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Cryptic Plasmid and Rifampin Resistance in *Rhizobium meliloti* Influencing Nodulation Competitiveness†

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An assessment was made of the relative contributions of a spontaneous mutation to rifampin resistance and a cryptic plasmid, pTA2, to competitive nodulation of *Medicago sativa* by a strain of *Rhizobium meliloti*. This was facilitated by use of rifampin-resistant derivatives of this strain in which pTA2 was originally present, cured, or reintroduced. Both curing of pTA2 and spontaneous mutation to rifampin resistance significantly influenced nodulating competitiveness, but the effect of rifampin resistance was greater and such that the contribution of pTA2 was evident only in cases in which paired competitors had the common rifampin resistance background. The data suggest that rifampin-resistant derivatives contain an altered RNA polymerase insensitive to the action of rifampin. All *R. meliloti* derivatives had symbiotic characteristics and phage susceptibility patterns similar to those of the wild type. Plasmid pTA2 transfer or other genetic interchange was not detected in nodules of *M. sativa* inoculated with paired competitors.

Legume inoculation in soils containing indigenous *Rhizobium* populations frequently results in only small proportions of nodules produced by introduced strains (27, 33). Therefore, the improvement of nodulating competitiveness in *Rhizobium* strains intended for legume inoculation is of considerable practical importance.

It has been reported that the symbiotic plasmid in *R. leguminosarum* (5), antibiotic resistance in *R. trifolii* (8), and genetic factors in the legume host (10, 17) influence competitive success in nodulation. However, the genetic basis of nodulating competitiveness in *Rhizobium* spp. is still not understood. Bromfield (6) reported that a derivative of a strain of *R. meliloti* obtained by spontanteous mutation to rifampin resistance and cured of a 135-megadalton cryptic plasmid (pTA2) was significantly less competitive in nodulation than the wild type. The main objective of this investigation was to assess the relative contributions of spontaneous mutation to rifampin resistance and plasmid pTA2 to competitive nodulation by this strain of *R. meliloti*. This was facilitated by use of rifampin-resistant derivatives with pTA2 originally present, eliminated, or reintroduced.

MATERIALS AND METHODS

***R. meliloti* and medium.** The *R. meliloti* strains used in this investigation are described in Table 1. All *R. meliloti* were characterized by the procedure of Lesley (20) with the same 16 distinct phages. *R. meliloti* IZ450(pTA2) and derivatives were grown on yeast extract-mannitol agar (YEM) modified from Fred et al. (16) by using 1 g of yeast extract (Difco Laboratories) per liter and omitting $CaCO_3$.

Competition experiment. Competition for nodulation between *R. meliloti* IZ450(pTA2) and derivatives was examined with *Medicago sativa* cv. Apollo grown in modified Leonard jars (32) containing quartz sand and supplied with nitrogen-free nutrient solution (22). Seedlings from surface-sterilized seed (32) were planted and inoculated with 1 ml of cell suspension washed from the surface of a YEM slope in sterile water to provide ca. 10^8 cells per ml. Inocula consisted of *R. meliloti* IZ450(pTA2) and derivatives individually and in combinations of intended 1:1 mixtures such that paired competitors in each mixture could be separately identified by differential antibiotic resistance. A standard derivative (IZ450rifΩ2::Tn*5*), which was ineffective in symbiosis (owing to a random Tn*5* insertion) and resistant to rifampin and kanamycin, was used to assess the relative competitiveness of derivatives marked only with rifampin resistance. To detect potential plasmid pTA2 transfer or other genetic interchange, we included a mixed-inoculation treatment consisting of IZ450rif/spc and IZ450rif (pTA2Ω1::Tn*5*A). The actual proportions of IZ450(pTA2) or derivatives in mixed inocula were estimated from colony counts on YEM (32). After emergence in a controlled-environment chamber at 200 μE/m^2 per s (22°C, 16-h day; 16°C, 8-h night), the seedlings were thinned to two per Leonard jar.

The experimental design was a randomized complete block with five replications of each inoculation treatment. Shoot dry weight and nodule numbers were determined at 6 weeks after planting, and roots were stored at −40°C for subsequent *R. meliloti* identification.

Identification of *R. meliloti* from nodules. As many nodules as possible (generally not less than 15) were typed from each plant. Excised nodules were surface sterilized (7), and isolations were made by a modification of the method of Franco and Vincent (15), such that each nodule was crushed in ca. 0.1 ml of sterile water between watchmakers' forceps, which were sequentially stabbed into plates of YEM with and without rifampin (100 μg/ml), kanamycin (50 μg/ml), or spectinomycin (200 μg/ml). Plates were incubated for 5 days at 28°C, after which they were read for *R. meliloti* strain or derivative identity. For each mixed inoculation treatment [except IZ450rif/spc plus IZ450rif(pTA2Ω1::Tn*5*A)], a random sample of 50 nodule isolates carrying the distinctive antibiotic resistance marker and growing on nonselective medium was used to determine the incidence of double infections by replica plating on YEM with and without appropriate antibiotics. All nodule isolates typed as double infections from the mixed inoculation treatment consisting of IZ450rif/spc and IZ450rif(pTA2Ω1::Tn*5*A) were tested for

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TABLE 1. *R. meliloti* and characteristics

R. meliloti[a]	Other designation	Characteristics and derivation[b]	Plasmids[a]	Source or reference
IZ450(pTA2)	IZ450	Natural isolate, Brazil	Sym; pTA2	1, 6, 31
IZ450rif(pTA2)	JJ1	Spontaneous Rif^r derivative of IZ450(pTA2)	Sym; pTA2	1, 30,31
IZ450rif	JJ1-C10, KN1001	IZ450rif(pTA2) cured of pTA2	Sym	1,6
IZ450rif(pTA2A)		pTA2 reintroduced into IZ450rif by mating with IZ450(pTA2)	Sym; pTA2	V. N. Iyer, Carleton University, Ottawa, Ontario, Canada
IZ450rif(pTA2Ω1::Tn*5*A)		As for IZ450rif(pTA2A), but pTA2 contained a random Tn*5* insertion	Sym; pTA2::Tn*5*	V. N. Iyer
IZ450rifΩ2::Tn*5*		Tn*5* inserted randomly into IZ450rif	Sym	R. J. Watson, Chemistry & Biology Research Institute, Ottawa, Ontario, Canada
IZ450rif/spc		Spontaneous Spc^r derivative of IZ450rif	Sym	This work

[a] IZ450(pTA2) and all derivatives contain a symbiotic (sym) plasmid (>500 megadaltons); pTA2 is a 135-megadalton cryptic plasimd. Explanation of designations for IZ450(pTA2) derivatives: pTA2A, pTA2 reintroduced; Ω1::Tn*5*, Tn*5* randomly inserted in pTA2; Ω2::Tn*5*, Tn*5* randomly inserted into genome; Tn*5* confers kanamycin resistance (100 μg/ml); rif, rifampicin resistance (200 μg/ml); spc, spectinomycin resistance (400 μg/ml).

[b] IZ450rifΩ2::Tn*5* is symbiotically ineffective; all others are effective on *M. sativa*.

genetic interchange (i.e., the presence of kanamycin and spectinomycin resistance) by replica plating on YEM with kanamycin (50 μg/ml) and spectinomycin (200 μg/ml).

Effect of rifampin on RNA polymerase. RNA polymerase from *R. meliloti* IZ450(pTA2) and IZ450rif was partially purified and tested in the presence and absence of rifampin. *R. meliloti* were grown in TY liquid medium (3) for 18 h at 28°C, and the enzyme was partially purified by ammonium sulfate precipitation (9) followed by elution on a DEAE 52-cellulose column (21). Enzyme activity per 100 μg of protein was assessed by incorporation of [^{14}C]ATP (specific activity, 2 mCi/mmol) into RNA (9).

RESULTS

Phage characterization of *R. meliloti* IZ450(pTA2) and derivatives showed that all rhizobia were sensitive to 4 of 16 typing phages. Phages 7a, N3, and SP produced identical lysis patterns with IZ450(pTA2) and derivatives, but phage N9 produced confluent lysis with IZ450(pTA2), IZ450rif (pTA2Ω1::Tn*5*A), and IZ450rif/spc and produced discrete plaques with the remaining derivatives. The plasmid profiles of IZ450(pTA2) and derivatives were confirmed by the Eckhardt (13) procedure as shown in Fig. 1. Data for shoot dry weight and nodule numbers of *M. sativa* cv. Apollo inoculated with *R. meliloti* IZ450(pTA2) and derivatives are presented in Table 2. The results indicate that IZ450(pTA2) and all but one derivative were approximately equal in symbiotic effectiveness (shoot dry matter production) and significantly differed ($P < 0.01$) from the uninoculated control. The exception (IZ450rifΩ2::Tn*5*), was confirmed as symbiotically ineffective, since shoot dry matter production did not significantly differ from that of the uninoculated treatment. *R. meliloti* IZ450(pTA2) and derivatives elicited similar numbers of nodules per plant, with the exception of IZ450rif(pTA2A), which induced more ($P < 0.01$) nodules than all *R. meliloti* strains except IZ450rif(pTA2Ω1::Tn*5*A).

Table 3 shows data for proportions of *R. meliloti* IZ450(pTA2) and derivatives in mixed inocula and nodules. The relative nodulating competitiveness of all *R. meliloti* strains was assessed by chi-square analyses of the proportions of IZ450(pTA2) or derivatives in the nodules and in each respective inoculum. In all mixed inoculation treatments, derivatives containing plasmid pTA2 [IZ450rif (pTA2), IZ450rif(pTA2A), IZ450rif(pTA2Ω1::Tn*5*A)] formed between 73 and 97% of the nodules and were more competitive ($P < 0.001$) than derivatives lacking this plasmid (IZ450rif, IZ450rifΩ2::Tn*5*, and IZ450rif/spc).

Transposon Tn*5* randomly inserted in pTA2 did not influence competitive ability, because IZ450rif(pTA2) and IZ450rif(pTA2A) (without Tn*5*) did not differ significantly in

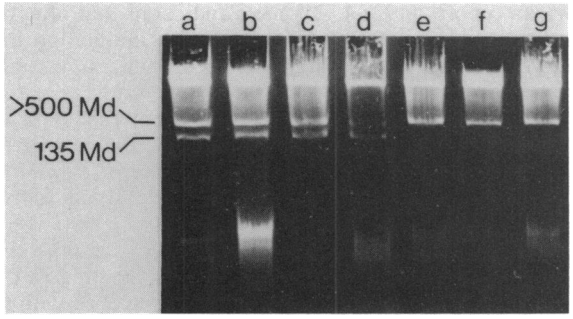

FIG. 1. Eckhardt gel analysis of *R. meliloti* IZ450(pTA2) and derivatives with cryptic plasmid pTA2 originally present [lane a, IZ450(pTA2); lane b, IZ450rif(pTA2)], cured (lane e, IZ450rif; lane f, IZ450rifΩ2::Tn*5*; lane g, IZ450rif/spc), or reintroduced (lane b, IZ450rif(pTA2A); lane c, IZ450rif(pTA2Ω1::Tn*5*A). Arrows marked >500 Md (megadaltons) and 135 Md indicate the positions of the symbiotic plasmid and pTA2, respectively.

TABLE 2. Response of *M. sativa* cv. Apollo to inoculation with *R. meliloti* IZ450(pTA2) and derivatives

Inoculum	Shoot dry wt (mg per plant)[a]	No. of nodules per plant[a]
IZ450(pTA2)	63.9a	43.8b
IZ450rif(pTA2)	45.0ab	70.5b
IZ450rif	70.9a	66.1b
IZ450rif(pTA2A)	83.7a	100.5a
IZ450rif(pTA2Ω1::Tn*5*A)	58.2a	72.4ab
IZ450rifΩ2::Tn*5*	9.4b	47.7b
IZ450rif/spc	61.3a	43.7b
Uninoculated	7.7b	

[a] Values are means of 10 plants; means with a letter in common do not differ at $P < 0.01$.

TABLE 3. Percentage representation of *R. meliloti* IZ450(pTA2) and derivatives in mixed inocula and nodules of *M. sativa* cv. Apollo

Composition of mixed inoculum[a]		% of *R. meliloti* A in inoculum[a]	% of nodules due to *R. meliloti* A alone	Ratio of doubly infected nodules to total nodules typed	χ_2 (df = 1)[b]
R. meliloti A	*R. meliloti* B				
IZ450rif(pTA2)	IZ450rif(pTA2Ω1::Tn*5*A)	60	48	74:202	2.90; NS[c]
IZ450rif(pTA2)	IZ450rifΩ2::Tn*5*	55	73	20:146	7.03; $P < 0.001$
IZ450rif(pTA2Ω1::Tn*5*A)	IZ450rif(pTA2A)	48	54	57:210	0.72; NS
IZ450rif(pTA2Ω1::Tn*5*A)	IZ450rif	49	77	29:187	16.82; $P < 0.001$
IZ450rif(pTA2A)	IZ450rifΩ2::Tn*5*	47	84	39:148	30.29; $P < 0.001$
IZ450rif	IZ450rifΩ2::Tn*5*	46	58	56:179	2.88; NS
IZ450(pTA2)	IZ450rif(pTA2)	27	99	8:167	111.20; $P < 0.001$
IZ450(pTA2)	IZ450rif(pTA2Ω1::Tn*5*A)	36	99	10:205	90.46; $P < 0.001$
IZ450(pTA2)	IZ450rif(pTA2A)	34	98	13:213	91.27; $P < 0.001$
IZ450(pTA2)	IZ450rif	35	99	7:162	92.63; $P < 0.001$
IZ450(pTA2)	IZ450rifΩ2::Tn*5*	31	99	10:169	101.63; $P < 0.001$
IZ450rif(pTA2Ω1::Tn*5*A)	IZ450rif/spc	40	97	31:267	75.29; $P < 0.001$

[a] For characteristics of *R. meliloti* IZ450(pTA2) and derivatives, see Table 1.
[b] Chi-square analyses relating proportions of *R. meliloti* IZ450(pTA2) or derivatives in each inoculum with those in nodules. df, Degree of freedom.
[c] NS, Not significant.

competitiveness from IZ450rif(pTA2Ω1::Tn*5*A) (with Tn*5*) in the respective mixed inoculation treatments. Further, results for mixed inoculum, IZ450rif plus IZ450rifΩ2::Tn*5*, indicated that random insertion of Tn*5* into the genome, inducing symbiotic ineffectiveness (IZ450rifΩ2::Tn*5*), had no significant effect on nodulating competitiveness. In inoculum mixtures with IZ450(pTA2), all rifampin-resistant derivatives were significantly less competitive ($P < 0.001$), forming no more than 2% of the nodules irrespective of the presence or absence of plasmid pTA2, clearly demonstrating that the common rifampin resistance marker had a major effect on competitive nodulation, which obscured the contribution of pTA2. Plasmid pTA2 transfer or other genetic interchange was not detected in any nodule isolates from the mixed inoculation treatment consisting of IZ450rif/spc and IZ450rif(pTA2Ω1::Tn*5*A). The incidence of doubly infected nodules, determined by replica plating, varied between 4 and 37% for all inoculation treatments.

The activities of RNA polymerase from IZ450(pTA2) and IZ450rif, assessed by incorporation of ^{14}C into RNA were, respectively (in disintegrations per minute): 1,560 ± 100 and 1,494 ± 78 (without rifampin); 116 ± 38 and 1,462 ± 57 (with 5 μg of rifampin per ml); and 120 ± 11 and 1,396 ± 66 (with 100 μg of rifampin per ml). These data indicate that RNA polymerase from IZ450rif was uninhibited by rifampin.

DISCUSSION

In this investigation, we assessed the relative contributions of a spontaneous mutation to rifampin resistance and cryptic plasmid, pTA2, to competitive nodulation by a strain of *R. meliloti*. Both pTA2 and spontaneous mutation to rifampin resistance influenced nodulating competitiveness, but the effect of rifampin resistance was greater and such that the contribution of pTA2 was evident only in cases in which paired competitors had the common rifampin resistance background. Rifampin resistance in *Escherichia coli* (2, 14) and *R. meliloti* (21) has been shown to be due to an altered RNA polymerase that is uninhibited by rifampin. The present data for *R. meliloti* suggest that the effect of rifampin resistance on competitive ability was also due to an altered RNA polymerase. The only previous report of an influence of antibiotic resistance on competitive nodulation was by Bromfield and Jones (8) using *R. trifolii*.

Considerable attention has been given to symbiotic plasmids in *Rhizobium* spp. in which determinants for biological nitrogen fixation have been located (26). These plasmids have also been implicated in the competitive nodulation of *R. leguminosarum* (5). However, many *Rhizobium* species contain additional (cryptic) plasmids whose functions are unknown (12). Our data show that a cryptic plasmid in *R. meliloti* influenced nodulating competitiveness but not susceptibility to lysis by phage or symbiotic properties. A previous report (1) also indicated that plasmid pTA2 was not essential for nodulation or nitrogen fixation in *R. meliloti*.

Reports by Schwinghamer (28, 29) and Bromfield and Jones (8) indicate that resistance to different antibiotics can affect symbiotic properties in several *Rhizobium* species. Our results show that spontaneous mutation to rifampin resistance in *R. meliloti* did not significantly influence symbiotic characteristics or phage susceptibility patterns relative to the wild-type strain.

Treatment of bacteria with heat to eliminate extrachromosomal DNA can result in genomic deletions (35). In the present study, heat treatment did not affect the competitive or symbiotic properties of *R. meliloti* because derivatives in which plasmid pTA2 had been eliminated and subsequently reintroduced [IZ450rif(pTA2A) and IZ450rif(pTA2Ω1::Tn*5*A)] did not significantly differ in this regard from a derivative in which pTA2 was originally present [IZ450rif(pTA2)].

Various reports (19, 24, 25) have indicated that effective *Rhizobium* strains are more competitive in nodulation than ineffective strains. However, instances of ineffective strains being more competitive (15, 34) suggest no consistent relationship between effectiveness and competitiveness in the legume-*Rhizobium* association. This is in agreement with the present data, in which an effective derivative of *R. meliloti* (IZ450rif) did not significantly differ in competitiveness from the ineffective derivative (IZ450rifΩ2::Tn*5*).

Dual-strain occupancy of nodules has been reported to occur in a substantial minority of nodules (up to 25%) on axenically grown *M. sativa* (6, 7). This is in accord with the present data for *M. sativa* grown in Leonard jars, in which between 4 and 37% of the nodules typed were doubly infected.

Plasmid transfer has been demonstrated by mating *Rhizobium* strains and species and selecting for transconjugants (1, 4, 11, 18, 23). However, in our experiments, cryptic plasmid transfer or other genetic interchange was not de-

tected in nodules of *M. sativa* inoculated with paired *R. meliloti* competitors.

This study was concerned with the contribution of two components of the *R. meliloti* genotype to competitive ability. The major influence exerted by determinants for rifampin resistance provides potential for further analysis of genetic factors involved in competitive nodulation by *R. meliloti*.

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