Effects of Supplemental Calcium or Calcium-binding Agents on Staphylococcal Bacteriophage Proliferation in Skim Milk

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Additions of 0.0005 N calcium borogluconate to Trypticase Soy Broth (TSB) produced an increase in phage titer about 1 million-fold, whereas its addition to skim milk resulted in about a 100-fold decrease in the maximal titer. Supplemental calcium had a stimulatory influence on bacterial growth in TSB but not in skim milk. Studies were made of the effect of binding of calcium of skim milk on the proliferation of staphylococcal bacteriophage. Sequestering the calcium with 2% phosphate mixture inactivated the phages without affecting the bacterial growth. However, chelation of calcium by 0.012% ethylenediaminetetraacetic acid produced an inhibitory effect on both the phages and the bacteria.

Rountree (7) showed that the staphylococcal typing phages require divalent cations (such as calcium and magnesium) in amounts varying from 5 to 400 µg/ml for a stage in phage multiplication which is probably that of penetration. Similar findings have been reported elsewhere (2, 9, 10). Moreover, the suppression or prevention of streptococcal bacteriophage in media caused by binding the calcium ions, or by the removal of calcium, is well recognized. Stassano and Beaufort (8) were perhaps the first to use citrate as a calcium-binding agent. Later there were other reports (3, 5, 6) which suggested the use of a medium low in calcium and supplemented with sufficient phosphate or other calcium-binding ions.

The present investigation was undertaken to observe the previously unreported effects of supplemental calcium and of calcium-binding agents on staphylococcal phage proliferation in skim milk.

Materials and Methods

Staphylococci. Staphylococcus aureus, phage type 79/54, was originally isolated from the milk of an infected cow. It was coagulase-positive; it produced β-hemolysis, fermented mannitol, and was resistant to penicillin and streptomycin. Propagating media were Trypticase Soy Agar (TSA; BBL) and Trypticase Soy Broth (TSB; BBL). Suspensions were prepared by transferring inocula from TSA slants to TSB and incubating for 8 hr at 37 C. After this, 0.01 ml of the growing culture was transferred by means of a standard wire loop to TSB which was incubated for 18 hr at 37 C. Serial dilutions were made in phosphate-buffered distilled water. Numbers of viable bacteria were determined by plating in duplicate as described in Standard Methods for the Examination of Dairy Products (1).

Bacteriophage. Staphylococcal bacteriophages, 79/54, were supplied by J. E. Blair, Hospital for Joint Diseases, New York, N.Y. Phage lysates were prepared by the double agar layer technique (2).

Bacteriophage titration. TSA (25 ml) was poured into sterile petri dishes (15 cm in diameter). Plates were dried for 24 hr in a low-humidity, 37 C incubator, which also provided a check on sterility. Plates were seeded with 1 ml of an 18-hr broth culture of the susceptible bacterial strain; then they were dried for 30 min at 37 C with lids open. After drying, serially 10-fold diluted samples of bacteriophage in TSB were spotted at specified places, in duplicate, on the seeded surface. The syringe used was equipped with a 27-gauge hypodermic needle which delivered 110 ± 10 drops per ml. Spots were allowed to dry to avoid spreading prior to incubating the plates for 1 hr at 30 C. After incubation, numbers of plaque-forming units (PFU) in each spot were determined with the aid of a Quebec counter.

Treatments of skim milk. A 12-mg amount of ethylenediaminetetraacetic acid (EDTA; Fisher Scientific Co., Pittsburgh, Pa.) was dissolved in 100 ml of skim milk to bind the calcium. The quantity required was calculated on the basis of the assumption that 1.2 mg of calcium is present per g of skim milk. After the addition of EDTA, the skim milk was autoclaved for 10 min at 121 C.

A phosphate mixture (36 g of KH₂PO₄ + 24 g of Na₂HPO₄ in 100 ml of distilled water) (6) was made

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and sterilized for 15 min at 121 C. A 2.5-ml portion of this mixture was added to 75 ml of skim milk, which had been previously sterilized for 10 min at 121 C, to give 2% phosphate. The phosphated skim milk was then steamed for 15 min.

Calcium borogluconate, sterile solution (0.83 ml of 26%; Jensen-Salsbery Laboratories, Kansas City, Mo.), was added to 100 ml each of sterile skim milk to provide 0.0005 lb calcium in the medium.

In every case, TSB was treated the same way as was the skim milk.

Each medium was dispensed in screw-capped tubes, 9 ml per tube.

RESULTS AND DISCUSSION

Experiments in TSB. The typical growth pattern of S. aureus in TSB with and without added calcium is shown in Fig. 1a. The culture reproduced more slowly but reached a higher population in the calcium-supplemented medium.

Figure 1b shows the influence of phage in the culture on the staphylococcal population when calcium was added. Early growth was unaffected, but the viable count decreased markedly during the next 12 hr. Evidently, considerable cell lysis occurred. Figure 1d indicates the time of maximal phage titer increase. However, lysis of staphylococci did not proceed to completion, and there was an increase in cell numbers from 24 to 48 hr of incubation such as to indicate a secondary logarithmic growth phase. The phage titer in the calcium-supplemented TSB reached a maximum of 2 x 10^12 PFU per ml, about 1 million times the maximal number when no calcium was added.

The lysis of staphylococci in TSB without added calcium was comparatively limited as indicated by the rapid increase in viable bacterial counts (Fig. 1c) and the relatively low phage titer (Fig. 1d). That lysis did occur during prolonged incubation is indicated by the approximate 100-fold decrease in viable count and the relatively stable phage titer.

Experiments in skim milk. The same experimental conditions resulted in different results when skim milk was the growth medium. Addition of calcium slowed reproduction of the staphylococci (Fig. 2a). Viable counts after 72 to 120 hr of incubation were about 50% lower in skim milk which contained added calcium.

Data in Fig. 2b and 2c show that supplemental calcium was the major depressant of early growth.
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TABLE 1. Effects of media and phosphates on staphylococcal phage proliferation and bacterial counts in skim milk

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Plaque-forming units/ml&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Bacteria/ml&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Skim</td>
<td>Skim + PO&lt;sub&gt;4&lt;/sub&gt;</td>
</tr>
<tr>
<td>0</td>
<td>1 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<tr>
<td>12</td>
<td>2 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
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<td>8 X 10&lt;sup&gt;6&lt;/sup&gt;</td>
<td>&lt;1 X 10&lt;sup&gt;6&lt;/sup&gt;</td>
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<sup>a</sup> Determined from duplicate spots in each observation.
<sup>b</sup> Determined by standard plate count.
<sup>c</sup> Phosphate (2%) added.

Lysis by phage was a secondary factor. Phage proliferation was hardly detectable after 12-hr incubation. The sharp rise in titer in plain skim milk at 24-hr (Fig. 2d) corresponds to the leveling off of the viable count curve (Fig. 2c). Viable staphylococci numbers remained relatively constant thereafter. Lysis probably continued at a faster rate in the calcium-supplemented skim milk after 72-hr incubation. Thus, it appears that phages were being inactivated by the skim milk to which no calcium was added, because the viable count continued to increase and to reach the same number as was found in the noninfected skim milk (Fig. 2a). This observation is supported by research reported earlier (4), which showed that phages were adsorbed to milk proteins. The addition of calcium probably decreased the amount of this adsorption. The highest phage titer was ob-

FIG. 2. Effects of calcium borogluconate on phage proliferation and bacterial counts in skim milk. (B = Staphylococcus aureus, P = homologous phage, Ca = calcium-borogluconate.)

TABLE 2. Effects of media and ethylenediaminetetraacetic acid (EDTA) on the proliferation of staphylococcal bacteriophage

<table>
<thead>
<tr>
<th>Time (hr)</th>
<th>Plaque-forming units per ml&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Trypticase Soy Agar (TSB)</td>
</tr>
<tr>
<td>0</td>
<td>5 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>12</td>
<td>2.5 X 10&lt;sup&gt;3&lt;/sup&gt;</td>
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</tr>
<tr>
<td>96</td>
<td>4 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
<tr>
<td>120</td>
<td>1.2 X 10&lt;sup&gt;4&lt;/sup&gt;</td>
</tr>
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</table>

<sup>a</sup> Determined from duplicate spots in each observation.
<sup>b</sup> Disodium EDTA, 1.2%.
tained in skim milk without added calcium, 5.0 \times 10^{10} \text{ PFU/ml}, but in TSB with supplemental calcium the titer reached 2.0 \times 10^{14} \text{ PFU/ml}.

Effects of binding calcium on proliferation of staphylococcal phages. Addition of calcium-binding agents to skim milk stops phage proliferation of streptococcal bacteriophage. Use of the recommended formulation of phosphates in the presence of staphylococcal phages also stopped proliferation (Table 1). The fact that viable staphylococcus counts were from two to five times higher in the phosphate-treated skim milk at and after 48 hr of incubation (Table 1) gives evidence that the effect was on some aspect of phage infectivity or reproduction.

In a second experiment, calcium was chelated with 1.2\% EDTA. The same effect on phage proliferation was observed (Table 2). Both TSB and skim milk were inoculated with the same quantity of suspensions of phage and bacteria. Initially, it was possible to recover phages from the skim milk but not from the TSB. This situation was opposite from that expected because of phage adsorption by milk proteins. It appears that residual unassociated EDTA in the TSB was carried over to the titration medium. Skim milk probably contained many more chelatable ions. It is also likely that chelation was completed sooner in TSB.

Not only was there complete inhibition of phage proliferation after the initial observation in both media containing EDTA, but there was a pronounced inhibitory effect on the multiplication of the bacteria. Since organism multiplication was not affected in phosphate-treated skim milk, the question arises as to why the two methods of calcium binding should produce different responses by the bacteria. The most obvious explanation is that sufficient calcium or other inorganic elements or both needed for staphylococcal growth were left unbound by the phosphates but not by the EDTA.

LITERATURE CITED