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Purification and Properties of a Cell-Bound Bacteriocin from a *Bacteroides fragilis* Strain

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A cell-bound bacteriocin was extracted from a *Bacteroides fragilis* BF-11 strain by treating the cells with a low-molarity buffer (0.01 M Tris-hydrochloride, pH 8.0). Sucrose osmotic shock experiments and ultrasonic lysis of whole cells indicated that the majority of the bacteriocin was located at the cell surface. Culture supernatants contained no significant bacteriocin activity. The bacteriocin was purified by DEAE-cellulose and Sephacryl S200 chromatography and had an apparent molecular weight of approximately 7,000. It was relatively heat stable and was inactivated by proteases. There was a delay of approximately 3.5 h before DNA, RNA, and protein synthesis were inhibited by the bacteriocin. Inhibition of macromolecular synthesis coincided with lysis of the susceptible indicator strain.

There are numerous reports on the characterization of bacteriocins produced by aerobic bacteria (7, 9, 11, 22), but the bacteriocins produced by strains of the anaerobic genus *Bacteroides* have not been extensively studied. Booth et al. (1) have investigated the production of a high-molecular-weight bacteriocin (>300,000) by a *Bacteroides* strain and the role of this strain in the ecology of the colon. Mossie et al. (12) purified a lower-molecular-weight bacteriocin (about 16,000) from a *Bacteroides fragilis* Bf-1 strain and reported that it inhibited RNA synthesis. Nakamura et al. (15) studied a cell-bound bacteriocin-like protein (melaninocin) which had an apparent molecular weight of about 105,000 and was produced by *Bacteroides melaninogenicus*.

Since the production of more than one bacteriocin by a single bacterial strain is not uncommon (22), we investigated the production of a cell-bound bacteriocin by a derivative of the *B. fragilis* Bf-1 strain Bf-11 which had lost the ability to produce the cell-free bacteriocin described by Mossie et al. (12).

MATERIALS AND METHODS

Bacterial strains. A derivative of *B. fragilis* Bf-1 (designated Bf-11) which had lost the ability to produce the cell-free bacteriocin which was studied by Mossie et al. (12–14) was used for the production of the bacteriocin. The susceptible indicator strain (Bf-2) and the 10 rifampin-resistant Bf-2 mutants have been described previously (13).

Media and bacteriocin assay. The bacteriocin was produced in a complex medium which contained, in grams per liter, Difco tryptic soy broth, 24; Difco yeast extract, 10; glucose, 1; and L-cysteine-hydrochloride 0.5. The bacteriocin was assayed with porcelain beads (18) containing 20-μl samples which were placed on the surface of a 3-ml seeded soft agar overlay on brain heart infusion agar plates which contained, in grams per liter, Difco brain heart infusion, 37; Difco yeast extract, 5; L-cysteine-hydrochloride, 0.5; and agar, 15. Bacteriocin titers in arbitrary units (AU) were expressed as the reciprocal of the highest doubling dilution that gave a zone of inhibition surrounding the bead. The minimal medium of Varel and Bryant (23) was used for the analysis of macromolecular synthesis. Hemin and menadione (Sigma Chemical Co.) were added to all the media (5 and 0.5 mg/liter, respectively). Protein concentrations were estimated by the method of Bradford (2).

Bacteriocin purification. Overnight cultures (100 ml) of *B. fragilis* Bf-11 were harvested by centrifugation, and the cells were suspended in 10 ml of extraction buffer (0.01 M Tris-hydrochloride, pH 8.0) for 30 min at 20°C. The bacteriocin was also extracted by the sucrose osmotic shock method of Willis et al. (24). The cells were disrupted by ultrasonication (Heat Systems-Ultrasonics Inc.) at 4°C. All subsequent operations were performed at 4°C. The supernatant obtained after centrifugation was dialyzed in dialysis tubing with a molecular cutoff of 6,000 to 8,000 against distilled water for 16 h.

The bacteriocin was adsorbed onto a column (25 by 200 mm) of freshly regenerated DEAE-cellulose (DE-52; Whatman, Inc.) equilibrated with 0.01 M Tris-hydrochloride (pH 8.0) and washed with the same buffer containing 0.01 M KCl. The activity was eluted with a linear gradient of 0.01 to 0.2 M KCl in the same buffer, and fractions were collected and assayed for bacteriocin activity. Fractions showing bacteriocin activity were pooled, dialyzed against distilled water for 6 h, lyophilized, reconstituted in about 1/10 of the original volume, and loaded onto a Sephacryl S200 (Pharmacia) column (25 by 850 mm) equilibrated with 0.1 M Tris-hydrochloride buffer (pH 8.0) containing 0.5 M NaCl. This buffer was used to elute 5-ml fractions from the column at a flow rate of 30 ml/h. Active fractions were pooled, filter sterilized (0.22-μm Millex-GS; Millipore Corp.), and lyophilized.

The molecular weight of the bacteriocin was determined by Sephacryl S200 chromatography (two 10-by-400-mm columns connected in series) and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The column was calibrated with cytochrome *c* (12,400 molecular weight), ovalbumin (45,000 molecular weight), and Transferrin (90,000 molecular weight). Discontinuous SDS-PAGE was carried out by the methods described by Laemmli (10) and O'Farrell (17), and 10 to 20% polyacrylamide gradient gels gave the best resolution of the bacteriocin.

Bacteriocin stability. The effect of pH on the stability of the purified bacteriocin was determined in 0.1 M citrate-phosphate-borate-hydrochloride buffer (3). A purified bacteriocin

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TABLE 1. Purification and specific activities of bacteriocin preparations

Purification step	Vol (ml)	Protein (μg/ml)	Total protein (mg)	Bacteriocin activity (AU/ml)	Total activity (AU)	Sp act (AU/mg)	Purification (fold)
Culture supernatant	1,800	<10	<18	<2			
20% Sucrose–2 mM EDTA extract	60	<10	<0.6	2			
Osmotic shock fluid after sucrose	50	1,400	70	32	1,600	22.8	
Extract of ultrasonicated cells after sucrose osmotic shock	50	2,280	114	8	400	3.5	
Extract of ultrasonicated whole cells	50	3,680	184	32	1,600	8.6	
0.01 M Tris extract	50	290	14.5	32	1,600	110.3	1
Extract of ultrasonicated cells after 0.01 M Tris extraction	25	3,550	177.5	16	400	2.25	0.02
DE.52 activity peak, pooled	38	83	3.154	16	608	192	1.74
S200 activity peak, pooled	11	8	0.088	32	352	4,000	36.2

preparation was dialyzed against distilled water, and 10 μl was added to 90 μl of the various pH buffers in steps of 1 pH unit from pH 3 to 10. After 4 h at 8 to 10°C, the samples were assayed for residual activity.

The susceptibility of the bacteriocin to proteolytic enzymes was tested by incubation of a purified preparation of bacteriocin with protease from *Streptomyces griseus* (Sigma) at a concentration of 1 mg/ml (pH 7.6) at 37°C for 30 min. Trypsin (Difco, 1:250), DNase and RNase (Miles Laboratories, Inc.) were also tested under the same conditions. After incubation, the mixtures were heated at 65°C for 20 min to reduce any possible interference by the enzymes in the assay of the bacteriocin.

This heat treatment reduced the activity of the *S. griseus* protease by 50% and trypsin by 90%. The sensitivity of *B. fragilis* Bf-2 cells to the bacteriocin was not affected by pretreatment with 0.5 mg of the proteases per ml for 10 min, followed by washing in growth medium.

Synthesis of proteins and nucleic acids. Syntheses of DNA, RNA, and protein by the indicator strain (Bf-2) in the presence of bacteriocin were determined under stringent anaerobic conditions by the incorporation of [2-^{14}C]thymidine (5 μg/ml, 3 μCi/ml), [^{3}H]uracil (20 μg/ml, 15 μCi/ml), and [^{35}S]methionine (25 μg/ml, 20 μCi/ml), respectively, into cold trichloroacetic acid-precipitable material. Exponential-phase cells were labeled for 30 min before the addition of purified bacteriocin (8 AU/ml).

RNA polymerase assay. RNA polymerase was purified from the *B. fragilis* Bf-2 indicator strain by the method of Davison (4). The RNA polymerase assay has been described previously (19).

RESULTS

Bacteriocin purification. Culture supernatants of *B. fragilis* Bf-11 contained very low levels of bacteriocin activity. Relatively high levels of bacteriocin activity were obtained after treatment of *B. fragilis* Bf-11 with the low-molarity buffer (Table 1). The bacteriocin was eluted from DEAE-cellulose by a 0.01 to 0.2 M KCl gradient and was purified further by Sephacryl S200 chromatography. SDS-PAGE of the purified active preparation revealed a single protein band (Fig. 1 and 2).

Since SDS interfered with the bacteriocin assay, the activity of the single band after SDS-PAGE could not be determined. PAGE of an undenatured sample of the bacteriocin on a 5 to 15% polyacrylamide gradient (pH 8.25) was carried out. Under these conditions, the bacteriocin was not resolved as well as on SDS-PAGE, and a broad band which was shown to have bacteriocin activity was obtained.

A 36-fold purification was achieved after Sephacryl S200 chromatography (Table 1 and Fig. 2). The bacteriocin was totally retained by dialysis tubing with a molecular cutoff of 6,000 to 8,000 and was not detected in the dialysate after 16 h of dialysis. However, it was not retained by dialysis tubing with a 12,000 to 14,000 molecular weight cutoff and was estimated to have a molecular weight of 7,200 or 6,400 by Sephacryl S200 chromatography and SDS-PAGE, respectively. Bacteriocin activity was not obtained in higher-molecular-weight fractions after Sephacryl S200 chromatography (Fig. 3).

Cellular localization of the bacteriocin. Culture supernatants of *B. fragilis* Bf-11 contained very low levels of bacteriocin (Table 1). The majority of the bacteriocin was released from the cells either by treatment with the low-molarity buffer or by sucrose osmotic shock (Table 1). Treatments of the cells with 20% sucrose and 2 mM EDTA

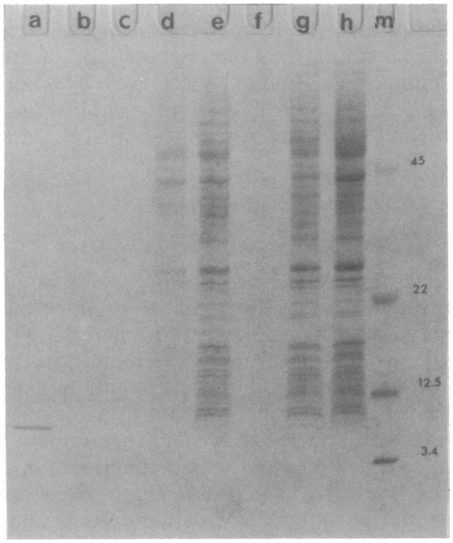

FIG. 1. Bacterial extracts analyzed by SDS-polyacrylamide gradient (10 to 20%) electrophoresis and stained with Coomassie blue R250. Lane a, Purified and concentrated bacteriocin; lane b, culture supernatant; lane c, 20% sucrose–2 mM EDTA extract; lane d, osmotic shock fluid after sucrose; lane e, extract of ultrasonicated whole cells; lane f, 0.01 M Tris extract; lane g, supernatant from ultrasonic lysate of osmotically shocked (sucrose) cells; lane h, extract of ultrasonicated cells after 0.01 M Tris extraction; lane m, marker proteins ovalbumin, human growth hormone, cytochrome *c* (bovine), and insulin B chain (molecular weights shown in thousands). Equal volumes of the cell extracts described in Table 1 were applied to lanes b through h.

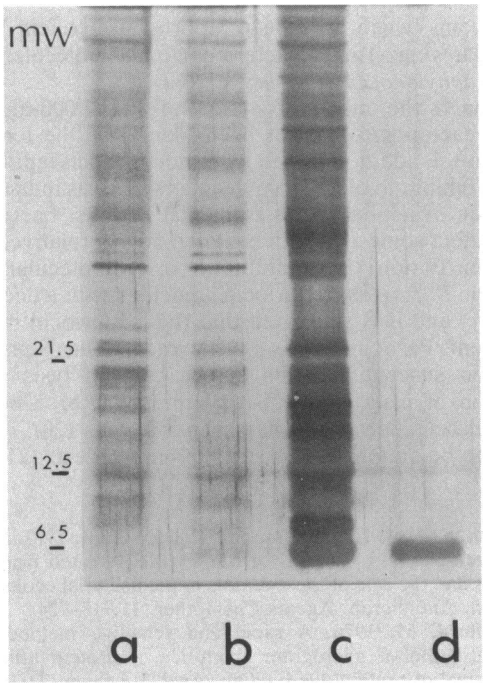

FIG. 2. SDS-PAGE of extracts before and after purification by DEAE-cellulose and Sephacryl S200 chromatography. Lanes show Tris (0.01 M) extract of the *B. fragilis* Bf-2 indicator strain (a) and the Bf-11 producer strain (b); fractions showing bacteriocin activity after DEAE-cellulose (c) and Sephacryl S200 (d) chromatography. MW, Molecular weight ($\times 10^3$) markers (Pierce Lo-Ranger) aprotinin (6.5), cytochrome *c* (12.5), and soybean trypsin inhibitor (21.5). Lowest band was associated with fractions which showed bacteriocin activity. Stained with Biorad silver stain.

did not release the bacteriocin, and the appropriate bacteriocin band was not present after SDS-PAGE (Fig. 1, lane c). Extraction with the low-molarity buffer resulted in a higher specific activity of the bacteriocin than extraction by sucrose osmotic shock, which released more contaminating non-bacteriocin proteins (Table 1 and Fig. 1). Examination of the cells by phase-contrast microscopy indicated that they were not lysed or disintegrated by these extraction procedures. This is also shown by the difference in the protein profiles between the extracts and the extracted cells after ultrasonication (Fig. 1, lanes f and h).

Ultrasonicated cell preparations did not result in an increased yield of bacteriocin (Table 1). Although ultrasonication disrupted the cells, it did not inactivate the bacteriocin.

Bacteriocin stability. The optimum pH for the stability of the purified bacteriocin was between pH 6 and 9. There was a marked reduction in activity above pH 9 and some reduction in activity below pH 6. The bacteriocin was stable at 50 and 60°C for 5 and 3 h, respectively. At 70°C, there was an initial 50% loss in activity after 1 h but no further loss in activity over 4 h. After autoclaving at 121°C for 15 min, 25% of the initial activity was retained.

The bacteriocin was inactivated 85% by *S. griseus* protease but only 30% by trypsin at a similar concentration. DNase and RNase did not have any affect on activity. Untreated bacteriocin retained its titer after the heat treatment used to reduce the activity of the enzymes.

Specificity of the bacteriocin. The bacteriocin susceptibility of 31 *B. fragilis* isolates from different clinical samples was determined. These isolates had been characterized as different from each other on the basis of phage susceptibility, bacteriocin production, and bacteriocin susceptibility. Of these isolates, eight were susceptible to the bacteriocin. *B. fragilis* ATCC 29771 was resistant to the bacteriocin. The bacteriocin did not affect isolates of *Escherichia coli* or of *Klebsiella, Acinetobacter, Pseudomonas*, and *Staphylococcus* species.

Since *B. fragilis* Bf-11 was a derivative of *B. fragilis* Bf-1, which was shown by Mossie et al. (12–14) to produce an extracellular low-molecular-weight bacteriocin which inhibited RNA polymerase activity, it was important to establish that the cell-bound bacteriocin was a different bacteriocin. Mossie et al. (13) isolated 10 rifampin-resistant *B. fragilis* Bf-2 mutants from the indicator strain which were shown to be resistant to the bacteriocin described by Mossie et al. (12). These rifampin-resistant mutants were susceptible to the cell-bound bacteriocin, which did not inhibit *B. fragilis* RNA polymerase in the in vitro RNA polymerase assay.

Nucleic acid and protein synthesis. There was a delay of approximately 3.5 h before DNA, RNA, and protein synthesis was inhibited at about the same time, and the inhibition of macromolecular synthesis coincided with a decrease in turbidity of the cultures (Fig. 4).

DISCUSSION

The low-molecular-weight bacteriocin we have described was extracted by suspending the cells in low-molarity buffer and by osmotic shocking. These treatments did not lyse the cells, and it is concluded that the bacteriocin is cell-bound.

The inhibitor we have described can be considered to be a bacteriocin since it is a bactericidal protein with a narrow activity spectrum and is inactive against the producer strain (9, 22). The *B. fragilis* bacteriocin differs from most other

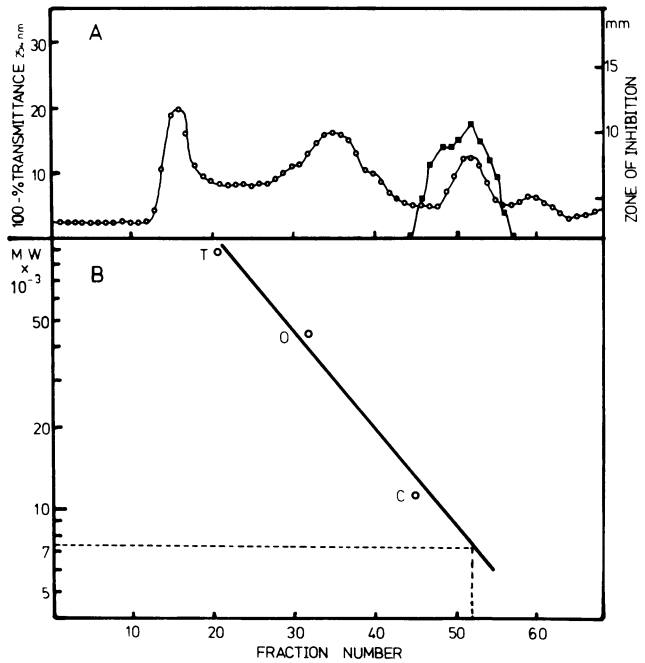

FIG. 3. Gel filtration chromatography of bacteriocin preparations on Sephacryl S200. A, purification of a concentrated sample from DEAE-cellulose showing percent transmittance at 254 nm (○) and bacteriocin activity (■). B, Molecular weight estimation using transferrin (T, 90,000), ovalbumin (O, 45,000), and cytochrome *c* (C, 12,400). The column was eluted with 0.1 M Tris-hydrochloride (pH 8.0) containing 0.5 M NaCl at a flow rate of 10 ml/h.

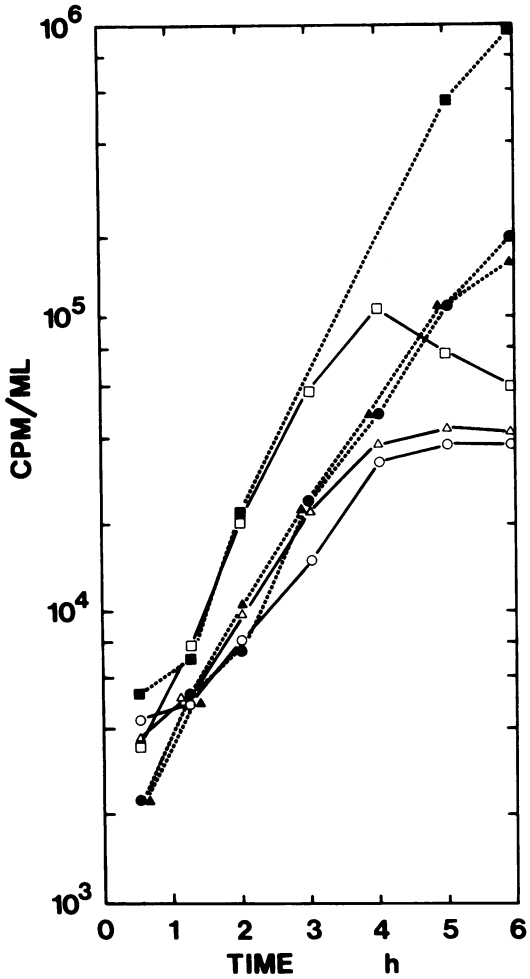

FIG. 4. Effect of bacteriocin on DNA, RNA, and protein synthesis in *B. fragilis* Bf-2 cultures. Cells were labeled with [^{14}C]thymidine with (○) and without (●) bacteriocin; [^{3}H]uracil with (□) and without (■) bacteriocin; and [^{35}S]methionine with (△) and without (▲) bacteriocin. The bacteriocin was added at 30 min.

bacteriocins reported to date in that it has a very low molecular weight (approximately 7,000) (23).

The next smallest native *Bacteroides* bacteriocin reported is produced by *B. fragilis* Bf-1 and has a molecular weight of approximately 16,000 (12). The 16,000-molecular-weight bacteriocin inhibited RNA polymerase activity. Although the 7,000-molecular-weight bacteriocin is produced by a derivative of the strain which produced the 16,000-molecular-weight bacteriocin, it is a different bacteriocin. The 16,000-molecular-weight bacteriocin was detected in culture supernatants and inhibited RNA synthesis immediately. DNA, RNA, and protein synthesis were inhibited simultaneously by the 7,000-molecular-weight bacteriocin after a 3.5-h delay. Furthermore, this bacteriocin was active against *B. fragilis* Bf-2 rifampin-resistant mutants which were isolated from the Bf-2 indicator strain and were resistant to the 16,000-molecular-weight bacteriocin. The *B. fragilis* bacteriocin described by Booth et al. (1) is completely different in that it is very large, with a molecular weight of 300,000. The 7,000-molecular-weight *B. fragilis* bacteriocin resembles melaninocin from *B. melaninogenicus* in that both bacteriocins were not detected in culture supernatant and had to be extracted from the cells (15). Other low-molecular-weight bacteriocins which have been reported are colicin V (4,000 molecular weight) and a bacteriocin (5,000 molecular weight) from *Staphylococcus aureus* (6, 16).

Although the mode of action of the 7,000-molecular-weight bacteriocin has not been identified, the long delay between the addition of the bacteriocin to susceptible cells and the inhibition of macromolecular synthesis indicates that the mode of action differs from that of most bacteriocins, which affect some aspect of cell metabolism relatively rapidly (within 10 min) (9). Inhibition of macromolecular synthesis by the *B. fragilis* bacteriocin coincides with a decrease in turbidity, and it is suggested that the bacteriocin may be a lytic agent. Pesticin acts as a lysozyme and has a protracted effect on susceptible cells before causing lysis and the inhibition of macromolecular synthesis (5, 8). Colicin M, which also induces lysis of susceptible cells (20), has been shown to be an inhibitor of murein biosynthesis (21).

LITERATURE CITED

1. **Booth, S. J., J. L. Johnson, and T. D. Wilkins.** 1977. Bacteriocin production by strains of *Bacteroides* isolated from human feces and the role of these strains in the bacterial ecology of the colon. Antimicrob. Agents Chemother. **11**:718–724.
2. **Bradford, M.** 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein-dye binding. Anal. Biochem. **72**:248–254.
3. **CIBA-GEIGY Corp.** 1975. Documenta Geigy, p. 280. *In* K. Diem and C. Lentner (ed.), Scientific tables, 7th ed. CIBA GEIGY Corp., Basel.
4. **Davison, B. L., T. Leighton, and J. C. Rabinowitz.** 1979. Purification of *Bacillus subtilis* RNA polymerase with Heparin-Agarose. J. Biol. Chem. **254**:9220–9226.
5. **Ferber, D. M., and R. R. Brubaker.** 1979. Mode of action of pesticin: *N*-acetylglucosaminidase activity. J. Bacteriol. **139**:495–501.
6. **Frick, K. K., R. L. Quackenbush, and J. Konisky.** 1981. Cloning of immunity and structural genes for colicin V. J. Bacteriol. **148**:498–507.
7. **Hardy, K. G.** 1975. Colicinogeny and related phenomena. Bacteriol. Rev. **39**:464–515.
8. **Hu, P. C., and R. R. Brubaker.** 1974. Characterization of pesticin: separation of antibacterial activities. J. Biol. Chem. **249**:4749–4753.
9. **Konisky, J.** 1982. Colicins and other bacteriocins with established modes of action. Annu. Rev. Microbiol. **36**:125–144.
10. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680–685.
11. **Mayr-Harting, A., A. J. Hedges, and R. C. W. Berkeley.** 1972. Methods for studying bacteriocins. Methods Microbiol. **7A**:316–363.
12. **Mossie, K. J., D. T. Jones, F. T. Robb, and D. R. Woods.** 1979. Characterization and mode of action of a bacteriocin produced by a *Bacteroides fragilis* strain. Antimicrob. Agents Chemother. **16**:724–730.
13. **Mossie, K. J., D. T. Jones, F. T. Robb, and D. R. Woods.** 1980. Rifampin and bacteriocin resistance in *Bacteroides fragilis*. Antimicrob. Agents Chemother. **17**:838–841.
14. **Mossie, K. J., F. T. Robb, D. T. Jones, and D. R. Woods.** 1981. Inhibition of ribonucleic acid polymerase by a bacteriocin from *Bacteroides fragilis*. Antimicrob. Agents Chemother. **20**:437–442.
15. **Nakamura, T., S. Fujimura, N. Obata, and N. Yamazaki.** 1981. Bacteriocin-like substance (melaninocin) from oral *Bacteroides melaninogenicus*. Infect. Immun. **31**:28–32.
16. **Nakamura, T., N. Yamazaki, H. Taniguchi, and S. Fujimura.** 1983. Production, purification, and properties of a bacteriocin from *Staphylococcus aureus* isolated from saliva. Infect. Immun. **39**:609–614.
17. **O'Farrell, P. H.** 1975. High resolution two-dimensional electrophoresis of proteins. J. Biol. Chem. **250**:4007–4021.

18. **Richardson, H., A. H. Emslie-Smith, and B. W. Senior.** 1968. Agar diffusion method for the assay of colicins. Appl. Microbiol. **16**:1468–1474.
19. **Robb, S. M., D. R. Woods, F. T. Robb, and J. K. Struthers.** 1977. Rifampicin-resistant mutant supporting bacteriophage growth on stationary phase *Achromobacter* cells. J. Gen. Virol. **35**:117–123.
20. **Schaller, K., R. Dreher, and V. Braun.** 1981. Structural and functional properties of colicin M. J. Bacteriol. **146**:54–63.
21. **Schaller, K., J.-V. Höltje, and V. Braun.** 1982. Colicin M is an inhibitor of murein biosynthesis. J. Bacteriol. **152**:994–1000.
22. **Tagg, J. R., A. S. Dajani, and L. W. Wannamaker.** 1976. Bacteriocins of gram-positive bacteria. Bacteriol. Rev. **40**:722–756.
23. **Varel, V. H., and M. P. Bryant.** 1974. Nutritional features of *Bacteroides fragilis* subsp. *fragilis*. Appl. Microbiol. **28**:251–257.
24. **Willis, R. C., R. G. Morris, C. Cirakoglu, G. D. Schellenberg, N. H. Gerber, and C. E. Furlong.** 1974. Preparation of the periplasmic binding proteins from *Salmonella typhimurium* and *Escherichia coli*. Arch. Biochem. Biophys. **161**:64–75.