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“FINGERPRINTING” β -HAEMOLYTIC STREPTOCOCCI BY THEIR PRODUCTION OF AND SENSITIVITY TO BACTERIOCINE-LIKE INHIBITORS

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THE BACTERIOCINES are a group of antibiotic substances produced by bacteria (Hardy 1975; Tagg, Dajani and Wannamaker, 1976) with characteristic properties that include (Tagg *et al.*, 1976): (i) a narrow spectrum of activity centred about the homologous species; (ii) the presence of an essential, biologically active protein; (iii) a bactericidal mode of action; (iv) attachment to specific cell receptors; and (v) plasmid-borne genetic determinants of bacteriocine production and of host-cell immunity to bacteriocines.

Bacteria may produce a variety of other inhibitory substances, including metabolic products (e.g., acids, hydrogen peroxide, ammonia), low-molecular-weight “classical” antibiotics, lytic enzymes and defective bacteriophages. Intra-species microbial antagonism produced by any of these substances may, on cursory examination, appear attributable to bacteriocines. For this reason, it has become customary to describe antagonism as “bacteriocine-like” until the inhibitory substances have been characterised (Tagg *et al.*, 1976).

Bacteriocine production and bacteriocine sensitivity have been used as strain markers in epidemiological studies. Typing schemes based upon the detection of bacteriocine production by test strains have usually been adaptations of the “scrape and streak” method used by Abbott and Shannon (1958) for the colicine typing of *Shigella sonnei*. Perhaps the most widespread and successful application of this method has been the pyocine typing of strains of *Pseudomonas aeruginosa* (Darrell and Wahba, 1964; Gillies and Govan, 1966; Tagg and Mushin, 1971). The specificity of the results of typing may be greatly enhanced by the combined testing of strains for production of and sensitivity to bacteriocines. Farmer and Herman (1969) have used the term “epidemiological fingerprinting” to describe such combined typing systems.

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The production of bacteriocine-like substances by streptococci of several Lancefield groups has been described: group A (Sherwood *et al.*, 1949; Kuttner, 1966; Overturf and Mortimer, 1970; Kolesnichenko and Totolyan, 1971; Prakash, Ravindran and Sharma, 1973; Tagg, Read and McGiven, 1973a); group B (Kuttner, 1966; Kramer and Brandis, 1972; Prakash *et al.*, 1973; Tzannetis, Poulaki-Tsontou and Papavassiliou, 1974; Tagg, Dajani and Wannamaker, 1975); and group D (Brock, Peacher and Pierson, 1963; Tzannetis, Leonardopoulos and Papavassiliou, 1970; Kékessy and Piguët, 1971; Pleceaş, Bogdan and Vereanu, 1972). Typing schemes have been established for the differentiation of group-D streptococci on the basis of their production of (Tzannetis *et al.*, 1970) and sensitivity to (Pleceaş *et al.*, 1972) bacteriocine-like substances or both (Kékessy and Piguët, 1971).

In the present study, we tested a large collection of β -haemolytic streptococci for mutual inhibition, and explored the possibility of using the production of bacteriocine-like substances (P typing) and sensitivity to them (S typing) as a means of labelling these streptococci for epidemiological purposes.

MATERIALS AND METHODS

Bacterial strains. Four hundred and fifty strains of streptococci were used in the present study. Most of them had been isolated from human clinical specimens in New Zealand. All of the strains selected formed a Lancefield-group antigen, as shown by precipitation tests in capillary tubes with group-specific rabbit antisera. A single representative of each of the recognised M types of group-A streptococci (Lütticken *et al.*, 1976) was obtained from the culture collection of the Pediatrics Department, University of Minnesota, by courtesy of Dr L. W. Wannamaker. Organisms in regular use were stored at 4°C on blood agar. Stock cultures of all strains were stored at -70°C or in the lyophilised state.

Staphylococcus epidermidis strain T-18 (previously called *Staph. aureus* strain CIT) had been found (Tagg and Wannamaker, 1976) to be particularly sensitive to certain streptococcal bacteriocines, and was used in this study.

Culture media and chemicals. Media and medium components obtained from Difco Laboratories included Todd-Hewitt Broth, Tryptic Soy Agar, Brain Heart Infusion, Columbia Blood Agar Base, Neopeptone, Yeast Extract, Proteose Peptone No. 3, Trypsin (1:250) and Bacto-Agar. Mitomycin C, glucose, catalase (from bovine liver), iodoacetic acid, TES {N-tris (hydroxymethyl) methyl-2-aminoethane sulphonic acid} and haemoglobin (type IV) were from Sigma Chemical Co., St Louis, Mo, USA. Defibrinated sheep blood was purchased from Laboratory Services Ltd, Auckland, and human blood was obtained from healthy donors.

Screening tests for simultaneous and deferred antagonism. The methods of Tagg *et al.* (1973a) were used. Briefly, the tests were performed on plates of human-blood agar that were incubated at 32°C. *Simultaneous-antagonism tests* were made by inoculating potential producers either as stab inocula on sterile tooth-picks from growth on solid media or as 0.05-ml surface drops from overnight (32°C; 18 h) Todd-Hewitt broth cultures on to the surface of medium recently spread with a lawn of the indicator strain. *Deferred-antagonism tests* were performed as in the standard method described below.

The standard "fingerprinting" method. Preliminary experiments indicated that the most suitable medium was Columbia Blood Agar Base with 5% human blood, poured as a layer (20 ml) on a base of peptone-saline agar (20 ml) in glass petri dishes of diameter 9 cm. This will be referred to as the "typing medium".

The method used to determine inhibitor patterns is similar to that previously used in a pyocine-typing scheme (Tagg and Mushin, 1971). Strains to be tested for inhibitor production (producer strains) were propagated on blood agar. A cotton swab was charged with growth and used to inoculate a 1-cm-wide diametric streak across the surface of the typing medium, which was then incubated at 32°C for 18 h. Macroscopically visible growth was removed by scraping with the edge of a glass slide. The plate was then inverted over a circle of chloroform-soaked filter paper in the lid of the petri dish. After 30 min. the plate was removed from the lid and

exposed to the air for 15 min. Then, overnight (18 h; 32°C) Todd-Hewitt-broth cultures of the indicator strains were streaked at right angles to the line of the original producer growth by the use either of cotton swabs or a mechanical contrivance (the "broomette"; Tagg and Mushin, 1971). After incubation at 32°C for 18 h, reduced growth of indicator strains in the vicinity of the area originally occupied by the growth of the producer strain provided evidence of inhibitor production. To record the result, the signs +, +/- and - were used: + indicated definite interference with the growth of an indicator; +/- an equivocal reaction of partial inhibition and - no reduction in the growth of the indicator. Strains giving +/- reactions were retested.

To simplify the recording of inhibition patterns we adopted the code described by Al-Jumaili (1975). The standard indicator strains (and the standard producer strains) were considered as a series of triplets (e.g., I1, I2, I3; I4, I5, I6; I7, I8, I9). A positive reaction for the first member of a triplet (e.g., I1, I4 or I7) was given a score of 4, for the second member a score of 2 and for the third member a score of 1. Negative reactions were scored as zero. By summing the scores within each triplet a number was obtained that uniquely defined the reactions within that triplet. For example, the P-typing pattern of strain P1 reads + + - + - + - + giving a P-typing code of 655, and the S-typing pattern of this strain (- + + + + - - +) an S-typing code of 371. Thus the inhibitor "fingerprint" of this strain may be recorded as 655/371.

Physicochemical properties of the inhibitors. The dialysability of the inhibitors was tested on blood agar. A 1-cm² sheet of sterile dialysis tubing was placed on the surface of a blood-agar plate and a drop of molten blood-agar medium seeded with the test strain was placed centrally on the dialysis tubing. A similar (control) drop was placed directly on the surface of the medium adjacent to the dialysis membrane. After incubation at 32°C for 18 h, the membrane and the two agar-drop cultures were removed, the surface of the medium was exposed to chloroform vapour and an appropriate susceptible indicator strain was seeded as a lawn culture. Dialysability of the inhibitor(s) was indicated by zones of reduced indicator growth in the areas originally occupied by both drop cultures. The heat stability of the inhibitors within the blood-agar medium was tested by heating the medium at 80°C for 30 min. in the course of testing the strains by the deferred-antagonism method. This heating step was inserted after the scraping off of the producer growth and before exposure to chloroform.

RESULTS

Detection of inhibitory streptococci and selection of standard indicator and producer strains

Strains of β -haemolytic streptococci (100 of group A, 20 of group B, 10 of group C, 10 of group D and 10 of group G) were selected at random and each was tested against all the others for production of inhibition by established techniques of simultaneous and of deferred antagonism (see *Materials and methods*, and Tagg and Wannamaker, 1976). In the simultaneous-antagonism tests, the method of stab inoculation of the producer strains proved simpler to execute and resulted in a higher frequency of detection of inhibitory strains than the surface-inoculation method. However, the method of deferred antagonism proved to be a considerably more sensitive procedure for the detection of inhibitors than that of simultaneous antagonism. Only about one-third of the strains found to produce inhibitors in the deferred-antagonism test were positive when tested by simultaneous antagonism. All of the findings described subsequently are based upon the use of the deferred-antagonism method.

These preliminary screening studies enabled a tentative selection of strains for use as standard indicators and producers to be made. As additional

TABLE I
Standard indicator and producer strains used for the "fingerprinting" of streptococci

Indicator (for P typing)	Strain specification
I1	<i>Staphylococcus epidermidis</i> , strain T-18
I2	Group-A streptococcus, strain FF22, M-type 52, T-pattern 3/13
I3	Group-F streptococcus, strain T-29
I4	Group-E streptococcus, strain T-6
I5	Group-A streptococcus, strain 71-679, M-type 4, T-pattern 4
I6	Group-N streptococcus, strain T-21
I7	Group-A streptococcus, strain 71-698, M-type 28
I8	Group-A streptococcus, strain W-1, T-pattern 6
I9	Group-C streptococcus, strain T-148
Producer (for S typing)	Strain specification
P1	Group-A streptococcus, strain 71-679, M-type 4, T-pattern 4
P2	Group-A streptococcus, strain FF22, M-type 52, T-pattern 3/13
P3	Group-B streptococcus, strain 74-628, type II
P4	Group-G streptococcus, strain W2580
P5	Group-A streptococcus, strain M28689, T-pattern 25
P6	Group-F streptococcus, strain T-29
P7	Group-A streptococcus, strain 71-724, M-type 57
P8	Group-D streptococcus, strain T-142
P9	Group-A streptococcus, strain 71-722, M-type 55

TABLE II
Characteristic P-type patterns of standard producers

Producer strain	Inhibition* of indicator									Code
	I1	I2	I3	I4	I5	I6	I7	I8	I9	
P1	+	+	-	+	-	+	+	(-)	+	655
P2	+	-	-	(+)	+	+	+	+	-	476
P3	+	+	(+)	-	+	+	+	+	-	736
P4	-	+	-	-	+	-	(+)	+	-	226
P5	+	+	+	+	+	+	+	+	+	777
P6	-	+	-	-	+	-	+	+	-	226
P7	+	(+)	-	-	-	+	(+)	-	-	614
P8	+	+	+	-	(-)	+	+	-	-	714
P9	+	+	-	-	(+)	-	+	(+)	-	626

* + = Present; - = absent; () = weak activity may sometimes occur.

streptococci became available, these too were evaluated as potential standard strains. At present we are using sets of nine indicator and nine producer strains (table 1) for P typing and S typing respectively. One of the indicators (see *Materials and methods*) is a strain of *Staph. epidermidis* and the rest—and all the producer strains—are streptococci. Three strains (FF22, 71-679 and T-29) are used as indicators and as producers. The inhibition patterns characterising the standard producer strains when tested against the set of nine standard indicator strains are shown in table II.

TABLE III
The effect of cultural conditions on inhibitor yield*

Modification to standard procedure	Amount of inhibition by producer strain								
	P1	P2	P3	P4	P5	P6	P7	P8	P9
None	++	++	++	++	++	++	++	++	++
Columbia Blood	++	+	+	+	+	+	+	+	+
Agar Base (BAB) (no blood)	++	+	+	+	+	+	+	+	+
Tryptic Soy Agar	+	+	+	+	+	+	+	+	+
Todd-Hewitt agar	++	++	++	++	++	++	++	++	++
Brain Heart Infusion agar	++	++	++	++	++	++	++	++	++
Todd-Hewitt agar + human blood	++	++	++	++	++	++	++	++	++
Brain Heart Infusion agar + human blood	++	++	++	++	++	++	++	++	++
BAB + sheep blood	++	++	++	++	++	++	++	++	++
BAB + heated human blood†	++	++	++	++	++	++	++	++	++
BAB + haemoglobin (1%)	++	++	++	++	++	++	++	++	++
BAB + Neopeptone (1%)	++	++	++	++	++	++	++	++	++
BAB + iodoacetate (10 ⁻⁵ M)	++	++	++	++	++	++	++	++	++
BAB + yeast extract (1%)	++	++	++	++	++	++	++	++	++
BAB + Proteose Peptone No. 3 (1%)	++	++	++	++	++	++	++	++	++
Supplement of 0.4M TES (pH 7)	++	++	++	++	++	++	++	++	++
Supplement of catalase 1000 units/ml	++	++	++	++	++	++	++	++	++
Anaerobic incubation	++	++	++	++	++	++	++	++	++
Incubation at 40°C	++	++	++	++	++	++	++	++	++
Incubation at 37°C	++	++	++	++	++	++	++	++	++
Incubation at 25°C	++	++	++	++	++	++	++	++	++
Ultraviolet irradiation (30 sec.)	++	++	++	++	++	++	++	++	++

* The amount of inhibitor produced is qualitatively estimated relative to the amount of activity (+ + +) observed under the standard conditions (see text); + + + + = increased activity (wider zones or increased spectrum); + + and + = degrees of reduced activity; - = no inhibitor detected.
† To 80°C for 30 min.

The effect of cultural conditions on inhibitor production and detection

This was examined by comparing the standard "fingerprinting" procedure with various modifications of it in tests of the inhibitory activity of the nine producer strains each on an indicator strain strongly inhibited by it in the standard test (table III).

The composition of the medium was a critical factor. The single most important component seemed to be the blood. In the absence of this, inhibitor production could be detected only in strains P1, P2 and P3. Moreover, the blood factor required for inhibitor production by strains P4, P6, P7 and P8 (but not strains P5 and P9) was inactivated when the blood-agar medium was heated at 80°C for 30 min. In the absence of blood, supplementing the basal medium with yeast extract, haemoglobin, Proteose Peptone No. 3 or with known inhibitors of streptococcal proteinase such as Neopeptone and iodoacetate did not provide suitable conditions for inhibitor production. The use of human blood instead of sheep blood appeared to favour inhibitor production by strain P5 and to a lesser extent by strains P8 and P9. It was also observed that, particularly when freshly isolated, several other strains giving P5-like inhibitory patterns (i.e., 777) produced detectable inhibition only on media supplemented with human blood. Todd-Hewitt agar, Tryptic Soy Agar and Brain Heart Infusion Agar were tested as possible alternatives to Columbia Blood Agar Base as a basal medium; none of these proved to be completely satisfactory even when supplemented with human blood.

The production of an acidic pH did not seem to be an important factor for the inhibitory action of the standard producer strains; only strains P2 and P9 seemed to produce somewhat less detectable inhibition when the medium was buffered at pH 7. Anaerobic incubation of the producers was found generally to enhance inhibitor production. This effect was particularly noticeable with strains P1, P2 and P7 and resulted in apparent changes in the P type of these strains. Production of the inhibitors in anaerobic culture indicated that this activity was unlikely to be due to the formation of hydrogen peroxide. Further support for this was obtained by demonstrating good production of all inhibitors on blood-agar medium supplemented with catalase 1000 units/ml.

For some producer strains the temperature of incubation appeared to be of importance. Elevated temperatures promoted production of inhibitors by strains P6 and P7, whereas incubation at 25°C enhanced inhibitor production by strains P1, P2, P5 and P9. An incubation temperature of 32°C was found to be satisfactory for demonstration of inhibitor production by most of the strains examined. The time of incubation of the producers was varied from 12 to 48 h without marked effect. Incubation for 18 h was convenient for routine laboratory work and gave satisfactory results with all the inhibitors.

None of the inhibitors showed any evidence of being inducible by exposure of the freshly inoculated producer streak cultures to a 90% killing dose of ultraviolet irradiation. On the contrary, less inhibitory action was observed; this may be attributed to reduced growth of the producer after irradiation.

Removal of the producer growth from the medium by scraping with the

edge of a glass slide was compared with a modification (Kohn, 1966) in which the producer streak was grown on a strip of sterile filter paper (Whatman No. 1 or No. 50) on the surface of the medium. Although rather similar results were obtained, the matt surface on the blood agar caused by the filter paper occasionally made the interpretation of inhibitory patterns more difficult.

Todd-Hewitt broth was found to be a suitable growth medium for all of the indicator strains. Comparable inhibition patterns were obtained by use of either 18-h or 4-h cultures, but interpretation of the patterns was often easier with the denser (18-h) inocula. Inoculation by means of swabs and the "broomette" gave identical results; nine indicators could be applied to a single plate with the "broomette", but only six or seven could conveniently be applied manually.

Characterisation of strains by inhibitor production (P typing)

The P-typing procedure was used to test 450 strains of streptococci (table IV). Approximately 80% of all the strains, and nearly 95% of the group-A strains, produced detectable inhibitors; 17 different inhibitory patterns were recognised. Inhibitory patterns 226 and 714 seemed most characteristically to be associated with strains of group-G and group-D streptococci respectively.

Table V lists the P types of a set of strains of group-A streptococci comprising one representative of each of 54 different M types. Some P-typing patterns (e.g., 004) were given by representatives of several M types, but others (e.g., 655 and 614) were given by only a single type-representative. The

TABLE IV
Relationship of P type to serological group

P type	Number of strains in the stated P type belonging to serological group						
	A (350)*	B (34)	C (34)	D (12)	E (3)	F (1)	G (16)
777	32	0	0	0	0	0	0
774	10	0	0	0	0	0	0
736	0	1	0	0	0	0	0
724	0	0	0	0	2	0	0
714	0	1	0	4	0	0	0
674	4	0	0	0	0	0	0
655	12	0	1	0	0	0	0
626	2	0	0	0	0	0	0
614	6	0	0	0	0	0	0
604	4	0	0	0	0	0	0
476	4	0	0	0	0	0	0
410	0	0	0	2	0	0	0
226	0	0	1	0	0	1	7
222	1	0	0	0	0	0	0
214	3	0	0	0	0	0	0
204	68	1	2	1	0	0	0
004	184	1	3	0	0	0	3
000	20	30	27	5	1	0	6

* Number of strains tested.

TABLE V
Relationship of P type to serological M type of group-A streptococci

P type	M-type numbers of strains belonging to the stated P type*
777	60
774	2
674	34, 39, 42
655	4
626	33, 55
614	57
222	28
214	61
204	15, 25, 58, 63
004	1, 3, 5, 6, 8, 9, 11, 12, 14, 17, 18, 19, 22, 23, 24, 26, 27, 29, 30, 31, 32, 37, 38, 40, 41, 46, 47, 48, 49, 50, 52, 53, 54, 59, 62
000	13, 43, 51, 56

* One representative of each M type was tested.

association of M-type 4 with the P-typing pattern 655 was investigated further; all 12 available representatives of this type had this pattern (see Johnson, Tagg and Wannamaker, 1979). The only other strain found in the present study to be of P-type 655 was an M-negative group-C streptococcus. Similarly, all six of the available M-type 57 strains had the P-typing pattern 614.

Some of the observed inhibitory patterns appeared to represent the combined action of two or more distinct inhibitory substances. Strains P1 and P2 could, by prolonged incubation *in vitro* (Tagg and Wannamaker, 1976), each be "cured" of the ability to produce one inhibitory substance characteristic of each strain. The "cured" derivatives of strains P1 and P2 were all found to be of P-type 004. The production of P-type 004 inhibitory activity is a characteristic of 42% of the streptococci examined, and this inhibitor may also contribute to the total inhibitory activity of strains having other P-typing patterns. Strains of P-type 004 were observed often to convert to P-type 204 when the producers were grown anaerobically. This indicates that P-typing patterns 004 and 204 may possibly represent the action of a single inhibitor that is produced in greater amount during anaerobic growth of the producer strain.

Characterisation of strains by sensitivity to inhibitors (S typing)

This gave a greater variety of patterns than did P typing. Some apparent associations between the streptococcal group and the S type of the test strains were evident (table VI). Table VII lists the relationship between S type and M type of the set of group-A streptococcal prototype strains. The S pattern of a strain appeared generally to be independent of its P type. Strains producing a particular inhibitor usually exhibited producer-cell immunity to the standard producer strain of the homologous inhibitor (table VIII). The only exception to this was strain P5 which appeared to be auto-inhibitory under the test conditions. Strains P1 and P2 displayed the phenomenon of producer-cell

TABLE VI
Relationship of S type to serological group

S-type	Number of strains of the stated S type belonging to serological group						
	A (350)*	B (34)	C (34)	D (12)	E (3)	F (1)	G (16)
777	184	0	1	0	0	0	0
776	5	0	0	0	0	0	0
774	1	0	0	0	0	0	0
773	41	0	0	0	0	0	0
772	10	0	0	0	0	0	0
771	28	0	0	0	0	0	0
770	13	0	0	0	0	0	0
767	4	0	0	0	0	0	0
760	0	0	0	0	1	0	0
722	0	0	5	0	0	0	0
720	0	2	1	0	2	0	0
670	0	0	1	0	0	0	0
660	0	0	1	0	0	0	0
577	2	0	0	0	0	0	0
571	2	0	0	0	0	0	0
570	0	0	0	0	0	0	1
562	1	0	1	0	0	0	0
560	0	0	1	0	0	0	0
535	3	0	0	0	0	0	0
532	0	0	0	0	0	0	1
531	4	0	0	0	0	0	0
470	0	0	1	0	0	0	0
462	0	0	1	0	0	0	0
460	0	0	1	0	0	0	7
430	0	0	1	0	0	0	0
420	0	0	2	0	0	0	3
377	3	0	0	0	0	0	0
376	2	0	0	0	0	0	0
375	7	0	0	0	0	0	0
373	10	0	0	0	0	0	0
372	2	0	0	0	0	0	0
371	15	0	0	0	0	0	0
330	0	0	1	0	0	0	0
324	3	0	0	0	0	0	0
320	0	6	0	0	0	0	0
170	5	0	0	0	0	0	0
160	0	0	2	0	0	0	0
140	0	0	1	0	0	0	0
134	2	0	0	0	0	0	0
124	2	0	0	0	0	0	0
122	0	0	0	0	0	1	0
120	0	24	12	0	0	0	0
110	1	0	0	0	0	0	0
020	0	1	1	0	0	0	4
006	0	0	0	1	0	0	0
002	0	0	0	2	0	0	0
000	0	1	0	9	0	0	0

* Number of strains tested.

immunity, and their cured derivatives each exhibited increased susceptibility only to the specific inhibitor produced by its own parental strain. One particularly interesting relationship observed was that the inhibitor produced by strain P6 is active almost exclusively against strains of group-A streptococci. In all, 97% of group-A streptococci were susceptible to the P6 inhibitor,

TABLE VII
Relationship of S type to serological M type of group-A streptococci

S type	M-type numbers of strains belonging to the stated P type*
777	1, 2, 3, 5, 6, 8, 9, 11, 12, 13, 14, 15, 19, 23, 24, 25, 26, 28, 30, 31, 32, 37, 39, 40, 41, 42, 43, 46, 48, 49, 51, 52, 53, 54, 56, 58, 59, 61, 63
773	17, 33, 50, 60, 62
772	29, 55
771	18, 22, 34, 57
770	38, 47
375	27
371	4

* One representative of each M type was tested.

TABLE VIII
Characteristic S-type reactions of standard producer strains of streptococci

Test strain	Inhibition* of test strain by producer strain									Code
	P1	P2	P3	P4	P5	P6	P7	P8	P9	
P1	—	+	+	+	+	+	—	(—)	(+)	371
P2	+	—	+	+	+	+	(+)	+	+	577
P3	—	—	—	—	+	—	—	—	—	020
P4	+	—	—	—	+	—	—	—	—	420
P5	—	+	+	+	+	+	—	—	+	371
P6	—	—	(+)	—	+	—	—	+	—	122
P7	+	+	+	+	+	+	—	—	(+)	771
P8	—	—	—	—	—	—	—	—	—	000
P9	+	+	+	+	+	+	(+)	(+)	(—)	776

* See footnote to table II.

whereas only 7% of non-group-A streptococci (five group C and two group G) were susceptible.

Consistency of results

The P-typing patterns of strains appeared to be remarkably stable. The standard producer strains showed unchanged inhibitory spectra after storage at -70°C for 18 months, and after weekly serial subculture on blood agar for up to 3 months.

In practice, we found it necessary to repeat the typing of about 5% of the strains tested because the initial results were difficult to interpret. Weakly positive reactions sometimes varied from test to test, but strong reactions very seldom changed to negative reactions. In general, S-typing results showed more variation than P-typing results.

Dialysability and heat stability of inhibitory agents

Strains P1, P2, P3 and P5 produced inhibitors that were dialysable and heat stable (at 80°C for 30 min.), and strains P4, P6, P7, P8 and P9 produced inhibitors that were non-dialysable and heat labile.

DISCUSSION

Previous studies (Tagg *et al.*, 1976) showed clearly that the effective yield of streptococcal inhibitors is often controlled by ill-defined cultural conditions; optimal conditions for inhibitor production must be determined empirically for each strain. In the present study we found that blood was the most important single constituent of the medium for the production of most of the inhibitors, but that the blood constituent required differed from strain to strain. Supplementing the basal medium (Columbia Blood Agar Base) with haemoglobin did not appear to enhance inhibitor yield, neither did the addition of various supplementary growth factors. In a recent study of bacteriocine production by "viridans" streptococci, Dajani and Veres (1977) reported that viridin B is inactivated by mammalian haemoglobin, but we found no evidence that haemoglobin inactivated any of the inhibitors we investigated. The possibility that blood components inhibited the action of streptococcal proteinase and so increased the yield of proteinaceous bacteriocine-like substances was excluded by tests in basal medium supplemented with inhibitors of proteinase such as Neopeptone (Cohen, 1969) and iodoacetic acid (Elliott, 1945). In studies of bacteriocine production by strains of *Pseudomonas aeruginosa* it had been observed that chemical inhibitors of proteases eliminated the requirement for blood in the typing medium (Darrell and Wahba, 1964; Gillies and Govan, 1966; Tagg and Mushin, 1971).

The apparent preference of some strains (P5, P8 and P9) for human blood as substrate for inhibitor production is of particular interest. It is tempting to suggest that this requirement, which is particularly marked for many P5-type strains, may indicate that these inhibitors could be produced naturally only in the course of human infections. Such inhibitors may prove particularly interesting to study in relationship to the hypothetical role of bacteriocine-like substances in the group-A streptococcal sequelae, rheumatic fever and glomerulonephritis (Tagg and McGiven, 1972).

The production of inhibitors does not seem directly attributable to the creation of a low pH. Production of inhibitors by strains P2 and P9 was nevertheless somewhat reduced by incubation at a more neutral pH. Previous studies (Tagg *et al.*, 1973a) indicated that the production of streptococcin A-FF22 in solid medium by strain P2 was enhanced at lower pH.

The apparent promotion of inhibitor production by many strains during anaerobic incubation is possibly a reflection of the enhanced growth of streptococci under this condition. Anaerobic incubation was not used routinely as part of the typing procedure because it often resulted in marked increases in the spectra, and thus changes in P type, of some of the producers. Nevertheless,

anaerobic incubation of producers may have application in special circumstances to help clarify P types when inhibitor production is rather weak.

The temperature of incubation of the producer strains was of importance; unless this was carefully controlled, a significant number of strains gave aberrant results. Some strains (e.g., P6, P7) produced optimum yields of inhibitors at 37°C whilst others (e.g., P1, P2, P5 and P9) were more productive at 25°C. A compromise temperature of 32°C was chosen, because all strains seemed to produce adequate, if not optimal, levels of inhibitors during incubation for 18 h at this temperature. Similarly, Gillies and Govan (1966) found incubation at 32°C to be preferable to 37°C in pyocine typing. Lesser proteolytic activity by streptococci at 32°C than at 37°C (Elliot, 1945) may contribute to the apparent increase in the yield of some inhibitors when the temperature is lowered. In some studies (Tripathy and Chadwick, 1971; Tagg and Mushin, 1973), it was found that the time required for bacteriocine typing could be shortened and the proportion of untypable strains reduced by the use of inducing agents. We did not observe any promotion of inhibitor production by exposure of the producer strains to ultraviolet irradiation, nor has any other evidence been produced of streptococcal inhibitors being inducible (Tagg *et al.*, 1976).

There were a few indications of a relationship between P-typing pattern and serological group, and two clear instances of a close correspondence between P-typing pattern and M serotype among group-A streptococci; one of these is described in detail elsewhere (Johnson *et al.*, 1979). Others may be revealed by subsequent studies. Some of the observed inhibitory patterns may have been attributable to the combined action of two or more distinct inhibitory substances. Studies of the production of bacteriocine-like substances by α -haemolytic streptococci (Kelstrup and Gibbons, 1969; Rogers, 1976) have also indicated that the observed spectra of inhibitory activity may represent the combined action of two or more distinct inhibitors.

Labelling of the streptococcal test strains according to their S type gave a larger number of distinct types than did P typing. Investigations of inhibitory interactions between the producer strains demonstrated that under the standard test conditions all strains other than P5 appeared to be immune to their own inhibitors. However, in other strains, breakdown of producer-cell immunity sometimes occurred when the producer strains were incubated anaerobically. Some S types seemed to be characteristic of particular serological groups of streptococci. An observation of particular interest was the apparent high specificity of action of the inhibitor(s) of strain P6 against group-A streptococci. Bacitracin also shows high specificity of action against group-A streptococci, but our unpublished observations indicate that among non-group-A streptococci susceptibility to bacitracin and to the P6 inhibitor occur independently.

P types were more consistently stable than S types, in which variability on repeated testing occasionally caused problems. Similar difficulties have been reported by others in the susceptibility typing of *Shigella* (Abbott and Shannon, 1958), *Proteus* (Senior, 1977) and *Serratia* (Farmer, 1972). Fluctuations

in the production of the inhibitors caused by ill-defined changes in incubation conditions, and differing sensitivities of colonial variants, may in part explain the variability sometimes observed in P and S typing. It would be highly desirable to obtain standardised cell-free preparations of each of the inhibitors so that these could be used in controlled doses in a S-typing scheme. Recently, Farkas-Himsley and Pagel (1977) have attempted to make bacteriocine typing more quantitative by mixing suspensions of test strains with standardised bacteriocine preparations and detecting the presence of leaked ultraviolet-light-absorbing material. Similar principles might eventually be applicable to the inhibitor typing of streptococci, but so far it has been possible to make cell-free preparations only of the inhibitors produced by strains P1 (Johnson *et al.*, 1979), P2 (Tagg *et al.*, 1973*b*) and P3 (Tagg *et al.*, 1975). Similar difficulties have been encountered in other investigations of inhibitor production by gram-positive bacteria (Tagg *et al.*, 1976).

Our present information on the nature of the inhibitory substances indicates that they exhibit a wide range of different physico-chemical properties. Bradley (1967) categorised bacteriocines into two broad groups: high-molecular-weight heat-labile and low-molecular-weight heat-stable substances. Examples of both were encountered in this study. According to Malke *et al.* (1974), many group-A streptococci produce inhibitory levels of hydrogen peroxide that may mimic that action of bacteriocines in simultaneous-antagonism tests. However, the inhibitors described in the present study appear to be quite distinct from hydrogen peroxide, because they can be detected in deferred-antagonism tests on blood agar supplemented with catalase and also after anaerobic incubation.

Our "fingerprinting" scheme for streptococci is easily performed, reliable and inexpensive. We do not suggest that it should replace serological typing, but it might find application in non-specialist laboratories that do not have ready access to typing sera. In specialist streptococcal laboratories it may be useful as a means of reducing the percentage of strains at present serologically untypable, and it may provide a means of subdividing strains of the same serotype. Although the present "fingerprinting" scheme was devised for typing β -haemolytic streptococci, we have found that the same set of standard indicator and producer strains can also be used to label α -haemolytic streptococci, lactobacilli and actinomycetes.

SUMMARY

A scheme for the "fingerprinting" of streptococci according to their production of (P typing) and sensitivity to (S typing) bacteriocine-like inhibitory substances has been developed.

P typing of 450 β -haemolytic streptococci by their action on a set of nine standard indicator strains revealed that 80% of strains produced one or more detectable inhibitors, and that 17 different P types could be recognised. Production of some inhibitors seemed to be a property of strains of a particular

serological group or type. Bacteriocine-like substances were produced by streptococci of serological groups, A, B, C, D, E, F and G.

Nine strains were selected as standard producers for S typing. These strains differed in their spectra of inhibition, but all seemed to be active only against gram-positive bacteria. One producer, a group-F streptococcus, specifically inhibited group-A streptococci.

The conditions of incubation were critical for demonstration of inhibitor production. A requirement for blood and for incubation at 32°C were important factors. None of the inhibitors was induced by ultraviolet irradiation. The observed inhibitory effects were not attributable to either hydrogen peroxide or low pH, but to the production of a variety of substances having diverse physicochemical properties and production requirements. Most of the inhibitors do not seem to be produced in liquid media.

The "fingerprinting" procedure is simple and inexpensive, and provides a reliable means of subdividing streptococcal strains that may find application as a supplement to the existing serological typing schemes.

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