trifolii* (Thurman et al. 1985; Harrison et al. 1988); *R. leguminosarum* bv. *phaseoli* (Lamb et al. 1982; Martínez et al. 1987); *R. leguminosarum* bv. *viciae* (Hirsch et al. 1980; Buchanan-Wollaston et al. 1980; Hombrecher et al. 1981); *Rhizobium* sp. (*Cicer*) (Broughton et al. 1984; Cadahía et al. 1986); *Rhizobium* sp. (*Hedysarum*) (Mozo et al. 1988); *R. tropici* (Martínez-Romero et al. 1991; Pardo et al. 1994); *R. etli* (Quinto et al. 1982; Segovia et al. 1993); *R. galegae* (Lindström 1989); *R. fredii* (Masterson et al. 1982; Prakash and Atherley 1984) and *Rhizobium* and *Bradyrhizobium* mesquite (*Prosopis glandulosa*) nodulating strains (Thomas et al. 1994).

It was reported that the large plasmids of *R. meliloti* do not seem to share more homology among themselves than they do with other plasmids of Rhizobiaceae (Huguet et al. 1983). Recent studies from our laboratory (Toro and Burgos, unpub-

lished) suggest that some plasmid origin of replication are conserved and widely distributed within *R. meliloti* native populations and homologs were found in other rhizobia species such as *R. fredii* and *R. tropici.*

Large plasmids have also been detected in *R. loti* (Pankhurst et al. 1986), *Rhizobium* sp. (*Cajanus*) (Sharma and Laxaminarayama 1989) and in *Bradyrhizobium* (Haugland and Verma 1981; Haugland et al. 1984), although they do not harbor symbiotic genes. No large plasmids have been detected in *Azorhizobium* (van den Eede et al. 1987), suggesting that symbiotic genes could be located in the chromosome of this species.

We define here as pSym those plasmids containing genes that are essential to establish a complete symbiotic state. It should be noticed that some plasmids that were originally considered by the corresponding authors as cryptic or non-pSym plasmids, are considered in this report as pSym because they carry essential functions for the symbiotic process. Plasmid curing or deletion of specific DNA region of the pSym plasmids leads to the loss of a complete or partial symbiotic performance, respectively. Among others, *nod, nif, exo,* and *fix* genes are harbored by pSyms (Johnston et al. 1978; Nuti et al. 1979; Hirsch et al. 1980; Banfalvi et al. 1981; Rosenberg et al. 1981; Hooykaas et al. 1982; Huguet et al. 1983; Finan et al. 1986; Hynes et al. 1986). In the case of *R. meliloti* pSym megaplasmids, it has been shown that they cannot be cured, presumably because they carry genes essential for free-living growth (Finan et al. 1986). Since the megaplasmids constitute nearly half of the *R. meliloti* genome and are essential for viability, they are considered small chromosomes (Sobral et al. 1991).

Symbiotic genes are not always located in the same plasmid. Thus, genes involved in exopolysaccharide (EPS) and lipopolysaccharide (LPS) synthesis and symbiotic nitrogen fixation are harbored by the larger pSym in *R. meliloti* (Finan et al. 1986; Hynes et al. 1986; Long et al. 1988; Watson et al. 1988; Glazebrook and Walker 1989; Zhan et al. 1989; Williams et al. 1990; Charles and Finan 1991), whereas *nod* and *nif* genes are in the smaller pSym (Banfalvi et al. 1981; Rosenberg et al. 1981). In *R. etli* strain CFN42, in addition to plasmid **d** (known to contain the *nod* and *nif* genes), plasmid **b** is required for nodule formation on *Phaseolus vulgaris*, partly owing to the presence of genes involved in LPS synthesis (Cava et al. 1989; Brom et al. 1992). Similarly, in strain VF39 of *R. leguminosarum* bv. *viciae* LPS genes are located in plasmid pRleVF39c, whereas *nod* and *nif* genes are in plasmid pRleVF39d (Hynes and Mcgregor 1990). Another example is a megaplasmid present in *R. leguminosarum* bv. *trifolii* ANU1173 (Chen et al. 1993). This strain has a number of different phenotypes, among them the presence of a slow-migrating LPS that is present in the parental strain but not in the cured derivative P22. This derivative had a reduced EPS production and cell motility in TY medium as well. Finally, the authors concluded that this megaplasmid, also involved in pH tolerance, is also required for the establishment of nitrogen-fixing nodules on clover species.

On other occasions, genes required for the formation of an effective nitrogen-fixing nodule are located on the chromosome (Forrai et al. 1983; Leigh et al. 1985; Dylan et al. 1986; Long et al. 1988; Müller et al. 1988).

As a logical consequence of their large size, nonsymbiotic genes are also found in pSyms. Thus, pSyms also contain *trc* (trigonelline catabolism) genes that enable the strains which harbor them to utilize this compound as the sole carbon and nitrogen source (Boivin et al. 1990) and osmoprotectant (Bernard et al. 1986; Le Rudulier and Bernard 1986). *mos* and *moc* genes, responsible for the synthesis and catabolism of rhizopine (3-*o*-methyl-scylo-inosamine), respectively (Murphy and Saint 1992), have been located in the pSym as well as genes essential for the catabolism of proline betaine, or stachydrine (Goldman et al. 1994). The complete *nos* region essential for dissimilatory nitrous oxide reduction by *R. meliloti* has been recently located in the *nod* pSym (Holloway et al. 1996). In some *R. leguminosarum* strains H_2-uptake hydrogenase genes (*hup*) were located on a plasmid that also contain *nif* genes (Leyva et al. 1987). The rizosphere expressed genes *rhiABC* are adjacent to the transcriptional activator *rhiR* in *R. leguminosarum* Sym plasmid pRL1J1 (Gray et al. 1996). A *R. tropici* pSym gene encoding a citrate synthase (*pcsA*) has been also described. Insertional mutations in this gene simultaneously reduce nodulation ability and citrate synthase activity. A possible role of pSym in iron uptake has been proposed as well (Pardo et al. 1994). Genes for thiamine biosynthesis (*thi*), lactose utilization (*lac*) as a sole carbon source, and an unidentified dehydrogenase activity have been linked to the *exo R. meliloti* pSym (Finan et al. 1986; Charles and Finan 1991). In the same plasmid, C_4-dicarboxylate transport genes (*dct*) are present (Finan et al. 1988; Watson et al. 1988). Likewise, genes required for utilization of the aromatic acids (*pca*) protocatechuate and quinate, α-galactosides (*mel*) melibiose and raffinose, β-hydroxybutyrate and acetoacetate (*bhb*), and dulcitol (*dul*) as sole carbon sources are located in the same pSym (Charles and Finan 1991). The pSym of *R. leguminosarum* bv. *trifolii* WE14-2 is involved in the utilization of the aromatic compound catechol (Baldani et al. 1992). Defined enzymatic activities and nitrate utilization are linked to pSym plasmids in *R. leguminosarum* bv. *trifolii* strains (Baldani et al. 1992). Finally, genes involved in melanin biosynthesis have been found on pSyms. Two loci involved in melanin biosynthesis have been localized on the symbiotic plasmid pRP2J1 of *R. leguminosarum* bv. *phaseoli* strain 8002: The putative structural gene for tyrosinase *melA*, and another one that seems to correspond to *nifA*, inducing transcriptional activation of *melA*. There is still a third gene (*melC*), with a chromosomal location. Several lines of evidence suggest that, in this case, melanin biosynthesis is under the control of the RpoN-NifA regulatory system (Borthakur et al. 1987; Hawkins and Johnston 1988; Hawkins et al. 1991). Genes for production of this trait are found on cryptic plasmids in other rhizobia and they are not influenced by the RpoN-NifA regulatory network.

FUNCTIONS ENCODED BY THE NONSYMBIOTIC PLASMIDS

We consider here non-pSym, those plasmids that are not necessary for the establishment of a complete symbiotic state, although in some cases, they can modulate the interaction between the symbionts either positively or negatively. They also may encode traits that confer phenotypic advantages to the rhizobial cells that harbor them (Table 1). Little is known about the role played by this "silent" DNA, although now it is

feasible to combine defined features with the presence of a determined plasmid. Interestingly, complex interactions are probably taking place among all genomic (chromosome and plasmid) sequences. Coupling a defined trait, important for either symbiosis or free-living growth, to the presence of a plasmid requires a complicated analysis. Thus, some plasmid-borne characteristics have been revealed in trials of inter-strain competition involving parent and cured derivatives, or pairs of cured derivatives. These assays can uncover features that are otherwise difficult to detect only by an individual capacity of a defined cured strain.

non-pSym borne traits related with symbiotic characteristics: competitiveness, infectivity, and effectiveness.

There are several examples indicating a link between the occurrence of non-pSym plasmids and certain symbiotic characteristics. Large self-transmissible nonsymbiotic plasmids are linked to bacteriocin synthesis that most likely influences specific competence (Hirsch 1979; Johnston et al. 1982). Nodulation competitiveness can also be affected by non-pSym plasmids. Competitive ability for nodulation of beans is influenced by a non-pSym (Martínez-Romero and Rosenblueth 1990). Bromfield et al. (1985) linked the action of rifampin resistance and the presence of cryptic plasmid pTA2 in nodulation competitiveness of a *R. meliloti* strain. The effect of rifampin resistance was dominant, and the contribution of pTA2 was evident only when paired competitors had the common rifampin resistance background. Likewise, non-sym plasmids of *R. tropici* strain CFN299 enhanced nodulation of *A. tumefaciens* transconjugants. In fact, these transconjugants nodulated better and fixed more nitrogen when harboring the whole set of CFN299 plasmids than transconjugants carrying only the pSym plasmid (Martínez et al. 1987). Brom et al. (1992) have demonstrated that in *R. etli* strain CFN42, plasmid **e** is required for the competitive ability exhibited by the wild-type strain. Cryptic plasmids in strains of *R. leguminosarum* bv. *trifolii* affect symbiotic characteristics. Thus, plasmid pRtrW14-2a seems to enhance nodulation ability, and curing of plasmid pRtrW8-7b resulted in a delay in nodule formation. Non-pSym plasmids pRtrW14-2a and pRtrW8-7b of *R. leguminosarum* bv. *trifolii* strains are also involved in cell motility. Cured-derivatives that have lost these plasmids also showed the absence of the O antigen-containing LPS band, which may pleiotropically affect motility (Baldani et al. 1992). In *R. leguminosarum* bv. *trifolii* an EPS region has been localized in a non-pSym plasmid (Skorupska et al. 1991).

Cryptic plasmid-borne genes directly involved in nodulation efficiency have been described in *R. meliloti* GR4 (Toro and Olivares 1986). Those genes located on plasmid pRmeGR4b (Fig. 1) were named as *nfe* (nodule formation efficiency) genes (Sanjuan and Olivares 1989; Soto et al. 1993). Expression of *nfe* genes is controlled by the NifA-RpoN regulatory system (Sanjuan and Olivares 1991) although some of these genes may possibly be expressed before the onset of nitrogen fixation (Soto et al. 1993). Three genes have been described so far in the *nfe* region of strain GR4, *nfeA* and *nfeB* which have promoters showing the highly conserved acting sequence for RpoN (Soto et al. 1993), and *nfeD,* which encoded product shows homology with the ornithine cyclodeaminase (OCDs) of *A. tumefaciens* (Soto et al. 1994). Although the functions of the *nfe* genes remain unknown, the homology to catabolic genes as *ocd* suggests that they may be involved in the catabolism of specific compounds, which in turn could provide a selective advantage during early stages of inter-strain competition. The presence of this type of genes also has been recently reported in *B. japonicum.* However, the *nfe* gene detected (*nfeC*) like the *nod* genes, has a chromosomal location in this symbiont (Chun and Stacey 1994).

The symbiotic effectiveness, in some cases, may be affected by plasmids others than the pSym. An example of such phenomena is the bacteriocinegic plasmid pRL3J1 which decreases effectiveness in the host strain (DeJong et al. 1981). A similar behavior has been observed in *R. loti* strains where plasmid cured derivatives showed enhanced competitiveness and effectiveness (Pankhurst et al. 1986). In *R. meliloti* strain SAF22, it has been recently reported that the presence of a cryptic plasmid attenuated its ability to promote normal nod-

Table 1. Nonsymbiotic plasmids borne traits

1. Bacteriocin synthesis
2. Nodulation efficiency
3. Enhanced/decreased effectiveness
4. Reiterated symbiotic genes
5. Exopolysacharide and Lipopolysacharide synthesis
6. Utilization of carbon sources
7. Melanin synthesis
8. Other enzymatic activities
9. Enhanced/decreased bacterial growth and survival under different environmental conditions
10. IS elements

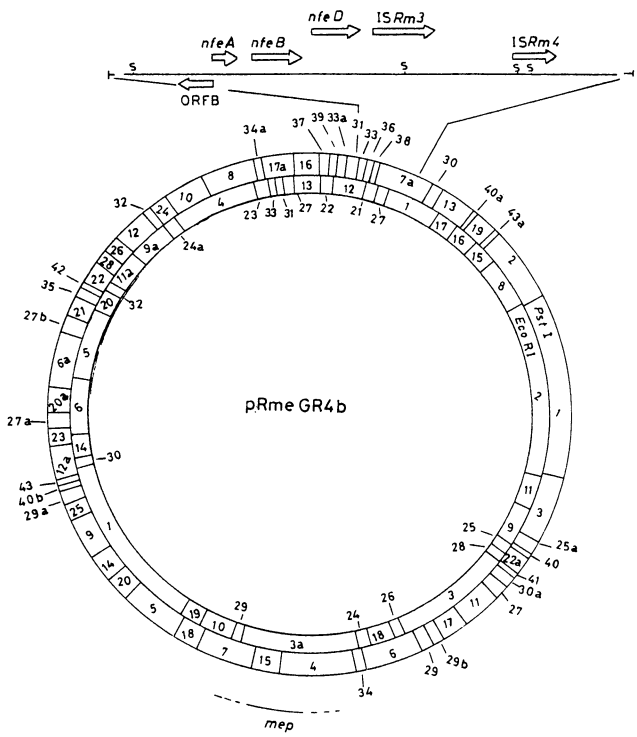

Fig. 1. *Eco*RI-*Pst*I restriction map of *Rhizobium meliloti* strain GR4 non-pSym plasmid pRmeGR4b. Location of the nodulation formation efficiency genes (*nfe*) and the melanin biosynthesis gene (*mep*) are indicated. An enlarger *Sal*I (S) restriction map of the *nfe* region is also shown.

ule development and its symbiotic effectiveness on alfalfa (Velázquez et al. 1995). Selbitschka and Lotz (1991) have found that genes present in nonsymbiotic plasmids of *R. leguminosarum* bv. *viciae* strains reduced the symbiotic nitrogen fixation efficiency with *Pisum sativum* but did not with *Vicia faba*. These authors have postulated that such sequences harbor the *hrf* determinants (host-dependent reduction of nitrogen fixation) which contribute to the uneven symbiotic effectiveness observed on different hosts.

The presence of reiterated *nod* and *nif* genes in the non-pSym has also been reported (Barran and Bromfield 1988). In *R. meliloti* 1076, plasmid pSV1 shows reiterations of *nodB* and *nodC* genes, although only the latter one is functional. Reiterations of *nifE* and *nifB* have been found as well (Rastogi et al. 1991). In the same way, only the reiterated *nifE* gene is functional. Two possible causes of gene reiteration have been proposed. One possible explanation is that cryptic plasmids harboring reiterations may represent the capture of pSym portions from other species due to their transmissibility events (Young and Wexler 1988). Alternatively, the reiterated symbiotic sequences of pSV1 plasmid might be originated by recombination events from the *R. meliloti* pSym (Brom et al. 1991; Romero et al. 1991). Reiterations are not rare phenomena, since they have been found in many strains (Flores et al. 1987). It has been postulated that such iterated regions are involved in the generation of gene rearrangements (Kaluza et al. 1985; Hahn and Hennecke 1987). These rearrangements appear at high frequency, as it has been described for *R. leguminosarum* bv. *phaseoli* (Brom et al. 1991; Romero et al. 1991). Gene rearrangements due to the presence of iterated sequences may cause amplification, deletion, or plasmid cointegration.

Utilization of carbon sources.

As we indicated above, pSym plasmids harbor genes that confer the ability to utilize particular organic compounds (Finan et al. 1988; Watson et al. 1988; Charles et al. 1990; Charles and Finan 1991). However, non-pSym plasmids may carry genes for this purpose as well. A good example is plasmid pRme41a of *R. meliloti* 41 and its involvement in calystegins metabolism (Tepfer et al. 1988; Boivin et al. 1990). Caslystegins constitute a group of secondary metabolites found in root exudates of different plants which can be utilized by strain 41, conferring a relative advantage of this strain in the rhizosphere of these plants. *cac* (calystegins catabolism) genes were located in plasmid pRme41a. Plasmid-cured derivatives of this strain were impaired in growth on minimal medium only when amended with calystegins. However, these genes have no direct implication in the symbiotic process. This phenotype seems to confer to the *Rhizobium* population a selective advantage during the saprophytic stage in the absence of the host legume (Boivin et al. 1990). These authors attribute the catabolism of root exudates to the presence of nonsymbiotic plasmids.

The alteration in ability to utilize several compounds as a C source after the curing of non-pSym plasmids of different strains of *R. leguminosarum* bv. *trifolii* has been studied by Baldani et al. (1992). These authors have found that plasmid pRtrW14-2a confers to strain W14-2 the ability to grow on medium containing malate or lactose. Plasmid pRtrW14-2b allows growth on the sugar alcohol adonitol, whereas plasmid pRtrW14-2c allows growth on rhamnose and sorbitol. A pRtrW11-9b cured derivative of strain W11-9 could not utilize inositol, malate, or arabinose. Finally, plasmid pRtrW8-7b affects the metabolism of malate and glycerol in strain W8-7. Utilization of catechol as a sole carbon and energy source is linked to the presence of cryptic plasmid pAMG1 in *Rhizobium* sp. isolated from *Lablab purpureus* (Gajendiran and Mahadevan 1990). However, this trait is harbored by the pSym pRtrW14-2d in *R. leguminosarum* bv. *trifolii* W14-2 (Baldani et al. 1992). As for other compounds, utilization of catechol or related molecules that are frequently found in soil organic matter may be advantageous for survival of rhizobia. The ability to utilize these and/or other compounds that are likely to be present in soil organic matter or root exudates leads to the idea that plasmids are playing an important role in the saprophytic competence of rhizobia in soil.

Bacterial growth and survival.

Some plasmids can influence growth and survival of rhizobia under environmental stress. Plasmids in *R. etli* CFN42 seem to be important for free-living growth of the cell (Brom et al. 1992). Baldani and Weaver (1992) studied the influence of plasmids of *R. leguminosarum* bv. *trifolii* strains on the cell survival, looking at two important parameters: high temperature and low soil moisture. Plasmids seem to play a minor role in the ability of cells to survive drought conditions. Moreover, cured derivatives survived better than the wild-type parent, suggesting that in fact they can be detrimental to the cell, perhaps because of the extra metabolic cost necessary to maintain the plasmids. Involvement of some plasmids in tolerance to heat is more relevant, although this varies according to the plasmid eliminated and stress imposed. For instance, it has been suggested that plasmids **b, c,** and **e** of strain W8-7 carry genes involved in tolerance to heat. The mechanism how plasmids influence cell survival at high temperature is unknown. However, a study by Sen et al. (1990) demonstrated that elimination of plasmids resulted in loss of expression of heat-shock proteins.

The relationship between the plasmid content of rhizobia and their ability to proliferate in the rhizosphere is poorly understood. Moënne-Loccoz and Weaver (1995), studying plasmid-cured derivatives of *R. leguminosarum* bv. *trifolii* W14-2, made an attempt to elucidate the influence of plasmids on the growth of this strain in the clover rhizosphere. They found that single plasmid-cured derivatives reached the same population as wild type when inoculated alone. However, differences were found in coinoculation experiments involving the wild type and cured derivatives. Under these conditions, cured derivatives showed a decrease in population density. These data led to the conclusion that all the plasmids contributed to the growth of strain W14-2 in the rhizosphere of clover. On this basis, one can expect that some plasmids in other rhizobia may play a similar role.

Production of melanin.

Production of melanin is widespread among rhizobia. Nevertheless, this trait is randomly distributed in rhizobial genomes and it is difficult to assess its implication in symbiotic nitrogen fixation, according to the data available. Cubo et al. (1988) established that production of melanin is found in a wide range of rhizobia species. Location of the responsible genes is variable, being either on symbiotic plasmids, as de-

scribed above or on cryptic plasmids, as occurs in *R. fredii* USDA205, or in *R. leguminosarum* bv. *trifolii* strain RS24. Plasmid pRj206b of *R. fredii* USDA206 appears to encode for repression of melanin synthesis (Barbour and Elkan 1990). The first report where the production of melanin was associated with the presence of a nonsymbiotic plasmid was for plasmid pRleVF39a of *R. leguminosarum* bv. *viciae* (Hynes et al. 1988). Production of melanin by strain GR4 of *R. meliloti* is also related to the presence of nonsymbiotic plasmid pRmeGR4b (Mercado-Blanco et al. 1993). Only one locus is involved in melanin synthesis, and unlike strain 8002, there is no link between melanin production and symbiotic nitrogen fixation in strain GR4. The structural gene for melanin biosynthesis has been characterized in this strain, and protein sequence analysis showed strong similarities with tyrosinases of both eukaryotic and prokaryotic organisms.

The biological significance of melanin synthesis is not clear. Hawkins and Johnston (1988) have pointed out a protective effect for melanin in senescent nodules of bean plants, as detoxifying phenolic compounds. On the other hand, unknown protective effects in the saprophytic state of the bacteria cannot be ruled out, on the basis of the chemical properties of this compound.

IS elements.

IS elements are small (size < 2.5 Kb), mobile genetic entities which do not contain selectable genes. In rhizobia these elements are widely spread being located in the pSym and non-pSym plasmids as well as into the chromosome. They tend to be restricted in their occurrence to just one or a few species (Ruvkun et al. 1982; Dusha et al. 1987; Wheatcroft and Watson 1988; Hartman and Amarger 1991; Simon et al. 1991; Wheatcroft and Watson 1988; Wheatcroft and Laberge 1991). It has been hypothesized that because the non-pSym plasmids carry unessential functions and due to their large size, they could be a safe genetic material to harbor insertion sequences. Following this hypothesis, Wheatcroft and Laberge (1991) isolated IS*Rm3* from the cryptic plasmid pTA2 of *R. meliloti* strain 102F70. Sequence analysis of *R. meliloti* strain GR4 plasmid pRmeGR4b revealed an IS element roughly situated every 2.5 kb. IS*Rm3*, IS*Rm4*, IS*Rm6*, IS*Rm7*, and ORFB showing homology transposases have been found within a DNA region of 13 kb (Soto et al. 1992a, 1992b; Soto et al. 1993; Toro and Zekrí, unpublished). The presence of IS elements in plasmids can explain their wide distribution in natural populations by means of conjugal transfer events.

Recently, the transposable element Tn*163* has been found in two strains of *R. leguminosarum* bv. *viciae*. These strains contain one copy of the transposon localized in a non-pSym plasmid. This constitutes the first report of native transposons in the genus *Rhizobium* (Ulrich and Pühler 1994).

Other traits linked to the presence of non-pSym plasmids.

Baldani et al. 1992 have correlated the presence of certain plasmids with specific enzymatic activities. Thus, curing of plasmids pRtrW8-7e, pRtrW11-9b, pRtrW11-9a (pSym), and pRtrW11-9b, and pRtrW14-2d (pSym) resulted in loss of superoxide dismutase activity in gels. Similarly, curing of pRtrW11-9b alone or together with pRtrW11-9a (pSym) eliminated a faint electrophoretic band corresponding to hexokinase and carbamate kinase activities.

Nitrate utilization is linked to the presence of pRtrW8-7e plasmid in strain W8-7, and pRtrW11-9b and pRtrW11-9a (pSym) plasmids in strain W11-9 (Baldani et al. 1992).

TRANSMISSIBILITY, STABILITY, AND REPLICATION OF RHIZOBIA PLASMIDS

Replication of the rhizobial chromosome and the harbored plasmids must be coordinately regulated and common sequence elements should probably be implicated. However, it is not known how rhizobial plasmids coordinate their replication with that of the host chromosome or how they partition during cell division. Little is known about other general features such as stability or conjugal transfer of rhizobial plasmids. The isolation and characterization of these plasmid replication origins would provide insight into these mechanisms.

Transmissibility.

Mobilization of rhizobial plasmids has been achieved repeatedly (Hooykaas et al. 1982; Kondorosi et al. 1982; Truchet et al. 1984; Hooykaas et al. 1985). However, some plasmids present in rhizobial species are reported to be self-transmissible. This characteristic is hardly detected in nodules for pSyms of *R. meliloti* (Pretorius-Güth et al. 1990), although pSyms of *R. leguminosarum* bv. *trifolii* have been demonstrated to be self-transmissible in vitro at frequencies of 10^{-4} and also in soil microcosms to native soil bacteria (Rao et al. 1994). However, this trait is frequent for cryptic plasmids (Johnston et al. 1982; Huguet et al. 1983). In some cases self-transmissible plasmids are able to promote the cotransference of other resident plasmids. Thus, self-transmissible plasmid pRmeGR4a of *R. meliloti* GR4 is able to induce cotransference of plasmid pRmeGR4b, a non-self-transmissible resident plasmid of strain GR4 (Mercado-Blanco and Olivares 1993). It has been recently reported that ammonia used as nitrogen source inhibits pRmeGR4a and, therefore, pRmeGR4b conjugal transfer to *R. meliloti* strains but not to *A. tumefaciens* (Herrera-Cervera et al. 1996).

Self-transmissibility and mobilization of plasmids may play important roles in the dispersion of both symbiotic and nonsymbiotic properties among different strains and species of rhizobia. This is also important from the evolutionary point of view, since conjugation and recombination events can play an active role in the evolution of the characteristics harbored by plasmids. The presence of extensive homology among rhizobial plasmids could indicate a phylogenetic relationship as well as serve to establish incompatibility groups, not only among *Rhizobium* plasmids but also in other members of Rhizobiaceae such as *Agrobacterium* (Huguet et al. 1983; Tepfer et al. 1988).

Replication and stability.

Despite the fact that most of the DNA carried by rhizobial plasmids is of unknown function, these replicons show a high degree of stability. Most of rhizobial plasmids, both symbiotic and nonsymbiotic, are stably maintained through generations without detectable loss, suggesting the presence of very accurate maintenance mechanisms. Nevertheless, there is little information about replication and stabilization mechanisms acting in these plasmids. So far, only five regions involved in replication have been isolated.

Regarding pSyms, only very recently has it been possible to isolate and characterize the megaplasmid pSym-b origin of replication from *R. meliloti* (Margolin and Long 1993). These authors have cloned and sequenced a 0.8-kb fragment that contains sequences sufficient for replication in a *recA* derivative of *R. meliloti*, although another portion of the megaplasmid is required in *trans* for replication in *A. tumefaciens*. This region contains motifs that are found in other well-known replication origins: several stretches of AT-rich sequences, a *E. coli oriC* 13-mer-like sequence (Bramhill and Kornberg 1988), and DNA-boxes. However, the described origin of replication has no significant overall similarities to bacterial chromosomal origins nor to other plasmid origins of replication. Because this origin seems to be dispensable, these authors suggest that another quite different replication origin exists on the megaplasmid, since the cloned minimal origin did not hybridize to sequences elsewhere on pSym-b (Margolin and Long 1993).

Only four regions involved in plasmid replication and stable maintenance of nonsymbiotic plasmids have been described. The first one resulted from the cloning of a 15-kb restriction fragment of a resident plasmid from *R. leguminosarum* bv. *trifolii* in a ColE1 replicon (Neilan et al. 1986). This fragment has not been further characterized. Mozo et al. (1990) reported the isolation of a 5.4-kb fragment responsible for the stable maintenance of the cryptic plasmid pHc23a from *Rhizobium* sp. (*Hedysarum*) UPM-Hc23. Besides the *oriV*, these authors localize in this fragment the replication and maintenance functions as well as determinants of incompatibility.

The third cloned and characterized region involved in replication and stable maintenance was that of plasmid pRmeGR4a from *R. meliloti* GR4. A 4.8-kb *Pst*I fragment is responsible for the autonomous replication in different hosts of Rhizobiaceae (Mercado-Blanco and Olivares 1993). Sequence analysis of the cloned fragment revealed the presence of several ORFs. However, only one of these is necessary for replication as revealed by deletion mutation studies. The amino acid sequence of this ORF showed some degree of homology with RepC proteins coded by plasmid pRiA4b of *A. rhizogenes* (Nishiguchi et al. 1987) and plasmid pTiB6S3 of *A. tumefaciens* (Tabata et al. 1989; Mercado-Blanco and Olivares 1994b). This fact is interesting from the evolutionary point of view and indicates a certain degree of phylogenetic relationship. Replication mechanisms of these plasmids could be derived from a common ancestor, as it was already pointed out for *Agrobacterium* plasmids (Otten et al. 1992). Despite the similarity of *Agrobacterium* RepC proteins, it was not possible to complement Rep mutants of different species, probably indicating a high degree of functional specialization (Tabata et al. 1989). The involvement of the other ORFs detected in this fragment is not clear. ORF2 seems to play a role in stabilization, and moderate homology with cytoskeletal proteins and DNA binding proteins have been detected (Mercado-Blanco and Olivares 1994a). Some proteins involved in stabilization of plasmids or chromosomes showed these characteristics (Williams and Thomas 1992 and references therein). Studies on sequence homology along with other experiments have led to the suggestion that partition mechanisms involving some of these proteins may operate in prokaryotic cells in a similar way to that in eukaryotic organisms.

In *Agrobacterium* plasmids, RepA and RepB proteins are similar to the maintenance function proteins IncC and KorB of RK2, respectively (Nishiguchi et al. 1987; Tabata et al. 1989; Williams and Thomas 1992). RepA and RepB proteins seem to play a role in plasmid stabilization, such as IncC and KorB (Motallebi-Veshareh et al. 1990). However and despite the similarities between Ti/Ri plasmids and IncP plasmids, the replication protein RepC has no similarity at all with the TrfA replication proteins of IncP (Pansegrau et al. 1994). Homologies with Ti/Ri RepA and RepB proteins have not been detected in the replication region of plasmid pRmeGR4a but homologous sequences have been recently found in a plasmid origin of replication derived from *R. leguminosarum* bv. *viciae* (Turner and Young 1995) in addition to a *repC* locus.

Hybridization experiments using an internal *Eco*RV restriction fragment that contains almost the entire coding sequence of pRmeGR4a *repC* as a probe showed homology with a high molecular weight *Eco*RI restriction fragment of plasmid pRmeGR4b. Moreover, homology was also found in other *R. meliloti* strains from distinct geographical locations as in some *R. fredii* strains (Toro et al., unpublished). These results suggest that the *repC* locus is quite well conserved and may characterize closely related origin of replications. The use of primers derived from the *repC* locus and upstream DNA region in PCR experiments is being a useful tool for the fast characterization of a large number of origin of replications for rhizobial plasmids (Villadas et al. 1995).

USE OF RHIZOBIA PLASMID REPLICATION ORIGINS IN VECTOR TECHNOLOGY

Some naturally occurring plasmids have a broad host range and can be easily transmitted in a controlled way to different heterologous organisms. After convenient manipulation, these plasmids are efficient cloning vectors. There are plasmids with replication and stable maintenance capabilities in a wide range of different Gram-negative bacteria. These plasmids belong to different incompatibility groups, and the most commonly used plasmid vectors are from IncP, IncQ, and IncW. However, it was necessary to introduce several modifications to improve them in order to get the typical characteristics of a good cloning vector: small size, presence of several unique restriction sites, presence of one or more markers for easy selection in different organisms, and ability to detect recombinant molecules by insertional inactivation (Simon and Priefer 1990). Furthermore, the presence of conjugative mechanisms to make them effective in transmission is also desirable. To avoid the risk of uncontrolled propagation it is advisable to keep replication and mobilization systems separated. Finally, the use of environmentally friendly markers is recommended, so that the spreading of undesirable markers is circumvented (for revision on vector technology in Rhizobiaceae, see Simon and Priefer 1990 and references therein).

Despite the great utility of the widely used vectors, problems appear that in some cases are difficult to overcome. Perhaps the most important undesired effect is the occasional low stability. Manipulation and reduction in size lead to the loss of important functions that are scattered throughout the plasmid genome and are implicated in stable maintenance. In some cases, instability is a consequence of vector genome deletion or insertion of exogenous DNA (Meyer et al. 1982). In fact, causes of vector instability are multiple and can vary from one

species to another for a given plasmid. This negative effect not only causes the loss of the plasmid, but also the generation of rearrangements in the vector or in the recombinant plasmid will undoubtedly lead to other important problems.

Additional problems in the use of vectors are the presence of inadequate selective markers. For example, ampicillin and chloramphenicol show an erratic behavior when used in *Rhizobium*. Finally, copy number of a vector and gene dosage can be inconvenient, especially when research is focused on regulatory genes.

The development of exclusive vectors for *Rhizobium*, and in a general sense for Rhizobiaceae, constitutes an attempt to subdue some of the problems that well-known vector systems have. Regardless, and as we already discussed above, little is known about replication systems in *Rhizobium*, despite the high stability showed by resident plasmids. In *A. tumefaciens*, studies on plasmid pTAR (Gallie et al. 1984; Gallie and Kado 1987) showed that some derivative recombinant plasmids were able to replicate in *A. tumefaciens*, *A. rhizogenes*, and *Rhizobium* strains (Gallie and Kado 1987), conferring upon them the potential to be used as cloning vectors.

Only a few attempts in this way have been made in *Rhizobium*. Cloned mini-pSym-b, which is stable in the presence of antibiotic selection, makes it possible to use a small easily transmissible plasmid (Margolin and Long 1993). The pBR322 origin allows the mini-pSym-b vector to exist at a high copy number in *E. coli*, thus facilitating cloning experiments. The very low copy number of mini-pSym-b in *R. meliloti* should be useful for gene expression studies, in which a simulation of chromosomal dosage is important. However, in *trans* functions present in pSym-b should be provided for the complete stabilization of this hybrid plasmid.

Mozo et al. (1990) have constructed several recombinant plasmids that contain a ColE1-type replicon and the *oriV* from plasmid pHc23a from *Rhizobium* sp. (*Hedysarum*). Some of these plasmids showed stabilization rates comparable to or higher than RK2 derivatives. Nevertheless, stability is strongly dependent on host genetic background and on the particular construction. In addition, stability is mediated by the influence of the internal fragments present in the recombinant plasmid as well as their relative position. Although *oriV* from pHc23a is not able to replicate in *E. coli,* the host range of the replicon can be considered broad among Rhizobiaceae. Vectors can replicate in strains of *Rhizobium* sp. (*Hedysarum*), *R. meliloti, R. leguminosarum* bvs. *phaseoli, trifolii,* and *viciae*, *Rhizobium* sp., (*Cicer*) and *A. tumefaciens*. However, use of these plasmids as vectors is limited due to the lack of adequate cloning sites.

Isolation of the minimal replicon of plasmid pRmeGR4a of *R. meliloti* GR4 led to the construction of several recombinant plasmids that retain a high level of stability (Mercado-Blanco and Olivares 1993). Differences in maintenance in different hosts were found. Stability of the hybrid plasmids depended on the cloning vector used. Again, interactions among the different genes present in the construction or their relative position can affect the stability of the recombinant plasmids. An interesting result found was the stabilization of some of the constructions with the *oriV* of pRmeGR4a when it carried the Par/Mrs region of plasmid RK2. This region contains an efficient stabilizing ability and a multimer resolution system, and can stabilize heterologous plasmids in many Gram-negative bacterial species (Roberts et al. 1990; Gerlitz et al. 1990; Saurugger et al. 1986). This region is able to stabilize RK2 derived plasmids in *R. meliloti* isolated from alfalfa nodules as well (Weinstein et al. 1992). Finally, it should be mentioned that stability of the vectors is affected by the genetic background of the bacterial host. Furthermore, better stability rates can be achieved in heterologous backgrounds, as is the case for plasmids pJM401 and pJMB40, which are more stable in *R. leguminosarum* bv. *trifolii* (Mercado-Blanco and Olivares 1993). Recently, several cloning vectors were constructed from the 7.2-kb pRme1132f *R. meliloti* cryptic plasmid (Froissard et al. 1995). The cloning of ColE1-based replicons and the *oriT* from RK2 made possible the extension of its host range. A high degree of stability was achieved from some of the vectors, and compatibility with a RK2-based replicon was demonstrated.

CONCLUDING REMARKS

The definition of pSym and non-pSym plasmids is very often unclear in the literature and some examples have been described above. In this review, we propose to name pSym to those rhizobial plasmids that harbor genes essential for the establishment of a complete symbiotic state resulting in nitrogen-fixing nodules. Then, non-pSym plasmids are defined as those which are not necessary to establish a successful symbiosis, although they can modulate the symbiotic interaction. Our knowledge about rhizobial non-pSym plasmids is increasing quickly. In some cases, symbiotic dispensable functions are encoded, whereas in others they encode functions involved in their survival under free-living conditions. Nevertheless, pSym and non-pSym plasmids still have a large amount of DNA with unknown functions. The existence of the cryptic plasmids raises the question of whether plasmids always need to confer a selective advantage in order to be maintained in the population. We believe that the non-pSym plasmids in rhizobia may confer to the native population not only a selective advantage during establishment of their ecological niches, but also (and maybe even more significantly) an important source of genetic variation and therefore of bacterial evolution.

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