Bacteriocin Typing of Streptococcal Isolates from Endocarditis

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A typing system based on bacteriocin production and sensitivity for *Streptococcus sanguis* strains was devised. Bacteriocin producer strains were grown (37°C) anaerobically on brain heart infusion-yeast extract agar for 18 h. Bacteriocin indicator strains were suspended to molten brain heart infusion-yeast extract agar and then overlaid onto the producer strain. After an additional 18 to 24 h of incubation, zones of bacteriocin inhibition were recorded. After establishment of the typing system, eight *Streptococcus* strains from bacterial endocarditis patients were characterized by the typing system. Four patient strains had identical bacteriocin reduction patterns but could be differentiated by differences in their bacteriocin sensitivity. Two isolates from the same patient had identical production and sensitivity patterns. By including both bacteriocin production and bacteriocin sensitivity, all seven epidemiologically unrelated isolates could be differentiated. On the basis of these results, there does not appear to be a correlation between bacteriocin production and infectivity, but the usefulness of the application of a typing system to a clinical situation is demonstrated.

Typing systems based on bacteriocin production and sensitivity have been developed for many bacteria (1). Similarly considerable attention has also been given to the interreactions between *Streptococcus sanguis* and other closely related oral streptococcal species and the need for a practical typing system. In this regard Kelstrup et al. have predicted the utility of bacteriocins for differentiation of oral streptococcal species (8).

The purpose of this study was to develop a typing system based on bacteriocin production and sensitivity for a set of *S. sanguis* strains and to use this system in an epidemiological study of streptococcal strains isolated from patients with bacterial endocarditis.

MATERIALS AND METHODS

Bacterial strains. There were 21 streptococcal strains obtained from the Medical College of Virginia (MCV) and two streptococcal strains obtained from the University of Alabama (UAB). The strains were labeled as follows: *S. sanguis* strains: Enole, Channon, K-208, ATCC 10556, ATCC 10557, ATCC 10558, Challis, Wicky, Thompson, Blackburn-Cole, Blackburn-Rotta, 410, 174-P, and F90A; *S. mutans* strains: JC43, AHT, HHT, KIR, FAI, E49F, and SS26 (MCV); *S. mutans* strains: AHT and GS5 (UAB).

Clinical isolates of alpha or gamma hemolytic, nonenteric streptococci were obtained from blood cultures of patients with documented bacterial endocarditis. These strains were obtained from the

Medical College of Virginia Clinical Pathology Laboratory and were labeled as follows: patients 1, 2, 3, 4, 5a, 5b, 6, and 7. Strains labeled patients 3, 4, and 6 were physiologically similar to *S. mutans*. The strain labeled patient 7 was similar to *S. sanguis*. The other strains did not definitively resemble either *S. mutans* or *S. sanguis*.

Stock cultures were transferred every 3 weeks into brain heart infusion-yeast extract broth (BHI-YE; Difco) and incubated in a Torbal jar (Torsion Balance Co.) for 18 to 24 h at 37°C in an atmosphere of 10% CO₂-90% H₂ gas. The stock strains were stored at 4°C in BHI-YE broth. Culture purity was periodically examined by streaking an inoculum of each organism onto a BHI-YE agar plate and then Gram staining for typical morphological characteristics.

Bacteriocin assay. A modification of the stab-overlay technique utilized by Fredericq (2) was used to demonstrate bacteriocin production and sensitivity. An overnight culture of the "producer" organism was grown in BHI-YE broth at 37°C in a Torbal jar under anaerobic conditions (10% CO₂-90% H₂ gas). These overnight cultures were stabbed into a sterile BHI-YE agar base with eight different organisms per plate. These plates were incubated for 18 h under the above conditions.

Sensitive indicator organisms were grown overnight in BHI-YE broth as above. A 0.1-ml sample of the overnight culture was inoculated into 4.9 ml of BHI-YE broth and incubated aerobically at 37°C until it reached a standard turbidity (40 Klett units on the Klett-Summerson colorimeter with the green filter [Klett Manufacturing Co.]). A 0.1-ml portion of a 1:10 dilution of this culture was inoculated into

TABLE 1. *Bacteriocin production and sensitivity tests for the known streptococcal strains*

Indicator	Producer ^a													
	Channon	JC43	Wicky	Blackburn-Rotta	Challis	ATCC 10558	ATCC 10556	ATCC 10557	147P	K208	F90A	Thompson	Enole	410
Thompson	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Enole	0	0	0	0	0	0	0	0	0	0	0	0	0	0
410	1, S	2, S	2, S	0	1, S	2, S	1, S	0	0	1, S	0	1, S	0	0
F90A	0	0	2, S	0	0	1, D	1, S	0	0	1, S	0	0	0	0
174P	0	0	0	0	0	0	1, S	0	0	0	0	0	1, S	0
Blackburn-Cole	0	0	0	0	0	0	0	0	0	0	0	0	0	0
K208	0	0	0	0	0	0	0	0	0	0	0	1, S	1, S	0
Channon	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Wicky	0	0	0	0	2, S	0	0	0	0	0	0	0	0	0
Blackburn-Rotta	0	2, S	0	0	0	0	0	0	0	0	0	0	0	0
ATCC 10558	0	0	0	0	0	0	1, S	0	0	0	0	0	0	0
ATCC 10556	1, S	2, S	0	0	0	0	1, S	0	0	0	0	1, S	0	1, S
ATCC 10557	0	2, S	0	1, S	0	0	1, S	0	1, S	0	0	0	0	0
Challis	0	2, S	0	0	0	0	0	0	0	0	0	0	0	0

^a The zones of inhibition were scored according to their size as follows: 0, No inhibition, 1, radius of inhibition, 1 to 3.9 mm; 2, radius of inhibition, 4 to 6 mm; 3, radius of inhibition >6 mm; D, diffuse border; S, sharp border.

1.9 ml of melted and cooled BHI-YE agar. The agar was poured onto the stabbed plate, evenly distributed over the surface, and allowed to dry. These plates were incubated for 18 to 24 h under anaerobic conditions at 37°C; after incubation the plates were read. The zones of inhibition were scored according to their size: 0, a zone of less than 1 mm; 1, a zone of 1 to 3.9 mm; 2, a zone of 4 to 6 mm, and 3, a zone of greater than 6 mm. Also, the periphery of the zone was recorded as sharp (s) or diffuse (d).

Assay for bacteriophages. A piece of the agar from the inhibition zones of the bacteriocin assay plate was aseptically removed and placed on a sterile BHI-YE agar plate. A sensitive organism was overlaid, incubated anaerobically for 18 h, and observed for plaques.

RESULTS

To differentiate strains, both production and sensitivity patterns were necessary. Reproducibility was established by performing the same test five times with at least four of the five trials in agreement. The results for the known oral streptococcal strains are summarized in Table 1.

Eight bacterial endocarditis isolates were obtained from January 1973 to June 1973. Biochemical profiles were performed on these isolates, and the strains were incorporated into the typing system. The sensitivity patterns

from the endocarditis strains are shown in Table 2, and the production patterns are shown in Table 3. Patients 5a and 5b had the same sensitivity patterns, whereas E49F, SS26, and patients 1 and 7 demonstrate identical production patterns. However, the combination of production and sensitivity patterns gave a distinctive pattern for four of these strains. Patients 5a and 5b, from the same individual, gave the identical pattern for production and sensitivity. Patient 5a was isolated prior to antibiotic therapy, and patient 5b was isolated a month after an apparent cure of bacterial endocarditis had relapsed. Both organisms had identical biochemical profiles and bacteriocin production and sensitivity patterns.

DISCUSSION

The properties and characteristics of bacteriocins have been delineated by Jacob et al. (7) and are summarized in the reviews of Fredericq (3), Ivánovics (6), and Reeves (9). Although biochemical analyses are required to prove the presence of bacteriocin, characteristics typical of bacteriocins were observed in this study. All of our strains produced a substance that inhibited one or more indicator strains, but none produced an inhibitor against itself. These properties are typical of bacteriocins and are

TABLE 2. *Sensitivity patterns of endocarditis isolates*

Indicator	Producer ^a															
	HHT	SLI	GS5	E49F	KIR	AHT	SS26	Patient 7	Patient 1	Patient 5a	Patient 5b	Patient 6	Patient 4	Patient 2	Patient 3	
Patient 1	2, S	2, S	2, S	0	2, S	2, S	0	0	0	2, S	2, S	3, S	1, S	0	0	
Patient 2	2, S	0	2, S	0	2, S	2, S	0	0	0	0	0	3, S	0	0	0	
Patient 3	2, S	0	2, S	0	2, S	2, S	0	0	0	1, D	1, D	3, S	0	0	0	
Patient 4	0	0	2, S	0	2, S	2, S	0	0	0	0	0	3, S	0	0	0	
Patient 5a	0	0	0	0	2, S	2, S	0	0	0	0	0	3, S	0	0	0	
Patient 5b	0	0	0	0	2, S	2, S	0	0	0	0	0	3, S	0	0	0	
Patient 6	0	0	0	0	1, S	0	0	0	0	0	0	0	0	0	0	
Patient 7	0	0	2, S	0	2, S	2, S	0	0	0	0	0	3, S	0	0	0	

^a The zones of inhibition were scored according to their size as follows: 0, no inhibition; 1, radius of inhibition, 1 to 3.9 mm; 2, radius of inhibition, 4 to 6 mm; 3, radius of inhibition >6 mm; D, diffuse border; S, sharp border.

TABLE 3. *Production patterns of endocarditis isolates*

Indicator	Producer ^a							
	Patient 7	Patient 1	Patient 5a	Patient 5b	Patient 6	Patient 4	Patient 2	Patient 3
HHT	0	0	0	0	3, S	0	0	0
SLI	0	0	1, S	1, S	0	1, S	0	0
GS5	0	0	1, S	1, S	3, S	0	0	0
E49F	0	0	0	0	3, S	0	0	0
KIR	0	0	0	0	2, D	0	1, D	0
AHT	0	0	1, D	1, D	0	1, D	0	0
SS26	0	0	1, D	1, D	0	1, S	0	1, D

^a The zones of inhibition were scored according to their size as follows: 0, no inhibition; 1, radius of inhibition, 1 to 3.9 mm; 2, radius of inhibition, 4 to 6 mm; 3, radius of inhibition >6 mm.

suggestive that the inhibitor is a bacteriocin. Holmberg and Hollander (4) attributed an inhibitory effect to bacteriocin production which they subsequently proved (5) to be due to peroxide production. The use of anaerobic conditions eliminated peroxide accumulation in the media. Thus, anaerobic conditions were used in this study to eliminate peroxide accumulation.

The bacteriocin producer and sensitive organisms used in the typing system can be increased. Only a limited number of producer organisms were utilized in this study. An ideal producer organism will inhibit 10 to 80% of the sensitive organisms. This range of inhibition would give the best results for a typing system.

The usefulness of a typing system to solve clinical problems was demonstrated in this study. The recurrent infection in one patient (patient 5a) was shown to be due to the original strain rather than to a new organism. Two important conclusions from a diagnostic and therapeutic viewpoint could be drawn based upon these typing results. (i) The source of reinfection is endogenous rather than exogenous. This endogenous focus most likely was from the heart valve itself, unless embolization from a monoinfected, distant site occurred. (ii) One can conclude that the original antibiotic therapy was inadequate. In this case, an experimental antibiotic was used in the therapeutic regimen, and it is possible that this drug did not reach the viable organisms within the valvular vegetations.

Rogers found a rather stable *S. mutans* populations for a given mouth, and it appears that one type predominates. Based on this information, he suggested that bacteriocin typing of *S. mutans* may be useful in epidemiological studies in caries production. He also suggested an alteration in bacteriocin sensitivity when grown in the presence of sucrose, but not glucose. These findings may be of great importance when considering disease caused by these organisms outside the oral cavity (10, 11).

Since the toxicity of bacteriocin on mammalian cell membranes has not been fully elucidated, screening the organisms involved in bacterial endocarditis for bacteriocin production might be useful to answer two questions: do all the organisms produce bacteriocins, and do some strains infect patients with greater regularity than others? The results of this study indicate that some endocarditis streptococci do not produce bacteriocins, at least for the indicator strains used here.

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LITERATURE CITED

1. Farmer, J. J. 1972. Epidemiological differentiation of *Serratia marcescens*: typing of bacteriocin production. *Appl. Microbiol.* 23:218-225.
2. Fredericq, P. 1946. Action bactéricide de la colicine K. *C. R. Soc. Biol.* 140:1295-1296.
3. Fredericq, P., and M. Betz-Barreau. 1948. Présence régulière et constante d'*Escherichia freundii* dans l'urine des malades atteints de fièvre typhoïde. *C. R. Soc. Biol.* 142:1078-1080.
4. Holmberg, K., and H. O. Hollander. 1972. Interference between gram-positive microorganisms in dental plaque. *J. Dent. Res.* 51:588-595.
5. Holmberg, K., and H. O. Hollander. 1973. Production of bacteriocidal concentrations of hydrogen peroxide by *Streptococcus sanguis*. *Arch. Oral Biol.* 18:423-434.
6. Ivánovics, G. 1962. Bacteriocins and bacteriocin-like substances. *Bacteriol. Rev.* 26:108-118.
7. Jacob, F. A., A. Woff, A. Siminovitch, and E. Wollman. 1953. Définition de quelques termes relatifs à la lysogénie. *Ann. Inst. Pasteur Paris* 84:222-224.
8. Kelstrup, J., S. Richmond, C. West, and R. J. Gibbons. 1969. Fingerprinting human oral streptococci by bacteriocin production and sensitivity. *Arch. Oral Biol.* 14:251-258.
9. Reeves, P. 1972. *The Bacteriocins*. Springer-Verlag, New York.
10. Rogers, A. H. 1975. Bacteriocin types of *Streptococcus mutans* in human mouths. *Arch. Oral Biol.* 20:853-858.
11. Rogers, A. H. 1974. Bacteriocin production and susceptibility among strains of *Streptococcus mutans* grown in the presence of sucrose. *Antimicrob. Agents Chemother.* 6:547-550.