Characterization of Temperate *Bacillus* Bacteriophage ϕ 105

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Temperate *Bacillus* phage ϕ 105 is serologically unrelated to previously described virulent *Bacillus* phages. Phage ϕ 105 is incapable of generalized transduction. Prophage ϕ 105 is inducible with mitomycin C. Phage ϕ 105 contains double-stranded deoxyribonucleic acid (DNA) with a molecular weight of about 25×10^7 as determined by band sedimentation and electron microscopy. The per cent guanine plus cytosine of ϕ 105 DNA is 43.5 as determined by buoyant density in CsCl and by thermal denaturation. Phage ϕ 105 DNA may contain complementary single-stranded ends.

The genome of the temperate *Bacillus* phage ϕ 105 was recently shown to become linearly integrated at a specific site in the chromosome of its host bacterium, *B. subtilis* 168, after lysogenization (13). Linkage between prophage and bacterial markers has been demonstrated in transfection with deoxyribonucleic acid (DNA) extracted from lysogenic cells (10). Prophage, vegetative, and mature DNA from ϕ 105 are all infectious for competent cells, but the three classes of DNA show specific characteristics in transfection (14). These facts make the ϕ 105-*B. subtilis* 168 system very useful for studies of the integrated prophage as well as for studies on the mechanism of transfection with bacteriophage DNA.

The aim of the present work is to give a general characterization of phage ϕ 105 and its DNA.

MATERIALS AND METHODS

Bacteria and phage. The bacterial and phage strains employed are listed in Table 1.

Media and assay of phage and bacteria. Min-CH, LB broth, TBAB, and the top-layer agar used were all described recently (13). Tryptone broth contains Difco Tryptone (10 g) and NaCl (5 g) in 1,000 ml of distilled water adjusted to pH 7. Dilutions of phage and bacteria were made in broth containing 1 g of tryptone per liter.

Viable bacteria were determined by spreading dilutions on TBAB plates; total bacteria were counted in a Petroff-Hauser counting chamber. Culture densities were measured in a Klett-Summerson colorimeter with filter no. 66. Phage was assayed by mixing 0.1 ml

of phage suspension with three to six drops of log-phase indicator bacteria in 2.5 ml of melted top-layer agar and pouring the mixture on plates. Phage ϕ 105 was assayed on min-CH plates; all other phages were assayed on TBAB plates. Unless otherwise stated, *B. subtilis* W168 was used as indicator bacteria.

Preparation of phage stocks. Lysates of ϕ 105 were prepared on plates or from induced lysogenic bacteria, and the phage was purified by repeated cycles of high- and low-speed centrifugation as previously described (14). Purification of the phage by banding in CsCl often leads to large losses in plaque-forming units (PFU); electron microscopy of such suspensions revealed that a large proportion of the phage particles had abnormal morphology. Stocks of phages ϕ 14, ϕ 15, ϕ 25, SP3, and PK were prepared on plates with *B. amyloliquefaciens* strain H as indicator. SP10 was prepared as described by Taylor and Thorne (17). We are grateful to B. E. Reilly for generous gifts of PBS1 and antiserum against this phage.

Antiserum against ϕ 105 was prepared by injecting rabbits twice a week with about 10^{11} purified PFU for 3 weeks and then bleeding the animals 1 week after the last injection. The k value of this serum is about 200. Antisera against ϕ 14, ϕ 15, and ϕ 25 were kindly provided by B. Rutberg and J. A. Hoch.

One-step growth experiment. *B. subtilis* W168 was grown at 37°C with forced aeration to a density of about 5×10^7 bacteria per ml. The bacteria were centrifuged and resuspended in 0.5 volume of fresh medium at the temperature chosen for the subsequent growth of the phage. Phage was then added at the desired multiplicity of infection. After 5 min of adsorption, unadsorbed phage were measured, and ϕ 105 antiserum was then added to a final k value of 1 to 3. After 4 min in the presence of serum, the samples were diluted into the respective growth media, and infection centers were assayed at intervals. Growing the bacteria in min-CH before infection makes an estimation of the total number of bacteria in the culture pos-

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TABLE 1. *Bacterial and phage strains*

Strain	Source
Bacterial	
<i>Bacillus subtilis</i> W168	Our collection
<i>Bacillus subtilis</i> W23	Our collection
<i>Bacillus amyloliquefaciens</i> strain H	Our collection
Phage	
$\phi 14$	Our collection
$\phi 15$	Our collection
$\phi 25$	Our collection
SP3	Our collection
SP10	J. A. Hoch
PBS1	B. E. Reilly
PK	Our collection
$\phi 105$	Our collection

sible, since few and short chains are formed by *B. subtilis* in this medium, whereas very long chains are formed in broth. Viable counts and total counts performed on bacteria grown in min-CH do not differ by a factor of more than two.

Preparation of DNA from $\phi 105$. To purified phage was added an equal volume of phenol (Mallinckrodt, reagent grade) twice washed with buffer. The mixture was gently rolled by hand at room temperature for about 5 min. The two phases were separated by centrifugation, and the aqueous phase was collected and dialyzed at 4 C for 18 to 24 hr against two changes of 1,000 volumes of buffer. DNA concentrations were determined by reading the absorbancy at 260 nm, taking one unit to correspond to 50 μ g.

Electron microscopy. Phage was prepared for electron microscopy by the pseudoreplica technique and stained with uranyl acetate or phosphotungstic acid.

DNA was prepared for electron microscopy as described by Roth and Helinski (12). The micrographs were taken at an original magnification of 15,000 on a Hitachi HU11A electron microscope. The microscope was calibrated with a carbon replica of a diffraction grating (960 lines/mm) and checked periodically with phage particles of known dimensions. The length of the DNA molecules was measured by projecting the negatives and tracing the molecules on paper. Only molecules which could be completely traced were measured. The contour length of the molecules was then measured by laying a string along the contour of the tracing or by measuring the length of the tracing with a map measurer (Keuffel and Esser, Switzerland). The length of the molecules was then calculated from the known magnifications. The molecular weight was calculated from the length, assuming a mass of the DNA of 192 daltons per 0.1 nm (11).

Determination of T_m of $\phi 105$ DNA. The T_m of $\phi 105$ DNA was determined in a Gilford multiple-sample absorbance reader model 200 attached to a Beckman DUR spectrophotometer. The temperature in the cuvette chamber was controlled by circulating water of a controlled temperature through metal coils in the chamber. The temperature in the chamber was continuously recorded together with the absorbancy at

260 nm during the experiment. At the end of the experiment the absorbancy had increased 35 to 40%, indicating complete strand separation. The per cent guanine plus cytosine (GC%) of the DNA was calculated from the relation established by Marmur and Doty (7).

Determination of the sedimentation velocity and buoyant density of $\phi 105$ DNA. Sedimentation and buoyant density determinations were performed in a Spinco model E rotor equipped with absorption optics, monochromator, and photoelectric scanner (6). A multiple switching device and a Titanium ANF rotor were used for simultaneous determination of three samples. Buoyant density was determined as described by Meselson et al. (8). About 1 μ g of DNA was centrifuged together with *Mycoplasma laidlawii* marker DNA (density = 1.694; M. Pollock, *personal communication*). The centrifuge was run at 44,000 rev/min and the temperature was controlled at 20 C. A solution of CsCl (Harshaw, optical grade) served as a reference in one side of the 12-mm double-sector centerpiece, when the scanner was operated in a split-beam mode.

Vinograd (18) band sedimentation was performed in 1 M NaCl essentially as described by Studier (15). A 15- μ liter amount of a DNA solution in 0.5 M NaCl was carefully layered on top of the 1 M solution in the cell. Sedimentation was begun and, after observing the layer formation at 5,000 rev/min, the rotor was taken to speed. Scans were made every 8 min with the monochromator set at 265 nm; the scanner was operated in a split-beam mode, referencing each sample to a cell containing a 1 M NaCl blank. Temperature was controlled at 20 C. Measurements were taken from the tracings when the peak had moved completely free of the steep refractile gradient generated by the layering process. Sedimentation coefficients were calculated from the maximal positions and were corrected to water by using the equations of Studier (15). Molecular weights were calculated from the equations given by Studier (15). Alkali-denaturation of DNA and neutralization of denatured samples was done immediately before centrifugation, as described by Studier (15).

RESULTS AND DISCUSSION

Host range and serological specificity of phage $\phi 105$. Phage $\phi 105$ plated with equal efficiency on *B. subtilis* 168 and on *B. amyloliquefaciens* H but did not plate on *B. subtilis* W23. Anti- $\phi 105$ serum, tested at a final k value of 1 to 5, did not inactivate phage $\phi 14$, $\phi 15$, $\phi 25$, SP10, SP3, PBS1, or PK when the phage were incubated with the serum for 1 hr at 37 C. The above phages represent the seven serological groups of *Bacillus* phages established by B. E. Reilly (B. E. Reilly, Ph.D. Thesis, Western Reserve University, Cleveland, Ohio, 1965). Phage $\phi 105$ is similarly not inactivated by antiserum against phage $\phi 14$, $\phi 15$, $\phi 25$, PBS1, or PK.

Phage $\phi 105$ thus represents a new serological

group of *Bacillus* phages, unrelated to the previously described virulent *Bacillus* phages. Serological cross-reaction between $\phi 105$ and SPO2 was reported by Boice et al. (3).

Growth of phage $\phi 105$. The latent period and burst size of W168 singly infected with $\phi 105$ are shown in Table 2. About 10 to 30% of the infected bacteria gave a lysogenic response as determined by spreading dilutions from the infected culture on TBAB plates impregnated with phage antiserum and testing the colonies for phage production and immunity to $\phi 105$ (2).

The rate of spontaneous induction of prophage $\phi 105$ in W168 ($\phi 105$) was 10^{-4} to 10^{-5} per cell per generation as determined from the amount of free phage found in a logarithmically growing culture in broth or min-CH. More than 90% of the lysogenic cells were induced to produce infectious phage after exposure of the bacteria to mitomycin C. The concentration of antibiotic used and the time of exposure of the cells to the antibiotic are critical. Optimal results in terms of production of plaque formers were obtained when bacteria grown in tryptone broth were exposed to 0.5 to 1 μg of mitomycin C per ml for 10 min, washed free of unbound antibiotic, and suspended in fresh medium (Fig. 1). Lysates of $\phi 105$ prepared either by lytic infection of sensitive cells or by induction of lysogenic bacteria were tested for transduction of bacterial markers try-2, phe-1, and ilvA1 in strain 168. Less than one transduced cell per 10^{10} plaque formers was found.

Phage $\phi 105$ is thus an inducible temperate phage incapable of generalized transduction.

Morphology. Electron micrographs and a schematic picture of phage $\phi 105$ are shown in Fig. 2. The phage has an icosahedral head with a diameter of about 52 nm and a flexible, non-contractile tail about 225 nm long with about 50 rows of subunits. The base plate has a central hole surrounded by six appendages. We did not observe tail fibers. The particles have a tendency to adsorb to membrane fragments (Fig. 2C). This suggests that the particles have a membrane site as well as a cell wall site for adsorption. It is difficult to understand from the structure of the phage how the DNA is injected upon infection.

FIG. 1. *B. subtilis* W168 ($\phi 105$) was grown in tryptone broth at 37 C with forced aeration to a density of about 50 Klett units. Mitomycin C was then added at a final concentration of 0.5 $\mu\text{g}/\text{ml}$. After 10 min, the bacteria were sedimented, resuspended in fresh broth at 37 C, and incubated until lysis. At intervals, samples were assayed for infectious centers, and the turbidity of the culture was determined. (●) Klett units; (○) infectious centers.

Phage $\phi 105$ is morphologically distinct from the phages of the seven serological groups of *Bacillus* phages; it resembles SPO2 (3). By following Bradley's (4) classification of bacteriophages on the basis of morphology, $\phi 105$ belongs to group B. The presence of phage particles resembling PBSX (9) in lysates of $\phi 105$ (Fig. 2A) is not unique to this phage system. In fact, approximately 75% of the 35 phage lysates examined (from both temperate and virulent phage infections) in the course of isolating new *B. subtilis* phages contained these particles (Hoch and Birdsell, unpublished data).

T_m and buoyant density of $\phi 105$ DNA. The T_m and buoyant density of $\phi 105$ DNA were determined as described. Four determinations gave a T_m of $87.2^\circ\text{C} \pm 0.3$. The buoyant density of the DNA is 1.703, i.e., identical to that of *B. subtilis*

TABLE 2. Growth parameters of W168 infected with phage $\phi 105^a$

Parameter	30 C		37 C		41 C	
	Min-CH	LB	Min-CH	LB	Min-CH	LB
Latent period (min)	70	70	40	40	35	35
Burst size	50-100	150-300	30-50	100-200	30-50	100-200

^a Multiplicity of infection was about 0.2.

FIG. 2. Electron micrographs and schematic representation of the structure of $\phi 105$. (A) Phage $\phi 105$ and PBSX particle; (B) schematic diagram of $\phi 105$; (C) $\phi 105$ particle adsorbed to membrane fragment; (D) end-on view of baseplate. Micrographs $\times 230,000$.

168. The GC% as calculated from T_m or buoyant density is 43.5; this indicates the absence of unusual bases in $\phi 105$ DNA (16).

Molecular weight of $\phi 105$ DNA. The molecular weight of $\phi 105$ DNA was determined by band sedimentation as described. Three determinations performed on native DNA at a final rotor speed of 24,000 rev/min gave an S value of 32 ± 0.5 , which corresponds to a molecular weight of 24×10^6 to 26×10^6 . The S value for alkali-denatured DNA is 36 in alkaline solution and 81 in neutral solution. Both values correspond to a molecular weight of the single strand of 12×10^6 to 13×10^6 . Only one peak of ultraviolet-absorbing material was observed in these experiments, indicating homogeneity of the preparations (Fig. 3). The close agreement between the molecular weight as calculated from the native or denatured molecule indicates that $\phi 105$ DNA does not contain any internal single-stranded breaks, although the existence of such breaks toward the end of the molecule cannot be excluded.

The molecular weight of $\phi 105$ DNA was next determined by electron microscopy. The contour

FIG. 3. Sedimentation pattern of $\phi 105$ DNA. (a) A 1.0- μ g amount of native DNA in 1 M NaCl; (b) 0.8 μ g of denatured DNA in 0.9 M NaCl-0.1 N NaOH; (c) 0.6 μ g of denatured and neutralized DNA in 1 M NaCl. The intervals between scannings were 16 min for samples a and b, 8 min for sample c.

lengths of 38 molecules were measured. The average length of the molecules was 11.6 μ m, with a standard deviation of 0.6 μ m. This corresponds to a molecular weight of the native molecule of 21×10^6 to 24×10^6 daltons, in good agreement with the values obtained from the band sedimentation.

Among the 38 molecules traced, 8 had no visible ends (Fig. 4B). The finding of apparently circular molecules might indicate that $\phi 105$ DNA contains complementary single-stranded ends.

It is characteristic of DNA extracted from all temperate coliphages, which possess complementary single-stranded ends, that it will form circles or multimers (1, 5, 19). The formation of these structures is enhanced by moderately high temperature, high salt, and high DNA concentrations (5). We tried, without success, to demonstrate the formation of circular or aggregated molecules from $\phi 105$ DNA in the following types of experiments. In the first type, heavy (5-bromo-deoxyuridine-labeled) $\phi 105$ was mixed with light $\phi 105$ particles, and the particles were lysed and incubated exactly as described by Baldwin et al. (1). After "annealing," the lysate was centrifuged to equilibrium in CsCl; only two peaks of ultraviolet-absorbing material at densities 1.703 and 1.738, respectively, with no intermediate absorbing material, were observed.

In the second type of experiment, ^3H -labeled DNA from $\phi 105$ was heated to 75 C for 10 min in 0.6 M NaCl at a concentration of 20 μ g/ml. One part of the DNA was diluted into ice-cold buffer; the other part was transferred to 45 C and kept at this temperature for 2 hr. The samples were then centrifuged in 15 to 30% linear neutral sucrose gradients. Both samples gave one sharp peak at identical positions. We recently sheared $\phi 105$ DNA into half and quarter molecules (L. Rutberg, *in preparation*) and incubated the sheared molecules in 0.6 M salt under annealing conditions followed by centrifugation in sucrose gradient. No increase in the sedimentation velocity of the molecules was observed.

These negative results, of course, do not exclude the existence of complementary single-stranded ends in $\phi 105$ DNA. In fact, the predominant DNA configurations observed in an electron microscope (55%) were molecules with ends in close opposition (*see* Fig. 4C). The negative results do suggest, however, that if the $\phi 105$ DNA molecule contains such ends these must be of a type different from those found in, e.g., coliphage lambda (19). This difference may be trivial, e.g., relate to the length or base composition of the single stranded regions. Further work is needed to elucidate fully the anatomy of the $\phi 105$ DNA molecules.

FIG. 4. Electron micrographs of $\phi 105$ DNA. (A) Open (linear) molecule; (B) closed (circular) molecule; (C) molecule with adjacent ends (see arrows). The bar represents $1 \mu\text{m}$.

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