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Use of Two-Dimensional Polyacrylamide Electrophoresis to Demonstrate that Putative *Rhizobium* Cross-Inoculation Mutants Actually Are Contaminants

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Two-dimensional polyacrylamide electrophoresis was used to determine that mutants of *Rhizobium trifolii* DT6, claimed to be capable of effectively nodulating soybeans, were actually *Rhizobium japonicum* 110 contaminants isolated from the parent DT6 culture.

Rhizobium strains have been classified into two broad groups (1). Group I consists of fast-growing species such as *R. leguminosarum, R. phaseoli, R. trifolii,* and *R. meliloti.* Group II comprises the slow-growing species, *R. japonicum* and *R. lupini.* Cross inoculation within these two groups has been shown to occur (reviewed in reference 6). However, a strain from a fast-growing species (e.g., *R. trifolii,* which nodulates clover) has never been found to nodulate a host plant that is normally infected by a slow-growing species (e.g., *R. japonicum,* which nodulates soybean).

Recently it was reported that after mutagenesis or by spontaneous resistance to L-methionine-D,L-sulfoximine (MSX), it was possible to isolate, from a strain of *R. trifolii* DT6, mutants capable of asymbiotic nitrogen fixation (3). The ability to fix high levels of nitrogen asymbiotically is a property of many strains of *R. japonicum,* but had not previously been reported for *R. trifolii.* Further work with these mutants indicated that perhaps one could still ineffectively nodulate clover but all could now effectively nodulate soybeans (4). It appeared that the barrier to cross-inoculation between group I host plants and group II host plants had been broken. However, other characteristics of these putative mutants such as slow growth on rich media, possession of an uptake hydrogenase, and a cytochrome spectrum resembling that of *R. japonicum* indicated that these mutants might in fact be *R. japonicum* isolates.

To test the possibility that these mutants actually were *R. japonicum* strains, we tested the cultures shown in Table 1, which were sent to us by K. T. Shanmugam. *R. trifolii* DT6 was the parent strain, and DT72 is a spontaneous isolate that is resistant to rifampin. Strain DT72 was derived from a rifampin-susceptible strain (DT71) that had been isolated from DT6 after nitrosoguanidine mutagenesis. Strain DT125 is a spontaneous mutant selected from DT6 on the basis of MSX resistance. DT130 is also resistant to MSX and was obtained from a strain (DT8) that had previously been selected from DT6 as being streptomycin and rifampin resistant. The parent strain, DT6, effectively nodulated clover. It was claimed that the mutants DT72, DT125, and DT130 could no longer effectively nodulate clover but that DT72 and DT130 could now effectively nodulate soybean. DT125 was not tested with soybean.

Immediately upon receipt of the strains, the cultures were streaked onto yeast extract-mannitol agar (5), and the plates were incubated at 30°C. After 3 days, it was possible to see two different colony types on plates streaked from the DT6 culture. One was a fast-growing slime producer that resembled a typical *R. trifolii* colony. The second type comprised very few of the total number of colonies on the plate and was a slow-growing non-slime producer. In older cultures, isolates of this type also produced a brown pigment. These are characteristics of *R. japonicum* colonies. Single colonies of each type were picked and restreaked for purity. The fast grower was labeled DT6-FG; the slow grower was labeled DT6-SG. Two colony types appeared on the plates streaked from the DT72 culture. Both were slow growing and did not produce slime. One type, labeled DT72-A, looked like the DT6-SG colonies, whereas the other was smaller and did not resemble either DT6-FG or DT6-SG. This isolate was labeled DT72-B. Both isolates were repicked and streaked for purity. Plates streaked from the DT125 and DT130 cultures had only one colony type on them. Single colonies of each were picked and repurified. These were labeled

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TABLE 1. *Nodulation of soybean or clover by isolates derived from a culture of R. trifolii DT6*[a]

Strain	Reported[b] to produce effective nodules on:	No. of nodulated plants	
		Clover	Soybean
Uninoculated		0	0
R. japonicum 110		0	10
R. trifolii 162P17		10	0
DT6	Clover	10	10
DT6-FG		10	0
DT6-SG		0	10
DT72	Soybean	0	10
DT72-A		0	10
DT72-B		0	0
DT125	NR	0	10
DT125-A		0	10
DT130	Soybean	0	10
DT130-A		0	10

[a] Plants were inoculated with cells grown in yeast extract-mannitol broth for 4 days, and then assayed after 2 weeks for acetylene reduction and nodule formation. Ten plants were assayed for each strain. All nodulated plants were effective. NR, Not reported.

[b] O'Gara and Shanmugam (4).

DT125-A and DT130-A, respectively. After a period of 2 months, we again streaked plates from the original cultures sent to us in order to see whether we could repeat these isolations. Strains obtained from the second isolation had the same properties as the first isolates; therefore, neither colony type arises from the other during storage.

Both the original cultures and strains that we isolated from those cultures were tested to see whether they could effectively nodulate soybean (*Glycine max* var. Corsoy) or clover (*Trifolium repens*). The nodulation tests were performed by the effectiveness assay (7) for soybean and the procedure of Leps et al. for clover (W. T. Leps, W. J. Brill, and E. T. Bingham, manuscript in preparation). The results of these tests are shown in Table 1. Uninoculated control plants had no nodules. *R. japonicum* 110 effectively nodulated soybean but not clover, whereas *R. trifolii* 162P17 was effective on clover but not soybean. These results show that our technique with the effectiveness assay was adequate to ensure aseptic conditions. The DT6 culture effectively nodulated both clover and soybean. In the original reports (3, 4), this strain had only been found to be effective on clover. The one isolate from the DT6 culture, DT6-FG, formed effective nodules on clover, whereas the other isolate, DT6-SG, nodulated soybean. The DT72 culture and the one isolate from that culture, DT72-A, both formed effective nodules on soybean. The other isolate, DT72-B, did not form nodules on either soybean or clover. Strains DT125 and DT130 and the single-colony isolates from those strains, DT125-A and DT130-A, all formed effective nodules on soybean. Since the putative mutants DT72, DT125, and DT130 were selected from the DT6 culture partly on the basis of being slow growing (a characteristic of *R. japonicum*), it can be concluded that they were not mutants of *R. trifolii* but merely *R. japonicum* contaminants.

To further indicate whether DT72, DT125, and DT130 were *R. japonicum* contaminants, two-dimensional polyacrylamide gel electrophoresis was used (5). If DT72, DT125, and DT130 are indeed mutants of DT6, then their gel patterns should look alike and be almost identical to gels obtained from DT6. This should be true particularly of DT125 and DT130, since they were reported to be spontaneous mutants and therefore should be altered at most in only a few proteins. If DT72, DT125, and DT130 are *R. japonicum* contaminants, then their gel patterns will not look like DT6 but will resemble those of typical slow growers (5). Figure 1A is a picture of a gel pattern from *R. trifolii* 162P17. Figure 1B is the pattern obtained from our isolate DT6-FG from the DT6 culture. It can be seen that the gels are similar, indicating that DT6-FG is a strain of *R. trifolii*. Figure 1C is a gel pattern obtained from *R. japonicum* 110 and shows the typical pattern for a slow grower. Figure 1D shows the gel pattern obtained with strains DT6-SG, DT72-A, DT72-B, DT125-A, and DT130-A. These strains all gave an identical pattern that is typical for a slow-growing strain. Among all the slow-growing strains examined (5; this paper), the above strains are most like *R. japonicum* 110. It can be concluded that DT6-SG, DT72-A, DT72-B, DT125-A, and DT130-A may be *R. japonicum* strain 110, a strain previously studied in that laboratory (2). Therefore, it appears that the original culture used by O'Gara and Shanmugam (3, 4) was contaminated with *R. japonicum*, and from this culture they isolated contaminants and used them for their nodulation studies.

The results from this paper demonstrate a practical use of two-dimensional polyacrylamide gels to identify and classify *Rhizobium*.

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ADDENDUM

After this paper was submitted for publication, two

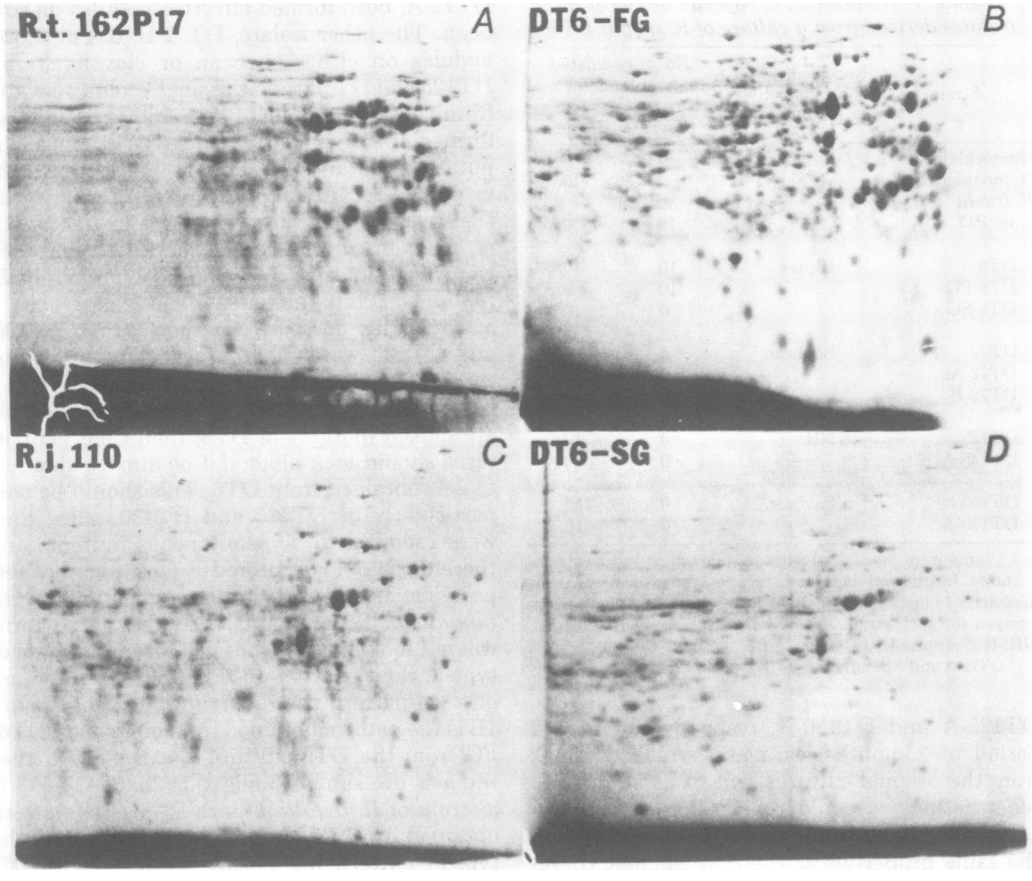

FIG. 1. *Two-dimensional polyacrylamide gel protein patterns of R. trifolii and R. japonicum strains. Exponentially growing cells were pelleted by centrifugation from yeast extract-mannitol broth and washed once in phosphate-buffered saline before being extracted for gels (5). In each gel, proteins on the left side are basic (pH ~8); those on the right side are acidic (pH ~4); those at the top are high molecular weight (~150,000); and those at the bottom are low molecular weight (~15,000).*

other reports appeared that reach the same conclusion as this report (J. R. Ludwig, E. A. Raleigh, M. J. Duncan, E. R. Signer, A. H. Gibson, W. F. Dudman, E. A. Schwinghamer, D. C. Jordan, E. L. Schmidt, and D. T. Tran, Proc. Natl. Acad. Sci. U.S.A. **76**:3942–3946, 1979; J. R. Mielenz, L. E. Jackson, F. O'Gara, and K. T. Shanmugam, Can. J. Microbiol. **25**:803–807, 1979).

LITERATURE CITED

1. **Jordan, D. C., and O. N. Allen.** 1974. Family *Rhizobiaceae*, p. 261–267. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
2. **O'Gara, F., and K. T. Shanmugam.** 1976. Regulation of nitrogen fixation by *Rhizobia*. Export of fixed N_2 as NH_4^+. Biochim. Biophys. Acta **437**:313–321.
3. **O'Gara, F., and K. T. Shanmugam.** 1977. Regulation of nitrogen fixation in *Rhizobium* spp. Isolation of mutants of *Rhizobium trifolii* which induce nitrogenase activity. Biochim. Biophys. Acta **500**:277–290.
4. **O'Gara, F., and K. T. Shanmugam.** 1978. Mutant strains of clover rhizobium (*Rhizobium trifolii*) that form nodules on soybean (*Glycine max*). Proc. Natl. Acad. Sci. U.S.A. **75**:2343–2347.
5. **Roberts, G. P., W. T. Leps, L. E. Silver, and W. J. Brill.** 1980. Use of two-dimensional polyacrylamide gel electrophoresis to identify and classify *Rhizobium* strains. Appl. Environ. Microbiol. **39**:414–422.
6. **Vincent, J. M.** 1974. Root-nodule symbiosis with *Rhizobium*, p. 265–341. *In* A. Quispel (ed.), The biology of nitrogen fixation. North-Holland Publishing Co., Amsterdam.
7. **Wacek, T. J., and W. J. Brill.** 1976. Simple, rapid assay for screening nitrogen-fixing ability in soybean. Crop Sci. **16**:519–522.