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# Production of Bacteriocins in a Liquid Medium by *Streptococcus mutans*

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A sterile-filtered, liquid medium composed of one-half-strength APT broth and 4% (wt/vol) yeast extract was found to support the production of bacteriocins by *Streptococcus mutans* strains BHT and GS-5. Culture supernatants, adjusted to pH 7.0 and sterilized by filtration, contained bacteriocin-like activity, which could be demonstrated by spotting dilutions onto top agar lawns seeded with *Streptococcus pyogenes* as the sensitive indicator and by adding dilutions to log-phase indicator broth cultures. A quantitative assay was developed for BHT bacteriocin, based on its lethal effects. Bacteriocin production did not occur until after the log phase of growth had ceased and was not inducible by ultraviolet irradiation or treatment with mitomycin C. Non-bacteriocinogenic clones of strain BHT occurred spontaneously at high frequency, suggesting control by a plasmid, but this frequency was not increased by treatment with the plasmid-curing agents acridine orange and ethidium bromide.

*Streptococcus mutans* is cariogenic in experimental animals (7, 8, 17) and is believed to be important in the initiation of smooth surface dental caries in humans (5, 6, 14). Bacteriocins produced by *S. mutans* are therefore of considerable interest since, by inhibiting other organisms, they may play important roles in establishing and/maintaining this pathogenic species in the oral cavity.

Whereas several strains of *S. mutans* are known to produce bacteriocins that are active against a number of related and unrelated gram-positive organisms (10-12), previous studies on these substances have been limited to systems involving agar-solidified media, because it has not been possible to produce these bacteriocins in liquid media, free of cells, or to extract active bacteriocins from agar media (11, 16). This inability to obtain active, cell-free bacteriocin preparations has hampered attempts to purify and characterize these interesting substances.

This paper reports the development of a simple, liquid medium which supports the production of active bacteriocins by two strains of *S. mutans*, describes methods suitable for detecting and quantitating these bacteriocins, and presents information on the synthesis and lethal activity of one of these bacteriocins.

## MATERIALS AND METHODS

**Bacteria.** *S. mutans* strain GS-5, a cariogenic human isolate (8) which belongs to Bratthall's serological group *c* (1), was obtained from R. J. Gibbons, Forsyth Dental Center, Boston, Mass. Strain BHT, another cariogenic human isolate (18), belongs to serological group *b* and was obtained from D. D. Zinner, University of Miami Institute of Oral Biology, Miami, Fla. The bacteriocin-sensitive indicator is a beta-hemolytic, group A clinical isolate of *Streptococcus pyogenes* which has been maintained in our department culture collection for several years.

**Media.** All bacteriological media, including yeast extract and various nitrogen sources, were obtained from BBL, Division of Becton, Dickinson & Co., Cockeysville, Md. Broth media included Trypticase Soy Broth (TSB), APT, Todd-Hewitt (TH), brain heart infusion, and nutrient broth. When used for top agar overlays and bottom agar plates, agar was added to the appropriate broth to a final concentration of 0.7 and 1.5% (wt/vol), respectively. TH-S is TH broth containing 4% NaCl. Purity and identity of the *S. mutans* was periodically checked by streaking them on Mitis-Salivarius agar; on this medium extracellular dextrans are produced which give each strain a distinctive, unique colonial morphology (13). Media were sterilized by autoclaving for 20 min at 15 lb/in$^2$ or by filtration (0.45-$\mu$m pore size, disposable filters, Nalgene Corp.). The liquid medium finally developed for bacteriocin production (see Results) is one-half-strength APT broth (22.5 g/liter) containing 4% (wt/vol) yeast extract (APT-YE).

**Chemicals.** Acridine orange was obtained from Calbiochem. LiCl was obtained from ICN-K & K Laboratories. Ethidium bromide, mitomycin C, and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine were obtained from Sigma Chemical Co. Other inorganic salts, alcohols, organic solvents, and sugars (all

analytical grade) were obtained from Fisher Scientific Co.

**Preparation of cell-free bacteriocins.** After overnight growth in 100 ml of APT-YE at 37 C (in stationary Pyrex milk dilution bottles), cultures were sonicated in 40-ml amounts by exposing them to four 15-s bursts of ultrasonic waves generated by the microtip probe of a Branson model LS75 Sonifier (power setting, 6). The cells were removed by centrifuging for 10 min at 12,100 × *g*, and the supernatant fluids were then adjusted to pH 7.0 with 0.1 N NaOH, sterile filtered, and stored at 4 C.

**Bacteriocin detection and assay.** The following semiquantitative spot test was used to detect bacteriocin activity and to compare the effects of various supplements and growth conditions on bacteriocin production. Two to three drops of an overnight 37 C TH broth culture of *S. pyogenes* were added to 2.5 ml of molten TH-S top agar, and the mixture was overlaid on a previously prepared TH-S agar plate. After solidifying, 0.02-ml aliquots of serial dilutions of bacteriocin suspension (made in sterile APT-YE, TSB, or 0.05 M phosphate buffer, pH 7.0) were spotted on the overlay and allowed to soak in. The plate was then incubated for 3 to 4 h at 37 C, after which time zones of inhibition were visible; maximum sensitivity is obtained by observing plates when the developing lawns of indicator cells first become visible.

A more sensitive, quantitative method (4) was modified for assaying BHT bacteriocin. One milliliter of log-phase indicator cells (ca. $10^8$ cells/ml) was added to 3.0 ml of TH-S and 1.0 ml of various dilutions of bacteriocin. The cultures were incubated for 2 h in a water bath at 37 C and chilled to stop further growth, and their optical density was read at 660 nm (Spectronic 20 colorimeter). A control culture, containing no bacteriocin, was used to determine the 100% increase in optical density, and the dilution of bacteriocin causing a 50% reduction was then taken as the number of killing units (KU) per milliliter.

**Induction and curing methods.** Attempts to induce bacteriocin production in log-phase APT-YE and TSB cultures of strain BHT were made by irradiating aliquots with ultraviolet light (35 cm below two G.E. 15 W germicidal lamps) for 0.5- to 5-min periods and by adding mitomycin C to final concentrations of 1 to 50 μg/ml. Treated cultures were incubated at 37 C for 4 h, with optical density readings being taken at 30-min intervals, adjusted to pH 7.0, and then sterile filtered and immediately tested for bacteriocin activity.

To determine the effects of plasmid-curing agents on bacteriocinogeny, log-phase cultures of strain BHT were diluted $10^6$, and 0.1-ml aliquots were added to tubes of fresh broth (10 ml) containing 1 to 50 μg of acridine orange per ml or 0.1 to 5 μg of ethidium bromide per ml; each tube received an inoculum of approximately 100 cells. After 24 and 48 h at 37 C, the tubes with the highest concentration of each curing agent which showed visible growth were diluted and spread on TSB agar plates and incubated for 24 h. Approximately 1,000 isolated colonies were then picked to fresh plates (10 per plate), incubated overnight, and then overlaid with *S. pyogenes*. After another 24-h incubation period, colonies that did not inhibit the indicator were picked and purified by restreaking on TSB agar and then tested again for loss of bacteriocin-producing ability. For comparison, untreated cultures (controls), cultures irradiated with ultraviolet light for 1.5 min, and cultures treated with 50 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 30 min were handled in the same manner.

**Extraction of cell-bound bacteriocin.** BHT cells grown overnight in APT-YE were collected from 100 ml of medium by centrifuging for 30 min at 8,000 × *g*, and the supernatant fluids were discarded. The pellets were then resuspended in 5 ml of various solvents and salt solutions for several hours at room temperature, diluted in phosphate buffer, and tested for bacteriocin activity by the spot test. The following solutions were used: acetone, butanol, chloroform, ethanol (95%), methanol, pyridine (10%), dimethyl sulfoxide (20 to 70%), NaCl (0.1 to 3 M), $MgSO_4$ (25%), sodium acetate (0.2 to 2 M), urea (6 to 8 M), LiCl (4 to 6 M), and phosphate buffer (0.05 M, pH 7.0).

## RESULTS

**Development of liquid APT-YE medium.** Whereas bacteriocin production was readily observed in agar-containing, autoclaved media by the stab-overlay technique, repeated attempts to detect bacteriocins in a variety of commercial liquid media were unsuccessful. Since this bacterial species has complex nutritional requirements (2, 3), it appeared that production of bacteriocins might require as yet unidentified, heat-sensitive nutritional factors. A series of liquid media containing increasing amounts of yeast extract was therefore prepared and sterilized by filtration. Using the commercially prepared media TSB and APT as the starting points, it was found that high concentrations of yeast extract (greater than 1%, wt/vol) did in fact allow bacteriocin synthesis in broth cultures. Autoclaving these media, moreover, destroyed their ability to support bacteriocin production. Cultures of BHT and GS-5 grown in TSB or APT containing 3 to 6% yeast extract yielded supernatants which, when neutralized to pH 7.0 and sterile filtered, contained bacteriocin-like activity that was detectable by spotting onto lawns of indicator cells. Similar media made with nutrient broth, brain heart infusion, or TH as the principal ingredient resulted in cultures with no such activity.

Using the semiquantitative spot test described in Materials and Methods, maximum cell-free bacteriocin activity was obtained with a medium composed of one-half strength APT plus 4% yeast extract APT-YE. Brief dialysis (4

h in 50 volumes of phosphate buffer) of the complete medium, the APT, or the yeast extract prior to sterile filtration prevented bacteriocin production, but not growth, suggesting that low-molecular-weight substances in both APT and yeast extract are required for bacteriocin production. These unknown factors are also heat sensitive, since autoclaving the medium eliminates its ability to support bacteriocin production.

**Effects of supplements and growth conditions on bacteriocin production.** With the development of the APT-YE medium it became possible to test the effects of additional supplements and different growth conditions on bacteriocin production. Variables tested included different carbon sources (5 to 50 g of glucose, sucrose, fructose, galactose, and glycerol per liter), nitrogen sources (1 to 4% Casamino Acids, casein, proteose peptone, Trypticase, tryptone, and neopeptone), divalent cations ($10^{-4}$ to $10^{-1}$ M Mg, Mn, Fe, and Ca), temperature (20 to 42 C), pH (5.5 to 8.0), reducing agents (0.1 to 1.0 g of cysteine-hydrochloride and sodium thioglycolate per liter), and different levels of anaerobiosis (shake flask, stationary flask, candle jar, and Brewer jar with 95% $H_2$-5% $CO_2$). Neither the type nor the amount of carbon source, cation, or reducing agent had any stimulatory effect on bacteriocin production. None of the nitrogen sources, at the concentrations tested, could substitute for yeast extract in promoting bacteriocin synthesis. Optimum temperature and pH conditions for bacteriocin production in APT-YE were found to be 37 C and 6.0 to 7.4, respectively. The degree of anaerobiosis had no detectable effect on the amount of bacteriocin produced under these conditions.

**Release of bacteriocins.** When BHT and GS-5 were grown overnight at 37 C in APT-YE (initial pH 7.2 to 7.4), active bacteriocins were found free of cells in low-speed supernatants. Vortexing the cultures with glass beads or sonicating them prior to centrifugation increased the yield of bacteriocin roughly 50%, doubling the dilution showing positive inhibition in the spot test. Since sonicating the supernatants alone did not increase the yield, some bacteriocin must remain rather loosely bound to the cells. Attempts to increase the yield of bacteriocins further by extracting cell pellets with a number of solvents and salt solutions (see above) were unsuccessful.

By sampling growing cultures of BHT in APT-YE at various times it was found that bacteriocin synthesis (or release) occurs very late, being undetectable until well after the stationary phase of growth is reached. This observation suggests that bacteriocin synthesis may be lethal to the producer cell. Due to the relatively low yield of bacteriocin and the chain-forming properties of *S. mutans*, however, it was not possible to obtain a clear relationship between viable cells, total cells, and quantity of bacteriocin.

**Lethal action of BHT bacteriocin.** Using the spot test, sonicated APT-YE bacteriocin preparations from strain BHT usually showed inhibitory activity when diluted 1:5 or 1:6. Figure 1 shows the results of such a test. When serial dilutions of this same preparation were added to log-phase indicator cultures, the results shown in Fig. 2 were obtained. At high concentrations BHT bacteriocin caused complete cessation of growth, after a lag period of approximately 30 min, but it did not cause actual lysis of indicator cells. A reproducible, quantitative assay for this bacteriocin was developed by carefully standardizing the type of experiment shown in Fig. 2 (see above).

The results of a typical experiment performed to determine the kinetics of cell killing by BHT bacteriocin are shown in Fig. 3. In this experiment viable count samples were immediately diluted at least $10^3$ to stop further adsorption; thus the data show that the bacteriocin has lethal rather than merely bacteriostatic effects. Killing occurred at a logarithmic rate, with a destruction curve consistent with single-hit kinetics. From the data shown in Fig. 3 it was calculated that 1 KU kills $2.5 \times 10^7$ colony-forming units/ml in 30 min at 37 C; when corrections are made for the number of cells per colony-forming unit (determined by microscopic counts) this figure corresponds to approximately $10^8$ total cells. APT-YE bacteriocin preparations usually contain 8 to 10 KU/ml.

**Inducibility of bacteriocin synthesis.** Bacteriocin production was not induced by ultraviolet irradiation or by treatment with mitomycin C, in either TSB or APT-YE. Both treatments, at levels ranging from no detectable inhibition of growth to 99.9% killing, did not increase the amount of bacteriocin produced by strain BHT. The possible role of intracellular levels of cyclic nucleotides in controlling the synthesis and release of *S. mutans* bacteriocins is presently being investigated.

**Loss of bacteriocinogeny by curing agents.** Whereas a slight increase in the frequency of non-bacteriocinogenic colonies was found after treatment with ultraviolet irradiation and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, growth of strain BHT in the presence of acridine orange and ethidium bromide did not cause a loss of bacteriocin-producing ability (Table 1); using identical treatments, 50 to 80% of the cells of

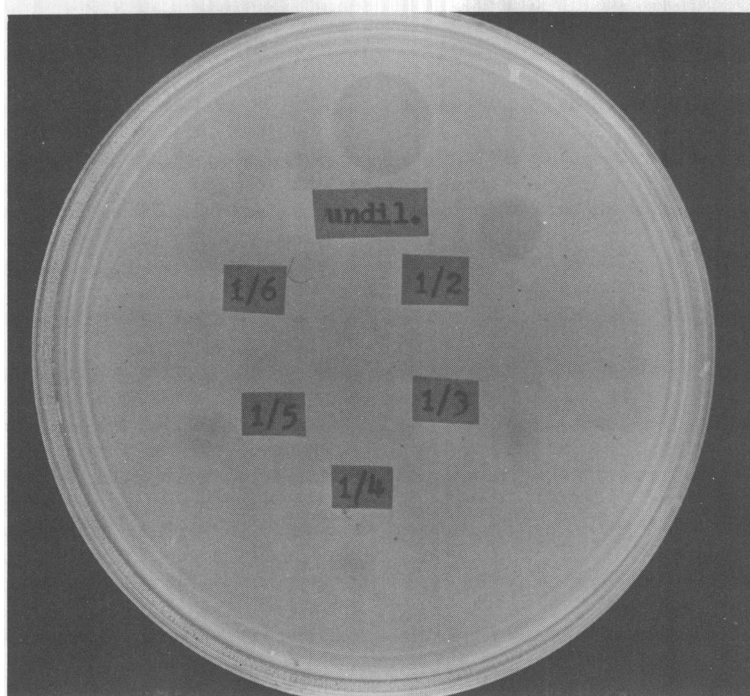


FIG. 1. *Spot assay of BHT bacteriocin. A 0.02-ml amount of various dilutions of bacteriocin (indicated on plate) was spotted onto a top agar lawn seeded with indicator cells. After drying the plate was incubated for 4 h at 37 C.*

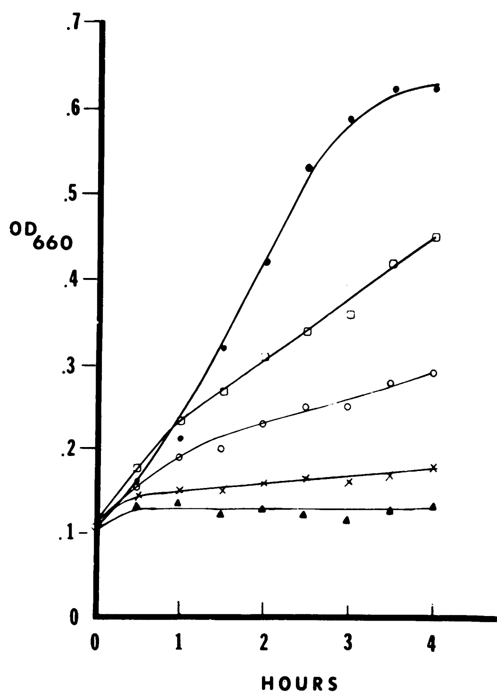


FIG. 2. *Effect of BHT bacteriocin on growth of S. pyogenes. At zero time, 1.0 ml of bacteriocin dilution or TSB (control) was added to identical 4.0-ml TSB log-phase cultures of S. pyogenes. The cultures were incubated at 37 C, and their optical density at 660 nm ($OD_{660}$) was read at the indicated times. Symbols: (▲) undiluted bacteriocin; (×) diluted 1:4; (O) diluted 1:8; (■) diluted 1:10; (●) control.*

gram-negative bacteria have been found to be cured of their extrachromosomal plasmids (15). Plasmids may be involved in *S. mutans* bacteriocin synthesis, but they are not curable by standard methods.

## DISCUSSION

Although the APT-YE medium described here allows one to obtain cell-free, aqueous preparations of bacteriocins from *S. mutans*, the yield of bacteriocin in such preparations is quite low. Sonicated supernatants generally contain only 8 to 10 KU/ml. In comparison, the yield of inducible bacteriocins from gram-negative bacteria is often several thousand-fold higher, for example, 50,000 KU/ml in the case of *Enterobacter cloacae* DF13 (4). This low yield makes it difficult to purify and characterize *S. mutans* bacteriocins. Inducible mutants are presently being sought to facilitate purification and to enable more critical studies on the relationship, if any, between bacteriocin synthesis and cell death.

The relatively high frequency of spontaneous

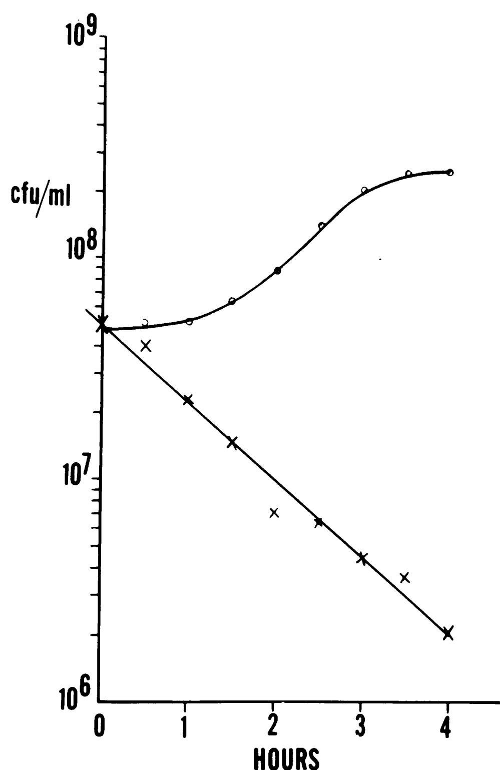


FIG. 3. *Lethal effect of BHT bacteriocin on S. pyogenes. Equal volumes of bacteriocin or sterile TSB were added to identical log-phase cultures at zero time, and samples for viable counts (determined by the pour plate method) were taken at the indicated times. Symbols:* (O) *control, untreated culture;* (×) *BHT bacteriocin added to give 0.4 KU/ml. cfu, Colony-forming units.*

TABLE 1. *Loss of bacteriocin-producing ability caused by mutagens and curing agents*[a]

| Treatment[b] | No. of colonies tested | Bacteriocin-negative colonies |
|---|---|---|
| None (control) | 1,006 | 2 |
| Acridine orange (40 μg/ml) | 985 | 4 |
| Ethidium bromide (1 μg/ml) | 1,155 | 3 |
| UV (1.5 min, 350 ergs/mm²) | 1,000 | 13 |
| NG (50 μg/ml, 30 min) | 1,000 | 27 |

[a] *S. mutans* strain BHT.

[b] UV, Ultraviolet irradiation; NG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine.

loss of bacteriocin-producing ability (Table 1) strongly suggests the involvement of a plasmid. Since no gene transfer processes are known for *S. mutans*, proof that plasmids are involved in bacteriocin synthesis will require isolation of extrachromosomal deoxyribonucleic acid from bacteriocinogenic strains and demonstration of non-bacteriocinogeny in strains lacking such deoxyribonucleic acid.

The mechanism by which the APT-YE medium promotes bacteriocin synthesis and/or release is unknown. Whereas bacteriocin inhibitors are not detectable in bacteriocin-inactive liquid cultures of *S. mutans*, the possibility that APT-YE represses the synthesis of such inhibitors cannot be ruled out. It should also be mentioned that not all strains of *S. mutans* produce bacteriocins in APT-YE. For example, strains AHT, OMZ 176, and P4 (which belong to Bratthall groups *a, d,* and *e*, respectively) were grown in APT-YE and did not produce any detectable bacteriocins. This failure is not due to the inability of these strains to synthesize any bacteriocins, since each produces inhibition zones against a variety of oral streptococci on suitable agar media (A. L. Delisle, unpublished data). The composition of APT-YE therefore lacks some factor(s) needed for bacteriocin synthesis by all strains of this species.

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