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Symbiotic effectiveness and bacteriocin production by *Rhizobium leguminosarum* bv. *viciae* isolated from agriculture soils in Faisalabad

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Abstract

Antagonism amongst mixtures of inoculant strains of *Rhizobiaceae* on the basis of bacteriocin production was assessed. A total number of 10 strains of *Rhizobium*, *Bradyrhizobium* and *Agrobacterium* were screened for their bacteriocin production ability. It was observed that *Rhizobium leguminosarum* bv. *viciae* strain LC-31 produced a medium typed bacteriocin that was found to be highly effective in growth inhibition of some strains of *R. leguminosarum* bv. *viciae* and *Agrobacterium* sp.

It was isolated and partially purified to homogeneity by chloroform extraction followed by ammonium sulfate fractionation. The bacteriocin fraction consistently migrated as a 50 kDa polypeptide on SDS-PAGE. Ex vivo assays were carried out using the partially purified bacteriocin protein fraction of *R. leguminosarum* bv. *viciae* strain LC-31 against related strains of the same species. It showed an activity pattern similar to that exhibited by strain LC-31.

Keywords: *Bacteriocin*; *Competition*; *Rhizobium leguminosarum* bv. *viciae*

1. Introduction

The *Rhizobium* legume symbiosis is the most promising plant bacterium association so far known. Inoculated *Rhizobium* spp. strains often fail to compete with indigenous soil rhizobia and do not increase nodulation (Bromfield et al., 1986; Singleton and Tavares, 1986). Thus the successful use of rhizobial inoculants requires the knowledge of factors affecting the effectiveness and competitive ability of the rhizobia. One of the major factors reported to be affecting competition among rhizobia are bacteriocins (Perveen et al., 1987; Schwinghamer, 1971; Oresnik et al., 1999). Bacteriocins are proteins or protein complexes with bacteriocidal activity directed against species that are usually closely related to producer bacterium (Tagg et al., 1976).

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Bacteriocins are ribosomally encoded peptide antibodies. Both Gram negative and Gram positive bacteria produce them. The Gram negative bacteriocins are well studied. These have characteristic structural domains involved in cell attachment, translocation and bactericidal activity. They bind to specific receptors on the outer membrane of the target cell. As a consequence, their range of activity tends to be narrow (Braun et al., 1994). *Rhizobium leguminosarum* strains have been shown to produce bacteriocins which have been characterized as small, medium or large based on their assumed size characteristics (Hirsch, 1979; Schwinghamer and Brockwell, 1978). Small bacteriocins were found to be chloroform soluble and have molecular mass less than 2000 Da (Hirsch, 1979; Van-Brussel et al., 1985). Very little is known about medium bacteriocins produced by *R. leguminosarum*. It has been shown that relatively few strains produce medium bacteriocins. Cross resistance pattern suggested that there may be several different bacteriocins within the medium bacteriocin family (Hirsch, 1979; Wijffelman et al., 1983). *R. leguminosarum* contains the symbiotic plasmid pRL1J1, which is one of the genetically best characterized nodulation plasmid. As well as containing genes necessary for nodulation and nitrogen fixation this plasmid has been shown to carry determinants for bacteriocin production (Hirsch et al., 1980). Oresnik et al. (1999) found that the bacteriocins appear to play a major role in determining competitiveness for nodulation when assayed against some strains. So the successful preparation of mixed inoculum requires the knowledge of bacteriocin producing ability of the inocula strains as well as their effect on the related rhizobia. In the present study some strains of *Rhizobium* and *Bradyrhizobium* has been investigated for their bacteriocin production and competition with the related microbes.

2. Materials and methods

2.1. Strains and media

Bacterial strains of *R. leguminosarum* bv. *viciae*, *R. leguminosarum* bv. *trifolii*, *Bradyrhizobium japonicum* and *Agrobacterium* sp. (Table 1) were obtained from the culture collection (BIRCEN) NIBGE, Faisalabad. The *Bradyrhizobium* strain TAL 102 and *Rhizobium trifolii* strain TAL 1826 was obtained from NifTAL, Hawaii, USA. The strains of *Rhizobium* and *Bradyrhizobium* were subcultured on YEM broth (Vincent, 1970) and confirmed as such by plant infectivity assays on the appropriate host plants (Hameed, 1986) (Table 1). The seeds of peas, alfalfa, mungbean, soybean and clover were obtained from the Ayub Agriculture Research Institute (AARI), Faisalabad and surface sterilized by 0.1% $HgCl_2$ and distil water. The plants were grown in growth pouches at 30 ± 2 °C. The plants were watered by N-free Hoagland nutrient solution (Arnon and Hoagland, 1940). The inoculum was given as 1 mL of broth culture per tube (10^9 cells mL^{-1}). Four-week-old nodules were picked and incubated with acetylene for 1 h at room temperature to determine the nitrogenase activity (Naeem et al., 2004). Two controls were used for the assay: (1) without any nodules containing pure acetylene only; (2) root nodules with acetylene. Trace Gas Chromato graph—GC 2000 (Thermo Quest-C.E. instrument Italiana) with a hydrogen flame ionization detector (FID) was used for acetylene reduction assay. The unchanged acetylene and ethylene produced were calculated

Table 1
Screening of efficient bacteriocin producing bacteria against various indicator strains

Strains	Species	Host	Presence/absence of bacteriocin activity	ARA (nmol h^{-1} g^{-1} nodule dry weight)
LC-31	*R. leguminosarum* bv. *viciae*	*Lens culinaris*	+++	224 ± 10 a
LC-21	*R. leguminosarum* bv. *viciae*	*Lens culinaris*	–	182 ± 9.5 b
LC-12	*R. leguminosarum* bv. *viciae*	*Lens culinaris*	++	182 ± 9.5 b
PS-1	*R. leguminosarum* bv. *viciae*	*Pisum sativum*	+/–	149 ± 3.51 c
PS-2	*R. leguminosarum* bv. *viciae*	*Pisum sativum*	+/–	143 ± 7.63 c
TAL 1826	*R. leguminosarum* bv. *trifolii*	*Trifolium* sp.	–	228 ± 18.47 a
TAL 1827	*R. leguminosarum* bv. *trifolii*	*Trifolium* sp.	–	248 ± 32.04 a
TAL 102	*B. japonicum*	*Glycine max*	–	224 ± 22.05 a
MnS	*B. japonicum*	*Vigna radiata*	–	0 d
Ca18	*Agrobacterium tumefaciens*	*Cicer arietinum*	–	0 d

+++: Strong bacteriocin producer; ++: moderate; –: non-producer. All the results are the means of triplicates. ARA: acetylene reduction assay.

as ratio on chrome card software. The nitrogenase activity was expressed as *n* moles of C_2H_4 produced h^{-1} g^{-1} nodule dry weight.

2.2. Bacteriocin activity assay

2.2.1. Simultaneous (direct) method

The antibiotic-producing ability for the isolates was bioassayed by the simultaneous (direct) antagonism method (Tagg et al., 1976). Indicator strains were grown in Tryptone yeast (TY) broth (Beringer, 1974) to an approximate cell concentration of 10^8 mL^{-1}. The number of cells were determined by viable cell count technique (Shah et al., 1995). These strains were diluted to 10^{-2} in TY broth. One milliliter of each diluted culture was added to approximately 20 mL of molten soft TY (0.6% agar, w/v) for preparing indicator plates. Single colonies of strain to be tested for bacteriocin activity were stab inoculated into the soft agar. Inoculation was carried out within 2 h after agar solidification and the plates were incubated at 28 ± 2 °C. The inhibitory spectrum was studied by using test strains belonging to different species (Table 1).

The plates were scored for inhibition zones 48 h at 28 °C after carrying out stab inoculation (Oresnik et al., 1999). Halos were visible as clear zones surrounding the stab inoculated cultures.

2.2.2. Preparation of cell-free supernatant (CSF)

Bacteriocin producing strains were grown in TY broth for 5 days and separated by centrifugation at 10,000 × *g* for 20 min. The supernatant was steamed in an autoclave for 5 min at 100 °C and ice cooled for 5 min. The supernatant was then filtered through 0.2 μm Millipore filter. The pH was adjusted at 6.5 with 1 M NaOH. The solution so prepared was designated as CFS (Hirsch, 1979; Oresnik et al., 1999; Delgado et al., 2001).

2.2.3. Quantification of bacteriocins

An adaptation of the critical dilution assay was used for the titration of bacteriocin activity. Diffusion assays in triplicate were used for the detection of antimicrobial activity and suitable negative controls were used for each test. The samples were added drop wise on agar or inside wells. In all the cases the plates were prepared by pouring 20 mL of soft TY agar (0.6%, w/v) with 1 mL of indicator strains at a density of 3×10^7 cfu mL^{-1} mixed in it. After solidification of the medium, wells of 5 mm diameter were prepared using a sterilized cork borer. The number of wells was kept limited to 4 per plate. These plates were then used for the quantification of bioactivity (Hirsch, 1979; Delgado et al., 2001).

The bacteriocin titer in arbitrary units per milliliter (AU/mL) is expressed as the reciprocal of the highest two-fold dilution showing a definite zone of growth inhibition on the indicator lawn, multiplied by a conversion factor (1 mL divided by the volume of the drop). The diameter of each inhibition zone was measured twice in perpendicular direction with vernier calipers of 0.1 mm precision on a colony counter (Gallenkamp, Leics., UK). The bioactivity of each sample was determined analytically by linear regression equation. Each assay was performed in duplicate. Analysis of regression was performed using program (Analyse-it® for Microsoft Excel) (Nunez et al., 1996).

2.3. Isolation and purification of bacteriocin proteins

2.3.1. Bacteriocin protein purification

Purification of proteins was carried out using the procedure of Yang et al. (1992). CFS was used to carry out protein extractions. Twenty percent chloroform was added to the CFS in a separatory funnel. The aqueous phase formed was separated and used for precipitating out the proteins. Protein precipitation was carried out on ice or at 4 °C by the addition of analytical grade ammonium sulfate. The aqueous phase was saturated with cold ammonium sulfate from 20 to 100% (w/v) saturations and gradually stirred with a glass stirrer for 10–15 min. The aqueous phase was kept overnight at 4 °C.

The precipitate was collected by centrifugation at 15,000 × *g* for 20 min. The solid pellete dissolved in distilled water and dialyzed against distilled water at room temperature for 24 h. The suspension obtained was designated as protienaceous fraction (or crude bacteriocin fraction). All the different dialysates obtained were lyophilized. Lyophilized material of 0.01 g was added in 100 μl Tris–HCl (pH 6.5) buffer and tested for inhibitory activity as described above. The quantification of protein concentration was done by standard Bradford method (Bio-Rad protein assay, Bio-Rad Laboratories, Hercules, CA, USA). Bovine serum albumin was used to construct the standard curve.

2.3.2. Polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) in the presence of 10% sodium dodecyl sulfate (SDS) was performed on a vertical slab gel (1 mm) (Laemmli, 1970). Electrophoresis was conducted at a constant current of 30 mA for 12 h at 30 °C. The gel was stained with Coomassie blue (Sigma). A 10 kDa protein ladder was used as protein standard (Gibco BRL®).

3. Results and discussion

Our results confirmed the bacteriocin production ability of the strains tested. An auto-antagonism re-

Table 2
Activity spectrum of bacteriocin of *R. leguminosarum* bv. *viciae* strain LC-31

Indicator strain	Potency	Diameter of inhibition area (mm)[a]	Inhibition area (mm)[a]
LC-31	–	0 d	0 f
LC-21	++	9.1 ± 0.1 a	65.0 ± 5.0 a
PS-1	+	8.5 ± 0.5 a	55.0 ± 4.9 b
PS-2	+	5.6 ± 0.7 b	24.0 ± 5.0 c
TAL 1826	+	3.0 ± 0.5 c	10.4 ± 1.0 de
TAL 1827	+/–	3.5 ± 0.5 c	10.4 ± 1.0 de
TAL 102	+/–	2.9 ± 0.5 c	5.0 ± 1.0 ef
MnS	+	2.8 ± 0.8 c	5.0 ± 1.0 ef
Ca18	+/–	3.2 ± 0.5 c	11.6 ± 1.52 d

–: Ineffective; +/–: less effective; +: effective; ++: highly effective.
[a] All the results are the means of triplicates. The correlation between the inhibition zone and area is 0.9, between inhibition zone and ARA is −0.03 and between area and ARA is 0.03. Values in columns followed by the same latter are not significantly different at $P = 0.05$ by Duncan's multiple range test.

lationship was not observed; no test strain inhibited its own growth, which is characteristic of the bacteriocin producers (Sidikaro and Nomura, 1974; Reeves, 1972; Hardy, 1975). Heteroantagonistic activity was quite heterogeneous. Five out of ten strains inhibited at least one of the indicator strains, in the heteroantagonistic assays. All strains of *Rhizobium* were found to produce antimicrobial activity, which inhibited the growth of the related strains on the agar medium. Marked bacteriocin production was observed by the *R. leguminosarum* bv. *viciae* strain LC-31, while there was progressively less production of bacteriocins in *R. leguminosarum* bv. *viciae* strains LC-12, LC-21, PS-1 and PS-2, respectively. *R. leguminosarum* bv. *trifolii* strains TAL 1826 and TAL 1827, *B. japonicum* MnS and TAL 102 and *Agrobacterium* strain did not show any bacteriocinogenic activity (Table 1).

The bacteriocin production was appeared after 48 h of incubation and reached at a maximum after 72 h of incubation. Further incubation does not affect the zone size, therefore, 72 h of growth of the producer colonies at 30 °C was considered as optimum conditions for bacteriocin production in this study.

Our results showed that when the sample is successively diluted, inhibition zone decreased until critical dilution is achieved when no inhibition of the sensitive organism observed (Table 2). The bacteriocin produced by the *R. leguminosarum* bv. *viciae* strain LC-31 showed a typical narrow spectrum activity, it was more effective against most closely related *R. leguminosarum* bv. *viciae* strains LC-12, LC-21 and PS-1 whereas the effect decreased against less related strains of *Agrobacterium* and *Bradyrhizobium* strains, respectively. These results supports the revised taxonomy of the family *Rhizobiaceae* in which the genus *Agrobacterium* has been incorporated with *Rhizobium* (Young et al., 2001), while the genus *Bradyrhizobium* has been allocated a separate family, i.e. *Bradyrhizobiaceae* (Garrity, 2001). The strains LC-12 and PS-1 also showed the bacteriocin production but these were effective only against the related strain LC-31. This may be due to the fact that different strains of *R. leguminosarum* bv. *viciae* have varied activity spectra which may be structurally and functionally variable (Oresnik et al., 1999; Wijffelman et al., 1983).

When the CFS bacteriocin produced in this study was tested against *Rhizobium*, *Brady rhizobium* and *Agrobacterium* strains. It showed the same activity pattern against these strains as the LC-31 strain itself (Table 3).

SDS-PAGE analysis of the protein isolated from LC-31 showed the presence of a 50 kDa which is associated with inhibitory activity as shown by well diffusion studies. This band was visible only in samples

Table 3
Purification of isolated bacteriocin protein from *R. leguminosarum* bv. *viciae* strain LC-31

Sample material	Potency	Diameter of inhibition zone	Inhibition area	Arbitrary units	Specific activity
Chloroform extract	+/–	12 ± 0.25	113	315	1.0
30% ammonium sulfate saturated pellet	+/–	7 ± 0.25	28	157	1.8
40% ammonium sulfate saturated pellet	+	8 ± 0.16	39	184	1.9
50% ammonium sulfate saturated pellet	+	11 ± 0.16	79	263	2.1
75% ammonium sulfate saturated pellet	++	12 ± 0.07	95	289	2.1
85% ammonium sulfate saturated pellet	–	0	0	0	0

–: Ineffective; +/–: less effective; +: moderately effective; ++: highly effective. All the results are means of triplicates.

isolated from 30 to 75% ammonium sulfate saturations of CFS (Table 3).

4. Conclusion

The results of this study has shown that bacteriocin production may play an important role in interspecific and intraspecific competition and also there great importance in the preparation of mix consortia to be used as biofertilizers. Any bacteriocin producing strain included in the commercial inoculants can inhibit the growth of all the other strains thus compromising the quality of the product. In this study, the *R. leguminosarum* bv. *viciae* strain LC-31 inhibited the growth of *Rhizobium* sp. and *Agrobacterium* sp. which is being added in the biofertilizers as phosphate solubilizer. This would be a disadvantage in the preparation of this type of consortia. So it is proposed in this study that the complete knowledge of bacteriocin production by the different strains of *Rhizobium* should be obtained before to be used in the fields as biofertilizers and also the effect of rhizobial bacteriocins on the microflora of the rhizosphere should also be investigated.

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