Purification and Certain Properties of a Bacteriocin from *Streptococcus mutans*

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An inhibition factor from *Streptococcus mutans* strain C3603 (serotype *c*) was purified and isolated, and its properties indicated that it was a bacteriocin. Bacteriocin C3603 is a basic protein with a pI value of 10 and a molecular weight of 4,800. The activity of this bacteriocin was not affected by pH over a range of 1.0 to 12.0 or by storage at 100°C for 10 min at pH 2.0 to 7.0 or storage at 121°C for 15 min at pH 4.0. Pronase; papain, phospholipase C, trypsin, and α -amylase had no effect on the activity of the bacteriocin, whereas α -chymotrypsin and pancreatin were partially active against it. Bacteriocin activity was greater against certain *S. mutans* strains of serotypes *b*, *c*, *e*, and *f* than against certain *S. mutans* strains of serotypes *a*, *d*, and *g*. Bacteriocin C3603 was also effective against selected strains of *S. sanguis*, *S. salivarius*, *S. bovis*, *S. faecium*, *S. lactis*, *Lactobacillus casei*, *L. plantarum*, *L. fermentum*, *Bifidobacterium bifidum*, *Bifidobacterium longum*, *Propionibacterium acnes*, and *Bacteroides melaninogenicus*, but it was not effective against certain strains of *Escherichia coli*, *Klebsiella pneumoniae*, *Corynebacterium parvum*, and *Candida albicans*. The inhibition of *S. mutans* strains BHT and PS-14 by bacteriocin C3603 was found to be due to the bacteriocidal activity of the bacteriocin. When water or a diet containing bacteriocin C3603 was consumed by gnotobiotic and specific pathogen-free rats infected with *S. mutans* PS-14, the caries score was found to be significantly reduced.

Streptococcus mutans, which is indigenous to the oral cavity, is generally accepted to be the principal etiological agent of dental caries in humans (5, 6, 9, 15-17). Bacteriocins produced by *S. mutans* are therefore of considerable interest since, by inhibiting sensitive bacteria, these agents may play an important role in the establishment of a given ecosystem in the oral cavity. Two recent surveys of clinical isolates and large collections of *S. mutans* indicate that there is a high percentage of bacteriocin-producing *S. mutans* strains (7, 27). Moreover, the bacteriocins produced by *S. mutans* have been found to be active not only against *Streptococcus* species but also against certain gram-positive bacteria (4, 7, 26).

Reports in the literature suggest that bacteriocin production by *S. mutans* is often affected by cultural conditions (11, 12) and that there are differences among the chemical and biological properties of various bacteriocins (4, 7, 23-26). This information demonstrates the multiplicity and complexity of bacteriocins and their production. With the exception of one work (23) which we know of, pure bacteriocins from *S. mutans*

have not been studied. We report here the isolation and purification of an inhibitory factor from *S. mutans* strain C3603 (serotype *c*) which, when subjected to sodium dodecyl sulfate (SDS)-disc gel electrophoresis, revealed a single protein band. In addition, we report certain physical, chemical, and biological properties of this factor which indicate that it is a bacteriocin.

MATERIALS AND METHODS

Microorganisms and growth media. *S. mutans* strain C3603 (serotype *c*), which was isolated from human dental plaque, was used to obtain the inhibitory factor employed in this study. Other microorganisms used are shown in Tables 3 and 4. *Streptococcus faecalis* C6SH was used as the standard indicator strain for the assay of bacteriocin C3603 activity; the minimum inhibitory concentration (MIC) of the bacteriocin for strain C6SH was 6 μ g/ml. Stock cultures of each strain were maintained at -80°C in brain heart infusion (Difco Laboratories, Detroit) agar slabs containing excess CaCO₃. The ingredients of the medium (LA medium) we used for the production of bacteriocin are as follows: glucose, 1%; peptone (Kyokuto; Tokyo, Japan), 2%; K₂HPO₄, 0.3%; KH₂PO₄, 0.2%; MgSO₄·7H₂O, 0.01%; MnSO₄·4H₂O, 0.002%; and

NaCl, 0.5% (pH 7.7). LA soft agar used for an assay of bacteriocin activity consisted of 1%; peptone, yeast extract (Difco), 0.5%; sodium acetate, 1%; glucose, 1%; and agar, 0.75% (pH 7.0). Other media used were partially defined medium with 0.5% glucose (PD-glucose) (10), tryptic soy broth (Difco), and mitis salivarius agar (Difco).

Purification of bacteriocin. Strain C3603, which was grown aerobically in 100 ml of LA medium at 37°C for 24 h, was used to inoculate 10 liters of LA medium, and the resulting culture was incubated aerobically at 37°C for 20 h. We centrifuged the culture at $20,000 \times g$ at 4°C, using a continuous centrifuge (Hitachi 20 PR-52), and then we added solid ammonium sulfate, to make the supernatant fluid 60% of saturation. The mixture was then allowed to stand at 4°C overnight. The precipitate was collected by continuous centrifugation at $20,000 \times g$ and then dissolved in a small volume of 0.01 M phosphate buffer, pH 7.0. Residual precipitate which did not dissolve was removed by centrifugation. The supernatant fluid was then applied to a carboxymethyl-Sephadex G-25 column (3.5 by 30 cm), and the column was washed first with approximately 850 ml of 0.01 M phosphate buffer, pH 7.0, and then with 0.05 M phosphate sodium hydroxide buffer, pH 9.5, until it was washed free of a mobile dark-brown band. Finally, the column was washed with 0.05 M phosphate sodium hydroxide buffer, pH 10.8, until a material which absorbed at 280 nm was eluted from the column (Fig. 1). The fractions containing bacteriocin-like activity were pooled, the pooled solution was applied to a Sephadex G-25 column (3.5 by 100 cm), and fractions containing bacteriocin-like activity were pooled and lyophilized. A summary of the purification of the bacteriocin-like material is presented in Table 1. Approximately 80 mg of the lyophilized preparation was obtained from the Sephadex G-25 step. This preparation, designated bacteriocin C3603, was employed in this study. Sterile culture medium was processed in the same manner as that described for the experimental culture medium. The sterile medium, the ammonium sulfate fraction (0 to 60%), and the carboxymethyl-Sephadex G-25 fraction did not contain any detectable bacteriocin activity (Table 1).

FIG. 1. Carboxymethyl-Sephadex-G25 elution profile of a dialyzed ammonium sulfate fraction of supernatant fluid from a culture of *S. mutans* C3603. The figure shows the elution profile of the phosphate-sodium hydroxide buffer (pH 10.8) step (see text). The pH of the collected fractions was adjusted to 7.0, and method A (see the text) was used to assay for bacteriocin activity.

Methods employed to measure bacteriocin-like activity. In this study, two methods were employed to measure the activity of the inhibitory factor. In method A, sterile solutions containing various concentrations of the bacteriocin-like material were prepared in 0.01 M potassium phosphate buffer, the pH of which was 7.0 to 8.0, depending upon the experiment. From each solution, 1 ml of material was taken and carefully layered onto LA soft medium seeded with *S. faecalis* strain C6SH. After 18 h of incubation at 37°C, the length of the zone of inhibition was measured. The log of bacteriocin C3603 concentrations was proportional to the length of the zone of inhibition. The LA soft agar medium was prepared in test tubes (12 by 105 mm) and inoculated with 2.5×10^5 colony-forming units (CFU) of *S. faecalis* strain C6SH. In method B, cells of test strains grown in tryptic soy broth culture (see Tables 3 and 4) were collected, washed three times with saline solution, and suspended in 0.05 M Tris buffer, pH 7.0 (ca. 10^6 CFU/ml). A 0.1-ml portion of the cell suspension was spread on PD-glucose agar plates containing different concentrations of bacteriocin C3603, and the plates were aerobically incubated at 37°C for 24 h. When *Propionibacterium*, *Corynebacterium*, and *Bacteroides* species were tested, tryptic soy broth agar was used instead of PD-glucose agar, and the plates were incubated anaerobically for 40 h. After incubation, the CFU count was determined. For both methods, the linear portion of standard curves (percent inhibition versus log of bacteriocin concentration) was used. Method B was used to determine the bacteriocin MICs necessary to inhibit 50 and 100% of the various test strains.

Effect of enzymes and diet components on bacteriocin C3603 activity. (i) **Effect of enzymes.** The effect of various enzymes on bacteriocin C3603 activity was determined by incubating 1 ml of enzyme solution (300 μg/ml in 0.01 M potassium phosphate buffer [pH 7.0 or 8.0, depending on the enzyme]) with 1 ml of bacteriocin C3603 solution (300 μg per ml of water) at 37°C for 1 h. We terminated the reaction by heating the solution at 100°C for 5 min. The enzyme-treated bacteriocin C3603 was assayed by method A. The enzymes tested were papain (Amano Pharmaceutical, Nagoya, Japan), pronase (Calbiochem, La Jolla, Calif.), lipase M-AP-10 (Amano), phospholipase C (Sigma Chemical Co., St. Louis, Mo.), trypsin type 1 (Sigma), α-amylase (Tokyo Kasei, Tokyo, Japan), α-chymotrypsin type 11 (Sigma), and pancreatin (Amano).

(ii) **Effect of diet components.** The effect of diet components on bacteriocin activity was determined by incubation of various amounts of test materials (see Table 7) with 2 ml of bacteriocin C3603 solution (50 μg/ml) for 1 h. After centrifugation at $12,000 \times g$ for 10 min, the activities of the supernatant fluids were measured by method A.

Bactericidal activity of bacteriocin C3603. *S. mutans* strains PS-14 and BHT were grown in tryptic soy broth, and 0.01 ml (optical density at 660 nm, 1.0) of the resulting culture was used to inoculate PD-glucose medium. The cells were collected, washed three times with saline solution, and suspended to an optical density at 660 nm of 1.0. From these suspensions, 0.1 ml of material was taken and added to 5 ml of 0.05 M Tris-hydrochloride buffer, pH 7.0, containing amounts of bacteriocin ranging from 5 to 120 μg/ml. The mixtures were incubated at room temperature for 20

TABLE 1. Purification of bacteriocin C3603

Purification step	Vol (ml)	Protein ^a (mg)	Activity (μg)	Sp act (μg/mg of protein)	Recovery (%)	Purification
Control						
Sterile medium	2,000	23,000	ND ^b			
(NH ₄) ₂ SO ₄ , 0–60%	600	1,640	ND			
CM–Sephadex G-25	90	ND	ND			
Experimental						
Culture supernatant fluid	2,000	23,800	53,700	2.4		
(NH ₄) ₂ SO ₄ , 0–60% fraction	600	1,740	30,600	17.6	57	7.3
CM–Sephadex G-25 fraction	90	15	12,600	824.0	23	343.0

^a Determined by the method of Lowry et al. (18); bovine serum albumin was used as the standard.

^b ND, Not detectable.

min, and then samples of the mixtures were immediately diluted and spread on tryptic soy agar plates. After an overnight incubation, the number of colonies was counted, and the percent kill was calculated from a control.

Molecular weight measurement. The molecular weight of bacteriocin C3603 was determined by sedimentation equilibrium with an analytical centrifuge, Hitachi model 282, equipped with an absorption scanning recorder, a DA-7 analyzer, an RAN 60 rotor, and a double-sector cell. The sample in sodium acetate buffer (pH 4.9; μ , 0.1) was centrifuged for 24 h at 40,000 rpm at 20°C. The density solvent and partial specific volume (v) were 0.9982 g/cm³ and 0.76, respectively.

Animal experiment. Two rat model systems were employed to test bacteriocin C3603 for its cariostatic effect. (i) Germfree 19-day-old Fisher rats were infected with streptomycin-resistant (0.5 mg/ml) *S. mutans* PS-14. The rats were fed a low-sucrose diet, diet 305 (19), and 50 ppm of bacteriocin C3603 (50 μ l/liter) in their drinking water and were housed under sterile conditions for 26 days. (ii) Specific pathogen-free, Sprague-Dawley rats whose oral microbiota were suppressed by ampicillin, carbenicillin, and cephalothin treatment for 4 days starting at 19 days of age (20) were infected when they were 24 days old with *S. mutans* strain PS-14. The rats were fed a high-sucrose diet, diet 2000 (14), plus 500 ppm of bacteriocin C3603 (500 μ l/liter) for 37 days. Control groups were fed in the same manner except that they did not receive bacteriocin. Oral swabs were collected once a week and cultured on mitis salivarius agar plus streptomycin (0.5 mg/ml).

RESULTS

Biochemical properties. Bacteriocin C3603 (after the Sephadex G-25 step) was subjected to SDS-polyacrylamide gel disc electrophoresis by the method of Weber and Osborn (31), and a single band was detected by a protein stain (Fig. 2). The molecular weight value of 4,800 (v = 0.76) was obtained by sedimentation equilibrium analysis. The isoelectric point value of 10.0 was obtained on polyacrylamide gels by the method of Wrigley (32). The inhibitory activity was stable when the bacteriocin was stored in aque-

ous solutions (500 μ g/ml) at pH values ranging from 1 to 12 at 37°C for 24 h. Bacteriocin C3603 was stable at 100°C for 10 min at pH 2 to 7 and also at 121°C for 15 min at pH 4.0. However, the

FIG. 2. SDS-polyacrylamide gel electrophoresis of purified bacteriocin C3603. Purified bacteriocin C3603 (after the Sephadex G-25 step) was applied to a 20% polyacrylamide gel containing 0.1% SDS. Electrophoresis was carried out for 1.5 h at a constant current of 8 mA per tube. The gel was stained for protein with Coomassie brilliant blue.

bacteriocin was inactivated by storage at 121°C for 10 min at pH 7.0. The activity was also stable when an aqueous solution (500 µg/ml, pH 4.0) was held at room temperature for 2 weeks and then heated at 121°C for 10 min. Papain, pronase, lipase, phospholipase C, trypsin, and α-amylase were inactive against the bacteriocin; however, α-chymotrypsin and pancreatin were only slightly active against it. The results are summarized in Table 2. Phagelike structures were not observed by electron microscopy.

TABLE 2. Properties of bacteriocin C3603

Property	Determination
Basic peptide	pI = 10.0
Mol wt	4,800
Solubility	500 µg/ml of water
pH stability (aqueous solution)	
pH 1.0–12.0	Stable
Heat stability (aqueous solution)	
pH 2.0	
100°C, 10 min	Stable
121°C, 10 min	Stable
pH 4.0	
100°C, 10 min	Stable
121°C, 15 min	Stable
pH 7.0	
100°C, 10 min	Stable
121°C, 10 min	Inactivated
Stability of aqueous solution ^a	Stable
Effect of enzyme treatment	
Papain (pH 7.0)	Stable
Pronase (pH 8.0) ^b	Stable
Lipase (pH 7.0)	Stable
Phospholipase C (pH 7.0)	Stable
Trypsin (pH 7.0)	Stable
α-Amylase (pH 7.0)	Stable
α-Chymotrypsin (pH 8.0) ^b	Partially inactivated
Pancreatin (pH 7.0)	Partially inactivated

^a The solution (500 µg/ml, pH 4.0) was stored at room temperature for 2 weeks and then at 121°C for 15 min.

^b See the text.

TABLE 3. Sensitivity of various streptococci to bacteriocin C3603

Indicator strain (serotype)	MIC (µg/ml) needed for:	
	100% Inhibition	50% Inhibition
<i>S. mutans</i>		
HS1 (a)	120	52
OMZ61 (a)	120	49
AHT (a/g)	130	65
BHT (b)	13	6
JC1 (c)	42	23
JC2 (c)	45	23
PS-14 (c)	32	15
Ingbritt (c)	25	13
B13 (d)	100	50
OMZ176 (d)	130	50
LM7 (e)	20	6
OMZ175 (f)	45	10
6715 (g)	100	55
K1R (g)	100	45
<i>S. sanguis</i>		
ATCC 10556	15	10
ATCC 10557	13	10
ATCC 10558	13	10
<i>S. salivarius</i>		
HHT	16	5
ATCC 9222	5	3
<i>S. bovis</i>		
ATCC 9809	6	3
<i>S. faecium</i>		
ATCC 6057	25	20
<i>S. lactis</i>		
ATCC 19435	7	4

Sensitivity of various bacteria to bacteriocin C3603. Several *Streptococcus* species, including seven serotypes of *S. mutans* and certain oral bacteria, were tested for their sensitivities to bacteriocin C3603. Results of an experiment to determine the bacteriocin MICs that inhibit 100 and 50% of the streptococci are shown in Table 3. In the case of *S. mutans*, the MICs for strains BHT (serotype *b*), JC1, JC2, PS-14, Ingbritt (serotype *c*), LM7 (serotype *e*), and OMZ175 (serotype *f*) were 45 µg/ml or less, and the MICs for strains HS1, OMZ61 (serotype *a*), B13, OMZ176 (serotype *d*), 6715, K1R (serotype *g*), and AHT (serotype *a/g*) were 100 µg/ml or greater. These results suggest that strains belonging to serotypes *b*, *c*, *e*, and *f* are more sensitive to bacteriocin C3603 than are the strains belonging to serotypes *a*, *d*, and *g*. A similar serotype grouping of strains sensitive to bacteriocin C3603 was obtained when the MICs needed to inhibit 50% of the strains were determined: for strains of serotypes *b*, *c*, *e*, and *f*,

these concentrations were equal to or less than 23 $\mu\text{g/ml}$, and those for strains of serotypes *a*, *d*, and *g* were equal to or greater than 45 $\mu\text{g/ml}$.

Table 3 also shows that the other streptococci tested were also sensitive and that the MICs needed to inhibit 50 and 100% of the strains were less than or equal to 25 and 20 $\mu\text{g/ml}$, respectively. The MICs for *Staphylococcus aureus* IAM307, *Leuconostoc mesenteroides* P-60 and IAM1151, *Lactobacillus plantarum* ATCC 8014, *Lactobacillus casei* ATCC 7469, *Lactobacillus fermentum* ATCC 9338 and IFO3954, *Bifidobacterium bifidum* RM1, *Bifidobacterium longum* RM20, *Propionibacterium acnes* ATCC 11827, ATCC 11828, and Exc-1, and *Bacteroides melaninogenicus* 3-8 were 25 $\mu\text{g/ml}$ or less (Table 4). However, *Corynebacterium parvum* ATCC 11829, *Escherichia coli* TW, *Klebsiella pneumoniae* NUD, and *Candida* species were not sensitive and grew in the presence of ≥ 100 μg of bacteriocin per ml.

Antibacterial effect. To obtain information on the nature of the inhibition by bacteriocin C3603, we treated cell suspensions of *S. mutans* BHT or PS-14 with increasing concentrations of the bacteriocin and then determined the number of surviving CFU. The results presented in Fig. 3 suggest that the inhibitory activity of bacteriocin C3603 for these strains is a bactericidal one. The bacteriocin concentration needed to kill 50% of the cells of *S. mutans* BHT or PS-14 was 6 or 15 $\mu\text{g/ml}$, respectively.

Inhibition of caries in rats. Experiments were performed to determine the effect of bacteriocin C3603 on the incidence of caries in rats fed a

TABLE 4. Sensitivity of various bacteria to bacteriocin C3603

Indicator strain	MIC ($\mu\text{g/ml}$)
<i>Staphylococcus aureus</i> IAM307	6
<i>Leuconostoc mesenteroides</i> P-60	25
<i>Leuconostoc mesenteroides</i> IAM1151	13
<i>Lactobacillus plantarum</i> ATCC 8014	6
<i>Lactobacillus casei</i> ATCC 7469	13
<i>Lactobacillus fermentum</i> ATCC 9338	25
<i>Lactobacillus fermentum</i> IFO3954	25
<i>Bifidobacterium bifidum</i> RM1	3
<i>Bifidobacterium longum</i> RM20	3
<i>Propionibacterium acnes</i> ATCC 11827	3
<i>Propionibacterium acnes</i> ATCC 11828	3
<i>Propionibacterium acnes</i> Exc-1	3
<i>Corynebacterium parvum</i> ^a ATCC 11829	100
<i>Bacteriodes melaninogenicus</i> OK-2	50
<i>Bacteriodes melaninogenicus</i> 3-8	3
<i>Escherichia coli</i> TW	>100
<i>Klebsiella pneumoniae</i> NUD	>100
<i>Candida albicans</i> N2	>100
<i>Candida tropicalis</i> S1	>100
<i>Candida krusei</i> YS1	>100

^a Listed as *Propionibacterium acnes* in the 1978 ATCC catalogue.

cariogenic diet. When gnotobiotic rats infected with *S. mutans* PS-14 were fed diet 305 with 50 ppm of bacteriocin in the drinking water for 26 days, the incidence of caries in these rats was significantly lower than that of rats receiving no bacteriocin (Table 5). The incidence of caries in specific pathogen-free rats infected with *S. mutans* PS-14 and fed diet 2000 plus 500 ppm of bacteriocin for 37 days was also significantly lower than that of control rats (Table 6). Buccal and proximal caries were reduced by 46.4 and 64.0%, respectively.

Inhibition of activity by diet components. The administration of bacteriocin C3603 to rats via drinking water or diet significantly reduced the incidence of caries; however, this reduction was not as great as one might have expected from the in vitro results. Accordingly, experiments were performed to investigate this lower activity of bacteriocin, and the results obtained suggested that certain diet components were found to be inhibitory. Treatment of bacteriocin C3603 with diet 305 or 2000 reduced the inhibitory activity by 86 or 83%, respectively, and certain proteins and carbohydrates also inhibited the activity (Table 7). However, several hydrolyzed protein and carbohydrate products did not inhibit the activity of bacteriocin.

DISCUSSION

This report describes the isolation and certain properties of a highly purified bacteriocin produced by *S. mutans* strain C3603 (serotype *c*).

FIG. 3. Bactericidal activity of bacteriocin C3603.

TABLE 5. Mandibular caries scores of gnotobiotic rats infected with *S. mutans* PS-14 and fed diet 305 with or without 50 ppm of bacteriocin C3603 in drinking water for 26 days

Diet	No. of rats	Mean caries score for indicated surface ^a			
		Buccal	Proximal	Sulcal	Total
Diet 305	10	42.6 ± 3.4	19.1 ± 2.0	51.1 ± 6.2	112.8 ± 8.3
Diet 305 + bacteriocin	12	32.9 ± 2.7	16.5 ± 3.9	45.4 ± 4.3	94.8 ± 9.2

^a Determined by the Keyes method (13). The comparison of the mean caries score of sulcal, buccal, and total surfaces shows significant differences; i.e., $P \leq 0.05$, $P \leq 0.01$, and $P \leq 0.01$, respectively.

Bacteriocin C3603 is a basic protein with a molecular weight of 4,800, as determined by sedimentation equilibrium. By using *S. faecalis* strain C6SH as the indicator strain, we found that bacteriocin C3603 was stable to pH changes over a wide range; resistant to heat treatments (100°C for 10 min), papain, pronase, phospholipase C, trypsin, and α -amylase; and partially resistant to α -chymotrypsin and pancreatin. A comparison of bacteriocin C3603 with other reported bacteriocins produced by *S. mutans* suggests that these inhibitory factors vary widely in their chemical, physical, and biological properties. Preliminary composition studies indicate that bacteriocin C3603 contains aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, methionine, isoleucine, tyrosine, phenylalanine, tryptophan, lysine, and arginine. Carbohydrate was not detected by a phenol sulfuric acid method. Additional analyses are currently being performed.

It is evident from several reports that proteolytic enzyme-treated bacteriocins are inactive, partially active, or active against indicator strains. Paul and Slade (23), employing *Streptococcus pyogenes* as the indicator strain, found that a bacteriocin obtained from *S. mutans* strain GS5, unlike the one produced by *S. mutans* strain C3603, is sensitive to trypsin and pronase. Rogers (26), on the other hand, using *S. pyogenes* as the indicator strain, reported that bacteriocins from serotype *c* strains of *S. mutans* are either sensitive, partially sensitive, or insensitive to treatment by either trypsin or pronase. Results from other reports on trypsin- or pronase-treated bacteriocins from serotype *c* strains of *S. mutans* also indicate various sensitivities (12; R. E. Morhart, M. H. Schenkman,

and R. R. Fitzgerald, *J. Dent. Res.* **54A**:129, 1975). These results suggest that the bacteriocins produced by serotype *c* strains of *S. mutans* differ from one another, and these differences are reflected in the action of proteolytic enzymes. Much is known about the amino acid residues and the positions of peptide bonds of proteins attacked by various proteolytic enzymes (1, 22, 28), but to date, there have been no studies that have investigated the products of enzyme treatments of bacteriocins from *S. mutans*. There is a discussion of one experiment, somewhat relative to this point, included in a report by Hamada and Ooshima (7), who, by using three indicator bacteria, tested the activity of a preparation of pronase-treated bacteriocin from a serotype *c* strain of *S. mutans*. They found that the activity of bacteriocin after pronase treatment is unaffected and that it inhibits *Streptococcus salivarius* strain HHT. However, when *S. salivarius* strain OMZ9 or *S. mutans* strain BHT is used as an indicator, the same preparation is inactive or partially inactive, respectively. This indicates that pronase does alter the bacteriocin in question and that the differences in sensitivities observed for different bacteria may be due to undigested bacteriocin possibly remaining. It is also possible that there may be differences in the sensitivities of the bacteria to small peptide products in the digest, which may still retain active sites for bacteriocin-like activity.

The sizes reported for bacteriocins produced by *S. mutans* have been estimated mainly by the ability of these inhibitors to diffuse through membranes. Of the bacteriocins produced by serotype *c* strains of *S. mutans*, one has been reported to have a molecular weight in excess of

TABLE 6. Mandibular caries scores of specific pathogen-free rats infected with *S. mutans* PS-14 and fed diet 2000 with or without 500 ppm of bacteriocin C3603 for 37 days

Diet	No. of rats	Mean caries score for indicated surface ^a			
		Buccal	Proximal	Sulcal	Total
Diet 2000	12	27.6 ± 2.4	5.1 ± 0.6	60.3 ± 4.8	92.8 ± 6.6
Diet 2000 + bacteriocin	12	14.8 ± 2.2	1.8 ± 0.5	45.2 ± 2.2	61.7 ± 4.0

^a Determined by the Keyes method (13). The comparison of the mean caries scores of buccal, proximal, and total surfaces shows significant differences; i.e., $P \leq 0.05$.

TABLE 7. Inhibition of bacteriocin C3603 by diet components

Inhibitor	Bacteriocin/ inhibitor ratio	Inhibition (%)
No addition	1/0	0
Diet		
2000	1/2,000	83
305	1/2,000	86
Diet component		
Lactalbumin	1/2,000 1/400	96 83
Skim milk	1/2,500 1/625	47 35
Cornstarch	1/2,000 1/1,000	68 54
Carbohydrate		
Wheat flour (low gluten)	1/2,000	70
Wheat flour (low gluten)	1/1,000	65
Wheat flour (high gluten)	1/1,000	77
Cellulose	1/1,000	85
Amicol no. 1 (dextrin)	1/1,240	0
Amicol no. 1B (dextrin)	1/1,240	0
Maltodextrin	1/1,240	0
Run (dextrin)	1/1,240	0
Polypeptide		
Casein	1/560	47
Na-caseinate	1/4,000	54
Peptone (18 kinds) ^a	1/4,000	0

^a Thytone, peptone, neo-peptone, Casitone, tryptose, proteose peptone, *n*-z-amine A, AF, NAK, B, E, YT, edamin S, HY SAY powder, and N-2 case.

20,000 and is not dialyzable (23), others have been reported to have molecular weights ranging from 3,500 and 6,000 when membranes with different molecular weight cutoff values are used (Morhart et al., J. Dent. Res.), and still others have been reported to diffuse or not diffuse through dialysis membranes (7, 12, 26). We have found that bacteriocin C3603, which is a cationic protein, adsorbed to at least one type of membrane (Amicon), and perhaps caution should be taken in interpreting the results of membrane studies.

Like the physical and chemical properties, the inhibition ranges of bacteriocins from *S. mutans* differ from one another. Although bacteriocin C3603 inhibited all serotypes of *S. mutans* tested, strains belonging to serotypes *b*, *c*, *e*, and *f* were more sensitive than were strains belonging to serotypes *a*, *d*, and *g*. Moreover, this bacteriocin, like others, inhibited other species of

Streptococcus, many gram-positive bacteria, and some gram-negative bacteria and did not inhibit certain species of gram-negative bacteria and *Candida*. On the other hand, the partially purified bacteriocin from *S. mutans* GS5 (23) is inactive against the strains that we tested which belonged to serotypes *a*, *b*, *c*, and *d*, *Bacillus subtilis*, *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli* but is active against certain streptococcal strains of Lancefield groups A, C, D, G, H, L, and O. Results reported by other investigators (7, 11, 26) differ from one another with respect to activity of bacteriocins from serotype *c* strains against different bacteria. One of the reasons for this lack of pattern may be the qualitative nature of assaying for the potency of bacteriocins.

A study (27) relating various serotypes of *S. mutans* with production of and sensitivity to bacteriocins suggests that serotypes *a*, *b*, and *g* are identifiable and that strains belonging to serotypes *c*, *d*, *e*, and *f* are heterogeneous and do not form well-defined groups. In the study reported here, the serotypes of *S. mutans* could be classified into two groups on the basis of sensitivity to bacteriocin C3603. The strains of serotypes *a*, *d*, and *g* studied were less sensitive to bacteriocin C3603 than were the strains of serotypes *c*, *e*, and *f*. It appears that this pattern of sensitivity is consistent with the proposal of genetic, biochemical, antigenic, and cultural relatedness already reported by others (2, 3, 8, 21, 29, 30). Accordingly, it would be interesting to determine whether a purified bacteriocin from serotype *a*, *d*, or *g* would be more effective against strains belonging to serotypes *a*, *d*, and *g* than against strains belonging to serotypes *c*, *e*, and *f*.

Paul and Slade (23) demonstrated that the bacteriocin from *S. mutans* GS5 is lethal for *S. pyogenes*. In this report, bacteriocin C3603 is shown to be lethal for *S. mutans* strains BHT and PS-14 (Fig. 3). Figure 3 also shows that the death curves for *S. mutans* strains BHT and PS-14 have shoulders and that killing begins at about 5 and 25 µg/ml, respectively. Furthermore, both curves are curvilinear. The presence of a shoulder suggests that multiple "hits" are required for killing (assessed by determining CFU), as is the case for streptococci, and the lack of linearity suggests that more than one type of sensitive cell may be present in both cell suspensions.

A variety of antibacterial substances have been tested in experimental animals to determine caries reduction activity; however, to date, bacteriocins have not been examined. Although bacteriocin C3603 significantly reduced the incidence of caries when provided to rats in drinking water or food, certain dietary components may

bind bacteriocin and thus may reduce its potential efficacy. Bacteriocin C3603 was found to lose activity in the presence of casein, skim milk, lactalbumin, wheat flour, cornstarch, and cellulose, and this loss of activity may be due to binding. Similarly, another bacteriocin has been found to be inactivated by its binding to cellular or medium components (7). Since dextrans and peptones were not found to reduce the activity of bacteriocin C3603, rat diets are being modified to obtain a nutritious, cariogenic, non-bacteriocin-binding diet.

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