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# Characterization of *Rhizobium* sp. (*Cicer arietinum* L.) by immunofluorescence, immunodiffusion, and intrinsic antibiotic resistance

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Twenty-nine cultures of *Rhizobium* spp. for chickpea (*Cicer arietinum* L.) from various culture collections were characterized by serological techniques and by intrinsic antibiotic resistance (IAR). Fluorescent antibodies (FAs) against the somatic antigens of eight of the inoculum-quality strains grouped all cultures into at least four distinct somatic serogroups. Serogroup I (Nitragin 27A8) contained the majority of the strains. Serogroups II (Nigragin 27A3) and III (TAL-480) each crossreacted with one strain, while serogroup IV (USDA 3HOa9) consisted only of the homologous strain. Nine of the chickpea strains and 55 other cultures from among strains of *R. leguminosarum*, *R. lupini*, *R. japonicum*, and *Rhizobium* spp. (*Vigna*, *Sesbania*, and *Stylosanthes*) did not react with any of the four specific FAs. Immunofluorescence – cross adsorption and immunodiffusion analyses of cultures within serogroup I revealed that four of the strains most commonly recommended for inoculum use were serologically identical. The intrinsic antibiotic resistance patterns of these four cultures were also identical. Serogroup II was shown to contain two distinct serotypes (Nitragen 27A3 and 27A16), while the two strains in serogroup III (TAL 480 and 622) were found to be the same. Although intrinsic antibiotic resistance could differentiate among the strains, some cultures that were completely different by serological means were found to group in the same IAR categories. Additionally, some cultures that were serologically identical were found to have different IARs. Our data on antigenic uniqueness of the chickpea rhizobia and their lack of serological crossreaction with any other *Rhizobium* species (especially with those in *R. leguminosarum*, where they had previously been classified), as well as the results of other workers on their nodulation specificity for *Cicer*, led support to the proposal that *C. arietinum* and its root-nodule bacteria constitute a separate cross-inoculation group.

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Vingt-neuf cultures de *Rhizobium* spp. pour le pois chiche (*Cicer arietinum* L.) regroupées à partir de diverses collections de cultures ont été caractérisées par des techniques sérologiques et par leur résistance intrinsèque aux antibiotiques (IAR). Des sérums fluorescents (FAs) dirigés contre les antigènes somatiques de huit souches de qualité d'inoculum ont permis de grouper toutes les cultures en un minimum de quatre sérogroupes somatiques différents. Le sérogroupe I (Nitragin 27A8) englobe la majorité des souches. Les sérogroupes II (Nitragin 27A3) et III (TAL-480) montrent tous deux une réaction croisée avec une souche, alors que le sérogroupe IV (USDA 3HOa9) ne comprend que la souche homologue. Neuf souches du pois chiche et 55 autres provenant de souches de *R. leguminosarum*, *R. lupini*, *R. japonicum* et *Rhizobium* spp. (*Vigna*, *Sesbania* et *Stylosanthes*) n'ont réagi avec aucun des quatre FAs spécifiques, L'immunofluorescence, l'adsorption croisée et l'immunodiffusion faites sur des cultures du sérogroupe I a révélé une identité sérologique chez quatre des souches les plus couramment recommandées comme inoculum. Les profils de résistance intrinsèque aux antibiotiques de ces quatre cultures étaient aussi identiques. Le sérogroupe II contenait deux sérotypes différents (Nitragen 27A3 et 27A16), alors que deux souches du sérogroupe III (TAL 480 et 622) se sont avérées semblables. Même si la résistance intrinsèque aux antibiotiques pouvait différencier les souches, quelques cultures qui étaient tout à fait différentes sérologiquement se sont retrouvées dans les mêmes catégories IAR. De plus, quelques cultures qui étaient sérologiquement identiques ont montré des profils IAR différents. Nos observations sur le caractère antigénique unique des rhizobium du pois chiche, leur absence de réaction sérologique croissée avec toute autre espèce de *Rhizobium* (surtout celles de *R. leguminosarum* où elles avaient été classées préalablement) ainsi que les travaux d'autres chercheurs sur la spécificité de la formation de nodules chez *Cicer* supportent l'hypothèse que *C. arietinum* et ses bactéries des nodules des racines constituent un groupe à part d'inoculation croisée.

[Traduit par le journal]

## Introduction

Serological methods provide very rapid and specific means for identifying bacteria. They have been used extensively in identifying rhizobia from cultures and from nodules of legumes (for reviews see Vincent 1982; Dudman 1977), as well as analyzing inter- and intra-specific relationships among rhizobia (Date and Decker 1965; Dudman 1964, 1971; Graham 1963, 1969; Skrdleta 1969). The fluorescent antibody (FA) technique is the most versatile of the serological techniques for ecological studies of *Rhizobium* (Schmidt 1973), since it permits simultaneous detection and identification of desired strains directly in the nodule, soil, and rhizosphere (see Bohlool and Schmidt 1980). The immunodiffusion technique has also been applied to taxonomic (Dudman 1964, 1971; Gibbins 1967;

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TABLE 1. Sources of chickpea *Rhizobium* cultures

| Cultures | Other designations | Source |
|---|---|---|
| Nitragin 27A2[a] | (USDA 691 = Rothamsted 3828 = ICRISAT 3828) | |
| Nitragin 27A3 | (USDA 3100) | |
| Nitragin 27A7 | (O. N. Allen 736) | Dr. J. C. Burton, Nitragin Co., WI |
| Nitragin 27A8 | (Mexico) | |
| Nitragin 27A11 | (Mexico) | |
| Nitragin 27A16[a] | (Ruiz-Argueso Ca 7) | |
| Nitragin 27A17[a] | (Ruiz-Argueso Ca 36) | |
| Nitragin 27A18 | (Ruiz-Argueso Ca 142) | |
| TAL-1008 | (= Dadarwal Ca 2) | |
| TAL-1010 | (= Dadarwal Ca 1002) | |
| TAL-1012 | (= Dadarwal Ca 141 St.) | |
| TAL-263 | (= Nu 191) | |
| TAL-385 | (= CB 1189 = Rothamsted 3827 = ICRISAT 3827 = TAL-619—origin USDA 3HOa1)[b] | NifTAL Culture Collection, University of Hawaii, HI. |
| TAL-480 | (= UASB67) | |
| TAL-619 | (same as TAL-385) | |
| TAL-620 | (= CC 1192 = Rothamsted 3889 = ICRISAT 3889, -ex CSIRO Canberra, -ex Israel)[b] | |
| TAL-621 | (= ICRISAT DNRa-1) | |
| TAL-622 | (= ICRISAT Cicer-5) | |
| TAL-623 | (= ICRISAT CA-7) | |
| Nitragin 27A5 ATCC 11444 | (= USDA 3HOa1) | Dr. A. H. Gibson, Canberra, Australia |
| USDA 3HOa1 | (obtained from M. S. Raju) | |
| USDA 3HOa2 | (Wis. No. 67, Univ. of Wisconsin) | |
| USDA 3HOa5 | (Univ. of Wisconsin, 1944) | |
| USDA 3HOa6 | (Ithaca, NY) | Dr. D. F. Weber, U.S. Department of Agriculture, MD |
| USDA 3HOa8 | (P. A. Ark, Univ. of Calif.) | |
| USDA 3HOa9[a] | (P. A. Ark, Univ. of Calif.) | |
| USDA 3HOa10 | (P.A. Ark, Univ. of Calif.) | |
| USDA 3HOa12 | (Univ. of Calif.) | |

[a]Cultures tested for growth characteristics (according to Vincent 1970) and found to be "fast-growing" acid-producing strains.
[b]Corbin *et al.* 1977.

Humphrey and Vincent 1965, 1969, 1975; Skrdleta 1969; Vincent and Humphrey 1968, 1970; Vincent *et al.* 1973) and ecological (Dudman and Brockwell 1968; Dudman 1977; Skrdleta 1980; Vincent 1977) studies of rhizobia. These serological investigations have emphasized the marked somatic heterogeneity of the *Rhizobiaceae*.

*Cicer arietinum* L. (chickpea, garbonzo) is an important pulse crop. It is the third most widely grown grain legume in the world (van der Maesen 1972). It is grown extensively in the Middle East; in many regions of India it is the most important legume grown (Medhane and Patil 1974; van der Maesen 1972). Simon in 1914 (see Fred *et al.* 1932) and later Vincent (1974) place the *Cicer* rhizobia in the pea (*R. leguminosarum*) inoculation group, although a number of other workers (for review see Guar and Sen 1979) recommended placement in a separate group. Guar and Sen (1979) tested 71 strains of *Rhizobium* spp. (*C. arietinum*) on 87 species of legumes and 287 *Rhizobium* strains from other legume species on four genotypes of *C. arietinum*. The lack of any apparent cross-nodulation led these authors to conclude that *C. arietinum* and its root-nodule bacteria should be considered in a separate cross-inoculation group.

In this study we apply immunofluorescence (IF), immunodiffusion (ID), and intrinsic antibiotic resistance (IAR) to characterize and group isolates of *Rhizobium* spp. (*C. arietinum*) obtained from several culture collections throughout the world.

## Materials and methods

*Source and maintenance of cultures*

Table 1 lists the cultures of the chickpea rhizobia used in this

study. The source and pedigree (if known) of the cultures are also given. All cultures were grown and maintained on a yeast extract mannitol medium (YEMS) (Bohlool and Schmidt 1970).

*Production of root nodules*

Seeds of *Cicer arietium* L. variety JG-62 (provided by Dr. P. J. Dart, International Crops Research Institute for the Semi-Arid Tropics, Hyderabad, India) were surface sterilized with 4% calcium hypochlorate for 15 min, rinsed six times in sterile water, and germinated aseptically in petri dishes containing 1% water agar. The seedlings were planted in modified Leonard jars (Leonard 1943), which contained sterile vermiculite and a nitrogen-free nutrient solution (Broughton and Dilworth 1971), adjusted to pH 7.0.

Each of three seedlings in a jar received 1 mL of a cell suspension containing $1 \times 10^6$ rhizobia/mL; some jars were left uninoculated. A 2-cm layer of sterile perlite was added to the surface of all jars. Plants were grown under controlled conditions in a growth chamber (model M-31, Environmental Growth Chambers, Chagrin Falls, OH) with a 14-h day and a temperature regime of 29:24°C (day:night). The nodules were harvested after 3 weeks and used immediately for immunofluorescence.

*Immunofluorescence*

Fluorescent antibodies were prepared from sera against the somatic components of TAL-480, USDA 3HOa9, TAL-619, and TAL-620, and Nitragin strains 27A3, 27A8, 27A11, and 27A16. Preparation of antisera and conjugation procedures were done according to Schmidt *et al.* (1968), except that cultures were grown in YEMS broth for 3 days. The same rabbits which were injected with somatic antigens were later injected with whole cells to develop antisera for immunodiffusion. IF microscopy was as described previously (Kingsley and Bohlool 1981); transmitted light microscopy, i.e., dark field or phase, was illuminated with a 12-V quartz-halogen lamp. Smears from cultures and nodules were stained by the method of Schmidt *et al.* (1968) at a 1:4 dilution of FA. Gelatin – rhodamine isothiocynate conjugate was used to control background fluorescence (Bohlool and Schmidt 1968).

Cross-reactions of FA-stained smears were assessed by subjective assessment of the degree of fluorescence, from – (no fluorescence) to 4+ (bright fluorescence with a brilliant glow). Immunofluorescence adsorption (Belly *et al.* 1973) was used for finer antigenic analysis and determination of serological relatedness. The somatic antigens used for adsorption were prepared from 100 mL of 3-day-old shake-flask cultures distributed to three tubes and pelleted by centrifugation. The pellet from one tube was resuspended in 6 mL of undiluted FA and incubated at room temperature. Thimerosal (0.01%) was added as a preservative. The fluorescent agglutinate was removed by centrifugation after 4 h, the supernatant transferred to a fresh pellet, and incubated as before. The adsorption cycle was repeated three times, with the final incubation proceeding overnight at 4°C. The adsorbed FA was used at a dilution of 1:4.

*Immunodiffusion*

Antisera for immunodiffusion were prepared from whole-cell antigens grown on solid, defined medium (Vincent 1970). The same rabbits used for production of somatic antisera for FA were injected intramuscularly with 1 mL (0.5 mL each hip) on an equal mixture of antigen and Freund's complete adjuvant (Difco). After 3 weeks, 2 mL of the same culture was injected intravenously without adjuvant. The rabbits were bled 1 week later.

Immunodiffusion was performed by the method of Dudman (1971). Dense cell suspensions were prepared by suspending growth from a solid defined medium (Vincent 1970) in enough saline to give approximately $15 \times 10^{10}$ cells/mL (25 mg dry weight/mL). For the study of heat-stable diffusable antigens, cell suspensions were heated in tightly capped tubes (Cryotubes, Vanguard International, Neptune, NJ) in a boiling water bath for the desired length of time. The gels were incubated at room temperature (about 23°C) for 1 week. Results were recorded photographically.

*Intrinsic antibiotic resistance*

The method was similar to that described by Josey *et al.* (1979). The use of a multiple inoculator allowed for the simultaneous inoculation of up to 27 cultures per petri plate. In practice, 13 cultures were replicated four times per antibiotic concentration used. Fresh solutions of antibiotics were added to cooled (48°C) YEMS medium to give the following final concentrations (milligrams per millilitre): chloramphenicol (Chl), 0.012 and 0.025; streptomycin sulfate (Str), 0.0025 and 0.010; tetracycline hydrochloride (Tet), 0.004 (antibiotics obtained from Calbiochem); kanamycin sulfate (Kan), 0.010; naladixic acid (Nal), 0.010; neomycin sulfate (Neo), 0.0025; polymyxin B sulfate (Pol), 0.020; rifampin (Rif), 0.006 and 0.010; and vancomycin (Van), 0.0015 and 0.005 (antibiotics obtained from Sigma). Antibiotic stock solutions were prepared in sterile distilled water at a concentration of 10 mg/mL, except chloramphenicol (10 mg/mL in 95% ethanol), naladixic acid (10 mg/mL in 1 *N* NaOH), and rifampicin (10 mg/mL in methanol).

## Results

The cultures of *Rhizobium sp.* (*C. arietinum*), obtained from collections worldwide (see Table 1) were tested with eight FAs against *Rhizobium* sp. (*C. arietinum*). The four serogroups defined by the resulting immunofluorescent reactions are listed in Table 2. Nine of these cultures failed to react with any of the FAs; there were no cross-reactions with 55 strains from several other species of *Rhizobium*. Serogroup I contained 15 cultures which cross-reacted at maximum fluorescence (4+) with FAs TAL-619, TAL-620, Nitragin 27A8 and 27A11. Serogroups II and III each contained one cross-reacting culture, while serogroup IV was highly specific for its homologous antigen.

Since four of the eight strains used to produce fluorescent antibodies fell into serogroup I (a serogroup with a large number of cross-reacting cultures), we employed immunofluorescence – cross-adsorption to discern finer antigenic differences. The results are given in Table 3. The four inoculum-quality strains from serogroup I (27A8, 27A11, TAL-619, and TAL-620) completely removed all reactive antibodies against themselves (homologous control), as well as against

TABLE 2. Immunofluorescence reaction of chickpea rhizobia[a]

| Strains | Fluorescent antibody/serogroup No. | | | |
|---|---|---|---|---|
| | Nitragin27A8/I[b] | Nitragin 27A3/II[c] | TAL-480/III | USDA 3HOa9/IV |
| Nitragin 27A8 | (4+) | – | – | – |
| Nitragin 27A2 | 4+ | – | – | – |
| Nitragin 27A5 | 4+ | – | – | – |
| Nitragin 27A7 | 4+ | – | – | – |
| Nitragin 27A11 | 4+ | – | – | – |
| ATCC 1144 | 4+ | – | – | – |
| TAL-385 | 4+ | – | – | – |
| TAL-619 | 4+ | – | – | – |
| TAL-620 | 4+ | – | – | – |
| TAL-621 | 4+ | – | – | – |
| USDA 3HOa1 | 4+ | – | – | – |
| USDA 3HOa2 | 4+ | – | – | – |
| USDA 3HOa5 | 4+ | – | – | – |
| USDA 3HOa6 | 4+ | – | – | – |
| USDA 3HOa8 | 4+ | – | – | – |
| Nitragin 27A3 | – | (4+) | – | – |
| Nitragin 27A16 | – | 4+ | – | – |
| TAL-480 | – | – | (4+) | – |
| TAL-622 | – | – | 4+ | – |
| USDA 3HOa9 | – | – | – | (4+) |
| TAL-263, TAL-623, TAL-1008, TAL-1010, TAL-1012, Nitragin 27A17, Nitragin 27A13, USDA 3HOa10, USDA 3HOa12 | – | – | – | – |
| Other rhizobia[d] | – | – | – | – |

[a]See Table 1 for pedigree of strains of *Cicer* rhizobia.
[b]FA's of Nitragin 27A11, TAL-619, and TAL-620 gave the same reactions as 27A8.
[c]FA of Nitragin 27A16 gave the same reaction.
[d]*Rhizobium leguminosarum* (25 strains); *R. japonicum* (10); *R. lupini* (9); *Rhizobium* spp. (*Sesbania*) (9); *Rhizobium* spp. (*Vigna*) (1); *Rhizobium* spp. (*Stylosanthes*) (1).
NOTE: Results in parentheses indicate the homologous strain.

each other. The cross-reactions in serogroups II and III were investigated also by this technique. Culture TAL-622 shared a full complement of antigens with TAL-480, as cross-adsorption of FA TAL-480 removed all material capable of reacting with the homologous strain. On the other hand, Nitragin 27A3 and 27A16, which belong to the same serogroup, could be differentiated from each other. Adsorption with the cross-reactive strain produced an FA still reactive against the homologous culture.

Immunodiffusion allowed for further resolution of shared antigenic components. Experiments using strains Nitragin 27A3, 27A11, TAL-619, and TAL-620 revealed that a heat treatment was required to visualize a greater number of antigenic components. If Nitragin 27A3, (serogroup II) grown on a solid, defined medium, were left untreated, no precipitin bands developed, while unboiled TAL-620 (serogroup I) produced one precipitin band close to the antigen well. Figure 1 illustrates the effect of duration of the heat treatment on development of precipitin bands from TAL-480. Similar results were obtained with the other cultures, except that the band closest to the antigen well diminished in intensity during the 2-h heat treatment. This was not the case with either TAL-620 or Nitragin 27A3. The results in Fig. 1 also illustrate that heat treatment alters the reactivity and the concentration–diffusibility of the somatic antigens. The arrow shows that the precipitin

TABLE 3. Measure of similarity of somatic antigens of eight strains of chickpea *Rhizobium* by immunofluorescence–cross-adsorption

| Fluorescent antibody | Serogroup No. | Absorbing antigen | Antigens tested | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 27A8 | 27A11 | TAL-619 | TAL-620 | 27A3 | 27A16 | TAL-480 | TAL-622 |
| 27A8[a] | I | 0 | 4+ | 4+ | 4+ | 4+ | – | – | – | – |
| | | 27A8 | – | – | – | – | | | | |
| | | 27A11 | – | – | – | – | | | | |
| | | TAL-619 | – | – | – | – | | | | |
| | | TAL-620 | – | – | – | – | | | | |
| 27A3 | II | 0 | – | – | – | – | 4+ | 4+ | – | – |
| | | 27A3 | | | | | – | – | | |
| | | 27A16 | | | | | 4+ | – | | |
| 27A16 | II | 0 | – | – | – | – | 4+ | 4+ | – | – |
| | | 27A16 | | | | | – | – | | |
| | | 27A3 | | | | | – | 4+ | | |
| TAL-480 | III | 0 | – | – | – | – | – | – | 4+ | 4+ |
| | | TAL-480 | | | | | | | – | – |
| | | TAL-622 | | | | | | | – | – |

[a]FA-27A11 and FA-TAL-619 produced identical patterns. (FA-TAL-620 was not used).

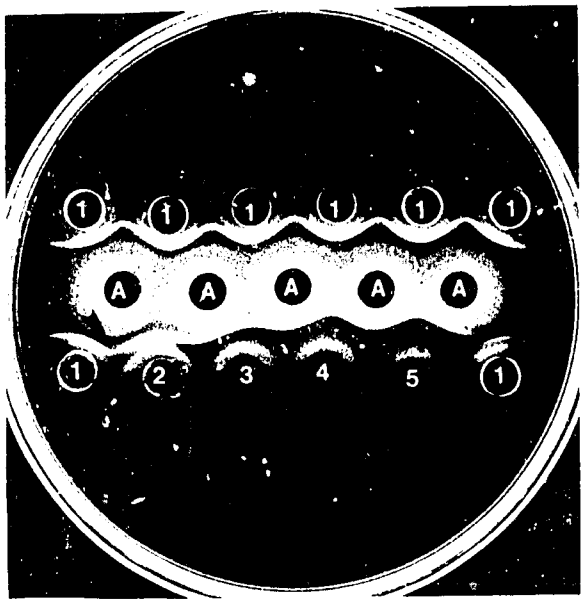


FIG. 1. Effect of heat treatment on release of heat-stable antigens of *Rhizobium* sp. (*C. arietinum*) strain TAL-480. Center wells (A) contain antiserum against whole cells of TAL-480; outer wells are antigens of TAL-480 boiled for the indicated period of time in minutes: (1) 0; (2) 15; (3) 45; (4) 90; and (5) 120. Arrow points to partial homology between the unboiled and boiled antigens.

bands formed against unboiled, and those against boiled, cells form only patterns of partial identity. Furthermore, as is shown in Fig. 1, the longer the antigens were boiled, the farther they formed bands from the antigen well. As a result of the time-course experiments, a 2-h heat treatment was adopted for treatment of the antigens for further immunodiffusion studies. Results in Figs. 2A and 2B further illustrate the antigenic similarity of the four cultures from serogroup I used for antiserum production. They all produced three identical bands against each others' antiserum. Strains that were negative by immunofluorescence (Table 2) were also shown to be negative by immunodiffusion.

The immunofluorescence reaction of bacteroids from nodules formed by different strains are illustrated in Figs. 3A, 3B, and 3C. The bacteroids of TAL-480 and Nitrogen 27A3 were thickened rods (Fig. 3A) of approximately the same dimensions as the respective culture-grown cells. Cells of Strain TAL-620, which were rod shaped in culture (Fig. 3C), appeared spherical (spheroplastlike) in nodule smears (Fig. 3B).

Sixteen cultures representing the four serogroups, as well as three serologically nonreactive cultures, were screened for intrinsic antibiotic resistance (IAR). A summary of the resistance patterns is given in Table 4. We found the 16 cultures to fall into six patterns. Seven of the nine cultures tested from serogroup I all produced an identical pattern (pattern 1), although strain 27A3 from serogroup II also displayed the same pattern of resistance. The other two cultures from serogroup I showed greater resistance to rifampicin and were grouped separately (pattern 2). The two strains from serogroup III (TAL-480, TAL-622) also produced different patterns of resistance.

## Discussion

All *Rhizobium* species or inoculation groups examined so far have been found to contain strains that

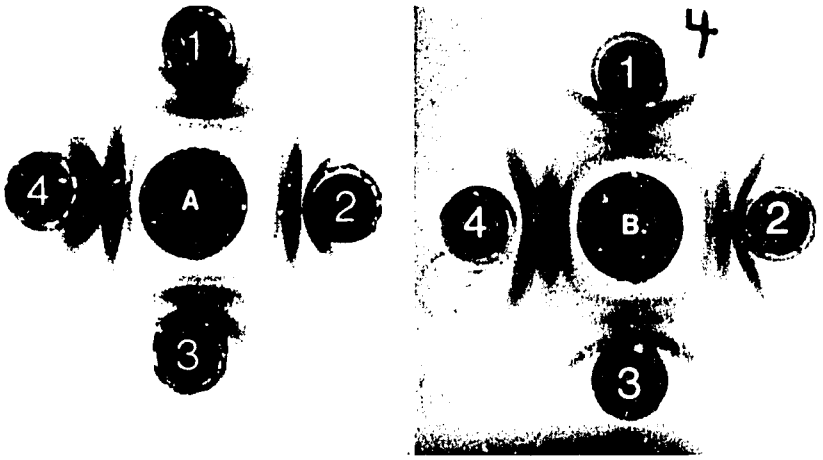


FIG. 2. Immunodiffusion pattern of heat-stable antigens of four cross-reacting cultures from serogroup I. Center wells are antiserum against whole cells of Nitragin 27A8 (A) and TAL-620 (B). Outer wells represent boiled (2 h) cell antigens of strains (1) Nitragin 27A8; (2) Nitragin 27A11; (3) TAL-619; and (4) TAL-620.

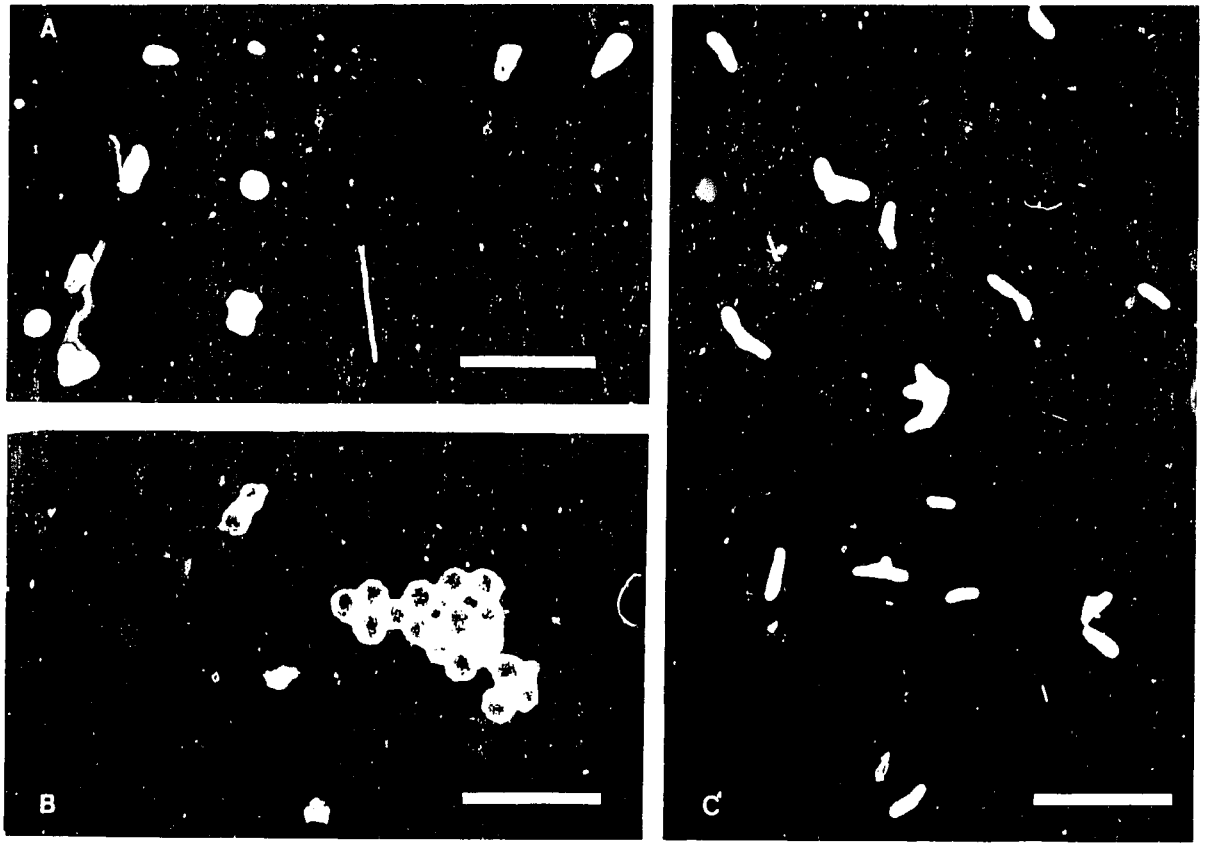


FIG. 3. Immunofluorescence reaction of root-nodule bacteroids and culture-grown cells of strains of *Rhizobium* spp. (*C. arietinum*). (A) Bacteroids of Nitragin 27A3; (B) bacteroids of TAL-620; and (C) culture-grown cells of TAL-620. Note pleomorphic (A) and spheroplastlike (B) morphology of the nodule bacteroids. Bar = 5 μm.

TABLE 4. Summary of intrinsic antibiotic resistance (IAR) patterns for some chickpea *Rhizobium* strains used in this study

| IAR pattern No. | Serogroup No. | Culture | Chl | | Kan | Nal | Neo | Pol | Rif | | Str | | Tet | Van | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0.012* | 0.025 | 0.010 | 0.010 | 0.0025 | 0.020 | 0.005 | 0.010 | 0.0025 | 0.010 | 0.004 | 0.0015 | 0.005 |
| 1 | I | – Nitragin 27A2, Nitragin 27A8, Nitragin 27A11, TAL-385/-619, TAL-620, Nitragin 27A7 | + | – | + | + | + | + | + | – | + | + | – | + | + |
| | II | Nitragin 27A3 | | | | | | | | | | | | | |
| 2 | I | – Nitragin 27A5, USDA 3HOa1 | + | – | + | + | + | + | + | + | + | + | – | + | + |
| 3 | III | TAL-480 | – | – | + | + | + | + | – | – | – | – | – | + | + |
| | III | TAL-622 | | | | | | | | | | | | | |
| 4 | US | USDA 3HOa10 | – | – | + | + | + | + | + | – | – | – | – | + | + |
| | US | USDA 3HOa12 | | | | | | | | | | | | | |
| 5 | IV | USDA 3HOa9 | – | – | + | + | + | + | + | + | + | + | – | + | – |
| 6 | US | TAL-1012 | + | – | + | + | – | + | – | – | + | + | – | sl | – |

*Antibiotic concentration in milligrams per millilitre.

NOTE: sl, Slight growth; +, growth indicating resistance; –, no growth indicating sensitivity; US, unknown serogroup, do not belong to serogroups I, II, III, or IV.

are serologically distinct, as well as strains that share cross-reactive antigens; no *Rhizobium* species is serologically homogenous (Dudman 1977). The chickpea rhizobia are no exception. However, the large number of cross-reactions between strains in serogroup I (Table 2) was quite unexpected. We especially did not expect the four strains used to produce the FAs (Nitragin 27A8, 27A11, TAL-619, TAL-620) to cross-react so strongly, for they purportedly represented different inoculum strains. Nitragin strains 27A8 and 27A11 (isolated in Mexico in 1971) and NifTAL strain TAL-619 (source, USDA 3HOa1, ex-India M. S. Raju, origin?) and TAL-620 (ex-Israel, origin?), although from different sources and isolated in different parts of the world, were identical by immunofluorescence, immunofluorescence–adsorption, immunodiffusion, and intrinsic antibiotic resistance. They were all probably derived from the same strain. This effective nodulator may have been disseminated around the world by various investigators and then given different numbers by the recipients. An alternative explanation is that this strain is extremely widespread ecologically. In addition, many of the other cultures in this serogroup came from collections in different parts of the world. However, we traced the histories of a number of these cultures and determined that several of them originated from one culture. The problem is caused by the use of multiple collection numbers for the same culture, a matter that should concern those working with *Rhizobium*, especially curators of culture collections. For example, six collection numbers were applied to USDA 3HOa1 (which itself probably originated in India). It is the same as ATCC 11444, Rothamsted 3827, ICRISAT 3827, CC-1189, TAL-385, and TAL-619. Therefore, six of the cross-reactions were only apparent. Several of the other strains in this serogroup may have also originated from the same source.

The two strains in serogroup III (TAL-480, TAL-622), though identical by immunofluorescence and immunofluorescence–adsorption (Tables 2, 3), were not identical by intrinsic antibiotic resistance. However, they still may be the same strain, but simply be physiological variants (mutants) of one another. Although Nitragin strains 27A3 and 27A16 were in the same serogroup, cross-adsorption produced two highly specific sera, indicating that these strains shared only a limited number of antigenic components.

The value of serological techniques in studies of *Rhizobium* stems from the fact that antigens of the bacteroids in the nodule are stable and react with antibodies produced against the culture-grown cells (See Bohlool and Schmidt 1980). This property accounts for the wide acceptance and broad-scale application of serology to the study of rhizobia in culture and in nodules. The chickpea rhizobia exhibited the same degree of antigenic stability. The nodule smears were of an equal intensity of fluorescence to the smears of their parent cultures (Fig. 3). The nodule bacteroids, however, had different morphologies than their respective cultures.

The identification of strains of *Rhizobium* in soil or nodules using serology is only reliable when no significant cross-reactions occur. Where strains do cross-react, other methods of identification are required. Intrinsic antibiotic resistance (IAR) (Josey *et al.* 1979) is a simple and useful characteristic which can be used for screening of cultures of *Rhizobium*. However, our data indicate that even very closely related cultures (i.e., identical by immunofluorescence, immunofluorescence–adsorption, and immunodiffusion (Tables 2, 3; Fig. 2)) can differ in their IAR patterns (Table 4). Cultures in the same somatic and whole-cell serogroups may be classified in different IAR groups. More importantly, cultures completely different by serological means could be classified in the same IAR categories.

We now apply IAR only as a preliminary screening of a large number of cultures to select strains for fluorescent antibody work. H. A. Moawad and B. B. Bohlool (unpublished data) have recently used IAR to categorize a large number of inoculum strains and nodule isolates from *Leucaena leucocephala*. The FAs produced against cultures with distinct IAR patterns were found to be highly specific serologically.

Guar and Sen (1979), based on their cross-nodulation studies, proposed that the *Cicer arietinum* symbiosis may constitute a separate cross-inoculation group. Our data on the serological specificity of the *Rhizobium* microsymbiont of *Cicer* provide further support for this proposal.

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