Characterization of an Effective Salt-tolerant, Fast-growing Strain of *Rhizobium japonicum*

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(Received 8 September 1982; revised 4 November 1982)

Rhizobium japonicum USDA191 is a member of a new group of *Rhizobium japonicum* strains found in China. This strain is one of several strains shown to be salt-tolerant and fast-growing; it is unique in being the only strain of this group that effectively nodulates American soybean cultivars. For these reasons strain USDA191 was chosen for further study and comparison to the common American *Rhizobium japonicum* isolate USDA110. Strain USDA191 has a doubling time of 3.2 h in complex medium and grows in concentrations of up to 0.4 M-NaCl, while strain USDA110, which has a doubling time of 12 h, is severely inhibited in media containing 0.1 M-NaCl. Under salt stress conditions, intracellular levels of K⁺ and glutamate were shown to increase. A comparison based on carbohydrate metabolism, DNA homology and protein patterns on polyacrylamide gels reveals that strain USDA191 is more closely related to the fast-growing rhizobia than to *Rhizobium japonicum*. However, the strain retains capacity to nodulate American soybean and cowpea cultivars effectively.

INTRODUCTION

A survey of strains of *Rhizobium japonicum* isolated from major production areas in the United States has shown that all the isolates are very sensitive to osmotic stress. Soybeans dependent on symbiotic nitrogen fixation are more salt sensitive than nitrogen-fertilized plants, limiting the soils where symbiotic nitrogen fixation is a useful means of fertilization (Bernstein & Ogata, 1966). The detrimental effects of salt stress on nodulation, plant growth and nitrogen fixation have been reported for many legumes including alfalfa, peas, mung beans and cowpeas (Balasubraminiam & Sinha, 1976; Upchurch & Elkan, 1977; Wilson & Norris, 1970; Yadav & Vyvar, 1971). The presence of high sodium chloride has been reported to reduce the number of rhizobia in legume inoculants (Steinborn & Roughly, 1974, 1975). Tolerance to salt stress may be an important part of saprophytic competence and competitiveness in rhizobia.

Rhizobium japonicum 191 is a member of a salt-tolerant group of *R. japonicum* recently described by Keyser *et al.* (1982). Strain 191 stands out among this group because it is the only one of this group which forms effective nodules on U.S. cultivars of soybeans. Keyser *et al.* (1982) point out that these strains seem similar to the fast-growing group of *Rhizobium* which are generally more salt-tolerant and produce an acid reaction when grown on mannitol media as opposed to sensitivity to 0.085 M-NaCl and an alkali reaction on mannitol media characteristic of slow-growing *Rhizobium japonicum* and *Rhizobium* spp. (cowpea). However, little further is known about the similarities strain 191 has with the fast-growing group or the mechanism involved in salt tolerance. We report here studies done on the characterization of this unique strain of *Rhizobium japonicum* and offer a preliminary step to understanding salt tolerance in *Rhizobium*.

METHODS

Bacterial strains and growth conditions. *Rhizobium japonicum* 110 was obtained from D. F. Weber, USDA, Beltsville, Md. *Rhizobium japonicum* 191 and *R. meliloti* 102F28 were obtained from J. C. Burton of the Nitragin Corp., Milwaukee, Wis. Strain 191 was isolated from nodules of 'Jupiter' soybeans (*Glycine max*) inoculated with soil 'A' obtained from Shanghai, China. Cultures were checked for uniform colony morphology using a Tween 40 medium (Lim, 1978) followed by repeated streaking for isolated colonies on mannitol/salts/yeast extract (MSY medium) plates containing bromothymol blue (Trinick, 1980; Vincent, 1970) to prevent the isolation of genetically mixed colonies.

Minimal media used for growth rate experiments contained glutamate (1.0 g l^{-1}) and the appropriate carbon source (4.0 g l^{-1}) unless otherwise indicated, as previously described (Trinick, 1980; Vincent, 1970). The pH of the medium was adjusted to 6.8 prior to autoclaving. The osmotic strength of the medium was increased by the addition of sodium chloride. Cultures were grown aerobically at 30°C .

Determination of growth rates. Liquid cultures were started using a 2% inoculum ($A_{420} = 0.1$, 1 cm length path) and incubated in a rotary shaker at 30°C . Growth was followed by measuring the absorbance at 420 nm in a Gilford model 300N spectrophotometer and also by the increase in cell protein, as described by Scott *et al.* (1979). Total protein was routinely assayed by the Lowry method using bovine serum albumin as a standard. Growth curve data were plotted on semilogarithmic paper and the mean generation times were obtained from the exponential phases of the growth curves.

Utilization of carbohydrates. Strains were tested for growth against each of 18 different carbohydrates. Cultures were grown in minimal media to the mid-exponential phase of growth, washed twice, and resuspended in sterile distilled water. A sample of each of the cultures (0.1 ml) (containing approx. 1×10^8 cells) was spread on plates containing the appropriate filter-sterilized carbon source (4.0 g l^{-1}). The nitrogen source used was KNO_3 (1.0 g l^{-1}). The plates were incubated at 30°C for 7 d and compared with control plates containing no carbon source.

Determination of intracellular potassium levels. The strains to be tested were grown in minimal medium (lactate/aspartate) containing the appropriate concentrations of NaCl in the medium. A 2 ml sample of each of the cultures were placed into test tubes in ice for protein determination. Portions (1 ml) were extracted for potassium analysis according to the method of Epstein & Schultz (1965). The samples were analysed as previously described (Sanui & Pace, 1968).

Enzyme assays. Acetylene reduction and hydrogen evolution in air by nitrogenase were determined under linear conditions on detached root systems as described by Bethlenfalvay & Phillips (1977). The relative efficiency (RE) was calculated as $\text{RE} = 1 - (\text{H}_2 \text{ evolution} / \text{C}_2\text{H}_2 \text{ reduction})$ (Schubert & Evans, 1976). Hydrogenase activity was determined by the tritium uptake assay as previously described (Lim, 1978).

Glucose-6-phosphate dehydrogenase (EC 1.1.1.49, D-glucose-6-phosphate:NADP⁺ 1-oxidoreductase) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44, 6-phospho-D-gluconate:NADP⁺ 2-oxidoreductase) activities were determined in crude cell-free extracts essentially as described by Martinez De-drets & Arias (1972). Assay mixtures contained 0.05 mM-NADP⁺, 0.5 mM-MgSO₄, 0.05 mM glucose-6-phosphate or 6-phosphogluconate (Na salt) and 80 mM-Tris/HCl pH 8.0. All assays were carried out at 25°C .

Polyacrylamide gels of *Rhizobium japonicum*. The method of Laemmli (1970), as previously described by Scott *et al.* (1979), was used for one-dimensional gel analysis.

DNA reassociation analysis. DNA was purified from 15.1 g of cells grown in MSY medium, washed twice with 0.85% (w/v) NaCl and the cell pellet stored at -20°C . Cells were resuspended in a minimal amount of lysis buffer (20% w/v, sucrose, 50 mM-Tris/HCl, 100 mM-NaCl, 5 mM-EDTA, pH 8.2) and 5 mg lysozyme ml^{-1} (Sigma) was added to initiate cell lysis. Cells were incubated for 30 min on ice. The lysate was brought to 0.5% (w/v) SDS using 20% (w/v) SDS-lysis buffer stock, followed by the addition of 10 μg RNAase ml^{-1} (Sigma) and incubated at 37°C for 24 h. The lysate was extracted once with phenol/chloroform (1:1, v/v) and twice with chloroform. The sample was run twice on a 49% (w/v) CsCl gradient at 47K r.p.m., 25°C in a VTI-65 Beckman rotor until equilibrium was reached (0.5 mg ethidium bromide ml^{-1} was added to enable monitoring of the DNA banding). The DNA was removed with a 20 gauge needle, ethidium bromide removed by mixing three times with equal volume of isoamyl alcohol followed by extensive dialysis against TNE buffer (10 mM-Tris/HCl, 10 mM-NaCl, 1.0 mM-EDTA pH 7.5). A 1 mg sample of this DNA was diluted to 100 ml in 60% (w/v) glycerol, 0.3 M-sodium acetate pH 5.0 and sheared to an average strand size of 580 nucleotides in a VirTis 60 homogenizer followed by precipitation with 1.1 vol. isopropanol at -20°C for 12 h. The DNA was collected by centrifugation for 30 min at 23000 g. The DNA pellet was dried under vacuum and resuspended in TNE to a concentration of 1 mg ml^{-1} . Alternatively, unsheared DNA was nick-translated using the method of Rigby *et al.* (1977) with ^3H -TTP or ^{32}P -TTP to a specific activity of 2.0×10^7 . Fold back sequences were removed by low C_0t incubation followed by hydroxyapatite fractionation. Reassociation analysis was performed essentially according to Britten *et al.* (1974) and has been described in detail (Timberlake *et al.*, 1977). In general, radioactively labelled DNA was reassociated with a large excess of unlabelled sheared DNA by denaturing samples and incubating to various C_0t values (mol nucleotides

l⁻¹ s) at either 60 °C in 0.12 M-phosphate buffer or at 68 °C in 0.41 M-phosphate buffer. The fractions of bound or unbound DNA were determined by scintillation counting. To determine the T_m and relative mismatch of the duplex DNA, gradual melting of the duplexes was observed by monitoring elution of tracer DNAs. Tracer and driver DNAs were incubated to a C_0t of 470 or greater and the sample was placed on a hydroxyapatite column, remaining single stranded DNA removed by extensive washing with 0.12 M-phosphate buffer at 60 °C. Points were taken by raising the column temperature at intervals of 3 °C and collecting fractions until no further counts were being eluted or counts stabilized at a low level. At 99 °C all DNA was denatured and completely eluted.

Plasmid analysis. Stationary phase cells (30 ml) grown in mannitol/salts/yeast (MSY) media were harvested by centrifugation and resuspended in 70 µl buffer (Triton X-100 5% (w/v); 15% sucrose; 50 mM-Tris/HCl pH 8.5 and 50 mM-Na₂-EDTA). A 5 µl portion of lysozyme (5 mg ml⁻¹, Sigma) was added to the above buffer, and cells were incubated on ice for 1 min followed by incubation for 1.5 min at 100 °C, then cooling on ice. The suspension was spun 10 min in a micro-centrifuge (Fisher model 235-A). The supernatant (≈ 70 µl) was transferred to a new tube and isopropanol precipitated at -20 °C for 16 h as described above. The precipitated plasmids were collected by centrifuging at 4 °C for 30 min. The pellet was drained thoroughly and resuspended in 20 µl 10 mM-Tris/HCl pH 7.5, 1 mM-Na₂EDTA, 1 mg RNAaseA ml⁻¹ (Sigma). A portion (10 µl) of the sample was run on 0.8% (w/v) agarose gel in 40 mM-Tris/OH; 20 mM-acetic acid; 2 mM-Na₂EDTA pH 8.1. Gels were stained with 0.5 µg ethidium bromide ml⁻¹ for 30 min before observation with UV light (Fig. 4).

Plant nodulation experiments. *Rhizobium japonicum* 110 and 191 were checked for effectiveness on Clark soybean (*Glycine max* L. Merr.), cowpea (*Vigna unguiculata* WALP), 'California blackeye' and alfalfa (*Medicago sativa*), peas (*Pisum sativum* L.), and other soybean cultivars (Harosoy, Dixon and the Chinese cultivar, Peking). Seeds were surface sterilized by washing with 95% ethanol, incubating 5 min in 0.1% (w/v) HgCl₂ followed by extensive washing with sterile distilled water. Plants were grown under control conditions (Williams & Phillips, 1980) in 1 litre modified Leonard jar assemblies (DeJong & Phillips, 1981). The light intensity in the growth chamber had a photon flux density of 650 µE⁻² s⁻¹. Plants were grown without combined nitrogen and harvested 28 d after planting. Appropriate positive controls were included in inoculation of identical plants.

Strains were reisolated from the nodules and checked for purity and authenticity by (i) their inability to grow on trypticsoy-agar (Difco) at 37 °C; (ii) morphology and growth characteristics in different media; (iii) serology with antisera against strains 110 and 191 prepared as previously described (Hua *et al.*, 1981); (iv) phage susceptibility tests (Vincent, 1977); (v) the presence of antibiotic markers where marked strains were used.

RESULTS

Survey of Rhizobium japonicum strains from the major soybean production areas for osmotic tolerance

A random survey of the osmotic (salt) tolerance capacity of *Rhizobium japonicum* isolates from soybean production areas in the U.S. has been done, and with results from 12 representative strains tested from nine soybean-producing states, only those from Ohio and Iowa were capable of growth at greater than 0.1 M-NaCl. No strains were capable of growth at 0.3 M-NaCl.

From the collection of *R. japonicum* isolates from China provided by Dr J. C. Burton, we identified an osmotic tolerant strain designated 191. The growth rates of strain 191 and the common U.S. strain 110 in the presence of various NaCl concentrations is summarized in Table 1. With no salt added, the doubling time for 191 was 3.2 h while the doubling time for 110 was 12 h. Strain 110 grew poorly at 0.075 M-NaCl and did not grow at 0.1 M-NaCl. *Rhizobium japonicum* 191 was still able to grow in the presence of 0.4 M-NaCl although at diminished growth rates. These results show that strain 191 is not only more osmotic tolerant, but also grows faster than strain 110 in minimal and NaCl supplemented media. Doubling times of 4.0 h, 4.0 h and 6.9 h were obtained for strain 191 in three minimal media that varied in carbon source, L-glutamate-mannitol, glutamate-succinate and aspartate-lactate, respectively. Strain 110 gave doubling times of 15.2 h, 16.0 h and 12.4 h in these same media, respectively. A fast growth rate is usually associated with an acid reaction in a mannitol medium (Vincent, 1977). *Rhizobium japonicum* 191 growing in such a medium has been reported to lower the pH from 6.8 to 5.2 (Keyser *et al.*, 1982) in contrast to the slow-growing *Rhizobium* which usually give an alkaline reaction.

Nature of osmotic tolerance of Rhizobium japonicum 191

When exposed to increased osmolarity in their growth medium, many micro-organisms adapt by elevating the intracellular osmotic tension. In certain Gram-negative bacteria, this is

Table 1. *Growth rates of R. japonicum 191 and 110 in MSY medium with various concentrations of sodium chloride*

Cultures were grown in MSY medium as described in Methods. Growth was followed both by the increase in absorbance at 420 nm and the increase in cellular protein ($\mu\text{g ml}^{-1}$). Values are the average of three experiments.

NaCl (M)	Doubling time (h)	
	Strain 191	Strain 110
0.00	3.5	12.0
0.05	ND	16.0
0.075	ND	36.0
0.1	4.0	NG
0.2	5.3	
0.4	17.0	
0.6	NG	

ND, not determined; NG, no growth.

Table 2. *Intracellular K⁺ levels in Rhizobium species under normal and NaCl stressed conditions*

K⁺ levels were determined as described in Methods. Values are the average of three experiments.

NaCl (M)	Intracellular K ⁺ level [nmol (mg protein) ⁻¹]		
	<i>R. japonicum</i> 110	<i>R. japonicum</i> 191	<i>R. meliloti</i> 102F28
0.00	423	575	263
0.05	533	ND	ND
0.1	NG	ND	ND
0.2		758	503
0.4		900	838

ND, Not determined; NG, no growth.

Table 3. *Enzyme activities in cell-free extracts of rhizobia*

Activities are expressed as nmol NADP reduced min⁻¹ (mg protein)⁻¹.

Strain	Glucose-6-phosphate	6-Phosphogluconate dehydrogenase
<i>Rhizobium japonicum</i>		
191	18.15	4.16
110	1.21	<0.05
<i>Rhizobium meliloti</i>		
102F28	11.75	6.96

accomplished by increasing the internal concentration of K⁺ and amino acids (Measures, 1975). The response of the steady state levels of K⁺ in three *Rhizobium* strains during growth under salt stress is shown in Table 2. *Rhizobium japonicum* 191 and *R. meliloti* 102F28 respond similarly to NaCl, increasing their intracellular K⁺ levels when the NaCl concentration in the medium is elevated. In the presence of 0.4 M-NaCl, internal K⁺ levels increased to 900 nmol (mg cell protein)⁻¹ in strain 191, as shown in Table 2. In the case of *R. japonicum* 110, intracellular K⁺ levels did not show a significant increase, and 0.1 M-NaCl was toxic to the organism. Under osmotic stress, intracellular glutamate levels were found to increase in *R. japonicum* 191. For example, amino acid analysis of the cell lysate showed that in the presence of 0.2 M-NaCl, glutamate levels in strain 191 increased fivefold (data not shown). This is consistent with the findings of the Hua *et al.* (1982) who showed increased glutamate levels under salt stress in the *Rhizobium* sp. WR1001.

Fig. 1. Kinetics of hybridization of *Rhizobium japonicum* DNA. ○, *R. japonicum* 191; ●, *R. japonicum* 110. (a) *R. japonicum* 191 was the driver DNA incubated to indicated C₀t values with *R. japonicum* 191 and 110 tracer DNA. (b) The converse experiment where *R. japonicum* 110 was the driver DNA.

Utilization of carbon sources

We have further characterized the strain 191 with respect to carbon sources for growth. Fast-growing strains of *Rhizobium* (e.g. *R. meliloti*, *R. leguminosarum*, *R. trifolii* and *R. phaseoli*) generally can use a much wider range of carbon sources than the more fastidious slow growers [*R. japonicum*, *Rhizobium* spp. (cowpea)] (Graham, 1964; Graham & Parker, 1964). Strain 191 resembles *R. meliloti* 102F28 in the type and range of carbon sources utilizing all carbon sources tested except dulcitol. Test carbon sources include: maltose, lactose, L-rhamnose, D-glucose, sucrose, D-mannose, galactose, D-fructose, sorbitol, D-xylose, L-arabinose, dulcitol, trehalose, sodium succinate, sodium fumarate, sodium malate and sodium citrate. Strain 191 is clearly different from *R. japonicum* 110, which utilized a more restricted range of carbon sources and cannot grow on maltose, lactose, cellobiose, sucrose, dulcitol and trehalose. These sugars are thus useful for the identification and classification of these strains.

Glucose-6-phosphate and 6-phosphogluconate dehydrogenases

Martinez De-drets & Arias (1972) have shown that fast- and slow-growing *Rhizobium* can also be differentiated by the levels of carbohydrate catabolic enzymes such as glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase. *Rhizobium japonicum* 191 possesses both these enzymes while only glucose-6-phosphate dehydrogenase is present in strain 110 (Table 3). In this respect, strain 191 resembles the fast grower *R. meliloti* more than the slow-growing strains which lack 6-phosphogluconate dehydrogenase (NADP⁺).

DNA homology

DNA homology has been used by many workers to confirm the relationships between the fast- and slow-growing strains (Crow *et al.*, 1981; Gibbins & Gregory, 1972; Jarvis *et al.*, 1980). DNA hybridization curves between *R. japonicum* 191 and 110 are shown in Fig. 1. Approximately 98% of the strain 191 DNA was associated with the strain 191 driver DNA after incubation at greater than C₀t 2 (Fig. 1a). However, only about 15% of the 110 DNA hybridized to the 191 driver DNA. Similar results were obtained in the converse experiment when 110 DNA was used as the driver (Fig. 1b). Genomes of the two strains were determined to be of similar size by comparison of C₀t 1/2.

Experiments to determine the melting temperature where 50% of the duplex DNA is denatured (*T_m*) and relative mismatch of the duplex DNA from *R. japonicum* 191 and 110 were also performed. The 191/191 DNA duplex formed stable hybrids while the 191/110 DNA hybrid was unstable, allowing for a mismatch of 25% (Fig. 2a). Similar results (Fig. 2b) were obtained in the converse experiment. The *T_m* values from these experiments of 96.5 for *R. japonicum* 191 and 94.5 for *R. japonicum* 110 indicate a difference in G + C content of 9.3%.

Fig. 2. Melting profile of *R. japonicum* DNA to determine T_m (temperature where 50% of the duplex DNA is denatured) and relative mismatch of duplex DNA. $\circ$, *R. japonicum* 191; $\bullet$, *R. japonicum* 110. (a) *R. japonicum* 191 was the driver DNA allowed to hybridize with both *R. japonicum* 191 and 110 tracer DNA. (b) The converse experiment where *R. japonicum* 110 was the driver DNA.

Fig. 3. Agarose gel electrophoresis of plasmid DNA. A, *R. japonicum* 191; B, *R. meliloti* 102F28.

Fig. 4. SDS-PAGE of cell-free extracts of rhizobia. Approximately 7 mg protein is in each lane, the gel is 10% polyacrylamide. A, *R. meliloti* 102F28; B, *R. japonicum* 191; C, *R. japonicum* 110. Molecular weight standards (BioRad) were: phosphorylase B, 92500; bovine serum albumin, 66200; ovalbumin, 45000; carbonic anhydrase, 31000; soybean trypsin inhibitor, 21500.

Plasmid analysis

Rhizobium japonicum 191 was shown to have two large plasmids of the same molecular weights as two of the three plasmids found in *R. meliloti* 102F28 (Fig. 3). *R. japonicum* 110 appears to have no plasmids under these conditions. Casse *et al.* (1979) examined the plasmids in *R. meliloti* 102F28 and found two plasmids, 73 ± 4 and 118 ± 3 MDal. In our study three plasmids were seen in *R. meliloti* 102F28, the two larger plasmids are of similar size and could have previously been interpreted as one plasmid. We estimate the plasmids in strain 191 to be of approximately the same size as those in strain 102F28. It may be possible that these strains have additional large plasmids which were lost in the plasmid preparation. Additional attempts were made to isolate plasmids by the methods described by Kado & Kiu (1981) and Davis *et al.* (1980) without success.

Polyacrylamide gel analysis

The protein patterns on polyacrylamide gels can give a measure of genetic relatedness between strains as shown by Roberts *et al.* (1980). Analysis of the protein patterns of cultures of *R. japonicum* 191 and 110 grown aerobically under identical conditions showed that at the gross level there is little similarity in the two-dimensional pattern of protein distribution between these two strains (data not shown). Most of the visible protein patterns in strain 191 were equally

Table 4. Nodulation experiments of 28 d old soybean (*Glycine max*) and cowpea (*Vigna unguiculata*) with *R. japonicum* 191 and 110

All results except those for specific nitrogenase activity and the relative efficiency are expressed per plant.

Host	Hydrogen evolution ($\mu\text{mol h}^{-1}$)	Specific nitrogenase		Relative* efficiency	Leaf area (cm^2)	Total plant weight (g)	Nodule weight (g)
		Total nitrogenase activity ($\mu\text{mol C}_2\text{H}_2 \text{ h}^{-1}$)	activity ($\mu\text{mol C}_2\text{H}_2 \text{ h}^{-1}$ (g nodules) $^{-1}$)				
<i>R. japonicum</i> 191							
'Clark' soybean	3.03 ± 0.68	6.36 ± 1.40	35.16 ± 4.72	0.51 ± 0.03	572.7 ± 45.1	3.19 ± 0.35	0.165 ± 0.022
'Calif. Blackeye 5' cowpea	18.51 ± 3.68	21.91 ± 3.76	47.07 ± 6.36	0.25 ± 0.06	600.0 ± 54.9	4.43 ± 0.57	0.435 ± 0.046
<i>R. japonicum</i> 110							
'Clark' soybean	0.16 ± 0.04	17.89 ± 2.68	103.63 ± 12.18	0.992 ± 0.022	568.4 ± 41.3	4.00 ± 0.20	0.167 ± 0.071
'Calif. Blackeye 5' cowpea	0.22 ± 0.06	40.06 ± 9.50	95.77 ± 16.95	0.984 ± 0.008	486.4 ± 50.8	3.96 ± 0.04	0.365 ± 0.050

* Expressed as $1 - (\text{H}_2 \text{ evolution}/\text{C}_2\text{H}_2 \text{ reduction or total nitrogenase activity})$.

Table 5. Summary of characteristics of *Rhizobium* 191 and 110

Characteristic	Strain 191	Strain 110
Doubling time (h)		
No NaCl	3.5	12.0
0.1 M-NaCl	4.0	No growth
DNA homology to 110	18%	98%
Plasmids	2 (50–150 MDal)	None found
6-Phosphogluconate dehydrogenase [nmol NADP reduced min ⁻¹ (mg protein) ⁻¹]	3.31	<0.05
Intracellular potassium level [nmol (mg protein) ⁻¹]		
No NaCl	575	423
0.4 M-NaCl	900	No growth
Tritium uptake* [10 ⁻³ × c.p.m. h ⁻¹ (mg protein) ⁻¹]	<5 (Hup ⁻)	638.7 (Hup ⁺)
Nodulation patterns	Soybean and cowpea	Soybean and cowpea
Acetylene reduction (as % of strain 110)	30%	100%

* Value given is the average of three experiments.

distributed between the basic and acidic regions, while those from strain 110 were concentrated in the basic and neutral regions. A few patterns in the high molecular weight basic region may be common to both strains indicating a degree of relatedness between the two strains. This difference can clearly be seen in Fig. 4 on one-dimensional gels. Some similarity can be seen between *R. japonicum* 191 and *R. meliloti* 102F28.

Nodulation experiments

Cross inoculation experiments of 'Clark' soybean (*Glycine max*) and 'California Blackeye 5' cowpea (*Vigna unguiculata*) were carried out with *R. japonicum* 191 and 110. The two strains were shown to be effective on both hosts. Nodules on both the legumes were similar, with an overall appearance much like those of typical *R. japonicum* soybean nodules. Although no major differences were detected in terms of nodule weight, leaf area and total plant weight, the specific nitrogenase activity (acetylene reduction capacity) of strain 191 was approximately 30% that of strain 110 on both legumes (Table 4). In addition, although H₂ evolution was detected from nodules formed by both strains, that from strain 110 was negligible when compared with strain 191. The relative efficiency values (Schubert & Evans, 1976) of strain 191 on both legumes also indicated that it lacks a hydrogen uptake system (Hup⁻), while strain 110 is Hup⁺ (Table 4), this is confirmed by tritium uptake values (Table 5) on whole cells. A streptomycin (100 µg ml⁻¹) resistant strain of 191 also nodulated the Harosoy, Dixon and Peking cultivars effectively. The bacteria isolated from the nodules retained the antibiotic marker and the faster growth rates of the strain used to inoculate the cultivars. Experiments to determine nodulation of pea (*Pisum sativum*) and alfalfa (*Medicago sativa*) with *R. japonicum* 191 were negative.

DISCUSSION

In this paper we have characterized the unusual *Rhizobium japonicum* strain 191. This new strain has more characteristics in common with the fast-growing groups of *Rhizobia* than the slow-growing group. The single characteristic that strain 191 has with the common American strain, *R. japonicum* 110, is the ability to form effective nodules on American soybeans. This nodulation pattern defines strain 191 as *R. japonicum*. The classification of rhizobia based on plant nodulation patterns is a controversial topic. Hollis *et al.* (1981) suggested that even within the slow-growing *R. japonicum* strains at least three distinct DNA homology groups exist. The discovery of strain 191 further supports the inadequacy of the current classification system since it differs not only by DNA homology, but by several other characteristics, summarized in Table 5.

Strains such as 191 seem to constitute an intermediate *Rhizobium japonicum* which, although

possessing most of the characteristics of the fast growers, has the ability to nodulate soybean and cowpea effectively. Recently, Brewin *et al.* (1980) and Hooykaas *et al.* (1981), have shown that genes influencing the host range of *R. trifolii* and *R. leguminosarum* are carried on large plasmids. The nodulation genes on one plasmid may suppress the nodulation genes on a second plasmid (Johnston *et al.*, 1978). These results suggest that a similar situation may have occurred in strain 191 where the two large plasmids present in strain 191 may alter host range.

Due to the fast growth rate and osmotic tolerance, strain 191 would be expected to have a competitive edge for survival in the environment. While the specific activity (acetylene reduction capacity) of strain 191 is only 30% of strain 110 on both soybeans and cowpea, there is very little difference between the two strains in terms of leaf area, and total plant and nodule weight. The economic value of strain 191, however, must be determined in field trials.

Until recently, researchers have had to choose between working with the more experimentally attractive fast-growing, salt-tolerant strains versus the more economically important slow-growing *R. japonicum* strains. Strain 191 may now offer a useful system for biochemical and genetic studies of *R. japonicum* in a fast-growing, salt tolerant strain effective on American soybean cultivars.

We would like to thank Dr R. C. Valentine, Dr D. A. Phillips and Dr W. E. Timberlake for helpful discussions. Appreciation is also extended to Ms Sandie Uratsu and Ms Sally Ju for expert technical assistance and Dr J. Meeks for critical reading of the manuscript. This work was supported by NSF grant AER 77-07301. S. T. Lim was supported by the University of Malaya under Vote F 153/79 and 137/80. S. S. Yang was supported by the Ministry of Education of the People's Republic of China.

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