KM1, a Bacteriophage of *Clostridium butyricum*

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Characteristics of bacteriophage KM1, which infects *Clostridium butyricum*, are described, and several important properties are compared with those of phage 5, which infects the same species. Phage KM1 has a hexagonal head and a tail with a contractile sheath. Agarose gel electrophoresis patterns of *Bgl*II, *Eco*RI and *Hind*III fragments of the two phage DNAs differed greatly. The G + C contents were 30.5 mol % for phage KM1 and 35.4 mol % for phage 5. Among the 32 strains of clostridia tested, phage KM1 infected only *C. butyricum* MII588. Other properties of phage KM1 are also discussed.

INTRODUCTION

Cowles (1934) isolated a bacteriophage which infected *Clostridium tetani*, and since then, the properties of phages infecting various species of *Clostridium* have been reported. The lysis of *C. butyricum* by *C. saccharoperbutylacetonicum* enzymes induced by the phages HM 3 and HM 7 was briefly mentioned by Tahara *et al.* (1975) and Hongo *et al.* (1974). In a report concerning phages of *C. butyricum* (Schlechte *et al.*, 1980), phage 5 was used to differentiate two oncolytic strains, *C. oncolyticum* M55 and *C. butyricum* H8. In this paper, we describe some morphological and physicochemical properties of phage KM1, which lyses *C. butyricum* MII588 and which is quite different from phage 5.

METHODS

Phages and bacterial strains. Phage KM1, which infects *C. butyricum* MII588, was isolated from soil. Phage 5, *C. oncolyticum* M55 (*C. butyricum* ATCC 13732) and *C. butyricum* H8 were kindly supplied by Dr H. Schlechte, Zentralinstitut für Krebsforschung der Akademie der Wissenschaften, Berlin-Buch, DDR. *C. butyricum* MII588 and *C. oncolyticum* M55 were used to prepare each phage lysate. Strains used for the host range test are given in Table 1.

Media. PYG broth, consisting (w/v) of 0.5% glucose (Wako Pure Chemical Industries), 0.5% polypeptone (Wako) and 0.3% yeast extract (Wako), was used for liquid medium. Glucose nutrient agar, consisting (w/v) of 0.5% glucose (Wako), 1.0% polypeptone (Wako), 0.5% meat extract (Wako), 0.3% NaCl, 0.3% CaCO₃ and 1.5% agar (Wako), was used for solid medium. Glucose nutrient agar without CaCO₃ and with 0.8% (w/v) agar was used for semisolid medium overlays.

Preparation of phage lysates. CaCO₃ (0.5%, w/v) was added to an early exponential phase culture of *C. butyricum* MII588 in PYG broth. After 5 min incubation, phage KM1 was added at an m.o.i. of about 0.001 and the culture was incubated at 37 °C overnight. The culture was centrifuged at 800 g for 20 min and the supernate was sterilized by filtration through a 0.45 µm Millipore membrane. With *C. oncolyticum* M55, the organism was grown in GAM broth (Nissui Seiyaku), phage 5 was added at an m.o.i. of about 0.001, and the mixture was incubated at 37 °C overnight. Phage KM1 was assayed for p.f.u. by the modified double layer method described by Hongo & Murata (1965).

Electron microscopy. Ammonium sulphate was added to lysates of phage KM1 or phage 5 at a concentration of 280 g l⁻¹. After overnight incubation at 4 °C, the resultant suspensions were centrifuged at 9000 g for 30 min. The

Table 1. *Strains of Clostridium used in this study*

Species	Strain no.*	Species	Strain no.*
<i>C. butyricum</i>	MII588†	<i>C. oncolyticum</i>	M55§
	IAM 19001	<i>C. butylicum</i> subsp. <i>convexa</i>	IAM 19092
	IFO 3315		IAM 19108
	IFO 3858	<i>C. beijerinckii</i>	ATCC 25752
	IAM 19225	<i>C. pasteurianum</i>	ATCC 6013
	ATCC 6015	<i>C. acetobutylicum</i>	IAM 19012
	ATCC 8260	<i>C. perfringens</i>	NCTC 8237
	ATCC 9690		NCTC 528
	ATCC 17791	<i>C. difficile</i>	IFM 2148†
	ATCC 859		ATCC 9689
	ATCC 860	<i>C. sporogenes</i>	ATCC 3584
	ATCC 19398	<i>C. paraputrificum</i>	ATCC 25780
	ATCC 25779	<i>C. ramosum</i>	ATCC 25582
	IAM 19004	<i>C. cadaveris</i>	ATCC 25783
	IAM 19016	<i>C. indolis</i>	ATCC 25771
	H8‡	<i>C. innocuum</i>	ATCC 14501

* Sources: IAM, Institute of Applied Microbiology, University of Tokyo, Japan; IFO, Institute of Fermentation, Osaka, Japan; ATCC, American Type Culture Collection, Rockville, Md., USA; NCTC, National Collection of Type Cultures, London, UK.

† This study.

‡ Schlechte *et al.* (1980).

§ *C. butyricum* ATCC 13732; Schlechte *et al.* (1980).

|| Matteuzzi *et al.* (1977).

pellet was dialysed against distilled water, mixed with 2% (w/v) phosphotungstic acid and placed on 200-mesh Formvar-coated copper grids. Electron micrographs were taken with an H-800 electron microscope (Hitachi Seisakusho).

Host range. The cross-streak method was adopted using lysates of phage KM1 or phage 5 (about 1.0×10^9 p.f.u. ml⁻¹) and clostridia strains (Table 1) to determine the host range. Plates were incubated at 37 °C for 48 h.

Isolation of phage DNA. The phage pellet, obtained by ammonium sulphate precipitation as described above, was dialysed at 4 °C for 7 h against 0.15 M-phosphate buffer (pH 7.0) containing gelatin (20 mg l⁻¹). DNAase I (Sigma) was added to a final concentration of 1 µg ml⁻¹ and the reaction mixture was shaken at 37 °C for 45 min. The phage pellet was obtained by centrifugation at 100000 g for 30 min and was resuspended in 2.0 ml SSC (standard saline citrate; 0.015 M-sodium citrate, 0.15 M-NaCl, pH 7.0). A 2.0 ml sample of 100% (v/v) phenol was added to the suspension and gently shaken at room temperature for 10 min. After centrifugation at 1000 g for 10 min, the lower phenol layer was removed and the phenol extraction process was repeated. The upper aqueous layer was dialysed against SSC at 4 °C for 48 h and stored at 4 °C.

G + C contents of phage DNAs. Melting temperature (T_m) of phage DNAs in SSC extracted as described above was measured with a Gilford spectrophotometer 2400S.

Restriction endonuclease digestion. λ phage DNA (Sigma) was used as a standard. Restriction endonucleases BglII, EcoRI and HindIII were obtained from Boehringer Mannheim, and digestion was done according to the manufacturer's directions.

Agarose gel electrophoresis of DNA fragments. Samples (20 µl) of phage DNA or endonuclease-digested phage DNA were mixed with 3 µl dye solution containing 0.175% (w/v) bromophenol blue and 60% (w/v) sucrose, and were applied to 0.7% (w/v) agarose gels. The electrophoresis was done at 110 V for 2.5 h in horizontal submarine gels in a buffer (pH 7.9) containing 40 mM-Tris, 5 mM-sodium borate, and 1 mM-EDTA. After electrophoresis, the gels were stained with ethidium bromide (0.5 µg ml⁻¹ in water) and then photographed under shortwave UV light with a Polaroid type high-speed no. 57 film.

Growth studies of phage-infected strain. A 3 ml sample of PYG broth prepared with 0.067 M-phosphate buffer (pH 7.0) in a 10 ml test tube, was overlaid with 2 ml liquid paraffin. Optical density growth curves of *C. butyricum* MII588 were recorded at 600 nm. When the OD₆₀₀ reached 0.3, phage KM1 was added at an m.o.i. of 1.0, 0.1 or 0.01 and the OD₆₀₀ was read at appropriate intervals.

Production of antisera. Two male albino rabbits, each weighing 2 kg, were injected subcutaneously into the dorsum and foot pad with a mixture of 0.2 ml phage KM1 lysate (about 1×10^6 p.f.u. ml⁻¹) and 0.2 ml Freund's complete adjuvant, followed by subcutaneous injection into the dorsum with 0.5 ml of the lysate (about $1 \times$

Fig. 1. Electron micrographs of phage KM1 (a) and phage 5 (b). Bar, 100 nm.

10^7 p.f.u. ml^{-1}) at 4-d intervals. At 20 and 24 d after the first injection, 0.8 ml of the lysate (about 1×10^8 p.f.u. ml^{-1}) was injected via the ear vein; 10 d after the last injection, the rabbits were bled from the carotid artery. The serum was absorbed with cells of *C. butyricum* MII588, and then inactivated at 56°C for 30 min.

Average burst size. A 0.8 ml sample of an exponential phase culture of *C. butyricum* MII588 (4.2×10^7 cells ml^{-1}) in nutrient broth was mixed with 0.1 ml phage KM1 lysate (6.4×10^8 p.f.u. ml^{-1}) and incubated at 37°C . After 10 min, 0.1 ml phage KM1 antiserum was added and after a further 5 min incubation, 0.1 ml of the mixture was added to 9.9 ml nutrient broth and incubated at 37°C . The samples were assayed for phage after 20, 30 and 40 min.

Effect of divalent cations on adsorption and release of phage KM1. By the procedure of Nakajima & Murooka (1983), CaCl_2 , MgCl_2 , sodium citrate or sodium oxalate was added to nutrient broth desalted with Amberlite IR-120B. The final concentration of the former two additions was 0.2% (w/v) and that of the latter two 0.5% (w/v). To 8.9 ml of each broth, 1 ml of an exponential culture of *C. butyricum* MII588 (9.2×10^6 cells ml^{-1}) and 0.1 ml phage KM1 lysate (9.2×10^6 p.f.u. ml^{-1}) were added and incubated at 37°C . After incubation for 7, 15, 20, 40 and 60 min, the cultures were centrifuged at 9000 g for 10 min and the supernatants were assayed for phage.

Isolation of phage KM1-resistant mutants. Spontaneous phage KM1-resistant mutants of *C. butyricum* MII588 were selected by mixing 1×10^6 endospores and 1×10^9 phages on plates. After incubation at 37°C for 48 h, colonies were picked and purified by three serial single colony isolations.

RESULTS

Morphology and host range. Typical plaques of phage KM1 after incubation for 48 h and phage 5 after incubation for 24 h at 37°C were similar, being clear and 1–2 mm in diameter. Electron microscopy showed phage KM1 particles to have hexagonal heads 60 nm in diameter and tails with a contractile sheath 90 nm long. Phage 5 particles have elongated heads measuring 50×80 nm and flexible, sheathless tails 125 nm long (Fig. 1). Phage KM1 infected only one strain, *C. butyricum* MII588, while phage 5 infected three strains, *C. butyricum* IAM 19004, *C. butyricum* H8 and *C. oncolyticum* M55 (*C. butyricum* ATCC 13732).

G + C contents of phage DNAs. The G + C contents were 30.5 mol % for phage KM1 and 35.4 mol % for phage 5. These values were slightly higher than that of *C. butyricum* (27–28 mol %; Smith & Hobbs, 1974).

Analysis of phage DNAs by agarose gel electrophoresis. Electrophoresis patterns of DNAs extracted from phages KM1, 5 and λ and treated with restriction enzymes *Bgl*II, *Eco*RI and *Hind*III showed marked differences. Phage 5 DNA was not digested by *Bgl*II. By comparison with λ phage DNA, the M_r both of phage KM1 and of phage 5 DNA was determined to be 31×10^6 .

Growth studies of phage-infected strain. Fig. 2 shows lytic curves of cultures infected with phage KM1. Infection at an m.o.i. of 1.0 or 0.1 resulted in a remarkable decrease in OD of about 0.2.

Fig. 2. Lysis of cultures infected with phage KM1. *C. butyricum* MII588 was infected with phage KM1 at an m.o.i. of 1.0 (■), 0.1 (▲) or 0.01 (●). Growth curves at OD₆₀₀ are compared with that of an uninfected control (○).

Average burst size. The change of p.f.u. ml⁻¹ at each incubation time was 8.4×10^4 for 0 min, 9.6×10^6 for 20 min and 2.6×10^7 for 30 min. At 30 min after incubation, the burst had almost finished and no further increase of p.f.u. occurred. This data indicated that the average burst size of phage KM1 was about 76.

Effect of divalent cations on adsorption and release of phage KM1. Neither Mg²⁺ nor Ca²⁺ had any effect on adsorption of phage KM1. However, in the presence of divalent cations, the phage was released after incubation for 20 min, but without the divalent cations, no release occurred even after 60 min incubation.

Preparation of phage KM1-resistant mutants. Stable phage KM1-resistant mutants of *C. butyricum* MII588, Res 1, Res 2 and Res 3, were obtained. Their biochemical and physiological properties, such as carbohydrate fermentation, gelatin liquefaction, nitrate reduction and behaviour with milk, were the same as those of the parent strain MII588.

DISCUSSION

Phage KM1 has a hexagonal head and a contractile sheath resembling phage $\alpha 1$, which infects *C. botulinum* (Kinouchi *et al.*, 1981). In contrast, phage 5 has an elongated head and a flexible, sheathless tail resembling phages which infect *C. sporogenes* (Betz, 1968; Beatriz *et al.*, 1981) or several strains of proteolytic types of *C. botulinum* (Eklund *et al.*, 1969; Dolman & Chang, 1972).

Among the 32 strains tested (Table 1), only *C. butyricum* MII588 was lysed by phage KM1. On the other hand, *C. oncolyticum* M55 (*C. butyricum* ATCC 13732), *C. butyricum* H8 and *C. butyricum* IAM 19004 were lysed by phage 5, and it should be emphasized that all three phage 5-sensitive strains formed rough colonies. *C. butyricum* ATCC 25779 and *C. butyricum* IAM 19016 also formed rough colonies, but they were not lysed by phage 5. The classification of all of these rough strains is doubtful because their cultures contain little butyric acid; they also digest gelatin and casein. When the cultures of *C. oncolyticum* M55 and *C. butyricum* IAM 19004 were analysed by steam chromatography, it was found that they produced acetic, propionic, butyric, isobutyric, isovaleric and isocaproic acids. When these two strains were tested for carbohydrate fermentation, they showed positive reactions with cellobiose, fructose, glucose, maltose, sorbitol and sucrose. *C. butyricum* typically produces smooth colonies (Smith & Hobbs, 1974), and thus the rough strains are assumed to be atypical. According to these morphological and biochemical characteristics, they might better be classified as *C. sporogenes*.

Agarose gel electrophoresis of the two phage DNAs digested with endonucleases showed completely different patterns; the two phages lacked DNA homology.

It is known that monovalent cations inactivate phages by the release of phage DNA and the denaturation of phage protein (Hongo & Ogata, 1969). Hongo *et al.* (1969) reported inactivation of phage HM 2 with various monovalent cations. However, phage KM1 was not inactivated by Na^+ or K^+ under the same conditions. Ca^{2+} and Mg^{2+} did not affect the adsorption of phage KM1 to the host. However, these divalent cations are indispensable for the release of the phage. Shew (1949) reported that Ca^{2+} was necessary for multiplication of a phage although its host bacteria did not require Ca^{2+} for growth.

The phage KM1-resistant mutants and the parent strain were treated with UV and mitomycin C, and no induction of phage KM1 occurred. This result confirmed that phage KM1 is a virulent type.

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