PROBABLE IDENTITY OF A GROUP D HEMOLYSIN WITH A BACTERIOCINE

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ABSTRACT

Brock, Thomas D. (Indiana University, Bloomington) and Joseph M. Davie. Probable identity of a group D hemolysin with a bacteriocine. J. Bacteriol. 86:708–712. 1963.—All strains of Streptococcus zymogenes (S. faecalis var. zymogenes) produce a bacteriocine which is active against lactic acid bacteria and most other gram-positive bacteria. Mutants which have lost the hemolytic characteristic lose at the same time their bacteriocine-producing ability. A strain which was resistant to the bacteriocine but which was nonhemolytic and nonbacteriocinogenic was irradiated, and two hemolytic mutants were isolated from it. These mutants were also bacteriocinogenic. Thus, the two activities are gained or lost together by mutation. Both activities are destroyed by chloroform vapors and are antagonized by lecithin. Both activities are destroyed at the same rate by treatment at 45°C under mildly acid conditions, and both activities are stable when heated in agar. The two activities are produced in parallel during the growth cycle, and disappear in parallel. The possible ecological role of a substance which is both a hemolysin and a bacteriocine is discussed.

The accompanying paper (Brock, Peacher, and Pierson, 1963) presents a survey of bacteriocine production in the enterococci. It was noted that all Streptococcus zymogenes (S. faecalis var. zymogenes) strains produced a characteristic bacteriocine. Since these strains also produced a hemolysin, it was natural to inquire as to whether the production of these two substances in the same strains was in any way related. The present paper presents physiological and genetic data which indicate that these activities are probably two aspects of a single substance. This leads to a consideration of the ecological advantage an organism might have by producing a single substance which is both a toxin and a bacteriocine.

Previous observations on antibiotic production by hemolytic enterococci were made by Stark (1960) and Sherwood et al. (1949). In neither of these reports was any comment made on the possible relationship between hemolysin and bacteriocine.

MATERIALS AND METHODS

Organisms used. The strains used and their sources are given in Table 1. Other strains that were used occasionally to establish the generality of the observations are listed in Table 1 of the accompanying paper. The characteristics defining the names of the various strains are given in Table 2 of the accompanying paper. Note that strain X54 was received as a hemolytic strain but was nonhemolytic in our hands. This strain was useful in certain key experiments described below.

Streptomycin-resistant mutants were isolated on plates containing 1000 µg/ml of antibiotic, and were used in certain experiments. The isolation of other mutants is described in Results.

Todd-Hewitt broth and agar (Difco) were used exclusively.

Phage and phage assay. A bacteriophage which attacks strains X14 and X54, designated here as P1, was obtained from H. D. Slade. This phage was propagated routinely in strain X14, and lysates assaying 10^9 to 10^10 plaque-forming units per ml were stored under chloroform. Phage assay was by the soft agar method, as described earlier (Brock et al., 1963).

Hemolysin assay. Because of certain peculiarities of this hemolytic system, it was not possible to assay hemolysin by measuring the amount of hemoglobin released from red cells. The alternative method of measuring the highest dilution of hemolysin that gave detectable hemolysis was not sufficiently quantitative for the present purposes. It was found, however, that a satisfactory assay could be obtained by
measuring the reduction in light scattering (turbidity) which took place when the red cells lysed. This method was simple and reproducible, and also gave a measure of the time course of lysis.

In preliminary work, the red blood cells were suspended in a saline-phosphate buffer at pH 6.8. When it was discovered that the hemolysin was more stable in the presence of calcium ions and ethylenediaminetetraacetate (EDTA), a buffer of the following composition was used: malonic acid \( (5 \times 10^{-2} \text{ M}) \), NaCl \( (0.15 \text{ M}) \), CaCl\(_2\) \( (10^{-2} \text{ M}) \), and EDTA \( (10^{-4} \text{ M}) \), at pH 6.9. The complete assay was as follows: (i) 1 ml of hemolysin-containing supernatant was placed in a screw-capped test tube and kept at 0 C, (ii) 4 ml of a solution of erythrocytes and saline-malate buffer were added, (iii) the solution was incubated in a water bath at 37 C, (iv) optical-density readings were taken periodically at 640 m\(\mu\) using as a blank a parallel sample in which the red cells had been lysed by about 0.01 g of saponin. The erythrocytes were prepared by diluting 1 ml of sterile, defibrinated horse blood (Colorado Serum Co., Denver, Colo.) in 4 ml of saline-malate buffer, centrifuging at 2000 rev/min, and resuspending the packed erythrocytes in 25 ml of buffer. A sufficient amount of this stock was added to the test solution to give a final optical density of 0.800 (usually a 1:10 dilution).

By use of this assay, the process of hemolysis showed two distinct phases: (i) a lag period during which no loss in optical density occurred, and (ii) a lysis period when the optical density decreased and approached zero. The lysis period generally took about the same length of time even with different concentrations of hemolysin, but the length of the lag period was directly proportional to the dilution of the hemolysin. When length of time for 50% lysis was plotted versus the dilution of the hemolysin, a straight line was formed. One unit is defined as that quantity of hemolysin which gives a 50% decrease in turbidity in 120 min at 37 C.

**Bacteriocine assay.** To assay quantitatively the amount of bacteriocine in a culture supernatant, strain X13 S (streptomycin-resistant) was used. A culture of X13 S in the late exponential phase of growth was diluted to about 5000 viable units per ml, and was mixed in equal parts with bacteriocine-containing supernatant or a broth dilution thereof. The mixture was incubated at 37 C for 30 min, and then 0.1-ml amounts were spread on agar plates containing 1000 \( \mu g/ml \) of streptomycin. After overnight incubation, the number of colonies was counted, and the per cent kill was calculated from a control of cells and bacteriocine plated directly without incubation. The use of streptomycin was necessary, because the bacteriocine was inactivated by chloroform or filtration, other procedures which might have been used to remove the bacteriocine-producing bacteria.

A dose-response curve obtained when per cent kill was plotted versus bacteriocine dilution on log probability graph paper gave a straight line, and a unit of activity could be defined which was equivalent to the hemolysin unit defined above.

**Qualitative tests for hemolysin and bacteriocine.** To determine whether a strain produced hemolysin, it was streaked on horse blood agar, or a loopful of broth culture was inoculated onto a plain agar plate, incubated overnight, and then overlaid with 2 ml of soft agar containing 5% horse blood; then the plate was incubated for an additional 5 to 6 hr, after which zones of hemolysis were read.

The qualitative bacteriocine assay was done by the method described in the accompanying paper (Brock et al., 1963).

In both the hemolysin and bacteriocine assays, some plates were treated with chloroform vapors.

**Table 1. Streptococcus strains used in this work**

<table>
<thead>
<tr>
<th>Code</th>
<th>Name</th>
<th>Source</th>
<th>Characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td>X13</td>
<td><em>S. faecium</em></td>
<td>I. U. stock</td>
<td>Indicator for bacteriocine</td>
</tr>
<tr>
<td>X14</td>
<td><em>S. zymogenes</em></td>
<td>I. U. stock</td>
<td>Bacteriocine producer</td>
</tr>
<tr>
<td>X22</td>
<td><em>S. zymogenes</em></td>
<td>NCTC 8176</td>
<td>Bacteriocine producer</td>
</tr>
<tr>
<td>X46</td>
<td><em>S. faecalis</em> 10 C 1</td>
<td>ATCC 11700</td>
<td>Indicator for bacteriocine</td>
</tr>
<tr>
<td>X54</td>
<td><em>S. zymogenes</em> 26 C 1</td>
<td>American Meat Institute</td>
<td>Nonhemolytic, resistant to bacteriocine</td>
</tr>
</tbody>
</table>

* *S. faecalis var. zymogenes.*
or heat before overlay to see whether the two activities were destroyed.

Results

Association of hemolysin and bacteriocine in different strains. As shown in the accompanying paper, all S. zymogenes strains produce a bacteriocine. The S. zymogenes bacteriocine is active against all other enterococci, as well as other lactic acid bacteria and most gram-positive bacteria. All S. zymogenes strains are resistant to the bacteriocine, including strain X54, which seems to have lost its ability to produce a hemolysin (but see below).

Genetic association of hemolysin and bacteriocine. If the two activities are associated, it might be expected that they would both be lost or gained together by mutation. However, to be certain that a mutant isolated is really derived from the parent, and is not a contaminant, it is essential that the parent be marked in other ways.

Strain X14 is hemolytic, bacteriocine-positive, streptomycin-sensitive, P1-sensitive, sorbitol-positive, and sensitive to X74 bacteriocine (hem+, bact+, SM*, P1*, sorb+, bactx74*). A streptomycin-resistant mutant was isolated which was also sorbitol-negative (not all SMR mutants are sorb−, but the one used here was), and this mutant was still hemolytic, bacteriocinogenic, and sensitive to X74 bacteriocine (hem+, bact+, SM*, P1+, sorb−, bactx74*). This mutant was then used to isolate a nonhemolytic mutant. This was done by a double ultraviolet irradiation procedure, the cells being irradiated to about 1% survival, grown up, irradiated again to 1% survival, and again grown up. These latter cells were then diluted, and samples were spread on 50 horse blood-streptomycin agar plates to obtain several hundred colonies per plate. About 16 nonhemolytic colonies were obtained, of which six were isolated and streaked to purify. Of these, three colonies remained nonhemolytic after repeated streaking. These were tested and found to be still P1-sensitive, sorbitol-negative, and sensitive to X74 bacteriocine. All three lacked ability to produce a bacteriocine.

Thus, these mutants, while retaining all other characteristics of the parent, lost both the hemolytic characteristic and the bacteriocine characteristic at the same time. It is unlikely, considering the rare occurrence of these mutants, that the changes were due to two separate mutational events.

The inverse experiment was performed with strain X54. This strain was neither hemolytic nor bacteriocinogenic, but was resistant to X14 bacteriocine; it was hypothesized that this strain, which had originally been hemolytic, had lost this characteristic during laboratory cultivation. Strain X54 did show extremely weak hemolysin and bacteriocine activities which were detectable occasionally, but these activities were too weak to be confused with those of the mutant obtained.

To avoid confusion from contaminants, a mutant was isolated which was streptomycin-resistant and P1-resistant. (Note that the X14 strain used was P1 sensitive.) The characteristics of this mutant were, therefore, hem−, bactx74*, bact−, SM*, P1*, sorb+. By the double-irradiation procedure, two hemolytic strains were isolated which retained all of the other characteristics of the parent. These two strains also produced a chloroform-sensitive bacteriocine typical of X14. Thus, by mutation X54 was able to gain at the same time both hemolysin and bacteriocine activities.

The above two experiments provide fairly firm genetic evidence of the identity of the two activities. In the absence of genetic recombination experiments, there would still be some lingering doubt only in the minds of the most critical individuals.

Stability. The bacteriocine activity of all S. zymogenes strains is destroyed when plates containing the substance are briefly exposed to chloroform vapors (see accompanying paper); the hemolytic activity is destroyed by the same chloroform treatment. The hemolytic activity of a culture supernatant is also destroyed by chloroform. In addition to the indication this observation gives of the identity of the two activities, it also suggests that the activity has a lipid component.

If lecithin (1 mg/ml) is incorporated into the agar used to test either bacteriocine or hemolytic activity, both activities are almost completely inhibited.

The hemolysin and bacteriocine activities were inactivated by heat; this inactivation was least rapid at pH 7.5 and more rapid below and above this value. The rate of inactivation of both activities is the same (see Fig. 1). When the hemolysin or bacteriocine is produced by bac-
bacteria growing on agar, both activities are stabilized and can withstand heating at higher temperatures, although certain other bacteriocines are inactivated under these conditions (see accompanying paper).

Thus, under all conditions that have been tested, both bacteriocine and hemolysin exhibit the same stability.

**Time course of production.** Samples of X14 culture supernatant were assayed simultaneously for hemolysin and bacteriocine at various stages of the growth curve. Both activities increase in parallel and decrease in parallel (Fig. 2). The decrease in the latter part of the growth cycle is probably due to the instability of the activities at the incubation temperature of 37 C at the point where the pH has fallen below 7.5.

**FIG. 1. Heat inactivation at 37 C.**

**FIG. 2. Time course of production of hemolysin and bacteriocine by strain X14.**

**DISCUSSION**

In this paper, genetic and physiological evidence has been advanced for the identity of a group D hemolysin with a bacteriocine. Although we feel the point has been adequately proved, a final case cannot be made until purification of the substance has been achieved. To date, low yields and instability have prevented any extensive attempts at purification.

The hemolysin in question differs in a number of ways from streptolysin S, and we have shown that purified streptolysin S (a gift of A. Bernheimer) has no bacteriocine activity. Certain group A streptococci do produce a bacteriocine with characteristics similar to the one discussed here (spectrum, chloroform sensitivity), but there is no evidence that this bacteriocine is an additional hemolysin in these strains. Interestingly, *S. pyogenes* strain C 203 S, which produces streptolysin S, shows a bacteriocine activity, whereas strain C 203 U, which does not produce streptolysin S, does not produce a bacteriocine. However, strain Blackmore produces streptolysin S but does not produce a bacteriocine. [See Bernheimer (1954) for the characterization of these strains.] Thus, it is seen that there is no correlation between streptolysin S and bacteriocine production, but that certain group A streptococci may produce small amounts of a bacteriocine which is like the group D bacteriocine. Further work on group A streptococci will be needed to clarify these relationships.

If we assume that the bacteriocine and hemolysin of *S. zymogenes* are two activities of the same substance, we might consider the possible role of such a material. Since the bacteriocine is active against a variety of gram-positive bacteria (see accompanying paper), it may play a role in the invasion by *S. zymogenes* strains into contaminated areas of the body, such as the throat, vagina, and intestine, where an invading organism would have to compete with already established bacteria for the available nutrients. If the bacteriocine is also a hemolysin, it would mean that the organism could use one and the same substance to combat resident bacteria as well as host defenses.

Preliminary experiments in vitro in mixed culture reveal that strain X14 is able to replace a bacteriocine-sensitive strain, even when the latter is present at 100 times the former (Pierson...
It remains to obtain similar data in the more complicated in vivo environment.

If the bacteriocine and hemolysin activities are two aspects of the same molecule, it is possible that the active site on this molecule is identical for both bacteria and red cells, and that these two diverse types of cells have some common receptor site. On the other hand, the active site for bacteria may be on a separate portion of the molecule from the active site for red cells, and the correspondence in the two activities may be merely accidental. Detailed genetic and biochemical studies will have to be done to distinguish between these two alternatives.

Earlier studies on a group D hemolysin were performed by Irwin and Seeley (1958) but, since the strain they used is no longer available (Seeley, personal communication), we cannot compare our results with theirs.

*S. durans* strains, which are hemolytic but which differ in many other ways from *S. zymogenes*, have not been shown to produce a bacteriocine. On this basis, we hypothesize that the *S. durans* hemolysin differs from the *S. zymogenes* substance.

### Acknowledgments

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### Literature Cited


and Brock, *unpublished data*.)