Bacteriophages of *Clostridium saccharoperbutylacetonicum*

Part VI. Further Characterization of HM-Phages

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Further characterization of the HM-phages of *Clostridium saccharoperbutylacetonicum* was described; plaque morphology, thermal inactivation, pH stability, inactivation by ultraviolet irradiation, and lysis of infected culture. Differences in the characteristics were observed among the three groups of HM-phages.

INTRODUCTION

On the characteristics of HM-phages of *Clostridium saccharoperbutylacetonicum* some studies were previously published.¹⁻⁴⁾

This paper presents further characterization of HM-phages; plaque morphology, thermal inactivation, pH stability, inactivation by ultraviolet irradiation, and lysis of infected culture.

Kinoshita and Teramoto⁵⁾ reported on these fundamental characteristics of phages of *Cl. acetobutylicum*.

MATERIALS AND METHODS

Phage and Bacterial Strains

Three representative phages of each group and their host strains were used^{1,3)}: phage HM 2 (group I) and host strain N1-4; HM 3 (group II) and N1-4; and HM 7 (group III) and N1-504.

Medium

TYA medium¹⁾ was used throughout the experiment.

Assay of Phages

The phages were assayed by the modified double-

layer method described previously.⁶⁾

Thermal Inactivation

To 9.9 ml of the medium at the test temperature, 0.1 ml of the phage suspension (1×10^6 PFU per ml) was added. Samples were removed at various time intervals, and assayed for phage survivals.

pH Stability

The medium was adjusted to pH values ranging from 3 to 10 by using sodium hydroxide or hydrochloric acid. pH was determined with a Hitachi-Horiba glass-electrode pH meter (model M3). To 9.9 ml of the medium at each pH value, 0.1 ml of the phage suspension (1×10^6 PFU per ml) was added. The broth was then incubated at 30°C for 60 minutes, and the phage survivals were assayed.

Inactivation by Ultraviolet Light Irradiation

Phages were suspended in phosphate buffer (0.002 M plus 10^{-3} M Mg^{++} or 0.067 M plus 10^{-3} M Mg^{++}) to obtain a final PFU of 10^4 per ml. The suspension (5 ml) was removed and irradiated for a given time in a petri dish (90 mm in diameter, less than 2 mm layer of liquid) placed at a distance of 40 cm from an ultraviolet lamp (Toshiba germicidal lamp, GL 15), while the dish was gently swirled by hand. The phage survivals were then assayed. All the experiments were carried out in dim light to avoid photo-reactivation.

Lysis of Multiply Infected Cultures

Four-hour broth culture of the host strain was ob-

1) M. Hongo and A. Murata, This Journal, 29, 1135 (1965).

2) M. Hongo and A. Murata, *ibid.*, 30, 292 (1966).

3) M. Hongo, T. Aono and A. Murata, *ibid.*, 30, 399 (1966).

4) M. Hongo, T. Miyamoto and A. Murata, *ibid.*, 30, 500 (1966).

5) S. Kinoshita and K. Teramoto, *J. Agr. Chem. Soc. Japan*, 29, 477 (1955).

6) M. Hongo and A. Murata, This Journal, 29, 1140 (1965).

tained in the usual manner.⁶⁾ To 18 ml of the medium in test tube (20 by 200 mm), 2 ml of the 4-hour broth culture was added, and this was infected with phage in the ratio of 10 phages to 1 cell. The infected culture was incubated anaerobically at 30°C by bubbling a highly purified nitrogen gas (purity over 99.995 percent, obtained commercially from Osaka-Oxygen Co.) into the culture, and the turbidity of the culture was measured at various time intervals at 660 m μ with a Tokyokoden photoelectric colorimeter (model 7A). For the blank reading uninoculated medium was used.

RESULTS AND DISCUSSION

Since the phages belonging to same group were similar to each other in all characteristics tested, only the results obtained with three representative phages of each group are presented.

Plaque Morphology

Typical plaque formation of HM-phages after 15 hours of incubation is represented in Fig. 1. The plaque formed by each phage had characteristic size and morphology. Phage HM 2 gave round plaques, with clear center and relatively sharp margins, of mean size of 2 mm in diameter. Plaques of HM 3 were similar to those of HM 2 in appearance, but had a mean size of 1 mm in diameter. HM 7

FIG. 1. Plaques of HM-Phages.

- (a) Phage HM 2 (on host NI-4)
 - (b) Phage HM 3 (on host NI-4)
 - (c) Phage HM 7 (on host NI-504)
- Magnification 1 $\times$.

produced pin-hole plaques.

Thermal Inactivation

Fig. 2 shows the stability of HM-phages various temperatures, ranging from 45° 65°C. All the phages in TYA medium

FIG. 2. Thermal Stability of HM-Phages.

In TYA medium (pH 6.2), heated at each temperature 10 min.

- HM2 ○ HM3 ■ HM7

FIG. 3. Thermal Inactivation Curves of HM-Phag

In TYA medium (pH 6.2), at 55°C.

- HM2 ○ HM3 ■ HM7

stable below 50°C, but were less stable above that point and were completely inactivated when exposed to 65°C for 10 minutes. Among the three phages, HM 2 was the most heat-stable and HM 3 the most heat-labile.

Fig. 3 shows the rates of inactivation of HM-phages in TYA medium at 55°C. The kinetics of inactivation of all the phages followed first-order reactions.

With respect to thermal stability of the phages for butanol-producing bacteria, some reports have been published.^{5,7-9} The phages of *Cl. saccharoperbutylacetonicum* seem to be more sensitive to thermal inactivation than those of other butanol-producing bacteria.

pH Stability

Fig. 4 shows the stability of HM-phages in TYA medium at various pH values, ranging from 3 to 10. All the phages were stable over the pH range of 5 to 8, but were less stable outside of this range. At pH 3 and pH 10 the phages were very unstable. There were

FIG. 4. pH Stability of HM-Phages.

In TYA medium, at 30°C for 60 min.

● HM2 ○ HM3 ■ HM7

7) J. C. Sylvester, Ph. D. Thesis of Univ. of Wisconsin (1943).
8) B. M. Nakhmanovich, S. G. Malinkin and V. V. Senkevich, *Mikrobiologiya*, 25, 77 (1956).

9) J. Kovats, *Przemysł Spożywczy*, 11, 113 (1957).

no large differences in stability among the three phages. On the acid side, however, HM 3 was more stable than the other two phages.

FIG. 5. Ultraviolet Light Inactivation Curves of HM-Phages.

In phosphate buffer (0.067 M or 0.002 M, $+10^{-8}$ M Mg^{++} , pH 6.2)

● HM2 ○ HM3 ■ HM7

FIG. 6. Lysis Curves of Cultures Infected with HM-Phages.

The multiplicity of infection: ca. 10.

△ uninfected control
● infected with HM2
○ infected with HM3

Inactivation by Ultraviolet Light Irradiation

Fig. 5 shows the ultraviolet light inactivation curves of HM-phages. Of the three phages, HM 2 was the most resistant to ultraviolet light, and HM 7 was the most sensitive. The kinetics of inactivation of HM 2 and HM 7 followed first-order reactions, but that of inactivation of HM 3 did not; the curve of HM 3 bended downward.

Lysis of Multiply Infected Cultures

Cultures of host bacteria were multiply infected, and the turbidity of the culture was measured continuously. The results are presented in Fig. 6. The turbidity of the culture infected with HM 2 remained constant after infection, began to decrease after about 50 minutes and reached its minimum after 120 minutes. Decrease in turbidity was small.

With the culture infected with HM 3, the turbidity began to decrease after about 100 minutes and reached its minimum after 180 minutes, and decrease in turbidity was large. These lysis curves are in good agreement with the result of each phage multiplication (the minimum latent period of HM 2 is about 45 minutes, and that of HM 3 about 90 minutes).⁴⁾ HM 7-infected culture of host strain N1-504 showed a marked agglutination just prior to and during lysis. This interfered with turbidity measurement, because the clumps were not dispersed by ordinary shaking. Consequently, the turbidity of HM 7-infected culture was not measured.

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