Bacteriocin Produced by *Bordetella pertussis*

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Of the 24 strains of *Bordetella pertussis* examined, 2 produced bacteriocins that inhibited the growth of all but 2 other strains of this species. The two strains producing the bacteriocin and the two resistant strains were rough, whereas all susceptible strains were smooth. The bacteriocin was not active on the *B. parapertussis* or *B. bronchiseptica* strains tested. These bacteriocins appeared to be protein in nature, since they were heat-labile and partially inactivated by trypsin. They were antigenic but the neutralizing antibodies did not precipitate the antigens. Absorption of the antiserum with homologous cell suspensions removed the agglutinating, but not the neutralizing, antibody.

A substance produced by *Escherichia coli* which inhibited only related organisms was first described by Gratia (7); the term colicin was later applied to this material (4). The inhibitory characteristic has been investigated in other families of bacteria, and there are now 15 known groups of bacteriocins (14).

Jacob et al. (11) advanced the following definition. "Bacteriocins are protein-like substances whose activity is restricted to related species and whose activity is conditioned by the presence of specific receptors. Their biosynthesis is associated with a lethal consequence for the producing organisms."

During a survey for phage of *Bordetella pertussis*, an inhibitory substance resembling a bacteriocin was observed. Since a *Bordetella* bacteriocin had not been previously described, the nature of this substance was investigated.

**MATERIALS AND METHODS**

**Organisms.** Strains of *B. pertussis* used were as follows: 190, 6627, 8467, 9306, 9797, 9832, 10380, 11615, 12741, 12742, 12743 (received from American Type Culture Collection); 10536, 28497, 28507 (kindly supplied by Grace Eldering, Michigan Department of Health); 1508-57, W2000, W2019, L92, 18.323R, G1-353, 2 atox variant 5 (received from Lister Institute of Preventive Medicine).

Strains of *B. parapertussis* used were: 17903, 21838, 31032 (Grace Eldering); E.958, B.S. 462 (Lister Institute of Preventive Medicine).

Strains of *B. bronchiseptica* used were: 262, 899, 22067 (Grace Eldering); 95, 106 (Lister Institute of Preventive Medicine); CDC 3847 (Microbiology Department, University of Louisville, Louisville, Ky.).

*B. pertussis* strains 190, 6627, 9306, and 11615 were found to be rough strains, based on their nonvirulence to mouse and nutritional requirements (15), whereas other strains were smooth, virulent organisms in phases 1 to 3.

**Survey techniques.** Two survey techniques were used with Bordet Genteng Broth Base (BG, Difco), containing 2% agar, 6 ml of glycerol (Baker-analyzed reagent) per 1,000 ml of broth, and 20% citrated whole human blood serving to support growth. In the spot technique, a heavy loopful of the strain being tested was spotted on a base culture of an indicator strain which had been applied in an even layer with a sterile swab. The plates were incubated for 48 hr at 37 C.

By use of the perpendicular streak technique developed by Frederich (4) for colicin survey, a streak of the test strain was made with a sterile swab across a plate of BG and the plate was incubated for 48 hr at 37 C. At this time, streaks of indicator strains were made perpendicular to the original streak. The plates were incubated another 48 hr and checked for lysis.

Ultraviolet irradiation was attempted to induce the production of the bacteriocin. Cultures (48-hr) of *B. pertussis* 190, 6627, 8467, 9306, 9797, 9832, 10380, 11615, 12741, 12742, 12743, and *B. parapertussis* 3847 were washed from the plate with 5 ml of saline and placed in a sterile plate. Each culture was irradiated for 15, 30, 45, and 60 sec in the dark with a 15-w General Electric ultraviolet lamp at a distance of 56 cm and was then tested by the spot technique.

**Assay of bacteriocin.** Wells 11 mm in diameter and approximately 5 mm deep were made in the assay medium, which consisted of 25 ml of BG medium which had been incubated for 24 hr at 37 C and used without further storage. A 0.1-ml amount of a standardized and frozen cell suspension of the indicator culture (*B. pertussis* 8467, approximately $9.7 \times 10^8$ cells per milliliter) was streaked on the plate with a sterile swab. A capillary pipette was used to place 4 drops (approximately 0.2 ml) of the material to be

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1 Based on portions of a dissertation submitted by Claudia Litkenhous to the University of Louisville, in partial fulfilment of the requirements for the M.S. degree.
assayed into the well. The plates were incubated at 37°C for 48 hr. The entire zone of lysis was measured, 11 mm of the well was subtracted, and the remainder was divided by 2. A 2-mm zone was arbitrarily chosen as an end point and was designated as 1 unit of activity. A serial twofold dilution of the extract was made in saline, and each dilution was tested. The reciprocal of the dilution that showed 1 unit of activity was taken as the titer of the preparation.

Methods for determination of protein. A modification of the Folin-Wu method (13) was used for the measurement of protein in the samples obtained during the various stages of purification of the bacteriocin. Crystalline bovine albumin served as the standard.

The protein content of the fractions obtained during chromatography was estimated according to Kalcarr's (12) variation of the method developed by Warburg and Christian (17). The same molarity of buffer which was used to obtain the fraction was used as a blank for that fraction.

Cell density determination. The cells were diluted in saline, and the optical density (OD) was read at 575 nm on a Coleman Junior spectrophotometer. An OD of 0.30 equaled a viable-cell count of 2.2 × 10⁸ cells per milliliter.

Production and concentration of the bacteriocin. Dialyzed Brain Heart Infusion (BHI, Difco) was used for the production of the bacteriocin of *B. pertussis* 190 and 6627. A 37-g amount of BHI was dissolved in 100 ml of distilled water and dialyzed overnight at 4°C against 400 ml of distilled water. The dialysate was replaced with 200 ml of distilled water, and dialysis was continued overnight. An addition of 1% agar was made to the combined dialysates, which were then autoclaved and distributed in 50-ml amounts into large petri plates (150 × 20 mm). The plates were inoculated with 2 drops of heavy cell suspension, incubated at 37°C for 24 hr, placed in a freezer (−20°C) for 48 hr, and thawed at 37°C, followed by removal of the fluid from the collapsed agar by centrifugation at 10,000 × g for 30 min at 4°C. A sample of the supernatant fluid was removed for the determination of protein and lytic activity, and another sample was concentrated with Carbowax (Union Carbide Corp., New York, N.Y.) overnight at 4°C. The remainder of the supernatant fluid was precipitated with 50% ammonium sulfate overnight at 4°C. After resuspension of the precipitate in a minimal amount of saline, the material was dialyzed in cellophane tubing (Visking Co., Div. of Union Carbide Corp., Chicago, Ill.) overnight at 4°C against phosphate buffer (pH 7.2), concentrated with Carbowax, and redialyzed against phosphate buffer. The material was then filtered with sintered-glass filters.

Chromatography. A DEAE (diethylaminoethyl) cellulose column (10 × 1 cm) was used in the chromatography. The sample containing 12.6 mg of protein was equilibrated overnight in a large volume of 0.01 M tris-(hydroxymethyl)aminomethane (Tris) buffer (Fisher Scientific Co., Pittsburgh, Pa.) at pH 8.0. Six 5-ml fractions were collected for each of the following molarities of Tris buffer: 0.01, 0.03, 0.05, 0.10, 0.30, 0.50, and 1.0.

Serological studies. Albino rabbits weighing approximately 2 kg each were used in the preparation of antiserum. The first two injections, consisting of 4 and 12 units, respectively, of bacteriocin in 0.5 ml, were mixed with equal amounts of Freund's incomplete adjuvant (composed of Arlacel and Bayol, 1:5:8.3) and were administered intradermally 11 days apart. The remaining four injections (24, 64, 128, and 256 units in that order) were administered intravenously over a period of 3 weeks. The bacteriocin used for all injections was partially purified by chromatography. The rabbits were bled twice during the injections and were exsanguinated 1 week after the final injection. The sera were sterilized by filtration with membrane filters (GS type, Millipore Corp., Bedford, Mass.) and were stored at 4°C.

To test for agglutinating antibody, the sera were diluted 1:10, and twofold serial dilutions were made with saline. To 0.5 ml of the diluted sera was added 0.5 ml of cell suspensions of *B. pertussis* strains 190 or 6627 at a concentration of 9.0 × 10⁸ cells per milliliter (tube no. 3 of the McFarland nephelometer series). Normal rabbit serum was used as a control. After overnight incubation at 37°C, the agglutination titer was determined by recording the highest dilution showing visible agglutination.

Before testing for neutralizing antibody, the sera were heated at 56°C for 1 hr since the unheated serum was inhibitory for the indicator strain, possibly due to complement. After making twofold serial dilutions with saline, an equal amount of bacteriocin containing 128 units was added, and the mixture was incubated at 37°C for 1 hr. An untreated sample of bacteriocin was included as a control.

The cells to be used for absorption were 48-hr cultures of *B. pertussis* strains 190 and 6627 were inhibitory to all other strains of *B. pertussis* except strains 9306, 10390, and 11615. The results of a typical experiment are shown in Fig. 1. Both producing strains were resistant to the bacteriocins. At no time did these two strains show any inhibitory effect on strains of *B. parapertussis* or *B. bronchiseptica*.

Of the strains exposed to ultraviolet light, no new bacteriocinogenic strains were found, nor were the spectra of the bacteriocinogenic cultures increased after ultraviolet irradiation.

After concentration of extracts of strains *B. pertussis* 190, 6627, 9306, and 11615; *B. parapertussis* 17903; *B. bronchiseptica* 262, 899, 22067, 2347, 95, and 106, only the *B. pertussis* extracts of strains 190 and 6627 produced any inhibition.
Once again, this inhibitory effect was observed only on strains of *B. pertussis* and not *B. parapertussis* or *B. bronchiseptica*. All the strains which were inhibited in the spot and streak surveys and strain 10380 were inhibited by the extracts, but strains 9306 and 11615 were still resistant to the bacteriocin. Strains 190 and 6627 had the same activity spectrum and remained unaffected by the extracts.

Table 1 summarizes the efficiency of the concentration technique. The activity increased from 0.025 to 4.5 units per mg of protein after the ammonium sulfate precipitation and Carbowax concentration.

The bacteriocinic activity obtained with liquid BHI aerated on a shaker was less than that obtained with solid media. Two types of cell disruption, sonic treatment and amyl alcohol-chloroform treatment, were unsuccessful in attempts to increase the titer.

There was some absorption of the bacteriocin with the sintered-glass filter used for sterilization.

The pattern of protein distribution and activity after chromatography is shown in Fig. 2. The peak of activity in the 1.0 M fraction did not coincide with the protein peak for that fraction. As shown in Table 2, the purification was fourfold since the activity increased from 30 to 120 units per mg of protein.

The bacteriocin did not diffuse through cellophane. Heating at 56 C for 30 min produced no loss of activity, whereas heating at 65, 75, and 100 C produced a loss of approximately 85% of the activity in all three cases.

The activity of the bacteriocin was destroyed at extreme pH values at 37 C; only residual activity remained after 24 hr of exposure to pH 3, 4, 9, and 10 and readjustment of the pH to 7.0 for testing. It was more stable near a neutral pH; by the 7th day, 75 to 80% of the activity had been destroyed.

Trypsin only partially inactivated the bacteriocin, with 33% being destroyed. There was no difference in the loss of activity in the 1- and 3.5-hr exposures. Neither ribonuclease nor deoxyribonuclease had any effect on the activity of the bacteriocin. The bacteriocin was stable at -70 C and no loss of activity was noted after lyophilization.

Intraperitoneal injection of the bacteriocin (25.6 units in 0.2 ml) into mice produced no ill effect, and intradermal injection of 1.6 units in 0.2 ml in rabbits failed to produce any skin lesion. It appeared that this bacteriocin was nontoxic.

The final agglutinating titer of the antiserum prepared against the extract of strain 190 was 20,480 for the cells of the homologous strain and 2,560 for the cells of strain 6627. A 0.5-ml amount of a 1:256 dilution of the same antiserum completely neutralized 128 units of the homologous bacteriocin, whereas the same serum neutralized 128 units of bacteriocin of strain 6627 at a 1:64 dilution. After the four absorptions with cells of strain 190, the agglutinating titer was reduced from 20,480 to 640 (32-fold decrease). The neutralizing antibody titer was reduced from 256 to 128 (twofold decrease).

In the agar gel diffusion experiments, multiple lines of precipitation were formed with the un-

![Fig. 1. Streak technique. Inhibitory effect of Bordetella pertussis 190 on B. pertussis strains (left to right) 8467, 8478, 9306, 10336, 28497, and 28507. Strain 9306 is resistant.](http://jb.asm.org/)

<table>
<thead>
<tr>
<th>Pregen</th>
<th>Lytic activity</th>
<th>Protein</th>
<th>Bacteriocin activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fluid...</td>
<td>&lt;1.0 (0.75)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate (50%) precipitation...</td>
<td>5.2</td>
<td>2.0</td>
<td>2.6</td>
</tr>
<tr>
<td>Ammonium sulfate (50%) precipitation and Carbowax</td>
<td>6.4</td>
<td>1.4</td>
<td>4.5</td>
</tr>
</tbody>
</table>

*Expressed as units per milligram of protein.*

*bActivity of the original supernatant fluid was less than 1 unit per ml. It was estimated to be about 0.75 unit per ml after concentration.*
treated antiserum and the antiserum precipitated with 33% ammonium sulphate. However, there were no lines of precipitation with the normal or the absorbed serum.

**Discussion**

The spectrum of the bacteriocin produced by two strains of *B. pertussis* was limited to other strains of *B. pertussis* and had no inhibitory effect on *B. parapertussis* or *B. bronchiseptica*. The two producing strains were resistant to the bacteriocins.

Compared with some other bacteriocins investigated, the number of bacteriocinogenic strains of *Bordetella* is relatively low. Only 2 of the 24 strains tested produced the bacteriocin, and these had the same activity spectrum. In the work with *Pasteurella*, for example, 23 of 24 strains of *P. pestis* were found to produce pesticin (2).

It was also noted that the two strains producing the bacteriocin were rough; they were neither mouse-virulent nor dependent upon the presence of blood for growth. The two strains of *B. pertussis* which were resistant to the bacteriocin were also rough, whereas all the susceptible strains were smooth.

These observations suggest that specific receptors may be necessary for attachment, and only the smooth strains possess these receptors. The bacteriocin may be a part of the somatic antigen of the cell which is not incorporated into the rough strains owing to some defect of synthetic mechanism. Colicin K (5, 6, 16) and colicin V (9) have been purified and found to be a part of the somatic antigen of the producing organism.

The proteinaceous nature of the bacteriocin produced by *Bordetella* was demonstrated by the following studies: precipitation by ammonium sulfate, partial inactivation by trypsin, and inactivation by incubation at 65°C and above.

Two types of antibody, agglutinating and neutralizing, were produced after injections of partially purified extracts of *B. pertussis* 190. After absorption of the agglutinating antibody with cells of strain 190, the neutralizing antibody was still active but did not precipitate with the antigen. This observation is similar to that of Amano et al. (1), who found that antiserum to colicin K contained both a precipitating and a nonprecipitating antibody, the latter being the neutralizing antibody.

There was moderate cross-reaction of the antiserum of the bacteriocin of strain 190 with the bacteriocin of strain 6627. There was also agglutination of the cells of 6627 but to a lesser extent. This indicates that the bacteriocins are serologically related but not identical—at least not in the partially purified stage. In the study of the antigenic relationships of bacteriocins within other species, there was no cross-reaction within megacins (10) but complete cross-reaction within monocins (8).

**Table 2. Purification of bacteriocin of Bordetella pertussis 190 by DEAE cellulose chromatography**

<table>
<thead>
<tr>
<th>Prepn</th>
<th>Total lytic activity mg</th>
<th>Total protein</th>
<th>Bacteriocin activitya</th>
<th>Bacteriocin activitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>Originala..........</td>
<td>384</td>
<td>12.6</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>Tris fraction, 1.0 M...</td>
<td>185</td>
<td>1.7</td>
<td>120</td>
<td></td>
</tr>
</tbody>
</table>

*a* Expressed as units per milligram of protein.

*a* A 2-ml amount of the preparation obtained by 50% ammonium sulfate precipitation and Carbowax concentration.
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Literature Cited


