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Characterization and Mode of Action of a Bacteriocin Produced by a *Bacteroides fragilis* Strain

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A *Bacteroides fragilis* strain produces a low-molecular-weight (13,500 to 18,700), proteinaceous bacteriocin during the stationary growth phase. The extracellular bacteriocin is not inducible by ultraviolet light or mitomycin C and is stable between pH 7.5 and 8.2. The majority of the bacteriocin is thermolabile, but a small proportion (3%) of the bacteriocin is stable after autoclaving at 121°C for 15 min. Killing of sensitive bacteroides cells follows single-hit kinetics, and the interaction of a single molecule of bacteriocin with a target cell occurs in two stages. The killing of susceptible cells is affected by temperature and the growth state of the susceptible cells. The bacteriocin is unusual in that the primary event in its mode of action is the inhibition of RNA synthesis. The bacteriocin inhibits RNA synthesis immediately but has no effect on DNA synthesis or intracellular ATP levels. Protein synthesis is inhibited after a delay of 20 min, presumably as a result of the initial inhibition of RNA synthesis.

Since the discovery of bacteriocins by Gratia (10), much work has been done on the bacteriocins produced by aerobic bacteria (11, 19, 22, 23). The colicins produced by *Escherichia coli* have been particularly well studied, and their modes of action have been identified (11, 19, 23). However, little work has been carried out on the bacteriocins produced by *Bacteroides fragilis*, which is the most frequently isolated anaerobe species from clinical specimens (13, 18). Booth et al. (3) investigated bacteriocin production by *Bacteroides* strains and the role of these strains in the ecology of the colon. Although Booth et al. (3) characterized and partially purified one of the bacteriocins, the mechanism of killing was not determined. We describe the characterization, purification, and unusual mode of action of a *B. fragilis* bacteriocin.

MATERIALS AND METHODS

Media and anaerobic techniques. Brain heart infusion (BHI) broth and agar (17) were used for bacterial growth and bacteriocin production. The glucose minimal medium was similar to that described by Varel and Bryant (28), except that 0.1 M phosphate buffer was used instead of 0.4% Na_2CO_3. The anaerobic glove box and techniques described by Moodie and Woods (17) were used, and incubation was at 37°C.

Bacterial strains. A *B. fragilis* strain (Bf-1) which produced a bacteriocin and a susceptible indicator strain (Bf-2) were isolated from human fecal samples. The bacteria were identified as *B. fragilis*, and this was confirmed by L. V. Holdeman, Virginia Polytechnic Institute and State University Anaerobic Laboratory, Blacksburg.

Bacteriocin assay. The bacteriocin was assayed by the well method (16) in 10-ml BHIA plates containing 1% agar and seeded with *B. fragilis* indicator cells. The wells were filled with 25 μl of the test solutions. Bacteriocin titers in arbitrary units (AU) were expressed as the reciprocal of the highest doubling dilution that gave a zone of inhibition surrounding the well.

Bacteriocin production. The producer strain was inoculated into BHI broth, and samples of the culture supernatant were assayed for bacteriocin at different time intervals.

Induction experiments. Mitomycin C (Calbiochem, La Jolla, Calif.) and ultraviolet irradiation were used to test for induction of the bacteriocin. Exponential-phase cells were added to BHI media containing 0.1, 0.2, 0.3, 0.4, 0.8 and 1.0 μg of mitomycin C per ml and incubated for 6 h. Cultures were either assayed for bacteriocin directly or harvested by centrifugation, washed, and suspended in BHI broth for a further 6 h before assaying. Induction by ultraviolet irradiation was carried out on exponential-phase cells in BHI broth. Samples (7.5 ml) were irradiated with a Hanovia ultraviolet lamp at a distance of 25 cm and a dose rate of 0.45 $J/m^2\ s^{-1}$ for 0, 20, 40, and 120 s. The irradiated cultures were centrifuged, the pellets were suspended in fresh BHI broth, and the bacteriocin titers were assayed after 6 h of incubation.

Localization studies. Cell-bound bacteriocin was determined by washing the bacterial cells in 1 M NaCl and assaying the supernatant. Intracellular toxin was determined in the supernatants of sonically disrupted

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cell samples that had been clarified by centrifugation at 12,000 rpm for 20 min.

Bacteriocin purification. The bacteriocin was concentrated by fractional precipitation with $(NH_4)_2SO_4$. The fraction precipitated between 30 and 60% $(NH_4)_2SO_4$ saturation was collected by centrifugation, dissolved in 0.02 M tris(hydroxymethyl)-aminomethane (Tris)-hydrochloride buffer (pH 8.2), and dialyzed against the Tris-hydrochloride buffer. The concentrated bacteriocin was initially purified by DEAE-cellulose (Whatman) chromatography on a column (2.5 by 36 cm) which was eluted with a 500-ml linear gradient of 0 to 0.3 M KCl. After DEAE-cellulose chromatography, the bacteriocin was lyophilized and further purified by Sephadex G-100 chromatography (column, 1 by 30 cm). The column was calibrated with bovine serum albumin (67,000 molecular weight), ovalbumin (43,000 molecular weight), cytochrome *c* (12,400 molecular weight), and insulin (6,000 molecular weight). The degree of purification was determined after each fractionation step by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Protein and nucleic acid synthesis. Since the *Bacteroides* strains were unable to incorporate labeled amino acids (28; unpublished data), protein synthesis could not be determined by the incorporation of ^{3}H-amino acids. The method of Elgat and Ben-Gurion (6) was used and involved precipitation with cold 50% trichloroacetic acid. The precipitate was collected, suspended in 5% trichloroacetic acid, and extracted at 90°C for 30 min to obtain nucleic acid fractions. The final precipitates were washed with 5 ml of 80% ethyl alcohol and were boiled with 2 ml or 1 N NaOH for 10 min; after centrifugation, protein was determined by the Folin-Ciocalteu reaction (15), and ribonucleic acid (RNA) was determined by the orcinol method (27). RNA and deoxyribonucleic acid (DNA) synthesis were determined by difference after incorporation of [^{3}H] adenine (2 μg/ml, 0.4 μCi/ml) into trichloroacetic acid-precipitable material (total nucleic acid) and into NaOh-hydrolyzed trichloroacetic acid-precipitable material (DNA) (9).

Estimation of intracellular levels of ATP. Intracellular levels of adenosine triphosphate (ATP) were determined 10 min after the addition of bacteriocin or after the cultures had been vigorously aerated for 10 min and compared with the relevant controls. Intracellular ATP was measured by counting the flashes of light emitted by mixtures of cell extracts and firefly lantern extracts (Sigma Chemical Co.) (8). A Beckman scintillation counter was used, and 0.05 nmol of ATP was produced at 100,000 cpm.

RESULTS

Production of the bacteriocin. The production of bacteriocin by Bf-1 in BHI broth is shown in Fig. 1. The highest levels of bacteriocin (60 to 70 AU) were routinely obtained after 6 to 10 h of growth when the cells entered the stationary growth phase. The production of the bacteriocin did not result in a decrease in the turbidity of the culture. Treatment with mitomycin C or ultraviolet irradiation did not result in induction and increased bacteriocin titers.

Localization studies carried out at 8 h indicated that the majority of the bacteriocin was extracellular (64 AU) compared to 2 AU intracellular and 1 AU cell bound.

Effects of physical and chemical agents and bacteriocin stability. The rate of thermal inactivation of the bacteriocin was determined by incubating crude samples of the bacteriocin at various temperatures. The bacteriocin was stable for 40 min at 37°C but was partially and rapidly inactivated at 60°C (half-life = 2.5 min at 60°C). However, the bacteriocin was not completely inactivated by heating at 60°C for 40 min, and it retained 3% of its activity. The 3% stable fraction was not inactivated by autoclaving at 121°C for 15 min.

The optimum pH for the stability of the bacteriocin was determined by adding crude samples of the bacteriocin to buffers between pH 4 and 10 and assaying the activity after 2 h at 37°C. The optimum pH for the stability of the bacteriocin was between pH 7.5 and 8.2, and a 50% reduction in activity resulted at pH 6.5 and 9.0.

The bacteriocin was inactivated by the proteolytic enzymes trypsin (Difco Laboratories) and pronase (Miles-Seravac) (100 μg/ml) but was not affected by the nucleases deoxyribonu-

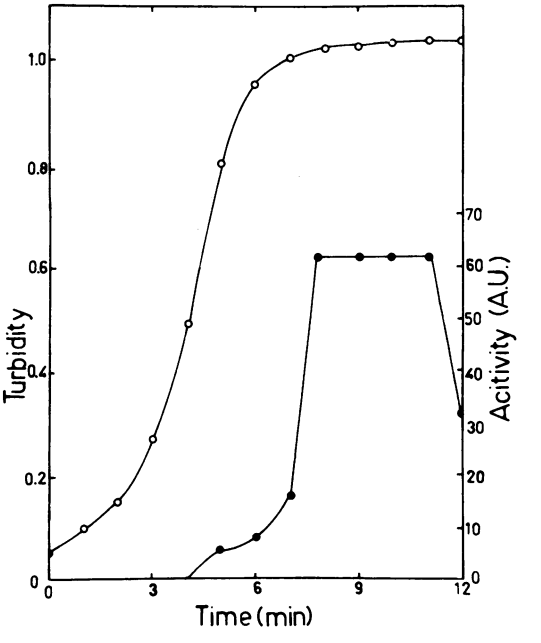

FIG. 1. *Production of bacteriocin in BHI broth. Turbidity* (○); *bacteriocin concentration in AU* (●).

clease (Miles-Seravac) and ribonuclease (Miles-Seravac) (100 μg/ml). Incubation was at 37°C for 2 h.

Purification of the bacteriocin. The degree of purification and specific activities of the bacteriocin preparations after fractional precipitation with $(NH_4)_2SO_4$, diethylaminoethyl (DEAE)-cellulose chromatography and Sephadex G-100 chromatography are shown in Table 1. A 320-fold purification was achieved after Sephadex G-100 chromatography, and a single peak of activity was obtained after chromatography on both DEAE-cellulose and Sephadex G-100. From the gel filtration studies, the molecular weight of the bacteriocin was estimated to be 13,500 (Fig. 3). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the concentrated fractions after gel filtration showed a single band and indicated that the bacteriocin had been purified to homogeneity. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis studies with known markers indicated that the bacteriocin had a molecular weight of 18,700.

Killing kinetics. Addition of the bacteriocin (4 killing units) to exponentially growing Bf-2 caused a rapid decrease in viability (Fig. 4). The killing kinetics were biphasic and a resistant population was selected after 20 min. The action of the bacteriocin showed single-hit kinetics when exponentially growing Bf-2 cells were treated with various bacteriocin concentrations at 37°C for 20 min (Fig. 5). The turbidity of a susceptible culture treated with bacteriocin remained constant over 60 min, indicating that the action of the bacteriocin is bactericidal but not bacteriolytic. The killing of susceptible cells was markedly affected by temperature. Susceptible cultures treated with bacteriocin (64 AU) for 30 min at 42, 37, 30 and 24°C before diluting, plating, and incubation at 37°C showed 10, 51, 100, and 100% survival, respectively. The effect of temperature on bacteriocin adsorption was determined by incubating cells and bacteriocin at 20 and 37°C for 30 min. The cells were removed by centrifugation, and the concentration of bacteriocin in the supernatants was estimated by treating fresh cells for 20 min at 37°C and plating for survivors. After adsorption at 20 and 37°C incubation of fresh cells resulted in 11 and 33% survival, respectively. Exponential-phase cells were more sensitive to killing by the bacteriocin than stationary-phase cells.

Trypsin rescue of bacteriocin-treated cells. Studies on the effect of treatment of sus-

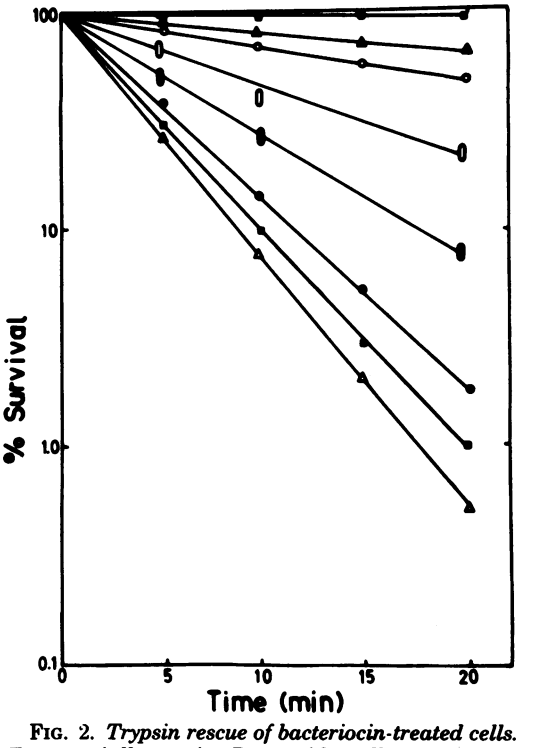

FIG. 2. *Trypsin rescue of bacteriocin-treated cells. Exponentially growing Bacteroides cells were treated with bacteriocin (4 killing units per colony-forming unit). At intervals, samples were plated for survivors* (■) *or diluted with trypsin (5 mg/ml in 20 mM Tris-hydrochloride, pH 8) and held for 30 min at 37°C before plating* (○). *Cells pretreated with trypsin for 30 min at 37°C before exposure to bacteriocin* (△). *Cells pretreated with 20 mM 2,4-dinitrophenol for 30 min at 37°C before exposure to bacteriocin* (0) *and rescued with trypsin* (□) *or 2,4-dinitrophenol removed before treatment with bacteriocin* (0). *Cells pretreated with 100 mM sodium arsenate for 20 min at 37°C before exposure to bacteriocin* (●) *and after trypsin rescue* (▲).

TABLE 1. *Purification and specific activities of bacteriocin preparations after fractional precipitation with $(NH_4)_2SO_4$, DEAE-cellulose chromatography, and Sephadex G-100 chromatography*

Purification step	Activity (AU/ml)	Protein (mg/ml)	Recovery (%)	Sp act (Au/mg)	Purification (fold)
Culture supernatant	2,560	12.0	100	213	
$(NH_4)_2SO_4$ precipitate	20,480	15.0	58	1,365	6.4
DEAE, peak	40,960	4.2	34	9,752	45.7
Sephadex G-100, peak	20,480	0.3	13	68,270	320.0

ceptible cells with trypsin, 2,4-dinitrophenol and sodium arsenate indicated that, in common with other bacteriocins (21, 24), the bacteriocin-treated cells were trypsin rescuable, and the lethal interaction between the bacteriocin and the susceptible cells occurred in two stages (Fig. 2). There is a stage after adsorption of the bacteriocin when the cells are trypsin rescuable and physiologically unaffected, followed by a transition to a second stage when the cells are physiologically damaged and trypsin unrescuable. Treatment with trypsin did not overcome the RNA inhibition in bacteriocin-treated cells after transition to stage II.

Effect on protein and nucleic acid synthesis. Since the *Bacteroides* strain did not incorporate labeled amino acids, protein synthesis was determined by measuring the protein con-

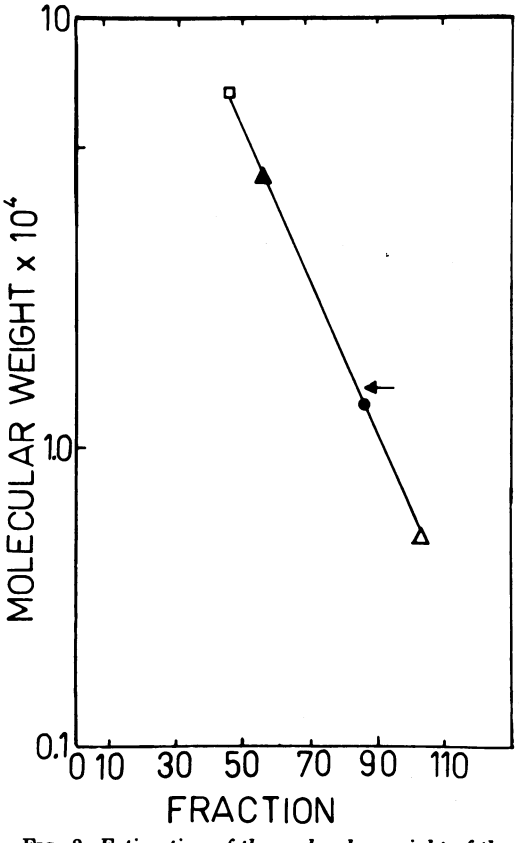

FIG. 3. *Estimation of the molecular weight of the bacteriocin by gel filtration on Sephadex G-100. The column was calibrated with serum albumin (67,000 molecular weight)* (□); *ovalbumin (43,000 molecular weight)* (▲); *cytochrome c (12,400 molecular weight)* (●); *and insulin (6,000 molecular weight)* (△). *Arrow indicates the position of the bacteriocin.*

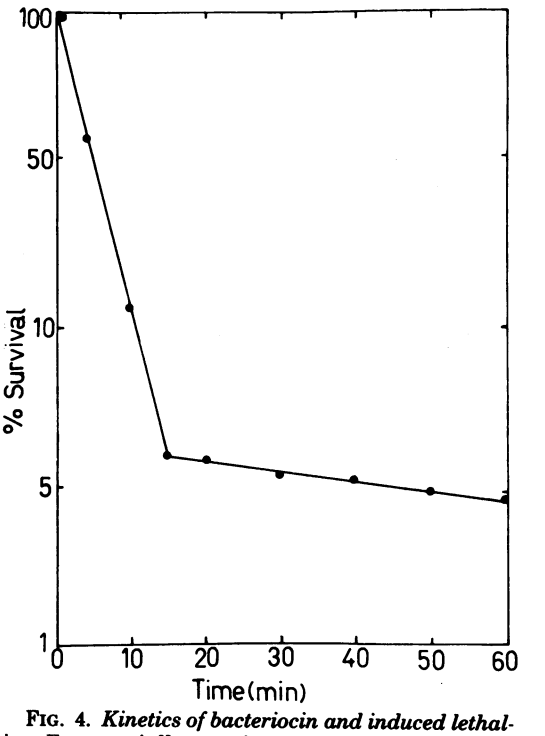

FIG. 4. *Kinetics of bacteriocin and induced lethality. Exponentially growing Bacteroides cells were treated with 4 killing units of bacteriocin and plated for survivors.*

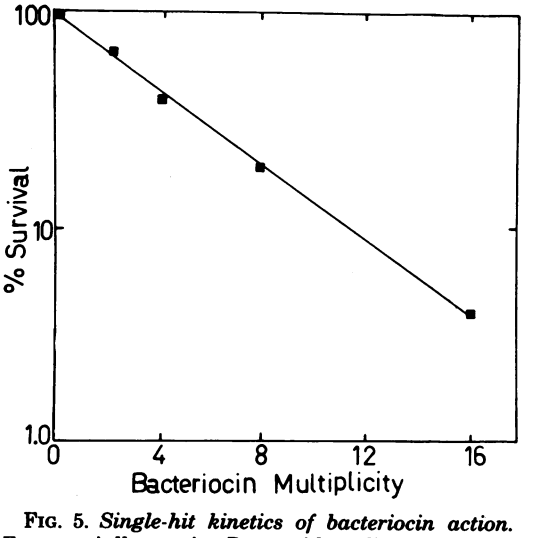

FIG. 5. *Single-hit kinetics of bacteriocin action. Exponentially growing Bacteroides cells were treated with various bacteriocin concentrations at 37°C for 20 min. The bacteriocin concentration was expressed as bacteriocin multiplicity where a multiplicity of 16 = 128 AU and a multiplicity of 2 = 16 AU.*

centration of trichloroacetic acid-precipitable material (Fig. 6). To obtain a comparison between RNA synthesis and protein synthesis, RNA synthesis was also determined in the same experiment by an analogous method. The results indicate that in cells treated with 64 AU of bacteriocin there was a rapid and complete inhibition in RNA snythesis but that protein synthesis was only partially inhibited after 20 min. At higher concentrations of the bacteriocin (256 AU) the inhibition of protein synthesis was more marked, but this inhibition still lagged behind the inhibition of RNA synthesis. Studies on the effect of bacteriocin on DNA and RNA synthesis in susceptible cells, estimated by the incorporation of labeled bases into trichloroacetic acid-precipitable material, showed that DNA synthesis was not affected but RNA synthesis was inhibited (Fig. 7). The 3% heat-stable fraction after autoclaving had the same effect on macromolecular synthesis as the untreated bacteriocin.

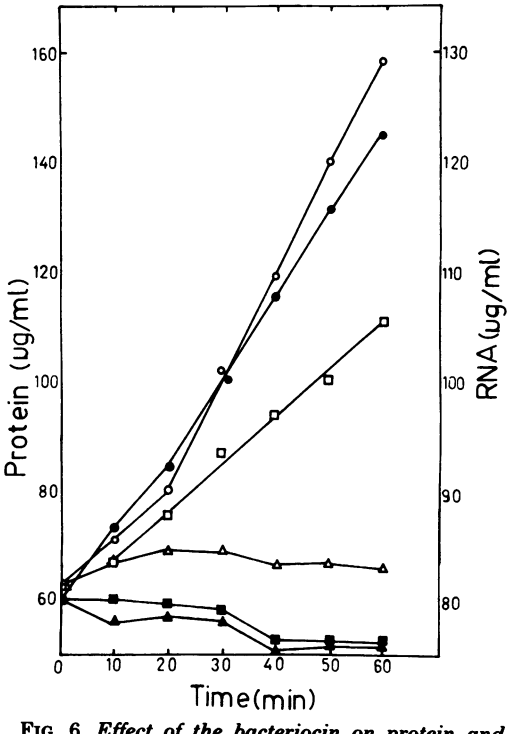

FIG. 6. *Effect of the bacteriocin on protein and RNA synthesis. Bacteriocin was added to exponential cultures at time zero. Protein synthesis control culture* (○); *with 64 AU of bacteriocin* (□); *and with 256 AU of bacteriocin* (△). *RNA synthesis control culture* (●); *with 64 AU of bacteriocin* (■); *with 256 AU of bacteriocin* (▲).

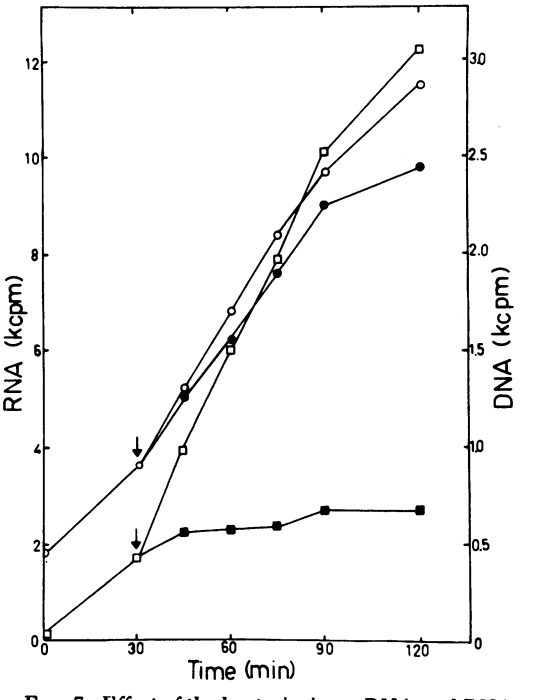

FIG. 7. *Effect of the bacteriocin on DNA and RNA synthesis. Exponential cultures were grown for 30 min before the addition of bacteriocin (64 AU) (arrow). DNA synthesis control culture* (○); *with bacteriocin* (●). *RNA synthesis control culture* (□); *with bacteriocin* (■).

Effect on intracellular ATP levels. The bacteriocin had no effect on intracellular ATP. Ten minutes after the addition of bacteriocin, the level of ATP was 97% of that of untreated control cultures. When the *Bacteroides* strain was aerated for 10 min, in the absence of the bacteriocin, the ATP levels fell to 63% of that of anaerobic control cultures. Aeration of the anaerobic *Clostridium acetobutylicum* is known to result in a marked decrease in intracellular ATP (20).

DISCUSSION

The proteinaceous *Bacteroides* bacteriocin we have described differs markedly from the only other *Bacteroides* bacteriocin, which has been fully characterized by Booth et al. (3). The bacteriocin from the *Bacteroides* strain T1-1 (3) is produced during the exponential growth phase and is a high-molecular-weight (>300,000) protein which is stable from pH 1 to 12 and only 50% inactivated by autoclaving for 15 min at 121°C. The Bf-1 bacteriocin is released during the stationary growth phase and is a small protein with a molecular weight between 13,500 and

18,700. The bacteriocin is stable over a narrow pH range and is markedly more thermolabile than the *Bacteroides* T1-1 bacteriocin. A 3% fraction of the *B. fragilis* bacteriocin is stable after autoclaving. It is interesting that the small bacteriocin which eluted as a single peak after DEAE-cellulose and Sephadex G-100 chromatography was composed of a thermolabile component (majority) and a thermostable component (minority). The bacteriocins from several other *Bacteroides* strains appear to be totally heat labile (1, 2).

As with many other bacteriocins, the killing of the *Bacteroides* cells follows single-hit kinetics, and the interaction of a single molecule of bacteriocin with a target cell occurs in two stages. Pretreatment with 2,4-dinitrophenol inhibits uptake of the bacteriocin, and because it is a known uncoupler of oxidative phosphorylation and dissipator of membrane energy it is suggested that 2,4-dinitrophenol prevents the transmission of the bacteriocin by interfering with the energy supply (14, 29). However, a direct interaction of 2,4-dinitrophenol with some membrane component essential for bacteriocin action cannot be ruled out (19). The bacteriocin action is affected by the temperature and growth phase. It seems that the temperature effect is due to the more rapid growth of the cells at higher temperatures and is different from the unusual temperature effect observed with pesticin (6). Although temperature affected adsorption of the bacteriocin to the cells, the effect on adsorption was markedly less than the effect of temperature on killing.

The *Bacteroides* bacteriocin completely inhibited RNA synthesis and had no effect on DNA synthesis or intracellular ATP levels. At low bacteriocin concentrations, protein synthesis was partially inhibited after a delay. It is concluded that the primary event in the mode of action of the *Bacteroides* bacteriocin is the inhibition of RNA synthesis. At higher bacteriocin concentrations, the inhibition of RNA synthesis is followed by the inhibition of protein synthesis. The inhibition of protein synthesis is presumably as a result of the inhibition in RNA synthesis. The mode of action of the *Bacteroides* bacteriocin is most interesting and somewhat unusual in that it specifically inhibits RNA synthesis but has no effect on DNA synthesis and a delayed effect on protein synthesis. One other bacteriocin, pneumocin G196, inhibits protein and RNA synthesis without affecting DNA synthesis (5). A bacteriocin from *C. septicum* (25) inhibits protein and RNA synthesis immediately and DNA synthesis after 10 min. The mode of action of the *Bacteroides* bacteriocin is different from that of other colicins which have been well studied and shown to affect either DNA synthesis (endonuclease activity) (26), protein synthesis (endonucleolytic ribosomal RNA cleavage) (4), or energy metabolism (7, 12). The *Bacteroides* bacteriocin may be useful as a specific inhibitor of RNA synthesis in studies on transcription in this important anaerobe.

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