Streptococcus faecium-Derived Antibacterial Substance Antagonistic to Bifidobacteria

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An antagonistic strain of Streptococcus faecium was isolated from human feces, and it displayed a marked inhibition of bifidobacteria on agar plates. In liquid culture this isolate produced an antibacterial substance that can be partially purified by ammonium sulfate precipitation followed by gel filtration and ion-exchange chromatography. Its activity was assayed by the inhibition of growth of Bifidobacterium longum. The substance was sensitive to digestion by proteolytic enzymes and α-amylase, but was resistant to treatment with 6 M urea, dithiothreitol, 2-mercaptoethanol, ethyl ether, chloroform, and lysozyme. It was also stable to heating at 100°C for 60 min. Its molecular weight was estimated to be about 50,000 by gel filtration.

Bacterial antagonism has long been recognized and usually attributed to such products as antibiotics, bacteriocins, metabolic wastes, and bacteriophages. This phenomenon has sometimes been observed in the course of ecological studies of microflora in human feces. During the studies of intestinal microflora, Hoogkamp-Korstanje et al. found Streptococcus faecium antagonistic to bifidobacteria, but they failed to demonstrate the inhibitory substance in supernatant of culture fluids (5). Various strains of S. faecium have shown to produce bacteriocins active against a wide range of bacteria (1, 3, 6, 10), but little is known about antibacterial substance in streptococci that is antagonistic to bifidobacteria. This report deals with the isolation and preliminary characterization of the antibacterial substance produced by a human fecal isolate of S. faecium antagonistic to bifidobacteria.

MATERIALS AND METHODS

Bacterial strains and cultivations. An inhibitor-producing coccus isolated from feces of a healthy adult was identified as Enterococcus (group D Streptococcus) on the basis of its Gram-staining reaction, microscopic morphology, growth in the presence of 6.5% NaCl, growth at 45°C, growth at pH 9.6, and lack of catalase activity. The isolate was further characterized as S. faecium by virtue of its fermentation of lactose, mannitol, arabinose, and melibiose; nonfermentation of sorbitol, glycerol, and melezitose; reduction of methylene blue; nonreduction of tetrazolium; nonhemolysis on 5% human and horse blood agar plates; and survival at 60°C for 30 min. A wide variety of organisms listed in Table 1 were obtained from various culture collections and were tested for susceptibility to inhibitory action of the isolated coccus.

The organisms were all grown anaerobically at 37°C on glucose-blood-liver (BL) agar medium, of which the composition was described elsewhere (8). Liquid medium was provided by removing agar from BL agar medium and by substituting Fildes solution for blood (4).

Assay of antibacterial substance. A 0.1-ml amount of the broth culture of indicator strain (Bifidobacterium longum) was pipetted onto the surface of plates and was spread to obtain a uniform bacterial lawn. After drying the surface of the agar medium at 37°C for 30 min, a loopful drop of twofold dilutions containing antibacterial substance was spotted on the surface of each plate. By this technique, the inoculum of the indicator strain was kept at a constant size (approximately 10⁷ cells per 0.1 ml). After incubation at 37°C overnight, the plates were examined for inhibition zones.

Molecular weight estimations. The molecular weight of the antibacterial substance was estimated by gel filtration on a Sephadex G-75 (Pharmacia Fine Chemicals, Upplands Väsby, Sweden) column (3 by 45 cm). Bovine serum albumin (molecular weight, 67,000), ovalbumin (molecular weight, 45,000), and lysozyme (molecular weight, 14,400) were used as reference proteins to calibrate the column. Bovine serum albumin and ovalbumin were obtained from Wako Pure Chemical Industries, Osaka, Japan, and lysozyme was from Seikagaku Kogyo Co., Tokyo, Japan.

Enzymes and chemicals. Various reagents were used to treat the partially purified preparation in an effort to define the nature of the antibacterial substance. Urea, dithiothreitol, 2-mercaptoethanol, ethyl ether, chloroform, trypsin, and α-amylase were obtained from Wako Pure Chemical Industries, Osaka, Japan; papain was from E. Merck AG, Darmstadt, Germany; protease (type V) was from Sigma Chemical Co., St. Louis, Mo.; and pronase E was from Kaken Chemical Co., Tokyo, Japan. The reagent-treated preparation was then measured for activity.

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Detection of glycoprotein. By using p-dimethylaminobenzaldehyde (Wako Pure Chemical Industries, Osaka, Japan) reagent, the hydrolysate of the antibacterial substance was tested by the method of Morgan and Elson (9).

RESULTS

Spectrum of antagonistic action. Various gram-positive and gram-negative bacteria were tested for susceptibility to inhibition by the isolated coccus. Antagonistic action was determined in cross-streaks of the isolate against horizontal streaks of organisms to be tested on BL agar plates. The isolate displayed a marked inhibition of bifidobacteria on agar plates (Fig. 1). It was also antagonistic to some lactobacilli, anaerobic gram-positive cocci, and Bacteroides melaninogenicus (Table 1), but no streptococci, with the exception of Streptococcus morbillorum, were susceptible.

Purification of antibacterial substance. BL broth (2,000 ml) was inoculated with 1% inoculum obtained from a 18-h culture of the antagonistic isolate. After incubation at 37°C for 18 h, the bacterial cells were removed by centrifugation at 10,000 rpm for 30 min. Solid ammonium sulfate was added to the supernatant fluid with constant stirring until the solution was 50% saturated. The precipitate was collected by centrifugation at 10,000 rpm for 30 min and discarded. Ammonium sulfate was added again to the supernatant fluid to bring it to 80% saturation, and the resulting precipitate was collected by centrifugation at 10,000 rpm for 30 min. This precipitate was dissolved in 80 ml of 0.05 M Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer (pH 7.8) and dialyzed in the cold against the same buffer for 18 h.

After filtration through a 450-nm pore size membrane filter (Millipore Corp., Bedford, Mass.), the solution of antibacterial substance was placed on a Sephadex G-75 column (3 by 45 cm) previously equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.8). The column was eluted with the same buffer at a flow rate of 35 ml/h. Effluent fractions of 5 ml were collected, monitored for absorbance at 280 nm, and assayed for antibacterial activity (Fig. 2).

The active fractions were pooled and applied to a column (1.5 by 30 cm) of diethylaminoethyl-Sephadex A-50 (Pharmacia Fine Chemicals, Uppsala, Sweden), previously equilibrated with 0.05 M Tris-hydrochloride buffer (pH 7.8). The antibacterial substance was eluted as a single peak with a linear gradient (0 to 0.5 M NaCl with the same buffer) at a flow rate of 35 ml/h (Fig. 3). These procedures are summarized in Table 2.

The resulting preparation of antibacterial substance was stored at 4°C and used for further characterization.

The purity of antibacterial substance was examined by high-performance liquid chromatography with a ConstaMetric II (Milton Roy Co., Riviera Beach, Fla.) with a Shodex OHpak B-804 (Showa Denko, Tokyo, Japan) column and a model 1202 SpectroMonitor II (Milton Roy Co., Riviera Beach, Fla.) (Fig. 4 and 5).

Properties of antibacterial substance. An estimation of the chemical nature of the antibacterial substance was obtained by testing its sensitivity to various reagents (Table 3). Partially purified preparation of antibacterial substance was sensitive to digestion by proteolytic enzymes and α-amylase. Incubation with either papain (1 mg/ml), pronase (1 mg/ml), protease (1 mg/ml), trypsin (2 mg/ml), or α-amylase (5 mg/ml) showed loss of activity, the titer decreasing from 32 to 16 or 8 at 37°C for 60 min.

Urea (6 M), dithiothreitol (3 × 10⁻² M), 2-mercaptoethanol (6 × 10⁻³ M), ethyl ether (50%), chloroform (50%), and lysozyme (1 mg/ml) had no effect on its activity. The substance was particularly stable to heating, with no apparent decrease in activity on heating at 100°C for 60 min.

The antibacterial substance revealed a positive reaction in the Morgan-Elson test. This substance appeared to have a molecular weight of about 50,000, as determined by its position of elution from the Sephadex G-75 column relative to the standard marker proteins.

DISCUSSION

The present investigation has shown that antagonistic action of S. faecium against bifidobacteria is due to an antibacterial substance. Some strains of S. faecium have been shown to produce bacteriocins. Pleceas examined 28 strains...
Table 1. Spectrum of inhibitory activity of the isolated coccus against various bacterial species

<table>
<thead>
<tr>
<th>Test organism</th>
<th>Inhibition zone (mm)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bifidobacterium bifidum ATCC 15696$^b$</td>
<td>2</td>
</tr>
<tr>
<td>B. infantis ATCC 15697</td>
<td>5</td>
</tr>
<tr>
<td>B. breve ATCC 15701</td>
<td>5</td>
</tr>
<tr>
<td>B. adolescens ATCC 15703</td>
<td>3</td>
</tr>
<tr>
<td>B. longum ATCC 15707</td>
<td>5</td>
</tr>
<tr>
<td>Bifidobacterium sp. ATCC 27538</td>
<td>1</td>
</tr>
<tr>
<td>Lactobacillus acidophilus ATCC 332</td>
<td>0</td>
</tr>
<tr>
<td>L. leichmannii ATCC 4797</td>
<td>0</td>
</tr>
<tr>
<td>L. delbrueckii ATCC 9649</td>
<td>2</td>
</tr>
<tr>
<td>L. bulgaricus ATCC 7993</td>
<td>2</td>
</tr>
<tr>
<td>L. lactis ATCC 8000</td>
<td>0</td>
</tr>
<tr>
<td>L. jugurti ATCC 8001</td>
<td>2</td>
</tr>
<tr>
<td>L. helveticus ATCC 10997</td>
<td>4</td>
</tr>
<tr>
<td>L. salivarius subsp. salivarius ATCC 11741</td>
<td>0</td>
</tr>
<tr>
<td>Streptococcus faecalis ATCC 19433</td>
<td>0</td>
</tr>
<tr>
<td>S. faecium ATCCd 19434</td>
<td>0</td>
</tr>
<tr>
<td>S. liquefaciens Jitsu-17</td>
<td>0</td>
</tr>
<tr>
<td>S. zymogenes Jitsu-16</td>
<td>0</td>
</tr>
<tr>
<td>S. durans Jitsu-18</td>
<td>0</td>
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<tr>
<td>S. intermedius ATCC 27335</td>
<td>0</td>
</tr>
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<td>S. morbillorum ATCC 27824</td>
<td>6</td>
</tr>
<tr>
<td>Staphylococcus albus Jitsu-1</td>
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</tr>
<tr>
<td>Listeria monocytogenes Jitsu-4</td>
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<tr>
<td>Pediococcus sp. Jitsu-9</td>
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<td>Peptococcus saccharolyticus ATCC 14953</td>
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<tr>
<td>P. anaerobius ATCC 14956</td>
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</tr>
<tr>
<td>P. niger ATCC 27731</td>
<td>2</td>
</tr>
<tr>
<td>Peptostreptococcus anaerobius ATCC 27337</td>
<td>3</td>
</tr>
<tr>
<td>Ruminococcus torques ATCC 27756</td>
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</tr>
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<td>Coprococcus eutactus ATCC 27759</td>
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<tr>
<td>Salmonella typhi H901W Jitsu-23</td>
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</tr>
<tr>
<td>S. paratyphi A Jitsu-25</td>
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</tr>
<tr>
<td>S. paratyphi B Jitsu-26</td>
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<td>Esherichia coli K-12 Jitsu-49</td>
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<tr>
<td>E. coli B Stnd. VI-107</td>
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<td>Bacteroides melaninogenicus subsp. melaninogenicus ATCC 15930</td>
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<td>B. melaninogenicus subsp. intermedius ATCC 25611</td>
<td>4</td>
</tr>
<tr>
<td>B. melaninogenicus subsp. intermedius NCTC 9336</td>
<td>8</td>
</tr>
<tr>
<td>B. asaccharolyticus ATCC 25260</td>
<td>8</td>
</tr>
<tr>
<td>B. asaccharolyticus NCTC 9337$^c$</td>
<td>8</td>
</tr>
<tr>
<td>B. microfurans ATCC 29729</td>
<td>5</td>
</tr>
<tr>
<td>B. bivius VPI 6318$^d$</td>
<td>1</td>
</tr>
<tr>
<td>B. disiens VPI 8057</td>
<td>1</td>
</tr>
<tr>
<td>B. fragilis NCTC 9344</td>
<td>0</td>
</tr>
<tr>
<td>B. distasonis ATCC 8503</td>
<td>0</td>
</tr>
<tr>
<td>B. ovatus ATCC 8483</td>
<td>0</td>
</tr>
<tr>
<td>B. thetaiotaomicron ATCC 29148</td>
<td>0</td>
</tr>
<tr>
<td>B. multiacidus ATCC 27723</td>
<td>0</td>
</tr>
<tr>
<td>B. hypermegans Stnd. VI-36</td>
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</tr>
<tr>
<td>Clostridium perfringens CCM 5744$^*$</td>
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<tr>
<td>C. septicum NCTC 504</td>
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<td>C. histolyticum CCM 5943</td>
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</tr>
<tr>
<td>C. ramosum VPI 8546</td>
<td>0</td>
</tr>
<tr>
<td>C. indolis ATCC 25771</td>
<td>1</td>
</tr>
<tr>
<td>C. oroticum ATCC 13619</td>
<td>1</td>
</tr>
<tr>
<td>C. innocum ATCC 14501</td>
<td>0</td>
</tr>
<tr>
<td>C. nexilis ATCC 27757</td>
<td>2</td>
</tr>
<tr>
<td>C. butyricum ATCC 19098</td>
<td>0</td>
</tr>
<tr>
<td>C. cocoides ATCC 29296</td>
<td>1</td>
</tr>
</tbody>
</table>

*a Inhibition was tested in cross-streaks of the isolated coccus against horizontal streaks of test strains.

$^b$ ATCC, American Type Culture Collection, Rockville, Md.

$^c$ NCTC, National Collection of Type Cultures, London, England

$^d$ VPI, Virginia Polytechnic Institute and State University, Blacksburg, Va.

$^* CCM, Czechoslovak Collection of Microorganisms, Brno, Czechoslovakia. All remaining strains are from our collection.
of *S. faecium* for production of bacteriocins, and found 50% of them to be bacteriocinogenic (10). Brock et al. (3) and Pleceas et al. (11) observed that *S. faecium* displayed a broad spectrum of activity against gram-positive bacteria, in contrast to the classical description of bacteriocins as being active against other strains of the same or closely related species. The antagonistic action of our isolate is effective against some strains of lactobacilli and *Bacteroides melaninogenicus* as well as against bifidobacteria. However, the overall spectrum of the antibacterial activity that we investigated was not always comparable to the bacterial species surveyed by Brock et al. (3) and Pleceas et al. (11), because these investigators did not include bifidobacteria among their indicator strains.

Hoogkamp-Korstanje et al. found a strain of *S. faecium* antagonistic to bifidobacteria on agar plates, but they failed to demonstrate the inhibitory substance in supernatant of culture fluids (5). Although no culture supernatant had inhibitory activity when tested directly in our studies, the active substance was precipitated with ammonium sulfate largely within the range of 50 to 80% saturated. The substance that we isolated seems to lack hydrophobic bonds and disulfide bonds.

![Fig. 2. Gel filtration of 50 to 80% ammonium sulfate-precipitated inhibitory substance on Sephadex G-75. The elution occurred in 0.05 M Tris-hydrochloride buffer (pH 7.8). Each 5-ml fraction was assayed for inhibitory activity (□) and protein (○).](image)

**Fig. 2.** Gel filtration of 50 to 80% ammonium sulfate-precipitated inhibitory substance on Sephadex G-75. The elution occurred in 0.05 M Tris-hydrochloride buffer (pH 7.8). Each 5-ml fraction was assayed for inhibitory activity (□) and protein (○).

**TABLE 2. Purification of the antibacterial substance**

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Vol (ml)</th>
<th>Activity/ml</th>
<th>Sp act</th>
<th>Total activity</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant fluid</td>
<td>2,000</td>
<td>ND&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium sulfate precipitate 50–80%</td>
<td>80</td>
<td>64</td>
<td>48</td>
<td>5,120</td>
<td>100</td>
</tr>
<tr>
<td>Dialysis</td>
<td>120</td>
<td>32</td>
<td>47</td>
<td>3,840</td>
<td>75</td>
</tr>
<tr>
<td>Sephadex G-75 gel filtration</td>
<td>180</td>
<td>16</td>
<td>326</td>
<td>2,880</td>
<td>56</td>
</tr>
<tr>
<td>Diethylaminoethyl-Sephadex A-50 chromatography</td>
<td>160</td>
<td>8</td>
<td>467</td>
<td>1,280</td>
<td>25</td>
</tr>
</tbody>
</table>

<sup>a</sup> Activity is expressed as the reciprocal of the highest dilution that formed inhibition zones. Specific activity is determined antibacterial titer per unit of absorbance at 280 nm.

<sup>b</sup> ND, Not determined.

![Fig. 4. High-performance liquid chromatography of the antibacterial substance after elution from Sephadex G-75. Column: Shodex OHPak B-804; eluent: 0.02 M H<sub>3</sub>PO<sub>4</sub>; flow rate: 1.0 ml/min; pressure: 17 kg/cm<sup>2</sup>; detector: ultraviolet, 270 nm.](image)

**Fig. 4.** High-performance liquid chromatography of the antibacterial substance after elution from Sephadex G-75. Column: Shodex OHPak B-804; eluent: 0.02 M H<sub>3</sub>PO<sub>4</sub>; flow rate: 1.0 ml/min; pressure: 17 kg/cm<sup>2</sup>; detector: ultraviolet, 270 nm.
bonds because of its stability against 6 M urea, dithiothreitol, and 2-mercaptoethanol. The substance may lack an active lipid component because it was resistant to chloroform and ethyl ether. Greater than 50% activity was lost when the substance was incubated with proteolytic enzymes or α-amylase at 37°C for 60 min. Besides, this substance showed a positive reaction in the Morgan-Elson test. The above characteristics may indicate that the substance is glycopeptide. Its molecular weight was estimated to be about 50,000 by gel filtration. Kremer and Brandis reported that the bacteriocin produced by S. faecium strain E1 could be separated into two distinct substances which differ in physicochemical characters; the predominant type was trypsin sensitive and had a molecular weight of about 10,000, and another type was trypsin resistant and had a molecular weight of above 100,000 (7). These lead us to speculate that S. faecium includes various strains which produce different active substances.

The bacteriocinogeny of S. faecium strain E1 could be eliminated in the presence of acridine orange, proflavin, or trypanflavin (2). Any definitive conclusion with regard to our antibacterial substance as a bacteriocin must await until its production is demonstrated to be controlled by a plasmid gene.

**LITERATURE CITED**


**FIG. 5.** High-performance liquid chromatography of the antibacterial substance after elution from diethylaminoethyl-Sephadex A-50. Specifications as in the legend to Fig. 4.