Properties of some Bacteriocins Produced by *Rhizobium trifolii*

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SUMMARY

Bacteriocins produced by six strains of *Rhizobium trifolii* were found to be of the relatively low molecular weight, non-phage type. The molecular weights ranged from approximately 1.8×10^5 to 2.0×10^5 . All were of protein composition, as indicated by buoyant density (1.32 to 1.34 g/cm³) in CsCl and by sensitivity to proteolytic enzymes. They were resistant to RNAase but sensitive to DNAase. The six bacteriocins could further be separated into two subgroups on the basis of sensitivity to extremes of pH, binding to filter membranes, activity spectrum on sensitive strains of *R. trifolii*, and possibly mode of action on sensitive bacteria. Bacteriocin production occurred spontaneously during the early- to mid-exponential phase of bacterial growth in broth culture.

INTRODUCTION

There have been several reports of bacteriocin production by the rhizobia (Roslycky, 1967; Schwinghamer, 1971; Lotz & Mayer, 1972; Schwinghamer, Pankhurst & Whitfeld, 1973) but little is known about the genetic determinants or about the nature of the substances. Two of the reports dealt with the structural nature of single bacteriocins, which were shown to be phage tail-like particles (Lotz & Mayer, 1972) or defective phage particles (Schwinghamer *et al.* 1973).

This paper examines some characteristics of other, smaller bacteriocins produced by *Rhizobium trifolii* which are clearly different from the phage-like types and may be a commonly occurring form in this species.

METHODS

Bacteria and media. The bacteriocinogenic (bacteriocin-producing) strains of *Rhizobium trifolii* and their sources are listed in Table 1. Indicator strains of *R. trifolii* are TA1, CC241 and CC276 from a strain collection maintained at C.S.I.R.O., Canberra, and T15 isolated from a clover nodule near Sutton, New South Wales, Australia. The one strain of *R. leguminosarum*, L6, was originally obtained as No. 128C5 from the Nitragin Co., Milwaukee, Wisconsin, U.S.A. All strains were subcultured and confirmed as rhizobia in nodule tests on the appropriate host plants.

The temperate phage, ϕ T10, was obtained from lysogenic strain T10 of *R. trifolii* and subsequently maintained on another strain of *R. trifolii*, T1.

A modified form of Bergersen's synthetic medium (Bergersen, 1961) was used for maintenance of strains, production of bacteriocins and assay of bacteriocin activity. The amounts of sodium glutamate and mannitol/l medium were reduced to 0.5 and 2.0 g, respectively, and yeast extract (Difco) was added (0.4 g/l) to stimulate growth.

Table 1. *Source and specificity of action of some bacteriocins (small, non-phage type) produced by Rhizobium trifolii*

Subgroup	Bacteriocin	Strain of bacteriocinogenic bacteria	Source (all in Australia) of bacterial strain	Activity of bacteriocin, on indicator strains:		
				TA1 or CC276	T15 or CC241	L6
Ia	782	CB782	CSIRO Canberra collection	+	—	+
	52	R52	Clover nodule, New South Wales	+	—	+
Ib	611	RO611	Clover nodule, Victoria	—	+	+
	111	RO111	Clover nodule, Victoria	—	+	+
	4	Tr4	Clover nodule, New South Wales	—	+	+
	11	Tr11	Clover nodule, New South Wales	—	+	+

Growth and bioassay of bacteriocins. For production of bacteriocins, bottles of broth were inoculated with fresh agar-grown bacteria and incubated for about 21 h on a shaker (110 rev./min at 29 °C). Cultures with satisfactory levels of bioactivity could be stored at 4 °C for up to several months with little loss of activity.

Samples to be assayed for bacteriocin activity were diluted, exposed to chloroform (1 drop/1 to 2 ml), and tested on appropriate indicator bacteria by standard spot-test procedures (Mayr-Harting, Hedges & Berkeley, 1972). Total activity of samples was expressed in arbitrary units (u) corresponding to the reciprocal of the dilution end-point for detectable spot activity. Occasional checks for possible phage activity were made for all bacteriocin-producing strains by standard phage plaque tests.

Concentration and partial purification of bacteriocins. Cultures with a satisfactory level of activity (i.e. dilution end-point ≥ 1000) were centrifuged at 10000 g for 25 min. Bacteriocins of subgroup Ib (Table 1) were precipitated from supernatant fluids by ammonium sulphate, although at much lower levels of activity than previously obtained with a phage-like bacteriocin (Schwinghamer *et al.* 1973). Solid ammonium sulphate was stirred into the chilled supernatant to the 45 to 48 % saturation level. After overnight storage at 4 °C the bacteriocins were sedimented by centrifugation at 24000 g for 1 h and resuspended in a small volume of 0.1 M-ammonium acetate. Ammonium sulphate concentrations below 45 % saturation gave lower yields of bacteriocin; concentrations greater than 48 to 50 % saturation gave improved yields but were unsatisfactory because of excessive precipitation of polysaccharides or other polymeric substances.

Bacteriocins produced by CB782 or R52 were only poorly precipitable by ammonium sulphate and were concentrated as follows: (i) rotary-vacuum evaporation (35 °C maximum) of the supernatant fluid to approximately one-eighth of the original volume; (ii) removal of the bulk of the polysaccharide material to reduce the viscosity of the concentrate, by treatment with DEAE-dextran (mol. wt 2 000 000; Pharmacia); (iii) further concentration (about fivefold) by membrane ultrafiltration, using XM-100A or XM-300 Diaflo membranes (Amicon Corp., Lexington, Massachusetts, U.S.A.); (iv) for maximum concentration, repetition of steps (ii) and (iii), with distilled water or phosphate buffer (0.005 M, pH 7.2) as diluent for the filtration steps. In the DEAE-dextran treatment, a 1.0 % aqueous solution of DEAE-dextran was added dropwise to the chilled viscous concentrate at about 1 drop/ml, with mild swirling, to induce 'thread' formation and aggregation of anionic polymers. The mucilaginous precipitate was removed by centrifugation at 12000 g for 15 min. In all of the above concentration procedures except rotary evaporation, the samples were maintained at

about 5 °C. Activity of these non-sterile concentrates could be maintained for several days at 4 °C, but for longer periods samples were stored frozen.

Equilibrium density gradient centrifugation in CsCl. Preliminary experiments had indicated that several of the bacteriocins banded near the centre of the tube when 30 % (w/w) CsCl was used, but prolonged exposure to this concentration of the salt resulted in loss of activity. A two-step, 4 ml initial gradient (Brunk & Leick, 1969) was therefore used to minimize inactivation of bacteriocins. The lower 2.0 ml step contained 40 % (w/w) CsCl (Sequanal Grade, Pierce Chemical Co., Rochford, Illinois, U.S.A.) in 0.015 M-tris-HCl buffer, pH 7.2; the upper 2.0 ml step contained 20 % (w/w) CsCl in tris-HCl buffer plus bacteriocin sample. The tubes were topped with a 1.2 ml layer of paraffin oil and centrifuged (Spinco SW 50 rotor, temperature 9 °C) at 165 000 g for 22 to 24 h. Fractions (9 drops) were collected through the bottom of tubes mounted in a puncturing device. Small portions of each fraction were diluted in broth and assayed to determine the bacteriocin peak. Buoyant density determinations were based on refractive index measurements (Abbe refractometer) taken for fractions on both sides of the activity peak. Fractions representing the peak of activity, as well as 'pre-peak' and 'post-peak' portions of the gradient, were pooled for further examination by electron microscopy or analytical ultracentrifugation.

Density-gradient centrifugation in glycerol. Glycerol density gradients (8 to 32 %, w/w) were prepared by carefully pipetting 1.15 ml layers of 32, 24, 16, and 8 % glycerol (in 0.01 M-tris-HCl, pH 7.3) into 5 ml cellulose nitrate tubes and allowing the solutions to diffuse at 4 °C overnight to produce a nearly linear gradient of density. Samples (0.3 ml) of bacteriocin concentrates or of reference enzymes were layered and the tubes centrifuged for the indicated time in an SW 50 rotor at 151 000 g and a rotor temperature of 9 to 10 °C. Fractions (8 to 10 drops, depending on the experiment) were collected and assayed as described for CsCl gradients.

The two reference enzymes used for molecular weight estimation – yeast alcohol dehydrogenase (2 × crystallized; Sigma) and bovine liver catalase (Sigma) – were dissolved in 0.02 M-phosphate buffer pH 7.2 at 1.5 mg/ml; the amount of enzyme added per gradient was therefore 450 µg. Alcohol dehydrogenase (ADH) activity in glycerol gradient fractions was measured by adaptation of a method used for detection of dehydrogenases in gels (see Vesell & Bearn, 1962). Enzymic oxidation of ethyl alcohol was followed colorimetrically (550 nm) in the presence of 0.6 mM-β-nicotinamide adenine dinucleotide (Sigma), 0.3 mM-*p*-nitroblue tetrazolium (Calbiochem), and 0.2 mM-phenazine methosulphate (Calbiochem) in 0.01 M-tris-HCl buffer, pH 8.8. Catalase activity was assayed by measuring the decrease in extinction at 240 nm per 30 s interval in a reaction mixture containing 0.2 % H₂O₂, 0.01 M-phosphate pH 7.0, and diluted samples of fractions being tested.

Sensitivity of bacteriocins to enzymes. Enzymes used for sensitivity tests were the following: lysozyme (3 × crystallized; Pentex Inc., Kankakee, Illinois, U.S.A.); ribonuclease (RNAase I, 1 × crystallized, beef pancreas; Sigma); deoxyribonuclease (DNAase I, beef pancreas; Sigma); protease (type VI, from *Streptomyces griseus*; Sigma); pronase (B grade; Calbiochem). Enzymes were added to supernatant fluids containing bacteriocins or to supernatant fluids concentrated 3- to 4-fold by ultrafiltration, with buffers and salts at concentrations as given in Results. Samples were incubated for 70 min at 31 °C, then assayed for residual bacteriocin activity. Control treatments included buffers alone and heat-treated (85 °C for 15 min) or autoclaved (103.5 kPa, 10 min) enzymes.

Sensitivity of bacteriocins to ultraviolet light. Bacteriocins were irradiated with u.v. light by exposing stirred suspensions for various times (0.33, 0.75, 1.5, 4 and 8 min) at a distance of 25 cm from a 15 W germicidal lamp (90 % emission at 253.7 nm).

Electron microscopy. Droplets of samples from CsCl or glycerol gradients were deposited on copper grids covered with carbon-coated parlodion film. These preparations were air-dried and negatively stained with 1.0 % phosphotungstic acid at pH 5.3 or with 1.0 % uranyl acetate at pH 4.4. Bacitracin (Nutritional Biochemical Co.) at 50 µg/ml was also used in some preparations as a low molecular weight spreading agent (Gregory & Pirie, 1973). Samples from peak activity fractions from CsCl gradients were examined directly, without dialysis pre-treatment, after 'washing' the air-dried grid preparations once with a droplet of 0.1 M-ammonium acetate to remove most of the CsCl. Samples from glycerol gradients could be examined directly without this wash treatment. Grids were examined in a Philips EM 200 electron microscope at 80 kV.

RESULTS

Production of bacteriocins and strain sensitivity

The six strains of *R. trifolii* listed as bacteriocinogenic in Table 1 have all been shown in repeated tests to produce antibacterial (growth-inhibiting) substances which are active against at least one-third of the strains of *R. trifolii* tested as indicators but, as expected, do not inhibit the producing strains. Previous work (Schwinghamer, 1971) had suggested these substances to be bacteriocin-like in that they were not dialysable and did not produce plaques. The results summarized here mainly compare substances produced by the first four strains given in Table 1, particularly CB782 and RO611. Bacteriocins produced by these two strains are considered to be representative of the two subgroups, Ia and Ib, which are readily distinguishable by their reciprocal specificity of action on some indicator strains (e.g. TAI and CC276 as compared with T15 and CC241).

Bacteriocin was readily produced during growth on agar or in broth, although the latter medium was used almost exclusively for harvesting purposes. There was no obvious increase of bacteriocin production after u.v. irradiation (253.7 nm) of strains CB782 or RO611, both of which thus appear to be non-inducible. Bacteriocin production in variously modified broth media appeared to parallel growth, being low in the synthetic medium without yeast extract and maximum when yeast extract was added at about 0.4 g/l. The level of mannitol was kept low and aeration was limited to shaking at 110 rev./min to minimize polysaccharide production.

Bacteriocin production by strains CB782 and RO611 was measured during growth in broth (Fig. 1). The doubling time for the bacteria under the conditions of this experiment was about 4.8 h for CB782 and 5.2 h for RO611. In contrast, the doubling time for bacteriocin production during the near-linear part of the total activity curve was only 2.2 h for CB782 and 2.5 h for RO611. In another experiment, with strain CB782 only, the total activity peaked similarly but showed no significant decline even when the culture was grown for 38 h. Most broth cultures, inoculated to a density of about 2×10^7 to 3×10^7 cells/ml, were therefore grown (at 29 °C) for only 19 to 21 h. Longer incubation not only failed to give higher yields of bacteriocin but also complicated bacteriocin concentration because of the accumulation of high levels of polysaccharides or debris in fully grown cultures.

Concentration of bacteriocins

Since these bacteriocins were smaller than the readily sedimentable phage-like bacteriocins, separation from polysaccharides (produced in abundance by rhizobia) was very difficult. More than 80 % of the active substance was sedimented in a fixed-angle rotor at 100 000 g for 8 to 10 h, but large amounts of polysaccharide-like material pelleted simultaneously and could not be effectively separated by differential centrifugation. Preliminary attempts to

Fig. 1

Fig. 1. Growth of bacteria and production of bacteriocin by *R. trifolii* strains CB782 and RO611. Bacteria were inoculated into broth (initial concentration about 2×10^7 cells/ml; see Methods) and grown in a shaker bath at 29 °C. ●, Growth; ▲, bacteriocin activity. Broken lines, 782 and CB782; solid lines, 611 and RO611.

Fig. 2

Fig. 2. Activity of bacteriocin 782 after centrifugation to equilibrium in a two-step (20 and 40 %, w/w) CsCl gradient. Centrifugation was in a Spinco SW 50 rotor at 165 000 g for 24 h. Fractions under braced zones were pooled for examination by electron microscopy. Vertical arrow indicates the peak of activity. ▲, Bacteriocin activity; ○, buoyant density.

remove the active agent selectively from culture supernatants by biphasic separation procedures (e.g. polyethylene glycol–dextran sulphate) or by ion exchange resins also failed, apparently due to strong binding properties of the bacteriocins, particularly those of sub-group Ib. Precipitation by ammonium sulphate, at a range of salt concentrations and pH levels, was poor with bacteriocins 782 or 52 and only moderately effective with 611, due to co-precipitation of some viscous materials and to partial loss of activity in the salt solutions.

Removal of polysaccharides by the polycation DEAE–dextran (Dudman, 1972) provided the most satisfactory means of reducing viscosity to workable levels, especially for the 782-type of bacteriocin, despite the associated loss of some activity. Under favourable conditions, by alternate DEAE treatment and concentration by ultrafiltration, 500 ml of culture supernatant fluid with an activity of about 2000 u/ml could be reduced to a 5 ml volume of moderate viscosity with an activity of about 40 000 u/ml (i.e. at an efficiency of about 20 %). At least 10 000 u/ml was considered desirable for monitoring the bioactivity of density gradient fractions, but a considerably higher concentration would be needed for spectrophotometric examination or other analytical procedures.

Filterability of bacteriocins

Filtration experiments with bacteriocins 782 and 611 (Table 2) as well as bacteriocins 52, 4 and 11 suggested a molecular weight of roughly 200 000 to 300 000 and a size of 10 to 20 nm, assuming reasonably spherical particles.

The portion of 782 activity which passed through XM-300 or XM-100A membranes was

Table 2. *Filterability of bacteriocins 782 and 611, with and without pretreatment*

Bacteriocin	Type of filter membrane	Pre-filtration treatment used to alter filterability	Activity in filtrate (%)
782	Millipore		
	0.45 μ m	None	100
	0.01 μ m	None	< 5
	Diaflo*		
	XM-100A	None	5
	XM-300	None	20
	Millipore	Filtrate from XM-100A or XM-300	< 5
	0.01 μ m	Sonication or blending	< 10†
	0.01 μ m	Triton X-100 or Brij-58 (0.3 %, v/v)	15-30†
	0.01 μ m	XM-100A filtrate of detergent-treated sample	70
	0.01 μ m	Urea, 5 M	< 5
	0.01 μ m	pH lowered to 4.2	< 5
611	Millipore		
	0.45 μ m	None	0‡
	0.45 μ m	pH lowered to 4.0	> 50
	0.01 μ m	pH lowered to 4.0	< 5
	Diaflo, * XM-100A	None	< 5

* Nominal molecular weight cut-off levels for XM-100A and XM-300 are approximately 100000 and 300000, respectively.

† Similar results obtained with the XM-100A membrane, for the sonication-blending or detergent treatments.

‡ Adsorbed.

retained by the 0.01 μ m Millipore filter, indicating variation in pore size of the former, rather than wide variation in size of the bacteriocin particles. Mild sonication or blending of samples did not induce filterability, but the non-ionic detergents Triton X-100 or Brij-58 increased filterability significantly and the fraction appearing in the filtrate appeared to filter even more readily. Urea did not increase filterability. It appears likely that at least a portion of the active material is associated with detergent-soluble bacterial fragments.

Bacteriocins of subgroup Ib differed clearly from those of subgroup Ia by binding strongly to Millipore membranes and were not recovered when 0.1 to 0.5 M-NaCl was passed through the inverted filter. This adsorption could, however, be minimized and the material made partly filterable through the 0.45 μ m filter by lowering the pH of the samples to 4.0.

Buoyant density in CsCl and electron microscopy of peak activity fractions

The bioactivity profile for fractions collected from a typical CsCl equilibrium centrifugation of a bacteriocin 782 sample is shown in Fig. 2. The accuracy of determination of buoyant density by bioassay monitoring was ± 0.01 g/cm³ for all experiments. The mean buoyant density values for bacteriocins 782, 52, and 611 are given in Table 3. These values indicate a protein composition, albeit a relatively 'heavy' protein particle which could contain a small amount of denser, non-protein material.

Peak activity fractions examined by electron microscopy contained large numbers of flagella and a very few phage-like particles, but no other identifiable particles. Pre-peak and post-peak pooled fractions were similar except for a much smaller number of flagella or flagellar fragments. However, the flagella and the active material were readily separable by glycerol gradient centrifugation. The buoyant density in CsCl of the active, flagella-free peak material obtained from a glycerol gradient containing bacteriocin 782 was found to be 1.35 g/cm³, almost identical with that of bacteriocins in crude concentrates containing flagella.

Table 3. *Buoyant density and approximate molecular weight of bacteriocins*

Details of procedure for determination of buoyant density in CsCl and of molecular weight by sedimentation in glycerol gradients are given in Methods. Molecular weights of yeast ADH and of catalase were taken as 150 000 and 250 000 respectively (Martin & Ames, 1961).

Bacteriocin	Buoyant density* (g/cm ³)	Molecular weight*	
		ADH as reference molecule	Catalase as reference molecule
782	1.34 ± 0.03 (11)	176 000 ± 3000 (2)	178 000 ± 9000 (4)
52	1.33	—	179 000 ± 6000 (2)
611	1.32 ± 0.01 (4)	212 000	196 000 ± 3000 (2)
4	—	207 000	195 000
Reference molecules ADH (yeast)	—	—	142 000 ± 1000 (3)
Catalase	—	265 000 ± 1000 (3)	—

* Standard error and number of determinations (in parentheses) are given for means; other values represent single determinations.

A high activity sample obtained from pooled peak fractions of several preparative CsCl centrifugation experiments was treated with sodium dodecyl sulphate (3 %, w/v) and examined for the presence of nucleic acid by analytical centrifugation in CsCl, a procedure used previously to detect DNA in bacteriocin 37 (Schwinghamer *et al.* 1973). No definite band of material absorbing at 260 nm could be detected, but a disperse background level of absorbance, presumably due to dissociated flagellar material, could have masked the presence of a small amount of nucleic acid.

Molecular weight of bacteriocins

Polysaccharide could be readily pelleted and separated from the bacteriocins in CsCl gradients but not in sucrose or glycerol gradients. However, when DEAE-dextran was used to remove the bulk of the interfering polysaccharides, sucrose (5 to 20 %) and glycerol (8 to 32 %) density gradients could be used successfully to determine approximate molecular size. Glycerol was used for all the experiments described because of the somewhat better retention of bacteriocin activity than in sucrose, and because it is more suitable for direct observation of non-dialysed fractions by electron microscopy.

The distribution of catalase, yeast ADH, and bacteriocin 782 was compared after 6 h of centrifugation in glycerol gradients (Fig. 3). Bacteriocin and enzyme samples were layered on separate gradient tubes to avoid interaction or interference between these materials. Adding a mixed sample of catalase and 782 (or 52) caused the bacteriocin peak to move much faster and to spread out over a wide range of density. The 'distance' of migration in these experiments was taken as the number of drops from the midpoint of the sample layer to the peak of activity (estimated to the nearest drop on the basis of the symmetrical part of the activity curve).

The relationship between migration distance and time of centrifugation under a given set

Fig. 3

Fig. 3. Activity of bacteriocin 782 (▲) and the two reference enzymes, catalase (●) and yeast ADH (■), after centrifugation for 6 h in a 8 to 32 % (w/w) glycerol gradient at 151 000 g. Arrows indicate the estimated peaks of activity, from which migration 'distance' (no. of drops between the peak and the mid-point of the added sample layer) was determined. Refractive index (x) measurements indicate linearity of density.

Fig. 4

Fig. 4. Migration 'distance' of bacteriocin 782 (▲) and two reference enzymes, catalase (●) and yeast ADH (■) in a glycerol density gradient, as a function of time of centrifugation. Samples (0.3 ml) were layered on separate glycerol gradients (8 to 32 %, w/w) and centrifuged in a Spinco SW50 rotor at 151 000 g and 9 °C. Each point represents the migration values as determined from the assay of fractions of one gradient, as in Fig. 3.

of experimental conditions (temperature, speed, type of rotor, type of gradient) is shown in Fig. 4 for bacteriocin 782 and the two reference enzymes. Migration was essentially linear for all three substances in the 4 to 8 h range, and further experiments (with 782 and other bacteriocins) were therefore done within this range of centrifugation time. The faster movement during the first 2 h may result from the relatively greater dilution of the upper portion of the gradient by the sample, an effect that became minimal after the materials had moved through this zone and linearity in glycerol density was established.

Approximate molecular weights were calculated from the ratio of the migration distance of a bacteriocin relative to that of the reference enzymes (Martin & Ames, 1961), for four bacteriocins (Table 3). Bacteriocins 782 and 52 (mol. wt about 180 000) appeared to be somewhat smaller than 611 and 4 (mol. wt about 200 000). These determinations are only approximations since they do not involve any measurements of partial specific volume or shape of molecule, but the reasonably close agreement of calculated and known molecular weight for the two reference enzymes indicates that the gradient conditions were suitable for estimation of bacteriocin size. The estimated molecular weights of the bacteriocins are also in reasonable agreement with the data from filtration experiments.

Sensitivity to enzymes

All of the bacteriocins were resistant to treatment with lysozyme (300 µg/ml; 0.02 M-NaCl + 0.01 M-phosphate buffer, pH 6.8) or RNAase (200 µg/ml; 0.01 M-tris-HCl, pH 7.2) for 70 min at 31 °C. They were fully sensitive to protease or pronase (20 µg/ml; 0.01 M-CaCl₂ + 0.005 M-tris-HCl, pH 7.3), as expected from the protein composition indicated by their buoyant density in CsCl but, unexpectedly, were also sensitive to DNAase (30 or 50 µg/ml;

Table 4. *Effect of buffer or salts background on the sensitivity of bacteriocin 782 to DNAase and protease*

The inhibitory effect of EDTA on activity and its prevention by prior exposure to CaCl_2 also occurred with bacteriocins 52, 611 and 4; the effect of Ca^{2+} or tris-HCl on DNAase action was tested only with 782.

Materials and sequence of addition*	Bacteriocin activity		
	Without enzyme	With enzyme	
		DNAase	Protease
Tris-HCl, enzyme	+	+	—
Phosphate buffer, enzyme	+	—	—
Phosphate buffer, CaCl_2 , enzyme	+	+	—
CaCl_2 , enzyme	+	+	—
Phosphate buffer, EDTA	—		
Phosphate buffer, EDTA, CaCl_2	—		
CaCl_2 , EDTA, enzyme	+	+	—
NaCl or KCl, EDTA	—		

* MgSO_4 was added to the bacteriocin in all treatments, before the addition of other materials. The reagents were used at the following concentrations: MgSO_4 , 5 mM; tris-HCl, 5 mM; phosphate buffer, 5 mM; CaCl_2 , 5 mM; EDTA, 1 mM; NaCl or KCl, 20 mM; DNAase, 50 $\mu\text{g}/\text{ml}$; protease, 20 $\mu\text{g}/\text{ml}$. All treatments at pH 7.2, 31 °C, for 70 min. Autoclaved enzyme controls did not inactivate the bacteriocins.

0.005 M- MgSO_4 + 0.005 M-phosphate, pH 7.2). Inactivation of bacteriocins 782 and 611 by DNAase was observed in repeated experiments involving two enzyme stocks and bacteriocin samples obtained from different subcultures of strains CB782 and RO611. The possibility of protease contamination in the DNAase samples was examined by comparing the enzymes in different buffer or salt backgrounds (Table 4). With tris buffer or CaCl_2 the bacteriocins were inactivated by protease but not by DNAase, suggesting that protease contamination was not involved in the DNAase results. EDTA could not be used as a chelator of Mg^{2+} ions to inhibit DNAase because it was strongly inhibitory to the bacteriocins; Ca^{2+} protected the bacteriocins when added before, but not after, the EDTA. The monovalent cations Na^+ or K^+ did not protect them.

Results very similar to those of Table 4 were obtained when no MgSO_4 was added to the bacteriocin-salt-enzyme mixtures. MgSO_4 was present in the original culture medium at a concentration of 0.4 mM, and apparently the level of Mg^{2+} remaining in the unconcentrated bacteriocin samples or even in samples concentrated by membrane ultrafiltration was adequate for DNAase activity.

Ethidium bromide binds to DNA and has been reported to inhibit DNAase I (Eron & McAuslan, 1966). Bacteriocin 782, incubated for 20 min in DNAase buffer with ethidium bromide at 10 or 100 $\mu\text{g}/\text{ml}$ (not inhibitory to the bacteriocin) before addition of DNAase, remained sensitive to the enzyme, suggesting that there is no DNA in the bacteriocin.

Some other properties of the bacteriocins

There was no apparent difference between bacteriocins of the two subgroups in their sensitivity to heat treatment. All were heat-labile, with at least 90 % of the activity being destroyed by heating at 60 °C for 15 min. Those of subgroup Ia were more tolerant to alkaline conditions and retained full activity within a pH range of about 4.2 to 10; bacteriocins of subgroup Ib were more tolerant to lower pH and retained their activity within a pH range of about 3.5 to 9.

The sensitivity of bacteriocins 782 and 611 to u.v. radiation was compared with that of

rhizobia phage ϕ T10. There was no measurable loss of bacteriocin activity even at the maximum exposure of 8 min where survival of the phage was less than 0.01 %, suggesting absence of functional nucleic acid in the bacteriocins.

Observations on mode of action of the bacteriocins were limited to a few experiments in which bacteria of sensitive strains were either grown in the presence of added bacteriocin or grown in mixed culture in the presence of bacteria of a bacteriocinogenic strain. Under these conditions, the action of bacteriocins 782 and 52 on bacteria of strain TAI appeared to be largely bacteriostatic. Bacteriocins 611 and 4 were strongly bactericidal to bacteria of strain T15. The two sub-groups of bacteriocins may therefore differ in mode of action as well as in other characteristics already noted (specificity of action, filterability, and tolerance to extremes of pH).

DISCUSSION

There are at least two types of bacteriocins or bacteriocin-like substances produced by *R. trifolii*, differing in structure or composition. The first type is represented by defective phage particles (Schwinghamer *et al.* 1973). The two strains producing these particles were both inducible by u.v. light and the particles were very similar in morphology and in buoyant density in CsCl. The second main type includes the lower molecular weight (about 180000 to 200000) bacteriocins described here. Although similar in size, buoyant density, and sensitivity or resistance to some enzymes, these bacteriocins differ sufficiently (e.g. in specificity of action and adsorption to filter membranes) to warrant further separation into two subgroups. The smaller type of bacteriocin occurs more commonly than the phage-like type among the strains of *R. trifolii* which were found to produce bacteriocin-like substances in culture, but nothing is known about the prevalence or relative importance of either type in nature.

The morphology of these 'small' bacteriocins could not be determined. Further concentration and purification will be needed for positive identification of the active substance by electron microscopy. The buoyant density and sensitivity to protease indicate protein (a characteristic of all known bacteriocins) but there may also be a minor non-protein moiety (carbohydrate, lipid or nucleic acid), as indicated for some bacteriocins of other bacteria (Bradley, 1967; Reeves, 1972). In particular, sensitivity to DNAase provides indirect evidence for DNA being present and essential for activity, although it is difficult to reconcile the presence of functional DNA in a molecule of molecular weight 200000 and of buoyant density (in CsCl) 1.34 g/cm³. The possibility of some proteolytic enzyme activity in the DNAase samples was discounted but not fully excluded in the experiments. Non-enzymic loss of bacteriocin activity as a result of non-specific complexing with nuclease, known to occur with RNAase and some RNA viruses (Loring, 1942), is possible even though dilution of DNAase-treated samples did not promote the expected recovery of bacteriocin activity. DNA has been shown to occur only in some of the large, phage-like bacteriocins, and bactericidal action appears to be restricted to the protein component of these particles (Bradley, 1967; Reeves, 1972).

Aside from sensitivity to DNAase, the bacteriocins described are exceptional also in their sensitivity to EDTA, in their interaction with catalase in glycerol gradients, in the effect of pH or non-ionic detergents on their membrane filtration behaviour, and in the apparently bacteriostatic effect of bacteriocins 782 and 52 on sensitive bacteria. A bactericidal mode of action is characteristic of most bacteriocins produced by other genera of bacteria.

The possibility that antibacterial substances may confer on the producing strains of rhizobia a competitive advantage under field conditions was briefly considered in the

previous reports for antibiotics, phage, or bacteriocins (Schwinghamer, 1971) and for a phage-like bacteriocin (Schwinghamer *et al.* 1973). The lower molecular weight forms of bacteriocins described in this paper could conceivably also play a significant role in the intense growth competition between rhizobia in the rhizosphere or in the rhizoplane of legume root systems. Production of these bacteriocins occurs spontaneously during growth of the bacteria in a comparatively non-rich medium, without the added requirement for some inducing agent normally present in the soil or excreted by the plant roots. Preliminary experiments with growth of a mixture of bacteriocinogenic bacteria and sensitive bacteria in broth suggest a considerable degree of suppression of the sensitive bacteria. It remains to be demonstrated, however, that these agents are produced abundantly under natural conditions and do in fact enhance the capacity of a strain to outgrow other rhizobia in the rhizosphere and nodulate the homologous legume host plant.

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