Inhibition of the Plaquing Efficiency of T4r+ Bacteriophage by Subtilisin

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The mechanism by which the replicative cycle of T4r+ phage is inhibited by certain nonhost bacterial systems was investigated. Some Bacillaceae, especially Bacillus subtilis, decreased the plaquing efficiency of this virus more than 95% within 24 hr of exposure. Sarcina lutea and Micrococcus sp. both failed to cause any significant change in the infectivity of T4r+ phage. Preliminary investigations into the nature of the inhibitory substance(s) suggested that an extracellularly elicited protein was at least partially responsible for this effect. Further analysis has implicated subtilisin, an exoprotease from B. subtilis, as the cause of some, if not all, of the observed decrease in plaquing efficiency. Gel-filtration chromatography of control and treated 14C-labeled T4r+ phage showed a wide dispersal of phage-specific material of these particles after 24 hr of exposure to pure subtilisin or to extended medium exoprotease from B. subtilis. It was concluded that B. subtilis exoprotease is capable of chemically altering the structure of the phage capsid, thus causing a decrease in its plaquing efficiency.

A variety of substances are produced extracellularly by Bacillaceae. Some of these are the normal growth products of catabolism (14). However, more complex substances, such as exoenzymes (3–6, 15, 19), are also found in abundance in the extended culture filtrates of these bacteria. In 1925, the culture filtrates of Bacillus subtilis were examined by Marie (12) for their antiviral capacity. It was noted that these filtrates exhibited inhibitory activity against rabies virus after 18 hr of incubation at 38 C. If the filtrate was heated to 100 C before it was mixed with the virus, however, the effect was lost. In 1936, Rakieten et al. (16) reported that staphylococcus phage and vesicular stomatitis virus were inactivated by various strains of bacteria belonging to the subtilis group. Ten years later, Remlinger and Bailly (18) found B. subtilis filtrates effective not only in combating rabies virus but also in inactivating an encephalomyelitis virus.

Aside from the observation that staphylococcus phage was inactivated due to a nonspecific adsorption phenomenon, only Marie (12) recognized the possibility that proteolytic enzymes in the expended culture medium might demonstrate such a widespread antagonistic effect. It is now known that up to 1.0 g of exoprotease per liter can be produced by B. subtilis (6). In fact, B. subtilis possesses the ability to secrete multiple proteolytic enzymes (11, 13, 17). B. megaterium and B. cereus secrete a single protease active only at a neutral pH. This research has been concerned primarily with the phage-inhibitory relationship existing between the culture filtrates of B. subtilis and T4r+ bacteriophage. The effects of other bacteria on this virus were also studied.

MATERIALS AND METHODS

Cultivation of bacteria. The preparation of Escherichia coli B as host bacterium in all T4r+ phage infectivity studies was done in the following manner. Either a loopful of stock E. coli B, obtained from a slant of this bacterium, or 0.3 ml from a stock culture of E. coli B in MS broth (MSB; 10) was placed into 30 ml of sterile MSB in a 125-ml Erlenmeyer flask. This bacterial suspension was cultivated aerobically overnight at 37 C on a New Brunswick Gyrotory Water Bath Shaker, model G76, at a speed of 180 to 200 rev/min. Amounts of 0.1 ml (ca. 5 X 10^4 cells/ml) from this culture per 10 ml of fresh MSB were then incubated at 37 C under these same conditions for various time periods.

E. coli B was also cultivated in M-9 broth (1) in the manner described above for E. coli B in MSB. In each case, the inoculum for final cultivation origi-
nated from a culture previously incubated overnight at 37°C in the respective growth medium under aerobic conditions. The various bacteria used throughout this study were cultivated in MSB as described above for *E. coli* B in MSB.

Preparation of T4r+ bacteriophage. *E. coli* B was cultivated in MSB as described above at 37°C. When the bacterial concentration reached ca. 5 × 10^8 cells/ml, 0.1 ml of T4r+ bacteriophage (final concentration, 10^9 particles per ml) per 10 ml of *E. coli* B was introduced. The suspension was reincubated at 37°C with aeration for 6 to 7 hr. Chloroform (1 ml) was added to the culture to artificially disrupt any cells that had failed to undergo phage-induced lysis. The flask was shaken vigorously by hand for 30 to 60 sec and refrigerated at 4°C overnight. The contents of the flask were transferred into 50-ml polypropylene or polycarbonate centrifuge tubes. These were centrifuged at 12,500 × g for 10 min in either a Sorvall RC2-B refrigerated centrifuge or an IEC model B-20 refrigerated centrifuge to remove the bacterial debris. The pellet was discarded. The supernatant fluid was saved and assayed for plaque-forming units (PFU) per milliliter according to the method of Hershey et al. (8). Prior to assay, the phage suspension was subjected to moderate aeration for 2 hr. This removed any excess CHCl₃. The phage suspension was stored at 4°C and was periodically assayed for PFU per milliliter to insure titer stability. Assay of the virus was always done according to the method of Hershey et al. (8).

Preparation of pure T4r+ bacteriophage. *E. coli* B was cultivated in M-9 broth at 37°C as described above. At the end of 3 to 3.5 hr, stock T4r+ phage was introduced and the suspension was reincubated at 37°C with shaking for 8.5 to 9 hr. The phage was then purified according to the method of Albertsson (2) with the modification that polyethylene glycol 20,000 and sodium dextran sulfate 2,000 were used instead of the compounds recommended. No alteration in the resultant titer was caused by this change.

Preparation of 14C-L-leucine-labeled T4r+ bacteriophage. *E. coli* B was cultivated in M-9 broth as explained above. After 3 to 3.5 hr at 37°C, the bacteria were sedimented at 11,000 × g for 10 min. The pellet was resuspended in 100 ml of sterile M-9 broth. To this culture was added pure T4r+ phage (4 × 10^10 phage/ml), and the suspension was placed at 37°C. The infected culture was aerated for at least 12 min but no longer than 15 min. At some time during this 3-min interval, 1.0 ml of uniformly labeled 14C-L-leucine (original activity, 5 μCi) plus 2.5 ml of L-leucine (stock solution = 0.06 mg of L-leucine/ml of distilled water) per 100 ml of culture were added. The flask was reincubated at 37°C with shaking for 2 hr, 3 ml of prechilled CHCl₃ was added, and the flask's contents were shaken vigorously as above. The CHCl₃ was allowed to settle to the bottom of the flask at 0°C. The clear phage lysate was carefully decanted into a 500-ml sterile separatory funnel, and the 14C-labeled virus (*T4r+* phage) was purified according to the method of Albertsson (2) as described above. The purified labeled phage was suspended in 10 ml of 0.15 M NaCl plus 10^-4 M MgCl₂·6H₂O (pH 7.0) and was stored at 0°C. Counts per minute were determined by suspending 0.1 ml of sample in 10 ml of counting solution (4 g of Omnifluor per 700 ml of toluene plus 300 ml of anhydrous methanol) and counting duplicate samples in a Nuclear-Chicago liquid scintillation counter.

Anti-T4r+ phage screening of bacteria. Overnight cultivation of *B. megaterium*, *B. cereus*, *B. subtilis*, *Sarcina lutea*, and *Micrococcus* sp. was done as described in a previous section. From each culture, 0.4 ml was introduced into 40 ml of fresh MSB and incubated at 37°C for 3 hr. At the end of this time, 20-ml samples from each culture were withdrawn and centrifuged at 12,500 × g for 10 min. Each pellet was resuspended in 20 ml of sterile 0.1 M tris-(hydroxymethyl)aminomethane (Tris), pH 7.2. To both the growing cultures of bacteria and their respective pellets resuspended in 0.1 M Tris, 1 ml of T4r+ phage was added to a final concentration of 10^6 particles/ml. A 1-ml amount of virus was also added to 20 ml of sterile MSB and 20 ml of sterile Tris. These suspensions were placed at 37°C for 24 hr. Supernatant fluids were collected after centrifugation at 12,500 × g for 10 min. Pellets were resuspended in 20 ml of either fresh MSB or Tris and shaken slightly. Phage assays according to the method of Hershey et al. (8) were done on all samples after 1, 4, and 24 hr.

Relation of sporulation to phage inhibition. *B. subtilis* was cultivated in 200 ml of MSB in the manner described above. At 4-hr intervals from 0 to 32 hr, 10-ml samples were withdrawn and centrifuged at 12,500 × g for 10 min. The pellets were resuspended in 10 ml of sterile distilled water and heat-shocked at 80°C for 30 min in a constant-temperature water bath. Samples of 0.1 and 1 ml were spread on MSB agar and incubated for 24 hr at 37°C.

Relation of α-amylase and protease to phage inhibition. From a culture of *B. subtilis* cultivated as described above, appropriate samples were withdrawn at 4, 8, 12, 16, 20, 24, and 28 hr after time zero and tested for α-amylase and protease (subtilisin) activity according to the methods of Street (20) and Hagihara et al. (7), respectively. The pH of the culture was also frequently determined.

Membrane-filtered expended media from the *B. subtilis* culture, harvested at 1, 4, 12, 19.5, and 24 hr, had T4r+ phage added to each sterile sample (final phage concentration, 10^9 phage/ml), and each of these five suspensions was incubated at 37°C for 24 hr. Plaque assays were done after this period on all five samples.

Activity of pure subtilisin on T4r+ bacteriophage. Various concentrations of subtilisin (10 to 10^4 μg/ml) were made in MSB, pH 7.5. T4r+ phage was added to each subtilisin-MSB solution, which had been membrane-filtered. The final phage concentration was 3.1 × 10^6 PFU/ml in each flask. Control flasks containing MSB (pH 7.5) plus T4r+ phage, autoclaved 24-hr *B. subtilis* expended media plus T4r+ phage, and membrane-filtered 24-hr *B. subtilis* expended medium plus T4r+ phage were included. All samples were incubated at 37°C for a total of 72 hr. Phage assays, according to the method of Hershey
et al. (8), were done on most samples at the end of 24 and 72 hr.

Column chromatography of pure subtilisin and B. subtilis expended media. A 1-ml amount of pure commercial subtilisin (Nutritional Biochemicals Corp., Cleveland, Ohio) in 0.1 M NaCl, pH 7.4 (1.0 mg/ml), or 1.0 ml of 24-hr membrane-filtered B. subtilis expended media was applied to the top of a Bio-Gel P-100, 100 to 200 mesh, gel column (Bio-Rad Laboratories, Richmond, Calif.) that had the following specifications: dimensions, 1.3 X 28 cm; buffer, 0.05 M Tris plus 0.15 M NaCl plus 0.001 M MgCl2·6H2O (pH 7.4); gel, Bio-Gel P-100, 100–200 mesh, hydrated for 24 hr in the above buffer; total bed volume, 22 ml; void volume (V0), 8.3 ml; total volume (Vt), 42 ml; flow rate, 1.9 ml/hr. After the respective sample entered the gel matrix, the glass sides of the upper column well were rinsed with buffer, with care taken not to disturb the gel bed. A 15- to 20-ml amount of buffer was carefully pipetted onto the top of the gel, and a buffer reservoir was attached to the column. Samples of 0.5 ml were collected by use of a model 321 Isco Fraction Collector equipped with a model 600 drop-detecting photocell assembly. Samples were read spectrophotometrically in a Beckman DU spectrophotometer equipped with a microcell attachment in a 1-cm light path at absorbencies of 260 and 280 nm. The amount of protein (milligrams per milliliter) present in each fraction was determined as described by Kalckar (9).

Column chromatography of T4r+ phage after treatment with above fractions. Those samples comprising any single peak from either P-100 column fractionation explained above were collected in suitable vessels. The sample of pure subtilisin was diluted to 30 ml in sterile MSB. A 1-ml amount of pure T4r+ phage was added to this suspension which was subsequently membrane-filtered. A 0.1-ml amount was withdrawn, and the radioactivity (counts per minute) was determined as described earlier. A similar procedure was followed with those samples obtained from the B. subtilis expended medium fractionation. A control of autoclaved expended medium plus T4r+ was also included. All samples were incubated at 37°C for 24 hr. Samples (1 ml) were removed and chromatographed on an A5m 100 to 200 mesh agarose column (Bio-Rad Laboratories) with the following specifications: dimensions, 1.3 X 27 cm; buffer, as above; gels, Bio-Gel P30, 100 to 200 mesh hydrated for 24 hr in the above buffer (0.5 ml at the top and base of the A5m column bed), and Bio-Gel A5m, 100 to 200 mesh (20.2 ml as the major bed); total bed volume, 21.2 ml; void volume (V0), 8.05 ml; total volume (Vt), 24.2 ml; flow rate, 5.7 ml/hr. Samples (1 ml) were collected, and the distribution of radioactive counts was determined as described above.

RESULTS

Effects of bacteria on the plaquing efficiency of T4r+ phage. It can be seen in the lower portion of Fig. 1 that growing cultures of B. megaterium, B. cereus, and B. subtilis demonstrated 45, 50, and 95% antagonism, respectively, against T4r+ phage, whereas neither S. lutea nor Micrococcus sp. elaborated any activity of this type. Although not illustrated in Fig. 1, the pellets that were resuspended in either fresh MSB or 0.1 M Tris after sedimentation and then tested for the presence of T4r+ phage showed virtually no such phage activity on E. coli B. Examination of those phage samples that had been incubated with 3-hr-old bacterial pellets resuspended in 0.1 M Tris, as well as the control samples, yielded data which showed that essentially no inhibition of T4r+ phage occurred during 24 hr of incubation (upper and lower portions of Fig. 1).

Phage inhibition, exoenzyme activity, sporulation, and pH. Determining the number of spores per milliliter, the production of α-amylase and protease (subtilisin), and the pH, as well as analyzing for the anti-T4r+ phage effect elaborated by B. subtilis expended growth media at different times during 32 hr of growth, yielded the results shown in Fig. 2. It is apparent that α-amylase and protease activity increased similarly with time and that maximal sporulation occurred between 16 and 24 hr after time zero.

![Fig. 1. Effect of growing bacterial cultures on T4r+ bacteriophage. Initial observations on the antagonistic nature of some Bacillaceae toward this virus.](image-url)
under these conditions of growth. Furthermore, both enzyme activities rose significantly during this time period with a concomitant upward shift in pH. Close examination of the inhibition of the plaquing efficiency of T4r+ phage reveals that its decrease paralleled, to some extent, the increase in exoenzyme activity as well as the increase in pH. This per cent change in phage infectivity is virtually identical to the results shown in Fig. 1.

Effect of pure subtilisin on T4r+ bacteriophage. Table 1 shows the effect that pure subtilisin, an exoprotease elicited by *B. subtilis*, had on the plaquing efficiency of T4r+ bacteriophage. All concentrations of subtilisin elaborated an inhibitory effect within 24 hr after initial incubation. The presence of 10 μg of the protease per ml renders 98.5% of the virus incapable of a normal infectivity cycle within this time period. It can also be seen that, as the concentration of the enzyme or the time of exposure to a certain concentration of enzyme was increased, there was a slight but concurrent increase in phage inhibition. Although pure subtilisin elicited a more rapid decrease in T4r+ phage titer than did *B. subtilis* expended media, a similar effect was seen when the virus was exposed to expended medium from *B. subtilis* (Table 1).

Activity of P-100 column fractions on the gel profile of pure 14C-labeled T4r+ phage. Pure subtilisin or 24-hr *B. subtilis* expended medium was chromatographed on a Bio-Gel P-100 column as described in the previous section. The results of these fractionations are shown in Fig. 3. The amount of protein (milligrams per milliliter) was determined as described by Kalckar (9). As Fig. 3 illustrates, the fractionation of 24-hr expended medium resulted in the appearance of two distinct protein peaks at elution volumes (Ve) of 11 and 22 ml, as well as peaks at Ve of 40 and 44 ml. One of these peaks (Ve = 22 ml) corresponded to the profile peak of commercial subtilisin. The peak at fraction 11, according to the known molecular weight pattern associated with this gel material, fell in a range

![Graph showing relationship of exoenzyme production, pH, and sporulation to the inhibition of T4r+ phage plaquing efficiency.](image)

**Fig. 2. Relationship of exoenzyme production, pH, and sporulation to the inhibition of T4r+ phage plaquing efficiency.**

### Table 1. Effect of pure subtilisin on the plaquing efficiency of T4r+ bacteriophage

| Enzyme concn* (μg/ml) | Residual T4r+ phage titer (PFU/ml) | Decrease in plaquing efficiency (%) |
|-----------------------|-----------------------------------|------------------------------------|
|                       | 24 hr                             | 72 hr                              | 24 hr | 72 hr |
| 10                    | 4.8 × 10⁴                         | 1.4 × 10⁸                          | 98.5  | 99.5  |
| 50                    | 3.4 × 10⁸                         | --                                 | 98.9  | --    |
| 100                   | 2.7 × 10⁹                         | 4 × 10⁶                            | 99.0  | >99.9 |
| 200                   | 3.36 × 10¹⁰                       | 2 × 10⁴                            | 99.8  | >>99.9 |
| 400                   | 2.1 × 10¹⁰                       | 7 × 10³                            | 99.9  | >>99.9 |
| 600                   | 4 × 10⁶                           | --                                 | >>99.9| >>99.9|
| 800                   | 3 × 10⁶                           | 1 × 10⁴                            | >>99.9| >>99.9|
| 1,000                 | 2 × 10⁶                           | 1 × 10⁴                            | >>99.9| >>99.9|
| Control A             | 2.9 × 10¹⁰                       | 3.1 × 10¹⁰                        | 7     | 0     |
| Control B             | 3.0 × 10¹⁰                       | 3.0 × 10¹⁰                        | 2.5   | 2.5   |
| Control C             | 3.8 × 10⁹                        | 7.8 × 10⁷                          | 88    | 99.75 |

* Control A is MSB at a pH of 7.5 containing 1.0 ml of T4r+ phage. Control B is autoclaved expended medium from a 24-hr culture of *Bacillus subtilis* containing 1.0 ml of T4r+ phage. Control C is 24-hr *B. subtilis* sterile expended medium containing 1.0 ml of T4r+ phage.  

* The presubtilisin and control initial T4r+ phage titer was 3.1 × 10⁹ PFU/ml.
comparable in molecular weight to α-amylase. Furthermore, the peaks found at other points in the elution profile most likely contained ribonuclease and its hydrolysis products from protease action on it, as well as other protein material.

Each of these fractions was tested against *T4r*+ phage which was subsequently analyzed chromatographically as described above (Fig. 4). There was a close similarity between the profiles of pure *T4r*+ phage exposed to commercial subtilisin and to the protease from the expended medium of *B. subtilis* from the P-100 column. After 24 hr, only 41% of the total radioactivity, initially associated with *T4r*+, was still associated with the phage in the control peak when treated with the protease peak from *B. subtilis* expended medium. A slightly larger fraction (56%) of the 14C label was found to be associated with the control phage peak when *T4r*+ was treated with commercial subtilisin. Nevertheless, between 50 and 60% of the phage-specific label had been released after treatment with these fractions. This does not, however, parallel the 90 to 95% decrease in phage plaquing potential shown in Fig. 1 and 2 during this same incubation time period. Exposure of *T4r*+ phage to the other fractions from the P-100 column did not produce such a response in the gel pattern. In fact, a very close similarity to the control peak was actually observed.

**DISCUSSION**

The results of this investigation demonstrate that an inhibition of *T4r*+ coliphage does, in fact, occur when it is treated with expended medium from *B. subtilis*. Growing cultures of *B. megaterium*, *B. cereus*, and *B. subtilis* exert 45, 50, and 95% antagonism toward this phage within 24 hr of exposure. Neither *S. lutea* nor *Micrococcus* sp. demonstrates this type of antagonism. It appears that *T4r*+ phage is not adsorbing to a nonspecific host which might cause this loss in plaquing efficiency, because virtually no change in virus titer was observed when the phage was mixed with 3-hr-old bacterial pellets resuspended in buffer. It appears, however, that the detrimental effect demonstrated against the phage is due to the action of an exoprotease produced by *B. subtilis*. Moreover, it is indicated that subtilisin, an extracellular *B. subtilis* protease, can be implicated as being at least partially responsible for this plaque potential decrease. This is evidenced in Table 1, which shows that a significant decrease in the activity of *T4r*+ phage occurs after 24 hr of incubation with several concentrations of the pure enzyme. The
effect produced by this enzyme appeared to be a hydrolytic one.

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