

Bacteriocin-Like Substances Produced by *Rhizobium japonicum* and Other Slow-Growing Rhizobia†

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Bacteriocin-like substances were commonly produced by slow-growing *Rhizobium japonicum* and cowpea rhizobia on an L-arabinose medium. Antagonism between strains of *R. japonicum* was not detected in vitro; however, such strains were often sensitive to some bacteriocins produced by cowpea rhizobia. Inhibitory zones (2 to 8 mm from colony margins), produced by 58 of 66 *R. japonicum* test strains, were reproducibly detected with *Corynebacterium nebraskense* as an indicator. Quantitative production was not related to symbiotic properties of effective strains, since nine noninfective strains and one ineffective strain produced bacteriocin. Eight *R. japonicum* strains that did not produce bacteriocin nevertheless formed effective nodules on soybeans. *R. japonicum* strains that produced bacteriocin in vitro had no antagonistic effect on nonproducer strains during soybean nodulation. Under controlled conditions, a nonproducer (3I1b135) predominated over a bacteriocin producer (3I1b6) when inoculated at 1:1 and 1:9 ratios. Depending on the particular ratio, up to 38% of the total nodules formed were infected with mixed combinations. The bacteriocin(s) had a restricted host range and antibiotic-like properties which included the ability to be dialyzed and resistance to heat (75 to 80°C, 30 min), Pronase, proteinase K, trypsin, ribonuclease, and deoxyribonuclease. *R. japonicum* strains representing genetic, serological, cultural, and geographic diversity were differentiated into three groups on the basis of bacteriocin production.

Strains of *Rhizobium japonicum* inhabiting a single soil type may differ in symbiotic properties and other characters. The successful establishment of highly infective and effective nitrogen-fixing strains in soil is of practical importance for successful soybean production. Dominance of certain strains in various areas producing soybeans is known (7, 31), but explanations for this phenomenon are incomplete. The ability of strains to persist or reside in soil has been studied in relation to soil fertility levels, variations in resistance to antagonistic soil microflora, and variations in growth rate, soil pH, soil temperature, and planting date (4-7, 29, 31, 38, 39). Relatively little is known about *R. japonicum* in association with the soybean rhizosphere or rhizoplane. Bacteriocins or antibiotics produced by *R. japonicum* may be one of the antagonistic or competitive factor(s) responsible for dominance of particular strains in soybean fields.

Roslycky's short report (23) described bacteriocin production by 13 of 27 strains of *R. japonicum* and 4 of 15 cowpea rhizobia. In vitro production was highly irregular and depended

upon cultural conditions. Several bacteriocins of fast-growing rhizobia have been described; these are phagelike bacteriocins (11, 14, 19, 26). However, Schwinghamer (25) noted that smaller (1.8×10^5 to 2×10^5 daltons), protease-sensitive bacteriocins were produced by *R. trifolii* and that these were more prevalent than phagelike bacteriocins. Earlier, Schwinghamer (24) described the production of diffusible antibiotic-like substances by *R. trifolii*; these antibiotics could be filtered through a 0.01- μ m membrane filter or dialyzed, but were not sedimented by high-speed centrifugation, induced by UV irradiation, or inactivated by heat (70°C, 15 min).

The coexistence of more than one *Rhizobium* strain in a single nodule has been described for a number of legumes (3, 8-10, 20), including soybean (13, 17, 27, 28). However, there have been no reports on whether bacteriocin production affected mixed infections in nodules or imparted a competitive advantage over nonproducer strains at root hair infection sites.

In this investigation, *R. japonicum* strains having diverse serological, cultural, and genetic differences were compared for bacteriocin production and sensitivity. Assay conditions described here enabled reproducible detection of

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bacteriocins produced by most strains of *R. japonicum*. The results showed three major groups of *R. japonicum*. Bacteriocin production is also discussed in relation to competition or antagonism among soil bacteria (rhizobia and non-rhizobia) in association with soybeans.

MATERIALS AND METHODS

Culture media. Stock cultures were maintained on L-arabinose agar (34), which contains, per liter: 0.5 g of K_2HPO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of NaCl, 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.), 5 g of L-arabinose, and 15 g of agar (Difco). The L-arabinose (10%, wt/vol) and magnesium sulfate (1 M) were added as sterile solutions after autoclaving (121°C) and cooling the basal medium to 50°C. Cycloheximide (stock solution, 10 mg/ml) was routinely incorporated into L-arabinose agar to a final concentration of 40 µg/ml.

Media used to test for bacteriocin production by *Rhizobium* species included: L-arabinose agar as described above; Bergersen synthetic medium (2) as modified by Schwinghamer (25); 0.22 g of K_2HPO_4 was substituted for $Na_2HPO_4 \cdot 12H_2O$; Campbell and Hofer's medium modified by Roslycky (23); the Roslycky medium supplemented with 5 g of L-arabinose per liter; *Rhizobium* medium 1 (RM1) (12), designed for fast-growing rhizobia; *Rhizobium* medium 2 (RM2) (12), designed for slow-growing rhizobia; potato extract-L-arabinose medium, containing 0.5 g of K_2HPO_4 , 0.2 g of $MgSO_4 \cdot 7H_2O$, 1.0 g of Difco yeast extract, 5.0 g of L-arabinose, broth from 300 g of cooked, peeled potatoes, 15 g of Difco agar, and deionized water to 1 liter; a Soytone medium containing 1.0 g of K_2HPO_4 , 3.0 g of Difco Soytone, 2.0 g of Difco Casitone, 2.5 g of L-arabinose, 0.2 g of $MgSO_4 \cdot 7H_2O$, 0.004 g of $FeCl_2 \cdot 6H_2O$, 15 g of Difco agar, and deionized water to 1 liter. Stock solutions of L-arabinose (10%, wt/vol), glycerol (10%, vol/vol), magnesium sulfate (1 M), ferric chloride (4%, wt/vol), calcium chloride (10%, wt/vol), and calcium glycerophosphate (10%, wt/vol) were each sterilized separately and added after autoclaving (121°C) to the medium as required.

Agrocin 84 production by *Agrobacterium radiobacter* strain 84 was on AB minimal medium (37).

Bacterial strains. All strains were checked for uniformity of colony type upon receipt. Single colonies were cultured and lyophilized. Cultures used routinely were stored on L-arabinose agar at 4°C. *R. japonicum* strains Hill 1, Hill 2, Hill 3, Hill 5, Moor 3, Lamb 1-2, Lamb 2, WA-16-1, WA-1, WA5099-1-1, WA5084-3-2, WA5036-3-2, and WA5077-3-3 were received from G. Ham (University of Minnesota, St. Paul). Strains 3I1b6, 3I1b34, 3I1b94, 3I1b85, 3I1b110, 3I1b117, 3I1b130, 3I1b140, 3I1b24, 3I1b75, 3I1b10, 3I1b122, 3I1b135, 3I1b61, 3I1b76, and 3I1b123 were received from D. Weber (U.S. Department of Agriculture, Beltsville, Md.). Strains 61-A-72, 61-A-96, 61-A-101, 61-B-8, and 3164 were received from L. Moore (Oregon State University, Corvallis). Strain 10324 was from the American Type Culture Collection (Rockville, Md.). Strains L-240, L-245, L-247, L-243, L-248, L-236, L-249, L-242, and L-246 were from the Northern Re-

gional Research Laboratory (Peoria, Ill.). Strains 1BB, 1A, 2AS, 2BA, 3BA, 3AS, 3C, 4BCS, 4C, 4BAL, 4A, 5AA, 5BA, 6AB, 6BB, RJ17WFG, RJ17WSG, RJ19FY, and RJ20FY were isolated from soybean nodules collected in Nebraska, and R-64 and D345 were from our lyophilized stock collection. *R. japonicum* 61A76; cowpea rhizobial strains 41D1, 32C2, 8A9, 32F1, 61-B-8, 177A6, 32H1, 176A16, 127N1, 127N2, and 41F1; *R. trifolii* strains 162P17 and 162P30; *R. leguminosarum* strains 128C53 and 128A12; *R. meliloti* strains 176A22 and 102F28; *R. phaseoli* strains 127K14, 127K17, and 3610; and *R. lupini* 96B15 were all a gift from the Nitragin Co., Milwaukee, Wis.

Bacterial species and genera tested for sensitivity to *R. japonicum* and cowpea rhizobia bacteriocins were from our lyophilized stock collection. These were: *Escherichia coli* K12A35; *Klebsiella pneumoniae* 5374; *Salmonella typhimurium* RC903; *Erwinia carotovora* 312; *E. amylovora* 598; *E. stewartii* 8199; *Flavobacterium* sp. 2297LC; *Pseudomonas aeruginosa* PA038; *P. syringae* 5D430; *P. allicola* 19802; *P. cepacia* PC142 and Hines no. 2; *Xanthomonas campestris* 528; *Serratia* sp. Sp ST7 and S1760; *Agrobacterium tumefaciens* 2437 and C5/73; *A. radiobacter* AB11/73; *A. rhizogenes* A-1; *Bacillus subtilis* ST168; *B. cereus*; *Streptococcus* sp. X22, X47, X13, and X4; *Micrococcus luteus* 12698; *Corynebacterium nebraskense* CN74-1 and CN76-1; *C. michiganense* 829S and 3D21; *C. sepedonicum* CScA; *C. insidiosum* P2; *C. fascians* 6D21; *C. rathayi* CR1 and CR101; *C. flaccumfaciens* CV6 and 2AdK; *C. poinsettiae* no. 13; *C. oortii* CO101; *C. betae* CB101; and *C. tritici* CT101. *A. radiobacter* K84 and *A. tumefaciens* C58 and NT1/C58 were received from D. Merlo, University of Washington, Seattle.

Bacteriocin production and detection. The procedures of Vidaver et al. (33) were modified to test for production of bacteriocins on solid and in liquid media. Plates containing 25 ml of medium were inoculated with five rhizobial strains. After incubation at 30°C for 4 days, they were then transferred with a multiple replicator to fresh plates and incubated for 7 days at 30°C. Test producer colonies were killed by exposing the inverted plates to the vapor of chloroform (2 ml) for 2 h. Test indicator cultures in log-phase growth were adjusted between 5×10^7 and 1×10^8 colony-forming units per ml, added to 2.5 ml of the appropriate 0.7% melted agar medium, and poured over the agar surface of the producer plate. Plates were incubated at 30°C until indicators grew sufficiently to read the final result. The type of zone was noted and measured as the distance from the margin of the producer colony to the point of normal growth of the indicator.

In liquid, strains were tested in duplicate for bacteriocin production by growing in 10 ml of medium to mid-log phase (approximately 2×10^8 to 6×10^8 colony-forming units per ml) in DeLong culture flasks at 30°C on a rotary shaker (250 rpm). To one set of cultures, mitomycin C was added to a final concentration of 1 µg/ml. Growth was monitored turbidimetrically, and, after growth to late log phase, cultures were centrifuged at $12,100 \times g$ for 15 min at 4°C. The supernatant fluid was decanted and mixed with 1 ml of chloroform in sealed tubes and stored at 4°C. Sam-

ples were tested on the appropriate indicator strain as described above for bacteriocin production. *R. japonicum* strains 3I1b6, 3I1b122, 3I1b110, 3I1b10, and R-64, along with cowpea rhizobial strain 32F1, were tested for bacteriocin production at final concentrations of 1, 2, 5, and 10 μg of mitomycin C per ml.

All *Rhizobium* strains were tested as a producer against all rhizobial strains, as well as *C. nebraskense* CN74-1 and CN76-1, as indicators. Various bacterial genera and species were tested as indicators for bacteriocin production by *R. japonicum* strains 3I1b6, 3I1b10, 3I1b110, 3I1b122, 3I1b135, R-64, D345, 4BAL, WA5099-1-1, and 5BA; cowpea rhizobial strains were 32F1, 32H1, and 8A9.

Agrocin 84, produced by *A. radiobacter* strain K84, was detected by using the procedure of Watson et al. (37) with *A. tumefaciens* C58 as indicator.

Bacteriocin properties. Heat inactivation was determined by exposing plates containing chloroform-killed producers to 75 to 80°C for 30 min in a preheated oven. The plates were allowed to cool for an additional 30 min and then overlaid with the indicator as described above. Unheated plates, otherwise treated identically, served as controls.

The following enzymes were assessed for the ability to inactivate the bacteriocins: Pronase, nuclease-free grade (Calbiochem, San Diego, Calif.); trypsin (Worthington Biochemical Corp., Freehold, N.J.); proteinase K (E. M. Laboratories, Inc., Elmsford, N.Y.); ribonuclease A and deoxyribonuclease 1 (Sigma Chemical Co., St. Louis, Mo.). Proteolytic enzymes [diluted in 0.05 M tris(hydroxymethyl)aminomethane (pH 7.3)-0.01 M CaCl_2] and ribonuclease [diluted in 0.05 M tris(hydroxymethyl)aminomethane, pH 8.1] were tested at concentrations of 100 and 10 $\mu\text{g}/10 \mu\text{l}$, and deoxyribonuclease [diluted in 0.05 M tris(hydroxymethyl)aminomethane (pH 8.1)-10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$] was tested at 20 $\mu\text{g}/10 \mu\text{l}$. Test enzymes were spotted in 10- μl amounts to duplicate chloroform-treated producer plates, incubated for 4 h at 30°C, overlaid with the appropriate indicator, and incubated further, as before. Buffer solutions were substituted for enzyme preparations as negative controls. *C. nebraskense* bacteriocins CN1 and CN2 (D. C. Gross and A. K. Vidaver, Ann. Proc. Am. Phytopathol. Soc. 4: 138, 1977), which are sensitive to proteolytic enzymes, were used as positive controls.

Bacteriocin size. Bacteriocins were tested for ability to diffuse through dialysis membranes by using the technique of Schwinghamer (24). Producer strains 3I1b6, 3I1b122, 8A9, and 32F1 were grown on washed sterile dialysis membrane (6,000 to 8,000 molecular weight cutoff) overlaid on L-arabinose agar. All other conditions remained the same. After the producers were killed with chloroform, the dialysis membrane was removed, and the agar was overlaid with the indicator.

Alternatively, attempts were made to concentrate the 3I1b6 bacteriocin by membrane ultrafiltration using XM300 and PM10 Diaflo membranes (Amicon Corp., Lexington, Mass.). Because none of the bacteriocin(s) produced by *R. japonicum* could be produced in liquid, agar from bacteriocin diffusion zones was removed from the plate and freeze-thawed (16). The liquid was decanted, centrifuged (12,100 $\times g$, 15 min)

to remove cells and agar, and then passed through Diaflo membranes. Both retained and filtered fluids were tested for bacteriocin activity.

Soybean nodulation tests. All rhizobial strains were tested for infectivity and effectivity on soybean (*Glycine max* L. Merrill) cultivar Amsoy 71. Seeds were surface sterilized (36) and then germinated on moistened filter paper in sterile petri plates held in the dark at 30°C for 2 days. Two germinated seeds were planted per 10-ounce (ca. 0.3 kg) styrofoam container containing Perlite. After watering with a nitrogen-free nutrient solution (1), each seedling was inoculated with approximately 10^8 cells (determined by viable plate counts) of the test *Rhizobium* strain, previously washed and diluted in 12.5 mM potassium phosphate buffer (pH 7.2), per ml. Inocula were prepared from log-phase cultures. Three replicate containers were prepared for each strain. Control plants were inoculated with sterile phosphate buffer. Plants were grown in an environmental growth chamber at a day-night regime of 16 h of light at 24°C and 8 h of dark at 18°C. Four weeks after inoculation, nodulation and nodule effectiveness were determined.

Isolation of strains from nodules. Procedures for inoculum preparation and conditions for growth of Amsoy 71 after inoculation with various proportions of 3I1b135 and 3I1b6 were as described above. Four weeks after inoculation, the rate of nitrogen fixation per plant was determined by the acetylene reduction assay (18). Nodules were harvested, washed, and surface-sterilized as above. Isolation from randomly selected, single nodules (1 to 3 mm in diameter) was completed for each treatment by grinding 10 nodules in 0.5 ml of 12.5 mM potassium phosphate buffer, followed by standard \log_{10} dilutions and plating 0.1-ml samples of the appropriate dilution(s) on L-arabinose agar with and without 10 μg of kanamycin (Sigma) per ml. In addition, 40 nodules were cut, and the internal tissue was directly streaked onto L-arabinose agar without kanamycin. Plates were incubated at 30°C for 10 days. The incorporation of kanamycin was useful for selecting strain 3I1b135 in the presence of strain 3I1b6. Along with this selective advantage, strain 3I1b135 was readily distinguished from 3I1b6 by its slow-growing, nonmucoid colonies. From all nodules resulting from mixed infections, the two colony types were picked onto duplicate plates, restreaked for colony uniformity, and confirmed as to type by bacteriocin production on L-arabinose agar. Colonies from single infected nodules served as controls.

RESULTS

Production and detection of bacteriocins.

R. japonicum strains did not produce an inhibitory substance against other *R. japonicum* strains or other *Rhizobium* species on L-arabinose agar (Table 1). In contrast, several cowpea *Rhizobium* strains generally produced inhibitory substances active against other cowpea rhizobia. Three cowpea strains (32F1, 8A9, 127N1) inhibited most strains of *R. japonicum*, with inhibitory zones ranging from 1 to 12 mm; zones were often turbid. Only two fast-growing *Rhi-*

TABLE 1. Bacteriocin production by *Rhizobium* sp.^a

Indicator	Producer ^b								
	<i>R. japonicum</i> ^c		Cowpea rhizobia ^d					<i>R. phaseoli</i> ^e 127K14	<i>R. lupini</i> 96B15
	3I1b6	3I1b135	32H1	32F1	177A6	127N1	8A9		
<i>R. japonicum</i> ^f	-	-	-	+	-	+	+	-	-
Cowpea rhizobia 32H1 ^g	-	-	-	+	+	-	+	-	-
Cowpea rhizobia 32F1	-	-	-	-	+	-	-	-	-
Cowpea rhizobia 177A6 ^h	-	-	-	+	-	+	-	-	-
Cowpea rhizobia 127N1	-	-	-	-	-	-	+	-	-
Cowpea rhizobia 8A9 ⁱ	-	-	-	+	+	-	-	-	-
Cowpea rhizobia 32C2 ^j	-	-	-	-	-	-	+	-	-
<i>C. nebraskense</i> CN74-1	+	-	+	+	+	+	+	+	+

^a *R. meliloti*, *R. leguminosarum*, and *R. trifolii* did not produce bacteriocins.

^b +, Sensitive; -, insensitive or not detected.

^c Strains WA5077-3-3, WA5084-3-2, 4A, 4BAL, RJ17WSG, RJ19FY, and RJ20FY gave the same pattern as strain 3I1b135; all other 58 *R. japonicum* strains gave the same pattern as strain 3I1b6.

^d Cowpea rhizobia strains 32C2, 176A16, 61-B-9, 41D1, 127N2, and 41F1 gave the same bacteriocin pattern as strain 32H1.

^e *R. phaseoli* strains 3610 and 127K17 gave the same pattern as 127K14.

^f Most *R. japonicum* strains, including bacteriocin producers and nonproducers, generally gave the same bacteriocin sensitivity pattern. Cowpea strains 61B9 and 41F1 along with *R. meliloti* 176A22 gave the same reaction as *R. japonicum*.

^g Strain 176A16 gave the same sensitive reaction.

^h Strain 127N2 gave the same sensitive reaction.

ⁱ Strain 41D1 gave the same sensitive reaction.

^j *R. lupini* 96B15 gave the same sensitive reaction.

zobium strains (*R. lupini* 96B15 and *R. meliloti* 176A22) were sensitive to some of the bacteriocins produced by cowpea rhizobia. Although bacteriocin production by *R. japonicum* could not be detected when tested against other strains of *R. japonicum*, bacteriocins produced by 58 of 66 strains (Table 1) were inhibitory to *C. nebraskense*. After 2 days of growth of the indicator, clear zones of inhibition were usually visible. Figure 1 shows the typical bacteriocin produced by two strains of *R. japonicum* and the lack of production by a third. The cell concentration of the indicator was an important variable, since zones were reduced or turbid when the indicator cell concentration was too high. *R. japonicum* strains WA5077-3-3, WA5084-3-2, 4A, 4BAL, RJ17WSG, RJ19FY, RJ20FY, and 3I1b135 did not produce bacteriocin. All cowpea strains inhibited *C. nebraskense*. Strains of *R. phaseoli* and *R. lupini* also inhibited *C. nebraskense*, but *R. meliloti*, *R. leguminosarum*, and *R. trifolii* did not. While results were generally reproducible, occasional variability was observed. Whereas *R. japonicum*, *R. phaseoli*, or *R. lupini* may produce the same bacteriocin(s), cowpea rhizobia produced a diversity of inhibitory substances.

The 66 *R. japonicum* strains tested could be classified into three groups based primarily on inhibition of *C. nebraskense* (Table 2). Group 1 consisted of the majority of strains that generally

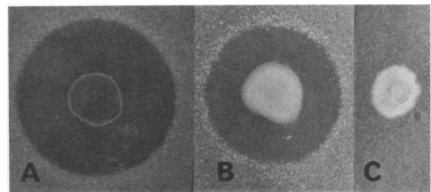


FIG. 1. Bacteriocin diffusion zones produced by *R. japonicum* on *L*-arabinose agar with *C. nebraskense* CN74-1 as the indicator. Producer strains 3I1b6, a pigmented strain (A), and RJ17WFG, a nonpigmented strain (B), produced zones 8 and 5 mm, respectively, from the margin of the producer colony. Strain 3I1b135 was not a producer (C).

had a doubling time less than 14 h and had a typical mucoid growth type. These strains produced bacteriocin zones of inhibition varying from 2 to 9 mm (Table 2). Many of these strains represent the common serogroups found in the U.S.A. (Table 3). Nine strains did not nodulate Amsoy 71; one strain (3I1b24) did so, but formed ineffective nodules. Plants inoculated with sterile phosphate buffer were not nodulated and became chlorotic. Group 2 (Table 2) consisted of four strains belonging to serogroup 123 that had a doubling time and growth type similar to group 1 but did not produce bacteriocin. However, other strains within serogroup 123, including 3I1b10 and 3I1b123, produced bacteriocin. Group 2 strains all had decreased culture viabil-

TABLE 2. Characteristics of *R. japonicum* groups tested for bacteriocin production

Group	No. of strains	Doubling time (h) ^a	Growth type	Culture viability	Pigmentation	Bacteriocin production
1	58	<14 ^b	Mucoid	Good	±	+
2	4 ^c	<14	Mucoid	Poor	—	—
3	4 ^d	>14	Dry	Good	—	—

^a Calculated from values interpolated between the beginning and the end of the logarithmic growth phase for strains grown in L-arabinose broth.

^b Strain WA5099-1-1 had a doubling time of 24 h.

^c Strains WA5077-3-3, WA5084-3-2, 4A, and 4BAL. All strains group in serogroup 123.

^d Strains 3I1b135, RJ17WSG, RJ19FY, and RJ20FY.

TABLE 3. Characteristics of different serogroups of *R. japonicum*^a

Strain	Sero-group	Source and year of origin	Group	Bacteriocin zone ^b (mm)
3I1b6	c1	Japan, 1929	1	8
3I1b94	94	North Carolina, 1956	1	2
3I1b110	110	Florida, 1959	1	6
3I1b117	76	Mississippi, 1959	1	5
3I1b130	130	Maryland, 1961	1	4
3I1b140	62	Bolivia, 1959	1	5
3I1b24	c1	Wisconsin	1	4
3I1b61	c3	North Carolina, 1946	1	6
3I1b74	Neg	Yugoslavia, 1955	1	5
3I1b10	123	Iowa, 1927	1	6
3I1b122	122	Mississippi, 1960	1	6
4BAL	123	Nebraska, 1975	2	0
3I1b135	135	Iowa, 1962	3	0

^a All strains listed nodulated soybean cultivar Amsoy 71, except strain 3I1b94. All nodulated plants formed effective nodules, except soybeans nodulated with strain 3I1b24.

^b Zone of diffusion from producer colony margin with *C. nebraskense* CN74-1 as the indicator.

ity compared with groups 1 and 3; after 3 months of storage on L-arabinose agar at 4°C, culture viability was either lost or at a low level. The group 3 rhizobia were readily distinguishable by their dry colony morphology, long doubling time, and lack of bacteriocin production. All strains in groups 2 and 3 formed effective nodules on soybeans.

Conditions for bacteriocin production. Optimum conditions for bacteriocin production include incubation for 7 days at 30°C on L-arabinose agar. Bacteriocin was produced at 20 and 25°C, but zone size generally coincided with the overall growth rate of the producer. Production occurred as early as day 4, but some poor producers required 7 days for reproducible results. After 14 days, zones were comparable to day 7; no loss in bacteriocin activity was noted.

The medium used for bacteriocin production is an important variable. Similar production pat-

terns were detected on L-arabinose agar, Schwinghamer medium, and RM2; only quantitative differences were observed. Bacteriocin zones were generally two to three times larger on L-arabinose agar. Only the better bacteriocin producers, such as 3I1b6, produced bacteriocin on Soytone agar, potato-L-arabinose agar, and the Roslycky agar supplemented with L-arabinose. No production was detected on the Roslycky and RM1 media.

Production was not detected in L-arabinose broth regardless of the time of harvest, growth stage, or aeration. Mitomycin C at various concentrations (1, 5, 10, and 20 µg/ml) did not stimulate production, but did not inhibit growth. Bacteriocin production occurred only on solid media at or above 0.6% agar; no production was detected at or below 0.3%.

Bacteriocin sensitivity of other bacteria.

The species of *Corynebacterium* sensitive to *Rhizobium* bacteriocins are listed in Table 4. *C. michiganense*, *C. sepedonicum*, and *C. insidiosum*, taxonomically closely related to *C. nebraskense* (32), gave similar quantitative and qualitative reactions to the bacteriocins. However, *C. fascians* and *C. rathayi*, distantly related to *C. nebraskense*, were sensitive to the bacteriocins produced by 3I1b6 and not those of other *R. japonicum* strains (Table 4). Although this might be a quantitative phenomenon, this was the only indication of more than one type of bacteriocin produced by *R. japonicum*. All other gram-positive and gram-negative bacteria tested were insensitive to *R. japonicum* bacteriocin(s). In addition, 78 of 80 coryneform soil isolates from three soybean fields were not sensitive to *R. japonicum* bacteriocins. Coryneform bacteria in these soils comprised 1 to 3% of the aerobic bacteria capable of growth on a complex medium (D. C. Gross and A. K. Vidaver, *Phytopathology*, in press). Cowpea strains 8A9 and 32F1 inhibited *B. subtilis* St168, and 32F1 inhibited *Serratia* sp. strain Sp ST7; in these cases, all zones (8 to 10 mm) were turbid.

Characterization of bacteriocin(s). The bacteriocins produced by strains 3I1b6, 3I1b122,

TABLE 4. *Corynebacterium* species sensitive to bacteriocins produced by slow-growing rhizobia

Indicator ^a	Producer ^b				
	<i>R. japonicum</i>			Cowpea rhizobia	
	3I1b6	3I1b122	3I1b135	32F1	8A9
<i>C. nebraskense</i> CN74-1	+	+	-	+	+
<i>C. michiganense</i> 829S	+	+	-	+	+
<i>C. sepedonicum</i> CSca	+	+	-	+	+
<i>C. insidiosum</i> P2	+	+	-	+	+
<i>C. fascians</i> 6D21	+	-	-	+	+
<i>C. rathayi</i> CR101	+	-	-	+	-

^a *C. michiganense* 3D21, *C. flaccumfaciens* CV6 and 2AdK, *C. poinsettiae* no. 13, *C. oortii* C0101, *C. betae* CB101, *C. rathayi* CR1, and *C. tritici* CT101 were all insensitive to these bacteriocins.

^b +, Sensitive; -, insensitive or not detected.

3I1b110, 3I1b10, R-64, and 32F1 were heat resistant, retaining all activity after exposure to 75 to 80°C for 30 min. Pronase, proteinase K, and trypsin had no effect on activity at concentrations of 100 µg/10 µl; deoxyribonuclease and ribonuclease also had no effect.

The bacteriocins produced by strains 3I1b6, 3I1b122, 8A9, and 32F1 readily passed through a dialysis membrane (6,000 to 8,000 molecular weight cutoff). Also, bacteriocin preparations of 3I1b6, obtained by freeze-thaw methods, were not concentrated by XM300 or PM10 Diaflo membranes (molecular weight cutoffs of 10⁵ and 10⁴, respectively). Freezing had no effect on bacteriocin activity.

Many *C. nebraskense* survivor colonies were evident within the bacteriocin zone after 5 days of incubation. Ten random colonies within the zone of inhibition were picked, cloned, and compared with the parent strain for sensitivity to the bacteriocin produced by strain 3I1b6. All were equally sensitive to 3I1b6 bacteriocin. Further tests showed that the number of viable bacteria within a bacteriocin zone was comparable to the number of bacteria outside the bacteriocin zone. Therefore, the bacteriocin appeared to have a bacteriostatic effect on *C. nebraskense*.

Comparison with agrocin 84. The bacteriocin produced by *R. japonicum* was compared with agrocin 84 because of taxonomic relatedness of the genera (35) and because agrocin 84 has been chemically characterized (22). *A. radiobacter* K84 produced agrocin 84 on AB medium, as detected by inhibition (15-mm zone) of *A. tumefaciens* C58. *R. japonicum* 3I1b6 pro-

duced bacteriocin (2-mm zones) on AB medium with *C. nebraskense* as an indicator. *R. japonicum* strains 3I1b6 and 3I1b135 on AB medium did not inhibit growth of *A. tumefaciens* C58 or NT1/C58; the same qualitative results were observed on L-arabinose agar. Agrocin 84 was not produced on L-arabinose agar. The *A. tumefaciens* strain cured of the Ti plasmid (NT1/C58), *R. japonicum* strains 3I1b135 and 3I1b6, and *C. nebraskense* CN74-1 were all resistant to agrocin 84. These results clearly show that agrocin 84 and the *R. japonicum* bacteriocin are different.

Mixed inoculation with bacteriocin producers and nonproducers. Strain 3I1b135, a bacteriocin nonproducer, was more competitive than strain 3I1b6, a good bacteriocin producer (Table 5). At ratios of 9:1 or 1:1 (3I1b135:3I1b6), most nodules were singly infected with strain 3I1b135. At inoculum ratios of 1:9, however, strain 3I1b6 formed 22% of the singly infected nodules and was present in 38% of nodules resulting from mixed infection. Since rhizobia from nodule surfaces were not detected after surface sterilization procedures, all the strains isolated were internal. Before reisolation from the nodules, plants were assayed for acetylene reduction to determine whether the nitrogen fixation rates reflected the presence of the predominant strain and whether strain dominance could be predicted. Small differences (not shown) were detected; these were not statistically significant ($P = 0.05$).

Strain interactions within nodules containing both strains were not apparent. The average nodule populations resulting from mixed infection, as compared to populations resulting from single infections, were similar, averaging 2×10^8 for strain 3I1b135 and 5×10^7 for strain 3I1b6. However, it is not known whether bacteriocin production occurs in nodules.

DISCUSSION

Production of bacteriocin-like substances by *R. japonicum* is common, regardless of serotype

TABLE 5. Dominance of strain 3I1b135 in nodules arising from mixed strain combinations

Ratio of 3I1b135 to 3I1b6 ^a	Nodules with mixed infections			Nodules with single infections		
	Total	3I1b135 dominant	3I1b6 dominant	Total	3I1b135 only	3I1b6 only
1:9	19	15	4	31	20	11
1:1	4	4	0	46	44	2
9:1	0	0	0	50	50	0
1:0	0	0	0	50	50	0
0:1	0	0	0	50	0	50

^a Total inoculum added per plant was 1.3×10^8 colony-forming units.

or geographic distribution. The *R. japonicum* bacteriocin(s) was not inducible by mitomycin C, but passed through dialysis membranes and was resistant to heat and proteolytic enzymes. These characteristics are similar to those antibiotic-like substances reported for a limited number of *R. trifolii* strains (24) and agrocin 84 (15) (produced by *A. radiobacter*).

We were unable to obtain bacteriocin production by *R. japonicum* against other *R. japonicum*; *C. nebraskense*, however, served as a reproducible indicator. All of our *R. japonicum* producing strains had the same activity spectrum and characteristics. Based on zones of inhibition of *R. japonicum*, Roslycky (23) inferred bacteriocin production by *R. japonicum* and cowpea rhizobia. Neither indicator nor producer strains were identified (23), and these were no longer available for comparison with our strains (Roslycky, personal communication). In contrast to *R. japonicum*, the cowpea rhizobia produced antibiotic-like bacteriocins inhibitory to most strains of *R. japonicum*. These bacteriocins were more diverse as determined by differential indicator patterns.

The antibiotic-like substances produced by *R. japonicum* and some cowpea rhizobia are termed bacteriocins, using the broad definition of Reeves (21), primarily based on their restricted host range. Recently, Roberts et al. (22) characterized agrocin 84 (produced by *A. radiobacter*, a species closely related taxonomically to fast-growing rhizobia [35]) as a substituted adenine nucleotide (molecular weight 1,100). They suggested classification of agrocin 84 as a new type of bacteriocin. Although the activity spectrum for agrocin 84 has not been reported, on the basis of our results its activity spectrum is different from that of bacteriocins produced by *R. japonicum*. Agrocin 84 had no effect on *R. japonicum* or *C. nebraskense*, and the *R. japonicum* bacteriocin had no effect on *A. tumefaciens*. It will now be of interest to determine whether the antibiotic-like bacteriocins produced by *R. trifolii* (24) and *R. japonicum* are chemically similar to agrocin 84 (22).

The assay methods and choice of indicator largely determine the number of antagonists detected and the type of antagonism involved (24). Protein-containing bacteriocins were not detected in slow-growing rhizobial strains. Since representative strains of *R. japonicum* were tested under a variety of cultural and experimental conditions, our report and Roslycky's (23) suggest that macromolecular and smaller protein-containing bacteriocins are rarely produced by slow-growing rhizobia. This slow-growing rhizobial class differs from the fast growers

in a number of characteristics besides growth rate (35).

Competitive superiority of *R. japonicum* strains in nodule formation did not appear to be related to the ability of a strain to produce bacteriocin *in vitro*. For example, bacteriocin producers and nonproducers coexisted in 33% of the nodules collected and assayed from five soybean-growing areas in Nebraska (unpublished data). Growth chamber studies of soybeans inoculated with strain mixtures also showed that dominance depended on factors other than the relationship to bacteriocin production. Strains 3I1b135 and 3I1b6, with similar and moderate efficiencies of nitrogen fixation on Amsoy 71, represent a nonproducer and good producer of bacteriocin, respectively. But even at a low input ratio 3I1b135 coexisted with strain 3I1b6 in up to 38% of the nodules formed by mixed infections.

Although coryneform bacteria can form a sizable proportion of the soil microflora (30), they were essentially insensitive to *R. japonicum* bacteriocins. Sensitivity generally was restricted to some phytopathogenic *Corynebacterium* species. The results suggest that the inhibition of coryneform bacteria in soil, if any, by *R. japonicum* may have no relationship to bacteriocin production and ability of *R. japonicum* to persist and survive in soil.

Bacteriocin production appears to be a common property regardless of ability to nodulate or fix nitrogen. Quantitative production was not related to symbiotic properties of strains or with a particular serotype. However, bacteriocin production, along with other characteristics, was useful in subdividing *R. japonicum* into three distinct groups. The importance and prevalence of these groups in nature are not known, but they exemplify genetic diversity within the species.

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