Bacteriocins and Phages Produced by *Serratia marcescens*

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SUMMARY

One hundred and thirty-nine strains of *Serratia marcescens* were investigated for bacteriocin and phage production. Two types of bacteriocins were detected. Group A were all active on serratias and were produced by 71 strains. Some of these bacteriocins also attacked *Salmonella*, *Escherichia* and *Aerobacter* strains. They were produced spontaneously in broth, and higher titres were produced by induction with ultraviolet (u.v.) radiation. These bacteriocins are divided into eight subgroups by their spectrum of activity and resistant mutants of indicator strains. Group B bacteriocins are not active on serratias. Their action is restricted to *escherichias*, *hafnias* and *aerobacters*. Mutant bacteria resistant to any one of these B agents are resistant to all other B bacteriocins. The B agents are serologically similar and are produced in broth by 54 strains. They are produced spontaneously and higher titres are produced after u.v. induction. With one exception the B bacteriocins may be identical. Thirty-five strains produced both types of bacteriocins. The A bacteriocins are resistant to chloroform, trypsin and the proteolytic enzymes of certain organisms, are heat-stable and non-dialysable. Group B bacteriocins are heat-labile, non-dialysable and inactivated by chloroform and the above enzymes. Agents A and B differ from colicins. Thirty-seven of the strains were lysogenic for *Serratia* species and 19 of the phages productively lysed *Salmonella* species. Although overlaps did occur it was always possible to distinguish the bacteriocins from the corresponding temperate phage produced by a strain, by means of their spectra of activity.

INTRODUCTION

Strains of *Serratia marcescens* may produce at least three antibacterial substances. Fuller & Horton (1950) described an antibiotic-like substance, marcescin, which has an inhibitory action on *Corynebacterium diphtheriae* and *Staphylococcus aureus*. Hamon & Peron (1961) and Mandel & Mohn (1962) described bacteriocinogenic activity among strains of *S. marcescens*; Prinsloo & Coetzee (1964) found 15 of 31 strains of *S. marcescens* to be lysogenic for other *S. marcescens* and *Salmonella* strains. Fredericq (1954) showed that colicinogenic factors were transmissible to strains of other *Escherichia coli* and shigellas while Amati & Ozeki (1962) transmitted colicinogenic factors E₁, E₂ from *Salmonella typhimurium* to serratias. In view of the genetic importance of phage and bacteriocinogenic factors, these agents were investigated in a series of 139 *Serratia marcescens* strains.

METHODS

Bacteria. These comprised 15 strains of *Serratia marcescens* obtained from the National Collection of Type Cultures, NCTC 1377, 2446, 2847, 4618, 8015, 3084, 8706, 8900, 9493, 9940, 9741, 9743, 10036, 10211, 4619; one American Type Culture strain ATCC 9986; 123 local strains isolated from human and insect sources. The 123 strains could be differentiated by lysogenicity and phage-susceptibility (Prinsloo & Coetzee, 1964; H. E. Prinsloo, unpublished). The strains were biochemically typical (Martinec & Kocur, 1961) except for sm8 which was maltose-negative, and sm52 which was sucrose-negative, and were either non-pigmented or pigmented. Standard colicinogenic strains which produced colicins A to K, V and S1-4 were obtained from Professor P. Fredericq. The indicator used for colicins was *Escherichia coli* ϕ , while the following bacterial strains were used to determine the spectrum of activity of the antibacterial agents produced by *S. marcescens*: all the *Serratia* strains, the colicinogenic strains of Fredericq, *Hafnia* spp (H_{1-6}), *Aerobacter aerogenes* (x_{1-7}), *Alcaligenes faecalis* (NCTC 8769), *Proteus hauseri*, *P. morganii*, *P. rettgeri* and the providence cultures previously used (Coetzee, 1963*a*). In addition, 12 *Salmonella typhi* strains of different Vi phage types were used as well as 28 *Salmonella* serotypes belonging to each of the Kauffman-White groups and 10 unclassified strains, 24 *Shigella* strains of different serotypes, 10 strains of *Staphylococcus pyogenes*, 10 *Bacillus* strains, and one toxigenic *Corynebacterium diphtheriae*. The following proteolytic organisms were used: *Bacillus cereus* strain B2, *S. marcescens* sm1, 5, 8, 9, 11, 41, 52, NCTC 2847, *P. mirabilis* strain 13-0 (Coetzee, 1963*b*). The bacteria were maintained on nutrient agar slopes at 4°. Cultures were incubated at 32°.

Media. Oxoid No. 2 nutrient broth was used. The solid media contained 1.1% (w/v) agar, 0.8% NaCl (w/v), 1.3% (w/v) tryptone, 0.2% (w/v) glucose. The soft top layer contained 0.6% (w/v) agar. When *str-r* mutants were used as indicators for bacteriocins streptomycin sulphate 1 mg./ml. was added to media.

Ultraviolet radiation source. A 30 W. Hanovia germicidal lamp, which delivered more than 80% of its output at a wavelength of 2537Å, was used at a distance of 12 inches.

Procedures. Streptomycin-resistant mutants of sensitive bacterial strains were selected by plating about 10^{11} organisms on streptomycin agar. After incubation resistant colonies were restreaked on this medium for purification. The optimal temperature for bacteriocin production was determined by incubating 0.1 ml. of overnight cultures of sm1, 5, 8, 9, 11, 41, 91, 99, ATCC 9986, NCTC 2847 in 7.5 ml. broth for 25 hr at 20°, 23°, 26°, 28°, 30°, 34°, 37°. After centrifugation, dilutions of the clear supernatant fluids were spotted on streptomycin agar which had previously been spread with the streptomycin-resistant indicator in a soft agar overlay; the plates were incubated at 32°.

Induction by ultraviolet radiation. Five ml. samples of broth cultures were u.v.-irradiated in Petri dishes in the dark; 0.1 ml. of the u.v.-irradiated suspensions was then added to 7.5 ml. broth and incubated in the dark at 30° for 24 hr. The general phage techniques used were those of Adams (1959). Turbidity measurements were made with a Fisher Electrophotometer, with a 425 m μ filter. Colonies resistant to inhibitory substances were picked from the zones of inhibition in the lawns of growth. The bacterial action of inhibitory substances was determined by subculturing.

ing from the clear zones to nutrient agar; when no growth occurred it was taken that the substances were bactericidal. Heat inactivation was studied by heating 1 ml. samples of the inhibitory solutions in a water bath at various temperatures for various intervals of time, and then testing for activity.

Inactivation by trypsin was determined by adding 0.2 ml. of a 1 % (w/v) trypsin solution (British Drug Houses) in a 0.1 M-phosphate buffer (pH 7.4) to 1 ml. of bacteriocin solution. The mixture was kept at 37° for 1.5 hr and then tested for activity of the bacteriocin.

Many *Serratia* strains are lysogenic (Prinsloo & Coetzee, 1964) and bacteriocin preparations may therefore also contain phage. Bacteriocins were demonstrated by their greater diffusibility as follows. Clear supernatant fluids obtained after induction were placed in holes in nutrient agar. After 18 hr at room temperature the surface was covered with soft agar which contained the indicator organism and incubated. Samples were then also taken from the periphery of these zones of inhibition, suspended in 1 ml. broth and retested. The absence of transferable activity confirmed the bacteriocin-like nature of the substances. Bacteriocin anti-serum was obtained by injection of 2 ml. bacteriocin broth solution (not necessarily free from phage) subcutaneously into rabbits twice weekly for 6 weeks. Neutralization tests with the antisera were done by mixing 0.1 ml. antiserum with 0.9 ml. bacteriocin solution. The mixture was kept at 37° for 1 hr and then tested for activity. Controls had normal rabbit serum in place of antiserum. Chloroform sensitivity was tested by adding 0.5 ml. chloroform to 2.5 ml. antibiotic solution; the mixture was shaken and left at room temperature for 15 min. After the bacteriocin solution was decanted and bubbled with sterile air at 37° to free it from chloroform, its activity was tested.

Chloroform sterilization of antibacterial solutions was not generally used. Instead, clear supernatant fluids from u.v.-induced suspension were spotted on streptomycin-resistant indicator organisms on streptomycin agar.

Inactivation of the antibacterial solutions by gelatin-liquefying strains was tested by the method of Wahba (1963). Proteolytic strains were grown in nutrient agar for 24 hr at 32°. Bacteriocin solutions were spotted over the proteolytic strains and were incubated overnight. The plates were covered with *str-r* indicator organisms in the top layer containing streptomycin. The presence of bacteriocins in broth cultures of SM1, 8, 9, 11, 41, 91, 99, NCTC 2847 incubated at 30° were tested after 24, 72, 144 hr for autodestruction by autologous proteolytic enzymes. Bacteriocin solutions were dialysed against distilled water for 24 hr at room temperature.

The lysogeny of *Serratia* strains was investigated as before (Prinsloo & Coetzee, 1964) and all strains were tested for phage susceptibility. Attempts were made to lysogenize susceptible *Salmonella* strains with *serratia* phages. These procedures and also tests for immunity of clones to superinfection with phage were done according to Levine (1957). Dilutions of phage suspension and host bacteria were plated on nutrient agar with phage antiserum. Individual colonies were tested for phage production and resistance to phage. Phage multiplicities of input of 1, 5 and 10 were used; the experiments were done at 32°.

RESULTS

According to the spectrum of activity of the bacteriocins two groups were distinguished.

Group A. These agents resembled bacteriocins in their narrow spectrum of activity and were produced by 71 strains in titres up to 1/100. Some strains were more active in the production of bacteriocins than others. They were all active on *Serratia* and some of them were also active against some salmonella strains and *Escherichia coli*.

Group B. These substances were not active on *Serratia marcescens*. They have a spectrum of activity restricted to *Escherichia coli*, *Aerobacter aerogenes* and the *Hafnia* species. They are produced by 54 strains. Titres up to 1/100 were obtained. Strain ATCC 9986 differed in that its bacteriocin did not attack *E. coli* strains.

Table 1. *Bacteriocinogenic activity of Serratia marcescens strains*

A group activity	A and B group activity	B group	Non-bacteriocinogenic
Strain no.			
48, 52, 53, 58, 59,	1, 4, 6, 7, 9, 11,	3, 5, 10, 12, 14, 15,	51, 56, 57, 60, 61,
73, 101, 102, 103,	19, 37, 38, 41, 42,	18, 39, 40, 45, 47,	62, 63, 64, 65,
107, 108, 109, 112,	43, 44, 91, 99, 111,	74, 75, 130, 161,	66, 67, 68, 69, 70,
118, 119, 120, 121,	116, 125, 127, 139,	172, NCTC 2446,	71, 72, 76, 77, 78,
122, 123, 124, 129,	140, 149, 150, 157,	9493, 9743	79, 104, 105, 106,
131, 132, 133, 134,	160, 170, 174, 190,		108, 113, 114, 115,
135, 136, 137, 144,	200, 207, ATCC		117, 138, 143, 146,
146, 148, 156, 176,	9986, NCTC 1377,		159, 171, 178, 180,
177, NCTC 8015,	2847, 9940, 10211		184, 185, 194, 201,
9741			202, 205, 208, 209,
			NCTC 8900, 3084,
			4618, 8706, 10036,
			4619

The agents of groups A and B were produced in broth at an optimum temperature of 30°. Bacteriocins were also produced at 20, 23, 26, 28, 34°, and most strains did not produce bacteriocins at 37°. Group B bacteriocins were produced spontaneously in broth but the concentration was greatly increased by ultraviolet irradiation. Suspensions were u.v.-irradiated for periods of from 5 sec. to 10 min. In no instance was mass lysis observed and turbidity measurements increased steadily with time. This also applied to strains which were both bacteriocinogenic and lysogenic. It was found that 45 sec. of u.v.-irradiation of an overnight broth culture incubated in broth in the dark usually yielded maximal titres. Only 32 of the 73 bacteriocinogenic *Serratia* strains investigated by Hamon & Peron (1961) produced detectable bacteriocins in agar. The remaining 41 strains only liberated bacteriocins after u.v.-induction of broth cultures. As seen in Table 1, 35 strains which produced A agents also produced B agents. This double activity was determined by the use of appropriate indicator organisms. Thus in the case of the A bacteriocins *Serratia* indicators were used, and for group B agents *Hafnia* indicators. Overnight as well as exponentially growing cultures were used. With strains which produced both A and B bacteriocins the B agent was usually detectable in supernatant fluids about 1 hr before A activity appeared. In no instance did a strain produce an agent which

was active on itself like the colicin which Ryan, Fried & Mukoi (1955) encountered, and the strains of *Pasteurella pseudotuberculosis* and *P. pestis* which are pestinogenic for pesticin II were also sensitive to it.

The bacteriocins were bactericidal. The bacteriocins of group A were resistant to trypsin and chloroform and withstood 60° for 30 min. They were non-dialysable. These agents were resistant to the action of proteolytic organisms but the bacteriocin liberated by sm 52 was thermolabile, sensitive to trypsin, and completely inactivated by proteolytic organisms. Group B substances were sensitive to trypsin and chloroform and inactivated at 60° for 30 min. They were non-dialysable. These B substances were inactivated by autologous proteolytic enzymes when tested by the method of Wahba (1963). In broth cultures the titre of the B bacteriocins was decreased after 72 hr incubation and could not be demonstrated after 144 hr.

Table 2. *Subdivision of A group bacteriocins of Serratia marcescens*

Bacteriocin produced by	Indicator strains of <i>S. marcescens</i>				Mutants of indicator strains				Sub- division
					SM. 42 resistant to		SM. 8 re- sistant to		
	SM. 42	8	NCTC 8900	1	41	41 + 8	11	99	
1, 6, 125, 127	+++	.	+++	.	+++	+++	.	.	A ₁
9, 91, 199, 122, 139, 177, 200, NCTC 10211	+++	.	.	.	+++	+++	.	.	A ₂
8, 44, 48, 101, 116, 121, 123	+++	.	.	.	+++	—	.	.	A ₃
11, 38, 43, 58, 73, 99, 137, 144, 146, 148, 157, 176, 190, 207	.	+++	+++	.	.	.	—	—	A ₄
41, 136, 140	+++	+++	+++	+++	—	—	.	.	A ₅
NCTC 2847, 37	+++	+++	+++	.	+++	+++	.	.	A ₆
ATCC 9986	+	.	++	+++	.	.	.	.	A ₇
52	+	+	.	+++	.	.	.	.	A ₈

+++ , clear spots; ++ , slightly turbid spots; + , turbid spots; — , negative.

Subdivision of antibiotics

Group A bacteriocins. Resistant mutants of indicator strains were obtained against most of the A bacteriocins apart from those which produced turbid areas of inhibition from which it was not possible to select mutants. Table 2 shows that the A bacteriocins can be differentiated into eight types by their activity on various resistant mutants. This division is supported by the results given in Table 3. Thus A₆ is the only bacteriocin active on salmonellas, A₁ and A₈ are active on *Aerobacter aerogenes* x₁, and bacteriocin A₁ kills *Escherichia coli* ø. The bacteriocins of 40 of the 71 strains which produce the A substances have been subdivided. The remaining strains either produce the bacteriocin in low titres or they do not correspond to any of the eight subtypes.

Group B bacteriocins. Resistant mutants selected from the indicator organism *Hafnia* H₁ to the B bacteriocins produced by sm 5, 6, 9, NCTC 1377 were resistant to

all other bacteriocins of group B. Resistant mutants of *Escherichia coli* ϕ to bacteriocins produced by SM 2, 5, 11, 43, 44, 45, NCTC 9940, 9743 were also resistant to all the bacteriocins of group B. Strain SM 5 produced only a B bacteriocin; antiserum against this bacteriocin inactivates all the B-group agents. Antiserum was prepared only against the SM 5 bacteriocin. These results indicate possible identity of the group B bacteriocins produced by the 54 strains. There is one exception: the product of ATCC 9986 is similar to the other B bacteriocins by the above criteria, but *E. coli* strains were not susceptible.

Table 3. *Spectrum activity of bacteriocins of Serratia marcescens*

Inhibitory agent	Susceptible strains
A ₁	<i>Serratia</i> , <i>Aerobacter aerogenes</i> x ₁ , <i>Escherichia coli</i> ϕ
A ₂	<i>Serratia</i>
A ₃	<i>Serratia</i> , <i>A. aerogenes</i> x ₁
A ₄	<i>Serratia</i>
A ₅	<i>Serratia</i>
A ₆	<i>Serratia</i> , <i>Salmonella</i>
A ₇	<i>Serratia</i>
A ₈	<i>Serratia</i>
B	<i>E. coli</i> ϕ , <i>E. coli</i> ca58, <i>A. aerogenes</i> x ₂ , <i>Hafnia</i> H ₁ -H ₆
B (produced by ATCC 9986)	<i>A. aerogenes</i> x ₂ , <i>Hafnia</i> H ₁ -H ₆

Distinction between colicins and bacteriocins A, B

Hafnia H₁ was susceptible to the B bacteriocins and not to the various colicins. *Serratia* strains susceptible to the A bacteriocins were not susceptible to colicins. *Escherichia coli* ϕ was susceptible to *serratia* bacteriocins B, A1 and all colicins. Colicin-resistant mutants of *E. coli* ϕ were still susceptible to the bacteriocins B, A1, except *E. coli* ϕ mutants resistant to colicin *k*. These mutants were resistant to bacteriocin B, although susceptible, to bacteriocin A1. However, resistant mutants of *E. coli* ϕ to bacteriocin B were susceptible to colicin K. Colicin K was not neutralized by antiserum to the bacteriocin B liberated by strain SM 5.

Lysogeny

Strains SM 8, 37, 41, 56 liberated phage spontaneously in high titres (1×10^9). Phages derived from the first two strains were active on these strains. These phages may be virulent mutants of prophages carried by these organisms (Jacob & Wollman, 1961) and capable of overcoming homologous immunity. The corresponding temperate phages have, however, not been demonstrated. Thirty-three out of the remaining 139 strains liberated phage spontaneously or after u.v.-induction. As in the case of inducible bacteriocins, mass lysis was never observed and the turbidity readings simply increased with time. Cultures were u.v.-irradiated in various phases of the growth cycle for various periods of time. A period of 1 min. of u.v.-irradiation of an overnight culture usually gave maximal titres after 18 hr incubation. Phages liberated by strains SM 8, NCTC 2847 had similar host ranges, and phages derived from strains SM 9, 41, 52, ATCC 9986 were also similar by this criterion. The results are too unwieldy for presentation but the remaining 10 phages could be differentiated from one another by their host ranges, and 19 phages plated equally well on various *serratias* and *salmonellas* (Prinsloo & Coetzee, 1964). The plaques which the 19

phages formed on the 15 *Salmonella* strains were slightly turbid but attempts to lysogenize three of the *Salmonella* strains were uniformly negative and the clones remained sensitive to the phages.

Strains which produced phage and an A type bacteriocin often attacked a particular *Serratia* strain but these agents could invariably be distinguished by using additional indicator organisms. The phage and the bacteriocin type A produced by a single strain could also be distinguished by the selection of mutants of the indicator organism resistant to the bacteriocin. The phage was invariably still active on the mutant.

DISCUSSION

The trypsin-resistant and trypsin-sensitive bacteriocins described previously (Hamon & Peron, 1961) correspond to the A and B bacteriocins described here. The bacteriocins described by Mandel & Mohn (1962) are similar to our B agents. It has been confirmed that the trypsin-sensitive B bacteriocins do not attack *Serratia marcescens* and shown that some of the A subtypes also attack *Escherichia coli*, *Aerobacter* and *Salmonella* strains while the B group kill *E. coli*, *Aerobacter* and *Hafnia* strains. Mandel & Mohn (1962) showed that a mutant of *E. coli* Row which was resistant to colicin K was also resistant to the substances produced by their *Serratia* strains. They designated the latter substances sub-species of colicin K. Colicin K resistant mutants of *E. coli* ϕ are also resistant to bacteriocin B but it has been possible to differentiate between the latter and colicin K. The two bacteriocins also have different electrophoretic mobilities (Prinsloo, Maré & Coetzee, 1965). Hamon & Peron (1961) demonstrated that 86% of their 85 *Serratia* strains were bacteriocinogenic. The investigation of Mandel & Mohn (1962) showed 58% of 31 strains of *S. marcescens* to be bacteriocinogenic for *E. coli* strains. The figure found in the present work is 64.7% for 139 strains of *S. marcescens*. Our strains do not undergo clearing after u.v.-induction and resemble the colicinogenic strains of *E. coli* (Fredericq, 1955), a pyocin-producing strain of *Pseudomonas aeruginosa* (Hamon, 1956), and pestocinogenic strains of *Pasteurella pestis* (Ben-Gurion & Hertman, 1958) in this respect.

Like the strains of Hamon & Peron (1961) many of our strains produce more than one antibiotic material. This has been confirmed (Prinsloo *et al.* 1965) by the demonstration that the A bacteriocins are electrophoretically mobile, while B agents produced by the same strain are stationary. A particular *Escherichia coli* strain may produce two or more distinct colicins (Fredericq, 1958), individual strains of *Enterobacter cloaca* (Hamon & Peron, 1962) are also believed to be multiple cloacinogenic, and strains of *Salmonella typhimurium* have been rendered doubly colicinogenic (Smith & Stocker, 1962).

The mole % guanidine + cytosine of the genera *Serratia* and *Salmonella* are 58 and 50%, respectively (Marmur, Falkow & Mandel, 1963). Despite this difference, 19 of the *Serratia marcescens* phages multiplied in various *Salmonella* species, but lysogenization by the *serratia* phages could not be demonstrated in salmonellas. Phages may multiply in bacteria with base compositions very different from their own (Lanni, 1960), and the present examples may only indicate that some *Serratia* and *Salmonella* strains share phage receptors (Meynell, 1964).

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