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Possible Involvement of a Megaplasmid in Nodulation of Soybeans by Fast-Growing Rhizobia from China

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Several isolates from a newly described group of fast-growing acid-producing soybean rhizobia, *Rhizobium japonicum*, were analyzed for plasmid content. All contained from one to four plasmids with molecular weights of 100×10^6 or larger. Although most of the isolates shared plasmids of similar size, the restriction endonuclease (*Bam*HI, *Eco*RI, and *Hin*dIII) patterns of the plasmids from three of the isolates were vastly different. Growth in the presence of acridine orange was effective in producing mutants cured of the largest plasmid in one of the strains. These mutants also lost the ability to form nodules on soybeans. High-temperature curing of a smaller plasmid in another strain did not lead to loss of nodulating ability or alteration of symbiotic effectiveness on soybean cultivars. The identities of all of the isolates and mutants were ascertained by immunofluoresence and immunodiffusion. The new fast-growing strains of *R. japonicum* may provide a better genetic system for the study of the soybean symbiosis than the slow-growing *R. japonicum*, not all of which can be shown to contain plasmids.

Members of the genus *Rhizobium* can best be described as a group of gram-negative, heterotrophic, non-spore-forming rods which form nodules on the roots of leguminous plants. Species designation within the genus is based exclusively on the host specificity of the bacteria (12). Rhizobia have also been separated into two groups depending on their growth rates and acid production in yeast extract-mannitol (YEM) medium (17, 24). The fast-growing species, *Rhizobium leguminosarum, Rhizobium meliloti, Rhizobium phaseoli*, and *Rhizobium trifolii*, have mean generation times of 2 to 4 h and produce a net decrease in the pH of the culture medium. Those referred to as slow growing, *Rhizobium japonicum, Rhizobium lupini*, and *Rhizobium* spp. of the "cowpea miscellany," have mean generation times of 6 h and longer and do not lower the pH of YEM medium (23).

The soybean-nodulating bacteria, *R. japonicum*, belong to the slow-growing group. However, Keyser et al. (13) have recently reported the isolation of a unique group of fast-growing, acid-producing rhizobia from soybean root nodules collected in the People's Republic of China (PRC). These new isolates are physiologically distinct from the typical slow-growing, alkaline-producing soybean rhizobia and belong to at least four different serological groupings (B. B. Bohlool, H. H. Keyser, and M. Sadowsky, unpublished data). Furthermore, although these isolates form nodules on all the soybean varieties tested, their symbiotic effectiveness is markedly different from the typical slow-growing *R. japonicum* (13).

Genetic studies involving plasmid transfer in *Rhizobium* have almost exclusively been done with the fast-growing species (1). This may in part be due to the fact that nitrogen fixation genes and some of the symbiotic genes have been shown to be plasmid borne in this group (2, 10, 11). The genetics of the root nodule bacteria of such an important crop as soybean have been neglected due to difficulties in consistently demonstrating plasmids in all the slow-growing *R. japonicum* strains and in identifying the location of the symbiosis-related genes. Recently, Pilacinski and Schmidt (19) demonstrated plasmid transfer within and between serologically distinct strains of *R. japonicum*. However, the plasmids used in their study were R68.45 and RP4 (originally from *Pseudomonas* spp.) and not rhizobial symbiotic plasmids. Gross et al. (5) examined plasmids in several isolates of *R. japonicum* (primarily from one serogroup). Although all isolates were obtained from soils with similar characteristics and were predominantly from the same serogroup (135), they could be subdivided in four groups on the basis of plasmid number and size. Recently, Masterson et al. (15) reported that the structural nitrogen fixation genes (*nifD, nifH*, and *nifK*) are located on large plasmids in several of the fast *R. japonicum* isolates. In this report we demonstrate the presence of large plasmids (molecular weight, > 100 $\times 10^6$) in 10 fast-growing, acid-producing *R.*

japonicum isolates from China and show that curing of one of these plasmids results in the loss of nodulating ability by the mutant.

MATERIALS AND METHODS

The *Rhizobium* cultures used in this study and their sources are listed in Table 1. Isolates were maintained on YEM agar slants (22). The fast-growing *R. japonicum* isolates chosen for this study fall into at least four somatic serological groups (B. B. Bohlool, H. H. Keyser, and M. Sadowsky, unpublished data).

For plasmid screening, plasmid DNA was isolated essentially by the method described by Hirsch et al. (8). Cultures were grown for 48 h in 200 ml of PA medium (8) supplemented with 0.20 g of K_2HPO_4 per liter and washed and suspended in 16 ml of TEN buffer (50 mm Tris, 20 mM disodium EDTA, 50 mM NaCl, pH 8.0).

For preparative plasmid extractions, all isolation steps were scaled up 10-fold, and the plasmids were further purified by CsCl-ethidium bromide equilibrium density gradient centrifugation ($n_D = 1.3925 \pm 0.001$) two times at 36,000 rpm and 17°C for 48 h in a type 40 (Beckman) rotor (3). Ethidium bromide and CsCl were removed from the plasmid preparation as outlined by Hirsch et al. (8) except that CsCl-saturated *n*-butanol was used as the ethidium bromide extractant.

For plasmid screening, samples were mixed with tracking dye (50% [wt/vol] glycerol, 0.125% [wt/vol] bromphenol blue, 50 mM disodium EDTA, pH 8.0), and plasmids were resolved by electrophoresis on horizontal 0.7% agarose (standard low; Bio-Rad Laboratories, Richmond, Calif.) gels (18 by 13 by 0.6 cm) at 60 mA for 6 h at 4°C with Tris-borate buffer (8).

For restriction enzyme analysis, 60 μl of purified plasmid DNA was digested with either *Bam*HI, *Eco*RI, or *Hin*dIII (Bethesda Research Labs, Inc., Gaithersburg, Md.) for 1 h at 37°C according to the directions of the supplier. After digestion, samples were heated at 65°C for 3 min to inactivate endonucleases, and 15 μl of tracking dye was added. Digested samples were electrophoresed on 0.7% agarose gels at 50 mA for 16 h at 4°C with Tris-acetate buffer (10). Gels were stained for 20 min in Tris-acetate or Tris-borate buffer containing 5 μg of ethidium bromide per ml, and bands were visualized by reflective, shortwave, UV light and photographed with Kodak Pan-X film and a yellow K2 filter (Hoya Co., Tokyo, Japan). For molecular weight estimations, the electrophoretic mobilities of plasmid bands were compared to the reference strain, *R. leguminosarum* 6015(pJB5JI) (8, 20), and the *Bam*HI, *Eco*RI, and *Hin*dIII digests of λ DNA (4).

Plasmid curing was done by the acridine orange procedure of Zurkowski et al. (25) or by the heat-treatment curing procedure of Zurkowski and Lorkiewicz (26). For acridine orange curing, overnight tryptone yeast extract (TY) (8) cultures were diluted to approximately 10^4 cells per ml in YM medium (25) containing 1, 3, 7, 15, or 20 μg of acridine orange per ml (Sigma Chemical Co., St. Louis, Mo.). Cultures were incubated at 25°C in the dark for 6 days. After incubation, cultures were diluted and spread plated onto YEM agar plates, and the resulting colonies which formed were streaked for purification two consecutive times on the same medium. For heat curing, overnight TY cultures were inoculated to approximately 10^6 cells per ml into PA medium. Cultures were incubated at 37, 40, and 42°C and transferred at weekly intervals. After heat treatment, cultures were spread plated on YEM agar plates, and isolates were purified as outlined above.

The identity of all cultures was ascertained by immunodiffusion (22) and immunofluoresence (21), using strain-specific antisera prepared by the method of Schmidt et al. (21).

Plant infection assays were performed in modified Leonard jar assemblies (14) containing one-quarter strength Hoagland plant nutrient solution (9). Soybeans (*Glycine max* cv. Peking and Chippewa) were surface sterilized by immersion in 4% (wt/vol) calcium hypochlorite for 20 min followed by exhaustive washing in sterile distilled water. Sterile seeds were aseptically transferred to plant growth vessels and after germination were inoculated in triplicate with 2-day-old YEM cultures. After incubation for 1 month, nitrogenase activity was determined by the acetylene reduction technique of Hardy et al. (6).

TABLE 1. Bacterial cultures

Organism	Strain designation
R. japonicum	
Fast growing	PRC OB2 (USDA 192)[a]
	PRC OB3 (USDA 193)
	PRC 194 (USDA 194)
	PRC 201 (USDA 201)
	PRC 205 (USDA 205)
	PRC 206 (USDA 206)
	PRC 208 (USDA 208)
	PRC 214 (USDA 214)
	PRC 217 (USDA 217)
	PRC 440 (USDA 191)
Slow growing	USDA 110[b]
	PRC 121-6
R. leguminosarum	6015(pJB5JI)[c]

[a] PRC, People's Republic of China. See Keyser et al. (13).

[b] From the U.S. Department of Agriculture Culture Collection, Beltsville, Md.

[c] From P. Hirsch, Max Planck Institut, Cologne, Germany.

RESULTS

All of the 10 fast-growing PRC isolates examined contained from one to four plasmids of approximately 100 megadaltons and greater. Figure 1 shows the relative electrophoretic mobilities of plasmids from PRC isolates 217, 208, 206, OB3, 440, 201, OB2, 214, 205, and 194 (lanes A, D, E, F, G, H, J, K, L, and M, respectively). The reference plasmids from *R. leguminosarum* 6015(pJB5JI) appear in Fig. 1, lanes C and I. All of the fast-growing PRC isolates with the exception of PRC 194 and PRC 440 shared a plasmid of similar size (ca. 200×10^6 molecular weight). Isolates 217, 440, 201, OB2, 205, and 194 each had two to three demonstratable plasmids (Fig. 1, lanes A, G, H, J, L,

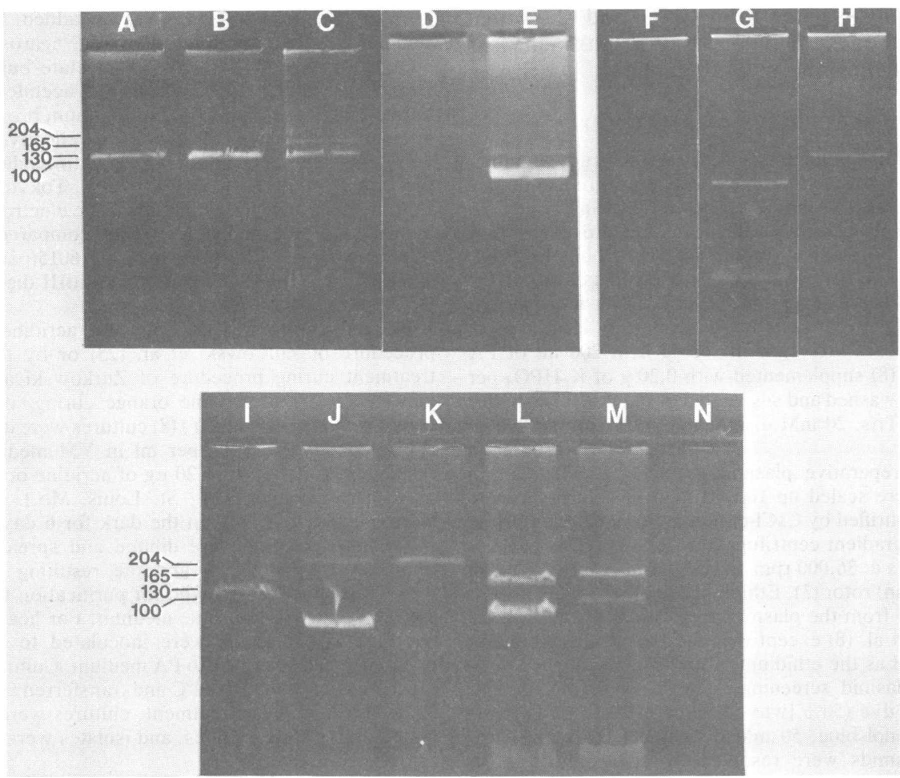

FIG. 1. Agarose gel electrophoresis of plasmid DNA from fast- and slow-growing rhizobia. Lanes A, D, E, F, G, H, J, K, L, and M, fast-growing *R. japonicum* PRC isolates 217, 208, 206, OB3, 440, 201, OB2, 214, 205, and 194, respectively. Lanes C and I, *R. leguminosarum* 6015(pJB5JI). Lanes B and N, slow-growing *R. japonicum* PRC isolates 121-6 and USDA 110, respectively. Numbers in margin refer to the size of the reference plasmids from isolate 6015(pJB5JI) in megadaltons (8, 20).

and M, respectively). One isolate, PRC 206, contained four plasmids (Fig. 1, lane E), whereas the other PRC isolates (Fig. 1, lanes D, F, and K) contained one plasmid. Isolate PRC 440 (Fig. 1, lane G) contained two plasmids, one of which exhibited the highest electrophoretic mobility (lowest molecular weight) of all the PRC isolates examined. No plasmids could be detected in the slow-growing *R. japonicum* isolate USDA 110 (Fig. 1, lane N). However, another slow-growing *R. japonicum*, PRC isolate 121-6 (Fig. 1, lane B), consistently exhibited one plasmid with a molecular weight of about 100×10^6.

Purified plasmid DNA from PRC isolates OB2, 194, and 205 (each a member of a separate serological group) was used for restriction enzyme analysis with three restriction endonucleases. Results in Fig. 2 show that the *Eco*RI, *Hin*dIII, and *Bam*HI (e, h, and b, respectively), restriction patterns of plasmids from the three isolates were vastly different from one another. For molecular weight comparisons, the *Eco*RI, *Hin*dIII, and *Bam*HI digests of λ DNA are presented in Fig. 2 lanes IVe, h, and b, respectively. In all instances, restriction endonuclease digestion gave rise to fragments with molecular weights less than 33 megadaltons. The *Bam*HI digest of plasmids from PRC 205 (Fig. 2, lane Ib) and the *Hin*dIII digest of plasmids from PRC 194 (Fig. 2, lane IIb) gave fragments with the highest molecular weights. Although PRC isolates 205 and 194 (Fig. 2, lanes I and II, respectively) contain plasmids of similar number and size on Tris-acetate gels (Fig. 2), their restriction digest profiles clearly show them to be different. These differences are most evident by the absence of restriction fragments in several regions of the *Eco*RI, *Hin*dIII, and *Bam*HI digests of plasmids from PRC 205 (Fig. 2, lanes Ib, e, and h). Similarly, the restriction digest profile of plasmids from PRC isolate OB2 is different from either of the other two isolates. It should also be noted that after plasmid purification by CsCl centrifugation, one extra plasmid band of approximately 33 megadaltons became evident on Tris-acetate gels (Fig. 2, lanes I and II) but was absent on Tris-borate gels run with crude lysates (Fig. 1).

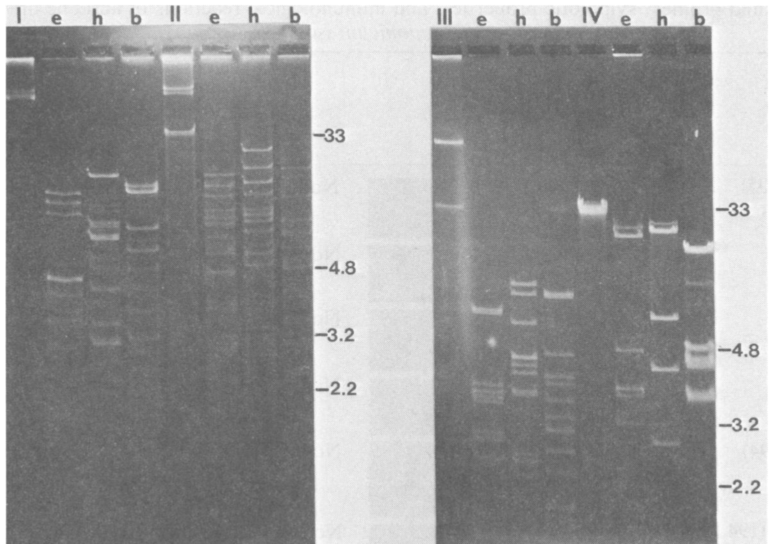

FIG. 2. Restriction endonuclease digest of plasmids from the fast-growing PRC *R. japonicum*. The undigested samples are given in lanes I (PRC 205), II (PRC 194), and III (PRC OB2). The three lanes to the right of each number are digest patterns of: e, *Eco*RI; b, *Bam*HI; h, *Hin*dIII restriction endonucleases. Intact λ DNA and the respective endonuclease digests are shown in lanes IVe, IVh, and IVb, for comparison. Numbers in the margin refer to the size of several λ fragments in megadaltons (4).

Three of the fast-growing PRC isolates, PRC OB2, 194, and 205, were used for acridine orange and heat curing experiments. All three of the isolates examined failed to grow in acridine orange at concentrations greater than 1 μg/ml. At an acridine orange concentration of 3 μg/ml or greater, viable cell numbers either decreased slowly or remained the same from the initial inoculum level. Several isolates which were recovered from growth-permissive (1 μg/ml) and -restrictive (>3 μg/ml) concentrations of acridine orange were examined for their plasmid profiles and for their nodulation responses on soybeans.

The plasmid pattern of the cured mutants is shown in Table 2A. The results show that the acridine orange mutants of PRC 205 (205 1AO1, 1AO3, and 1AO5) are devoid of the largest (molecular weight, ca. 200×10^6) of the two plasmids.

The three PRC 205 mutants were isolated from cultures grown in the presence of 1 μg of acridine orange per ml. All three of the isolates failed to nodulate (Nod^-) both soybean cultivars (Table 2). However, all of the isolates recovered from acridine orange at 3 μg/ml and greater effectively nodulated (Nod^+ Fix^+) soybean cultivar Peking. Likewise, all of the PRC 194 and OB2 isolates from cultures with 1 μg of acridine orange per ml retained their nodulating ability.

Immunofluorescence analysis of the Nod^- mutants indicated that the mutants were serologically identical with the parent strain. Immunodiffusion analysis with antisera produced against PRC 205 whole-cell antigens and PRC 205 somatic-cell antigens also indicated that the nodulation-deficient mutants were serologically identical to the parent (Table 2). Both somatic- and whole-cell antigens from the mutants showed patterns of identity with the parent strain.

Incubation of PRC OB2 and 205 at elevated temperatures did not alter the plasmid profiles of the recovered isolates. Organisms recovered from growth medium incubated at 37, 40, and 42°C for various periods of time effectively nodulated both soybean cultivars, and plasmid profiles from several of the isolates were identical to those of their respective parents. Similarly, incubation of PRC 194 at 37 or 42°C did not alter the plasmid profiles of the recovered mutants. However, one PRC 194 isolate, 194 1X40 (Table 2A), which was incubated at 40°C for 7 days, had a plasmid profile different from the parent strain. This isolate was missing its lowest molecular weight plasmid band (molecular weight, ca. 100×10^6). However, it retained the ability to nodulate soybeans. Immunofluorescence and immunodiffusion analysis of its somatic- and whole-cell antigens showed that it was serologically the same as the parental strain (Table 2).

DISCUSSION

In this report, we demonstrated the presence of from one to four plasmids in 10 fast-growing strains of soybean rhizobia. The recent results of

TABLE 2. Plasmid profiles, symbiotic properties, and immunological reactions of acridine orange- and heat-cured *R. japonicum* isolates

Culture	Plasmid profile	Symbiotic properties on cultivar		Immunological reactions[a]	
		Peking	Chippewa	Immuno-diffusion	Immuno-fluorescence
Parental (PRC 205)		Nod^+ Fix^+	Nod^+ Fix^-	4	4+
Cured mutants					
205 1AO1		Nod^-	Nod^-	4	4+
205 1AO3		Nod^-	Nod^-	4	4+
205 1AO5		Nod^-	Nod^-	4	4+
Parental (PRC 194)		Nod^+ Fix^+	Nod^+ Fix^-	4	4+
Cured mutant (194 1X40)		Nod^+ Fix^+	Nod^+ Fix^-	4	4+

[a] Antiserum for immunodiffusion was prepared against whole- and somatic-cell antigens. Antiserum for immunofluorescence was prepared against somatic antigens. For immunodiffusion, the values are the number of precipitin bands formed with whole-cell antiserum; each culture formed two bands with somatic antiserum. For immunofluorescence, the intensity of the reaction was expressed as: 4+, very intense; −, no reaction.

Masterson et al. (15) have indicated that several of the fast-growing isolates contain plasmids which hybridize to the structural *nifKDH* genes of *Klebsiella pneumoniae*. However, these authors did not show the location of nodulation genes in these isolates. Our results suggest that in at least one of these isolates, a large plasmid may also be the location of some of the nodulation genes. All of the acridine orange-generated, nodulation-deficient mutants of PRC 205 lost their largest plasmid. Zurkowski and Lorkiewicz (27) showed a correlation between the loss of nodulation character and the elimination of a plasmid in *Rhizobium trifolii* T12 and 24. Morrison et al. (16) have recently demonstrated that the *Lablab purpureus* isolate NGR234, which can nodulate some legumes and the nonlegume *Parasponia* sp., could be cured of its nodulating ability by treatment at elevated temperatures. In this isolate, the loss of nodulating ability was found to be due to the elimination of its largest resident plasmid, on which genes involved in nodulation and nitrogen fixation reside. In our study the heat-treated PRC 194 mutant, missing a smaller plasmid, was still able to effectively nodulate soybeans. Masterson et al. (15) indicated that in PRC 194, the structural *nif* genes were not located on any of the plasmids.

We also used the cured Nod^- mutants of 205 in blocking experiments to see whether they would inhibit nodulation by Nod^+ parental strain as well as other fast-growing *R. japonicum* (data not shown). We did not observe any significant suppression of nodulation, even when the Nod^- mutant was given a 48-h head start.

Zurkowski and Lorkiewicz (26) have shown that *R. trifolii* strains can easily lose their nodulation plasmid at elevated temperatures. The fast-growing *R. japonicum* isolates that we examined did not lose nodulating ability at restrictive temperatures of 37°C for up to 7 weeks, or at 40 and 42°C for 7 days. These isolates seem to be resilient to the heat-curing treatments used effectively for other species of *Rhizobium*. However, at levels of acridine orange that allowed some growth, one isolate was cured of its nodulating plasmid, whereas in bacteriostatic concentrations, no curing of any of the plasmids could be demonstrated. These results are in agreement with those of Pariiskaya (18) who found that *R. meliloti* L-1 was only significantly cured of nodulating ability in subbacteriostatic levels of acridine orange. However, Higashi (7) and Zurkowski et al. (25) cured different *R. trifolii* isolates of their nodulating ability in bacteriocidal concentrations of acridine orange.

In this report we showed that the loss of a large plasmid from the fast-growing *R. japonicum* leads to loss of nodulating ability. These nodulation-deficient *R. japonicum* mutants are an essential tool for genetic manipulations in the soybean-*Rhizobium* symbiotic system.

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