Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Protein Banding Patterns among *Rhizobium leguminosarum* biovar phaseoli Strains Isolated from the Mexican Bean *Phaseolus coccineus*

R. ARREDONDO-PETER†* AND E. ESCAMILLA

Departamento de Microbiología, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-242, 04510 México, D.F., México

Received 28 December 1992/Accepted 11 August 1993

Several rhizobial strains were isolated from *Phaseolus coccineus* root nodules and were determined to be *Rhizobium leguminosarum* biovar phaseoli strains after reinfection of the same host plant. These strains were characterized by cultural procedures (growth on different carbon sources and intrinsic antibiotic resistance) and electrophoretic procedures (sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total proteins). Our results showed that these rhizobia are very similar to each other, especially in their electrophoretic protein banding patterns, suggesting that they might belong to isolated populations.

Soil bacteria belonging to the genera *Rhizobium* and *Bradyrhizobium* induce the formation of nodules on the roots of legume plants. These root nodules are formed by a specialized tissue in which nitrogen fixation occurs. This symbiotic association is usually specific, and in the past the nomenclature of the symbiotic bacteria was based on the infected host plants (2). The taxonomic system has been changed to a more natural (phylogenetic) system (1); the slowly growing rhizobia are placed in the genus *Bradyrhizobium*, and the fast growers are placed in the genus *Rhizobium*, which includes the species *Rhizobium leguminosarum*. Several other genera and species are included in the family *Rhizobiaceae* in addition to these two genera. These taxonomic propositions resulted from the high level of diversity found among the strains isolated from new and well-known host plants.

Currently, it is accepted that *R. leguminosarum* should be divided into the following three biovars on the basis of the host plants which are infected: *R. leguminosarum* biovar phaseoli, *R. leguminosarum* biovar trifolii, and *R. leguminosarum* biovar viciae. *R. leguminosarum* biovar phaseoli has been widely studied because it is the symbiont of *Phaseolus vulgaris* (common bean). This legume is an important source of food.

To our knowledge, few systematic studies have been performed with rhizobia isolated from the bean *Phaseolus coccineus* (known as ayocote, bótil, and frijolón), which is an important legume food source and green fertilizer in Mexico. This bean is commonly intercropped with *Zea mays* (corn).

In this paper we describe the sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) protein banding patterns, as well as some cultural features, of rhizobia isolated from *P. coccineus* collected in the Mexican state of Chiapas.

P. coccineus plants were collected at several places

around San Cristobal Las Casas in the southwestern Mexican state of Chiapas (17°N, 92°W). After collection, the plants were transported to a laboratory, where the rhizobia were immediately isolated from the nodules or the root nodules were kept at –20°C until they were used. It is important to note that no inoculation programs with any *Rhizobium* strains took place in this locality before this work.

Rhizobia were isolated from the root nodules by standard procedures and were grown in YMA media (8). Preliminary characterization was accomplished by determining colony morphology, cell morphology (with a light microscope), growth rate in liquid YM media (8), Gram stain reaction, and growth in YMA media containing bromothymol blue to monitor pH changes that occurred in the media. Finally, the original host plant was reinfected by inoculating *P. coccineus* seedlings in Leonard jars with strains grown in liquid YM medium (8). The inoculated seedlings were grown in controlled environment cabinets programmed with a photoperiod of 13 h of light (300 microeinsteins m⁻² s⁻¹) at 20°C. Root nodule development was recorded 6 to 8 weeks after inoculation. Cultures confirmed to be rhizobia were kept in YMA media and were deposited in the culture collection of the Centro de Investigaciones Ecológicas del Sureste (CIES), San Cristobal Las Casas, Chiapas, Mexico.

In addition to the rhizobia isolated from *P. coccineus*, other rhizobia were included in this study for comparative purposes. These strains were isolated from other *Phaseolus* species collected at several Mexican localities and in Brazil (5). The rhizobial strains used are shown in Table 1.

The following carbon sources were used: the sugars arabinose, glucose, lactose, maltose, raffinose, rhamnose, starch, sucrose, and xylose; the polyalcohols glycerol and myo-inositol; the organic acids acetic acid and citric acid; and the amino acids arginine, glycine, lysine, and methionine. Rhizobia were grown on solid Vincent's minimal media supplemented with thiamine and biotin (8). Small pieces of sterilized filter paper impregnated with a 25% filter-sterilized solution of each carbon source were placed on the surface of medium previously inoculated with bacteria. Growth was recorded 48 h after inoculation.

* Corresponding author.

† Present address: Department of Biochemistry, Institute of Agriculture and Natural Resources, University of Nebraska—Lincoln, Lincoln, NE 68583-0718.

TABLE 1. *Rhizobium* strains used in this study

Strain	Host plant	Origin ^a	Reference
CIES strains			
CIES 68	<i>P. coccineus</i>	Belizario Domínguez	This work
CIES 69	<i>P. coccineus</i>	Belizario Domínguez	This work
CIES 71	<i>P. coccineus</i>	Bautista Chico	This work
CIES 75	<i>P. coccineus</i>	Bautista Chico	This work
CIES 78	<i>P. coccineus</i>	Bautista Chico	This work
CIES 110	<i>P. coccineus</i>	Bautista Chico	This work
CIES 111	<i>P. coccineus</i>	Bautista Chico	This work
CIES 113	<i>P. coccineus</i>	Bautista Chico	This work
CIES 87	<i>P. coccineus</i>	Las Ollas	This work
CIES 90	<i>P. coccineus</i>	Las Ollas	This work
CIES 96	<i>P. coccineus</i>	Experimental field ^b	This work
CIES 106	<i>P. coccineus</i>	Experimental field ^b	This work
CIES 112	<i>P. coccineus</i>	Experimental field ^b	This work
CIES 115	<i>P. coccineus</i>	Experimental field ^b	This work
CIES 98	<i>P. coccineus</i>	Jocosic	This work
CIES 134	<i>P. coccineus</i>	Elambó	This work
CIES 136	<i>P. coccineus</i>	Yalbanté	This work
CFN strains			
CFN 7	<i>P. vulgaris</i>	Guanajuato, Mexico	5
CFN 42	<i>P. vulgaris</i>	Guanajuato, Mexico	5
CFN 226	<i>Phaseolus acutifolius</i>	Morelos, Mexico	5
CFN 229	<i>P. vulgaris</i>	Brazil	5

^a All of the localities for the CIES strains are in the Mexican state of Chiapas.

^b This experimental field is located at the Centro de Investigaciones Ecológicas del Sureste, San Cristobal Las Casas, Chiapas, Mexico.

Rhizobial strains were grown in antibiogram dishes (Bigaux Diagnóstica, Mexico City, Mexico) containing solid YMA media supplemented with the following antibiotics: ampicillin (10 µg), cephalosporin (30 µg), nalidixic acid (30 µg), carbenicillin (100 µg), furadantin (300 µg), gentamicin (10 µg), cefotaxime (30 µg), amikacin (30 µg), colimycin (10 µg), tetracycline (30 µg), and sulfamethoxazole-trimethoprim (25 µg). Growth was recorded as described above.

FIG. 1. SDS-PAGE total protein band patterns on a Coomassie blue-stained gel for representative *Rhizobium* strains. The small arrows indicate major protein band differences; the large arrow indicates the oversynthesized 40-kDa protein found in strain CFN 7. The arrowheads indicate the positions of the following molecular weight markers: bovine serum albumin (molecular weight, 68,000), horseradish peroxidase (44,000), and horse cytochrome *c* (13,000).

FIG. 2. (A and B) Silver-stained SDS-PAGE gel comparison of protein band patterns for CIES strains isolated from diverse localities (see Table 1). The small arrows indicate major protein band differences; the arrowheads indicate the positions of the molecular weight markers described in the legend to Fig. 1 and the molecular weight marker carbonic anhydrase (molecular weight, 29,000). (C) LMWP close-up for the CIES strains shown in panel B. The large arrows indicate the enhanced LMWP observed in strains CIES 110 and CIES 113. The arrowhead indicates the position of horse cytochrome *c* (molecular weight, 13,000).

Cells grown in liquid YM media for 24 to 48 h were harvested by centrifugation at 4,000 rpm for 5 min at 4°C. The cells were suspended in 10 mM Tris-HCl (pH 8.3) and enzymatically disrupted by the TMK-20 procedure (6) to

avoid protein denaturation by mechanical procedures. The total protein contents of the extracts were determined by the method of Lowry et al. (4). The protein patterns of the extracts were determined by SDS-PAGE, using 50 µg of protein per lane, and the proteins in the gels after electrophoresis were stained by using Coomassie blue R-250 (3). For silver staining, the gels were loaded with 5 µg of protein per lane, and the procedure described by Schipper et al. (7) was used.

A total of 17 isolates were obtained from *P. coccineus* root nodules and were confirmed to be *R. leguminosarum* biovar phaseoli strains by performing reinfection experiments in which the same host plant was used. The isolates had morphological and cultural features typical of the genus *Rhizobium*; all strains appeared to be gram-negative rods that grew rapidly and acidified YMA, as determined by using bromothymol blue as the indicator. Similar results were obtained with the reference strains.

The growth rates observed with different carbon sources were similar for the strains (data not shown). Most of the strains were able to grow on the sugars, polyalcohols, and amino acids tested; the exceptions were strains CIES 69, CIES 71, CIES 96, CIES 106, CIES 110, CIES 113, and CIES 136, which were not able to use either raffinose or starch. A large proportion of the rhizobia were not able to grow on citrate and acetate; the exceptions were strains CIES 90 and CIES 113. The growth capabilities of strains CFN 7, CFN 42, CFN 226, and CFN 229 and the CIES strains were similar. The intrinsic antibiotic resistance patterns of the isolates were similar. Most of the strains were sensitive to gentamicin, amikacin, and tetracycline; the exceptions were strain CIES 112 (sensitive to gentamicin), strains CIES 69 and CIES 112 (sensitive to amikacin), and strains CIES 69 and CIES 112 (sensitive to tetracycline). Variability in resistance to colimycin was observed. Most strains were resistant to the other antibiotics.

As an initial approach for comparing the SDS-PAGE banding patterns, total proteins from some of the CIES strains and strains CFN 7, CFN 42, CFN 226, and CFN 229 were electrophoresed in 10% slab gels and stained with Coomassie blue (Fig. 1). Several proteins having molecular masses in the range from 60 to 30 kDa were highly conserved among the CIES strains; few, if any, differences were observed among these strains. In contrast, some minor differences were detected when the CIES strains and strains CFN 7, CFN 42, CFN 226, and CFN 229 were compared, particularly in the oversynthesized 40-kDa protein present in strain CFN 7 (Fig. 1). In this gel, a large smear occurring after the 13- to 14-kDa band was present in all of the strains studied; this suggested that protein degradation occurred. However, the smear was determined to be well-defined low-molecular-weight peptides (LMWP) when the gels were prerun overnight (see below).

A high degree of similarity in protein banding patterns was observed when CIES strains from different localities were compared (Fig. 2A and B). Minor variations were observed for some protein bands (Fig. 2A and B, arrows). The LMWP that were detected when the protein samples were loaded after the gel was prerun were highly conserved in the CIES strains collected at the Yalabá, Elambó, Jocosic, and

Botanical Garden localities (Fig. 2A). In contrast, strains CIES 110 and CIES 113, which were collected at the Bautista Chico locality, contained a major LMWP (Fig. 2C, arrows) that was not detected in any other *Rhizobium* strain used in this study.

In this work we studied rhizobia that belong to two variability categories. *R. leguminosarum* biovar phaseoli CFN 7, CFN 42, CFN 226, and CFN 229 were originally isolated from diverse geographic areas (5) and exhibited some degree of variability when the SDS-PAGE total protein band patterns were compared. This result was expected because Martínez et al. (5) showed that rhizobia isolated from Mexican *P. vulgaris* host plants are a diverse group. In contrast, the CIES strains produced similar SDS-PAGE protein band patterns, which suggests that these rhizobia belong to an isolated population that may have a low level of genetic exchange or no genetic exchange with other soil bacterial populations. Moreover, the CIES strains were collected from a narrow geographic area, as was the case with the rhizobia studied by Young (9); however, in this case the topography of the localities is such that they are isolated, which may favor diversification of populations and speciation.

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