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Further examination of presumptive *Rhizobium trifolii* mutants that nodulate *Glycine max*

(host specificity/DNA analysis/carbon source utilization/phage susceptibilities/immunological properties)

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ABSTRACT **Two recent reports described the isolation of derivatives of a *Rhizobium trifolii* strain that had gained the ability to nodulate *Glycine max* and *Vigna radiata* and that had demonstrated altered patterns of carbon source utilization, free-living nitrogen fixation, and hydrogen uptake. More extensive characterization of these strains now supports the conclusion that these strains are *R. japonicum* and are not derived from the putative parent *R. trifolii*.**

Two recent communications (1, 2) described derivatives of *Rhizobium trifolii*, strain T1, that appeared to have acquired several unusual properties simultaneously through a single-step selection for decreased assimilation of ammonium. Because these derivatives were also found to be resistant to the antimetabolite L-methionine-D,L-sulfoximine, additional isolates were selected as being resistant to this compound. These new isolates were also initially characterized as defective in ammonium assimilation, but in fact they lacked none of the enzymes of this pathway—namely, glutamine synthetase (EC 6.3.1.2), glutamate synthase (EC 2.6.1.53), and glutamate dehydrogenase (EC 1.4.1.4). These strains also grew much more slowly than the putative parent strain DT6, an isolate of *R. trifolii* T1, and metabolized a different range of carbon compounds. Furthermore, these isolates could reduce dinitrogen to ammonium (fix nitrogen) in appropriate culture conditions (2) even though neither *R. trifolii* nor any other fast-growing *Rhizobium* has yet been found to do so.

Subsequently, these same isolates were reported to form fully effective N_2-fixing nodules on both *Glycine max* and *Vigna radiata*. This report was highly promising because *R. japonicum* strains are normally specific for the legume hosts with which they form effective symbioses (3), although some *Rhizobium* strains exhibit broader specificity. The previous report suggested a means of selecting *Rhizobium* derivatives with a greatly altered host range, and it offered a potential insight into the factors involved in the determination of specificity for both nodulation and nitrogen fixation.

The potential significance of these observations prompted us to further examine strains DT71, DT72, DT125, DT128, DT129, and DT130 (1, 2) by various procedures: DNA equilibrium density centrifugation, phage susceptibility, carbon source utilization, immunofluorescence, immunodiffusion, and nodulation specificity. We show that these isolates behaved similarly to each other, but differed from the presumed parental strain, *R. trifolii* DT6, in all of these tests. At the same time, they showed a strong similarity with the *R. japonicum* strain 3I-1b-110 (USDA 110).

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MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. (See Table 1.) Methods of culture and derivation of *R. trifolii* T1, its derivatives, and *R. japonicum* 3I-1b-110 (USDA 110) have been described (1, 2, 4–6). Strain DT71 was isolated after selection for ammonium-growth-defective derivatives of DT6. Strain DT72 is a spontaneous *rif*^{r} mutant of DT71. DT125 was isolated as a methionine sulfoximine-resistant (Msx^r) derivative of DT6. DT128, DT129, and DT130 resulted from methionine sulfoximine-resistant selections of DT8, which was a *rif*^{r} *str*^{r} derivative of DT6. DT6 was isolated from a mutagenized culture of T1. The strains were grown in YAP medium (0.1% yeast extract/0.1% L-arabinose/0.1% L-glutamate/7.5 mM potassium phosphate, pH 6.3) or YM (yeast mannitol) medium (4). Solid media were supplemented with 1.2% or 1.5% agar. For tests of growth on various carbon sources the average diameters (mm) of well-isolated single colonies were measured after 8 days at 30°C in defined medium (2). Carbon sources were added to 0.2% except for acetate, which was added to 0.075%.

Enzyme Assays. Strains were grown in YAP medium at 30°C and cell extracts were prepared and assayed for enzyme activities as described (7).

Propidium Bromide/CsCl DNA Equilibrium Density Centrifugation. DNA (20 μg) from phenol-extracted cell pellets of strains DT6, DT72, and DT125 were mixed with 20 mM Tris·HCl, pH 8.2/0.1 mM EDTA to which CsCl was added to achieve a density of 1.71 g ml^{-1} and 5 ml total volume (8). Propidium bromide was added to 0.05 mg ml^{-1} and solutions were centrifuged to equilibrium at 150,000 × *g* for 72 hr at 25°C. Photographs were taken directly from CsCl gradient tubes containing fluorescent DNAs banded to their equilibrium buoyant densities.

Phage Susceptibilities. Phage φT10 is a temperate phage that infects *R. trifolii* T1 with relatively high specificity (9). Phages F2, F4, F5, F6, F8, F14, F15, F17, P311, P19F, φR1, and φjE were isolated from soil by E. R. Signer, R. A. Ludwig, and E. A. Raleigh. Phages F2 through P19F infect various fast-growing *Rhizobium* strains; φR1 and φjE infect *R. japonicum* R54a (from J. Döbereiner). Bacterial host strains used for phage tests were *R. trifolii* T1, *R. phaseoli* 127K26, *R. leguminosarum* 128C53, and *R. japonicum* R54a. Bacteria were grown in YM or YAPcm (YAP supplemented with 1 mM $CaCl_2$

Abbreviations: FA, fluorescent antibody; FITC, fluorescein isothiocyanate.

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Table 1. Bacterial strains

R. trifolii	T1	E. Schwinghamer
	DT6	(From T1)
	DT8	DT6 Str^r Rif^r
Derivation uncertain	DT71	DT6 Asm$^-$
	DT72	DT71 Rif^r
	DT125	DT6 Msx^r
	DT128, 129, 130	DT8 Msx^r
R. japonicum	3I-1b-110	USDA 110
	61A76	Nitragin
	R54a	J. Döbereiner
R. leguminosarum	128C53	Nitragin
R. phaseoli	127K26	Nitragin
Rhizobium sp. "cowpea"	CB1809	
	CB756	
	32H1	Nitragin

and 0.8 mM $MgSO_4$) medium. To test phage susceptibility, we harvested bacterial cultures in exponential growth phase and spotted phages onto lawns of bacteria plated in soft agar (0.65%). Phage suspensions were at 1–2 × 10^9 plaque-forming units ml^{-1}, except for ϕT10 at >10^8 plaque-forming units ml^{-1}, and produced definitive zones of lysis in sensitive bacterial lawns; all tests were done in duplicate.

Immunodiffusion. Procedures have been described (10). Cell suspensions were used without heat treatment in diffusion against a concentrated immunoglobulin preparation made from *R. trifolii* T1 antiserum by ammonium sulfate precipitation (11). Before diffusion against specific antisera for *R. japonicum* strains CB1795, CB1809, USDA 110, USDA 123, and CC709 (equivalent to 61A77) and for *Rhizobium* sp. 32H1 and CB1923, samples of each suspension were immersed in a boiling water bath for 20 min (10).

Immunofluorescence. An isolated colony of each strain to be tested was transferred to a slant which subsequently served as inoculum for growth in YM medium. Each isolate was grown for 5 days and then tested for fluorescent antibody (FA)- and lectin-binding ability. Reference strains representative of different serogroups of *R. japonicum* were included. All strain-specific antibodies included were prepared and used for immunofluorescence as described (12, 13). Lectin from soybean seed was prepared by affinity chromatography (14), labeled with fluorescein isothiocyanate (FITC), and observed for bacterial binding by fluorescence microscopy (15). In the Canberra laboratory, dried suspensions were treated with antiserum prepared against T1 or USDA 110, throughly washed, and then treated with FITC-labeled goat anti-rabbit antiserum. In addition to T1, DT6, DT72, DT125, and DT128-DT130, a number of single colony isolations from DT6 streak plates were also examined.

Plant Nodulation Tests. Surface-sterilized seeds of *Trifolium subterraneum*, *Glycine ussuriensis*, and *Macroptileum atropurpureum* were germinated on agar plates and sown into foil-capped tubes with an agar slope (4, 16). Similarly treated *T. repens* were sown into cotton-plugged tubes with a short agar slope (17). The tubes were inoculated with the test *Rhizobium* strain 3 days after sowing. Plants in foil-capped tubes were grown in a controlled environment cabinet (18) with (*i*) 22°C root temperature and 22°C/15°C shoot temperature regime (*T. subterraneum*) or (*ii*) 28°C root temperature and 28°C/23°C shoot temperature regime (*G. ussuriensis* and *M. atropurpureum*). The daily light period was 14 hr and the light intensity was 540 μE m^{-2} sec^{-1}. *T. repens* seedlings were grown beneath a light bank at 25°C constant temperature and

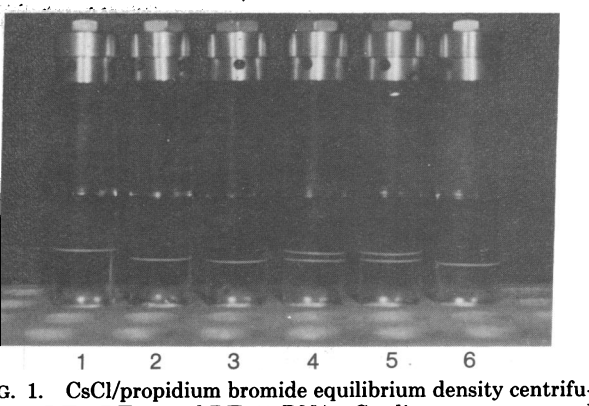

FIG. 1. CsCl/propidium bromide equilibrium density centrifugation of DT6, DT72, and DT125 DNAs. Gradients were prepared and centrifuged. Tube 1, DT6 DNA; tube 2, DT72 DNA; tube 3, DT125 DNA; tube 4, DT6 and DT72 DNAs; tube 5, DT6 and DT125 DNAs; and tube 6, DT72 and DT125 DNAs.

plants were examined for nodulation at 2-day intervals commencing 7 days after inoculation. The three first-mentioned plant species differed from those previously described (1) and were chosen for their ease of culture (i.e., small seeded) in foil-capped tubes and their similarity of nodulation patterns with *T. repens*, *G. max* (19), and *V. radiata*, respectively. Surface-sterilized seeds of *G. max* (cv. Lincoln), *V. unguiculata* (cv. Poona), and *V. radiata* were sown into pots of vermiculite/perlite. Inocula were added at sowing. *G. max* was grown in controlled environment with a 25°C/20°C temperature regime and 14-hr light period at 500 μE m^{-2} sec^{-1}. *Vigna* species were grown under similar conditions except for a 30°C/25°C regime. Most seedlings had emerged in 4 days and were examined for nodulation 9 days later. In order to examine reported differences (1) in H_2 evolution between the DT strains and the effectiveness of these strains on soybean, cowpea, and mung bean, nodulated roots were tested for H_2 evolution in a 30-min assay and companion plants were used in an acetylene reduction assay. Both H_2 and C_2H_4 were determined by gas chromatography. Shoot dry weights were determined.

RESULTS

Fig. 1 shows results of density gradient centrifugation and demonstrates that DNAs of strains DT6, DT72, and DT125, centrifuged to equilibrium singly, exhibited one fluorescent band at density approximately 1.7 g ml^{-1} in CsCl/propidium bromide solution. When parallel gradient tubes were compared, DT6 DNA exhibited a lighter buoyant density than did either DT72 or DT125 DNA. When DT72 and DT125 DNAs were centrifuged to equilibrium in the same gradient tube there was only a single fluorescent band, whereas when DT6 DNA was centrifuged together with either DT72 or DT125 DNAs two

Table 2. Growth on various carbon sources after 8 days at 30°C

	Colony size, mm		
Carbon source	DT6	DT72	DT125
None	1.0	0.5	0.4
L-Arabinose*	3.8	1.5	0.9
Glucose	5.0	0.8	0.9
Ribitol	3.0	0.5	0.4
Malate	3.9	0.5	0.5
Gluconate	1.0	0.8	1.1
Pyruvate	0.9	0.5	1.1
Citrate	0	0.7	0.9

* Results for fructose, mannitol, xylose, ribose, D-arabinose, and glycerol were similar to those found for L-arabinose. Acetate and lactose completely inhibited growth.

Table 3. Key enzymes of the L-arabinose pathway in DT6, DT72, and DT125*

Strain	L-Arabinose dehydrogenase	2-Keto-3-deoxy-L-arabonate aldolase	2-Ketoglutarate semialdehyde dehydrogenase	Glycolaldehyde dehydrogenase
DT6	220	0	85.5	0
DT72	20	46.5	11.6	2.0
DT125	31.4	54	35.6	14.7
R. trifolii T1	184	0	375	0
R. japonicum 61A76	23	65	28	28

* Activities were measured in extracts of cultures grown with L-arabinose as the carbon source and are expressed as nmol min^{-1} mg $protein^{-1}$.

distinct fluorescent bands were evident in each case. The difference in buoyant densities between DT6 and either DT72 or DT125 does not support the previous contention that strains DT72 and DT125 are derivatives of strain DT6.

Table 2 shows growth on different carbon sources. There is some growth (presumably on the glutamate) even without an added carbon source. Growth was stimulated by a variety of carbon sources, but the pattern was different in strains DT72 and DT125 from that in strain DT6, the former strains being "slow growers." Growth of strain DT6 was inhibited by lactose, acetate, and citrate; gluconate and pyruvate had no effect; and the other carbon sources were stimulatory. [Succinate was also stimulatory, with maximal colony size reached in 3 days (not shown).] Strains DT72 and DT125 were also inhibited by lactose and acetate. In contrast to strain DT6, DT72 and DT125 used gluconate and citrate but failed to use ribitol, malate, or succinate.

Table 3 shows results of enzyme assays in the L-arabinose pathway. DT6, like *R. trifolii* T1, had high levels of L-arabinose dehydrogenase activity and appreciable 2-ketoglutarate semialdehyde dehydrogenase activity, but neither 2-keto-3-deoxy-L-arabonate aldolase activity nor glycoaldehyde dehydrogenase activity was detectable. On the other hand, DT72 and DT125 had aldolase activities comparable to the control *R. japonicum* 61A76. Fast- and slow-growing classes of *Rhizobium* have different pathways for the utilization of L-arabinose (7). DT6 appears to metabolize L-arabinose like fast-growing rhizobia whereas the presence of the aldolase activity in DT72 and DT125 suggests that they metabolize L-arabinose via a different pathway, like that of slow-growing strains of rhizobia, such as *R. japonicum*. Therefore, both growth tests and assays of L-arabinose catabolic enzymes indicate that, whereas DT6 behaves as a typical *R. trifolii* strain, DT72 and DT125 show substantially different patterns of carbon metabolism.

Results of phage susceptibility tests are shown in Table 4. *R. leguminosarum* 128C53, *R. phaseoli* 127K26, and *R. trifolii* T1 and DT6 were all sensitive to 10 phages that grew on fast-growing strains of rhizobia, and DT6 and T1 were also sensitive to the T1-specific phage ϕT10. Strains DT72 and DT125 were resistant to ϕT10 and, like *R. japonicum* R54a, were also resistant to the other 10 phages specific for the fast-growing strains; these strains were sensitive to phages ϕjE and ϕR1. Strains DT71 and DT128–DT130 were resistant to ϕT10.

Table 4. Sensitivity of *Rhizobium* strains to bacteriophage

Phage	Bacterial strain 128C53	127K26	T1	DT6	DT72	DT125	R54a
ϕT10	NT	NT	+	+	−	−	NT
F2*	+	+	+	+	−	−	−
ϕR1	−	−	−	−	+	+	+
ϕjE	−	−	−	−	+	+	+

NT, not tested.

* Identical results with phages F4, F5, F6, F14, F15, F17, P311, and P19F.

Immunodiffusion tests (Fig. 2) showed that only DT6 reacted with antibodies specific for *R. trifolii* T1; additional precipitin bands were formed by DT6 but were attributed to the dense suspensions used. DT72, DT125, DT128, DT129, and DT130 reacted with antibodies specific for *R. japonicum* USDA 110 to form continuous bands between all of these strains and the homologous standard (Fig. 3); all these strains reacted strongly with the antiserum prepared against *R. japonicum* CC709 but lacked one precipitin band present in the CC709 standard (Fig. 4). No reactions were detected with any of the other antisera used. Furthermore, after preadsorption with cells of DT125, anti-USDA 110 antiserum reacted with neither USDA 110 nor any of the DT isolates. In a subsequent test (results not shown), DT71 (from F. O'Gara) also reacted with USDA 110 antiserum. Strain CC714 (R54a from J Döbereiner) did not react with USDA 110 antiserum.

The results of immunofluorescence and lectin-binding tests, obtained immediately after receipt of the cultures (in Minnesota), are summarized in Table 5. DT strains 72, 125, 128, 129, and 130 reacted strongly with FA prepared against *R. japonicum* strain USDA 110, but failed to react with any of the other *R. japonicum* FAs used. Staining in each of the crossreactive instances was equivalent to that of the homologous *R. japonicum* control strain, USDA 110. All microscope fields were examined carefully by both phase and fluorescence systems to detect evidence of mixed cultures. No 110-FA stained cells were seen in any DT6 preparation after initial receipt of the cultures. No 110-FA nonreactive cells were observed in DT125, DT128, DT129, and DT130. In preparations of DT72 stained with 110-FA, nonfluorescing rods were observed with a frequency of less than 1%. Adsorption of 110-FA with DT128 cells rendered the FA completely unreactive to both DT128 and *R. japonicum* 110. All DT strains crossreactive to 110-FA also bound soybean lectin as actively as did *R. japonicum* 110. Binding of soybean lectin as a possible determinant in soybean

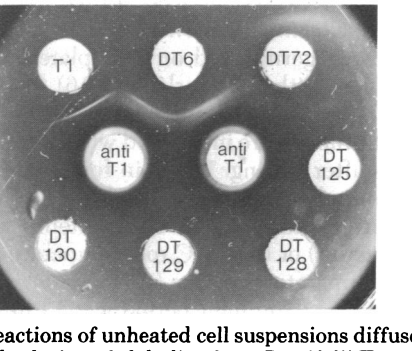

FIG. 2. Reactions of unheated cell suspensions diffused against a concentrated solution of globulins from *R. trifolii* T1 antiserum.

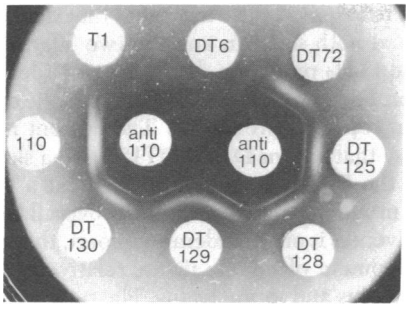

FIG. 3. Immunodiffusion of heated cell suspensions against *R. japonicum* USDA 110 antiserum.

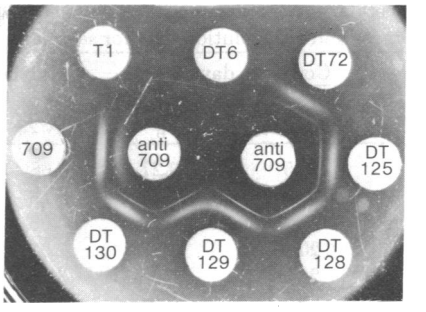

FIG. 4. Immunodiffusion of heated cell suspensions against *R. japonicum* CC709 antiserum.

nodulation specificity has been associated with strains of *R. japonicum*, but not with *R. trifolii* (15).

DT6 was reexamined for immunofluorescence 10 weeks after initial receipt of the cultures. Examination of the same microscope fields by alternate immunofluorescence and phase contrast showed that this preparation now included 50–60% 110-FA reactive cells. Apparently, the 110-FA binding component of the DT6 culture must have been present initially in low concentration and have become dominant during the 10-week period proceding subsequent reexamination.

In the Canberra laboratory, the results of fluorescent antibody tests with USDA 110 antiserum were similar to those from Minnesota. In addition, strain DT71 from F. O'Gara gave a positive result with this antiserum, whereas T1 and cultures of DT6 from both Davis and F. O'Gara were the only ones to react with T1 antiserum. A low proportion of cells in an older culture of DT6 reacted with 110 antiserum.

Plant nodulation tests were conducted on a number of isolates. The *Trifolium* species were nodulated promptly by T1, DT6, three cultures derived from fast-growing, single-colony isolates of DT6, and one slow-growing isolate from DT6 (DT6-21). *Trifolium* was not nodulated by three other slow-growing single-colony isolates from DT6, nor by any cultures in the group DT72, DT125, DT128, DT129, and DT130, nor by *R. japonicum* USDA 110 or CB1809, nor by *Rhizobium* sp. 32H1, CB756, or CB1024. All these cultures, except CB1024 and CB756, nodulated *Macroptileum atropurpureum* and *G. ussuriensis* promptly; strains CB1024 and CB756 were 2–3 days slower in nodulating *G. ussuriensis*. Uninoculated controls of all species failed to nodulate. Only one culture, DT6, nodulated all four species, albeit somewhat tardily with *M. atropurpureum* and *G. ussuriensis*, which suggested that DT6 was, in fact, a mixed culture. After 12 days, pot-grown plants (see *Materials and Methods*) failed to show any nodules when inoculated with T1, DT6-21, or a fast-growing, single-colony isolate from DT6. Those plants inoculated with two slow-growing, single-colony isolates from DT6, with DT72, DT125, DT128, DT129, and DT130, with *R. japonicum* USDA 110, or with DT6 not purified by single-colony isolation, all showed good nodulation in the crown region of the plants. Uninoculated plants were not nodulated. Immunofluorescence tests on single-colony isolates from DT6 cultures showed that those nodulating *Trifolium* species reacted only with T1 antiserum, whereas those nodulating *G. max*, *M. atropurpureum*, and the two *Vigna* species only reacted with 110 antiserum. DT6-21 reacted with T1 antiserum despite being isolated as a slow grower. These results confirm the conclusion that DT6 was a mixed culture containing both *R. trifolii* and *R. japonicum* when received in Australia.

Hydrogen evolution from nodules formed by DT6 and its derivatives on cowpea was low (less than 0.2 μmol $g^{-1}hr^{-1}$), and C_2H_2 reduction rates, also expressed on a nodule fresh weight basis, were uniformly high (Table 6). Compared with the shoot weight data for plants nodulated by *Rhizobium* sp. strains CB756 and 32H1, the level of symbiotic effectiveness of DT6 and putative derivatives was moderate. Similar results were obtained with these strains on *V. radiata* and *M. atropurpureum*. With soybeans, all DT cultures formed an effective symbiosis, comparable to that achieved by the known effective *R. japonicum* strain CB1809. There was a marked decline in the rates of H_2 evolution between 28 and 30 days after sowing, with occasional plants showing no H_2 evolution, suggesting the development of uptake hydrogenase activity. Acetylene reduction rates were similar at both sampling harvests. These observations could explain the apparent lack of H_2 evolution previously reported for DT130. Uptake hydrogenases are limited largely to *Rhizobium* sp. (cowpea rhizobia), but it is of interest that strain USDA 110 is one of the few *R. japonicum* strains possessing this enzyme (20).

Table 5. *R. japonicum* FA- and lectin-binding reactions of *R. trifolii* strains (6) and reference strains of *R. japonicum*

	Immunofluorescence*							Lectin
Cultures 1	31-FA	Web 48-FA	71A-FA	110-FA	117-FA	123-FA	138-FA	binding†
"Mutant" strains‡								
DT-6	—	—	—	—	—	—	—	—
DT-72	—	—	—	3+	—	—	—	4+
DT-125	—	—	—	3+	—	—	—	4+
DT-128	—	—	—	4+	—	—	—	3+
DT-129	—	—	—	4+	—	—	—	3+
DT-130	—	—	—	3+	—	—	—	4+
Homologous *R. japonicum* strains§	4+	3+	4+	4+	4+	3+	4+	NT

* Symbols: —, no fluorescence; 3+ to 4+, bright specific fluorescence equivalent to homologous reaction.
† Binding of FITC-labeled soybean seed lectin to bacteria as observed by fluorescence microscopy. Symbols: —, no binding; 3+ to 4+, strong yellow-green fluorescence associated with most cells; NT, not tested.
‡ Subcultures from single colony isolations made from cultures immediately after receipt from K.T. Shanmugam
§ E. L. Schmidt culture collection. These strains reacted only with their own FA.

Table 6. Acetylene reduction and shoot dry weight data for cowpeas and H_2 evolution data for soybeans

Strain	Cowpeas, day 24		Soybean H_2,	
	C_2H_4,	Shoot,	μmol $g^{-1}hr^{-1}$	
	μmol $g^{-1}hr^{-1}$	g	Day 28	Day 30
"DT6"	20.5	0.27	0.66	0.16
DT6-11*	19.2	0.27	5.18	ND
DT72	17.9	0.19	0.08	0.02
DT125	20.8	0.45	2.92	0.47
DT128	23.7	0.28	3.65	1.03
DT129	22.1	0.24	3.83	0.54
DT130	24.3	0.15	4.17	1.06
32H1	24.3	0.56	ND	ND
CB756	25.5	0.55	ND	ND
CB1809	ND	ND	0.17	ND
LSD ($P = 0.05$)	NS	0.17	1.71	0.55

All rates were based on nodule fresh weight. "DT6", not purified; ND, not done; NS, not significant.
* Slow-growing isolate reacting with USDA 110 antiserum.

DISCUSSION

On the basis of the marked differences between the fast-growing DT6 and the slow-growing putative derivatives of DT6 in such characteristics as DNA buoyant density, carbon source utilization, the enzymes of carbon metabolism, phage sensitivity, lectin binding, and antigenic features, there seems little possibility that such derivatives are genuine mutants of DT6. Change in one, or possibly more, characteristics may be expected after mutagenesis, but the chance that all characteristics would be affected, in addition to the other differences previously reported (1, 2), is exceedingly remote. For example, extensive experience with *Rhizobium* phages has indicated that spontaneous or induced mutants resistant to a variety of antibiotics and antimetabolites rarely show any loss of sensitivity to ϕT10 or other phages (E. A. Schwinghamer, unpublished results). In all the experiments reported herein, the results are consistent with the interpretation that DT6 is *R. trifolii*, whereas DT71, DT72, DT125, DT128, DT129, and DT130 are closely related to each other and to *R. japonicum* strain USDA 110.

It seems likely that the source of confusion was contamination of the *R. trifolii* strain DT6 culture with a strain similar to *R. japonicum* USDA110. This is indicated from the immunofluorescence assays and the plant nodulation tests with single-colony isolations from DT6 grown on normal YMA medium. While it may be argued that DT8, derived from DT6, and the parental culture for DT128-DT130, was a *rif*^{r} *str*^{r} mutant of *R. trifolii* DT6 and that contaminants should have been eliminated during selection for antibiotic resistance, some *R. japonicum* and *Rhizobium* sp. (cowpea rhizobia) strains have a high inherent level of resistance to streptomycin and rifampicin (B. L. Dreyfus and A. H. Gibson, unpublished data). Furthermore, they are completely resistant to methionine sulfoximine (6 mg ml^{-1}) used to select DT128, DT129, and DT130 (R. A. Ludwig, unpublished results). Therefore, the selections for antibiotic resistance were probably inadequate to rid DT8 of contaminating *R. japonicum* and these were then enriched after methionine sulfoximine selection. Unfortunately, strain DT8 was not available for examination. The observation that DT6 was a mixed culture, and that in the Canberra laboratory, 2 of 12 slow-growing single colony isolates exhibited both T1 and 110 serotypes, could provide an explanation for the apparently anomalous nodulation of *T. repens* by DT71 and DT72 (1).

The previous report (1) also indicated that the DT6 derivatives nodulated both soybean and the two *Vigna* species effectively, and C_2H_2 reduction data were provided to support this conclusion. Such broad specificity in symbiotic effectiveness had not been reported previously. The current results (e.g., Table 5) show that strains DT72–DT130 and a slow-growing isolate from the DT6 culture reduce C_2H_2 at a high rate in cowpea and mung bean nodules, but the overall level of symbiotic effectiveness is only moderate. In part, this is due to the slower development of nodule tissue, relative to CB756 and 32H1, on cowpeas and mung beans. The results also point up the inherent danger of using a single C_2H_2 reduction assay alone to assess symbiotic effectiveness.

In conclusion, we believe that there are sufficient uncertainties surrounding the true orgin of the slow-growing cultures designated DT71–DT130 to make the earlier reports, indicating that they were derived from *R. trifolii*, open to serious doubt. At the same time, the results reported in this paper indicate the difficulty in positively identifying cultures supposedly derived from a particular strain, especially when a parent strain exhibits an undistinguished phenotype.

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