Interaction Between Streptococcal Bacteriophage and Milk

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Abstract

The effect of pH on recovery of streptococcal bacteriophage e2 from skim milk was determined. Titers were determined by taking sample aliquots from trypticase soy broth or skim milk which had been adjusted to pH levels of 4.0 to 9.0. Significantly higher titers were obtained from skim milk at pH 4.0 than at pH 7.0. Comparisons of recoverability were made using suspending mediums of 3% casein, skim milk, whey, and trypticase soy broth at pH 6.9 ± 0.2. Resulting titers from whey were highest, while those of trypticase soy broth, skim milk, and 3% casein were successively and significantly lower. As the pH of skim milk was adjusted to below the isoelectric point of casein, more phage particles became titratable, possibly because of the change in charge on the casein micelles.

The effect of pH on streptococcal phages has been studied by several workers (2, 5, 6, 8). Their results concerned the maximum, minimum, and optimum pH levels for phage proliferation. Prouty (7) determined the pH levels which would completely inactivate three specific phage strains during an exposure period of 1 min.

In recent work with staphylococcal host-phage systems, Das and Marshall (3) demonstrated reduced recovery of phage due to suspension in skim milk. Recoverability increased when the pH of skim milk was lowered below the isoelectric point of casein (pH 4.7). Through other in vitro studies it was concluded that interactions between phage and casein resulted in apparently lower titers of phage in skim milk.

In our research a streptococcal host-phage system was studied using a modification of the method of Das and Marshall (3).

Experimental Procedure

In all experiments Streptococcus lactis, Strain C2, and its homologous phage e2 were used. Cells were transferred daily in trypticase soy broth (TSB: Baltimore Biological Laboratory, Inc.) at a temperature of 30-32 C.

Phage was propagated on its homologous host using TSB medium supplemented with 0.0005 M calcium. Phage suspension was added at a multiplicity of infection of approximately one-tenth while the culture was in the log phase of growth. This mixture was incubated on a mechanical shaker at a slow speed until the broth cleared. After storage overnight at 4 C, the lysate was passed through a 0.45 µ membrane filter to remove residual cells and cell debris, then stored at 4 C.

Spot plate phage titration. Approximately 35 ml of trypticase soy agar (TSA:BBL), supplemented with calcium and amino acids (4), was poured into 15-cm petri plates and allowed to dry overnight at 37 C. This procedure proved sterility of the medium. The surface of the dried medium was seeded with approximately 1 ml of a 5-hr culture (slightly turbid), after which the seed layer was dried for 30 min at 37 C with the petri cover removed. This plate was divided into four to six sections, depending on the number of phage dilutions made. Serial dilutions of the phage suspension were made in trypticase soy broth. A small aliquot of sample was removed from each dilution blank with a tuberculin syringe (1-ml) attached to a 27-gauge needle. From each dilution five to seven drops were deposited individually on a section of the dried seeded surface and allowed to dry. The plate was inverted and incubated at 30 to 32 C for 7 to 8 hr, and then plaques within each drop area were counted. The number of plaque forming units (pfu) per drop was multiplied by 140, the number of drops in 1.0 ml, to determine the pfu/milliliter.

Preparation of casein and whey. Ultracentrifuged casein was prepared using a modification of the method of Bohren and Wenner (1). Fresh, raw skim milk was centrifuged at 50,000 × g for 3 hr at 23 C. The sedimented casein fractions were resuspended in sufficient distilled water to produce half the original volume. To this suspension was added an equal amount of 0.02 M phosphate buffer of pH 7.0. This soluble suspension was dialyzed against distilled water at 4 C for 24 hr. The
resulting liquid was lyophilized and stored at 4 C in a desiccator. Three per cent suspensions in distilled water were prepared from this lyophilized casein. They were sterilized by autoclaving for 10 min at 121 C.

Whey was prepared by lactic acid precipitation of the casein, which was removed by filtration with Whatman no. 30 filter paper. After adjusting the pH of the filtrate to 7.0, it was passed through a 0.45 μ membrane filter for sterilization.

Effect of pH on phage recoverability from skimmilk. Experiments to determine recoverability of phage from skimmilk by the spot plate method were performed over a pH range of 4.0 to 9.0, and the results were compared to recoverability of phage from trypticase soy broth over the same pH range.

A mixture of phage and skimmilk or phage and trypticase soy broth was placed in a beaker containing a magnetic stirrer bar. Acid or base was added while stirring, and the temperature maintained at 0-4 C to prevent casein precipitation.

Sample aliquots were removed from this phage suspension after the pH had been adjusted to 7.0, 8.0, 9.0, 5.0, 4.5, and 4.0, respectively. These pH adjustments were made using solutions of 1 x NaOH and 10% lactic acid. Additions of the acid and base changed the phage concentration so that the following equation was used for making titer corrections:

\[
\text{Dilution factor} = \frac{\text{Original volume} \times \text{total milliliters of acid or base added}}{100} \\
\text{pfu/drop} = \text{Dilution factor} \times \text{observed pfu/drop}
\]

After each pH adjustment, five drops of the sample were placed individually on a separate section of the seeded TSA medium. This titration procedure took approximately 20 min. From this point incubation and counting were done as previously stated.

Recovery of phages from various suspending media. Equal numbers of phage were added to trypticase soy broth, whey, skimmilk, and a 3% casein suspension to determine the recoverability of phage from these suspending mediums.

Aliquots were taken from each of the four mediums and titrated by the spot plate method using seven individual drops per sample. This experiment was replicated six times.

Results and Discussion

Effect of pH on phage recoverability from skimmilk. Numbers of plaque-forming units recovered from skimmilk and TSB at various pH levels are shown in Figure 1. Significant differences (P < 0.05) between numbers of pfu recovered were observed due to type of suspending medium and due to pH (Table 1). Because maximum numbers of pfu were recovered at pH 7.0 from trypticase soy broth, this titer was considered to be the true one. Therefore, recovery percentages were calculated based on this titer.

There was no significant difference between the true titer and the titer observed in skimmilk adjusted to pH 4.0. Although about 97% of the phage was recovered from the skimmilk at pH 4.0, when compared to the true titer, at pH 9.0 only 51% was recovered.

Lowering the pH of skimmilk caused higher titers of phage, but lowering the pH of trypticase soy broth had an opposite effect. Single-drop aliquots of the soy broth or skimmilk were placed on the surface of trypticase soy agar plates containing pH indicators. The final pH at the site of the spots ranged from 5.0 to 5.4 when the suspending mediums were adjusted to pH 4.0. Data of other workers indicate that phage proliferation does not take place below a pH of approximately 5.0. However, a higher titer observed in skimmilk at the low pH tends to negate this as an explanation for the low titer from the soy broth at low pH levels.

These observations indicate that some constituent of milk decreased the plaque-forming ability of this phage at normal pH values. Previous work by Das and Marshall (3) indicated that casein influences the recovery of phages.

Recovery of phage from various suspending media. To determine the effect milk proteins
TABLE 1. Phage recoverability from skim milk and trypticase soy broth as influenced by pH.

<table>
<thead>
<tr>
<th>Medium</th>
<th>pH</th>
<th>Mean number* of pfu/drop</th>
<th>Phage recovery (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Trypticase soy broth</td>
<td>7.0</td>
<td>7.600</td>
<td>100</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.0</td>
<td>7.400</td>
<td>87</td>
</tr>
<tr>
<td>Trypticase soy broth</td>
<td>9.0</td>
<td>6.436</td>
<td>84</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.5</td>
<td>6.312</td>
<td>83</td>
</tr>
<tr>
<td>Trypticase soy broth</td>
<td>8.0</td>
<td>6.064</td>
<td>80</td>
</tr>
<tr>
<td>Skim milk</td>
<td>5.0</td>
<td>6.056</td>
<td>80</td>
</tr>
<tr>
<td>Trypticase soy broth</td>
<td>5.0</td>
<td>5.128</td>
<td>67</td>
</tr>
<tr>
<td>Skim milk</td>
<td>4.5</td>
<td>5.060</td>
<td>67</td>
</tr>
<tr>
<td>Skim milk</td>
<td>7.0</td>
<td>4.852</td>
<td>64</td>
</tr>
<tr>
<td>Trypticase soy broth</td>
<td>4.0</td>
<td>4.620</td>
<td>61</td>
</tr>
<tr>
<td>Skim milk</td>
<td>8.0</td>
<td>4.344</td>
<td>57</td>
</tr>
<tr>
<td>Skim milk</td>
<td>9.0</td>
<td>3.920</td>
<td>52</td>
</tr>
</tbody>
</table>

* Numbers bordered by the same line are not significantly different (P < 0.05).

have on recoverability of streptococcal phages, an experiment using different suspending mediums was performed. Casein was prepared by ultracentrifugation. Whey was prepared and sterilized without heat, to retain the natural properties of the protein fractions. There were significant differences (P < 0.05) between means of numbers of pfu/drop for all mediums. The suspending medium which allowed for maximum recovery of phage was whey, whereas that which produced the poorest was 3% casein. Results are shown in Table 2.

The proliferation of phage in host-phage systems is often dependent upon concentration of calcium. Lowering of the pH of milk increases the concentration of soluble calcium. The possibility that this factor influenced our results is considered nil, because Mikolajcik (4) demonstrated that this particular host-phage system has a comparatively low requirement for calcium, and our titrations were performed on a solid medium supplemented with calcium.

It appears from these results and those of the experiment in which pH was the variable, that at pH levels above its isoelectric point, casein influences the infectivity of this streptococcal bacteriophage on its host. When the isoelectric point is approached, its infectivity increases, as is shown by an observable rise in titer (Fig. 1). This is possibly due to changes in the electrical changes of the casein micelles.

References

4. Mikolajcik, E. M. 1964. Some factors which govern plaque formation by lactie


