Characteristics of a Bacteriocin Derived from
Streptococcus faecalis var. zymogenes
Antagonistic to Diplococcus pneumoniae

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A bacteriocin-producing strain of Streptococcus faecalis var. zymogenes (E-1) was isolated from clinical material (conjunctiva). The active substance differed from bacteriocins described by other investigators primarily in its spectrum of antibacterial activity, especially by its marked inhibition of Diplococcus pneumoniae. The E-1 bacteriocin also inhibited nonhemolytic strains of enterococci as well as one-third of the Viridans group of streptococcal strains investigated. The degree of inhibition, however, as indicated by the size of the zones against the latter organisms, was significantly reduced. No activity was detected against any of the strains belonging to the following groups of bacteria: hemolytic enterococci, beta-hemolytic streptococci, nonhemolytic streptococci, staphylococci, and various gram-negative species. Similarly, three strains each of Bacillus cereus and Listeria monocytogenes and one strain of Erysipelothrix insidiosa were not inhibited. The bacteriocin was able to diffuse through bacterial membranes as well as cellulose dialyzer tubing. It was inactivated by heating to 80°C for 20 min but resisted inactivation by either trypsin or chloroform.

Bacteriocins are proteinaceous substances synthesized by certain strains of bacteria. They differ from antibiotics in chemical composition, in mode of action, and in their restricted spectrum of inhibition limited to the same or closely related species (13).

The physiological role of bacteriocins in the life of bacteria is unknown and has not been elucidated. Many differences exist between bacteriocins produced by gram-positive bacteria as contrasted to those obtained from gram-negative species (3, 8, 11).

Bacteriocin production is widespread in nature, and several types may be formed by the same bacterial strain (2, 7, 11, 13). Brock et al. (2) described five types of bacteriocins (enterococci) produced by group D streptococci on the basis of their activity against various microorganisms, susceptibility to proteolytic enzymes, and reaction to heat and chloroform.

A group of bacteriocinogenic strains of Streptococcus faecalis var. zymogenes were isolated in our laboratory which, in general, resembled those described by Brock et al. However, one of the strains studied, E-1, elaborated a bacteriocin which resembled type 5 described by these authors but which differed in several respects from the latter, particularly in its pronounced and selective activity against Diplococcus pneumoniae. All strains of nonhemolytic enterococci tested and some strains of streptococci of the Viridans group were also found to be susceptible to its action.

It is the aim of this communication to describe the activity and characteristics of the bactericidal substance produced by S. faecalis var. zymogenes (E-1).

MATERIALS AND METHODS

The bacteriocin-producing organism E-1 (producer strain) was recovered from the conjunctiva of a patient. Identification of the isolate as an enterococcus was established on the basis of its Gram staining reaction, microscopic morphology, growth in the presence of 6.5% NaCl, bile resistance, lack of catalase activity, and by the fermentation of glucose,
lactose, sucrose, maltose, salicin, and esculin. The organism was further characterized as *S. faecalis* var. *zymogenes* by virtue of its rapid fermentation of sorbitol and mannitol, reduction of litmus milk, and the production of large zones of beta hemolysis on 3% horse blood but not on sheep blood-agar. Casein was hydrolyzed but gelatin was not liquefied.

The 106 enterococci-sensitive pneumococcal strains (indictor strains) possessed characteristic Gram reaction and microscopic morphology, produced alpha hemolysis around colonies on sheep blood-agar, and were bile-soluble and Optochin (ethylhydrocuprein)-susceptible. The Neufeld queUlung reaction, with type-specific antiserum, was performed on representatives of the indicator pneumococci, and various different serological types were distinguished. Other microorganisms studied were identified on the basis of criteria outlined in *Bergey's Manual* (7th ed.) and by Edwards and Ewing (6).

Screening for E-1 bactericidal activity against various microorganisms was performed by the lawn-spotting technique (5) with 5% sheep blood-agar for all gram-positive species, Mueller-Hinton for gram-negative strains, and chocolate agar for *Haemophilus* and *Neisseria* isolates. In this technique, the inoculum of the producer strain was incubated at a constant size.

A type 3 pneumococcus was selected as an indicator strain to study the characteristics of the bacteriocin produced by strain E-1 by means of a modified associative culture technique in liquid medium (14). This strain was chosen because of the ease with which the mucoid colonies could be detected after subculture. The producer organism was grown together with the indicator pneumococcal strain, and the degree of inhibition was determined by colony count. For comparison with the producer strain, a control bacteriocin-negative, nonhemolytic strain of *S. faecalis* was selected because of its inability to inhibit the indicator strain. Producer, indicator, and control strains were grown individually for 18 hr in Trypticase Soy Broth (TSB; Difco) supplemented with yeast extract and dextrose (TSBY-D), after which the cells were harvested by centrifugation and then washed three times with sterile saline. These suspensions were adjusted to an optical density of 0.054 by using a Lumitron colorimeter operated at a wavelength of 580 nm. Bacterial suspensions at this density were equivalent to approximately 10⁶ cells/ml. A 1-ml amount of either producer or control organism was pipetted into each of seven tubes containing 10 ml of TSBY-D prepared in 0.15 M phosphate buffer (pH 7.2). Next, 1 ml of the pneumococcal indicator strain was added. Growth controls were prepared for each organism. All tubes were then incubated at 37°C. At hourly intervals for a 6-hr period and at the end of 24 hr, one tube was removed from each series, the pH was recorded, and viable counts were performed by plating serially diluted samples onto blood-agar. The plates were incubated at 37°C for 24 hr of incubation at 37°C for inhibition of the indicator strain. Counts of the indicator strain in the presence of the producer strain of *S. zymogenes* were compared to those of the indicator strain in the presence of the bacteriocin-negative strain of *S. faecalis*.

**Diffusibility.** For the agar overlay method (7), five plates of the producer organism were prepared with Trypticase Soy Agar (TSA) and incubated overnight at 37°C. After 24 hr of growth, the plates were covered with a thin layer of sterile TSA, maintained for 1 hr at 22°C to permit diffusion of the bacteriocin, and then streaked with the indicator strain. After an additional 24 hr of incubation at 37°C, the plates were examined for evidence of inhibition. Control plates which consisted of inoculating TSA with the bacteriocin-negative strain were employed for comparison.

Diffusibility of the enterococci was also investigated by using the method of Trust (15). A sterile membrane (0.45 µm; Millipore Corp., Bedford, Mass.) was placed onto the surface of blood- and chocolate-agar plates and overlaid with the producer organism in an agar suspension. After incubation for 24 and 48 hr, the membranes were removed, and the cell-free underlying agar surface was flooded with an indicator strain. The plates were then observed for inhibition after a subsequent 24 hr of incubation. Sterilized cellulose dialyzer tubing similarly treated was also used. Controls consisted of membranes layered with a bacteriocin-negative strain.

Susceptibility of the E-1 enterococci to chloroform, heat, and trypsin was determined by the methods of Datta and Prescott (5) and Brock (2), respectively.

**RESULTS**

As seen in Table 1 and Fig. 1, all 106 pneumococcal strains tested were susceptible to the bacteriocin of *S. zymogenes* strain E-1, displaying discrete and maximal zones of inhibition (approximately 14 mm). An inhibitory effect of the E-1 bacteriocin was also observed against the nonhemolytic enterococcal indicator strains tested and in 33% of streptococcal strains of the Viridans group, but the degree of inhibition of both these groups was significantly reduced (approximately 10 mm). No inhibitory activity was evident against the following bacterial species: beta-hemolytic streptococci of various groups, nonhemolytic streptococci, beta-hemolytic enterococci (*S. zymogenes*), staphylococci, other gram-positive bacteria, and numerous gram-negative species of different genera.

The inhibitory substance diffused through a thin sterile agar layer, eliciting its inhibitory effect upon the pneumococcal strains tested by the agar-overlay technique. In addition, the enterococci diffused freely through membranes (Millipore Corp.). Large zones of inhibition were observed when the membranes were removed and the cell-free underlying agar was tested with the indicator pneumococcal strain (Fig. 2). Similar results were obtained by using cellulose dialyzer tubing. No sensitive organisms
TABLE 1. Spectrum of bactericidal activity of E-1 enterococcin against various microbial species

| Species                  | No. of strains tested | No. of susceptible strains | Approximate inhibition zone (mm) |
|--------------------------|-----------------------|-----------------------------|----------------------------------|
| Sensitive strains        |                       |                             |                                  |
| Pneumococci              | 106                   | 106                         | 14                               |
| Nonhemolytic enterococci | 35                    | 35                          | 10                               |
| Viridans streptococci    | 39                    | 13                          | 10                               |
| Resistant strains        |                       |                             |                                  |
| Streptococcus zymogenes  | 79                    | 0                           | 0                                |
| Beta-hemolytic streptococci | 113               | 0                           | 0                                |
| Staphylococcus aureus    | 121                   | 0                           | 0                                |
| S. epidermidis            | 16                    | 0                           | 0                                |
| Bacillus cereus           | 3                     | 0                           | 0                                |
| Listeria                 | 3                     | 0                           | 0                                |
| monocytogenes            | 1                     | 0                           | 0                                |
| Erysipelothrix insidiosa | 43                    | 0                           | 0                                |
| Haemophilus influenzae   | 3                     | 0                           | 0                                |
| Neisseria gonorrhoeae    | 32                    | 0                           | 0                                |
| Escherichia coli          | 5                     | 0                           | 0                                |
| Herellea spp             | 22                    | 0                           | 0                                |
| Klebsiella spp           | 20                    | 0                           | 0                                |
| Proteus spp              | 43                    | 0                           | 0                                |
| Pseudomonas spp          | 8                     | 0                           | 0                                |
| Salmonella spp           | 8                     | 0                           | 0                                |

could be recovered from subcultures made from inhibition zones, and no inhibition was evident with the control bacteriocin-negative strain treated as above.

A bactericidal effect upon the growth of the pneumococcal indicator strain was demonstrated by the associative culture technique. No viable organisms could be recovered from samples subcultured as early as 1 hr postincubation with the E-1 producer strain. By contrast, many colonies of the indicator strain developed at hourly subcultures and after 24 hr in the case of associative growth with the bacteriocin-negative control strain (Fig. 3). This could not be attributed to a pH effect, since the pH of the test and control cultures dropped equally from pH 7.2 initially to 5.8 at the end of 24 hr.

The bactericidal substance derived from the producer strain was inactivated by 20 min of exposure to 80°C but not by trypsin. Figure 4 shows that chloroform exerted no effect upon the bactericidal activity of the enterococcin. A large uniform zone of growth inhibition of the indicator strain was observed surrounding the central well containing the 24-hr growth of the producer organism previously exposed to chloroform vapor for 1 hr. A smaller zone was observed when a sensitive enterococcus strain was utilized as an indicator. No zone was observed when the central well contained a bacteriocin-negative organism.

The bactericidal activity, when tested by the lawn-spotting technique, was evident at 4, 22, and 37°C as well as under 10%; CO₂ and anaerobic cultural conditions.

**FIG. 1.** Effect of E-1 bacteriocin on various gram-positive species by the lawn-spotting technique on 5% sheep blood-agar. (Upper center) Pneumococcus showing a maximal and discrete zone of inhibition. (Middle right) Nonhemolytic enterococcus showing a smaller zone of inhibition. (Bottom center and middle left) Resistant strains of Viridans and hemolytic streptococci.

**FIG. 2.** Inhibition zone of pneumococcal indicator strain after diffusion of E-1 bacteriocin through a bacterial membrane filter on chocolate-agar.
The latter molecular or observed the productizing strain having lesser zones negative species streptococci bacteriocin that control with Diplococcus (left) and against pneumococci (right). Indicator strain is completely inhibited by E-1 producer.

The bacteriocin, however, differs from the type 1 bacteriocin by virtue of its chloroform resistance and by having a narrower range of activity. The type 2, 3, and 4 bacteriocins of Brock et al. differ from E-1 in that they are produced by other enterococcal species (S. faecalis, S. liquefaciens, S. faecium). The type 5 bacteriocin produced by S. zymogenes strain X-74 of Brock et al. resembles E-1 in its reaction to treatment with chloroform, heat, and trypsin. It differs from E-1, however, by displaying activity only against enterococci including other S. zymogenes strains and by possessing two components, one chloroform-resistant and the other chloroform-sensitive. The E-1 bacteriocin, on the other hand, remains active after chloroform treatment and probably consists of a single bacteriocin that is chloroform-resistant and without activity against other S. zymogenes strains (Table 2). However, as the overall spectrum of E-1 bactericidal activity was not compared against the same microbial species surveyed by Brock and co-workers and as these investigators did not include pneumococci among their indicator strains, any definitive
conclusion with regard to E-1 as a new group D bacteriocin must await comparative studies.

The continued production of bacteriocin by *S. faecalis var. zymogenes* E-1 after innumerable subcultures indicates that this activity is a constant feature of this organism. Efforts at isolation and purification of E-1 have thus far proven unsuccessful. Lachowicz (12), working with staphylococci, and Hertman and Ben-Gurion (10), working with pesticin, encountered similar purification difficulties. Gagliano and Hinsdill (8) could not isolate staphylococcin from supernatant fluids of old broth cultures, and they attributed this failure to the possibility that the bacteriocin produced was quickly inactivated. They were able, however, to isolate the active substance by fragmenting the cells.

We have shown that the active principle of E-1 is separable from the cells which produce it by passage through membrane filters superimposed on agar substrates. Current efforts include attempts to isolate the bacteriocin from agar after diffusion. In addition, isolation of the active substance from disrupted cells will also be essayed.

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**TABLE 2. Comparative properties of bacteriocins synthesized by Streptococcus faecalis var. zymogenes**

| Properties                  | E-1             | Type 1a          | Type 5 (X-74)a   |
|-----------------------------|-----------------|------------------|------------------|
| Chloroform                  | Resistant       | Inactivated      | Resistant        |
| Heat                        | Inactivated     | Resistant        | Inactivated      |
| Trypsin                     | Positive        | Resistant        | Resistant        |
| Hemolytic Action against S. | Pneumococci, Viridans streptococci, nonhemolytic enterococci | Positive        | Resistant        |
| zymogenes                   |                 | Resistive        | Sensitive        |
| Gram-positive spectrum      |                 |                  | Limited to enterococci b |

a According to Brock et al. (2).
b Pneumococci not tested.