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Analysis of *Rhizobium meliloti* Sym Mutants Obtained by Heat Treatment†

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Deletions in the pSym megaplasmid of *Rhizobium meliloti* were produced at a high frequency, and their lengths varied according to incubation temperature. Morphological differentiation into large and small colonies occurred after heat treatment. Small colonies elicited pseudonodules on alfalfa roots.

Nodulation (*nod*) and nitrogen fixation (*nif-fix*) genes are located on large plasmids in most fast-growing *Rhizobium* strains. They are of greater (megaplasmid) size in *Rhizobium meliloti* (2, 19). The symbiotic gene sequence *nod fix nif fix* seems to be a general feature of all *R. meliloti* strains (7, 15, 17). Mutations in this symbiotic region obtained by different mutagenic treatments have been used to analyze the structural and physical organization of these genes (2, 24). After incubation of *R. meliloti* strains at elevated temperatures, a change in symbiotic properties appeared at a high frequency, but little is known about the molecular mechanisms involved (2). Zurkowski (25) studied the loss of *R. trifolii* nodulation ability and suggested that it derives from the temperature-sensitive replication of the plasmid carrying the nodulation genes. This paper reports studies on *R. meliloti* symbiotic (Sym) mutants obtained by heat treatment.

The bacterial strains and plasmids used are listed in Table 1. *Escherichia coli* was cultured on L broth (14), and rhizobia strains were cultured on yeast extract-mannitol medium (23). For incubation at high temperature, bacteria from late-exponential-phase growth were diluted in fresh medium to give 10^7 to 10^8 cells per ml and incubated without shaking at 37, 38.5, and 39.5°C (±0.2°C) for 12 days. At 2-day intervals, viable cell counts were done. At the end of the incubation time, a sample of each culture was plated onto agar medium and about 200 colonies were tested for nodulation and nitrogen fixation ability. The method of Eckhardt (8) as modified by Rosenberg et al. (20) was used for the physical analysis of plasmids. Purification of plasmid pID1, used as the *nif* structural gene probe, DNA hybridization, and autoradiography were performed as described by Toro et al. (21). Nodulation and nitrogen fixation tests were performed by the method of Olivares et al. (18) and Bedmar and Olivares (4), respectively. Nodules were prepared for light and electron microscopy as described by Herrera et al. (10).

The viability of strains GR4 and L5.30 decreased after the start of incubation and then stabilized. The survival of GR4 and its Sym mutants was essentially the same at elevated temperature (data not shown).

Strain GR4 Sym mutants appeared only at temperatures above 38°C, whereas those of L5.30, a *R. meliloti* strain used for comparative purposes, were obtained at 37°C. All mutants obtained from GR4 after incubation at 38.5°C were of the Nod^+ Fix^- phenotype, whereas at 39.5°C, both Nod^+ Fix^- and Nod^- Fix^- phenotypes were obtained, the latter in higher proportion (Table 2). None of the Sym mutants was auxotrophic.

After incubation of strain GR4 at 38.5°C (Table 2), two types of colonies were always obtained, namely large (L) colonies, that are morphologically similar to those of the parent strain and small (S), clearly differentiated colonies, in a ratio of 3:1. Cells from all the S colonies elicited on *Medicago sativa* roots nodules that were abnormal, appearing as ineffective pseudonodules (phenotype $Nod^{+/-}$); about 25% of the L colonies were Nod^+ Fix^-, and the rest of the L colonies were Nod^+ Fix^+. No type S colonies appeared at 39.5°C.

R. meliloti GR4 harbors four plasmids, two megaplasmids

TABLE 1. Bacterial strains and plasmids

Strains and plasmids	Relevant characters[a]	Source or reference
R. meliloti GR4	Wild type; Nod^+ Fix^+	This laboratory
Mutants		
GRO29	Nod^+ Fix^- [39.5°C]	This work
GRT3	Nod^- Fix^- (Δ*nod-fix*) [39.5°C]	This work
GRT21S	$Nod^{+/-}$ (elicits pseudonodules) [38.5°C]	This work
GRT64L	Nod^+ Fix^- [38.5°C]	This work
R. meliloti L5.30	Wild-type: Nod^+ Fix^+	6
E. coli HB101	*hsdS hsdM pro leu thi gal lacY recA* Sm^r	
Plasmids		
pID1	pBR322 carrying *nifHD* genes	2
pRmSL26	pLAFR1 carrying *nod* genes of *R. meliloti* 2011	16
pGR2	R-prime derivative of pJB3JI isolated from ZB277	1
pGR3	R-prime derivative of pJB3JI	1
pGMI42	R-prime derivative of RP4 isolated from *R. meliloti* 2011	13

[a] The temperatures at which mutants were obtained are in brackets.

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† This paper is dedicated to L. F. Leloir on the occasion of his 80th birthday.

TABLE 2. Effect of incubation at elevated temperatures on symbiotic characteristics of *R. meliloti*

Strain	Temp (°C)	% Sym mutants	% Nod⁻	% Fix⁻	Colony differentiation[a]
GR4	32	0			−
	37	0			−
	38.5	52.5	0	52.5	+
	39.5	2	1.5	0.5	−
L5.30	32	0			−
	37	1.5	0.5	0	−
	38.5	1.0	0	1	−

[a] +, Type S and L colonies were present.

and two large plasmids (N. Toro and J. Olivares, Mol. Gen. Genet., in press). In the plasmid analysis pattern of all Nod⁺ Fix⁻ mutants, neither loss nor deletion of any plasmid was detected by gel electrophoresis (Fig. 1, lanes B, C, and D). Hybridization with the *Pst*I-*nif* fragment of pID1 was obtained with Nod⁺ Fix⁻ (type L) and Nod$^{+/-}$ (type S) mutants obtained at 38.5°C. However, a small deletion in pSym occurred in Nod⁺ Fix⁻ mutants derived from the incubation at 39.5°C (GRO29), because no hybridization was detected by using the same probe (Fig. 1, lane b). This is an indication that at least the *nifH* and *nifD* genes had been lost. In contrast, a large deletion in pSym was observed by electrophoresis of Nod⁻ mutants obtained at 39.5°C (Fig. 1, lane E). This allowed us to detect in 0.7% agarose two megaplasmids greater than 1,000 megadaltons in GR4 (5); one of them was recognized as pSym, a general feature of *R. meliloti* strains. The two megaplasmids of the wild-type strain were also detected by electrophoresis on 0.3% agarose gels (data not shown). No hybridization with pID1 was detected in the Nod⁻ mutants (GRT3; Fig. 1, lane e). The results of hybridization experiments indicated that the homology between the large plasmid of 140 megadaltons, pRmeGR4b, and pID1 was unaltered in all defective symbiotic mutants obtained by heat treatment (Fig. 1). This homology is not due to a reiteration of the *nif* structural genes, because no hybridization is seen when the *Eco*RI-*nif* fragment of pSA30 is used as a probe (Toro and Olivares, in press).

Complementation studies were performed to determine the nature of alterations in the mutants obtained by heat treatment. The results show that although the presence of pGMI42 and pRmSL26 was detected in type S mutants such as GRT21S, no restoration of the mutation was found. On the other hand, pGR3 and pGMI42 but not pRmSL26 restored the Nod⁻ phenotype of GRT3. Type L mutants, such as GRT64L, were not complemented by either pGR3 that carries the *fix* genes mapped to the left of the *nif* structural genes in the pSym of Rm41 or pGMI42 that also carries *fix* genes 200 kilobases away from *nif* genes, as reported by Batut et al. (3). This suggests that other *fix* genes were deleted from GRT64L; perhaps *fix* genes locate to the right of the *nif* genes (15).

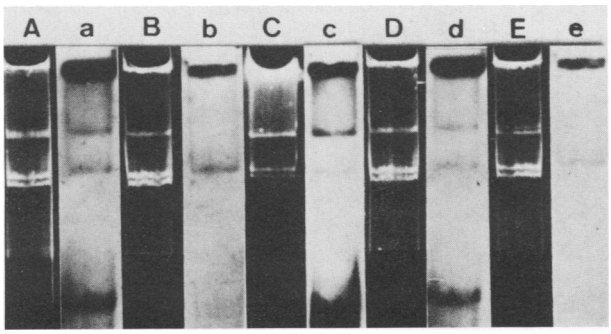

FIG. 1. Agarose gel electrophoresis of lysates from *R. meliloti* GR4 (A), GRO29 (B), GRT21S (C), GRT64L (D), and GRT3 (E). Lanes marked with small letters show the corresponding autoradiograms after hybridization with the *nif* fragment of pID1.

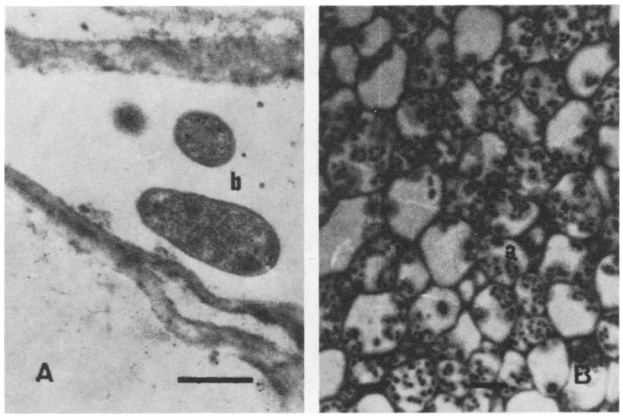

FIG. 2. Microscopic analysis of nodules induced on *M. sativa* by *R. meliloti* GRT21S. (A) Electron micrograph of a nodule section. Bacteria (b) were detected only in the intercellular space of the cortex cells. Bar, 1 μm. (B) Thin section of a nodule. Cells devoid of bacteria are filled with amyloplasts (a). Bar, 10 μm.

These results and the fact that at 38.5°C only Nod⁺ Fix⁻ mutants were obtained from GR4, whereas at 39.5°C Nod⁻ Fix⁻ mutants were also found, suggest that a common origin for all deletions is located to the right of *nif* genes on the basis of the general distribution of *nod*, *nif*, and *fix* genes proposed for other *R. meliloti* strains (14).

Nod⁻ mutants and those that elicited pseudonodules (Nod$^{+/-}$ phenotype) on *M. sativa* roots were used for a detailed study of the process of infection. None of the Nod⁻ mutants assayed produced any modification of the root hairs, and only nonspecific adhesion was observed. GRT21S, which produces pseudonodules, caused curling, but no infection thread was detected. By microscopic analysis, bacteria were observed only in the extracellular spaces of the root cortex (Fig. 2A). Cells of the central nodule (pseudonodule) zone did not contain any bacteria and were filled with amyloplasts (Fig. 2B). GRT21S seems to enter the root cortex through intercellular spaces. The behavior of GRT21S in the establishment of symbiosis with *M. sativa* and the results of complementation assays using different primes showed that both common *nod* and *hsn* genes were present. It was recently reported (11, 12, 22) that the introduction of pSym or only the *nod* genes mapping to the left of the *nif* genes into *Agrobacterium tumefaciens* or *E. coli* causes pseudonodule formation on *M. sativa* roots by these bacteria. Infection and the ultrastructure of nodules produced by these transconjugants look like those induced by GRT21S and the symbiotic mutants described by Finan et al. (9). We suggest that other *nod* genes, essential for functional nodulation and not yet mapped, are located away from this region of pSym or in another part of the *R. meliloti* genome. These type S mutants can be related to those

deficient in exopolysaccharide production described by Leigh et al. (16), who mapped the corresponding locus on a megaplasmid that is distinct from pSym.

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