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A bacteriocin produced by certain M-type 49 *Streptococcus pyogenes* strains when incubated anaerobically

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SUMMARY

Bacteriocin production (P)-typing of 75 M-type 49 group-A streptococci obtained from a variety of epidemiological incidents in different countries gave no evidence of production under the usual aerobic test conditions. However, with anaerobic incubation, 28% of the strains gave a pattern of inhibitory activity against the indicator strains which was indistinguishable from that previously attributed to the bacteriocin, streptococcin A-FF22 (SA-FF22). Isolation and partial purification of the M type 49 bacteriocin (SA-M49) by freeze–thaw elution from anaerobically grown lawn cultures, followed by ammonium sulphate precipitation and Sephadex chromatography, showed the activity to be associated with a heat-stable proteinaceous molecule of molecular weight approximately 8000 – properties similar to those of SA-FF22. SA-FF22 and SA-M49 were found to have identical inhibitory spectra including immunity of the producer strains to the inhibitory activity of both the homologous and heterologous bacteriocin preparations. SA-M49 production occurred in some strains of phage subtypes II, III and provisional VI and, since it was a consistent property for all isolates from single outbreaks of infection, it provides a means of discriminating between strains of each of these three phage subtypes. There was no evidence of any increased incidence of SA-M49 production in M-type 49 strains associated with nephritic sequelae.

INTRODUCTION

Application of an inhibitor 'fingerprinting' scheme based upon a deferred antagonism test on blood agar has shown that many group-A streptococcus (*Streptococcus pyogenes*) strains produce antibacterial agents (Tagg & Bannister, 1979). By use of this scheme, all of 12 M-type 4 streptococci tested produced an identical range of activity against nine standard indicator strains. In code form this inhibitory pattern is referred to as production (P)-type 655, a type which appears to be uniquely associated with M-type 4 strains. Similarly, only the six M-type 57 strains tested were found to produce P-type 614 inhibitory activity (Tagg & Bannister, 1979). Proteinaceous antibiotics isolated from prototype

strains representative of these two serotypes have subsequently been characterized as bacteriocins (Johnson, Tagg & Wannamaker, 1979; Simpson & Tagg, 1983).

The first reported group-A streptococcus bacteriocin was streptococcin A-FF22 (SA-FF22) (Tagg, Read & McGiven, 1973), produced by group A streptococcus strain FF22 (M type 52) and found in many respects similar to nisin, an antibiotic produced by *Streptococcus lactis* (Tagg & Wannamaker, 1978). As part of the inhibitor 'fingerprinting' scheme (Tagg & Bannister, 1979) an assessment is made of the sensitivity (S)-type of test strains, using a set of nine standard inhibitory streptococci. Strain FF22 is included as one of these standard inhibitor producers and has been found widely active against other group A streptococci, all of 54 prototype strains representing different M serotypes being sensitive (Tagg & Bannister, 1979). Other relevant characteristics include the immunity of strain FF22 to its own bacteriocin, an essential feature of any bacteriocinogenic strain (Tagg, Dajani & Wannamaker, 1976) and the enhanced production of SA-FF22 under conditions of anaerobic incubation (Tagg & Bannister, 1979). M type 49 streptococci have frequently been implicated in the development of pyoderma, sometimes complicated by ensuing acute glomerulonephritis (Maxted, Fraser & Parker, 1967). Discrimination between different M49 strains has been aided by the use of a phage-typing scheme, five different phage subtypes initially being reported amongst 72 strains representing a variety of epidemiological incidents from ten worldwide sources (Skjold & Wannamaker, 1976). In our preliminary studies (Tagg & Skjold, unpublished) using the standard inhibitor 'fingerprinting' scheme there was no evidence of bacteriocin-like inhibitor production by any of the available M type 49 streptococci; however, some strains were distinctive in that they were insensitive to SA-FF22 when S-typed. This apparent immunity to SA-FF22 action was the first indication of the possible production by these strains of an antibiotic substance which might be closely similar to SA-FF22. In the present study we have examined a large collection of M49 streptococci for inhibitor production and for its relationship to the phage subtype and the nephritis association of the strains.

MATERIALS AND METHODS

The nine standard indicator stains (I1-I9) and the basic P-typing procedure used in this study have been described previously (Tagg & Bannister, 1979). For purposes of the present study calcium carbonate (0·5%, w/v) was added to the typing medium to minimize inhibition due to the accumulation of acidic metabolites (Tagg & Martin, 1984). Most of the M-type 49 group-A streptococci tested for inhibitor production were selected from those used in previous phage-typing studies (Skjold & Wannamaker, 1976; Skjold *et al.* 1983) . Four strains provided by courtesy of Dr W. R. Maxted (Colindale) had been isolated in South Oxfordshire (Mayon-White & Perks, 1982). Anaerobic incubation was at 32°C for 18 h in an atmosphere of 85% nitrogen, 10% hydrogen and 5% carbon dioxide (Forma Scientific anaerobic glovebox). For production of partially purified SA-M49 and SA-FF22 the methods for inoculation of lawn cultures, freeze–thaw extraction of the culture liquor, precipitation with 80% saturated ammonium sulphate and chromatography on a calibrated column of Sephadex G100 were as previously described (Tagg, Read & McGiven, 1973*b*; Tagg *et al.* 1973*a*). Assay of preparations

Table 1. *Sensitivity to SA-FF22 and SA-FF22-like inhibitor production by M type* 49 *streptococci*

Strain designation	Strain characteristics	Sensitivity to SA-FF22*	Inhibitor production* (P-type)	
			Aerobically	Anaerobically
FF22	M52, SA-FF22 producer	−	476	476
EB1	SA-FF22 negative derivative of FF22	+	000	000
GT-9653	M49, phage subtype II	−	000	476
GT-9538	M49, phage subtype III	−	000	476
81-086	M49, phage subtype VI	−	000	476
GT-8760	M49, phage subtype I	+	000	000
GT-6481	M49, phage subtype V	+	000	000
GT-7903	M49, phage subtype IV	+	000	000

* Tested by deferred antagonism with producer growth at 32 °C on blood agar plus 0·5 % (w/v) calcium carbonate.

for inhibitory activity was by a well-diffusion method on Columbia blood agar base medium (Tagg *et al.*, 1973*a*). Tests for protease and heat susceptibility were by conventional techniques (Tagg *et al.* 1973*a*). Phage typing was by the method of Skjold & Wannamaker (1976). The types defined were the originally described types I–V (Skjold & Wannamaker, 1976), provisional VI and provisional VII, the latter representing sensitivity to phage 5 (Skjold *et al.* 1983).

RESULTS

In view of our preliminary observation that certain M type 49 streptococci were insensitive to SA-FF22, several of these strains were re-examined for inhibitor production under anaerobic conditions (Table 1). With anaerobic incubation all of the SA-FF22-resistant strains were found to be P-type 476, a P-type identical to that given by strain FF22. Neither the SA-FF22-sensitive M49 strains nor strain EB1, a SA-FF22-negative derivative of strain FF22, produced detectable inhibitors under these incubation conditions.

Clusters of epidemiologically related M49 strains were tested for P-type 476 inhibitor production both aerobically and anaerobically (Table 2). Approximately 28 % of the strains, including representatives from three of the seven phage subtype categories, had inhibitor-positive representatives. All of the strains within a defined infection cluster were found to be either inhibitor-positive or inhibitor-negative. Four of the seven clusters of inhibitor-positive strains and 12 of the 16 inhibitor-negative clusters were considered to have had a nephritic association. Inhibitor-positive strains of phage subtype III were present in nephritis-associated outbreaks in Trinidad in 1965–6 and in 1975–6. Also associated with the latter outbreak were two inhibitor-positive isolates of the provisional phage subtype VI.

Table 2. *P-type* 476 *inhibitor production by clusters* of M type* 49 *strains of various phage subtypes and epidemiological sources*

Phage subtype	Source†	Nephritis Association	Inhibitor production		No. of isolates tested
			Aerobically	Anaerobically	
I	Red Lake 1966	+	−	−	4
	Alabama 1964–5	+	−	−	4
II	The Netherlands 1960–2	+	−	−	4
	Great Britain, 1966?	−	−	+	4
	Chile (year unknown)	+	−	+	4
	Oxford‡, 1980–1	−	−	−	4
	Kent‡ 1980	−	−	+	2
III	Alabama, 1964–5	+	−	−	2
	Trinidad, 1965–6	+	−	+	4
	Trinidad‡, 1975–6	+	−	+	4
IV	Minneapolis/ Red Lake, 1953	+	−	−	3
	Alaska‡, 1975–9	+	−	−	1
V	Alabama, 1964–5	+	−	−	4
	Alaska,‡ 1975–9	+	−	−	4
VI (prov)‡	South Oxfordshire§ 1977	−	−	−	4
	Alabama 1964–5	+	−	−	2
	Czechoslovakia, 1961–2	+	−	−	5
	Lincolnshire,‡ 1980	−	−	−	6
	Cambridgeshire,‡ 1980	−	−	−	4
	Trinidad,‡ 1975–6	+	−	+	2
	Kent,‡ 1980	−	−	+	1
VII (prov)	Alaska,‡ 1975–9	+	−	−	2
	Alabama, 1964–5	+	−	−	1

* Each cluster of isolates was from a similar geographical source and date and had the same phage subtype.

† The strains were selected from those previously described (Skjold & Wannamaker, 1976) unless otherwise designated.

‡ Skjold *et al.* (1983).

§ Mayon-White & Perks (1982).

Although the observed cross-resistance in deferred antagonism tests between strain FF22 and the M49 inhibitor producers suggested that the M49 inhibitor was closely similar to SA-FF22, isolation and characterization of the inhibitor was required to substantiate this. Strain GT-9653 was selected as the prototype producer of SA-M49 activity for use in these studies.

Inhibitor production in liquid media was unreliable, only trace amounts being detected in pre-reduced, anaerobically incubated cultures in tryptic soy broth, Todd Hewitt broth or brain–heart infusion. Addition of 5% (v/v) blood and incubation at various temperatures (25, 32, 37°C) was tried, but found unhelpful in promoting consistent production. Low yields (titre 2) of SA-M49 activity were obtained by freeze–thaw extraction of lawn cultures grown anaerobically (24 h/32°C) on the P-typing medium. Greater recoveries (titre 4–8) were obtained with use of a sheep blood rather than human blood supplement to the medium.

Table 3. *Inhibitory activity of partially-purified preparations of SA-M49 and SA-FF22*

Test strain	Inhibitory activity* of partially purified SA-M49	Inhibitory activity* of partially purified SA-FF22
Group A streptococcus strain GT 9653	−	−
Group A streptococcus strain EB1	+ +	+ +
Group A streptococcus strain GT8760	+ +	+ +
Standard indicator strains		
Micrococcus luteus T18(I1)	+ + +	+ + +
Group A streptococcus FF22(I2)	−	−
Group F streptococcus T29(I3)	−	−
Group E streptococcus T6(I4)	+	+
Group A streptococcus 71-679(I5)	+ +	+ +
Group N streptococcus T21(I6)	+ + +	+ + +
Group A streptococcus 71-698(I7)	+ + +	+ + +
Group A streptococcus W1(I8)	+ +	+ +
Group C streptococcus T148(I9)	−	−

* Tested by the well-diffusion method. Zone diameter: + + + (>14 mm), + + (10–14 mm), + (6–10 mm), − (no inhibition), well diameter 6 mm.

Further studies showed that it was the plasma component of the blood that was important in promoting inhibitor production and heating the plasma at 56 °C for 30 min did not interfere with this effect. Subsequently, crude preparations of SA-M49 were prepared by freeze–thaw elution from batches of lawn cultures incubated 24 h/37 °C anaerobically on Columbia blood agar base plus 5 % (v/v) sheep plasma. Partial purification of SA-M49 by precipitation of the inhibitor with 80 % saturated ammonium sulphate followed by chromatography on a calibrated Sephadex G100 column allowed an estimation of the molecular weight of approximately 8000. The spectrum of activity of the partially purified SA-M49 against the standard indicators was the same as found in P-typing tests and was identical to that of a similarly purified preparation of SA-FF22 (Table 3). Both SA-M49 and SA-FF22 were stable to heating at 80 °C for 30 min and loss of activity followed treatment for 60 min with 1 mg/ml trypsin.

DISCUSSION

The production by certain M-type 49 isolates of SA-M49, a bacteriocin apparently identical to SA-FF22, provides a useful additional strain marker within this widely occurring nephritogenic serotype of group A streptococci. SA-M49 production did not appear to correlate with any increased association with nephritis. However, it was found to be a consistent property within clusters of epidemiologically-related isolates and since it seems to occur independently of the phage subtype of the strain, it offers a means of further subdividing M49 strains when used in conjunction with phage typing. Previously (Skjold *et al.* 1983) it had been suggested on the basis of phage typing data that the same strain may have persisted over a 9-year period to be responsible for two outbreaks of nephritis in Trinidad. Isolates from both of these outbreaks were found to be SA-M49 positive,

affording additional evidence for the close-relatedness of the strains involved in these two episodes.

None of the M49 strains of phage subtypes IV, V or PR VII obtained in 1975–9 in association with a high incidence of nephritis in an Alaskan Eskimo population were SA–M49 positive. Previously it has been reported (Skjold *et al.* 1983) that in association with this same outbreak a number of group A streptococci were isolated having T14 antigen, as did the M type 49 strains, but differing from the latter in being serum opacity factor-negative and non-typable with available phages. These strains, which appear to represent a new M type, have also been tested for inhibitor production (Tagg, unpublished) and all are P-type 226, an inhibitory pattern quite unlike that detected in any of the M type 49 strains.

An unusual feature of SA-M49 production was its apparent dependence upon anaerobic incubation of the producer strain. Recovery from liquid cultures was poor, extraction from lawn cultures on sheep-plasma-containing media being required to provide sufficient activity for purification and characterization studies. Partially purified SA-M49 was closely similar to a SA-FF22 in its molecular weight, heat-stability, sensitivity to trypsin and activity spectrum.

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