INFECTION AND IMMUNITY, Apr. 1984, p. 141–144
0019-9567/84/040141-04$02.00/0

Vol. 44, No. 1

Isolation of a *Streptococcus mutans* Strain Producing a Novel Bacteriocin

JEFFREY D. HILLMAN,* KRISTIN P. JOHNSON, AND BERNARD I. YAPHE
Forsyth Dental Center, Boston, Massachusetts 02115

Received 28 July 1983/Accepted 9 January 1984

A strain of *Streptococcus mutans* has been isolated that produces a bacteriocin with novel properties. Its antibiotic spectrum includes 123 of 124 strains of *S. mutans* tested and a variety of other gram-positive microorganisms. Experiments with dialysis membranes indicate that the molecular weight of the bacteriocin is less than 1,000. Mutants of the producer strain were obtained that are deficient in bacteriocin production or produce twofold elevated amounts. The ability of these strains to superinfect or preemptively colonize the oral cavities of rats correlated with the amount of bacteriocin they produced.

Since their discovery by Kelstrupp and Gibbons (10), bacteriocins produced by *Streptococcus mutans* have attracted interest as epidemiological tools (5, 11, 14, 15), as prospective anticaries agents (7, 9), and as factors possibly important in promoting colonization of the oral cavity (8, 17–19). Bacteriocinogeny is a common property of *S. mutans*. In some studies (5, 16), up to 80% of the strains examined demonstrated the ability to produce a bacteriocin. Most of these further demonstrated unique spectra of bacterial inhibition, thereby indicating marked diversity within this class of molecules. More extensive testing of some of these bacteriocins indicated that their diverse nature extends to their chemical and physical properties (9, 13, 18).

The diverse nature of *S. mutans* bacteriocins has provided a basis for various epidemiological studies, such as determining the mode of transmission of this organism (2, 14). However, their diversity provides an obvious source of confusion for studies concerned with the anticaries and colonization-promoting potential of bacteriocins; the same experiment performed with different bacteriocins is likely to yield different results. The ability of a bacteriocin to serve as a useful anticaries agent or as a factor in promoting colonization would depend, in large part, on both its potency and spectrum of bacterial inhibition. To date, no one has studied an *S. mutans* bacteriocin that is extreme in these properties.

The following report describes the isolation of a strain of *S. mutans* that inhibits the growth of virtually all other strains of this microorganism tested. Characterization of the inhibitory activity indicates that it is a bacteriocin with a molecular weight of less than 1,000. Studies involving animal models indicate that this particular bacteriocin is an important parameter in promoting colonization of the oral cavity by the producer strain.

MATERIALS AND METHODS

Microorganisms. Laboratory strains of *S. mutans* and other oral bacteria listed in Table 1 were obtained from the Forsyth Dental Center collection. Fresh isolates of *S. mutans* were obtained by streaking saliva samples from 115 adult subjects on mitis salivarius agar containing bacitracin (4). After 2 days of incubation in candle jars at 37°C, colonies with characteristic morphology were purified on the selective medium and tested for their ability to ferment mannitol and sorbitol and to produce dextran from sucrose. One freshly isolated strain, called JH1000, was selected for further study. Strain JH1001 is a spontaneous mutant of strain JH1000 resistant to 1 μg of tetracycline per ml. It was obtained by previously described methods (6). Strains JH1005 and JH1010 are ethyl methanesulfonate-induced (6) mutants of JH1001 that produce, respectively, elevated and undetectable amounts of bacteriocin. They were obtained by spreading appropriately diluted samples of a mutagenized culture of JH1001 on brain heart infusion agar to give ca. 200 colonies per plate. After 18 h of incubation in candle jars at 37°C, the plates were overlaid with 3 ml of molten top agar (brain heart infusion broth containing 0.8% agar) containing three drops of an overnight culture of the *S. mutans* indicator strain Jo. After an additional 24 h of incubation, colonies producing relatively larger and smaller zones of inhibition were picked and purified by streaking and tested for their ability to produce bacteriocin by methods described below.

All strains were maintained in 50% (vol/vol) glycerol stabs stored at −40°C.

Detection of bacteriocin activity. Bacteriocin production was assayed by a modification of the overlay technique of Fredericq (3). Single colonies of the strains to be tested were stab inoculated into brain heart infusion medium and incubated overnight in candle jars at 37°C. Three drops of an overnight Todd-Hewitt broth culture of the indicator strain were mixed with 3 ml of molten top agar and poured evenly over the surface of the plate. After an additional 24 h of incubation, clear zones surrounding the test strain were measured.

Cell-free culture liquors containing bacteriocin activity were prepared by growing the producer strain aerobically for 24 h in Todd-Hewitt broth containing 0.5% glucose. Cells were removed by filtration. Quantitative estimates of bacteriocin activity were made by preparing an overlay as described above, using *S. mutans* strain Jo as the indicator. Serial twofold dilutions of the culture liquor to be tested for activity were prepared with sterile Todd-Hewitt broth. Samples (20 μl) were spotted onto the overlay and allowed to air dry, and the plates were incubated for 24 h in candle jars at 37°C. Zones of growth inhibition were scored on a scale ranging from 1 to 4 depending on the extent of clearing.

Sensitivity of the bacteriocin to hydrolytic enzymes. A cell-free culture liquor of strain JH1005 was prepared as described above. The pH was adjusted to 7.1 with 10 N NaOH.

* Corresponding author.

TABLE 1. Sensitivity of various oral bacteria to the JH1000 bacteriocin

Organism	Strain (serogroup)	Sensitivity[a]
Streptococcus mutans	FA1 (a)	+
	BHT-2 (b)	+
	LM7 (e)	+
	Ingbritt (c)	+
	MT-3 (c)	+
	10449 (c)	+
	E49 (a)	+
	JC2 (c)	+
	GS5 (c)	+
	PK1 (c)	+
Streptococcus salivarius	SS2	+
	02	+
	04	+
Streptococcus sanguis	Fc-1	+
	KJ3	+
	Challis	−
Streptococcus mitis	MT	+
	RE-7	+
	26	+
Streptococcus pyogenes	STA628	+
Streptococcus faecalis	RF	−
Staphylococcus aureus	DC3	+
Lactobacillus casei	Lac-6	−
Lactobacillus salivarius	UCL-37	+
Actinomyces israelii	X523	+
	10048	+
Actinomyces naeslundii	12104	+
	N16	+
	6-60B	+
Actinomyces viscosus	W1528	+
	T6	+
	M100	+
Micrococcus luteus	207-79	−
Bacteroides gingivalis	381	−
Wolinella recta	371	−
Capnocytophaga sputigena	4	−

[a] Zones of inhibition measuring greater than 5 mm in diameter were taken as indicating sensitivity to the bacteriocin.

Various enzymes, including trypsin, pronase, thermolysin, DNase I, RNase A, lipase types I, VI, and XI, and lysozyme, were added from sterile stock solutions to 1 ml of the culture liquor to give a final concentration of 100 μg/ml. The mixture was incubated for 30 min at 37°C, and 20-μl samples of serial twofold dilutions were spotted on an overlay of the indicator strain Jo prepared as described above. Sterile Todd-Hewitt broth treated with these enzymes served as a control.

Animal studies. Three groups of 24 conventional, *S. mutans*-free Sprague-Dawley rats were infected at 21 days of age with 0.1 ml of an overnight culture of strain Ingbritt. The infection was repeated the following day. After 3 weeks, subgroups of three animals were challenged once with a 10-fold serial dilution of the parent strain JH1001, the high bacteriocin-producing mutant JH1005, or the bacteriocin-deficient mutant JH1010. After 4 weeks, oral swab samples were streaked on brain heart infusion medium containing 1 mg of streptomycin or 1 μg of tetracycline per ml to identify the presence of strain Ingbritt or the challenge strains, respectively. The minimal infectious dose (MID) was taken to be the lowest concentration which caused persistent colonization of the challenge strain in over half of the animals tested.

RESULTS

Survey of *S. mutans* strains for bacteriocin production. Ten laboratory and ten freshly isolated *S. mutans* strains were screened by the overlay technique for their ability to inhibit each others' growth. Eight of these strains were found to inhibit the growth of one to nine of the other strains (data not shown). One freshly isolated strain, called JH1000 (serogroup c), inhibited the growth of all 19 of the other strains. JH1000 was tested against an additional 105 freshly isolated strains of *S. mutans*. Of these, the growth of 104 was inhibited.

Characterization of the JH1000 inhibitory activity. Representative strains of various oral bacteria were tested for their sensitivity to the JH1000 inhibitory activity by using the overlay technique (Table 1). In addition to *S. mutans*, most gram-positive organisms were found to be sensitive, including *Streptococcus salivarius*, *Streptococcus sanguis*, *Streptococcus mitis*, *Streptococcus pyogenes*, *Staphylococcus aureus*, *Lactobacillus salivarius*, and *Actinomyces* species. Gram-negative bacteria were invariably resistant to inhibition by strain JH1000.

The inhibitory factor was produced in detectable amounts only during early stationary phase and could be recovered from Todd-Hewitt broth cultures of JH1000. Its effect on other strains of *S. mutans* was bacteriocidal, since loopfuls of agar taken from clear zones were found to be sterile. The inhibitory activity in cell-free culture liquors was completely inactivated by treatment with trypsin under the conditions tested. Incorporation of trypsin inhibitor into the reaction mixture at a concentration of 100 μg/ml prevented this inactivation. The inhibitory activity was inactivated ca. 50% by treatment with pronase. Higher concentrations of pronase (250 μg/ml) or more prolonged treatment (1 h) resulted in complete inactivation of the bacteriocin activity. It appeared to be completely resistant to inactivation by DNase I, RNase A, lipases, thermolysin, and lysozyme. The proteinaceous nature of the inhibitor, indicated by this experiment, plus its biological activity formally qualify it for inclusion in the broad family of bacteriocins.

Twenty-four-hour stabs of JH1000 covered with sterile dialysis membranes (Spectrapore) were found to inhibit the growth of overlays of strain Jo. The size of the clear zone varied in proportion to the pore size of the membranes (Table 2). Significant inhibition was observed with membranes that exclude molecules larger than 1,000 daltons. Similar results were obtained with cell-free culture liquors of strain JH1000 as the source of the bacteriocin. These results indicate an unusually small size for the JH1000 bacteriocin.

Influence of bacteriocin production on colonization. In repeated testing, strain JH1005 was found to produce ap-

TABLE 2. Diffusion of the JH1000 bacteriocin through dialysis membranes

Membrane exclusion limit (daltons)	Area of inhibition (cm^2)
Control[a]	3.14
12,000	2.26
6,000	1.54
1,000	0.64

[a] Control was without a membrane.

proximately twice as much bacteriocin as its parent, JH1001. Strain JH1010 was found to produce undetectable amounts of the bacteriocin when tested against strain Jo and the other *S. mutans* strains listed in Table 1. These strains were used to test the role of bacteriocins in promoting colonization of the oral cavity in the presence of an indigenous strain of *S. mutans*. The MID for superinfection by the bacteriocin-deficient strain JH1010 was greater than 10^{10} CFU/ml (Table 3). The MID for both the parent JH1001 and the high producer JH1005 was found to be 10^5 CFU/ml. Unlike the parent strain, animals challenged with 10^8 or 10^9 CFU of JH1005 per ml were found to be completely free of strain Ingbritt within 4 weeks of the challenge. Thus, the ability to superinfect Sprague-Dawley rats previously colonized by strain Ingbritt corresponded to the amount of bacteriocin produced by the challenge strain. In the absence of an indigenous strain of *S. mutans*, the MIDs for all three strains were found to be less than or equal to 10^3 CFU.

The opposite experiment was also performed, in which groups of animals were first infected with JH1001, JH1005, or JH1010 and then challenged with serial 10-fold dilutions of strain Ingbritt. The MID for strain Ingbritt in animals previously colonized by JH1010 was found to be 10^8 CFU/ml (Table 4). The MID for superinfection by Ingbritt in animals colonized either by JH1001 or JH1005 was greater than 10^{10} CFU/ml, and JH1005 was more efficient at excluding Ingbritt than was its parent. These results indicate a correlation between bacteriocin production and the ability of the producer strain to preemptively infect the oral cavities of rats.

TABLE 3. Relationship of bacteriocin production to potential for superinfection

Challenge strain	Challenge concn (CFU/ml)	Frequency of superinfection
JH1010	10^3	0[a]
	10^4	0
	10^5	0
	10^6	0
	10^7	0
	10^8	0
	10^9	0
	10^{10}	0
JH1001	10^3	0
	10^4	0
	10^5	2
	10^6	3
	10^7	3
	10^8	3
	10^9	3
JH1005	10^3	0
	10^4	1
	10^5	3
	10^6	3
	10^7	3
	10^8	3
	10^9	3

[a] Values represent the number of animals persistently colonized out of three animals challenged.

TABLE 4. Relationship of bacteriocin production to preemptive colonization

Challenge strain	Challenge concn (CFU/ml)	Frequency of superinfection[a]		
		JH1010	JH1001	JH1005
Ingbritt	10^5	0	0	0
	10^6	0	0	0
	10^7	2	0	0
	10^8	5	0	0
	10^9	5	2	0
	10^{10}	5	2	0

[a] Values represent the number of animals persistently colonized out of five animals challenged.

DISCUSSION

A strain of *S. mutans* has been isolated that produces a bacteriocin with certain novel properties. The spectrum of its activity includes representatives of many streptococcal species and a variety of other gram-positive organisms as well. This suggests that the target for the bacteriocin has been highly conserved throughout evolution. Further, the target probably serves an essential function, since spontaneous or mutagen-induced bacteriocin-resistant mutants of strain Jo were not obtainable.

The small size of the bacteriocin, its relatively broad antibiotic spectrum, and its protein nature are analogous to the class of microcins observed in enterobacteria (1). Unlike some of these microcins, the JH1000 bacteriocin is not antagonized by L-methionine at a concentration of 10 μg/ml, nor does it have any activity against gram-negative organisms.

In two separate animal studies, it was found that the ability of the producer strains to superinfect or preemptively colonize the oral cavities of rats corresponded with the amount of bacteriocin produced in vitro. A definitive relationship could not be established since wild-type revertants of the high-producing and bacteriocin-deficient strains were not obtained. The failure to obtain such revertants probably reflects the inadequacy of the screening methods employed for their isolation. Screening of over 100 biochemical and physiological properties with the rapid identification system of Newman et al. (12) revealed no differences between parent and mutants, thereby suggesting that the observed differences in their ability to superinfect and colonize the oral cavities of rats were due to differences in the amounts of bacteriocin produced.

The application of these findings are twofold. The small size of the bacteriocin indicates its potential for purification, sequencing, and in vitro synthesis for use as a direct anticaries agent. Also, the ability of strain JH1005 to preemptively colonize the oral cavity and to completely displace an indigenous strain of *S. mutans* makes it a logical candidate from which to derive a nonvirulent effector strain for use in the replacement therapy of dental caries.

ACKNOWLEDGMENT

This work was supported in part by grant DE04529 from the National Institute of Dental Research.

LITERATURE CITED

1. **Asensio, C., J. C. Perez-Diaz, M. C. Martinez, and F. Baquero.** 1976. A new family of low molecular weight antibiotics from enterobacteria. Biochem. Biophys. Res. Commun. **69**:7–14.
2. **Berkowitz, R. J., H. V. Jordan, and G. White.** 1975. The early establishment of *Streptococcus mutans* in the mouths of infants. Arch. Oral Biol. **20**:171–174.
3. **Fredericq, P.** 1957. Colicins. Annu. Rev. Microbiol. **11**:7–22.
4. **Gold, O. G., H. V. Jordan, and J. van Houte.** 1973. A selective medium for *Streptococcus mutans*. Arch. Oral Biol. **18**:1357–1364.
5. **Hamada, S., and T. Ooshima.** 1975. Production and properties of bacteriocins (mutacins) from *Streptococcus mutans*. Arch. Oral Biol. **20**:641–648.
6. **Hillman, J. D.** 1978. Lactate dehydrogenase mutants of *Streptococcus mutans*: isolation and preliminary characterization. Infect. Immun. **21**:206–212.
7. **Hillman, J. D., K. P. Johnson, and B. I. Yaphe.** 1983. Characterization of a *Streptococcus mutans* bacteriocin with novel properties. J. Dent. Res. **62**:241.
8. **Huis in't Veld, J. H. J., J. S. Drost, and R. Havenaar.** 1982. Establishment and localization of mixtures of *Streptococcus mutans* serotypes in the oral cavity of the rat. J. Dent. Res. **61**:1199–1205.
9. **Ikeda, T., T. Iwanami, M. Hirasawa, C. Watanabe, J. R. McGhee, and T. Shiota.** 1982. Purification and certain properties of a bacteriocin from *Streptococcus mutans*. Infect. Immun. **35**:861–868.
10. **Kelstrupp, J., and R. J. Gibbons.** 1969. Bacteriocins from human and rodent streptococci. Arch. Oral Biol. **14**:251–258.
11. **Kelstrupp, J., and R. J. Gibbons.** 1970. Fingerprinting human oral streptococci by bacteriocin production and sensitivity. Arch. Oral Biol. **15**:1109–1116.
12. **Newman, M. G., S. S. Socransky, E. Savitt, M. Krichevsky, M. A. Listgarten, and W. Lai.** 1974. Characterization of bacteria isolated from periodontosis. J. Dent. Res. **53**:136.
13. **Paul, D., and H. D. Slade.** 1975. Production and properties of an extracellular bacteriocin from *Streptococcus mutans* bacteriocidal for group A and other streptococci. Infect. Immun. **12**:1375–1385.
14. **Rogers, A. H.** 1975. Bacteriocin types of *Streptococcus mutans* in the human mouth. Arch. Oral Biol. **20**:853–857.
15. **Rogers, A. H.** 1976. Bacteriocin patterns of strains belonging to various serotypes of *Streptococcus mutans*. Arch. Oral Biol. **21**:243–249.
16. **Rogers, A. H.** 1976. Bacteriocinogeny and the properties of some bacteriocins of *Streptococcus mutans*. Arch. Oral Biol. **21**:99–103.
17. **van der Hoeven, J. S., and A. H. Rogers.** 1979. Stability of the resident microflora and the bacteriocinogeny of *Streptococcus mutans* as factors affecting its establishment in specific pathogen-free rats. Infect. Immun. **23**:206–212.
18. **Weerkamp, A., L. Bongaerts-Larik, and G. D. Vogels.** 1977. Bacteriocins as factors in the in vitro interaction between oral streptococci in plaque. Infect. Immun. **16**:773–780.
19. **Weerkamp, A. H., G. D. Vogels, and M. Skotnicki.** 1977. Antagonistic substances produced by streptococci from human dental plaque and their significance in plaque ecology. Caries Res. **11**:245–256.