

Effects of Homologous Bacteriophage on Growth of *Pseudomonas fragi* WY in Milk¹

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Pseudomonas fragi strain WY and its homologous bacteriophage were added in varying concentrations to sterile skim milk which was stored at 7 C for 72 hr. When the initial concentration of the bacterial host was 100,000/ml, addition of as few as 10 plaque-forming units per ml of bacteriophage resulted in significantly lower counts in treated skim milk than in the controls which contained no phage. There was no significant effect, however, when the phage input was 1 in 10 ml and the bacterial count was 1,000 or 100,000/ml. No differences in bacterial counts occurred even when the phage concentration was 1,000/ml if the initial bacterial concentration was only 1,000/ml.

Whitman and Marshall (2) isolated two strains of bacteriophages and their bacterial hosts from raw skim milk. They isolated 36 other strains from additional refrigerated foods. This created speculation that the growth of bacteria within refrigerated foods might be slowed by homologous bacteriophage, thus extending shelf life of the food. The present report describes conditions which had to exist if a homologous bacteriophage of *Pseudomonas fragi* WY was to significantly affect growth of the host bacterium in refrigerated milk.

MATERIALS AND METHODS

Bacteriophage and host. *P. fragi* WY and its homologous bacteriophage, wy, were isolated from ground beef, and their characteristics have been reported (3). A suspension (160 ml) of bacteriophage particles containing 2×10^{12} plaque-forming units (PFU) per ml was prepared by propagation on 25 large petri plates (150 mm) by the double-layer method (1). The semisolid layer of each confluent lysed plate was macerated in 6 ml of tryptic soy broth (TSB) and combined in a beaker. After 5 hr of incubation at 4 C to complete lysis, the slurry was centrifuged for 15 min at $10,000 \times g$, and the supernatant fluid was sterilized by consecutive filtration through membranes with pore sizes of 1.2, 0.45, and 0.22 μm , respectively.

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Anti-phage serum. Four adult rabbits were subcutaneously inoculated twice weekly for 3 weeks with 5 ml of the high-titer phage suspension. One week after the last injection, rabbits were bled by cardiac puncture. The blood was allowed to coagulate at 37 C in vaseline-lined centrifuge tubes before overnight storage at 4 C. After centrifuging for 10 min at $5,000 \times g$, the serum was drawn off, filtered through a membrane filter (0.45 μm diameter pore size), stored at 4 C, and assayed for anti-phage activity. After assay, the sera were pooled and stored frozen.

Assay of anti-phage activity. Serum was diluted 1:100 and 1:1,000 in TSB, and high-titer phage stock was diluted to 10^7 PFU/ml. Phage suspension (0.1 ml) was added to 0.9 ml of each dilution of antiserum at room temperature. At 5-min intervals, 0.1-ml samples of the phage-serum mixture were added to 9.9 ml of TSB to stop the antibody reaction, and 0.1-ml samples of this dilution were plated by the agar layer method (1). If phages were not inactivated, about 1,000 plaques appeared after incubation; 90% inactivation resulted in about 100 plaques, and 99% inactivation (the desired level) resulted in about 10 plaques.

Effect of phage on its host in skim milk. Skim milk, obtained from the University of Missouri Dairy, was sterilized by heating at 121 C for 15 min. The milk was divided into 400-ml lots and then inoculated with *P. fragi* WY in two concentrations: 1,000 and 100,000 cells/ml. (Previous observation showed that a 24-hr TSB culture contained approximately 2×10^8 cells/ml; this figure was used as a basis for dilutions prior to inoculation.) The host cells were mixed thoroughly with the milk by stirring 5 min with a Teflon spinbar driven by a magnetic stirrer. Each 400-ml lot of milk containing either 10^3 or 10^5 host cells per ml was subdivided into four 100-ml samples; these samples were inoculated with 0, 0.1, 10, or 1,000 PFU/ml. These ratios of phage to host were consid-

ered to be those which might occur in milk normally. Controls consisting of milk containing no phage particles or bacteria and one containing 100 phages per ml but no bacteria were also incubated.

All samples were incubated at 7 C for 72 hr (a time and temperature at which grade A milk might be held in practice). Samples were then plated in triplicate to determine bacteria and phage counts. Plates were incubated at 21 C; phage counts were made after 15 hr, and bacteria were counted after 48 hr. Antiserum was added at a 1:1,000 concentration to the first dilution bottle used in making bacterial counts to prevent additional infections of bacteria. These experiments were replicated three times.

RESULTS AND DISCUSSION

Anti-phage serum was used to preclude lysis of host cells during plating of samples to determine bacterial counts. Our preliminary experiments had suggested this as a possibility. Our serum inactivated 99% of the bacteriophages at room temperature in 5 min when diluted 1:100 and in 20 min when diluted 1:1,000. No bacteria were found in controls with only added phage, and no phage were found in controls with only added bacteria.

When the initial concentration of *P. fragi* WY was 100,000/ml, addition of 10 or 1,000 PFU/ml of homologous phages caused significantly lower bacterial counts after 72 hr at 7 C (Fig. 1). The count appeared to be lower when only 0.1 PFU/ml was added, but the difference was not statistically significant. Bacteriophage titers were significantly different at the end of incubation, with the lowest and highest counts corresponding to the lowest and highest inputs of phage, respectively.

Effects of added phages were much less pronounced when the initial bacterial concentration was 1,000/ml (Fig. 2). In fact, the bacterial count of the control was lower (insignificantly) than the average counts of each sample to which phage was added. Numbers of phages increased markedly in samples to which 10 and 1,000 PFU/ml were added. There was no detectable multiplication of phage in the samples to which only 0.1 PFU/ml was added.

These results indicate that bacteriophage can have an influence on shelf life of refrigerated milk, but the conditions necessary for significant effect are improbable. These conditions are (i) a relatively high population of the host bacterium, (ii) presence of its homologous

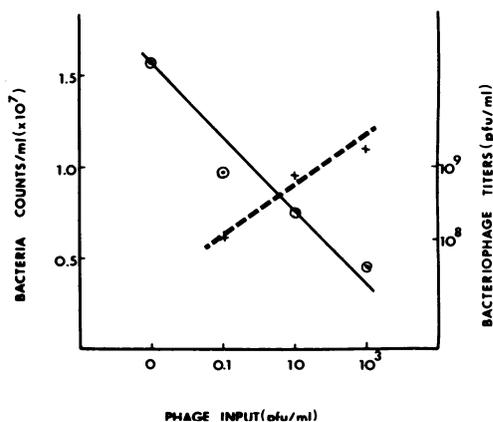


FIG. 1. Bacterial counts (solid line) and bacteriophage titers (broken line) in milk after inoculation with varying numbers of bacteriophage and incubation at 7 C for 72 hr. Initial bacterial concentration was 10^6 /ml.

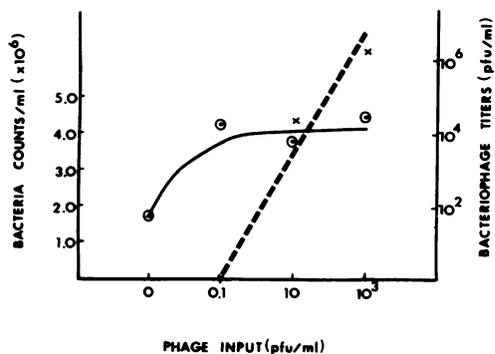


FIG. 2. Bacterial counts (solid line) and bacteriophage titers (broken line) in milk after inoculation with varying numbers of bacteriophage and incubation at 7 C for 72 hr. Initial bacterial concentration was 10^3 /ml.

phage, and (iii) the absence of significant numbers of other harmful psychrophilic bacteria which could not be lysed by the phage.

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