Production of Hemolysin and Bacteriolysin in a Synthetic Medium by *Streptococcus faecalis* var. *zymogenes*

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A synthetic medium has been developed in which *Streptococcus faecalis* var. *zymogenes* X-14 elaborates a lysin. The medium consists of 18 amino acids, lactose, six vitamins, adenine, guanine, uracil, and six salts. Glucose, and K$_2$HPO$_4$ in excess of 0.5% (wt/vol), were inhibitory to lyasin production; increasing concentrations of L-arginine-hydrochloride gave increasing yields of lytic activity.

Brock et al. (4), in a survey of the bacteriocins of the enterococci, described a bacteriocin from *Streptococcus faecalis* var. *zymogenes* X-14 that possessed hemolytic and bacteriolytic activities. Additional work by Davie and Brock (3, 6, 7) provided evidence favoring the notion that a single entity was responsible for both hemolytic and bacteriolytic activity. Using mutants of *S. faecalis* var. *zymogenes* X-14, Granato and Jackson (10) were able to demonstrate that the hemolysin exists as a bicomponent system, consisting of an A (activator) and an L (lytic) component. Upon purification from the respective mutant cultures, both components were shown to be acidic proteins, the A protein having a molecular weight of 27,000, and the molecular weight of the L component determined to be 11,000 (11, 12).

All reports concerning hemolysin production by the group D streptococci have dealt with preparations obtained from cells grown in complex media. For example, a serum-beef infusion broth was used by Plummer (18), whereas Irwin and Seeley (17) found that a polynucleotide from yeast autolysate could stimulate the activity of hemolysin when this polynucleotide was used to supplement their neopeptide-beef infusion medium. The studies reported by Brock and his co-workers and Jackson’s group have also dealt with hemolysin produced in other complex media: Todd-Hewitt and brain heart infusion (BHI) broths, respectively. Synthetic media are available for the growth of hemolytic enterococci (22), but no reports are extant with regard to their utilization for lyasin biosynthesis. This is to be contrasted with biosynthesis of enterococcal extracellular protease, which has been shown to be elaborated in a completely synthetic medium (15, 19). It was of interest to us, therefore, to determine if a synthetic medium could be developed for lyasin production and to compare these nutritional requirements with those of protease production. In any event, the definition of a synthetic medium for lyasin production might provide the conditions necessary to facilitate further experiments on the regulation of lyasin biosynthesis.

(Material included in this paper was taken from a thesis by B. A. submitted to the graduate School, The Pennsylvania State University, in partial fulfillment of the requirements for the degree of Master of Science.)

**MATERIALS AND METHODS**

**Bacterial strains.** *S. faecalis* var. *zymogenes* X-14, a hemolytic streptococcus, was obtained from R. W. Jackson, Syracuse University, and was the principal source of lyasin. Partially lytic and nonlytic mutants of this organism (*S. faecalis* var. *zymogenes* X-14/A18, X-14/NGBL2, X-14/132) also were obtained from Dr. Jackson. A preliminary screening to obtain an indicator organism showed that any one of a number of gram-positive organisms (including members of genera such as *Bacillus*, *Staphylococcus*, *Sarcina*, and *Lactobacillus* and many species of streptococci) were susceptible to the lyasin. (The lyasin was ineffectual on a number of gram-negative organisms tested, such as *Escherichia coli* B, *Pseudomonas aeruginosa*, *Proteus vulgarus*, and *Enterobacter aerogenes*.) *S. faecalis* var. *liquefaciens* 3, a non-proteolytic mutant derived by ultraviolet irradiation of *S. faecalis* var. *liquefaciens* 31, ultimately was chosen as the indicator organism for lyasin assay because of its high susceptibility to lyasin.

**Media.** The complex growth medium used throughout this study was brain heart infusion (Difco). Additional media were developed from an N-Z Case synthetic medium (2) and will be described in the next section.

**Temperature.** All experiments were carried out at 37°C unless otherwise stated.

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Production of crude lysin. One-tenth milliliter of an 8-h litmus milk stock culture of *S. faecalis* var. *zymogenes* X-14 was inoculated into 10 ml of test medium and incubated for 18 h. Ten milliliters of this culture was used to inoculate 90 ml of medium contained in a 500-ml Nephelo culture flask (Becton). The cells were grown to a turbidity corresponding to 90 Klett units (as measured with a no. 54 filter on a Klett-Summerson colorimeter), put on ice for 10 min to stop further growth, and then centrifuged at 4 °C for 20 min at 20,000 × g. The crude lysin in the supernatant fluid was decanted and kept on ice until assayed.

Preparation of indicator cells and bacteriolytic assay. Ten milliliters of an 18-h culture of the indicator organism, *S. faecalis* var. *liquefaciens* 3, was inoculated into a 500-ml flask containing 90 ml of BHI. Growth was terminated at mid-log phase (90 Klett units) by plunging the flask into an ice bath for 10 min and then centrifuging its contents. The supernatant fluid was decanted, and the cells were washed twice and resuspended in PBS-A buffer (0.01 M phosphate in 0.147 M NaCl, pH 6.8) to an optical density (OD) of about 0.3 at 540 nm as measured on the Evelyn photoelectric colorimeter. Crude lysin was added to a final concentration of 20% (vol/vol), the tubes were incubated at 37 °C, and loss of turbidity was measured (1). Controls consisted of a preparation of indicator cells suspended in PBS-A without lysin. After 3 h of incubation, the change in OD (ΔOD) was determined for each tube, and the ΔOD of the control tube was subtracted from the experimental value. The percent reduction in OD was calculated on the following basis: (ΔOD/ODt0) × 100 = % reduction in OD, where ODt0 = optical density at zero time.

Erythrocytes. Rabbit erythrocytes were purchased as 10% suspensions of washed, pooled red cells from BBL (BBL, Division of BioQuest, Cockeysville, Md.), refrigerated at 4 °C, and used within 1 week of receipt. Prior to their use in hemolytic assays, erythrocytes were washed three times in PBS-A buffer by centrifugation (1,500 × g) for 5 min. A 0.12% stock suspension was made in buffer and kept on ice until used.

Hemolytic assay. Hemolytic activity was measured (1) by the release of hemoglobin from a suspension of 0.08% rabbit erythrocytes in PBS-A containing 20% lysin (vol/vol). Samples (3.5 ml) were removed by centrifugation for 3.5 min, and the supernatant fluid was withdrawn with a Pasteur pipette and read for hemoglobin in the Beckman DU-2 spectrophotometer at 541 nm. Controls consisted of a suspension of 0.08% erythrocytes in buffer containing no lysin. After 2 h of incubation, the extent of lysis was expressed as the percentage of hemolysis compared with a control of 0.08% erythrocytes lysed in double distilled water. The control was arbitrarily called 100% hemolysis; on occasion this control value was exceeded by the experimental value, indicating that total lysis of red blood cells is not obtained by distilled water treatment.

RESULTS

Development of a synthetic medium. Previous experience in our laboratory had shown that it was possible for *S. faecalis* var. *liquefaciens* strain 31 to biosynthesize an extracellular protease when it was grown on a synthetic medium (15, 19). A less expensive, yet excellent, medium for preliminary nutritional experiments substitutes Sheffield's N-Z Case (2) for amino acids as a source of nitrogen. This latter medium, though highly conducive to extracellular protease biosynthesis, yielded negligible bacteriolysin (16% reduction in OD) compared with BHI (52% reduction in OD) when *S. faecalis* var. *zymogenes* X-14 was grown in it. By lowering the K2HPO4 concentration from 1.47 to 0.25% in the N-Z Case synthetic medium, bacteriolytic activity was increased to 44% reduction in OD or almost threefold. Further experiments relied on this effective phosphate concentration.

In an attempt to determine if glucose might have a negative impact on lysin production, the effectiveness of this sugar was compared with that of two sugars (lactose or galactose) that give maximal yields of protease in a synthetic medium (19). Table 1 shows that the results of such an experiment on lysin production are analogous to those obtained with protease.

Using the optimal phosphate concentration and 0.2% lactose, sources of amino acids were varied in the N-Z Case synthetic medium. Table 2 shows that acid-hydrolyzed casein plus tryptophan and arginine, as well as a mixture of 18 single amino acids, are able to sustain conditions for bacteriolysin biosynthesis. This situation is analogous to that of extracellular protease biosynthesis by *S. faecalis* var. *liquefaciens* (15, 19) where the enzyme is elicited in a completely synthetic medium. Also, as with the extracellular protease (5, 13, 14), the concentration of arginine appears to play a singular role (see media 2 and 3, Table 2).

If *S. faecalis* var. *zymogenes* X-14 is grown in a completely synthetic medium, in which 18 amino acids are used in place of the casein hydrolysate, the cells grow at a slower rate, and less lysin is obtained (medium 4, Table 2). However, Table 2 also shows (medium 5) that the lesser activity can be overcome by allowing the cells to incubate longer.

Time course of lysin production. To determine the time course of lysin production, a 10% inoculum was used to inoculate a flask of 90 ml of medium 3 (Table 2). Periodically, samples of 10 ml were withdrawn from the flask, the OD was determined, the samples were iced for 10 min and centrifuged, and the supernatant fluid was kept on ice until assayed.

The results (Fig. 1) indicate that lysin production reaches a peak when the culture has entered the mid-log phase and then declines.
TABLE 1. Effect of the carbohydrate source on bacteriolytic production

<table>
<thead>
<tr>
<th>Carbohydrate (% wt/vol)</th>
<th>% Reduction in OD</th>
<th>Final pH of assay mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (BHI medium)</td>
<td>62</td>
<td>6.65</td>
</tr>
<tr>
<td>Glucose, 0.20%</td>
<td>35</td>
<td>6.69</td>
</tr>
<tr>
<td>Lactose, 0.20%</td>
<td>53</td>
<td>6.81</td>
</tr>
<tr>
<td>Galactose, 0.20%</td>
<td>50</td>
<td>7.16</td>
</tr>
<tr>
<td>Glucose, 0.10%, and galactose, 0.10%</td>
<td>51</td>
<td>6.73</td>
</tr>
<tr>
<td>Galactose, 0.10% and lactose, 0.10%</td>
<td>50</td>
<td>6.84</td>
</tr>
<tr>
<td>Glucose, 0.10%, and lactose, 0.10%</td>
<td>50</td>
<td>6.66</td>
</tr>
</tbody>
</table>

* Cells were grown in N-Z Case synthetic medium containing 0.25% (wt/vol) K$_2$HPO$_4$, and varying sugar concentrations. Lysin was prepared and assayed as described in Materials and Methods.

FIG. 1. Time course of lysin production in medium 3, Table 2, by *S. faecalis* var. *zymogenes* X-14. Cells were grown in medium 3, and lysin was assayed against rabbit erythrocytes and indicator bacteria.

rapidly as the cells enter the stationary phase. This is in agreement with the data of Brock and Davie (3), whose data are reported for cells grown in Todd-Hewitt broth.

The similarity between the curves for lysin production in a nonsynthetic and a synthetic medium lends some credence to the notion that the lysins obtained from *S. zymogenes* X-14 under these two experimental growth conditions are identical. Further support for this unity was obtained from additional experiments.

Comparison of lysins from synthetic and nonsynthetic media. Reconstitution experi-

ments analogous to those of Granato and Jackson (10) were run with their mutant strains.

TABLE 2. Effect of amino acid source on bacteriolytic production

<table>
<thead>
<tr>
<th>Medium</th>
<th>Amino acid source</th>
<th>% Reduction in OD</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>N-Z Case synthetic medium*</td>
<td>60</td>
</tr>
<tr>
<td>2</td>
<td>0.2% Casein hydrolysate + 0.003% L-tryptophan + 0.193% L-arginine-hydrochloride</td>
<td>57</td>
</tr>
<tr>
<td>3</td>
<td>0.2% Casein hydrolysate + 0.003% L-tryptophan + 0.39% L-arginine-hydrochloride</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>18 Amino acids*</td>
<td>54</td>
</tr>
<tr>
<td>5</td>
<td>18 Amino acids*</td>
<td>62</td>
</tr>
</tbody>
</table>

* *S. faecalis* var. *zymogenes* X-14 was grown in the indicated medium, and the supernatant fluid was assayed for lysin.

The N-Z Case synthetic medium has been described previously (2) but was modified to contain 250 mg of K$_2$HPO$_4$. Constituents of this medium are: lactose, 200 mg; Sheffield N-Z Case, 200 mg; L-arginine-hydrochloride, 193 mg; NaCl, 200 mg; MgSO$_4$ - 7H$_2$O, 0.80 mg; FeSO$_4$ - 7H$_2$O, 0.40 mg; MnSO$_4$, H$_2$O, 0.16 mg; CoCl$_2$, 0.12 mg; CaCl$_2$, 1.50 mg; adenine, guanine, and uracil, 0.5 mg each; riboflavin, calcium pantothenate, pyridoxine-hydrochloride and niacin, 0.1 mg each; folic acid and biotin, 0.1 μg each. The pH was adjusted to 7.0, and the final volume was brought to 100 ml with double distilled water. For the other media tested (no. 2-5) the N-Z Case was omitted and replaced by acid-hydrolyzed casein (with the indicated supplements) (Nutrition Biochemicals) or by 18 amino acids, as described.

Concentrations of amino acids (as based on their percentage occurrence in 0.2% hydrolyzed casein, except for arginine, which is higher) are: L-alanine, 7.4 mg; L-arginine-hydrochloride, 405 mg; L-aspartic acid, 14.2 mg; L-cystine, 0.7 mg; L-glutamic acid, 46.6 mg; L-histidine-hydrochloride, 6.0 mg; L-isoleucine, 12.4 mg; L-leucine, 20.6 mg; L-lysine-2HCl, 16.8 mg; L-methionine, 6.8 mg; L-phenylalanine, 10 mg; L-proline, 21.2 mg; L-serine, 12.4 mg; L-threonine, 8.2 mg; L-tryptophan, 3.0 mg; L-tyrosine, 12.0 mg; L-valine, 13.6 mg; glycine, 4.0 mg. Lactose (200 mg), K$_2$HPO$_4$ (250 mg), and the salts, purines and pyrimidines, and vitamins were used at their concentrations in medium no. 1. The pH of the medium was adjusted to 7.0, and the final volume was brought to 100 ml with double distilled water.

In this experiment, cells were harvested at 120 Klett units, and the supernatant fluid was assayed for lytic activity.
Table 3, experiment 4, shows that an active bicomponent system is obtained with supernatant fluids from a synthetic medium just as from the nonsynthetic BHI. Neither the strain containing only activator (A) X-14/A18 nor the strain containing only the lytic component (L) X-14/NGBL2 had any effect (see experiments 2 and 3). Experiment 5 is the lysin-negative I mutant, and the negative data are similar for both media. Data analogous to that for bacteriolysin activity (as shown in Table 3) was also obtained from assays of hemolytic activity.

Additional evidence that demonstrates the similarity between lysin obtained from defined and nonsynthetic media is that the lysins are sensitive to the activity of trypsin, Pronase, and the extracellular protease of S. faecalis var. liquefaciens 31.

**DISCUSSION**

Previous studies on the hemolysin produced by S. faecalis var. zymogenes X-14 have all dealt with preparations obtained from cells grown in complex media. Referring to hemolysis by the lysin of S. faecalis var. zymogenes X-14, Werth and Jackson (21) state that BHI is an unknown manner, "...is slightly, but significantly, stimulatory to lytic activity." The stimulatory effects of peptone, deoxyribonucleic acid, and a component of yeast autolysate, presumed to be ribonucleic acid, were shown by Irwin and Seeley (17). Our report is believed to be the first of hemolysin production in a synthetic medium by a group D streptococcus.

If attempts had been made by other laboratories to develop a synthetic medium for hemolysin production, perhaps their lack of success may possibly be attributed to overlooking three important components: the sources of carbon and energy, the presence of an arginine supplement, and the phosphate buffer concentration. Previous experience in our own laboratory has indicated the critical nature of these components in any synthetic medium (14, 19).

In comparing nutritive requirements for extracellular protease production to extracellular hemolysin production, the only dissimilarity appears to be the inhibition of lysin production by concentrations of K₂HPO₄ in excess of 0.5% (wt/vol). All other attributes of the synthetic medium are strikingly similar. For example, lactose and galactose are both more effective sources of carbon for hemolysin than they are reported to be for protease (19). The negative effect of glucose on lysin production may stem from catabolite repression; Duncan and Cho (9) have reported this phenomenon with regard to glucose and staphylococcal alpha-toxin production. Alternatively, glucose may stimulate greater synthesis of a lysin inhibitor, which is also known to be synthesized by these organisms (3, 21). This lysin inhibitor has recently been characterized by Werth and Jackson (Abstr. Annu. Meet. Amer. Soc. Microbiol. 1973, P25, p. 145) as phosphatidylserine, an important constituent of cell membranes. Earlier work (6, 7) had demonstrated that the lysin itself was membrane specific. This is to be contrasted with the autolysin of S. faecalis described by Shockman et al. (20) whose substrate is cell wall peptidoglycan. These latter workers have reported that maximal autolysis is produced during the logarithmic growth phase (16). They have speculated also that the autolysin is functionally associated with the process of cell division through peptidoglycan cleavage (16). Since our bacteriolysin is also produced maximally during the exponential phase of growth, its cellular function may be membrane cleavage in a fashion analogous to the requirement of autolysin for division.

The beneficial effect of increased arginine concentrations for hemolysin production is in keeping with similar observations for enterococcal protease (14). We do not know yet if increased arginine is necessary as the sole source of adenosine 5'-triphosphate (8) to synthesize hemolysin, as has been reported for protease biosynthesis for S. faecalis var. liquefaciens (5); it is possible also to explain the increased lysin resulting from increased arginine in terms of the arginine repressing the synthesis of lysin inhibitor.

**Table 3. Comparison of bacteriolytic activity obtained from mutant strains grown on synthetic and nonsynthetic media**

<table>
<thead>
<tr>
<th>Expt no.</th>
<th>Strain of S. faecalis var. zymogenes</th>
<th>% Reduction in OD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Synthetic medium</td>
</tr>
<tr>
<td>1</td>
<td>X-14</td>
<td>71</td>
</tr>
<tr>
<td>2</td>
<td>X-14/A18</td>
<td>0</td>
</tr>
<tr>
<td>3</td>
<td>X-14/NGBL2</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>X-14/NGBL2 + X-14/A18</td>
<td>69</td>
</tr>
<tr>
<td>5</td>
<td>X-14/132</td>
<td>0</td>
</tr>
</tbody>
</table>

*Each strain was grown on the indicated medium, and the supernatant fluid was assayed for lysin. In experiment 4 the supernatant material of two cultures was used, employing a ratio of three parts X-14/A18 to 17 parts X-14/NGBL2. The total volumes of supernatant fluid were constant for all tubes as described in Materials and Methods.*
ACKNOWLEDGMENTS

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LITERATURE CITED