Inhibition of Ribonucleic Acid Polymerase by a Bacteriocin from *Bacteroides fragilis*

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The *Bacteroides fragilis* bacteriocin which inhibits ribonucleic acid (RNA) polymerase activity had a narrow activity spectrum in vivo and only inhibited the growth of certain *B. fragilis* strains. In vitro the bacteriocin was not specific and inhibited RNA polymerases from widely diverse bacterial genera. RNA polymerases from rifampin-resistant strains of *Bacteroides thetaiotaomicron* and *Clostridium acetobutylicum* were resistant to the bacteriocin in vitro. Purified bacteriocin bound to partially purified RNA polymerase, and both proteins were cosedimented in a glycerol gradient. In the RNA polymerase reaction, the bacteriocin acted as a competitive inhibitor for adenosine, cytidine, and uridine 5'-triphosphates and as a noncompetitive inhibitor for guanosine 5'-triphosphate. The bacteriocin did not inhibit RNA polymerase from chicken embryos.

Mossie et al. (11) described the production of a low-molecular-weight bacteriocin by a *Bacteroides fragilis* strain. The mode of action of the bacteriocin is unusual in that it inhibits ribonucleic acid (RNA) synthesis, and studies with crude extracts from *B. fragilis* cells indicate that it prevents RNA synthesis by inhibiting RNA polymerase activity (12). A similarity in the mode of action of the bacteriocin and rifampin was suggested by the isolation of 10 rifampin-resistant mutants which all showed varying degrees of susceptibility to the bacteriocin and differed from the susceptible parent strain (12). However, it is unlikely that the target sites for the bacteriocin and rifampin are identical, as the RNA polymerase mutants which were resistant to rifampin showed different responses to the bacteriocin. The present study was carried out to investigate the specificity and nature of the inhibition of RNA polymerase by the bacteriocin.

MATERIALS AND METHODS

Bacterial strains. The bacteriocin-producing *B. fragilis* Bf-1 strain and the susceptible indicator Bf-2 strain described by Mossie et al. (11) were used for the production and assay of the bacteriocin. The specificity of the bacteriocin in vivo and in vitro was tested against the following strains: *Escherichia coli* B, *Bacillus subtilis*, *Vibrio alginolyticus* (13), *Bacteroides thetaiotaomicron* (4), *B. thetaiotaomicron* rif^r, *Clostridium acetobutylicum* (1), *C. acetobutylicum* rif^r, and *B. fragilis* Bf-2 rif^r (12).

Media and anaerobic and bacteriocin tech- niques. Brain heart infusion broth and agar (9) were used for bacterial growth and the production and assay of the bacteriocin as described previously (11). The bacteriocin was purified by the method of Mossie et al. (11) and was homogeneous on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Bacteriocin titers in arbitrary units were expressed as the reciprocal of the highest doubling dilution that gave a zone of inhibition surrounding wells in brain heart infusion agar. The anaerobic glove box and techniques described by Moodie and Woods (10) were used, and incubation was at 37°C.

RNA polymerase assay. Crude bacterial extracts and partially purified RNA polymerase from *B. fragilis* cells were assayed for RNA polymerase activity as described previously (12). Control assays were performed in the presence of rifampin (30 µg ml^{-1}). One unit of activity was defined as the incorporation of 1 µmol of [^{3}H]uridine 5'-monophosphate into RNA per 10 min at 37°C. The specific activity of the RNA polymerase samples was calculated as activity units per milligram of protein, which was determined by the method of Lowry et al. (8), using bovine serum albumin as a standard. Chicken embryo RNA polymerase was assayed by the method of van der Westhuyzen (15).

Kinetics of incorporation of nucleoside triphosphates. Partially purified *B. fragilis* RNA polymerase (30 µg ml^{-1}) was assayed in the presence of three nucleoside triphosphates at 0.4 mM each and the fourth nucleoside triphosphate at a concentration that varied from 0.1 to 0.02 mM.

RNA polymerase purification. RNA polymerase from *B. fragilis* cells was partially purified by a method which was adapted from the techniques of Zillig et al. (17) and Burgess and Jendrisak (3). All purification procedures were carried out at between 1

and 4°C unless stated otherwise. Late-exponential-phase B. fragilis Bf-2 cultures (5 liters) were harvested by centrifugation, washed in grinding buffer, and frozen at −20°C. The grinding buffer (buffer A) contained: 50 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (pH 7.9), 5% (vol/vol) glycerol, 2 mM ethylenediaminetetraacetic acid (EDTA), 0.1 mM dithiothreitol, 0.23 M NaCl, 200 μg of lysozyme per ml, 1 mM 2-mercaptoethanol, and 50 μg of phenylmethylsulfonyl fluoride per ml. The frozen packed cells (ca. 80 g) were cut into small pieces, added to 240 ml of buffer A, and blended at low speed in a Sorvall Omni-Mixer 17106 (Du Pont Co.) for 3 min. The Omni-Mixer was then placed at 20°C for 20 min, and after the addition of 5 ml of 4% (wt/vol) sodium deoxycholate, the cells were blended at low speed for 30 s. After standing for a further 20 min, the DNA was sheared by high-speed blending for 30 s. The suspension was transferred to 320 ml of cold TGED buffer (10 mM Tris-hydrochloride [pH 7.9], 5% (vol/vol) glycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol) containing 0.2 M NaCl and blended at high speed for 30 s in a Waring blender. The suspension was clarified by centrifugation at 8,000 rpm for 45 min (fraction I), and 10% (vol/vol) Polymin P (BSAF, WHOZ Hauptlaboratorium B9, Ludwigshafen/Rhein, West Germany) prepared by the method of Burgess and Jendrisak (3) was added slowly with stirring at 4°C to give a final concentration of 0.6%. Stirring was continued for 5 min, and the suspension was centrifuged for 15 min at 6,000 rpm. The pellet was suspended in 300 ml of TGED plus 0.5 M NaCl in the Omni-Mixer and blended at low speed for 5 min. The suspension was centrifuged at 6,000 rpm for 15 min, and the pellet was suspended in TGED plus 1.0 M NaCl and again blended as described above. After centrifugation at 7,000 rpm for 30 min, the supernatant (fraction II) was brought to 55% saturation with (NH₄)₂SO₄. After being stirred for 30 min, the precipitate was collected by centrifugation, suspended in 20 ml of TGED buffer, and dialyzed against TGED plus 0.02 M KCl. The dialyzed enzyme preparation was diluted to 100 ml with TGED plus 0.02 M KCl and fractionated by diethylaminoethyl (DEAE)-cellulose column chromatography as described by Burgess (2). The column (2.5 by 20 cm) was eluted stepwise with TGED plus 0.1 to 0.4 M KCl. Fractions with high specific activity were precipitated by the addition of (NH₄)₂SO₄ (60% saturation) and dialyzed against TGED (fraction III). The degree of purification of fraction III was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Binding of ¹²⁵I-labeled bacteriocin to RNA polymerase. The bacteriocin produced by B. fragilis was purified to homogeneity as described previously (11). The purified bacteriocin was labeled with ¹²⁵I (Amersham Corp.) by the method of Greenwood et al. (5). The labeled bacteriocin was centrifuged in a Beckman SW27.1 rotor at 25,000 rpm for 24 h in a 15 to 35% glycerol gradient (38 ml) in TGED plus 0.4 M KCl. The partially purified RNA polymerase and a mixture of the RNA polymerase and the labeled bacteriocin were also centrifuged on the glycerol gradient. Fractions were collected and assayed for RNA polymerase activity; for ¹²⁵I, using a Packard gamma detector; and for protein by absorption at 280 nm.

RESULTS

Specificity of the bacteriocin. The bacteriocin did not affect the growth of the following strains: B. fragilis Bf-2 rif ʳ, B. subtilis, E. coli B, V. alginolyticus, B. thetaiotaomicron WT and rif ʳ, and C. acetobutylicum WT and rif ʳ (Table 1). The RNA polymerase activity in crude extracts from each of the rifampin-susceptible bacteria was inhibited by the bacteriocin and rifampin (Table 1). The bacteriocin and rifampin did not affect the in vitro activity of RNA polymerase from chicken embryos and the rifampin-resistant B. fragilis, B. thetaiotaomicron, and C. acetobutylicum strains. The extent of inhibition of RNA polymerase activity from E. coli depended on the concentration of the bacteriocin in the assay mixture (Fig. 1).

Partial purification of B. fragilis RNA polymerase. The purification and specific activity of RNA polymerase from B. fragilis are shown in Table 2. RNA polymerase after DEAE-cellulose chromatography (fraction III) which had a high specific activity was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and contained nine protein bands. The increased total activity in fraction III was presumably due to interference in the RNA polymerase assay by some component of the crude and less purified preparations which was removed by DEAE-cellulose chromatography.

Optimum conditions for RNA polymerase activity. The optimum pH and temperature for the activity of the partially purified B. fragilis RNA polymerase were pH 8.0 to 8.2 and 37°C. The enzyme reaction was affected by Mg^{2+}, Mn^{2+}, and KCl and was optimal at 0.2 mM Mg^{2+}, 0.4 mM Mn^{2+}, and 0.2 M KCl. The

TABLE 1. *Inhibition of growth of bacterial strains and inhibition of RNA polymerase activity in crude extracts by the B. fragilis bacteriocin*

Bacterial strain	Susceptibility (S) or resistance (R) of cells to bacteriocin	% Inhibition[a] of RNA polymerase activity by:	
		Bacteriocin (50 μl)	Rifampin (30 μg ml⁻¹)
B. fragilis Bf-2	S	60	65
B. fragilis Bf-2 rifʳ	R	0	0
E. coli	R	5	67
B. subtilis	R	60	69
V. alginolyticus	R	51	65
B. thetaiotaomicron	R	54	61
B. thetaiotaomicron rifʳ	R	0	0
C. acetobutylicum	R	57	65
C. acetobutylicum rifʳ	R	0	0
Chicken embryos	—[b]	0	0

[a] Percent inhibition relative to the total RNA polymerase activity of each strain.

[b] —, Not done.

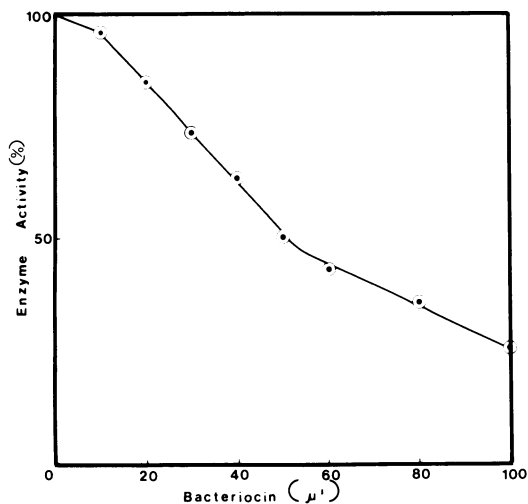FIG. 1. *Inhibition of E. coli RNA polymerase by B. fragilis bacteriocin. Different amounts of the bacteriocin were added to the reaction mixture (final volume, 0.2 ml) before the addition of the enzyme (80 μl).*

B. fragilis RNA polymerase did not show any specificity with regard to the nature of the template and was equally active with salmon sperm deoxyribonucleic acid (DNA), phage λ DNA, phage T4 DNA, and B. fragilis phage DNA.

Binding of ^{125}I-labeled bacteriocin to RNA polymerase. The purified bacteriocin which had been labeled with ^{125}I was not sedimented by centrifugation on the glycerol gradient and remained at the top of the tube (fractions 15 to 22, Fig. 2A). Partially purified B. fragilis RNA polymerase was sedimented in the glycerol gradient (Fig. 2B). The RNA polymerase activity was associated with the leading edge of the protein peak (absorption at 280 nm). When the labeled bacteriocin and the partially purified RNA polymerase preparation were mixed and centrifuged together on the glycerol gradient, a large proportion of the bacteriocin cosedimented with the partially purified RNA polymerase (Fig. 2C). The ^{125}I profile was similar to that obtained for the RNA polymerase activity in that it was also associated with the leading edge of the protein peak.

Kinetics of incorporation of nucleoside triphosphates. The kinetics of incorporation of nucleoside triphosphates by partially purified B. fragilis RNA polymerase into RNA in the presence and absence of purified bacteriocin (40 μg ml^{-1}) were determined (Fig. 3). The bacteriocin is a noncompetitive inhibitor for the binding of guanosine 5'-triphosphate (GTP) to RNA polymerase and decreased the V_{max} but did not affect the K_m. The double reciprocal plots for

adenosine and cytidine 5'-triphosphates (ATP and CTP) show competitive inhibition. In Fig. 3 the line which has been drawn for uridine 5'-triphosphate (UTP) involves the four points at the higher UTP concentrations and suggests that UTP shows competitive inhibition. The fifth point at the lowest UTP concentration was ignored as a considerable scatter was always obtained at this low UTP concentration and the reliability and reproducibility of the other points at the higher UTP concentrations were far greater.

DISCUSSION

In common with other bacteriocins the B. fragilis bacteriocin has a narrow activity spectrum in vivo and only inhibits the growth of certain B. fragilis strains. However, the bacteriocin in vitro is not specific and inhibits RNA polymerase from widely diverse genera. The lack of inhibition of RNA polymerase from chicken embryos suggests that the bacteriocin does not affect RNA polymerase from eukaryotic cells. The correlation between rifampin resistance and altered susceptibility to the bacteriocin does not only apply to B. fragilis rif^r strains (12), as a similar correlation was observed with the B. thetaiotaomicron rif^r and the C. acetobutylicum rif^r strains.

The correlation between rifampin resistance, which involves an alteration in the RNA polymerase (6, 14, 16), and altered bacteriocin susceptibility suggested that the bacteriocin binds to the RNA polymerase. In the RNA polymerase reaction the bacteriocin acts as a competitive inhibitor for ATP, CTP, and UTP and as a noncompetitive inhibitor for GTP. It is suggested that the mechanism of inhibition is unique and involves binding of the bacteriocin at the trinucleotide substrate binding site of RNA polymerase. Since a partially purified RNA polymerase was used, co-sedimentation in a glycerol gradient of the purified bacteriocin and the B. fragilis RNA polymerase preparation

TABLE 2. *Purification and specific activity of RNA polymerase from B. fragilis*

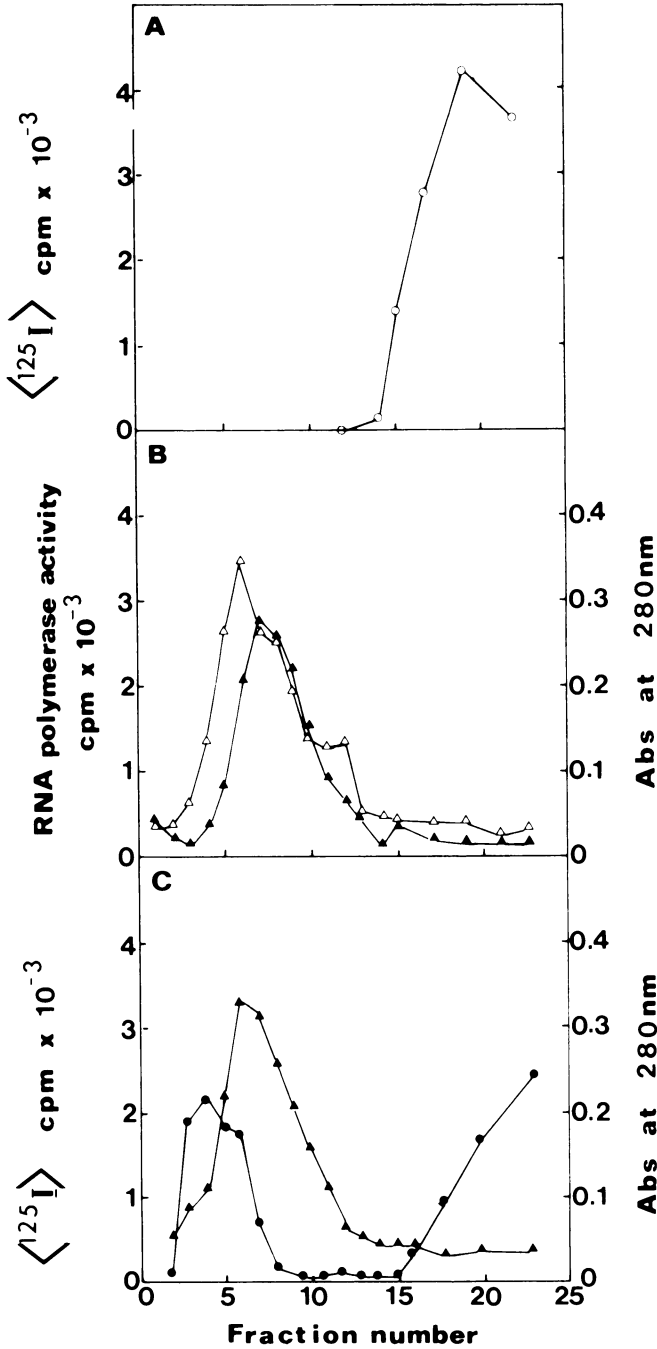| Fraction	Total protein (mg)	Total activity (U)	Sp act (U/mg)	Purification
Fraction I (low-speed supernatant)	5,510	15,015	2.7	1.0
Fraction II (Polymin P supernatant)	758	14,596	19.2	7.1
Fraction III (DEAE-cellulose column)	89	17,464	196.2	72.7

FIG. 2. *Binding of purified* ^{125}*I-labeled bacteriocin to partially purified B. fragilis RNA polymerase. (A) Centrifugation on the glycerol gradient of labeled bacteriocin; (B) RNA polymerase; and (C) a mixture of the labeled bacteriocin and the RNA polymerase. Symbols:* ○ *and* ●, *assay for* ^{125}*I in (A) and in (C) respectively;* △, *RNA polymerase activity in (B);* ▲, *absorbance (Abs) at 280 nm in (B) and (C). Fraction 22 is the top of the gradient.*

cannot be taken as direct evidence that the bacteriocin binds to a site on the RNA polymerase. However, had co-sedimentation not oc-

curred, it would have negated our conclusions from the kinetic studies. The co-sedimentation studies therefore do add support to the sugges-
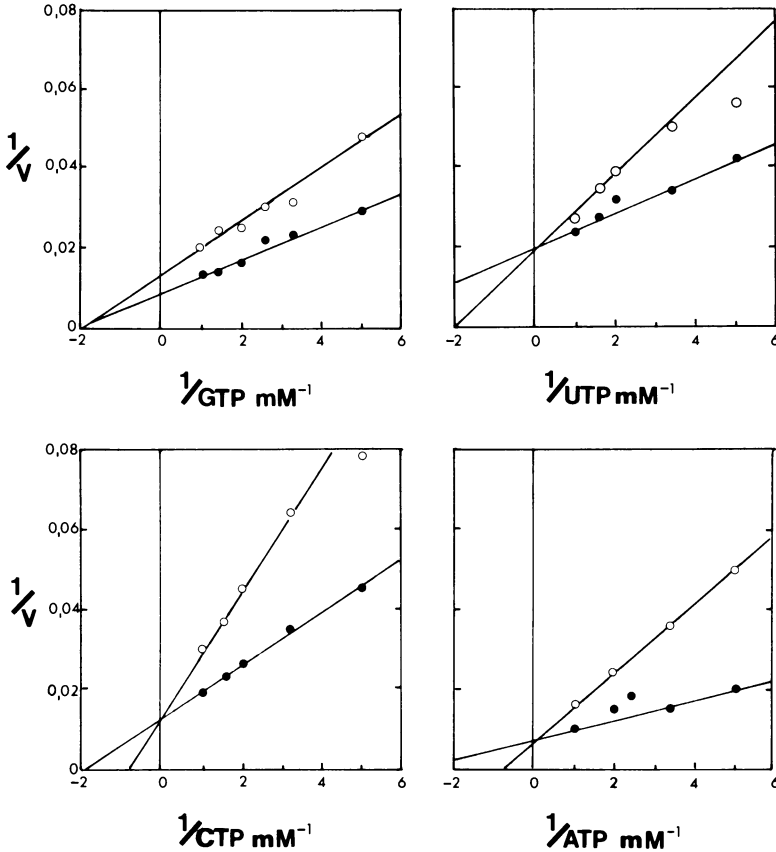
FIG. 3. *Kinetics of incorporation of nucleotide triphosphates by partially purified B. fragilis RNA polymerase (30 μg ml⁻¹) into RNA in the presence and absence of purified bacteriocin. Control without bacteriocin (●) and in the presence of 40 μg of bacteriocin per ml (○).*

tion that the bacteriocin binds to the RNA polymerase. The high degree of conservation that would probably exist at this site during evolution of RNA polymerase could explain the broad spectrum of action of the *B. fragilis* bacteriocin in vitro.

This is the first report of the partial purification of RNA polymerase with a high specific activity from the anaerobe *B. fragilis*. The requirements for activity of the RNA polymerase were similar to those for *E. coli* RNA polymerase activity (7). Further work regarding the characterization of the RNA polymerase subunits is in progress.

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