Biological and Biophysical Characteristics of Phages Isolated from *Clostridium botulinum* Type C and D Strains, and Physicochemical Properties of the Phage DNAs

Hiroyuki SUNAGAWA and Katsuhiro INOUE[1)]

Hokkaido Institute of Public Health, Kita-19, Nishi-12, Kita-ku, Sapporo 060 and [1)]Department of Bioindustry, Tokyo University of Agriculture, Yasaka, Abashiri 099-23, Japan

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ABSTRACT. Toxin-converting phages CEβ and d-16ϕ and non-converting phages CEγ and d-1', isolated from toxigenic strains C-468 and D-CB16 of *Clostridium botulinum* types C and D, respectively, were characterized biologically and biophysically. DNAs isolated from these four phages were studied physicochemically. Phages CEβ, d-16ϕ, CEγ and d-1' were adsorbed to their susceptible cells at the constant rates of 1.1×10^{-8}, 3.3×10^{-8}, 1.4×10^{-8} and 1.4×10^{-8} ml/min, and grew after latent periods of 35, 45, 35 and 35 min at the burst sizes of 38, 85, 35 and 35, respectively. Converting phages were more susceptible than non-converting phages to physical and chemical treatments such as temperature, pH, time, UV-irradiation and organic solvents. GC% of phage DNAs were determined by melting temperature, buoyant density and HPLC analysis to be 26, 26, 29 and 29% for CEβ, d-16ϕ, CEγ and d-1' phage DNAs, respectively. The restriction digestion profiles of phage DNAs with seven endonucleases were compared by agarose gel electrophoresis, revealing that the two converting phage DNAs were similar in regard to five enzyme digestion profiles, but different in the other two enzymatic profiles. Non-converting phage DNAs produced the same restriction digestion profiles with all seven endonucleases. The molecular sizes determined from the size of restriction enzyme digestion fragments were about 110 kb for converting phage CEβ and d-16ϕ DNAs and 65 kb for non-converting phage CEγ and d-1' DNAs. Dot blot hybridization experiments revealed that DNA homology between the converting phages CEβ and d-16ϕ was 50–75%, while that between non-converting phages CEγ and d-1' was about 100%. Converting phage and non-converting phage DNAs did not hybridize at all.—KEY WORDS: bacteriophage, biological characterization, biophysical characterization, *Clostridium botulinum*, phage DNA.

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All of seven types of *Clostridium botulinum*, A through G, are lysogenized with bacteriophages [9, 18, 31]. Toxigenicities of type C and D cultures are governed by their specific bacteriophages, known as converting phages [10, 11, 19, 20]. These phages are divided into three groups based on differences in antigenicity and host specificity as defined by Oguma *et al.* [25]. A new converting phage, which did not belong to any of Oguma's three groups, was isolated from one type D botulinum culture [29]. Most of the toxigenic *C. botulinum* type C and D strains were found to be lysogenized with non-converting phages, in addition to converting phages [28, 29]. These non-converting phages different from converting phages by having smaller virion size and not being involved in determining the toxigenicity of their parent organisms. Irrespective of isolation from type C or D, non-converting phages share identical antigenicity and host specificity [28, 29].

Recently, nucleotide sequences of botulinum type C and D neurotoxin genes have been determined [5, 16, 21, 30]. Several striking homologous amino acid sequences were found between deduced amino acid sequences of type C and D neurotoxins, and 53.2% identity was observed between these amino acid residues [30]. Type C and D neurotoxins possess common and specific antigenic sites [24, 26]. One of the type C neurotoxins was found to be a mosaic-like molecule composed of a type C light chain and a type D heavy chain [24, 32]. The heterogeneity and similarity of these two types of neurotoxins are considered to be responsible for the pseudolysogenic characteristics of the converting phages, and the mosaic-like neurotoxins might be formed by the recombination of phage genes that control type C and D neurotoxin production [26]. Since the role of lysogenic phages in determining the toxigenicity of their parent cells has not been studied, we began to investigate the relationships between parent strains and lysogenic phages. In this paper, converting and non-converting phages isolated from type C and D strains are characterized virologically, while the DNAs isolated from these phages are characterized physicochemically.

MATERIALS AND METHODS

Bacterial strains: The following *C. botulinum* strains were used; toxigenic type C strain 468 (a stock culture in our laboratory), toxigenic type D strain CB16 (provided by T. Itoh, Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan), non-toxigenic type C strain AO28 (provided by M. W. Eklund, National Marine and Fisheries Service, Seattle, Wash. U.S.A. [10]) and non-toxigenic type D strain D-16nt (isolated from D-CB16 treated with acridine orange [29]).

Bacteriophages: Phages CEβ and CEγ were isolated from a UV-induced lysate of strain C-468 by plating with indicator strain C-AO28 according to Eklund *et al.* [10]. Phages d-16ϕ and d-1' were isolated from a mitomycin C induced lysate of D-CB16 by plating with indicator strains D-16nt and C-AO28, respectively [29]. All of these phages were cloned by three successive single plaque isolations. Phage lambda (λ) of *Escherichia coli*, used as a standard for several tests, was provided by M. Hayashi, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan.

Media and phage assay: Bacterial strains were cultured and kept in Cooked Meat Broth (Difco) and transferred when required to TYG broth (pH 7.2) [19] consisting of 3% Trypticase Peptone (BBL), 2% Yeast Extract (Difco), 0.5% glucose and 0.15% cystein-HCl. Phage assay was achieved by the agar-overlay procedure according to Eklund *et al.* [10].

Adsorption experiments: One m*l* of phage solution (2×10^7 PFU; plaque forming unit/m*l*) was added to 4 m*l* of exponential phase cells (5×10^7 cells/m*l*) of the indicator strains, and the mixtures were incubated at 37°C. A 0.1 m*l* sample was taken from the mixture at various times, diluted 100-fold with 9.9 m*l* ice-cold TYG broth, and centrifuged at 10,000 rpm for 5 min at 4°C. The supernatants were assayed for the unadsorbed phages.

One step growth experiments: One step growth experiments were performed according to the methods described by Mahony and Kalz [23] for the lysogenic phage of *Clostridium perfringens* with slight modifications. Two m*l* of phage solution (1×10^8 PFU/m*l*) was added to 4 m*l* of exponential phase cells (5×10^7 cells/m*l*). After incubating for 10 min at 37°C, the mixture was centrifuged at 10,000 rpm for 5 min at 4°C to remove the unadsorbed phages, and the pellet was resuspended in 6 m*l* of prewarmed TYG broth. The cell suspension was further diluted 10-fold with prewarmed 4.5 m*l* TYG broth, and incubated at 37°C. At various intervals, some of the suspension was taken and assayed for the number of infectious phages.

Sensitivity of phages to physical and chemical treatments: Sensitivities of the phages to temperature, UV-irradiation, pH, storage period, chloroform, ether, Triton X-100, DNase and RNase were tested according to the methods described by Ackermann *et al.* [3].

Propagation and purification of phage particles and extraction of phage DNAs: Phages, cloned by three successive single plaque isolations on lawns of each of the indicator cultures, were propagated in TYG broth as described by Oguma *et al.* [25]. Purification of phage particles and extraction of phage DNAs were performed by the method of Davis *et al.* [7]. The DNAs were precipitated by adding two volumes of cold ethanol.

Determination of base composition of phage DNAs by HPLC: Phage DNAs were hydrolyzed with 98% formic acid for 30 min at 175°C. After drying, the products of hydrolysis were dissolved in distilled water and analyzed by High Performance Liquid Chromatography (HPLC, Hitachi model 655). The concentration of each of the bases was calculated from the chromatographic peak height with a Shimazu chromatopak (model C-R3A).

Melting temperature of DNAs: Melting temperature (Tm) was measured with a spectrophotometer (Hitachi model 200-10) at 260 nm in standard saline solution (SSC, 0.15 M NaCl, 0.015 M sodium citrate).

Buoyant density of phage particles and phage DNAs: Buoyant density (ρ) was determined by CsCl equilibrium centrifugation in a Hitachi RPS-65T rotor according to the method described by Kinouchi *et al.* [22]. Centrifugation was performed at 35,000 rpm for 48 hr at 5°C for phage particles and at 20°C for phage DNAs. Densities were monitored with an Abbe's refractometer (Atago model 3T). Absorbance of phage particles and phage DNAs were monitored with a spectrophotometer at 280 and 260 nm, respectively. λ phage particles and DNA having densities of 1.508 and 1.709 g/cm^3, respectively, were used as density markers.

Cleavage of DNA by restriction endonuclease and electrophoresis of fragments: Phage DNAs were digested with restriction endonucleases *Eco*RI, *Hin*dIII, *Bam*HI, *Hae*III, *Hpa*I, *Sau*3AI and *Pst*I

(Boheringer Mannheim). Digestions were performed as recommended by the manufacturer. Digests were analyzed by electrophoresis in 1.0% agarose gels in running buffer E (40 mM Tris-HCl, pH 7.7, 5 mM sodium acetate, 1 mM EDTA). After electrophoresis at 50 V for 1 hr, gels were stained in running buffer containing 1 μg/m*l* of ethidium bromide. DNAs in gels were visualized with UV transilluminator (UV Products) and photographed through a SC-58 filter onto Kodak Technical Pan film. Fragment lengths were estimated by comparing the immigration of fragments on gels with that of *Hin*dIII and *Hin*dIII-*Eco*RI digestion fragments of λDNA (Boheringer Mannheim).

Dot blot hybridization of DNAs: Phage DNAs were labeled with biotin-11-dUTP using a Nick Translation Reagent Kit (Bethesda Research Laboratories (BRL)). Phage DNAs were serially diluted 200 to 1 pg/μ*l*, and 5 μ*l* of each of the diluents were blotted onto a nitrocellulose membrane filter (Schleicher & Schuell, BA85) using Bio-Dot™ (Bio-Rad). Biotin-labeled DNAs were then hybridized with the dot-blotted DNAs on the filter according to the manual. The hybridized DNAs with biotin-labeled DNAs were detected using the DNA Detection System (BRL).

RESULTS

Adsorption of phage to host cell: Adsorption patterns are shown in Fig. 1. The adsorption constants were calculated according to the equation: $K=2.3/Bt\times\log(P_0/P)$ [2], in which K is the velocity constant, B is the bacterial concentration, t is the time in min, P_0 is the phage concentration at zero time, and P is the phage concentration after t min. The adsorption constant Ks for CEβ, d-16ϕ, CEγ and d-1' in TYG broth at 37°C with a bacterial concentration of 1×10^7 cells/m*l* were 1.1×10^{-8}, 3.3×10^{-8}, 1.4×10^{-8}, and 1.4×10^{-8} m*l*/min, respectively.

One step growth experiment: Plots of one step growth data of phages CEβ, d-16ϕ, CEγ and d-1' are shown in Fig. 2. Latent periods of CEβ, d-16ϕ, CEγ and d-1' were 35, 45, 35 and 35 min, respectively. The average burst sizes of CEβ, d-16ϕ, CEγ and d-1' were 38, 85, 35 and 35, respectively.

Buoyant density of phage particles: The buoyant densities in CsCl of phages CEβ, d-16ϕ, CEγ and d-1' were 1.50, 1.49, 1.45 and 1.45 g/cm^3, respectively.

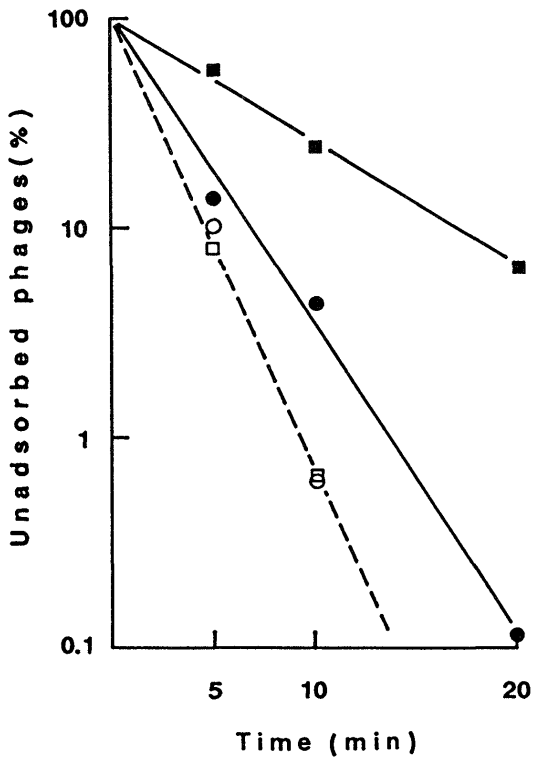

Fig. 1. Adsorption of phages CEβ, CEγ and d-1' to C-A028 cells and phage d-16ϕ to D-16nt cells. (●) CEβ, (■) d-16ϕ, (○) CEγ and (□) d-1'.

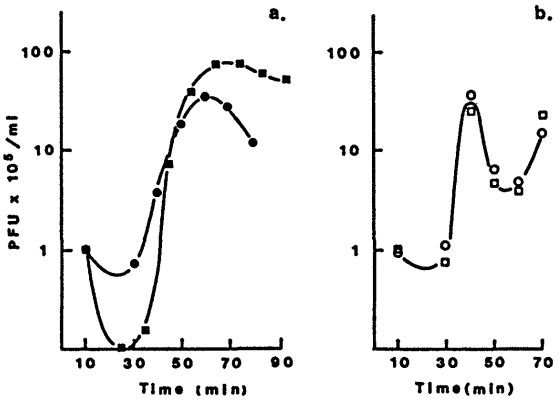

Fig. 2. One-step-growth curves of phages CEβ, d-16ϕ, CEγ and d-1' in TYG medium at 37°C. (a) (●) CEβ, (■) d-16ϕ, (b) (○) CEγ and (□) d-1'.

Stability of phages:

1) *Temperature*; Phages CEβ and d-16ϕ were stable for 10 min at 45°C, and about 1% of these phages survived for 10 min at 55°C. Phages CEγ and d-1' were stable for 10 min at 50°C, and about 1% of these phages survived for 10 min at 60°C (Fig. 3).

2) *pH*; Phages CEβ and d-16ϕ were stable for 1 hr at pH 4.5 to 9.0 and CEγ and d-1' were stable for 1 hr

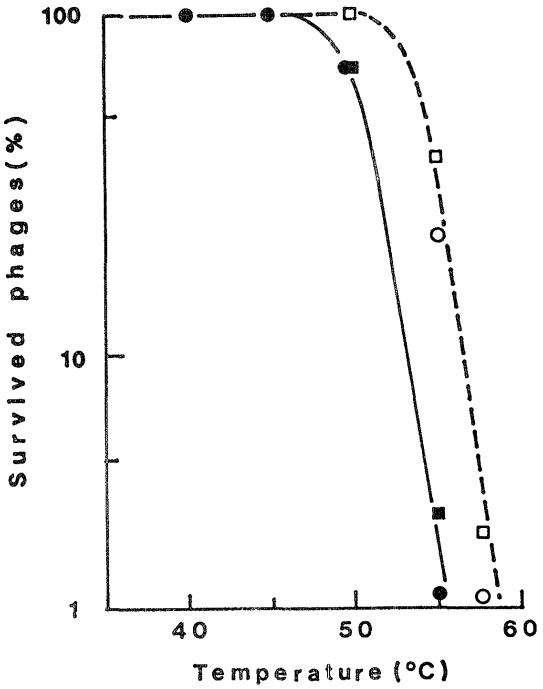

Fig. 3. Effect of temperature on phage viability. Phage lysates in TYG medium (2×10^7PFU/m*l*) were held at various temperatures for 10 min and the survived phages were assayed. (●) CEβ, (■) d-16ϕ, (○) CEγ and (□) d-1'.

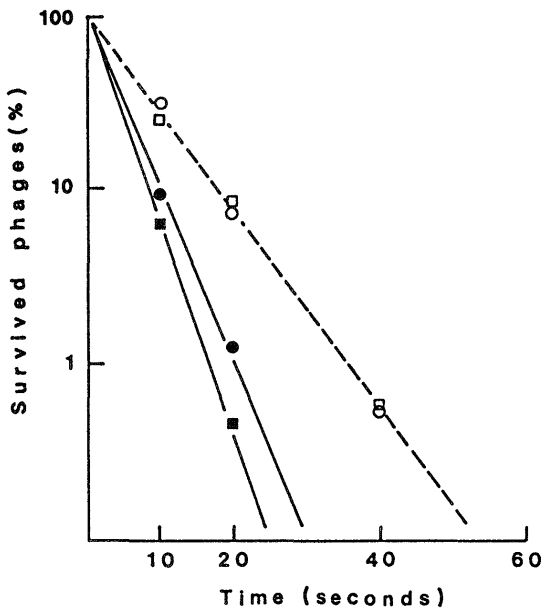

Fig. 5. Effect of UV-irradiation on phage viability. Phage particles were suspended in 10 mM Tris-HCl buffer (pH 7.2) containing 10 mM $MgCl_2$, 5 m*l* of suspension was poured into plastic petri-dish of 5 cm diameter, and irradiated with a UV-lamp (15W) at 50 cm distance, and then the survived phages were assayed. (●) CEβ, (■) d-16ϕ, (○) CEγ and (□) d-1'.

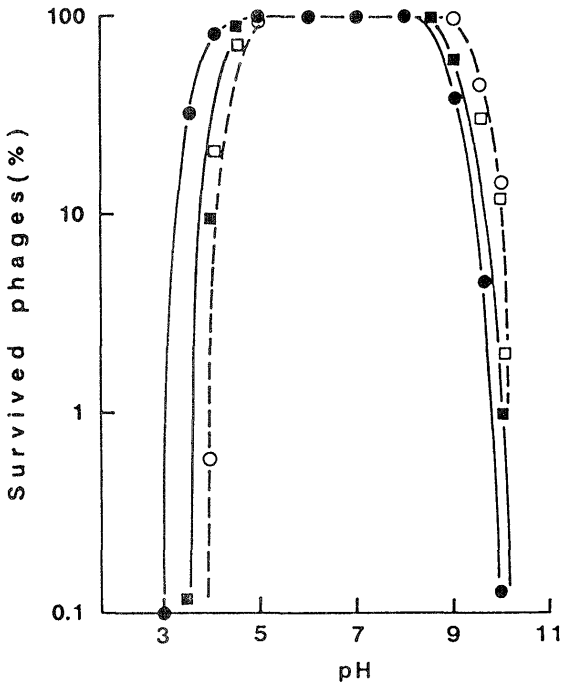

Fig. 4. Effect of pH on phage viability. Phage particles were suspended to 2×10^7PFU/m*l* in TYG medium, those were adjusted to the indicated pH, held for 1 hr at 25°C, and diluted 100 times with TYG medium (pH 7.2) to assay the survived phages. (●) CEβ, (■) d-16ϕ, (○) CEγ and (□) d-1'.

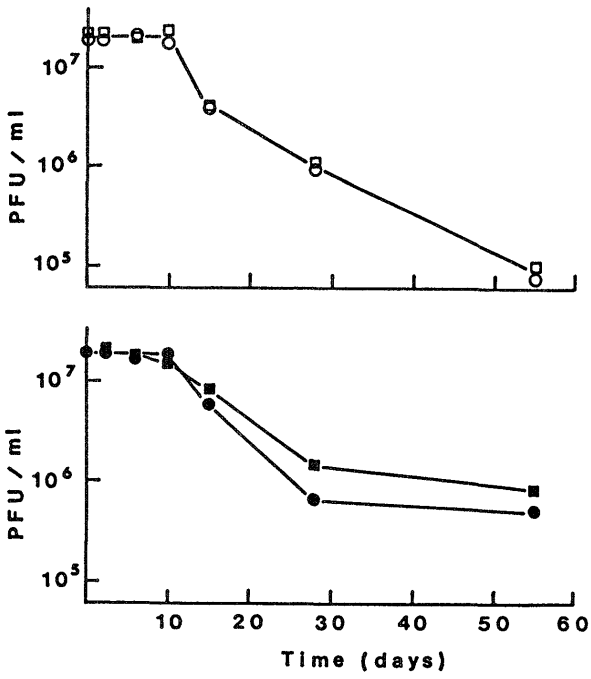

Fig. 6. Stabilities of phages CEβ, d-16ϕ, CEγ and d-1' during storage at 4°C in TYG medium (pH 7.2). (●) CEβ, (■) d-16ϕ, (○) CEγ and (□) d-1'.

at pH 5.0 to 9.0 at 25°C (Fig. 4).

3) *UV-irradiation*; About 1% of phages CEβ and d-16ϕ, and 10% of CEγ and d-1' survived UV-irradiation (15 W UV-lamp) at 50 cm distance for 20 sec in 10 mM Tris-HCl buffer (pH 7.2), containing 10 mM $MgCl_2$ (Fig. 5).

4) *Time*; When stored at 4°C in TYG broth, phages CEβ, d-16ϕ, CEγ and d-1' were stable for 10 days. However, after 30 days storage, the infectivity of these phage lysates decreased to about 10% of the original titers (Fig. 6).

5) *Chemical and biochemical agents*; Phages CEβ, d-16ϕ, CEγ and d-1' were inactivated at 25°C for 60 min in 10 mM Tris-HCl (pH 7.2) adding one third volume of ether. Sixty to 90% of these phages were inactivated in 10 mM Tris-HCl (pH 7.2) adding one third volume of chloroform. However 0.05% Triton X-100, 50 μg/m*l* DNase or RNase did not influence the viability of these phages at 25°C for 60 min (Table 1).

Table 1. Effect of chemical and biological agents on phage infectivity[a)]

Agents	Survival(%)			
	CE β	d-16ϕ	CE γ	d-1'
Chloroform[b)]	33	10	44	35
Ether[b)]	0.05	0.05	0.2	0.15
Triton X-100 (0.05%)	100	100	100	100
DNase (50 μg/m*l*)	100	100	100	100
RNase (50 μg/m*l*)	100	100	100	100

a) Phages (2×10^7 PFU/m*l*) were treated with each agent at 25°C for 60 min.
b) One volume of chloroform or ether was added to three volumes of phage solution.

Table 2. Thermostability of phage CEβ and CEγ in various solutions

Solutions	Survival (%)[a)]	
	CEβ	CEγ
TYG medium (pH 7.2)	5	10
TYG medium (pH 7.2) with 10 mM $MgCl_2$	100	100
Distilled water	<0.1	<0.1
0.1 M NaCl	<0.1	<0.1
10 mM Tris-HCl (pH 7.2)	0.3	1.0
10 mM Tris-HCl (pH 7.2) with 0.1 M NaCl	0.1	0.5
10 mM Tris-HCl (pH 7.2) with 10 mM $MgCl_2$	100	100
10 mM Tris-HCl (pH 7.2) with 10 mM $CaCl_2$	100	100
10 mM Tris-HCl (pH 7.2) with 20 mM EDTA	<0.1	<0.1

a) Survival (%) of phages after heating at 55°C for 10 min in various solutions.

Effect of divalent cations on the viability of phages: Stabilities of phages CEβ and CEγ in various solutions at 55°C are shown in Table 2. Phage CEβ was rapidly inactivated in distilled water and 0.1 M NaCl, whereas it was in 10 mM Tris-HCl (pH 7.2) buffer. The stability in Tris-HCl buffer or TYG broth was markedly improved by addition of 10 mM $CaCl_2$ or $MgCl_2$ and markedly decreased by addition of 20 mM EDTA. $CaCl_2$, $MgCl_2$ and EDTA had the same effect on the stability of phage CEγ as on that of CEβ (Table 2).

Buoyant density of phage DNAs in CsCl: The buoyant densities (ρ) of phage DNAs were determined by CsCl equilibrium density gradient centrifugation. The densities of phage CEβ, d-16ϕ, CEγ and d-1' DNAs were 1.687, 1.689, 1.678 and 1.677 g/cm^3, respectively. The GC% of these DNAs calculated from the buoyant density according to the De Ley's equation (GC% = 1038.47 (ρ −1.6616)) [8] were 26, 28, 17 and 16%, respectively.

Melting temperature (Tm) of DNAs: The melting temperatures of phage CEβ, d-16ϕ, CEγ and d-1' DNAs in SSC were 80.0, 80.0 81.3 and 81.1°C, respectively (Fig. 7). The GC% of these DNAs calculated from the melting temperatures according to the De Ley's equation (GC% = 2.44 (Tm−69.4)) [8] were 26, 26, 29 and 29%, respectively.

Base composition of DNAs: The elution patterns of hydrolyzed phage DNAs with HPLC are shown in Fig. 8. These results show that botulinum type C and D phage DNAs consist of the usual bases only. The

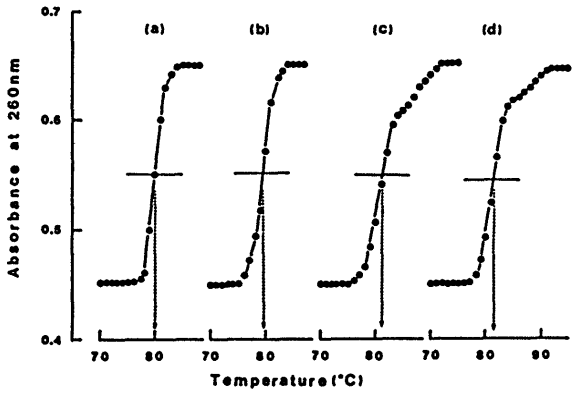

Fig. 7. Melting profiles of DNA preparations. DNA solution (5 μg/m*l* in SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0)) were measured absorbance at 260 nm and temperature in quartz cuvette with 1 cm path using spectrophotometer (Hitachi model 200–10) fitted with temperature controller and thermistor. (a) CEβ DNA, (b) d-16ϕ DNA, (c) CEγ DNA and (d) d-1' DNA.

Table 3. Physicochemical characteristics of phage DNAs

Characteristics	Measurement			
	CE β DNA	d-16ø DNA	CE γ DNA	d-1' DNA
Buoyant density in CsCl (g/cm^3)	1.687	1.689	1.678	1.688
Tm in SSC[a] (°C)	80.0	80.0	81.3	81.1
Molecular size calculated from nuclease fragment mobility (kb)	103–110	96–117	59–68	59–68
GC %				
from buoyant density in CsCl	26	28	17	16
from Tm in SSC	26	26	29	29
from HPLC	25.9	26.0	28.4	27.7

a) SSC; standard saline citrate (0.15 M NaCl, 0.015 M sodium citrate).

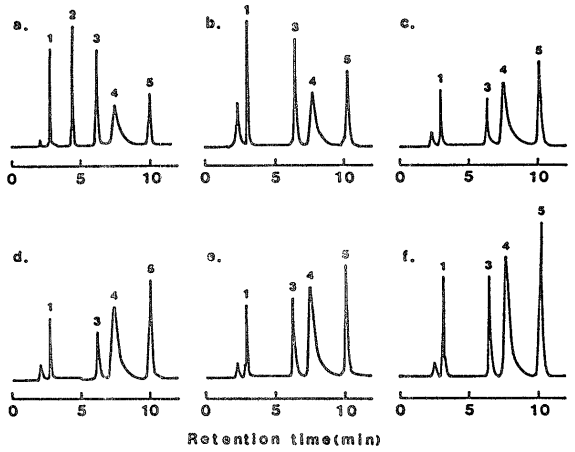

Fig. 8. HPLC chromatogram of phage DNAs hydrolyzed with formic acid. The HPLC condition is: column; Nucleosil $5C_{18}$ (4×250 mm), eluate; 2% $NH_4H_2PO_4$ (pH 3.4), flow rate; 1 ml/min, detection; 260 nm. (a) standard bases, (b) λDNA, (c) CEβ DNA, (d) d-16ϕ DNA, (e) CEγ DNA, (f) d-1' DNA. 1; cytosine, 2; uracil, 3; guanine, 4; adenine, 5; thymine.

GC% of phage CEβ, d-16ϕ, CEγ and d-1' DNAs were 25.9, 26.0, 28.4 and 27.7%, respectively. Base compositions of phage DNAs estimated from HPLC analysis, Tm and buoyant density are shown in Table 3.

Restriction analysis of DNAs: The DNAs of converting phage CEβ and d-16ϕ and non-converting phage CEγ and d-1' were compared after treatment with restriction endonucleases and electrophoresis on agarose gels (Fig. 9). The restriction profiles showed similarity between converting phage CEβ and d-16ϕ DNAs (Fig. 9, lane 2 and 3), except restriction enzyme *Sau*3AI. The restriction profiles of non-converting phage CEγ and d-1' DNAs were identical with all restriction enzymes used (Fig. 9, lane 4 and 5). The restriction profiles of converting phage DNAs differed from that of non-converting phage DNAs in fragment number and size. The molecular sizes of DNAs were calculated by adding the size of endonuclease digested fragments. Molecular sizes of phage CEβ and d-16ϕ DNAs calculated from *Eco*RI, *Hin*dIII and *Hae*III digestion, were 103–110 kb, average 110 kb and 96–117 kb, average 110 kb, respectively. Molecular sizes of phage DNAs CEγ and d-1' DNAs, calculated from *Eco*RI, *Hpa*I and *Pst*I digestion were 59–68 kb, average 65 kb.

Hybridization analysis of phage DNAs: To further evaluate the relationships between these phage DNAs, dot blotted phage DNAs on nitrocellulose filter were subjected to hybridization analysis with biotin labeled probes of each of the phage DNAs. As expected from the analysis of the restriction profiles, the DNAs of converting phages CEβ and d-16ϕ hybridized about 50–75%, and the DNAs of non-converting phages CEγ and d-1' hybridized about 100 %, while the DNAs of converting phages and non-converting phages did not hybridize at all (Fig. 10).

DISCUSSION

To clarify role of lysogenic phages in type C and D botulinum organisms, the biological and biophysical properties of two types of bacteriophages, converting and non-converting phages, have been investigated. In addition, the DNAs of these bacteriophages were studied physicochemically. Results revealed that these converting phages share similar

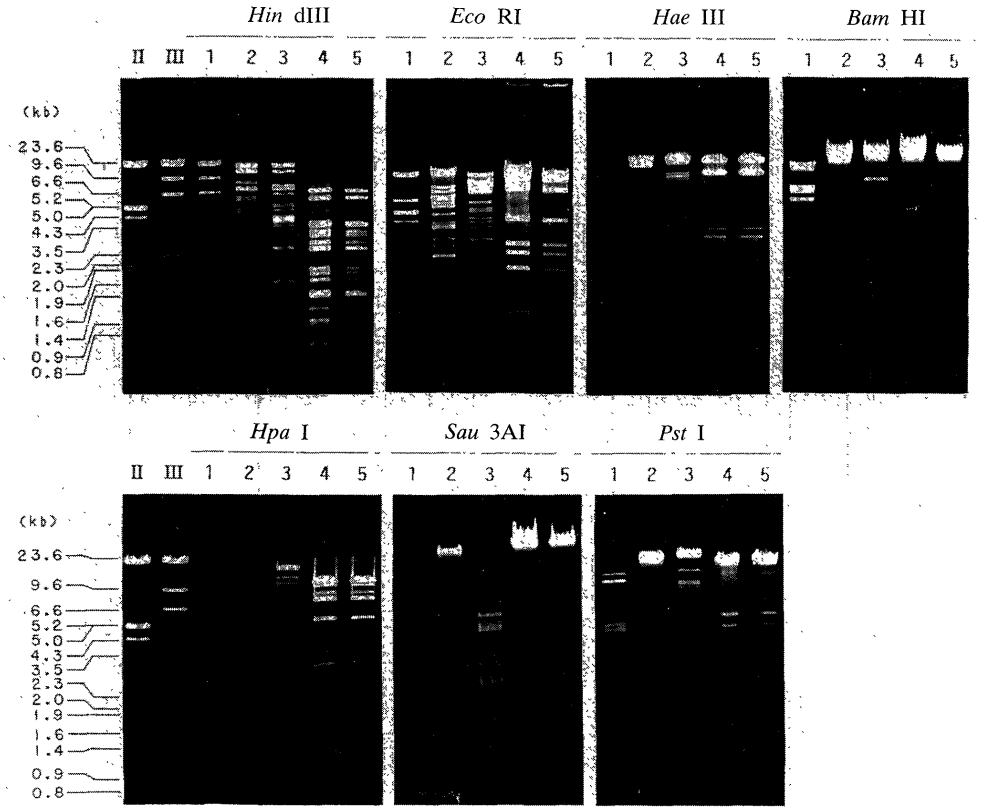

Fig. 9. Profiles of phage DNAs digested with restriction nucleases. DNA mixture (1 μg of DNA, 5 μl of 10× restriction buffer, 1 unit of restriction endonuclease, total volume 50 μl with distilled water) was incubated at 37°C for 60 min. Endonuclease-cleaved DNAs were loaded on 1.0% agarose gel and run in E buffer at 50 V for 1 hr. Lanes: II; λDNA doubly digested with *Eco*RI and *Hin*dIII, III; λDNA digested with *Hin*dIII, 1; λDNA, 2; CEβ DNA, 3; d-16ϕ DNA, 4; CEγ DNA, 5; d-1' DNA.

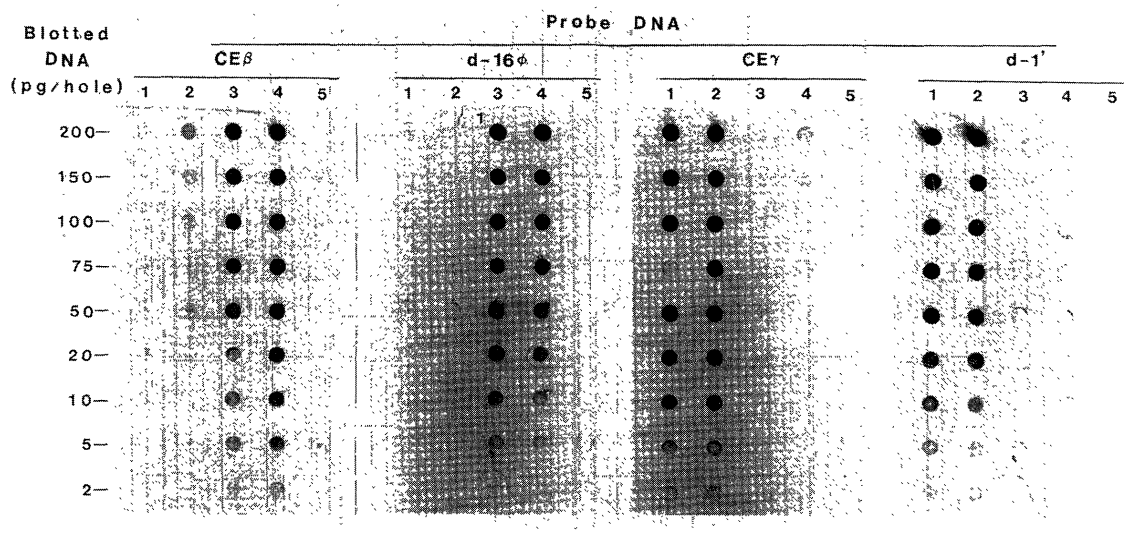

Fig. 10. Dot blot hybridization. Phage DNAs were serially diluted and 5 μl of each diluent was blotted onto a nitrocellulose membrane filter. These DNAs were separately reacted with phage DNA probes labeled with biotin-11-dUTP and washed, then the hybridization was detected with the DNA Detection Sytem. Blotted phage DNAs, 1; d-1', 2; CEγ, 3; d-16ϕ, 4; CEβ and 5; λ.

in their biological and biophysical properties, and have similar physicochemical characteristics and close homology of their DNAs. The non-converting phages also were confirmed to be identical under these characterizations. However, converting phages were demonstrated to be different from non-converting phages on their virological characteristics and physicochemical characteristics of their DNAs.

One step growth experiments were attempted by the methods recommended by Mahony and Kalz [23]. Since the results obtained by this method are not defined, the indicated curves in Fig. 2 were produced from the results of three to five experiments. Furthermore, the one-step curves of botulinum phages differed from those of the other bacteriophages, e.g. λ phage of *E. coli* [2]. After adsorption of phages to susceptible cells, during the eclipse period the number of plaque forming units decreased to one tenth or less of added phages. A stationary plateau was not obtained after the growth peak of phages. These phenomena had been observed for the other clostridial phages [4, 23]. The reason is not clear but the following points may influence the results. (1) Phage-infected cells during the eclipse period may be very susceptible to physiological and chemical agents [27], so they were easily killed by dilution treatment of samples. (2) The rapid adsorption of released phage particles to cell membranes may prevent the formation of the stationary plateau as indicated by Mahony and Kalz [23].

Botulinum phages were also shown to be more heat-stable in the solution which contained divalent cations $Mg^{2}+$ and $Ca^{2}+$ rather than in Mg^{2+} and Ca^{2+} free solution as already known for other phages [1]. This stability was abolished by the addition of a small amount of EDTA to the solution containing Mg^{2+} and Ca^{2+} solution.

Clostridium novyi type A phages can infect a non-toxigenic mutant strain of *C. botulinum* type C and convert it to a *C. novyi* alpha toxin producer [13]. These novyi phages were morphologically similar to the non-converting phages of *C. botulinum* types C and D [17], but they were remarkably different in their lability. The infectious titer of these phages decreased to 10^{-4}–10^{-6} of the original by overnight storage at 4°C [13]. Novyi phages were highly sensitive to heat inactivation at temperature over 45°C [17].

The GC% of type C and D phage DNAs determined by buoyant densities were different from those determined by Tm or HPLC analysis. Kinouchi *et al.* [22] studied the physicochemical properties of phages α1 and α2 isolated from *C. botulinum* type A strain 190L, and found a difference between the GC% determined from the buoyant density and Tm. They described that this discrepancy may possibly be due to the presence of abnormal bases or additional carbohydrates in the DNAs. De Ley [8] described that substitution of cytosine by small amounts of methylated cytosine had no effect on Tm, but did affect buoyant density. Botulinum type C and D phage DNAs were demonstrated by HPLC analysis to consist of normal bases. Therefore the GC% determined by Tm and HPLC analysis in this experiment seemed to be more accurate than that determined by buoyant density. The fact that the GC% of botulinum type C and D phages were less than 30% may be one of the reasons explaining the difference between the GC% determined from buoyant density and from Tm, since De Ley's equation was based on the data range from 30 to 75% GC. Comparing the physicochemical properties of DNAs of type A phages and type C and D phages, the GC% of each of the phage DNAs was about 30%, while the molecular size of type A phage DNA was about half that of type C and D converting phages and similar to that of type C and D non-coverting phages. These type A phages are not involved in determining the toxigenicity of their parent organisms, so type A strains may also be lysogenized with non-converting phages similar to those of type C and D strains.

Fujii *et al.* [14] isolated DNAs from five strains of botulinum type C and D converting phages, compared restriction endonuclease digestion profiles and studied the homologies of these phage DNAs. They indicated that the classification of these converting phages based on their conversion spectra and antigenicity agreed with the susceptibility of these phage DNAs to *Pst*I and *Sau*3AI digestion. The DNAs of group 1 phages were digested by *Pst*I and those of group 2 were digested by *Sau*3AI. Three strains of group 1 phage DNAs had the same molecular size about 110 kb and demonstrated a high homology with the dot blot hybridization test. However, molecular sizes of group 2 phage DNAs were not the same (110 or 150 kb) and there was little similarity between these DNAs. The molecular size of DNAs of converting phages CEβ and d-16ϕ use in this experiment was about 110 kb, the same as

group 1 of Fujii *et al.* [14], and rather high homology was demonstrated between these DNAs with the dot blot hybridization test. The restriction digestion profiles of these phages showed CEβ to belong to group 1 and d-16ϕ to belong neither to group 1 nor 2. These results suggest that the converting phages of type C and D have both diversity and similarity in their antigenic and genetic characteristics. Neurotoxin production is controlled by converting phages which also show diversity and yet possess similarities in antigenic and genetic characteristics. These diversity and similarity between phage virions and neurotoxins are thought to come from evolutionary and phylogenetic relationships between type C and D converting phages.

The concept of a family of phages was first defined for the lambdoid phages, which possess the ability to exchange genetic information by homologous recombination with themselves, by Campbell and Botstein [6]. This concept was also adopted for the beta-related phages of *Corynebacterium diphtheriae* by Groman [15]. According to this definition of phage families, it could be concluded that the converting phages of *C. botulinum* types C and D formed one family. These converting phages were thought to exist in their parent organisms in a pseudolysogenic state [10, 11], and some phages make interconversion between types C and D [12, 20]. Therefore, recombinations between these converting phages are thought to occur at times in the natural state. However, there is little information available to elucidate these interesting problems concerning the evolutionary history of botulinum type C and D converting phages. Further investigation of the genetic elements is required.

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