Bacteriophages of *Clostridium saccharoperbutylacetonicum*

Part XVI. Isolation and Some Characters of a Temperate Phage*

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A temperate phage (designated as HM T) was isolated from an abnormally-fermented broth of butanol fermentation simultaneously with the isolation of a virulent phage HM I described previously.

Phage HM T lysogenized *Clostridium saccharoperbutylacetonicum* strain N1-4, but the frequency of lysogenization was very low. The phage-bacterium combination in lysogenized cells was stable. HM T was not inducible, although the bacterial lysis was induced by UV and mitomycin C.

HM T was very similar to HM I in serology, morphology, host-range, DNA base composition and others. It may be concluded that HM I is a weak virulent mutant derived from HM T.

HM T had a DNA base composition that differed from that of its host.

We isolated a virulent phage (HM I) active against the parent strain N1-4 of *Clostridium saccharoperbutylacetonicum* from the first abnormally-fermented broth of butanol fermentation, as already described in the first paper of this series.¹⁾ Subsequently, we isolated from the same broth a temperate phage that could lysogenize strain N1-4.

Little is known about the lysogeny in butanol-producing bacteria; only a report on a temperate phage of *Cl. acetobutylicum* was published by Kinoshita and Teramoto.²⁾ Recently, Hongo *et al.*^{3,4)} reported on the studies

of lysogeny in nonpathogenic clostridial bacteria, including butanol-producing bacteria. A few studies were reported on the isolation and some characters of lysogenic systems in pathogenic *Cl. perfringens*.^{5~7)}

The present study was undertaken to elucidate the general characters of the temperate phage (designated as HM T) of *Cl. saccharoperbutylacetonicum* and to clarify the relationship between the temperate phage and the virulent phage of the same bacterium.

MATERIALS AND METHODS

Phage and bacterial strains. A temperate phage HM T and a strain N1-4 of *Cl. saccharoperbutylacetonicum*, an indicator strain, were used throughout the work. HM I and other HM-phages were also used in part

* A part of this work was presented at the Annual Meeting of the Agricultural Chemical Society of Japan, Fukuoka, April, 3, 1961.

1) M. Hongo and A. Murata, *Agr. Biol. Chem.*, **29**, 1135 (1965).

2) S. Kinoshita and K. Teramoto, *Nippon Nogei-kagaku Kaishi*, **29**, 487 (1955).

3) H. Hongo, A. Murata, K. Kono and F. Kato, *Agr. Biol. Chem.*, **32**, 27 (1968).

4) M. Hongo, A. Murata, S. Ogata, K. Kono and F. Kato, *ibid.*, **32**, 773 (1968).

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of the work for comparison.

Lysogenic strains N1-4 (HM T) were isolated from surviving colonies on an agar plate spread with phage lysate. They were made free from free phages by the serial single-colony isolation method and tested for their immunity and ability to produce phage. They were maintained in sand culture as spore.

Media and cultural condition. TYA medium¹⁾ was used throughout the work, and PG medium³⁾ was also used in part of the work for sporulation and germination of spores. A technique of anaerobiosis was adopted using ordinary anaerobic jars. Cultures in the logarithmic phase were prepared by adding a 10% inoculum of about 18-hr broth culture to a fresh TYA broth and incubating the culture for about 4 hr. All incubations were carried out at 30°C unless otherwise stated.

Assay of phage and bacteria. The phage and bacteria were assayed by the modified double-layer method described previously.⁸⁾

Responses of infected bacteria. Cells in the logarithmic phase were infected with phages and stood for 5 min for adsorption and the suspension was plated on the plain agar medium.

In order to distinguish lysogenized cells from uninfected or refractory cells, the immunity and ability for phage production of the colonies formed on the plates were studied.

Cells that give a lytic response form plaques on agar medium spread with indicator.

Solvent analysis. Butanol and ethanol were determined by the method of Johnson,⁹⁾ and acetone by the method of Goodwin.¹⁰⁾

Concentration and purification of phage. The phage was concentrated and purified from an about 10-hr broth culture of lysogenic strain (usually contained $1 \sim 3 \times 10^{10}$ PFU/ml) by differential centrifugation and treatment with enzymes as previously described.^{11,12)}

Irradiation with UV. A sample solution (5 ml) in a petri dish (90 mm in diameter) was irradiated for a given time at a distance of 400 mm from an ultraviolet lamp (GL-15, Toshiba germicidal lamp) with a gentle swirling by hand. The experiments were carried out under dim light.

Turbidity measurement. Turbidity of a culture was measured at 660 m μ in a photoelectric colorimeter (model 7A, Tokyokoden Co.). The blank reading was made by the use of an uninoculated broth.

Electron microscopy. Phage suspension was mixed with an equal volume of 2% phosphotungstic acid (pH 7.0) according to the method described by Brenner and Horne.¹³⁾ A droplet of the mixture was placed on a carbon-coated collodion-filmed grid and air-dried. The specimens were examined under an electron microscope (JEM-7A, Japan Electron Optics Laboratory Co.).

Adsorption and one-step growth experiment. The techniques were the same as described previously.¹⁴⁾

Host-range determination. Phage suspension (ca. 10^6 PFU/ml) was spotted on overlayer of an agar plate spread with testing bacteria. The plate was incubated at the temperatures (30~37°C) optimal for each testing bacteria and appearance of a lysis zone was examined after 15~24 hr.

Antiphage serum. Antiphage serum was prepared by repeated injection of the phage suspension into rabbits. Details of the preparation and measurement of velocity constant (k of neutralization) were given in a previous paper.¹⁵⁾

DNA. The isolation of DNA from phage and bacteria and the determination of DNA base composition were made by the method previously described.¹²⁾

RESULTS

Source of phage isolation

It was already described that frequent abnormal fermentations caused with phage in-

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9) M. J. Johnson, *Ind. Eng. Chem. Anal. Ed.*, **4**, 20 (1932).

10) L. F. Goodwin, *J. Am. Chem. Soc.*, **42**, 39 (1920).

11) M. Hongo and A. Murata, *Agr. Biol. Chem.*, **30**, 292 (1966).

12) M. Hongo, H. Ono, S. Ogata and A. Murata, *ibid.*, **30**, 982 (1966).

13) S. Brenner and R. W. Horne, *Biochem. Biophys. Acta*, **34**, 103 (1959).

14) M. Hongo, T. Miyamoto and A. Murata, *Agr. Biol. Chem.*, **30**, 501 (1966).

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

fection occurred in the industrial production of butanol with *Cl. saccharoperbutylacetonicum* and that a virulent phage designated as HM 1 was isolated from the first abnormally-fermented broth. Subsequent investigations revealed that another phage was present in the same abnormally-fermented broth, but a proportion of the number of the phage to HM 1 was very small. The phage was found to lysogenize the parent strain N1-4 of *Cl. saccharoperbutylacetonicum*, indicating that the phage was a temperate phage. This phage was designated as HM T.

Plaque morphology

After 15-hr of incubation round clear plaques with obscure margin were observed. The plaques with a peripheral ring appeared after prolonged incubation. However, the plaques with a turbid center which were common and characteristic for the plaques of phage KT of *Clostridium* sp.,⁴¹ were not observed. As is obvious from Fig. 1 which

FIG. 1. Plaques with and without a Peripheral Ring Formed by Phage HM T.

Magnification; $3\times$.

shows a plaque formation after 24-hr of incubation, the phage does not give plaques that are uniform in size (0.3~2 mm in diameter) and in morphology (with or without a

ring). Several plaques of each of the variants were picked and replated, but plaques uniform in size and morphology were not formed regardless of the plaque-type picked. The results show that the heterogeneity of plaque is not an indication of the presence of more than one phage type in the phage stocks.

Lysogenization

When bacteria were infected with phage HM T, most of the infected bacteria were lysed and only a small number of infected bacteria were lysogenized. The percentage of lysogenic responses was varied with each experiments, but was around 0.1 per cent. Namely, HM T was a temperate phage with a very low efficiency of lysogenization. The results account for the finding that the phage does not form turbid plaque.

It is known in some lysogenic systems that the frequency of lysogenization can be drastically modified by changing the conditions of infection and incubation.¹⁶¹ With the HM T system, however, the frequency of lysogenization could not be influenced by such conditions as temperature of incubation (20, 30 and 37°C), the multiplicity of infection (0.1, 1 and 10) or addition of chloramphenicol (30 μ g/ml). The percentage of lysogenic responses was around 0.1 per cent in all the conditions.

Characters of lysogenized bacteria

About twenty strains of bacteria lysogenized with phage HM T were obtained. All of them were not in a carrier state or pseudo-lysogenic state, but stable lysogeny. The stable phage-bacterium combination was ascertained as follows: (i) Lysogenic bacteria were incubated in PG broth for several days for sporulation at the maximum rate. The spores were then heated at 80°C for 10 min to inactivate free phages completely and to kill vegetative cells, and incubated in a fresh PG broth for several days. Such treatment,

16) A. Lwoff, *Bact. Revs.*, **17**, 307 (1953).

subculture of sporulation and heating, were repeated five times. Even after repeated subcultures all the lysogenic bacteria retained their capacity to produce phage HM T. (ii) When about twenty colonies derived from the cultures stated above were examined, all of them were found to be lysogenic. (iii) Lysogenic strains preserved their capacity to produce phage when cultivated in the presence of an antiphage serum that neutralized the infectivity of all free phage. (iv) They were immune against the homologous phage.

The spontaneous production of phage from the lysogenic strains was very high, and reached its maximum at about 10-hr of incubation (up to 3×10^{10} PFU/ml).

As a consequence of lysogenization with HM T, changes were hardly observed in properties of the host bacteria. As shown in Table I, solvents yield of the lysogenic strain

FIG. 2. Induction of Lysis of Lysogenic Strain N1-4 (HM T) by Ultraviolet Light.

Cells in logarithmic phase were harvested by centrifugation and suspended in a phosphate buffer (0.002 M, pH 6.2) (*ca.* 1×10^8 cells/ml). After exposure to UV, suspensions were diluted 1:10 in a fresh TYA broth and incubated in a test tube with a fermentation-pung.

● unirradiated control ○ 3 sec irradiation
■ 5 sec irradiation □ 7 sec " "
▲ 10 sec " △ 30 sec "

TABLE I. SOLVENTS YIELD OF LYSOGENIC STRAIN

Strain	Yield based on sugar added (%)				Time of fermentation (hr)
	Total solvents	Butanol	Acetone	Ethanol	
N1-4	36.9	29.0	6.6	1.3	<i>ca.</i> 30
N1-4 (HM T)	37.0	29.1	6.7	1.2	<i>ca.</i> 40

Medium, TYA broth (sugar 3.9%) 100 ml; inoculum 10 ml; incubated at 30°C.

was not differ from that of the parent strain. However, the lysogenic strain apparently grew considerably slower than the parent strain. Mass doubling time for the lysogenic strain was about 90 min, whereas that for the parent strain was about 60 min. One of the reason for long mass doubling time might be due to the high percentage of spontaneous lysis in the lysogenic culture.

Induction of bacterial lysis by ultraviolet light and mitomycin

When a culture of the lysogenic strain was exposed to an optimal dose of ultraviolet light, mass lysis was induced after about 4-hr of a

latent period as shown in Fig. 2. Induction of lysis could also be effected by exposure to mitomycin C. However, lysis was not accompanied by phage liberation; plaque assays showed less phage in the induced lysates than in the unexposed control. The results indicate that phage HM T is uninducible.

Studies on the lysis induction are in progress.

Host-range

A large number of strains belonging to butanol-producing clostridia and closely related bacteria were tested to determine the host-range of phage HM T. The list of the

bacteria tested was the same as previously presented.¹¹ Like phage HM 1, the host-range of HM T was restricted to a strain N1-4 of *Cl. saccharoperbutylacetonicum*.

Adsorption and one-step growth

Phage HM T was highly adsorbed on its sensitive bacteria at a rate of 90 per cent in 5 min.*

One-step growth of the phage was studied by exposing a sensitive strain to the phage. The minimal latent period was about 50 min and an average burst size was about 400.

Adsorption and growth characters are similar to those of phage HM 1. The large burst size and the high frequency of spontaneous lysis stated above might account for the high titer of free phage in the lysogenic culture.

Morphology

Phage HM T virion consisted of a head and a intricate tail. The head had a nearly regular hexagonal outline and was about 45 m μ in diameter. The tail showed many appendages symmetrically arranged around the central tail. The length of tail was about 30 m μ . The more detailed structure of the phage, together with that of other HM-phages, will be described elsewhere.¹⁷⁾

In size and shape, HM T is very similar to phage HM 1. However, HM T differs from any temperate phages reported so far in clostridial bacteria: Phage m of *Cl. acetobutylicum* was reported to have a spherical head (about 40 m μ in diameter) without tail;¹⁸⁾ a phage of *Cl. perfringens*, a hexagonal head (about 50 m μ in diameter) and a long flexible tail (about 200 m μ in length);⁷⁾ phage KT of *Clostridium* sp., a hexagonal outlined head (about 85 m μ in diameter) and a tail with contractile sheath (about 100 m μ in length).⁴⁾

* Phage (final 1×10^8 PFU/ml) and bacteria (final 1×10^8 cells/ml) were mixed in TYA broth at 30°C.

17) M. Hongo *et al.*, *Agr. Biol. Chem.*, in preparation.

18) S. Kinoshita, S. Itagaki, T. Okumura, M. Terada and T. Tomii, *Nippon Nogeikagaku Kaishi*, **26**, 104 (1952).

Serology

The K-value of phage HM T for its homologous antiserum was about 200. The serum was tested against phage HM 1 and other HM-phages to detect neutralization reactions. The results are shown in Table II. The

TABLE II. NEUTRALIZATION OF HM-PHAGES BY ANTI PHAGE HM T SERUM

Phage	K-value
HM T	230
HM 1 (group I)	230
HM 2 (")	210
HM 8 (")	240
HM 9 (")	190
HM 10 (")	190
HM 11 (")	200
HM 12 (")	240
HM 3 (group II)	0
HN 7 (group III)	0

K-values represent average of two or more trials. Standard error: ca. 10%.

serum reacted with HM 1 and other HM-phages of group I, but not with HM 3 of group II and HM 7 of group III. No significant difference was found in K-values of the serum when tested with HM 1 and other HM-phages of group I. The results indicate that HM T and HM 1 (and other HM-phages of group I) are very closely related.

Nucleic acid

The purified phage HM T was analyzed for DNA and RNA by the diphenylamine¹⁹⁾ and the orcinol reaction,²⁰⁾ respectively. Consequently, the phage was found to contain only DNA as the nucleic acid moiety. DNA was isolated from the phage by the phenol method.¹²⁾ It contained usual four bases and no unusual bases. The DNA base composition of the phage determined chemically, together with that of phage HM 1 and host bacteria, is listed in Table III.

The DNA base composition is very similar

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TABLE III. DNA BASE COMPOSITION OF PHAGE HM T AND HOST BACTERIA

DNA from	Mole per cent				
	Guanine	Adenine	Cytosine	Thymine	G+C
Phage HM T	17.5±0.3	33.0±0.8	17.1±0.5	32.4±0.6	34.6±0.5
(Phage HM 1)	(17.4±0.4)	(32.5±0.7)	(17.6±0.5)	(32.3±0.5)	(35.0±0.5)
Indicator strain	15.6±0.5	34.0±0.8	15.4±0.5	35.0±0.8	31.0±0.5
Lysogenic strain	15.8±0.6	34.2±0.7	15.5±0.5	34.5±0.7	31.3±0.6

Determined by paper chromatography.

in HM T and HM 1, confirming that the two phages are closely related. However, the base composition of the temperate phage differs significantly from that of the lysogenic or indicator host strains.

DISCUSSION

Temperate phage HM T and virulent phage HM 1 were isolated from the same abnormally-fermented broth of butanol fermentation. Therefore, it is one of the purposes of the present study to clarify the relationship between the two phages.

A comparison of the properties of the two phages gave the following results: (i) HM T and HM 1 are serologically indistinguishable; (ii) they are morphologically indistinguishable; (iii) they show similar growth characters; (iv) they have same host-range; (v) they have similar DNA base composition. In addition, lysogenized cells with HM T are immune against HM 1. HM 1 inactivated by ultraviolet light does not kill sensitive bacteria,²¹⁾ and the lytic activity of HM 1 is weak.²²⁾ It is known that temperate phages can give rise to virulent mutants.²³⁾ Therefore, it may be concluded that HM 1 is a weak virulent mutant derived from HM T. However, the

origin of the temperate phage is at present unknown.

Studies on nucleic acid of temperate phages and lysogenic bacteria of the genus *Clostridium* have not been published. The DNA base composition of temperate phages is, in general, very similar to that of the host bacteria.²⁴⁾ In this HM T system, however, the value of 35% G+C obtained for HM T phage DNA differs significantly from that of the DNA (31% G+C) of the lysogenic and indicator strains. There are a few instances, in phages of the genus *Bacillus*, that temperate phages have a DNA base composition which differs from that of its hosts.²⁵⁻²⁸⁾

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21) M. Hongo and S. Ogata, *Agr. Biol. Chem.*, **33**, 1 (1968).

22) M. Hongo and A. Murata, *ibid.*, **30**, 913 (1966).

23) F. Jacob, E. Wollman and L. Siminovich, *Compt. Rend.*, **236**, 544 (1953).