Production of Staphylococcal Enterotoxins A, B, and C Under Conditions of Controlled pH and Aeration

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The production of enterotoxins A, B, and C by nine strains of \textit{Staphylococcus aureus} has been studied under controlled conditions in a fermenter. The strain to strain differences between staphylococci producing a specific enterotoxin were very marked. Increasing aeration in shake flasks improved both growth and production of all extracellular proteins measured other than that of enterotoxin C, the yield of which was decreased in one strain at high aeration. Silicone antifoam decreased the production of extracellular proteins, although enterotoxin A production from three strains was much less affected than that of enterotoxins B and C. In a detailed study of three strains, production of enterotoxins A and C was considerably greater in a defined amino acid medium than in a casein hydrolysate medium and was optimal for all three enterotoxins between pH 6.5 and 7.3. Changes in the pH or medium used in the fermenter that led to increased enterotoxin production could generally be correlated with a change in growth pattern, showing an extended transition period from exponential to stationary phase. Three out of five enterotoxin-A producing strains produced significantly more enterotoxin at a controlled pH of 6.5 in the fermenter than in shake-flask cultures. The yields with strain 100 were about five times greater than hitherto reported. Since many foods are buffered at pH 6 to 6.5, some strains may, therefore, produce sufficient enterotoxin A to cause food poisoning, although little or none may be produced when grown under normal testing procedures.

There are at present many unexplained features about the production of enterotoxins by \textit{Staphylococcus aureus}. Food-poisoning incidents most commonly are caused by enterotoxins A and D, although these are produced in relatively small amounts when compared to enterotoxins B and C. Indeed, the low yields of enterotoxin A produced in various semisynthetic media, and hence its relative unavailability, have considerably impeded studies on its properties.

Although the effect of the initial pH of the medium on enterotoxin production has been studied (6, 7, 15), the effect of pH under controlled conditions has not been reported (2). This may be of special significance since most foods are naturally buffered. In the present investigation the production of enterotoxins A, B, and C in two different media was studied in a fermenter in which pH, gas flow, stirrer speed, temperature, and the level of dissolved oxygen in the medium were carefully controlled. Conditions were established which markedly increased the yield of enterotoxin A, particularly by strain 100.

Differences in the sites or mechanisms of synthesis of enterotoxins A and B have been postulated (14), but it is possible that such differences in the few strains investigated might be due rather to the characteristics of the particular strain under examination. For this reason, a number of different strains of enterotoxin-producing staphylococci were studied in this investigation, particular attention being given to two strains (S-6 and 22) which produce both enterotoxin B and a small amount of enterotoxin A. Total extracellular protein (TEP), lipase, lysozyme, and deoxyribonuclease were also assayed to determine whether the effect of different conditions on levels of en-
terotoxin production related specifically to that enterotoxin or was a more general effect on the production of other extracellular proteins.

MATERIALS AND METHODS

Staphylococcal strains. Strains 100, S-6, and 361 were supplied by M. S. Bergdoll, and 743 was supplied by R. W. Bennett. Strains 10, 22, and 30 are from the International Phage-typing set, and strains 3 and 37 are clinical strains. Enterotoxins A, B, and C and their corresponding antisera were kindly supplied by M. S. Bergdoll, and enterotoxin B antiserum was also purchased from Makor Chemical Co., Israel.

Media. The two media used were that of Hallander (8) consisting of casein hydrolysate (2% Difco, Casamino Acids, 2% BBL Trypticase) supplemented with vitamins and mineral salts (CH medium) and medium 4 (2.08%) as described by Wu and Bergdoll (17), which is a completely defined medium containing amino acids, vitamins, and mineral salts (AA medium).

Cultural conditions. The inoculum for the fermenter consisted of cells grown overnight on a New Brunswick gyratory shaker, washed, and resuspended, all of these operations being carried out in the medium to be used in the fermenter. Two liters of medium were maintained at 37°C in a 5-liter glass fermenter. The pH was automatically controlled by the addition of 1 N HCl or NaOH by Sigma pumps as required. The fermenter was aerated by means of a gas stream consisting of air and nitrogen maintained at a constant flow rate. The concentration of dissolved oxygen in the medium was controlled by intermittent additions of air into the nitrogen stream through a solenoid valve actuated by a Beckman oxygen sensor. Oxygen concentrations and pH were continuously recorded. Agitation was by a stirrer with 2-in (approximately 5.08 cm) impeller blades run at 1,000 rpm, unless otherwise specified. Samples were taken through a sampling port with a sterile syringe, and additions of sterilized silicone antifoam (Dow-Corning) were made as required through the same port. Growth was determined by measuring the optical density (OD) of an appropriate dilution on an SP 500 Unicam spectrophotometer which had been calibrated and shown to give a linear relationship between OD and the number of organisms from 0.0 to 0.7 OD units at 600 nm. OD readings were converted to whole numbers for ease of comparison. Samples were then centrifuged, and enterotoxin and other determinations were carried out on the supernatant extract. One hundred micrograms of Merthiolate (Eli Lilly Co.) per milliliter was added to the samples which were then stored at 4°C.

Determination of extracellular proteins. Enterotoxin assays were carried out on supernatant extracts by the single-tube gel-diffusion method (16) after appropriate dilutions in phosphate-buffered saline (pH 7.4). Under these conditions the pH of the medium did not affect the assay. Enterotoxin standards were dissolved in the appropriate medium for each test, the minimum level detectable being approximately 1 μg/ml. A new reference curve was prepared with each day's test by using enterotoxin values of 3 to 25 μg/ml. Samples to be tested were diluted with appropriate phosphate-buffered saline so that they fell within this range. Tubes were incubated for 20 h at 30°C. Deoxyribonuclease and lipase activities were determined by micro-slide gel-diffusion techniques. Relative values in samples were obtained by measuring the zones of activity against those produced by dilutions of known concentrations of a purified staphylococcal deoxyribonuclease and lipase (9, 11). Lysozyme and TEP determinations were carried out as described elsewhere (10).

RESULTS

Nine strains of S. aureus were used to study the effect of silicone antifoam and aeration on the production of enterotoxins A, B, and C in CH medium in shake-flasks. These nine strains were also used in a comparison of shake-grown and fermenter-grown cultures and, where specified in the results, were used to compare cultures with and without pH control. Three strains, 100, S-6, and 361, were then studied in detail in shake-flasks and in the fermenter at different pH levels in two different media. The effect of sodium chloride on the production of enterotoxins by these three strains was also examined.

In both AA and CH media (initial pH 6.5 and 7.3, respectively), the pH rose steadily during the growth of the cultures. For all strains tested, the yields of enterotoxins and TEP were almost invariably higher in shaker-grown than in fermenter-grown cultures (Table 1). Lipase, lysozyme, and deoxyribonuclease were also higher.

<table>
<thead>
<tr>
<th>Vessel and type</th>
<th>Strain and type</th>
<th>OD</th>
<th>Final pH</th>
<th>Enterotoxin (μg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Shake-flask</td>
<td>10 A</td>
<td>29</td>
<td>8.1</td>
<td>2.6</td>
<td>0.98</td>
</tr>
<tr>
<td>Fermenter</td>
<td>10 A</td>
<td>24</td>
<td>8.9</td>
<td>ND*</td>
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<td>Shake-flask</td>
<td>100 A</td>
<td>21</td>
<td>8.4</td>
<td>7.6</td>
<td>0.13</td>
</tr>
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<td>100 A</td>
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<td>9.3</td>
<td>8.2</td>
<td>0.04</td>
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<td>743 A</td>
<td>28</td>
<td>8.35</td>
<td>2.0</td>
<td>1.1</td>
</tr>
<tr>
<td>Fermenter</td>
<td>743 A</td>
<td>24.6</td>
<td>8.8</td>
<td>1.0</td>
<td>0.38</td>
</tr>
<tr>
<td>Shake-flask</td>
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<td>22</td>
<td>8.37</td>
<td>104</td>
<td>0.28</td>
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<tr>
<td>Fermenter</td>
<td>22 B</td>
<td>23</td>
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<td>66</td>
<td>0.17</td>
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<td>Shake-flask</td>
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<td>23.6</td>
<td>8.42</td>
<td>130</td>
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<td>8.95</td>
<td>52</td>
<td>0.15</td>
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<tr>
<td>Shake-flask</td>
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<td>16</td>
<td>8.15</td>
<td>290</td>
<td>2.96</td>
</tr>
<tr>
<td>Fermenter</td>
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<td>9.0</td>
<td>114</td>
<td>0.97</td>
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<td>Shake-flask</td>
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<tr>
<td>Fermenter</td>
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<td>22</td>
<td>8.9</td>
<td>11</td>
<td>0.27</td>
</tr>
<tr>
<td>Shake-flask</td>
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<td>8.32</td>
<td>86</td>
<td>0.22</td>
</tr>
<tr>
<td>Fermenter</td>
<td>37 C</td>
<td>23.6</td>
<td>8.87</td>
<td>3</td>
<td>0.71</td>
</tr>
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<td>Shake-flask</td>
<td>361 C</td>
<td>26</td>
<td>8.24</td>
<td>63</td>
<td>0.75</td>
</tr>
<tr>
<td>Fermenter</td>
<td>361 C</td>
<td>25</td>
<td>9.0</td>
<td>2.6</td>
<td>0.09</td>
</tr>
</tbody>
</table>

* ND indicates not detectable.
in shaker-grown than in fermenter-grown cultures. In shake-flasks the yields of these extracellular proteins were higher when CH rather than AA medium was used, with the exception of enterotoxin C production by strain 361. In the fermenter, however, yields of enterotoxins A and C from strains 100 and 361 were higher in AA medium than in CH medium.

In these experiments it was observed that increased yields of enterotoxins generally were associated with longer transition period from exponential to stationary phase and a significantly lower rate of increase in pH in the cultures during growth, as shown by S-6 (Fig. 1) and Table 1. Factors which could account for these differences between the shaker-grown and fermenter-grown cultures were therefore examined, particular attention being given to the effects of antifoam, aeration, sodium chloride, composition of growth medium, and pH. Each experiment was continued until growth and the production of enterotoxin and other extracellular proteins had reached their maximum values.

**Effect of antifoam.** In the fermenter, the amount of antifoam required depended both on the strain and the medium used. Strains such as S-6 and 361 required a total of 3 to 4 mg/ml in CH medium, whereas in the AA medium much smaller quantities (0.3 mg/ml) were necessary. Additions of antifoam caused an immediate decrease in oxygen in solution as recorded by the oxygen sensor, which was compensated for rapidly by increased additions of air. To determine the effect of antifoam on the production of enterotoxin and other extracellular proteins, shake-flasks were inoculated with overnight cultures of staphylococci, and additions of antifoam were made at times corresponding to additions on the fermenter (0, 4, 5, 7, and 10 h) to give final levels of 0, 1.5, and 5 mg of silicone antifoam per ml. Three strains producing enterotoxin A, three producing B, and three producing C were tested, and typical results with three of these nine strains are presented in Table 2. Growth was not affected by the additions of antifoam. The production of enterotoxins B and C was considerably reduced, but enterotoxin A production appeared to be less affected by the presence of antifoam, being unaffected by 1.5 mg/ml in the three strains.

![Fig. 1. Growth and enterotoxin B production by S. aureus strain S-6 in AA medium in shake-flask (closed symbols) and fermenter (open symbols) at uncontrolled pH. OD (Δ); enterotoxin B (Ο); and pH (□)](image)

**Table 2. Effect of silicone antifoam on the production of extracellular proteins in CH medium in shake-flasks after 24 h of incubation**

<table>
<thead>
<tr>
<th>Extracellular proteins*</th>
<th><strong>Strain 100</strong></th>
<th><strong>Strain S-6</strong></th>
<th><strong>Strain 361</strong></th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Antifoam added (mg/ml)</td>
<td>Antifoam added (mg/ml)</td>
<td>Antifoam added (mg/ml)</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>1.5</td>
<td>5.0</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>8.3</td>
<td>8.3</td>
<td>6.3</td>
</tr>
<tr>
<td>Lipase</td>
<td>260</td>
<td>170</td>
<td>100</td>
</tr>
<tr>
<td>Lysozyme</td>
<td>1.4</td>
<td>0.6</td>
<td>0.01</td>
</tr>
<tr>
<td>Deoxyribonuclease</td>
<td>112</td>
<td>108</td>
<td>51</td>
</tr>
<tr>
<td>TEP</td>
<td>0.04</td>
<td>0.03</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Enterotoxins are recorded as micrograms per milliliter. Lipase and deoxyribonuclease units were determined as in the text. Lysozyme is recorded in units equivalent to the activity of known dilutions of egg white lysozyme (micrograms per milliliter). TEP is total extracellular protein expressed in terms of bovine albumin (milligrams per milliliter).

* Strains 100, S-6, and 361 were tested for enterotoxins A, B, and C, respectively.
tested. Only one enterotoxin A-producing strain was affected by 5 mg/ml, and even then only 25% reduction in yield was obtained. With all nine strains, the addition of antifoam resulted in decreased production of the other extracellular proteins measured, i.e., lipase, lysozyme, deoxyribonuclease, and TEP.

**Effect of sodium chloride.** To maintain constant pH conditions during a fermenter experiment, HCl giving a final concentration of up to 0.1 M in the medium was required. Therefore, the effect on enterotoxin production was determined for equivalent concentrations of NaCl added to shake-flask cultures of strains 100, S-6, and 361. Additions of NaCl were made at intervals so that the molarity of NaCl in the shake-flasks was equal to the molarity of HCl in the fermenter at the same OD. This was in addition to 0.05 M NaCl present in the medium in Casamino Acids. NaCl reduced the production of enterotoxins A, B, and C by 7, 25, and 10%, respectively, and TEP by 0.29, and 12% in these three strains without affecting growth.

**Effect of aeration.** Preliminary experiments were carried out with CH medium in shake-flasks to determine the effect of increased aeration for 9 strains by using 1-1 flasks with and without side baffles. The former is said to increase oxygen absorption rate approximately threefold (5). To avoid excessive foaming in the baffled flasks it was necessary to reduce the shaker speed considerably. The exponential growth rate was almost doubled, and the final pH was higher in the more aerated flasks. Table 3 shows the effect of increased aeration on the level of enterotoxins and total extracellular protein produced during 24 h of incubation in CH medium, initial pH 6.5. For all but one of the nine strains, enterotoxin production was greater in the baffled flasks. The exception was enterotoxin C production by strain 361, which was reduced in the baffled flask. Similar experiments with initial pH 7.3 showed similar results for most strains. However, at the higher initial pH, S-6 produced considerably less enterotoxin B (196 μg/ml) even in the baffled flask than generally obtained in control flasks at higher shaker speeds. Also at initial pH 7.3, the decrease in enterotoxin C production by strain 361 in the baffled flask was more marked, being 28 μg/ml as compared with 67 μg/ml in the control flask. This was evidently not a characteristic of enterotoxin C production, as strains 3 and 37 showed an increase in growth and a corresponding increase in enterotoxin C production in baffled flasks. Nevertheless, considerable difficulty was experienced in obtaining high yields of enterotoxin C in the fermenter with any of these three strains. Whereas shake-flask yields

<table>
<thead>
<tr>
<th>Aeration</th>
<th>Strain and type</th>
<th>OD</th>
<th>Final pH</th>
<th>Enterotoxin (μg/ml)</th>
<th>TEP (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 A</td>
<td>20.4</td>
<td>8.08</td>
<td>2.05</td>
<td>0.48</td>
</tr>
<tr>
<td>Baffled</td>
<td>10 A</td>
<td>18.4</td>
<td>8.47</td>
<td>2.05</td>
<td>1.86</td>
</tr>
<tr>
<td>Control</td>
<td>100 A</td>
<td>19.2</td>
<td>8.28</td>
<td>6.5</td>
<td>0.09</td>
</tr>
<tr>
<td>Baffled</td>
<td>100 A</td>
<td>17.6</td>
<td>8.60</td>
<td>14</td>
<td>0.17</td>
</tr>
<tr>
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<td>16.0</td>
<td>8.09</td>
<td>NDa</td>
<td>0.44</td>
</tr>
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<td>8.68</td>
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</tr>
<tr>
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<td>8.03</td>
<td>85</td>
<td>0.41</td>
</tr>
<tr>
<td>Baffled</td>
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<td>16.4</td>
<td>8.64</td>
<td>90</td>
<td>1.13</td>
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<tr>
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<td>8.40</td>
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<td>1.91</td>
</tr>
<tr>
<td>Control</td>
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<td>7.92</td>
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<td>Baffled</td>
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<td>0.36</td>
</tr>
<tr>
<td>Baffled</td>
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<td>19.4</td>
<td>8.60</td>
<td>55</td>
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<tr>
<td>Control</td>
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<td>8.17</td>
<td>33</td>
<td>0.14</td>
</tr>
<tr>
<td>Baffled</td>
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<td>63</td>
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<tr>
<td>Control</td>
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<td>8.11</td>
<td>38</td>
<td>0.29</td>
</tr>
<tr>
<td>Baffled</td>
<td>361 C</td>
<td>23.2</td>
<td>8.60</td>
<td>28</td>
<td>2.8</td>
</tr>
</tbody>
</table>

*ND indicates not detectable.*
another enterotoxin C-producing strain, but with strain 3 both TEP and enterotoxin production were reduced in the fermenter.

**Effect of pH in CH medium.** The production of enterotoxins and other extracellular proteins by strains 100, S-6, and 361 was studied in the fermenter over a pH range of 6.0 to 8.0, by using CH medium, standardized additions of antifoam, air-N₂ flow of 2 liters per min, and a level of dissolved oxygen between 30 and 40 mm of Hg. Controlling pH between 6.0 and 7.3 caused a marked increase in enterotoxin A production by strain 100, with an optimal pH of 6.5 (Table 4). Strain S-6, which did not produce detectable quantities of enterotoxin A in the shake-flask or in the fermenter without pH control, produced 3 μg/ml in the fermenter at pH 6.5 (Table 4). Strains 10, 22, and 743 were then grown in the fermenter at pH 6.5 and uncontrolled pH. Strain 743 showed an increase in enterotoxin A production, but strain 10 did not show an increase in enterotoxin A production under controlled pH conditions. Similarly strain 22, which produced 2 to 3 μg of enterotoxin A per ml in the shake-flask, did not produce detectable enterotoxin A in the fermenter under any combination of aeration and pH. The effect of varying pH on enterotoxin A production was therefore a characteristic of the strain under examination.

Enterotoxin B production by strain S-6 was higher under conditions of controlled pH and showed an optimal pH of 6.5 to 7.3 (Table 4). Enterotoxin B production by strain 22 was not, however, higher under controlled pH conditions, in agreement with the finding that yields of enterotoxin A with this strain also did not increase under controlled pH.

Enterotoxin C production by strain 361 in CH medium was extremely low in the fermenter under all conditions as compared with shake-flask production. Therefore, the small increase at pH 8.0 over other pH values may not be significant (Table 4).

**Effect of pH in AA medium.** Growing the staphylococcal strains in AA medium had the great advantage over CH medium that very little foaming occurred, and consequently, antifoam was not needed. If no antifoam at all was added, however, the cells were very sensitive to the gas flow and lysis occurred. The following standard operating procedure was therefore adopted for all strains. When growth was established in the exponential phase and OD had reached approximately 1.0, 0.3 mg of antifoam per ml was added, and air and N₂ were turned

### TABLE 4. Effect of pH on the production of enterotoxins A, B, and C and other extracellular proteins in CH medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular proteins*</th>
<th>Shake-flask</th>
<th>pH in fermenter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NC</td>
<td>6.0</td>
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<tr>
<td>100</td>
<td>Enterotoxin A</td>
<td>7.6</td>
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<tr>
<td></td>
<td>Lipase</td>
<td>190</td>
<td>6.9</td>
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<td></td>
<td>Lysozyme</td>
<td>0.8</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Deoxyribonuclease</td>
<td>165</td>
<td>90</td>
</tr>
<tr>
<td></td>
<td>TEP</td>
<td>0.13</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Final OD</td>
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<td>16.2</td>
</tr>
<tr>
<td>S-6</td>
<td>Enterotoxin A</td>
<td>ND*</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>Enterotoxin B</td>
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<td>114</td>
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<tr>
<td></td>
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<td></td>
<td>Lysozyme</td>
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<td>0</td>
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<td></td>
<td>Deoxyribonuclease</td>
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<td>2600</td>
</tr>
<tr>
<td></td>
<td>TEP</td>
<td>2.95</td>
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</tr>
<tr>
<td></td>
<td>Final OD</td>
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<td>Lysozyme</td>
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<td></td>
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<tr>
<td></td>
<td>TEP</td>
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<td>0.09</td>
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<tr>
<td></td>
<td>Final OD</td>
<td>26</td>
<td>25</td>
</tr>
</tbody>
</table>

* Enterotoxin, lipase, lysozyme, and deoxyribonuclease units as in Table 1.
* ND indicates not detectable.
* NC indicates not controlled.
on and maintained at 0.3 liters per min. No further additions of antifoam were made.

Strain 100 grew more slowly in AA than in CH medium (Fig. 2) with a corresponding lesser increase in pH, and the final level of enterotoxin A was considerably higher. However, in general the level of extracellular proteins was lower in the AA medium. The optimal pH for enterotoxin A production was 6.5 in agreement with that obtained in CH medium (Table 5). Strain S-6 produced less enterotoxin B in AA medium, and again a pH of 6.5 to 7.0 was optimum. However, with this strain, for which the medium was originally developed, lipase, lysozyme, deoxyribonuclease, and TEP were similar to or higher than in CH medium. Strain 361 showed a similar effect to strain 100 in the AA medium, with considerably higher enterotoxin C production. Although the production of enterotoxin C in the AA medium was successively improved by reducing gas flow, using a minimum of antifoam and the optimal pH of 6.5, the yield was still only half that achieved in shake-flasks. In general, growth at controlled pH had a similar effect on the production of lipase and lysozyme to that on enterotoxins, maximum yield in both media being obtained between 6.5 and 7.3. This was not true, however, of deoxyribonuclease produc-

![Graph showing growth and enterotoxin A production by S. aureus strain 100 in shake-flasks in AA medium (closed symbols) and CH medium (open symbols). OD (Δ); enterotoxin A (○); and pH (□).](http://iai.asm.org/)

### Table 5. Effect of pH on the production of enterotoxins A, B, and C and other extracellular proteins in AA medium

<table>
<thead>
<tr>
<th>Strain</th>
<th>Extracellular proteins*</th>
<th>pH in fermenter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>NC*</td>
</tr>
<tr>
<td>100</td>
<td>Enterotoxin A</td>
<td>6.5</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Deoxyribonuclease</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>TEP</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Final OD</td>
<td>18</td>
</tr>
<tr>
<td>S-6</td>
<td>Enterotoxin A</td>
<td>ND*</td>
</tr>
<tr>
<td></td>
<td>Enterotoxin B</td>
<td>194</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>59</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>0.1</td>
</tr>
<tr>
<td></td>
<td>Deoxyribonuclease</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td>TEP</td>
<td>0.68</td>
</tr>
<tr>
<td></td>
<td>Final OD</td>
<td>17</td>
</tr>
<tr>
<td>361</td>
<td>Enterotoxin C</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Lipase</td>
<td>67</td>
</tr>
<tr>
<td></td>
<td>Lysozyme</td>
<td>1.1</td>
</tr>
<tr>
<td></td>
<td>Deoxyribonuclease</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>TEP</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>Final OD</td>
<td>24</td>
</tr>
</tbody>
</table>

* Enterotoxin, lipase, lysozyme, deoxyribonuclease and TEP units as in Table 1.
* ND indicates not detectable.
* NC indicates not controlled.
tion, since maximum yields were obtained at pH 8.0 in both media. These results are in
general agreement with the findings of Arvidsen
and Holme (1).

Relation to growth. In all strains studied
except S-6, enterotoxin production followed
growth in shake-flasks and fermenter
experiments. In shake-flasks only, S-6 showed a
marked increase in enterotoxin B production
during late exponential and early stationary
phase in AA medium (Fig. 1). A similar increase
was found in shake-flask cultures of S-6 in CH
medium.

In eight out of nine strains, a higher yield of
enterotoxin in shake-flask as compared with the
fermenter (Table 1) was associated with a long
transition period from exponential to stationary
phase and a less rapid increase in pH, as is
shown with strain S-6 in Fig. 1. The production
of lipase, lysozyme, deoxyribonuclease, and
TEP was generally greater in shake-flasks than
in the fermenter in CH medium, but this was
not so in AA medium. This apparent association
of increased enterotoxin production with a
slower rate of growth and a less rapid increase in
pH is also evident when the production of
enterotoxin in the AA and CH media is com-
pared (Fig. 2).

DISCUSSION

The pattern of production of enterotoxins
from nine different enterotoxigenic strains of
staphylococci in response to a wide range of
growth conditions varied considerably. The
strain-to-strain differences between staphylo-
occi producing a specific enterotoxin were
very marked. This was particularly dem-
onstrated by strains S-6 and 22 which produced
both enterotoxins A and B. Thus the yield of
both enterotoxins was increased by growth of
strain S-6 under controlled pH conditions,
whereas neither increased when strain 22 was
used. The present study therefore throws some
doubt on the validity of the conclusions from
some investigations that were carried out with
one strain producing one enterotoxin only.

The higher production of enterotoxins A, B,
and C by eight out of nine strains in shake-
flasks as compared with the fermenter may be
attributed to several factors. The addition of
antifoam depressed the production of all ex-
tracellular proteins, although the extent to
which this occurred depended on the strain.
Antifoam was found to decrease the diffusion of
oxygen into the medium and may have affected
extracellular protein production by lowering the
rate of diffusion of oxygen into the cell. The
production of enterotoxin A by three strains was
less sensitive to the presence of antifoam than
the production of enterotoxins B and C from six
other strains. Production of the other extracel-
lular proteins measured, however, were similarly
depressed for all nine strains. This suggests a
possible difference between the synthesis of
enterotoxin A and that of both enterotoxins B
and C. Other investigators suggested that the
mechanism of synthesis of enterotoxins A and B
is different (13, 14).

NaCl was found to decrease the production of
all 3 enterotoxins and the other extracellular
proteins measured in three strains at a lower
level than has previously been reported (7, 12).
If this effect is attributable to the chloride ion,
then similar additions of HCl made to the
fermenter to maintain a constant pH may have
lowered yields of enterotoxins and other ex-
tracellular proteins.

The finding that cultures in baffled flasks
produce more enterotoxin than their controls is
in general agreement with the findings of Die-
trich et al. (4) that increasing aeration increases
enterotoxin production. These workers found
that beyond an optimal shaking speed there was
a decrease in enterotoxin production. It may be
that the reduction in enterotoxin C production
by strain 361 in baffled flasks occurred because
this strain has a low optimal aeration. However,
since increases observed in baffled flasks are
significantly less than increases reported with
higher shaker speeds (4), it is possible that
baffles have some other effect on enterotoxin
production in addition to that due to increased
aeration.

In the fermenter the production of all three
enterotoxins in both media by the three strains
studied in detail was optimal between pH 6.5
and 7.3. The yield was higher at these controlled
pH values than in fermenter experiments where
the pH was uncontrolled. The increase in en-
terotoxin A which occurred with three out of five
strains at pH 6.5 in the fermenter as compared
to the commonly used shake-flask culture may
be of considerable significance. As many foods
are buffered at pH 6 to 6.5, strains of staph-
ylococci which produce little or no enterotoxin
A under the usual conditions of laboratory tests
may produce sufficient enterotoxin to cause
food poisoning. The low yields of enterotoxin
A (1-6 µg/ml) produced in various synthetic
media, even at different initial pH values (15),
and hence the relative unavailability of both
the toxin and its antiserum have greatly limited
studies on enterotoxin A. The use of the chem-
ically defined amino acid medium in a fermen-
ter as described in this study would be a suit-
able means of producing large volumes of high
titers of enterotoxin A.
Increased aeration in CH medium in shake-
flasks resulted in higher growth rates by all
strains and increased production of all extracel-
lar proteins except enterotoxin C. However,
under conditions in the fermenter where the
aeration was kept constant and the pH and type
of medium only were varied, increased produc-
tion of all three enterotoxins could be obtained.
These increases in yield were generally asso-
ciated with a change in growth pattern, show-
ing an extended transition period from exponen-
tial to stationary phase. In experiments without
pH control, the yield of enterotoxins was great-
est when the increase in pH during growth was
least. This may be of significance in food
poisoning where conditions are likely to be
conducive to slow growth under conditions of
buffered pH. It has been postulated (3) that a
decrease in the growth rate of Bacillus subtilis
results in the formation of exoenzyme messen-
ger ribonucleic acid and extracellular protein at
the maximum rate. If this were so for staphy-
lococci, it would be expected that conditions of
reduced growth leading to increases in en-
terotoxin production would invariably be asso-
ciated with increases in extracellular protein.
However, several instances have been given in
this paper where such changes in the production
of enterotoxins A, B, and C are not related to
changes in other extracellular proteins.

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