Plasmids, Biological Properties and Efficacy of Nitrogen Fixation in *Rhizobium japonicum* Strains Indigenous to Alkaline Soils

By DENNIS C. GROSS¹, ANNE K. VIDAVER¹ AND ROBERT V. KLUCAS²

¹Department of Plant Pathology and ²Laboratory of Agricultural Biochemistry, University of Nebraska, Lincoln, Nebraska 68583, U.S.A.

(Received 11 December 1978)

Plasmids were isolated from strains of *Rhizobium japonicum*, predominantly serogroup 135, obtained from soybean nodules collected at 15 sites in Nebraska, U.S.A. In addition to their serotype, these strains were indistinguishable from *R. japonicum* strain 3I1b135 in growth rate, sensitivity to phage Rhj781, antibiotic sensitivities, general colony characteristics and rates of nitrogen fixation per plant. All strains occupied soil habitats with similar characteristics, including a high pH (7.2 to 8.3), relatively high conductivity (0.04 to 0.32 mS), relatively high sodium saturation (0.32 to 12.7%), low iron content (3.2 to 14.8 p.p.m.) and low manganese content (5.1 to 18.7 p.p.m.). However, agarose gel electrophoresis analysis of plasmids enabled subdivision of these extra-slow-growing strains into four groups on the basis of differences in plasmid number and size. These strains carried combinations of two or more of four plasmids, ranging in mass from 49 to 118 megadaltons and comprising approximately 20% of the total DNA per cell. Biological and symbiotic data, along with plasmid analysis, were useful in identifying a wild-type strain (RJ23A) that shows potential as a soybean inoculant in alkaline soils.

INTRODUCTION

Inoculation of soybeans with a strain of *Rhizobium japonicum* does not necessarily ensure or augment nodulation of roots by that strain. Resident strains frequently induce formation of over 90% of the nodules despite prior inoculation of seed with agronomically desirable *R. japonicum* strains (Caldwell & Vest, 1970; Johnson *et al.*, 1965). Soil pH, soil temperature, planting date and variations in resistance to antagonistic soil microflora have been related to the persistence of particular strains of *R. japonicum* in soil (Caldwell & Weber, 1970; Drapeau *et al.*, 1973; Ham *et al.*, 1971; Smith & Miller, 1974; Weber & Miller, 1972). For example, Ham *et al.* (1971) substantiated and extended the work of Damirgi *et al.* (1967), showing that Iowa (U.S.A.) serogroup 135 *R. japonicum* strains were significantly associated ($P > 0.001$) with nodules produced by soybeans grown on alkaline soils (pH ≥ 7.5) while serogroup 123 predominated in Iowa soils below this pH.

Only two procedures have been commonly used to distinguish and identify strains of *R. japonicum* – serotyping (Ham *et al.*, 1971; Johnson & Means, 1963) and bacteriophage typing (Kowalski *et al.*, 1974). Thus, a more definitive means of strain differentiation is desirable. Plasmids from *R. japonicum* have been detected in ethidium bromide–caesium chloride and sucrose gradients (Klein *et al.*, 1975; Luyindula *et al.*, 1975; Nuti *et al.*, 1977), but the number and molecular weights of plasmids in any strain have not been characterized. No phenotypic character(s), including any associated with nodulating ability or

nitrogen fixation, has been correlated with the presence of plasmids. However, in various fast-growing *Rhizobium* species and a few cowpea rhizobia, extrachromosomal DNA has been physically isolated and characterized (Bechet & Guillaume, 1978; Nuti *et al.*, 1977; Tshitenge *et al.*, 1975; Zurkowski & Lorkiewicz, 1976).

This paper reports the physical characterization of plasmids from strains of *R. japonicum* taken from different alkaline soils in Nebraska, and compares the biological and symbiotic properties of these strains. Plasmid analyses promise to be a means of comparing and identifying strains of *R. japonicum*. Such information, coupled with data on desirable agronomic characteristics, for example, effectiveness of nitrogen fixation or a tendency to thrive in a defined soil habitat, may lead to improved management of seed inoculants.

METHODS

Bacterial strains. *Rhizobium japonicum* strains are listed in Tables 1 and 3. Strains 3I1b135, 3I1b117, 3I1b6 and 3I1b110 were from Dr D. Weber (USDA, Beltsville, Md., U.S.A.), strain WA5099-1-1 was from Dr G. Ham (University of Minnesota, St Paul, Minn., U.S.A.) and the other strains were isolated from nodules of commercially grown soybeans collected in Nebraska. *Escherichia coli* strains J53(RP1) and DT41(R27) were provided by Dr R. Olsen (University of Michigan, Ann Arbor, Mich., U.S.A.) and strain J53(R1drd19) by Dr S. Falkow (University of Washington, Seattle, Wash., U.S.A.).

Cultivation of bacteria. *Rhizobium japonicum* strains were maintained on Vincent's (1970) L-arabinose agar slants as described by Gross & Vidaver (1978). *Escherichia coli* strains J53(RP1) and DT41(R27) were maintained on nutrient broth/yeast extract (NBY) agar (Vidaver, 1967) containing 25 µg tetracycline ml⁻¹; strain J53(R1drd19) was grown on NBY agar containing 25 µg neomycin ml⁻¹.

For culturing rhizobia in liquid, L-arabinose broth (30 ml in 250 ml flask) was inoculated from slants and aerated on a rotary shaker at 25 °C and 250 rev. min⁻¹. *Escherichia coli* liquid cultures were grown in NBY broth at 37 °C.

Biological characterization of strains. Exponential phase *R. japonicum* strains in broth cultures were subcultured in duplicate into fresh medium and grown at 25 °C and 250 rev. min⁻¹. At 24 h intervals, samples were diluted in sterile buffer (12.5 mM-potassium phosphate pH 7.2), appropriate dilutions were plated (100 µl) on to L-arabinose agar, and after incubation and growth at 30 °C the number of colony-forming units (c.f.u.) ml⁻¹ was determined. Doubling times were calculated from values interpolated from the growth curves near the beginning and the end of the exponential growth phase (Meynell & Meynell, 1970).

Rhizobium japonicum strains were tested for bacteriocin production on L-arabinose agar by the procedure of Gross & Vidaver (1978).

Bacteriophage sensitivity of strains was tested with bacteriophage Rhj781, isolated from soil collected from a soybean field in Nebraska. A stock of purified phage [10⁸ to 10⁹ plaque-forming units ml⁻¹] was produced and stored as described by Kowalski *et al.* (1974). The phage-typing procedure of Kowalski *et al.* (1974) was modified by spotting the stock-phage preparation in 5 µl quantities on L-arabinose agar overlaid with 2.5 ml L-arabinose soft agar (0.7%, w/v) containing the test *R. japonicum* strain. The rhizobia were grown in L-arabinose broth to exponential phase, then diluted and mixed (100 µl) with the L-arabinose soft agar to give between 5 × 10⁶ and 1 × 10⁷ c.f.u. per plate.

Minimal inhibitory concentrations of antimicrobial agents (chloramphenicol, gentamicin, kanamycin, nalidixic acid, neomycin, penicillin G, streptomycin and tetracycline) were determined for *R. japonicum* strains by the agar dilution method (Washington & Barry, 1974) using L-arabinose agar medium.

The agglutination procedure of Damirgi *et al.* (1967) was used to classify strains serologically.

Isolation of rhizobia from nodules. Nodulated 6 to 8 week-old field-grown soybean plants were collected from 22 widely dispersed sites in Nebraska. Roots were rinsed in sterile water and nodules were then excised, surface-sterilized by immersion in 1.2% (v/v) sodium hypochlorite solution for 10 min, and rinsed with distilled water. As determined by independent checks, this procedure was effective in eliminating detectable surface rhizobia. From each of the 22 collection sites, six nodules were harvested and surveyed for *R. japonicum* type by culturing on L-arabinose agar. Individual colonies were picked and restreaked three times to ensure colony uniformity.

Inoculation and growth of soybeans. Surface-sterilized soybean (*Glycine max*, cv. Amsoy 71) was inoculated as previously described (Gross & Vidaver, 1978). One seedling was planted in each container and inoculated 2 d later with approximately 10⁸ bacteria. Control plants received only buffer. Preliminary experiments indicated that the initial inoculant strain could always be recovered and identified from the resulting nodules.

Acetylene reduction assays. Five weeks after inoculation, each *R. japonicum* strain was tested in triplicate on Amsoy 71 for nitrogenase activity by following acetylene reduction using the procedures of Pedersen

et al. (1978) and Klucas (1974). In these tests, plants grown from surface-sterilized seeds were enclosed in 30.5 × 61 cm polypropylene bags that were then injected with 42 µmol methane as an internal standard and approx. 0.1 atm (10 kPa) acetylene. Samples (500 µl) were taken at intervals and ethylene levels were measured using a Perkin-Elmer model 3920B gas chromatograph equipped with a flame ionization detector and a 3M Porapak N column. Rates of ethylene production per plant and per mg dry nodule were calculated from the slope of a graph of measurements taken at three 15 min intervals. All nodules were harvested and dried to constant weight in an 80 °C oven for determination of dry weight. Sterile 12.5 mM-potassium phosphate buffer, pH 7.2, was substituted for inoculant to obtain nodule-free checks.

In another experiment, relative nodule development was monitored by determining the rates of acetylene reduction at 3, 4 and 5 weeks after inoculation of Amsoy 71 with strains 3I1b135, RJ12S, RJ23A, RJ10B, RJ19FY, RJ17W and 3I1b6.

Soil analyses. Rhizosphere soil samples were obtained from the 22 soybean collection sites described above. After drying, the soil samples were analysed for pH, conductivity, sodium saturation, and manganese and iron concentrations (along with nine other soil characteristics) by the University of Nebraska Soil Testing Laboratory, basically as outlined by Black *et al.* (1965).

Cell lysis and plasmid isolation. Bacteria were grown in 30 ml medium to between 2×10^8 and 6×10^8 colony-forming units (c.f.u.) ml⁻¹. *Rhizobium japonicum* and *E. coli* cells were lysed (final vol. 12 ml) as described by Currier & Nester (1976) except that Sarkosyl NL30 (ICN Pharmaceuticals, Plainview, N.Y., U.S.A.) was substituted for sodium dodecyl sulphate (SDS). Lysates were sheared in 4 ml amounts and then denatured with alkali. Denatured DNA was removed by phenol treatment, with 1 M-LiCl substituted for 3% (w/v) NaCl. After two extractions with phenol, 2 vol. 95% ethanol at -20 °C were added to facilitate precipitation of plasmids from the aqueous phase followed by storage of samples for a minimum of 2 h at -20 °C.

Agarose gel electrophoresis. Precipitated plasmid DNA was collected by centrifugation (12100 g for 20 min) and the pellet was resuspended in 200 µl of a dye solution containing 25% (v/v) glycerol, 5% (w/v) SDS and 0.025% (w/v) bromophenol blue in water. Samples (5 to 30 µl) of plasmid preparations were applied to 0.7% (w/v) agarose (Sea Kem, Marine Colloids, Rockland, Me., U.S.A.) vertical slab gels and subjected to electrophoresis (ORTEC model 4200, Oakridge, Tenn., U.S.A.) according to the method of Meyers *et al.* (1976). The 8 × 10 × 0.3 cm gels had 12 slots for sample placement. Electrophoresis was complete after 3.5 h with 40 V supplied by a constant voltage power source. Gels were stained with ethidium bromide (1 µg ml⁻¹ in distilled water) for 1 h and visualized according to Hansen & Olsen (1978). The average molecular mass was determined according to Meyers *et al.* (1976) by comparing the relative mobility of *R. japonicum* plasmids to plasmids RP1 [39 megadaltons (Mdal) (Burkardt *et al.*, 1978)], R1 [62 Mdal (Meyers *et al.*, 1976)] and R27 [112 Mdal (Hansen & Olsen, 1978)].

Dye-buoyant density equilibrium centrifugation. Exponential phase cells of *R. japonicum* from liquid culture were subcultured in L-arabinose broth (5×10^7 initial c.f.u. ml⁻¹) containing 5 µCi [³H]adenine ml⁻¹ (20.2 Ci mmol⁻¹; New England Nuclear). Bacteria were then grown and lysed as described above.

Concentrated [³H]DNA was dialysed and prepared for caesium chloride-ethidium bromide gradients according to Currier & Nester (1976). The suspension was centrifuged at 15 °C in a Spinco Ti50 fixed angle rotor for 60 h at 40000 rev. min⁻¹. Gradients were fractionated from the bottom. Radioactivity was determined after alkali-treated fractions had been precipitated by trichloroacetic acid (Bollum, 1968).

RESULTS

Geographic distribution of extra-slow-growing R. japonicum strains in relation to the soil habitat

Extra-slow-growing (ESG), non-mucoid *R. japonicum* strains have doubling times of approximately 14 h or more (Table 1), while most strains have doubling times of 6 to 13 h (Vincent, 1977). An exception is the mucoid strain WA5099-1-1 that has a doubling time of approximately 24 h. The geographically diverse soils which yielded these ESG strains have similar mineral and chemical properties (Table 2). Sixteen of the 22 soils analysed were alkaline soils [saline and/or calcareous (Thompson & Troeh, 1973)], and ESG, non-mucoid *R. japonicum* strains were recovered from soybean nodules produced on 15 of these high pH soils. Mucoid rhizobia of usual colony morphology (Vincent, 1977) and noticeably faster growth on agar media (and commonly serotype 123) were exclusively recovered from the remaining soils. Nodules containing either or both colony types were found in some soils. However, the prevalence of non-mucoid, ESG strains was generally correlated with a

Table 1. *Characteristics of R. japonicum strains*

Strain no.	Serogroup	Bacteriocin group	Sensitivity to phage Rhj781	Doubling time (h)	Ethylene produced (nmol per plant min ⁻¹)*	Ethylene produced (nmol per mg dry nodule h ⁻¹)*
3I1b135	135	3	+	15.7	69.3 BC†	36.2 CD†
RJ10B	135	3	+	19.8	88.0 BC	45.0 CD
RJ12S	135	3	+	20.7	87.6 BC	53.3 BC
RJ19FY	135	3	+	30.1	82.0 BC	41.0 CD
RJ17W	135	3	+	22.6	73.2 BC	38.4 CD
RJ23A	135 and 117	3	—	13.4	162.4 A	67.0 B
WA5099-1-1	110	1	—	24.1	41.1 C	24.3 D
3I1b6	c1	1	—	10.6	112.0 AB	92.8 A
3I1b110	110	1	—	9.3	80.7 BC	69.4 B
4BAL	123	2	—	10.6	55.5 C	35.8 CD
3I1b117	117	1	—	11.6	ND	ND

ND, Not determined.

* Determinations completed 5 weeks after inoculation of cultivar Amsoy 71. Each rate is an average of three replications. Plants inoculated with sterile phosphate buffer remained non-nodulated.

† Values followed by identical letters are not significantly different ($P \leq 0.05$) using Duncan's new multiple-range test.

Table 2. *Association of extra-slow-growing (ESG) R. japonicum strains with alkaline soils*

Six nodules were obtained from different plants (6 to 8 weeks-old) for each soil site and used for isolation of *R. japonicum*. In nodules from soybeans grown in alkaline soils the ESG strains were either found exclusively or mixed in varying proportions (up to 50%) with mucoid strains. All *R. japonicum* isolates from nodules of soybeans grown in pH 5.5 to 6.9 soils were mucoid strains.

Soil pH range	No. of soils	No. yielding ESG <i>R. japonicum</i>	Conductivity (mS)	Sodium saturation (%)	Manganese (p.p.m.)	Iron (p.p.m.)
7.2-8.3	16	15	0.04-0.32	0.32-12.70	5.1-18.7	3.2-14.8
5.5-6.9	6	0	0.02-0.06	0.02-0.93	15.4-39.6	16.9-72.2

higher pH. All soils with resident ESG rhizobia had five common characteristics: high pH, high conductivity and high sodium content but low levels of iron and manganese.

The natural geographic distribution of ESG, non-mucoid strains in Nebraska (Fig. 1) was largely independent of plasmid size and number (determined and discussed below). Isolation from six nodules at each of 15 sites (Table 2) from plants chosen at random showed that the distribution ranged from approximately half to all ESG strains. Each collection site generally yielded ESG strains comprising a single plasmid group. For example, at one collection site, where isolates from each of six nodules were examined for plasmids, all isolates were indistinguishable in plasmid profile. No particular soil factor, such as pH or iron level, was correlated with the presence of a particular plasmid group.

Isolation and characterization of plasmids

All ESG strains examined, including strains not listed here, contained plasmids; a representative strain from each collection site is included in Table 3. Plasmids were readily and reproducibly isolated from both ESG and mucoid strains of *R. japonicum* (Table 3). These plasmids ranged between 48 and 130 Mdal, as assessed by relative electrophoretic mobility on agarose gels (Fig. 2). Of particular significance was the detection of four groups of strains of *R. japonicum* (exemplified by RJ10B, RJ12S, RJ19FY and RJ17W) that carried combinations of two or more of four plasmids (Table 3). The 118 and 91 Mdal plasmids were common to all strains within these four groups; the presence or absence of the 74 and

Fig. 1. Geographical distribution of ESG strains of *R. japonicum* in Nebraska, U.S.A. Symbols indicate the plasmid group for strains recovered at a particular site: ■, 49, 74, 91 and 118 Mdal plasmids; □, 74, 91 and 118 Mdal plasmids; ●, 49, 91 and 118 Mdal plasmids; ○, 91 and 118 Mdal plasmids.

Table 3. Plasmids from *R. japonicum* strains

Strain no.	No. of plasmids*	Plasmid size (megadaltons)*
3I1b135	4	48(±1.1), 73(±0.0), 111(±2.1), 115(±0.7), [2]
RJ10B†	4	49(±2.5), 74(±3.1), 91(±3.0), 118(±4.1), [10]
RJ12S	3	74(±3.1), 91(±3.0), 118(±4.1), [10]
RJ19FY‡	3	49(±2.5), 91(±3.0), 118(±4.1), [10]
RJ17W§	2	91(±3.0), 118(±4.1), [10]
RJ23A	2	86(±3.7), 99(±2.9), [8]
WA5099-1-1	2	83(±3.6), 116(±7.4), [6]
3I1b117	1	130(±7.5), [5]

* Determined by electrophoretic separation on agarose gels as described in Methods. Numbers in parentheses are the standard deviation of the molecular mass determined for each plasmid. Numbers in brackets refer to the number of replications. The average plasmid size was calculated from these gels.

† Strains RJ2M, RJ14C and RJ22AY were indistinguishable from RJ10B in the number and size of plasmids.

‡ Strains RJ5V, RJ6V, RJ7A, RJ8S, RJ9B, RJ20FY and RJ21NH were indistinguishable from RJ19FY in the number and size of plasmids.

§ Strains RJ4G and RJ22AH were indistinguishable from RJ17W in the number and size of plasmids.

49 Mdal plasmids differentiated these groups. Numerous plasmids were detected in other commonly found *R. japonicum* strains. For example, strain 3I1b135 had four plasmids but they differed in size from those in strain RJ10B. Only one strain (3I1b117) had a single plasmid; its molecular mass was 130 Mdal (Table 3).

The caesium chloride-ethidium bromide sedimentation profile of plasmids isolated from strain RJ19FY is shown in Fig. 3. More than half of the isolated DNA was separated into a satellite band of covalently closed circular DNA that clearly shows the selective loss of chromosomal DNA while enriching for plasmid DNA. Electrophoresis on agarose gels of the RJ19FY satellite DNA resulted in the separation of the three plasmids (Table 3). The ratio of plasmid DNA (recovered in the peak shown in Fig. 3) to total DNA (calculated from radioactivity in the cleared lysate subsamples prior to phenol treatment) was approximately 22% of the total DNA for strain RJ19FY. Assuming a genome size of approximately 2500 Mdal (Bak *et al.*, 1970) it was estimated there were approximately 1 to 3 copies per cell of each plasmid. Similar dye-buoyant density gradient profiles and plasmid yields were obtained for strain 3I1b135 (not shown). Strains 3I1b6, 3I1b110 and 4BAL also had plasmids, as determined by separation from chromosomal DNA on caesium chloride-ethidium bromide density gradients.

Fig. 2. Agarose gel electrophoresis of plasmids from ESG *R. japonicum* strains and reference plasmids from *E. coli*. Chromosomal fragments were the most rapidly migrating DNA (bottom band). Reference plasmids are: A, RP1 (39 Mdal); B, R1drd19 (62 Mdal); C, R27 (112 Mdal). Plasmid profiles of *R. japonicum* strains are: D, 3I1b135; E, RJ10B; F, RJ14C; G, RJ12S; H, RJ19FY; I, RJ21NH; J, RJ17W; K, RJ4G; L, RJ23A. Plasmid number and mass for these strains is summarized in Table 3.

Biological and symbiotic properties of R. japonicum strains differing in plasmid number and size

Rhizobium japonicum strains that had different plasmid complements (Table 3) varied in their biological and symbiotic properties (Table 1). Despite quantitative or qualitative plasmid differences in strains 3I1b135, RJ10B, RJ12S, RJ19FY and RJ17W, they were all serotype 135, in bacteriocin group 3 (non-producers), sensitive to bacteriophage Rhj781, and ESG (doubling times greater than 14 h). These strains also had indistinguishable sensitivity profiles to eight antibiotics, with no significant resistance to any individual antibiotic.

Rates of nitrogen fixation for soybean plants inoculated with strains RJ10B, RJ12S, RJ19FY and RJ17W were similar and appeared to have no relationship to the presence or absence of the 74 and 49 Mdal plasmids (Table 3). All strains identical in plasmid number and size also had approximately the same rate of ethylene production (Table 1). Other commonly used strains of *R. japonicum* that exemplify the diversity of *R. japonicum* by serogroup (four), efficacy of nitrogen fixation (41 to 112 nmol ethylene produced per plant min^{-1}) and plasmid size and number are also shown in Table 3.

Strain RJ23A was of particular interest because it had many characteristics in common with the majority of ESG strains including overall colony morphology, a slow growth rate, antibiotic sensitivity profile, lack of bacteriocin production and agglutination with 135 antiserum (Table 1). However, it also reacted with 117 antiserum, was insensitive to bacteriophage Rhj781 and differed in plasmid profile (Table 3). More significantly, it had approximately twice the rate of nitrogen fixation per plant and a greater rate of nitrogen fixation per nodule dry weight than any of the serogroup 135 strains.

Three different plasmid-containing types of ESG strains were compared for nodule development as measured by acetylene reduction (Fig. 4). Strain RJ12S exemplifies the similarity of increase in nitrogen fixation with time for strains RJ10B, RJ19FY and RJ17W. Strain RJ23A was superior in nitrogen fixation to all serogroup 135 strains throughout the 3 week period that represents the early but important stage of symbiosis. Both the ESG strains and the other *R. japonicum* strains formed effective nodules 3 weeks after inoculation.

Fig. 3

Fig. 4

Fig. 3. Caesium chloride-ethidium bromide density gradient profile of *R. japonicum* RJ19FY [^3H]-DNA. After reaching equilibrium the gradient was fractionated from the bottom. Fraction 32 contained the plasmid DNA peak and fraction 35 contained the chromosomal DNA peak.

Fig. 4. Relative increase in rate of acetylene reduction 3, 4 and 5 weeks after inoculation of Amsoy 71 with *R. japonicum* strains 3I1b135, RJ12S, and RJ23A. Each bar represents an average rate from four replicated plants.

DISCUSSION

Analysis of serogroup 135 strains of *R. japonicum* for differences in plasmid number and mass provides a rapid and reliable means of strain distinction. In Nebraska, strains of four plasmid groups were distributed in 15 geographically-distinct alkaline soil sites. Although they all had a 118 and 91 Mdal plasmid in common, they varied in the presence or absence of a 74 and/or 49 Mdal plasmid. The plasmid profiles were not associated with bacteriophage sensitivity, antibiotic sensitivity, doubling time or efficacy of nitrogen fixation on soybean. Phenotypic characteristics have not yet been definitively associated with any plasmids in other *Rhizobium* species (Nuti *et al.*, 1977; Zurkowski & Lorkiewicz, 1976), but there is evidence that bacteriocin production and nodulating ability may be plasmid-linked properties in a strain of *R. leguminosarum* (Johnston *et al.*, 1978). Conversely, Cole & Elkan (1973) inferred that transmissible antibiotic resistance of *R. japonicum* was due to a plasmid(s), but presented no physical evidence.

All strains of *R. japonicum* were easily lysed by the basic Currier & Nester (1976) technique and plasmids were readily isolated. These could be quantitatively differentiated. No difference in plasmid recovery from these strains occurred if Hansen & Olsen's (1978) technique designed for large plasmids was used (unpublished data).

Agarose gel electrophoresis can be used to determine plasmid molecular mass with less than 10% error (Meyers *et al.*, 1976) and there is linearity of large plasmid migration in gels to approximately 140 Mdal (Hansen & Olsen, 1978). Since the reference plasmids RP1 (39 Mdal), R1 (62 Mdal) and R27 (112 Mdal) formed a linear standard, an accurate determination of mass was possible for most of the *R. japonicum* plasmids, which range from 48 to 118 Mdal. In strains 3I1b135 and RJ10B, that had two plasmids of practically the same size, only further studies, such as by restriction endonuclease analysis, will show evidence of identity, if any.

The association of ESG strains of *R. japonicum* with alkaline soils substantiates earlier observations by Damirgi *et al.* (1967), Ham *et al.* (1971) and Kowalski *et al.* (1974). As shown here, wild-type strains isolated from surface-sterilized nodules could be cloned, inoculated on soybeans and re-isolated from surface-sterilized nodules. In addition, serological, bacteriophage and bacteriocin typing support the observation that these ESG, non-mucoid strains belong to an alkaline soil ecotype and are not mutants (Herridge & Roughley, 1975; Kuykendall & Elkan, 1976; Upchurch & Elkan, 1977). However, a soil of high pH is not considered to be the primary factor responsible for ESG strain success. Cationic effects on growth and viability are well documented (Upchurch & Elkan 1977; Vincent, 1977). In our study, concentrations of iron and manganese were lowest in fields harbouring ESG strains (alkaline pH having a negative effect on availability of these cations). Survival in such soils is perhaps related to greater sensitivity of common rhizobia to one or both of these cations. These cations may also affect competitive ability to nodulate soybean. In greenhouse experiments, nodules containing serogroup 135 decreased from 88 to 43% as iron concentrations in neutral pH nutrient solutions increased from 0 to 16 p.p.m. (Vest *et al.*, 1973). Our field studies support such results since serogroup 135 rhizobia were not recovered from soils in Nebraska with greater than 16 p.p.m. iron.

Nodules formed by serogroup 135 strains of *R. japonicum* generally had similar and moderate rates of nitrogen fixation. Although soybean cultivar, cultivar age and various environmental conditions can also profoundly affect fixation rates (Eskew & Schrader, 1977; Klucas & Arp, 1977), our observed range was consistent with these and other published reports (Carter *et al.*, 1978; Kuykendall & Elkan, 1976). Plants were inoculated at a neutral pH but the symbiotic characters effected by strains from alkaline soils were comparable to strains from other soils. Experiments at alkaline pH may show differences in comparison with common strains isolated from neutral or acidic soils.

Effective nitrogen fixation rates are an important prerequisite for commercial use of *R. japonicum* strains. Carter *et al.* (1978) found nodules formed by strain USDA 135 (3I1b135) had a moderate acetylene reduction rate and were moderately efficient (compared with 3I1b110) in conserving H₂, a by-product of nitrogenase activity. However, a strain must be adaptable to and compatible with the habitat in which it is introduced. For example, a phenotypically distinct strain (RJ23A), obtained from an alkaline soil, formed nodules that had a high rate of nitrogen fixation under our test conditions. On a dry nodule weight basis, it was not as effective as 3I1b6 or 3I1b110 but it supplied more fixed nitrogen to the plant, primarily by virtue of increased nodulation and nodule size. Strain RJ23A thus shows potential as an inoculant to be used in alkaline soils. Besides finding effective wild-type strains, effectiveness of some strains may be increased by mutagenesis (Maier & Brill, 1978).

Ecological adaptation by *R. japonicum* has not been widely considered in choosing soybean inoculant strains due to a lack of information and definitive tests to differentiate strains. Analysis of plasmids of natural rhizobial populations has provided a useful means to distinguish serogroup 135 strains adapted to an alkaline soil environment.

This work was supported by USDA-CSRS research agreement no. 516-15-115. We thank Dr R. A. Wiese for providing soil analyses, Dr G. E. Ham for serotyping and Dr C. F. Gonzalez for consultations on plasmid analyses. The skilful technical assistance of Michael B. Keralis, Mitchell Yakrus and Mark Widger is gratefully acknowledged. Published as Paper no. 5691, Journal Series, Nebraska Agricultural Experiment Station.

REFERENCES

- BAK, A. L., CHRISTIANSEN, C. & STENDERUP, A. (1970). Bacterial genome sizes determined by DNA renaturation studies. *Journal of General Microbiology* **64**, 377-380.
- BECHET, M. & GUILLAUME, J. B. (1978). Mise en évidence d'ADN extrachromosomique chez *Rhizobium meliloti*. *Canadian Journal of Microbiology* **24**, 960-966.
- BLACK, C. A., EVANS, D. D., WHITE, J. L., ENSMINGER, L. E. & CLARK, F. E. (editors) (1965). *Methods of Soil Analysis: Chemical and Microbiological Properties*, part 2, pp. 771-1572. Madison, Wisconsin: American Society of Agronomy.
- BOLLUM, F. J. (1968). Filter paper disk techniques for assaying radioactive macromolecules. *Methods in Enzymology* **12B**, 169-173.
- BURKARDT, H. J., MATTES, R., PUHLER, A. & HEUMANN, W. (1978). Electron microscopy and computerized evaluation of some partially denatured group P resistance plasmids. *Journal of General Microbiology* **105**, 51-62.
- CALDWELL, B. E. & VEST, G. (1970). Effects of *Rhizobium japonicum* strains on soybean yields. *Crop Science* **10**, 19-21.
- CALDWELL, B. E. & WEBER, D. F. (1970). Distribution of *Rhizobium japonicum* serogroups in soybean nodules as affected by planting dates. *Agronomy Journal* **62**, 12-14.
- CARTER, K. R., JENNINGS, N. T., HANUS, J. & EVANS, H. J. (1978). Hydrogen evolution and uptake by nodules of soybeans inoculated with different strains of *Rhizobium japonicum*. *Canadian Journal of Microbiology* **24**, 307-311.
- COLE, M. A. & ELKAN, G. H. (1973). Transmissible resistance to penicillin G, neomycin, and chloramphenicol in *Rhizobium japonicum*. *Antimicrobial Agents and Chemotherapy* **4**, 248-253.
- CURRIER, T. C. & NESTER, E. W. (1976). Isolation of covalently closed circular DNA of high molecular weight from bacteria. *Analytical Biochemistry* **76**, 431-441.
- DAMIRGI, S. M., FREDERICK, L. R. & ANDERSON, I. C. (1967). Serogroups of *Rhizobium japonicum* in soybean nodules as affected by soil types. *Agronomy Journal* **59**, 10-12.
- DRAPEAU, R., FORTIN, J. A. & GAGNON, C. (1973). Antifungal activity of *Rhizobium*. *Canadian Journal of Botany* **51**, 681-682.
- ESKEW, D. L. & SCHRADER, L. E. (1977). Effect of rj_1 rj_1 (non-nodulating) soybeans on nodulation of near isogenic Rj_1 Rj_1 plants in nutrient culture. *Canadian Journal of Microbiology* **23**, 988-993.
- GROSS, D. C. & VIDAVER, A. K. (1978). Bacteriocin-like substances produced by *Rhizobium japonicum* and other slow-growing rhizobia. *Applied and Environmental Microbiology* **36**, 936-943.
- HAM, G. E., FREDERICK, L. R. & ANDERSON, I. C. (1971). Serogroups of *Rhizobium japonicum* in soybean nodules sampled in Iowa. *Agronomy Journal* **63**, 69-72.
- HANSEN, J. B. & OLSEN, R. H. (1978). Isolation of large bacterial plasmids and characterization of the P2 incompatibility group plasmids pMG1 and pMG5. *Journal of Bacteriology* **135**, 227-238.
- HERRIDGE, D. F. & ROUGHLEY, R. J. (1975). Variation in colony characteristics and symbiotic effectiveness of *Rhizobium*. *Journal of Applied Bacteriology* **38**, 19-27.
- JOHNSON, H. W. & MEANS, U. M. (1963). Serological groups of *Rhizobium japonicum* recovered from nodules of soybeans (*Glycine max*) in field soils. *Agronomy Journal* **55**, 269-271.
- JOHNSON, H. W., MEANS, U. M. & WEBER, C. R. (1965). Competition for nodule sites between strains of *Rhizobium japonicum* applied as inoculum and strains in the soil. *Agronomy Journal* **57**, 179-185.
- JOHNSTON, A. W. B., BEYNON, J. L., BUCHANAN-WOLLASTON, A. V., SETCHELL, S. M., HIRSCH, P. R. & BERINGER, J. E. (1978). High frequency transfer of nodulating ability between strains and species of *Rhizobium*. *Nature, London* **276**, 634-636.
- KLEIN, G. E., JEMISON, P., HAAK, R. A. & MATTHYSSE, A. G. (1975). Physical evidence of a plasmid in *Rhizobium japonicum*. *Experientia* **31**, 532-533.
- KLUCAS, R. V. (1974). Studies on soybean nodule senescence. *Plant Physiology* **54**, 612-616.
- KLUCAS, R. V. & ARP, D. (1977). Physiological and biochemical studies on senescing tap root nodules of soybeans. *Canadian Journal of Microbiology* **23**, 1426-1432.
- KOWALSKI, M., HAM, G. E., FREDERICK, L. R. & ANDERSON, I. C. (1974). Relationship between strains of *Rhizobium japonicum* and their bacteriophages from soil and nodules of field-grown soybeans. *Soil Science* **118**, 221-228.
- KUYKENDALL, L. D. & ELKAN, G. H. (1976). *Rhizobium japonicum* derivatives differing in nitrogen-fixing efficiency and carbohydrate utilization. *Applied and Environmental Microbiology* **32**, 511-519.
- LUYINDULA, N., TSHITENGE, G., LURQUIN, P. & LEDOUX, L. (1975). Etude des plasmides de *Rhizobium japonicum*. *Archives internationales de physiologie et de biochimie* **83**, 199-200.
- MAIER, R. J. & BRILL, W. J. (1978). Mutant strains of *Rhizobium japonicum* with increased ability to fix nitrogen for soybean. *Science* **201**, 448-450.
- MEYERS, J. A., SANCHEZ, D., ELWELL, L. P. & FALKOW, S. (1976). Simple agarose gel electro-

- phoretic method for the identification and characterization of plasmid deoxyribonucleic acid. *Journal of Bacteriology* **127**, 1529–1537.
- MEYNELL, G. G. & MEYNELL, E. (1970). *Theory and Practice in Experimental Bacteriology*, 2nd edn. Cambridge: Cambridge University Press.
- NUTI, M. P., LEDEBOER, A. M., LEPIDI, A. A. & SCHILPEROORT, R. A. (1977). Large plasmids in different *Rhizobium* species. *Journal of General Microbiology* **100**, 241–248.
- PEDERSEN, W. L., CHAKRABARTY, K., KLUCAS, R. V. & VIDAVER, A. K. (1978). Nitrogen fixation (acetylene reduction) associated with roots of winter wheat and sorghum in Nebraska. *Applied and Environmental Microbiology* **35**, 129–135.
- SMITH, R. S. & MILLER, R. H. (1974). Interaction between *Rhizobium japonicum* and soybean rhizosphere bacteria. *Agronomy Journal* **66**, 564–567.
- THOMPSON, L. M. & TROEH, F. R. (1973). *Soils and Soil Fertility*, 3rd edn, pp. 203–204. New York: McGraw-Hill.
- TSHITENGE, G., LUYINDULA, N., LURQUIN, P. F. & LEDOUX, L. (1975). Plasmid deoxyribonucleic acid in *Rhizobium vigna* and *Rhizobium trifolii*. *Biochimica et biophysica acta* **414**, 357–361.
- UPCHURCH, R. G. & ELKAN, G. H. (1977). Comparison of colony morphology, salt tolerance and effectiveness in *Rhizobium japonicum*. *Canadian Journal of Microbiology* **23**, 1118–1122.
- VEST, G., WEBER, D. F. & SLOGER, C. (1973). Nodulation and nitrogen fixation. In *Soybeans: Improvement, Production, and Uses*, pp. 353–390. Edited by B. E. Caldwell. Madison, Wisconsin: American Society of Agronomy.
- VIDAVER, A. K. (1967). Synthetic and complex media for the rapid detection of fluorescence of phytopathogenic pseudomonads: effect of the carbon source. *Applied Microbiology* **15**, 1523–1524.
- VINCENT, J. M. (1970). *A Manual for the Practical Study of the Root Nodule Bacteria*. Oxford: Blackwell Scientific Publications.
- VINCENT, J. M. (1977). *Rhizobium*: general microbiology. In *A Treatise on Dinitrogen Fixation*, Section III: *Biology*, pp. 277–366. Edited by R. W. F. Hardy & W. S. Silver. New York: John Wiley.
- WASHINGTON II, J. A. & BARRY, A. L. (1974). Dilution test procedures. In *Manual of Clinical Microbiology*, 2nd edn, pp. 410–417. Edited by E. H. Lennette, E. H. Spaulding & J. P. Truant. Washington: American Society for Microbiology.
- WEBER, D. F. & MILLER, V. L. (1972). Effect of soil temperature on *Rhizobium japonicum* serogroup distribution in soybean nodules. *Agronomy Journal* **64**, 796–798.
- ZURKOWSKI, W. & LORKIEWICZ, Z. (1976). Plasmid deoxyribonucleic acid in *Rhizobium trifolii*. *Journal of Bacteriology* **128**, 481–484.