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Second Symbiotic Megaplasmid in *Rhizobium meliloti* Carrying Exopolysaccharide and Thiamine Synthesis Genes

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Using physical and genetic data, we have demonstrated that *Rhizobium meliloti* SU47 has a symbiotic megaplasmid, pRmeSU47b, in addition to the previously described *nod-nif* megaplasmid pRmeSU47a. This plasmid includes four loci involved in exopolysaccharide (*exo*) synthesis as well as two loci involved in thiamine biosynthesis. Mutations at the *exo* loci have previously been shown to result in the formation of nodules which lack infection threads (Inf$^-$) and fail to fix nitrogen (Fix$^-$). Thus, both megaplasmids contain genes involved in the formation of nitrogen-fixing root nodules. Mutations at two other *exo* loci were not located on either megaplasmid. To mobilize the megaplasmids, the *oriT* of plasmid RK2 was inserted into them. On alfalfa, *Agrobacterium tumefaciens* strains containing pRmeSU47a induced marked root hair curling with no infection threads and Fix$^-$ nodules, as reported by others. This plant phenotype was not observed to change with *A. tumefaciens* strains containing both pRmeSU47a and pRmeSU47b megaplasmids, and strains containing pRmeSU47b alone failed to curl root hairs or form nodules.

A great many natural isolates of rhizobia, agrobacteria, and pseudomonads have been shown to carry a variety of large plasmids (for examples, see references 8, 21, and 38). Some of these are megaplasmids, with molecular masses over 450 megadaltons (38). Genes for a few functions have been localized to these large plasmids, notably including pathogenicity genes for the Ti and Ri plasmids of agrobacteria (6, 21) and symbiotic nodulation (*nod*) and nitrogen fixation (*nif*) genes for the Sym megaplasmids of the fast-growing rhizobia (3, 8, 28, 33, 37, 38). Nevertheless, the significance of this genomic organization remains obscure, although the fact that these are all plant-associated soil bacteria does suggest an underlying evolutionary cause.

Rhizobial Sym megaplasmids are being characterized extensively with regard to symbiosis. In addition, *Rhizobium meliloti* 41 has recently been shown to carry a second megaplasmid, with a molecular weight very nearly that of pSym, on which a region for surface exclusion (although none for symbiotic functions) has tentatively been identified (2). A second megaplasmid has also been identified in *R. meliloti* L530 (34) and SU47 (22, 30, 41, 44) and found to carry genes for production of exopolysaccharide (T. M. Finan, G. F. De Vos, and E. R. Signer, Proc. 6th Int. Symp. Nitrogen Fixation, p. 135, 1985; M. F. Hynes, P. Müller, K. Niehaus, and A. Pühler, Proc. 6th Int. Symp. Nitrogen Fixation, p. 137, 1985). Thus, two megaplasmids may be a general feature of *R. meliloti* strains.

We and our collaborators have been studying a class of fixation-defective (Fix$^-$) mutants of *R. meliloti* SU47 that have a characteristic phenotype (15, 27). On alfalfa they form atypical nodules that lack infection threads (Inf$^-$) and have few bacteria in superficial intercellular spaces only; in culture they are deficient in an extracellular acidic heteropolysaccharide. The mutants have been subdivided by resistance to several phages and complementation with cloned DNA into six phenotypic groups (ExoA through ExoF). Here we show that, whereas the loci for two of the groups (*exoC* and *D*) are chromosomal, the remaining four (*exoA*, *B*, *E*, and *F*), as well as two loci for biosynthesis of thiamine, are located on a megaplasmid that is nearly the same size as but different from the *nod-nif* megaplasmid. Thus, for reasons that are by no means apparent, *R. meliloti* genes required for effective nodulation are located on more than one megaplasmid.

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MATERIALS AND METHODS

Bacterial strains, plasmids, and media. Table 1 lists the bacterial strains and plasmids used. Complex medium was LB (29) or LB containing 2.5 mM $CaCl_2$ and 2.5 mM $MgSO_4$ as previously described (14). Minimal medium M9 (29) was supplemented with 0.25 mM $CaCl_2$, 1 mM $MgSO_4$, 15 mM glucose or sodium succinate, and 0.5 mg of biotin per liter. Antibiotics were routinely used at the following concentrations (in micrograms per milliliter). For *R. meliloti* and *A. tumefaciens*: gentamycin (Gm), 20; neomycin (Nm), 100 and 200; oxytetracycline (Ot), 0.5; rifampin (Rf), 50; spectinomycin (Sp), 100 and 200; streptomycin (Sm), 200; and tetracycline (Tc), 10. For *E. coli*: streptomycin, 10; tetracycline, 10; kanamycin (Km), 20; carbenicillin (Cb), 200; and chloramphenicol (Cm), 20.

Bacterial matings and megaplasmid transfer. Equal volumes of log-phase to late-log-phase cultures of donor, recipient, and mobilizing strain [usually MT607(pRK600)] were mixed, and 1 ml was transferred to a 0.45-μm nitrocellulose membrane filter and washed with LB. The filter was placed on LB agar and incubated at 32°C for 4 to 18 h. In some experiments portions of the mating mix were spotted on LB agar and incubated at 32°C overnight. After mating, the cells were suspended and diluted in 0.85% NaCl and plated on the appropriate media. Controls routinely included donor and recipient both alone and also mixed without the mobilizing strain. Transconjugants were purified three times by single-colony isolation. Plasmid pRK600, which was used to mobilize the megaplasmids via *oriT*, does not replicate in *R. meliloti*: however, it evidently persists for a sufficient period of time to express the RK2 transfer genes (S. Klein, personal communication).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics[a]	Source[b] or reference
R. meliloti		
SU47	Wild type	48
Rm1021	SU47 *str-21*	31
Rm5000	SU47 *rif-5*	14
Rm5011	Rm1021, *suc*::Tn*5*	T. M. Finan, unpublished
Rm5012	Rm1021, *suc*::Tn*5*	T. M. Finan, unpublished
Rm5049	Rm1021, *thi-502*::Tn*5*	S. Klein and G. Walker
Rm5071	Rm1021, *nifH*::Tn*5*	14
Rm5079	Rm1021, Ω5007::Tn*5*-132	15
Rm5083	Rm1021, Ω5011::Tn*5*-233	15
Rm5158	Rm1021, Ω5007::Tn*5*-*oriT*	Tn*5*-*oriT* replacement of Ω5007::Tn*5*-132 in Rm5079
Rm5159	Rm1021, Ω5007::Tn*5*-*oriT*	Tn*5*-*oriT* replacement of Ω5007::Tn*5*-132 in Rm5079
Rm5209	SU47, Ω5007::Tn*5*-*oriT*	ϕM12 (Rm5158) → SU47 Nm^r
Rm5210	SU47, Ω5007::Tn*5*-*oriT*	ϕM12 (Rm5159) → SU47 Nm^r
Rm5213	Rm1021, *thi-504*::Tn*5*-mob	41; S. Klein
Rm5214	Rm1021, *thi-501*::Tn*5*	S. Klein and G. Walker
Rm5216	Rm1021, *thi-503*::Tn*5*-VB32	4; Kosluck and Finan, unpublished
Rm5293	Rm1021, Ω30::Tn*5* (pPH1JI)	W. Szeto (42)
EJ212	SU47, *str-3 thi-505*::Tn*5*	E. Johansen
EJ213	SU47, *str-3 thi-506*::Tn*5*	E. Johansen
EJ219	SU47, *str-3 thi-507*::Tn*5*	E. Johansen
Rm5295	Rm1021, *thi-505*::Tn*5*	ϕM12 (EJ213) → Rm1021 Nm^r
Rm5296	Rm1021, *thi-506*::Tn*5*	ϕM12 (EJ213) → Rm1021 Nm^r
Rm5297	Rm1021, *thi-507*::Tn*5*	ϕM12 (EJ219) → Rm1021 Nm^r
Rm5300	Rm1021, *thi-502*::Tn*5*-11	Tn*5*-11 replacement of *thi-502*::Tn*5* in Rm5049
S2A3	Rm1021, ΩS2A3::Tn*5*	S. Long (23)
Rm5305	Rm1021, ΩS2A3::Tn*5*-11	Tn*5*-11 replacement of ΩS2A3::Tn*5* in S2A3
Rm5307	Rm1021, Ω30::Tn*5*	ϕM12 Rm5293) → Rm1021 Nm^r
Rm5320	Rm1021, Ω30::Tn*5*-11	Tn*5*-11 replacement of Ω30::Tn*5* in Rm5307
Rm5334	Rm1021, Ω30::Tn*5*-11 *exoE*::Tn*5*	ϕM12 (Rm5320) → Rm7022 Gm^r Sp^r
Rm5336	Rm1021, *thi-502*::Tn*5*-11 *exoE*::Tn*5*	ϕM12 (Rm5300) → Rm7022 Gm^r Sp^r
Rm7013	Rm5000, *exoB*::Tn*5*	J. Leigh (27)
Rm7017	Rm1021, *exoD*::Tn*5*	27
Rm7022	Rm1021, *exoE*::Tn*5*	27
Rm7025	Rm1021, *exoC*::Tn*5*	27
Rm7053	Rm1021, *exoD*::Tn*5*	27
A. tumefaciens		
GMI9050	C58C1, Sm^r Rf^r pAtc58	J. Denarie, 39
GMI9023	GMI9050, cured of pAtC58	J. Denarie, 39
At104	GMI9050, pRmeSU47b Ω5007::Tn*5*-*oriT*	Rm5209 × GMI9050 Rf^r Nm^r
At115	At104, pRmeSU47a ΩS2A3::Tn*5*-11	Rm5305 × At103 Rf^r Gm^r Sp^r
At117	GMI9050, pRmeSU47a ΩS2A3::Tn*5*-11	Rm5305 × GMI9050 Rf^r Gm^r Sp^r
At125	GMI9023, pRmeSU47b Ω5007::Tn*5*-*oriT*	Rm5209 × GMI9023 Rf^r Nm^r
At128	GMI9023, pRmeSU47a Ω30::Tn*5*-11	Rm5320 × GMI9023 Rf^r Gm^r Sp^r
At135	At125, pRmeSU47a Ω30::Tn*5*-11	Rm5320 × At125 Rf^r Gm^r Sp^r
E. coli		
MM294A	*pro-82 thi-1 hsdR17 supE44*	G. Walker
A57	MM294A *recA56 srl*::Tn*10*	J. Kruger
MT607	MM294A *recA56*	*srl*$^+$ UV-sensitive strain from A57
KG33	*thiA32*	26; B. Bachmann
KG1673	*thiB33*	26; B. Bachmann
KG6593	*thiC34*	B. Bachmann via T. Kawasaki
Plasmids		
pGS330[c]	Cb^r	De Vos et al.[c]
pGS220	pGS330 Ω::Tn*5*, Cb^r Km^r	De Vos et al.[c]
pGS232	Cb^r Gm^r Km^r Sp^r Sm^r	De Vos et al.[c]
pEYDG1	pBR322 derivative containing Tn*5*-*oriT*	51
pTFM1	pGS330 Ω::Tn*5*-11, Cb^r Km^r Gm^r Sp^r Sm^r	This work
pRK2013	Nm^r, ColE1 replicon with RK2 transfer genes	13
pRK600	Cm^r Nm^s, pRK2013 Nm^r::Tn*9*	This work

[a] *suc*, Defect in succinate metabolism. Tn*5*-VB32 is a Tn*5* promoter probe (4). Symbols for insertions are explained in the text.

[b] As an example, Rm5209 was constructed by phage ϕM12 transduction (14) of Nm^r from Rm5158 to SU47.

[c] pGS330 is a deletion derivative of pBR322 lacking the entire Tc^r area including the single *Eco*RI, *Hin*dIII, *Bam*HI, and *Sal*I restriction sites (G. F. De Vos, G. C. Walker, and E. R. Signer, Mol. Gen. Genet., in press).

Tn*5* replacements. The replacement of Ω5007::Tn*5*-132 by Tn*5*-*oriT* is diagrammed in Fig. 2. (An insertion in no known gene is given an identification number preceded by Ω and followed by a double colon and the symbol of the transposon [1]. For replacements, the insertion number remains unchanged, and the symbol for the transposon changes to the new form.) *Escherichia coli* MT607 (pRK600) was used to mobilize plasmid pEYDG1 from MT607 to *R. meliloti* Rm5079 in a 4-h filter mating. Sm^r Nm^r colonies were selected and screened for Ot^s (frequency, approximately 2%).

Replacements of wild-type Tn*5* (Nm^r) inserts by Tn*5-11* (Gm^r Sp^r) were done as described above, except that plasmid pTFM1 was used instead of pEYDG1, and selected Gm^r Sp^r colonies were screened for Nm^s (frequency, approximately 2%).

DNA manipulations. Plasmid DNA was isolated from 5- to 25-ml *E. coli* cultures by the procedure of Rambach and Hogness (36) as modified by Winans and Walker (50). Lysozyme (0.1 ml, 25 mg/ml) was added to 0.6 ml of concentrated cells in 25% sucrose–50 mM Tris hydrochloride–40 mM EDTA, pH 8.0. After mixing, 0.5 ml of 0.2% Triton X-100 in 50 mM Tris hydrochloride–60 mM EDTA (pH 8.0) was added. The tubes were gently inverted and after lysis was evident were centrifuged in an Eppendorf centrifuge for 5 to 10 min. The supernatants were heated to 70°C for 5 min, and the aggregates were removed by centrifugation. Following polyethylene glycol precipitation, the nucleic acid was phenol extracted, ethanol precipitated, and suspended in 0.1 ml of 20 mM Tris hydrochloride–1 mM EDTA (pH 8.0). Plasmids used for cloning and labeling were isolated as described previously (10) and purified by CsCl-ethidium bromide equilibrium density gradient centrifugation. Total DNA was isolated from *R. meliloti* as described previously (31), and standard conditions were used for restriction endonuclease digestions, transformations, nick translations, and gel electrophoresis (29, 40).

Megaplasmid detection in agarose gels. Megaplasmid DNA was detected by gel electrophoresis by the in-well lysis procedure of Eckhardt (11). For vertical gels the modifications of Rosenberg et al. (38) were used. Horizontal gels with the double-well system (34, 41) and TBB buffer (38) ca. 2 mm above the gel were subjected to electrophoresis for 1 h at 10 mA and then for ca. 15 h at 35 mA. After staining with ethidium bromide, the DNA was visualized under UV light and photographed. DNA-DNA hybridizations were done either directly with dried gels (40) or by Southern blotting (29). Prehybridization in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt solution–50 mM sodium phosphate buffer (pH 6.5)–1 mg of sonicated salmon sperm DNA per ml was done overnight at 42°C. The hybridization solution was as listed above except 20 mM phosphate buffer and 1× Denhardt solution was used. Hybridization with ^{32}P-labeled plasmid pGS220 was done at 42°C for 48 h.

Construction of Tn*5*-11. Tn*5*-11 consists of a central region containing genes encoding Gm^r, Km^r, Sp^r, and Sm^r and the *oriT* of plasmid RK2 (51) flanked by the Tn*5* insertion elements IS*50*L and IS*50*R (25). It was made by using plasmid pGS232 (De Vos et al., manuscript in preparation), which is a pGS220 derivative (Table 1) in which the central *Bgl*II-*Bgl*II fragment of Tn*5* has been replaced by a *Bam*HI-*Bgl*II fragment encoding Gm^r, Km^r, Sp^r, and Sm^r. The 760-base-pair (bp) *oriT*-containing *Bam*HI fragment from pEYDG1 (51) was cloned into the single *Bgl*II site in pGS232 (thereby causing loss of both restriction sites) by ligation of *Bam*HI-restricted pEYDG1 DNA to *Bgl*II-restricted pGS232 DNA; following ethanol precipitation, the ligated DNA was restricted with *Bgl*II and transformed into *E. coli*, with selection for Cb^r. Plasmid pTFM1 was identified among the resulting tranformants, and its structure was confirmed by restriction analysis with *Bgl*II, *Eco*RI, *Hin*dIII, and *Pst*I.

FIG. 1. Order of *exo* and *thi* loci linked by transduction to transposon inserts in pRmeSU47b. The isolation and linkage of the inserts to *exoB* has been described previously (15); for the order of the other loci, see reference 27, the text, and de Vos et al., in press.

Plant tests. Plant nodulations were done by inoculating 2-day-old alfalfa seedlings (cv. Iroquois) growing on Jensen medium (49) in test tubes (18 by 150 mm) with ca. 10^7 bacteria. Plants (at least 10 for each strain tested) were examined for nodule formation over a 6-week period and were assayed for nitrogenase activity by the acetylene reduction assay (31). Plant nodulations were repeated at least twice, and two or more transconjugants from each cross were tested. Alfalfa roots were examined in the light microscope for root hair deformations and infection threads in a modified Fahraeus assembly (12, 19) after staining with 0.01% methylene blue (47).

RESULTS

Mapping of Ω5004::Tn*5*. We have previously described (15) the isolation of transposon inserts Ω5004::Tn*5* and Ω5011::Tn*5*-233 linked by transduction to and flanking the *exoB* locus (Fig. 1). Other work (27; De Vos et al., manuscript in preparation) has shown that two other loci, *exoA* and *exoF*, are linked to these markers in the order Ω5004-*ExoB-ExoF-exoA*-Ω5011. To determine where this region is in the *R. meliloti* genome, we mapped insert Ω5004::Tn*5*.

In RP4-mediated conjugation (31) we found no linkage of Ω5004::Tn*5* to known chromosomal markers, suggesting that the locus was on another replicon. To examine this, we separated plasmid and chromosomal DNA by gel electrophoresis with the in-well lysis procedure of Eckhardt (11). When gels of strains containing Ω5004::Tn*5* were probed with ^{32}P-labeled Tn*5*, hybridization occurred to the megaplasmid band. Horizontal Eckhardt gels (but not vertical gels) often revealed a doublet at the megaplasmid position, in which case the Tn*5* probe hybridized to the upper megaplasmid band; in contrast, with strains carrying a *nifH*::Tn*5* insert, the Tn*5* probe hybridized to the lower megaplasmid band (see Fig. 4). This and the genetic evidence below show that strain SU47 contains two megaplasmids differing only slightly in molecular weight. According to the proposal of Prakash et al. (35), we have designated the *nod-nif* megaplasmid identified previously (8, 38, 39) pRmeSU47a and the *exo* megaplasmid identified here as pRmeSU47b.

Of the remaining *exo* loci, *C* and *D* are not located on pRmeSU47b. For gels of *exoC*::Tn*5* and *exoD*::Tn*5* mutants (Table 1), the Tn*5* probe hybridized to the top of the gel and not to the megaplasmid bands, suggesting a chromosomal location.

For the *exoE*::Tn*5* mutant Rm7022, the Tn*5* probe hybridized to the megaplasmid region, but the two megaplasmids

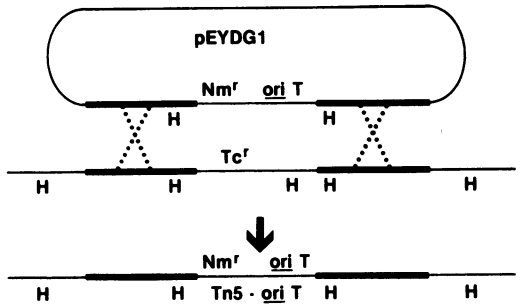

FIG. 2. Replacement of Tn*5*-132 by Tn*5*-*oriT*. Plasmid pEYDG1 is a deletion derivative of pBR322 containing Tn*5*, with the 760-bp *oriT* region of plasmid RK2 inserted in its *Bam*HI site (51). Sites of crossing-over are indicated. Tn*5*-132 contains a 2,700-bp *Bgl*II fragment encoding Tc^r from Tn*10* (24), which has an assymetric *Hin*dIII site (H). Both Tn*5*-132 and Tn*5*-*oriT* have single *Hin*dIII sites in their IS*50* elements (25).

TABLE 2. Conjugal linkage of Ω5007::Tn*5*-*oriT* inserts to *thi-501* and *thi-502*[a]

Donor strain	Recipient allele	Frequency of Thi$^+$ colonies/ donor cell	% Nm^r (no. screened)[b]
SU47	*thi-501*::Tn*5*	6.0×10^{-7}	0.0 (31)
	thi-502::Tn*5*	6.0×10^{-7}	0.0 (30)
Rm5209	*thi-501*::Tn*5*	1.5×10^{-5}	26 (185)
	thi-502::Tn*5*	1.3×10^{-5}	24 (251)
Rm5210	*thi-501*::Tn*5*	1.1×10^{-4}	4.7 (718)
	thi-502::Tn*5*	8.0×10^{-5}	5.5 (775)

[a] Recipient strains were Rm5049 and Rm5214, and *E. coli* MT607(pRK600) was the mobilizing strain. Thi$^+$ recombinants selected on minimal medium containing streptomycin (300 μg/ml) were screened for neomycin sensitivity by replica plating. Spontaneous donor Sm^r occurred at ca. 5×10^{-7}, while Thi$^+$ revertants of Rm5049 and Rm5214 occurred at $<10^{-7}$.

[b] The number of colonies screened (from three independent experiments) is shown in parentheses.

were not resolved. We therefore constructed strains containing both *exoE*::Tn*5* and the mobilizable RK2 origin of transfer *oriT* (Tn*5*-11; see below) in pRmeSU47a and pRmeSU47b (strains Rm5334 and Rm5336, respectively; see Table 1). Nm^r was transferred from Rm5336 to Rm5000 at a frequency of 10^{-4} per donor, whereas no transfer was observed from Rm5334 ($<10^{-8}$). Thus, the *exoE*::Tn*5* insert is located on pRmeSU47b.

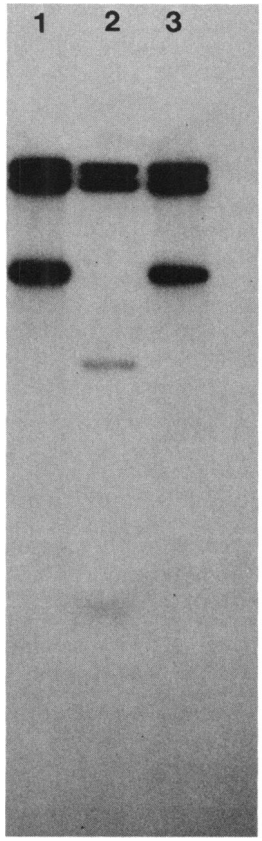

FIG. 3. Southern blot analysis of *Hin*dIII-restricted total DNA from *R. meliloti* Rm5079 (lane 2) and the two Ω5007::Tn*5*-132 replacement strains Rm5209 and Rm5210 (lanes 1 and 3). The blot was probed with labeled pGS220. The boundary fragments in the three strains are the same size, whereas Tn*5*-132 generates two internal fragments differing in size from the single internal fragment of Tn*5*-*oriT* (see Fig. 2).

Mobilization of pRmeSU47b. To manipulate megaplasmid pRmeSU47b genetically, we inserted into it the origin of transfer (*oriT*) of broad-host-range plasmid RK2 by replacing insert Ω5007::Tn*5*-132 with Tn*5*-*oriT* (51), as outlined in Fig. 2. Because the inverted insertion elements (IS*50*L and IS*50*R) on either side of Tn*5* are homologous, recombination between the IS*50*'s of the incoming Tn*5* and the resident Tn*5* derivative results in replacements in which the central region of the replacing transposon has an equal chance of being in either orientation (1, 5; De Vos et al., manuscript in preparation). Southern blot analysis of *Hin*dIII-restricted DNA from strains containing either insert Ω5007::Tn*5*-132 or one of two Ω5007::Tn*5*-*oriT* replacements (Rm5209 and Rm5210) showed that the sizes of the *Hin*dIII-generated border fragments were unchanged (Fig. 3). Furthermore, in transduction both of these replacements showed 100% linkage to Ω5007::Tn*5*-132 and also the expected linkage to the nearby insert Ω5011::Tn*5*-233 [6 and 1%, respectively, compared to ca. 2% for Ω5007::Tn*5*-132; unpublished data and reference 15]. Thus, these are precise replacements.

To determine the direction of *oriT*-mediated plasmid transfer, we transduced the linked insert Ω5011::Tn*5*-233 into the Ω5007::Tn*5*-*oriT* replacement strains and then examined transductants for subsequent transfer of Gm^r Sp^r (i.e., Tn*5*-233). Of the four Tn*5*-*oriT* replacements examined, two transferred Gm^r Sp^r at a frequency of 10^{-6} per recipient (e.g., Rm5209) and two at 10^{-4} (e.g., Rm5210). These data are consistent with the interpretation that Rm5209 transfers pRmeSU47b counterclockwise with respect to Ω5011::Tn*5*-233, whereas Rm5210 transfers it clockwise, as diagrammed in Fig. 1.

Thiamine biosynthetic genes on pRmeSU47b. To investigate whether biosynthetic genes are located on pRmeSU47b, we crossed the Ω5007::Tn*5*-*oriT* replacement strains Rm5209 (counterclockwise) and Rm5210 (clockwise) with 43 independent auxotrophs representing 19 nutritional classes. Only for two *thi*$^-$ (thiamine) auxotrophs were recombinants detected above background (Table 2), suggesting that these two *thi* loci are on pRmeSU47b. This conclusion was confirmed for *thi-502*::Tn*5* by hybridization to Eckhardt gels (Fig. 4).

Approximately sevenfold more Thi$^+$ recombinants were obtained with Rm5210 as donor than with Rm5209 (Table 2). Also, a higher percentge of the Thi$^+$ recombinants were Nm^r

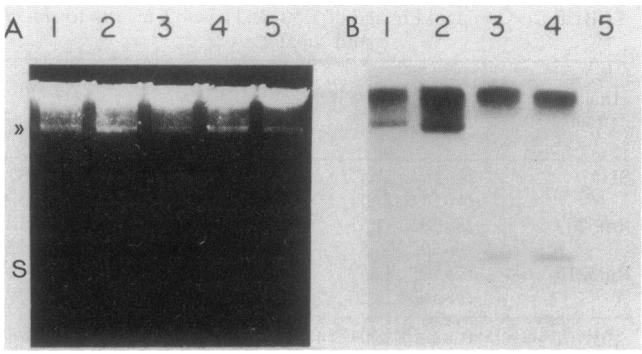

FIG. 4. (A) Horizontal Eckhardt gel of *R. meliloti* strains. Lanes: 1, Rm 5049 (*thi-502*::Tn*5*); 2, Rm5071 (*nifH*::Tn*5*); 3 and 4, Rm5011 and Rm5012 (chromosomal Tn*5* inserts), respectively; 5, Rm1021 (no Tn*5* insert). The positions of the megaplasmid bands (double arrowhead) and sheared DNA (S) are indicated. (B) Autoradiograph of the dried gel shown in panel A after hybridization with labeled plasmid pGS220.

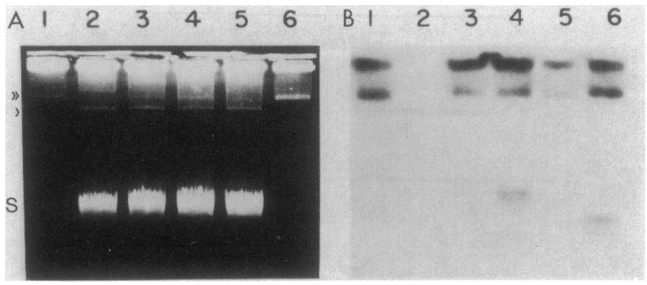

FIG. 5. (A) Horizontal Eckhardt gel of *R. meliloti* strains and *A. tumefaciens* transconjugants. Lanes: 1, Rm5305; 2, GMI9050; 3, At104; 4, At115; 5, At117; 6, Rm5209. The positions of the megaplasmid bands (double arrowheads), pAtC58 (single arrowhead), and the sheared DNA (S) are indicated. (B) Autoradiograph of a Southern blot of the gel shown in panel A after hybridization with plasmid pGS220.

with Rm5209 than Rm5210 as donor. These data suggest that *thi-501*::Tn*5* and *thi-502*::Tn*5* are more closely linked to Ω5007 clockwise than counterclockwise.

We subsequently examined a total of seven independent *thi* mutations. Recombinant plasmids which complemented individual mutations were isolated by transfer of an *R. meliloti* cosmid clone bank (16) into the auxotroph and selection for prototrophic transconjugants. The *thi* mutations fell into two classes based on complementation by the recombinant plasmids obtained (Table 3). In transduction, the class 2 mutation *thi-504*::Tn*5*-mob was 90% linked to Ω5011::Tn*5*-233 (Fig. 1), whereas no linkage to the class 1 mutations *thi-501*::Tn*5* and *thi-502*::Tn*5* was found (data not shown). Moreover, two class 2 mutant strains (Rm5295 [*thi-505*::Tn*5*] and Rm5296 [*thi-506*::Tn*5*]) failed to fluoresce when screened on LB containing Calcofluor (27), indicating that these strains were Exo$^-$. Fluorescence in strain Rm5295 was restored by plasmids pRmT9 and pRmT11 but not by pRmT1 or pRmT8, whereas none of the four plasmids restored fluorescence in strain Rm5296. Both pRmT9 and pRmT11 complemented the Exo$^-$ phenotype of strain Rm7061 (*exoA*::Tn*5*), and conversely the ExoA-complementing plasmid pD34 (27) complemented the auxotrophy of the class 2 *thi* mutation *thi-504*::Tn*5*-mob. These data show that the class 2 *thi* locus and *exoA* are tightly linked, whereas the class 1 *thi* locus is unlinked. *exoA*::Tn*5* insert mutants were Thi$^+$, and whether the two Thi$^-$ Exo$^-$mutants represent deletion or regulatory (e.g., polar) mutations has not been established.

TABLE 3. Complementation of *thi* mutations by recombinant plasmids from an *R. meliloti* clone bank

Strain	Allele	Growth response[a] to plasmid[b]			
		pT1	pT8	pT9	pT11
R. meliloti					
Class 1					
Rm5214	*thi-501*::Tn*5*	+	+	−	−
Rm5049	*thi-502*::Tn*5*	+	+	−	−
Rm5216	*thi-503*::Tn*5*-VB	+	+	−	−
Rm5297	*thi-507*::Tn*5*	+	+	−	−
Class 2					
Rm5213	*thi-504*::Tn*5*-mob	−	−	+	+
Rm5295	*thi-505*::Tn*5*	−	−	+	+
Rm5296	*thi-506*::Tn*5*	−	−	+	+
E. coli					
KG33	*thiA32*	+	+	−	−
KG1673	*thiB33*	±	±	−	−
KG6593	*thiC34*	+	+	−	−
MT607	*thi-1*	+	+	−	−

[a] +, Growth; −, no growth (glucose minimal medium).
[b] Plasmid pairs (pT1 and pT8, pT9 and pT11) are different but have several *Eco*RI, *Bam*HI, *Bgl*II, and *Hin*dIII restriction endonuclease fragments of the same size.

When the *thi*-complementing plasmids were introduced into *E. coli thi*-auxotrophs, the class 1 plasmids complemented *thiA*, *thiC*, and *thi-1* mutations (Table 3) and partially complemented a *thiB* mutation, whereas the class 2 plasmids did not complement any of those mutations.

Transfer of pRmeSU47b to *Agrobacterium tumefaciens*. *A. tumefaciens* strains containing pRmeSU47a form Inf$^-$ nodules on alfalfa which are structurally very similar to those formed by many Exo$^-$ mutants (15, 27, 45). We therefore examined the effect of pRmeSU47b-encoded products on the Inf$^-$ phenotype of *A. tumefaciens*(pRmeSU47a) transconjugants. To construct an *A. tumefaciens* strain containing both plasmids, we required a method of transferring and genetically identifying each plasmid independently (see reference 20). To mark pRmeSU47b, we used the insert Ω5007::Tn*5*-*oriT* and found that Nm^r transfer from Rm5209 to *A. tumefaciens* 9023 or 9050 occurred at frequencies of 10^{-6} per recipient. To mark pRmeSU47a we used Tn*5*-11, a derivative carrying Gm^r Sp^r and *oriT* (see Methods). The Tn*5* inserts Ω30 (42) and ΩS2A3 (23) in pRmeSU47a were replaced by Tn*5*-11 by a procedure similar to that described above for Tn*5*-*oriT* replacements (see Methods). The resulting strains (e.g., Rm5305 and Rm5320) were then used as donors for transfer of pRmeSU47a Ω::Tn*5*-11 to *A. tumefaciens* strains both with and without pRmeSU47b Ω5007::Tn*5*-*oriT*. In these matings Gm^r Sp^r transfer occurred at a frequency of 10^{-5} per recipient.

For both pRmeSU47a and pRmeSU47b, transfer was not detected ($<10^{-8}$) without the mobilizing plasmid, indicating that neither plasmid is self-transmissible. In each case Eckhardt gels of transconjugants showed a band which comigrated with the appropriate megaplasmid in *R. meliloti* (Fig. 5). Plasmid pRmeSU47b, unlike pRmeSU47a, was found to be unstable in *A. tumefaciens*, and under nonselective conditions transconjugants which lost pRmeSU47b formed larger colonies than those with the plasmid. Approximately 10% of colonies from an overnight culture were

Nm^s, regardless of the presence or absence of pRmeSU47a and the native *A. tumefaciens* plasmid pAtC58.

On alfalfa, *A. tumefaciens* strains containing pRmeSU47b alone failed to form nodules or curl root hairs. *A. tumefaciens* containing pRmeSU47a alone formed small white Fix^- nodules on ca. 50% of the plants and induced shepherd's crook formations in root hairs as early as 4 days after inoculation. No infection threads were seen.

With a single exception, strains containing both plasmids had the same phenotype as those containing pRmeSU47a alone. The exception was a plant examined 15 days after inoculation with strain At135(pRmeSU47a, pRmeSU47b), in which infection threads were seen within root hairs. However, in a repeat experiment no infection threads were found. Moreover, bacteria recovered from these nodules nearly always had lost pRmeSU47b. Thus, the significance of these findings is problematical.

DISCUSSION

Physical and genetic data show that in *R. meliloti* SU47 four loci involved in exopolysaccharide synthesis (*exoA*, *B*, *E*, and *F*), as well as two involved in thiamine biosynthesis (*thi*), map to a megaplasmid, pRmeSU47b, which is distinct from the *nod-nif* megaplasmid pRmeSU47a. Mutations at any of the six identified *exo* loci result in Fix^- nodules (15, 27). Thus, in strain SU47 both of the known megaplasmids contain genes required for normal nodule formation.

The precise biochemical step controlled by each of these *exo* loci is not known. However, we have shown that the remaining two *exo* loci (*C* and *D*) are chromosomal, and these two groups of mutations have previously been suggested on other grounds to be only indirectly involved with polysaccharide synthesis (27).

VandenBosch et al. (46) have described three chromosomal mutations of *R. phaseoli* which result in defective exopolysaccharide synthesis and a symbiotic phenotype similar to that of *R. meliloti* Exo^- mutants. Interestingly, in that strain of *R. phaseoli* symbiotic mutations have been mapped to the chromosome and to three different plasmids (32). In a different *R. phaseoli* strain, a gene (*psi*) which inhibits exopolysaccharide production and is required for symbiotic nitrogen fixation has been described (7). As the *psi* gene is located on the *nod-nif* plasmid, it will be of interest to determine whether a similar gene on the *nod-nif* plasmid (pRmeSU47a) in *R. meliloti* controls the *exo* genes on pRmeSU47b.

Like Truchet et al. (45), we find that *A. tumefaciens* (pRmeSU47a) transconjugants elicit a marked root hair curling response and Fix^- nodules but fail to form infection threads. This phenotype is also characteristic of Exo^- mutants, particularly those with mutations in the *exo* genes (*exoA*, *B*, *E*, and *F*) located on pRmeSU47b. Nevertheless, the *A. tumefaciens*(pRmeSU47a) phenotype does not appear to change after addition of pRmeSU47b (which we find to be somewhat unstable in *A. tumefaciens* for reasons not yet known). This result is difficult to interpret, particularly in light of evidence that this exopolysaccharide has the same primary glycosidic structure in both *A. tumefaciens* and *R. meliloti* (52). The explanation might lie in differences in distribution of the known acetyl, succinyl, and pyruvyl substituents, with which, moreover, the untransferred *exoC* and *exoD* loci could conceivably be involved.

The observation that thiamine biosynthetic genes are located on pRmeSU47b is interesting, and in *Rhizobium* and *Agrobacterium* species these genes do not appear to be essential in nature as several strains from each genus appear to naturally require thiamine for growth (18). Thiamine protrotrophy in association with yellow pigmentation has been reported to be plasmid-encoded in four strains in the *Erwinia herbicola* group (17, 43). The *Erwinia* plasmids (ca. 500 and 260 kilobases) appear to be smaller than *R. meliloti* megaplasmids, which have been estimated at as large as 1,500 kilobases (9). Although both rhizobia and erwinia are soil bacteria, the evolutionary relationship among their *thi* plasmids remains to be determined.

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