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Plasmid-determined Bacteriocin Production by *Rhizobium leguminosarum*

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Bacteriocin production by 97 isolates of *Rhizobium leguminosarum* was investigated and two types of bacteriocins were identified and designated *small* and *medium* on the basis of their size. Three isolates appeared to carry determinants of *medium* bacteriocin production which were self-transmissible at frequencies of 10^{-1} to 10^{-2}, suggesting the presence of bacteriocinogenic plasmids in these strains. The mobilization of chromosomal genes was associated with transfer of the bacteriocinogenic plasmids. Indirect evidence that the production of *small* bacteriocins may be determined by plasmids in three other strains is presented.

INTRODUCTION

Genetic evidence suggests that some of the genes involved in *Rhizobium*–legume symbiosis may be located on plasmids (Higashi, 1967; Dunican *et al.*, 1976; Johnston *et al.*, 1978). Recently, Prakash *et al.* (1978) found that a rough mutant of *R. leguminosarum* had become unable to form nodules on *Pisum sativum* and no longer carried a large plasmid. There is other physical evidence for endogenous plasmids in *Rhizobium* (Sutton, 1974; Tshitinge *et al.*, 1975; Zurkowski & Lorkiewicz, 1976; Nuti *et al.*, 1977) but no phenotypic characteristics have been correlated with their presence and no evidence for their transfer has been reported.

Conjugative plasmids of the P1 incompatibility group can be transferred into *Rhizobium* spp. (Datta *et al.*, 1971) where they have facilitated the construction of circular linkage maps for *R. meliloti* (Kondorosi *et al.*, 1977; Meade & Signer, 1977) and *R. leguminosarum* (Beringer *et al.*, 1978).

Many bacteriocins of enterobacteria are plasmid-determined (Hardy, 1975) and this may be a feature common to other bacterial genera. Bacteriocin production by *Rhizobium* spp. has been described (Roslycky, 1967; Schwinghamer & Belkengren, 1968; Schwinghamer, 1971, 1975; Lotz & Mayer, 1972; Schwinghamer *et al.*, 1973) but there are no data to suggest that it may be plasmid-determined. The term 'bacteriocin' is used here to describe any inhibitory agent which causes antagonism between closely related strains and which is neither self-propagating (i.e. bacteriophage) nor an antibiotic with activity against a wide range of micro-organisms.

This paper describes the characterization of bacteriocin production and sensitivity amongst a collection of wild-type isolates of *R. leguminosarum* and subsequent attempts to transfer bacteriocin production between bacteria.

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Table 1. *Bacterial strains and plasmids*

John Innes Institute strain no.	Bacteriocins produced* *Small*	*Medium*	Original designation or phenotype, where relevant	Source†	Reference
Rhizobium leguminosarum strains mentioned specifically					
238	+	+		1	Josey *et al.* (1979)
240	+	−		1	Josey *et al.* (1979)
248	−	+		2	Josey *et al.* (1979)
279	−	+		1	This paper
289	+	+		1	This paper
300	+	−		1	Johnston & Beringer (1975)
306	−	+		1	Josey *et al.* (1979)
307	+	−		1	This paper
309	−	+		1	Josey *et al.* (1979)
336	+	−	Rothamsted strain 1007	3	
1062	+	−	300 *ura-14 trp-16 str-86*		Johnston *et al.* (1978)
1027	+	−	300 *ade-27 phe-1 rif-45*		This paper
Other strains tested for bacteriocin production					
R. trifolii					
514	+	−		4	This paper
6599	+	+	Rothamsted strain 209	3	
6614	+	+	Rothamsted strain 26	3	
6661	+	−	*rif* derivative of Rothamsted strain 12	3	Johnston *et al.* (1978)
6710	+	−	*str* derivative of Rothamsted strain 204	3	Johnston *et al.* (1978)
R. phaseoli					
1233	+	−	*rif* derivative of Rothamsted strain 3644	3	Johnston *et al.* (1978)
Agrobacterium radiobacter					
1108	−	−	K84, produces agrocin 84	5	Kerr & Htay (1974)
A. tumefaciens					
1109	−	−	K23, sensitive to agrocin 84	5	Kerr & Htay (1974)
C58Ti$^+$	−	−		6	Hamilton & Fall (1971)
C58Ti$^-$	−	−		6	Hamilton & Fall (1971)
Escherichia coli strains which produce, or are sensitive to, colicins					
712(colB-K98)			produces colicin B	7	
J5-3*nal*(colV-CA7, R538*drd1*)			produces colicin V	7	
J5-3/V1			sensitive to colicin B, resistant to colicin V	7	
CL144			sensitive to colicin V, resistant to colicin B	7	

Plasmids	Antibiotic resistances	Reference
R68.45	Kanamycin, tetracycline, ampicillin	Haas & Holloway (1976)
pJB5JI	Kanamycin	Johnston *et al.* (1978)
pRL1JI	None known	This paper‡
pRL3JI	None known	This paper‡
pRL4JI	None known	This paper‡

* *Small* bacteriocin production was identified by inhibition of strain 248; *medium* bacteriocin production by inhibition of strain 336 – see text.

† Sources: 1, J. E. Beringer, John Innes Institute (isolated from *Pisum sativum* root nodules); 2, J. E. Beringer (*Vicia faba* root nodule); 3, Rothamsted Experimental Station, Harpenden, Herts; 4, J. E. Beringer (*Trifolium* sp. root nodule); 5, A. Kerr, Waite Agricultural Institute, University of Adelaide, S. Australia; 6, M. van Montagu, Rijksuniversiteit Gent, Belgium; 7, P. Cooper, University of East Anglia.

‡ These plasmids were characterized by their determination of *medium* bacteriocins; they conferred this ability on strain 1062 which previously produced only *small* bacteriocins and were subsequently transferred into other strains. pRL1JI was derived from strain 248, pRL3JI from strain 306 and pRL4JI from strain 309.

Table 2. *Bacteria used to test the range of activity of inhibitory substances produced by Rhizobium leguminosarum*

Species	Original designation (where known)	Source*
Bacillus megaterium		1
Bacillus mycoides		1
Bacillus cereus		1
Bacillus subtilis		1
Staphylococcus aureus	8325-4	1
Klebsiella aerogenes	A3SL	1
Proteus mirabilis	F67	2
Pseudomonas putida	AC4	3
Escherichia coli	J5-3	4
Agrobacterium tumefaciens	B6	5
Rhizobium trifolii	W19	6

* Sources: 1, John Innes Institute; 2, J. T. Smith, School of Pharmacy, University of London; 3, A. M. Chakrabarty, General Electric Company Research and Development Center, New York, U.S.A.; 4, E. Meynell, Lister Research Institute of Preventive Medicine, London; 5, D. N. Butcher, Unit of Developmental Botany, University of Cambridge; 6, G. J. Atkins, Department of Genetics, University of Liverpool.

METHODS

Bacterial strains and plasmids. These are shown in Tables 1 and 2.

Media and culture conditions. Complete (TY) and minimal (Y) media were described by Beringer (1974). VS medium was Vincent's (1970) yeast sucrose (phage broth). Soft Y and soft VS agar contained Difco Bacto-agar at 6 g l^{-1}.

Rhizobium and *Agrobacterium* strains were incubated at 28 °C and those of other genera at 37 °C, unless otherwise indicated. Liquid suspensions of *Rhizobium* and *Agrobacterium* were prepared by inoculating TY slants, growing for 2 d, then washing off the bacteria in 20% (v/v) glycerol. These were stored frozen until required. Strains of other genera were grown overnight in Oxoid nutrient broth before use.

Cultures used for crosses were grown on TY slants for 2 d and washed off with distilled water. Crosses were performed as described by Beringer *et al.* (1978).

Proteolytic enzymes. These were provided by M. W. Rees (John Innes Institute, Norwich) and made up as follows: trypsin, chymotrypsin and pronase at 4 mg ml^{-1} in 0·13 M-NH_4HCO_3, pH 8·5; thermolysin at 2 mg ml^{-1} in 0·2 M-ammonium acetate, 5 mM-$CaCl_2$, pH 8·5. The solutions were sterilized by membrane filtration (0·45 μm Millipore filters) and stored frozen.

Bacteriocin screening. Initial observations on bacteriocin production by some *R. leguminosarum* isolates were reported by Beringer (1973). These isolates were tested against each other by various methods, and strains which produced or were sensitive to different types of bacteriocin were identified by the appearance and width of their inhibition zones and ranges of activity. Using these strains, the following method gave clear and reproducible results.

Strains to be tested for bacteriocin production were stab-inoculated (from liquid cultures or single colonies) into two plates of Y and TY agar. When many identical test plates were needed, a multiple wire inoculator (Najfeld, 1973; Josey *et al.*, 1979) was used. The plates were incubated for 2 to 4 d until a patch of growth 1 to 3 mm in diameter was visible at the top of each stab. This was removed by pressing the plates over a replicating block covered with sterile velvet from which replica plates could be made if required. Re-growth was prevented by surface-sterilization – plates were inverted over glass Petri dishes containing 5 ml chloroform for 45 min then 'aired' for 30 min in a laminar flow cabinet. Each plate was overlaid with 2·5 ml soft Y agar held at 42 °C and inoculated with 0·1 ml of the culture to be tested as an indicator. After 2 to 3 d the indicators had grown into confluent lawns and the inhibition zones could be clearly seen. Streptomycin-resistant derivatives of indicator strains were also used, avoiding the need for surface-sterilization since streptomycin included in the top layer prevented re-growth of sensitive producer strains. Results obtained by this method were identical to those found using chloroform surface-sterilization.

The cultures used as indicators were liquid suspensions washed off TY slants and stored in 20% glycerol (see above). Some indicators were sensitive to many strains and gave large zones of inhibition which tended to overlap and obscure each other. Therefore, until the inhibition zones had been characterized, not more than nine strains were tested for production on each plate.

Four indicators (strains 240, 248, 300 and 366) were used to test our collection of *R. leguminosarum*

isolates for production of bacteriocins. Each isolate was also tested for sensitivity to bacteriocins produced by five isolates (strains 238, 248, 279, 300 and 309).

Strains were tested for antibiotic production against a range of indicators of different genera (Table 2). The procedure was the same as that described above except that plates overlaid with indicators other than *Rhizobium* or *Agrobacterium* were incubated overnight at 37 °C.

Testing for the presence of bacteriophage. Using a 4 mm diam. cork borer, plugs were removed from inhibition zones produced against indicator strain 336 (avoiding the producer stab) placed in 2 ml VS medium and left for 4 h. The infusions were diluted in VS medium; then 0·1 ml samples were added to soft VS agar inoculated with streptomycin (to kill any producer bacteria) and a streptomycin-resistant derivative of strain 336, and poured over plates containing TY agar. After 2 d incubation, the plates were examined for plaques.

Physical properties of bacteriocins. Heat stability of bacteriocins was investigated by incubating surface-sterilized bacteriocin test plates at 85 °C for 45 min before overlaying with indicator. Diffusion through cellophane was tested by placing sterile cut discs from Visking dialysis tubing (Scientific Supplies, London) over surface-sterilized test plates before overlaying with indicators. Visking tubing is reported by the U.K. distributors to exclude globular proteins with molecular weights greater than 12000 to 14000.

Sensitivity to proteolytic enzymes was examined as follows. Plates were inoculated with one to four producer strains, incubated and then surface-sterilized after removal of surface growth. Sterile wicks 30 × 3 mm of Whatman no. 1 filter paper were placed radially from the inoculation points and 10 μl of proteolytic enzyme solution was pipetted on to each wick. After incubation at 37 °C for 4 h, the wicks were removed and the plates were overlaid with indicators.

The use of these methods for characterizing *Rhizobium* bacteriocins was justified by testing them on *Escherichia coli* strains which produced, or were sensitive to, colicins B and V, using the media and methods described for *R. leguminosarum*. Colicin B was heat-labile and colicin V heat-stable; both were unable to diffuse through cellophane and were sensitive to trypsin. These observations agree with the properties of colicins B and V reported by Reeves (1972). The colicins were also sensitive to pronase, thermolysin and chymotrypsin. Strain J5-3/V1 (sensitive to colicin B) was sensitive to pronase and was inhibited in growth along the length of the wick. However, destruction of colicin B was apparent by growth of the indicator around the wick in the 'inhibition zone'.

Cell-free culture supernatants. Bacteriocinogenic strains of *R. leguminosarum* were grown in liquid Y medium for 7 d in an orbital shaker (150 rev. min^{-1}), then shaken with 5% (v/v) chloroform to enhance the release of cell-bound material, and centrifuged for 2 h at 20000 *g* to obtain cell-free supernatants. These were sterilized by shaking with 2% (v/v) chloroform and left to settle before testing for viable cells. They were stored at 4 °C, where no loss of activity was observed for several weeks.

Plates of Y or TY agar were overlaid with soft Y agar top layers inoculated with indicator strains, then wells were cut with a 5 mm diam. cork borer, and 70 μl of cell-free supernatant was pipetted into each. After 2 d incubation, inhibition zones were visible around some wells.

Samples (4 ml) of the cell-free supernatants were centrifuged for 18 h at 84000 *g* in a Beckman 50 fixed angle rotor. The upper 2 ml of supernant was removed from each tube and retained before resuspending the precipitate in the lower 2 ml. Both upper and lower fractions were compared with the original cell-free supernatant as above. The lowest sedimentation coefficient of molecules which would be completely sedimented by this centrifugation was calculated to be 3·7S (which corresponds to a molecular weight of about 40000 for a globular protein).

RESULTS

Bacteriocin screening

When the collection of 97 *R. leguminosarum* isolates was tested for bacteriocin production, the inhibition zones fell into two distinct classes; further tests showed them to represent different classes of bacteriocins. One class, giving wide inhibition zones (always more than 10 mm and usually 25 mm) against certain indicators, was produced by 83 isolates. Further testing of six of these (strains 238, 240, 289, 300, 307 and 336) revealed that their bacteriocins could diffuse through cellophane, were heat-labile and were resistant to proteolytic enzymes (although sensitivity of indicator strains to pronase made tests using this enzyme unreliable). Bacteriocins of this type were designated *small* because of their diffusion properties. All 83 isolates which produced *small* bacteriocins were resistant to *small* bacteriocins produced by strains 238 and 300, and several non-producers (e.g. strains 248, 279 and 309) were sensitive to all producers against which they were tested. Further investigations showed

ten isolates to be resistant to *small* bacteriocins produced by each other. Strain 248 was subsequently used as a diagnostic indicator for *small* bacteriocins.

The other class of bacteriocins, produced by 27 isolates, gave zones 2 to 10 mm wide, and further testing of six strains (238, 248, 279, 289, 306 and 309) showed that their bacteriocins were unable to diffuse through cellophane, were heat-labile but were not sensitive to proteolytic enzymes. These bacteriocins were designated *medium* since they were assumed to be of an intermediate size compared with the *small* bacteriocins described above and the large bacteriocins resembling defective phage particles reported by others (Lotz & Mayer, 1972; Schwinghamer *et al.*, 1973) which would not have diffused through agar sufficiently to have been detected by our screening method.

Inhibitory activity due to *medium* bacteriocins could be demonstrated in cell-free culture supernatants of the four producers tested (strains 238, 248, 279 and 289) and resulted in inhibition zones 2 to 10 mm wide around the wells. No activity remained in the upper fraction following further centrifugation, implying that the sedimentation coefficients of the *medium* bacteriocins were greater than 3·7S. No *small* bacteriocin activity could be detected in cell-free culture supernatants of producer strains 238, 240, 289, 300, 307 or 336, although the *medium* bacteriocins produced by strains 238 and 289 were present. (These were amongst the 19 isolates tested which produced both *small* and *medium* bacteriocins.)

There was little obvious cross-resistance between eight *medium* bacteriocin producers when tested against each other, although all were resistant to their own bacteriocins. It was not feasible to search for resistant mutants of the indicator strains since colonies appearing in the inhibition zones after about 1 week (up to five colonies per cm^2 in all zones) were found to be only phenotypically resistant.

The two indicator strains sensitive to most *medium* bacteriocins (strains 240 and 336) were not always inhibited by the same producers. For example, strain 279 (and two other isolates from the same location) inhibited strain 240 only on minimal (Y) agar although strain 336 was inhibited on both minimal and complete (TY) agar. Cell-free supernatants from cultures of strain 279, grown in liquid Y medium, gave the same results, implying that strain 240 was sensitive only on minimal media to a *medium* bacteriocin produced by strain 279. Several other strains were also sensitive to strain 279 only on Y agar (e.g. 306, 309 and W19). These were the only differences observed when screening bacteriocin production and sensitivity on Y and TY agar. Strain 336 was sensitive to more *medium* bacteriocins than was strain 240; it generally gave clearer zones and was subsequently used as the diagnostic indicator for *medium* bacteriocins.

One isolate, strain 307, gave clear inhibition zones with uneven edges, 0·5 to 2 mm wide after 2 d, against many indicators. These zones were shown to be due to a temperate bacteriophage produced by strain 307. The presence of phages was demonstrated in cell-free supernatants of cultures of strain 307 by plaque formation in top layers containing indicators. However, no inhibition zones were present around wells containing the preparation, confirming the inability of phage particles to diffuse through agar. No other inhibition zones contained phages.

Strains 238, 240, 279 and 300 did not inhibit any of the indicator strains of various genera shown in Table 2, except for *R. trifolii* strain W19 which was sensitive to *medium* bacteriocins produced by strains 238, 248 and 279.

Rhizobium trifolii strains 514, 6599, 6614, 6661 and 6710, *R. phaseoli* strain 1223 and *Agrobacterium* strains 1108, 1109, C58Ti$^+$ and C58Ti$^-$ were tested for bacteriocin production using indicator strains 336 and 248. All the *Rhizobium* strains produced *small* bacteriocins and strains 6599 and 6614 also produced *medium* bacteriocins, but the *Agrobacterium* strains did not inhibit either indicator.

Table 3. *Transfer of bacteriocinogenicity*

The recipient was strain 1062, a derivative of strain 300, and produced only *small* bacteriocin. Columns A and B represent two sets of crosses. In each case, 200 recipient colonies were tested for production of *medium* bacteriocins active against strain 336. There was no evidence for transfer of determinants of *small* bacteriocin production from strain 1062 to the donor strains (300 colonies were picked from plates selecting for the donors in each case, and overlaid with strain 248).

In crosses involving R68.45, 150 recipients selected on kanamycin (assumed to carry R68.45) and 150 colonies from selection for all recipients were tested for *medium* bacteriocin production against strain 336. Although R68.45 transferred at a frequency of 10^{-2} per recipient, no *medium* bacteriocin-producing recipients from the total recipient selection carried R68.45.

	Bacteriocins produced by donor		Percentage of recipients producing *medium* bacteriocin	
Donor strain	*Small*	*Medium*	A	B
248	−	+	15	5
306	−	+	1	10
309	−	+	10	15
300	+	−	<0·5	<0·5
			R^+ recipients	Total recipients
238(R68.45)	+	+	<0·7	<0·7
248(R68.45)	−	+	6·6	1·3
279(R68.45)	−	+	<0·7	<0·7
289(R68.45)	+	+	<0·7	<0·7
306(R68.45)	−	+	5·3	0·7
309(R68.45)	−	+	2·7	1·3

Transfer of determinants for bacteriocin production and mobilization of chromosomal markers

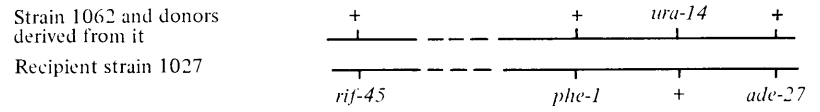
Three isolates (248, 306 and 309) could transfer the ability to produce *medium* bacteriocins to a derivative of strain 300 which previously produced only *small* bacteriocins (Table 3). The frequency of transfer of bacteriocinogenicity (10^{-1} to 10^{-2}) indicated that it was determined by conjugative plasmids. There was no evidence for transfer of the ability to produce *small* bacteriocins from strain 1062 to the donor strains.

Many recipients of the ability to produce *medium* bacteriocins had lost the ability to produce their *small* bacteriocins, to which they had become sensitive. Recipient colonies producing both types of bacteriocins gave rise to colonies producing one or the other type. There was no evidence for transfer of the ability to produce *medium* bacteriocins from three other isolates (238, 279 and 289) into which R68.45 had been transferred, although in crosses with R^+ derivatives of strains 248, 306 and 309 there was a slight enrichment for bacteriocin producers amongst recipients carrying R68.45 (Table 3).

Evidence for chromosome mobilization by the plasmids determining production of *medium* bacteriocins was obtained in crosses between differently marked derivatives of strain 300 (Table 4). The frequency of transfer of selected alleles, 10^{-7} to 10^{-8} per recipient, was lower than the frequency of 10^{-6} obtained in similar crosses involving R68.45 (Beringer *et al.*, 1978) although the frequency of transfer of bacteriocinogenicity (2×10^{-2} to 5×10^{-2}) was slightly higher than that of R68.45 (about 10^{-2}). The observed frequencies of transfer of the ability to produce *medium* bacteriocins were probably lower than the actual frequencies because of incompatibility with the resident determinants of *small* bacteriocin production in the recipients. However, the proportion of recombinants which had become *medium* bacteriocin-producers was much higher than that of the total recipient population.

Table 4. *Mobilization of chromosomal genes, associated with transfer of determinants for medium bacteriocin production*

Only the classes of recombinants with auxotrophic markers from both parents are shown; *rif-45* was the selected recipient marker. Strain 1062 and donors derived from it carried *str-86* and *trp-16* which were disregarded. The order of markers was deduced by Beringer *et al.* (1978). Figures in parentheses show the number of recombinants of each class which produced *medium* bacteriocin with activity against indicator strain 336. Strains 1062 and 1027 were derived from the same parent (strain 300) which normally produces only *small* bacteriocin.



Donor strain	Percentage of recipients producing *medium* bacteriocin	No. of recombinants: Selected donor marker *phe*$^+$. *rif phe*$^+$ *ura ade*	No. of recombinants: Selected donor marker *ade*$^+$. *rif phe ura ade*$^+$
1062	<0·5	0 (0)	0 (0)
1062(pRL1JI)	5	59 (54)	4 (4)
1062(pRL3JI)	5	65 (57)	7 (7)
1062(pRL4JI)	2	27 (25)	11 (10)

It was not possible to demonstrate cotransfer of resistance with the ability to produce *medium* bacteriocins in strain 300 derivatives because they were not initially sensitive to *medium* bacteriocins produced by the donor strains 248, 306 and 309. Instead, pJB5JI, a kanamycin-resistant derivative of the bacteriocinogenic plasmid pRL1JI derived from donor strain 248 (Johnston *et al.*, 1978), was transferred into a sensitive strain 336. Derivatives of strain 366 carrying pJB5JI were tested for sensitivity to various bacteriocinogenic strains (Table 5).

Since pJB5JI does not determine *medium* bacteriocin production (Tn5 probably inserted into and inactivated the relevant genes; Johnston *et al.*, 1978) it can be used to eliminate incompatible plasmids determining *medium* bacteriocin production. It was transferred into various bacteriocinogenic strains, selecting for transfer of kanamycin resistance, and these recipients were tested for bacteriocin production against indicator strain 336. The results (Table 5) show that pJB5JI confers resistance to the plasmid-determined *medium* bacteriocins derived from strains 248, 306, and 309 (i.e. to strain 1062 carrying pRL1JI, pRL3JI and pRL4JI) and to strain 248, although the indicator was still slightly inhibited. However, strains 306 and 309 gave clear inhibition zones against strain 336 (pJB5JI), implying that they produced *medium* bacteriocins other than those to which pJB5JI conferred resistance. Further evidence was obtained when pJB5JI was shown to eliminate the production of *medium* bacteriocins when transferred into strain 248 but not strains 306 and 309.

Strain 248 was sensitive to strains 306 and 309 but strains 306 and 309 were resistant to each other and to strain 248. Therefore strains 306 and 309 may produce similar *medium* bacteriocins other than those determined by pRL3JI and pRL4JI. Since strains 306 and 309 were isolated from different plants from the same source, they may be related although they have different endogenous antibiotic resistance levels (Josey *et al.*, 1979).

When pJB5JI was transferred into strain 289, which produces both *small* and *medium* bacteriocins, *medium* bacteriocin production was not eliminated. Since strain 289 is sensitive to *medium* bacteriocins produced by strains 248, 306 and 309 and strain 248 is sensitive to *medium* bacteriocins produced by strain 289 (shown by using cell-free supernatant from a culture of strain 289, since strains 248, 306 and 309 are all sensitive to *small* bacteriocins produced by strain 289), this probably represents another class of *medium* bacteriocins.

Table 5. *Medium bacteriocin production and resistance in strains carrying bacteriocinogenic plasmids*

Strain	Indicator strain: 336	Indicator strain: 336(pJB5JI)
1062	−	−
248	+	±
306	+	+
309	+	+
1062(pRL1JI)	+	−
1062(pRL3JI)	+	−
1062(pRL4JI)	+	−
1062(pJB5JI)	−	NT
248(pJB5JI)	−	NT
306(pJB5JI)	+	NT
309(pJB5JI)	+	NT

−, No inhibition zone; ±, very faint zone of inhibition;
+, clear zone of inhibition; NT, not tested.

However, *small* bacteriocin production was eliminated from strains 289, 336 and 1062 on the introduction of pJB5JI implying that the determinants were related.

DISCUSSION

All except five of the 97 *R. leguminosarum* isolates and all the *R. trifolii* and *R. phaseoli* isolates tested produced *small* or *medium* bacteriocins (or both). The ubiquitous *small* bacteriocins may be closely related since all producers tested were cross-resistant (unlike the *medium* bacteriocin producers).

The most obvious explanation for the high frequencies of transfer of the ability to produce *medium* bacteriocins by the three donor strains, and the correlation of transfer of this property with transfer of chromosomal genes, is that conjugative bacteriocinogenic plasmids are involved. Although no transfer of the ability to produce *small* bacteriocins was detected, the concomitant loss of production and resistance on the introduction of plasmids determining *medium* bacteriocin production implies that these properties could be determined by an incompatible plasmid.

The plasmid-determined *medium* bacteriocins produced by strains 248, 306 and 309 are probably closely related since pJB5JI (derived from strain 248) confers resistance to *medium* bacteriocins determined by pRL3JI and pRL4JI (derived from strains 306 and 309, respectively). The plasmids themselves are also presumed to be related since pJB5JI eliminates pRL3JI and pRL4JI; the same argument may be applied to the presumptive plasmids determining *small* bacteriocins. However, strains 306 and 309 also appear to produce *medium* bacteriocins other than those determined by pRL3JI and pRL4JI. Similarly, *medium* bacteriocin production by strain 289 was not eliminated on transfer to pJB5JI although recipients lost their ability to produce *small* bacteriocins. Production of more than one type of *medium* bacteriocin by some strains may account for the lack of cross-resistance observed between producers. It may be significant that all three isolates carrying conjugative bacteriocinogenic plasmids produced only *medium* bacteriocins. Further crosses using other isolates may show whether this is the general case.

Strain 300 carries at least three plasmids, with molecular weights of about 200×10^6, 150×10^6 and 100×10^6 (Prakash *et al.*, 1978). Whether or not any of these plamids is missing in *medium* bacteriocin-producing derivatives which have lost *small* bacteriocin production and resistance remains to be determined.

Johnston *et al.* (1978) showed that pJB5JI confers the ability to nodulate peas when

transferred into a non-infective mutant of *R. leguminosarum* strain 300, into *R. trifolii* strains 6661 and 6710, and into *R. phaseoli* strain 1233. This transfer was associated with loss of the ability to produce *small* bacteriocins, and resistance to them. Although the molecular nature of the *small* and *medium* bacteriocins is uncertain and their ecological significance unknown, their potential as markers on endogenous *Rhizobium* plasmids which may be involved in symbiosis warrants further investigation.

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