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Interconversion of Type C and D Strains of *Clostridium botulinum* by Specific Bacteriophages

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These studies show that *Clostridium botulinum* types C and D cultures can be cured of their prophages and converted to either type C or D depending on the specific phage used. Strains of types C and D were cured of their prophages and simultaneously ceased to produce their dominant toxins designated as C_1 and D, respectively. Cured nontoxigenic cultures derived from type C strain 162 were sensitive to the phages from the toxigenic type C strain 162 and type D strain South African. When cured nontoxigenic cultures derived from strain 162 were infected with the tox^+ phages from the 162 strain of type C and the South African strain of type D, they then produced toxin neutralized by types C and D antisera, respectively. Cured nontoxigenic cultures isolated from the type D South African strain were only sensitive to the parent phage, and, when reinfected with the tox^+ phage, they produced toxin neutralized by type D antiserum. Type C strain 153 and type D strain 1873, when cured of their respective prophages, also ceased to produce toxins C_1 and D, but, unlike strain 162 and the South African strain, they continued to produce a toxin designated as C_2. When the cured cultures from strains 153 and 1873 were infected with the tox^+ phage from type D strain 1873, the cultures simultaneously produced toxin that was neutralized by type D antiserum. When these cured cultures were infected with the tox^+ phage from type C strain 153, the cultures produced toxin that was neutralized by type C antiserum. These studies with the four strains of *C. botulinum* confirm that the toxigenicity of types C and D strains requires the continued participation of tox^+ phages. Evidence is presented that types C and D cultures may arise from a common nontoxigenic strain.

Based upon the antigenic specificity of its toxins, *Clostridium botulinum* has been divided into seven types designated A through G.

It was previously reported (9, 10, 16, 17) that changes from non-toxigenicity to toxigenicity in types C and D required the active and continued participation of specific bacteriophages. Subsequent studies (7) showed that some strains of types C and D cured of their prophages cease to produce their dominant toxins but continue to produce low levels of another toxin, designated as C_2, which does not appear to be governed by phages. These cured strains of types C and D therefore become indistinguishable with respect to the toxin produced. This suggested that types C and D cultures may arise from the same parent.

The possibility of changing type D to type C was first indicated by Inoue and Iida (17). In their studies, they were able to isolate a nontoxigenic culture from a strain of type D. When this nontoxigenic isolate was incubated in broth with filtrates of a toxigenic type C, toxigenic isolates producing type C toxin were recovered. This same nontoxigenic isolate, however, was apparently not sensitive to the phages of the parent strain, since it remained nontoxigenic when grown in broth with a filtrate from the toxigenic parent strain of type D.

In our studies, types C and D cultures were cured of their prophages and concomitantly ceased to produce their dominant toxins. These cured, nontoxigenic strains of types C and D could then be converted to either toxigenic type C or D depending upon the specific phage used.

This report describes the interconversion of these two types of *C. botulinum* by phages.

MATERIALS AND METHODS

Cultures. *C. botulinum* types C and D were obtained from the following sources: type D strain 1873 from H. Iida, University of Hokkaido, Japan; South African strain of type D from D. Boroff, Albert Einstein Center, Philadelphia, Pa.; type C strains 153

and 162 from A. Baillie, Unilever Research Laboratories, England.

All bacterial strains were maintained on SFEM medium (21) containing 0.5% glucose and 0.5% $(NH_4)_2SO_4$.

Nontoxigenic, phage-sensitive strains were isolated from spores that were heated at 70 C for 15 min or from vegetative cells grown in the presence of acridine orange. Strains AOA113, AO20, HS37, and HS15 were cured of their prophages and simultaneously ceased to produce their dominant toxins designated as C_1 for type C and as D for type D. Strains AOA113 and AO20 were cured of their prophages by culturing the toxigenic parental (1873 and South African, respectively) strain in medium containing acridine orange by following the procedures previously described (9, 10). Strains HS37 and HS15 were isolated from sporulated cultures (162 and 153, respectively) that were treated with heat. All phage-sensitive isolates were tested for toxigenicity.

Phages and preparation of phage stocks. The phages were isolated from the following toxigenic cultures: Phage $1D^{tox^+}$ (formerly designated as DEβ) from South African strain of type D; phage $2D^{tox^+}$ from strain 1873 of type D; phage $3C^{tox^+}$ from type C strain 162; phage $4C^{tox^+}$ from type C strain 153.

Phages were isolated from an 18-h broth culture of the toxigenic parent strain. The broth culture was centrifuged at 6,000 $\times$ *g* for 10 min and sterilized by filtration. The phages of each toxigenic strain were purified by five successive single-plaque isolations on the respective nontoxigenic, phage-sensitive strain.

Phage stocks were produced by propagating the purified phage with the phage-sensitive strain in TYG medium. Filtrates from an 18-h culture (phage-sensitive strain carrying the purified phage) were added to the actively growing indicator strain when the cell concentration was equivalent to optical density of 0.25 (Bausch and Lomb, Spectronic 20 at 525 nm). Phages were added to the bacterial culture at a ratio of 5 ml of filtrate to 15 ml of culture. The mixture was incubated for 5 to 6 h at 26 C, and phage titers of 10^7 to 10^8 plaque-forming units per ml were obtained. These stocks were filter-sterilized, refrigerated, and used within 4 days. The phage titers would often decrease when refrigerated for over 1 week.

Before using the phage stocks for studies of their relationship to toxigenicity of the bacterial culture, the stocks were treated with 40 μg of crystalline deoxyribonuclease II (Sigma) per ml for 3 h at 30 C and filter-sterilized.

All filtrates were checked to be bacteria-free by inoculating TYG broth and incubating for several weeks at 33 C.

Electron micrographs of the phages were prepared according to the procedures of Eklund et al. (8).

Phage assay. All phage experiments were carried out with bacteria-free filtrates. For assay of the phage, all indicator cells were grown in broth and plated while in the logarithmic phase of growth. The cultures were plated when the cell concentration was equivalent to an optical density between 0.25 and 0.30.

Base agar for strain HS37 was TYG (3% Trypticase, 2% yeast extract, 0.4% glucose, and 0.1% cysteine hydrochloride, pH 7.2). TPGY agar (2% Trypticase, 0.5% peptone, 0.1% glucose, 0.5% yeast extract, and 0.1% cysteine hydrochloride, pH 7.2) was used for strains HS15, AO20, and AOA113. The base agar was poured, and surfaces of the agar were dried in a 42 C drying oven.

All plates were pre-reduced in Brewer anaerobic jars by using hydrogen gas and were stored at 25 C for 3 days for sterility tests.

The soft agar overlay for strains HS15 and HS37 was EM agar (filtered infusion of 15 g of egg meat medium in 100 ml of water, and to the liquid portion were added 1% yeast extract, 0.5% $(NH_4)_2SO_4$, 0.5% glucose, 0.1% cysteine hydrochloride, and 0.7% agar, final pH 7.2). Overlays were dispensed in volumes of 3.3 ml per screw-capped tube. Before plating strain HS15, 0.1 ml of whole human citrated blood was added to the overlays. When strain HS37 was used, the following sterile solutions were added to each EM overlay: 0.6 ml of 20% sodium chloride and 0.2 ml of catalase (500 μg/ml, 3,600 U/mg; Sigma). The final pH of these overlays was 6.7. For strains AOA113 and AO20, TPGY (TPGY broth + 0.7% agar) overlays in 6-ml volumes were used. Before plating, the following sterile solutions were added to each TPGY tube: 0.8 ml of 20% sodium chloride, 0.6 ml of 12% lactalysate, and 0.2 ml of catalase.

Cultures were added to the molten agar in volumes of 1 ml for 3.3-ml overlays and 2 ml for 6-ml overlays. Phage stocks and dilutions thereof were added at a volume of 0.1 ml per overlay. The contents of the tubes were mixed and poured onto the surface of the base agar. All plates were placed in Brewer anaerobic jars with hydrogen gas and incubated overnight at 33 C.

Toxin assay and neutralization tests. The cultures were grown in SFEM or TYG medium to determine whether they produced toxin. All cultures were incubated at 30 or 33 C for 3 days. Culture supernatant fluids were assayed for toxin by injecting 0.5 ml intraperitoneally into Swiss Webster mice weighing 18 to 26 g. Toxin titrations were made by diluting samples in half-log intervals in gelatin-phosphate buffer and injecting mice with 0.5 ml of each dilution by the intraperitoneal route.

The effect of trypsin treatment on increasing the toxigenicity of the culture supernatant fluids was studied at 37 C by the procedures of Duff et al. (6) or Eklund and Poysky (7) by using a final concentration of 0.25% trypsin (Difco, 1:250).

Toxin neutralization tests were made by injecting pairs of mice with 0.6 ml of a mixture composed of a ratio of 0.5 ml of toxic fluid or dilutions thereof and 0.1 ml of a monovalent antitoxin. *C. botulinum* antitoxins were obtained from the Center for Disease Control, Atlanta, Ga. or were produced at our laboratory (7).

The C_2 and D antisera used in these studies were monospecific. The type C antiserum, however, contained approximately 10,000 anti-minimal lethal dose (MLD) of C_1 component and 10 anti-MLD of the D component per 0.1 ml. The type C antiserum would therefore neutralize low levels of D toxin produced by the type C strains. Therefore, whenever the C_1 toxin is

discussed, it is possible that low levels of D toxins may also have been present.

Relation of phages to toxigenicity. Two procedures were followed to test the relation of the phages to the toxigenicity of the nontoxigenic, phage-sensitive cultures. In the first procedure, dilutions of the filter-sterilized phages were plated with the nontoxic strain by using the agar-overlay procedure. Material from isolated plaques was transferred into TYG broth and incubated at 30 C for 3 days. The cultures were then tested for toxicity, phage production, and resistance to phage infection. In the second procedure, phages were added to actively growing, nontoxigenic cultures at different multiplicities of infection (MOI). After a 40-min exposure to the phage, the cultures were diluted in TYG broth and plated with TYG agar. Isolates were picked into TYG broth and incubated at 33 C for 3 days and assayed for toxin production and phage production and resistance. If the isolates were toxic, the type of toxin produced was determined by neutralization tests with the type C or D antisera.

The sensitivity of the bacterial culture was tested by adding the indicator culture to the overlay and spotting the surface of the overlay with the phage. When strains of AOA113, AO20, and HS15 were tested, the phage was spotted on the base agar and overlaid with soft agar and the indicator strain.

RESULTS

C. botulinum types C and D produce at least three toxins, which have been designated as C_1, C_2, and D (7, 18, 20). The C_1 toxin is the dominant toxin produced by type C strains, and the D toxin is dominant for type D strains. Some type D strains may produce low levels of C_1 and C_2 toxins, and type C strains may produce low levels of D and C_2 toxins (7, 18).

The results of curing type C and D cultures of their prophages and the effect on toxigenicity are summarized in Table 1. Cured, nontoxigenic cultures were isolated from the different toxigenic strains by culturing in acridine orange or by heating the spores. Cured isolates were not obtained when strain 1873 was treated with ultraviolet light. The effectiveness in the curing of these cultures is probably based upon the pseudolysogenic relationship of phages and bacteria of types C and D (9, 10). Each of the cured cultures was phage-sensitive and simultaneously discontinued the production of the C_1 and D toxins. Cured isolates from type D strain 1873 and type C strain 162, however, continued to produce C_2 toxin. All cultures were subcultured 12 times over a period of one year and they remained sensitive to the phages and did not produce the C_1 or D toxins.

The results of the studies on the relation of phages to the toxigenicity of the cured, nontoxigenic cultures are divided into two parts based upon the host range of the phages.

Type C strain 162 and type D strain South African. Strains HS37 (from 162) and AO20 (from South African) were cured cultures that simultaneously ceased to produce the C_1 and D toxins. The C_2 toxin was not detectable in any of these toxigenic or nontoxigenic cultures with or without trypsin treatment.

Phage 1D from the South African strain infected AO20 and HS37. Phage 3C from strain 162 infected HS37 but did not infect AO20.

Table 2 summarizes the results of the relation of Phage 1D to the toxigenicity of bacterial strain AO20. All broth cultures arising from plaque material were toxigenic, produced phage, and were resistant to phage 1D. After a 40-min contact with phage 1D, 40% of the 0.2

TABLE 1. *Curing C. botulinum types C and D cultures of their prophages and the effect on toxigenicity*

Bacterial strain	Type	Method of curing[a]	No. of surviving colonies	
			Cured and non-toxic[b]	Tested
South African	D	AO	5	127
South African	D	HS	19	39
1873	D	UV	0	110
1873	D	AO	23	214
162	C	AO	2	64
162	C	HS	1	40
153	C	HS	9	58

[a] Cultures were tested for phage sensitivity and toxicity after the following treatments: (AO) culture grown in TYG broth containing 15 μg of acridine orange; (HS) sporulated cultures heated at 70 C for 15 min; (UV) cultures spread on surface of TYG agar and irradiated with ultraviolet light for 60 s.

[b] Cured cultures of 1873 and 153 ceased to produce D and C_1 toxins but continued to produce C_2 toxin.

TABLE 2. *Reinfecting cured, nontoxigenic bacterial strain AO20 with phage 1D and effect on toxigenicity*[a]

Method of obtaining toxigenic cultures	No. of cultures	
	Toxigenic and phage producers	Tested
Plaques[b]	40	40
MOI[c] 0.2	16	40
MOI 2.0	28	40
MOI 5.8	30	40

[a] All toxins were neutralized by antiserum of type D.

[b] Broth cultures arising from plaque material.

[c] Cultures were exposed to different concentrations of bacteriophages for 40 min and isolates were tested.

MOI, 70% of the 2.0 MOI, and 75% of the 5.8 MOI cultures were converted to toxigenicity and produced phages. The cultures remaining nontoxigenic were sensitive to phage 1D, and, when infected, they simultaneously became toxigenic, produced phage, and were resistant to phage 1D. A cured culture reinfected with phage 1D was permitted to sporulate, and the spores were heated at 70 C for 20 min to inactivate the free phage. Isolated colonies were tested for toxigenicity and phage production. Of the 37 isolates tested, 29 were nontoxic and sensitive to phage 1D. When these nontoxigenic cultures were infected with phage 1D, they again became toxigenic and produced phage. The toxins produced by each of these cultures were neutralized only by type D antiserum.

Table 3 summarizes the results of the relation of phage 3C to the toxigenicity of strain HS37. Each culture started from plaque material was toxigenic and produced phage. The exposure of HS37 to phage 3C at an MOI of 0.2 resulted in 27.5% of the tested cultures being toxigenic. Increasing the MOI to 2.0 increased the toxigenic cultures to 35%. Cultures remaining nontoxigenic continued to be phage sensitive, and these cultures could be converted to toxigenicity when infected with phage 3C. The toxin produced by culture HS37(3C) was neutralized only by type C antiserum.

The data on the relation of phage 1D to the toxigenicity and type of toxin produced by HS37(1D) are shown in Table 4. Cultures from plaque material produced toxin and were resistant to both phages 1D and 3C. When HS37 was infected with phage 1D at different MOIs (no salt), none of the 0.2 and 3.0 MOI cultures tested produced toxin. Only two of the 40 cultures from the 5.0 MOI produced toxin. In earlier studies on procedures for assaying phage particles, plaques were obtained when the soft agar overlay contained 2.5% sodium chloride.

TABLE 3. *Reinfecting cured, nontoxigenic bacterial strain HS37 with phage 3C and effect on toxigenicity*[a]

Method of obtaining toxigenic cultures	No. of cultures	
	Toxigenic and phage producers	Tested
Plaques[b]	40	40
MOI[c] 0.2	11	40
MOI 2.0	14	40

[a] All toxins were neutralized by antiserum of type C.
[b] Broth cultures arising from plaque material.
[c] Cultures were exposed to different concentrations of bacteriophages for 40 min and isolates were tested.

TABLE 4. *Reinfecting cured nontoxigenic bacterial strain HS37 with phage 1D and effect on toxigenicity*

Method of obtaining toxigenic cultures	No. of cultures		Toxin neutralized by antiserum of
	Toxigenic and phage producers	Tested	
Plaques[a]	40	40	Type D
MOI 0.2 (2.0% salt)[b]	2	40	Type D
MOI 0.2 (no salt)	0	40	
MOI 2.0 (2.0% salt)	2	40	Type D
MOI 2.0 (no salt)	0	40	
MOI 5.0 (2.0% salt)	12	40	Type D
MOI 5.0 (no salt)	2	40	Type D

[a] Cultures started with plaque material were tested.
[b] Broth cultures contained 2.0% sodium chloride when mixed with bacteriophage 1D.

Therefore, an additional experiment was made exposing HS37 to phage 1D in broth containing 2.0% sodium chloride (Table 4). After a 40-min exposure to the phages, 5% of the 0.2 and 2.0 MOI cultures and 30% of the 5.0 MOI cultures were toxigenic. Nontoxic cultures were sensitive to phage 1D (and also phage 3C) and produced toxin when infected. The toxin produced by HS37(1D) was neutralized by type D antiserum. Some toxic samples contained only 10 MLD of toxin per ml. In this case, type C antiserum offered protection because of the presence of the D component. The toxicity of these cultures, however, was increased to 2,000 MLD by trypsin treatment. Then the trypsinized culture supernatant fluid was neutralized only by type D antiserum. Phage 1D was again isolated from HS37(1D), and it was used to infect strain AO20. The toxin titer of strain AO20(1D) was generally in the range of 10,000 MLD per ml of culture fluid. These titers were also increased 10-fold after trypsin treatment, and the toxin was neutralized by type D antiserum.

Strain HS37(1D) was permitted to sporulate, and the spores were heated at 70 C for 20 min. Of the 92 cultures tested, 16 were nontoxigenic and sensitive to phages 1D and 3C. When reinfected with these phages, they produced toxin neutralized by types D and C antisera, respectively.

Culture HS37(1D) often lost its toxigenicity after three or four transfers in TYG broth. The toxigenicity could be maintained longer when the SFEM medium containing 2.0% sodium chloride was used.

The relation of phages 1D and 3C to the toxigenicity and type of toxin produced by cured strains of types C and D is summarized further in Fig. 1 and 2. Culture AO20 could be infected only by the phage from the toxigenic

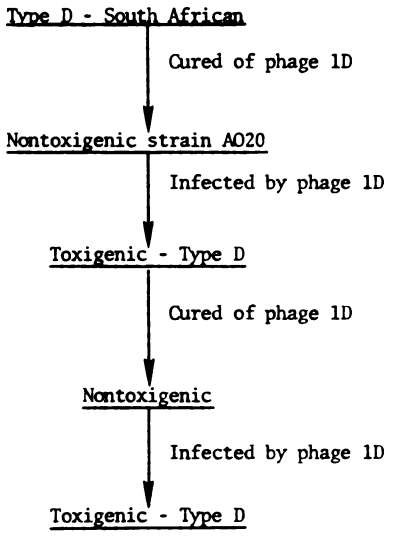

FIG. 1. *Relation of phage 1D^{tox+} to the toxigenicity and type of toxin produced by cured cultures of South African strain of type D.*

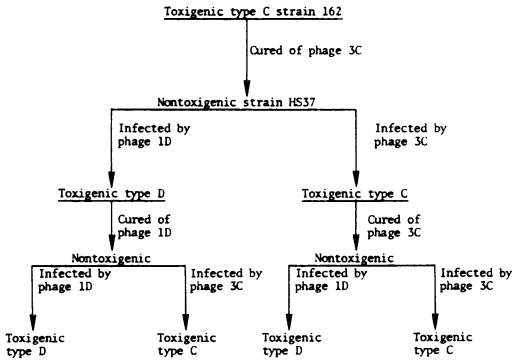

FIG. 2. *Relation of phages 1D^{tox+} and 3C^{tox+} to the toxigenicity and type of toxin produced by cured cultures of type C strain 162.*

parent. Culture HS37 could be infected with either phage 1D or 3C. The phage in each case governed the type of toxin produced. Cultures infected with phage 1D produced toxin neutralized by type D antiserum, and cultures infected with 3C produced toxin neutralizable only by type C antiserum.

Phage 1D produced turbid plaques 1.0 to 2.0 mm in diameter on lawns of strain AO20, whereas with strain HS37 the plaques were 0.5 to 1.5 mm in diameter. Phage 3C produced turbid plaques on lawns of strain HS37 that were variable in size (0.5 to 3.0 mm in diameter).

Photomicrographs of phages 1D and 3C are shown in Fig. 3. Phage 1D exhibited a polyhedral head measuring 100 nm in diameter and a tail 340 nm in length and 10 nm in diameter. The tail of phage 1D was surrounded by a sheath 140 nm in length and 28 nm in diameter. Phage 3C also exhibited a polyhedral head 110 nm in diameter and a tail 370 nm in length and 10 nm in diameter. The sheath surrounding the tail of phage 3C was 160 nm long and 35 nm wide.

Type C strain 153 and type D strain 1873. Strains HS15 (from 153) and AOA113 (from 1873) were cured cultures that simultaneously ceased to produce the C_1 and D toxins. These cultures, however, continued to produce C_2 toxin which was toxic only after trypsin treatment. Phage 4C from strain 153 infected both HS15 and AOA113. Likewise, phage 2D from strain 1873 would infect both of the cured cultures.

To study the relation of these phages to the toxigenicity, material from isolated plaques was transferred into TYG broth and incubated at 33 C for 3 days and assayed for toxin and phage production.

The relation of phages 2D and 4C to the toxigenicity and type of toxin produced by strains AOA113 and HS15 is summarized in Table 5 and Fig. 4. All cultures arising from plaque material of strains AOA113 and HS15 infected with phages 2D and 4C were toxic and produced phages. Cultures infected with either phage were resistant to infection from both phages. Both of the cured types C and D cultures produced toxin that was neutralized by type D antiserum when they were infected with phage 2D. When either of the cured strains was infected with phage 4C, then toxin neutralized only by type C antiserum was produced. The toxigenic cultures in each case also produced the C_2 toxin; however, this toxin was not detectable unless the culture fluid was treated with trypsin.

Each of these cultures, HS15(2D), HS15(4C) AOA113(2D), and AOA113(4C), was permitted to sporulate, and the spores were heated to 70 C for 20 min. Cultures sensitive to the phages and producing only the trypsin-activated C_2 toxin were isolated. These cultures in turn were then infected with either phage 4C or 2D, and they again produced toxin neutralized by types C and D antisera, respectively. The toxin titers of strain AOA113(4C) and HS15(4C) were 2,000 MLD per ml of culture supernatant fluid. In comparison, the toxin titers of strain AOA113(2D) and HS15(2D) were 100,000 MLD/ml. No significant increases in toxicity were obtained when these culture supernatants were treated with trypsin.

Phages 2D and 4C both produced turbid

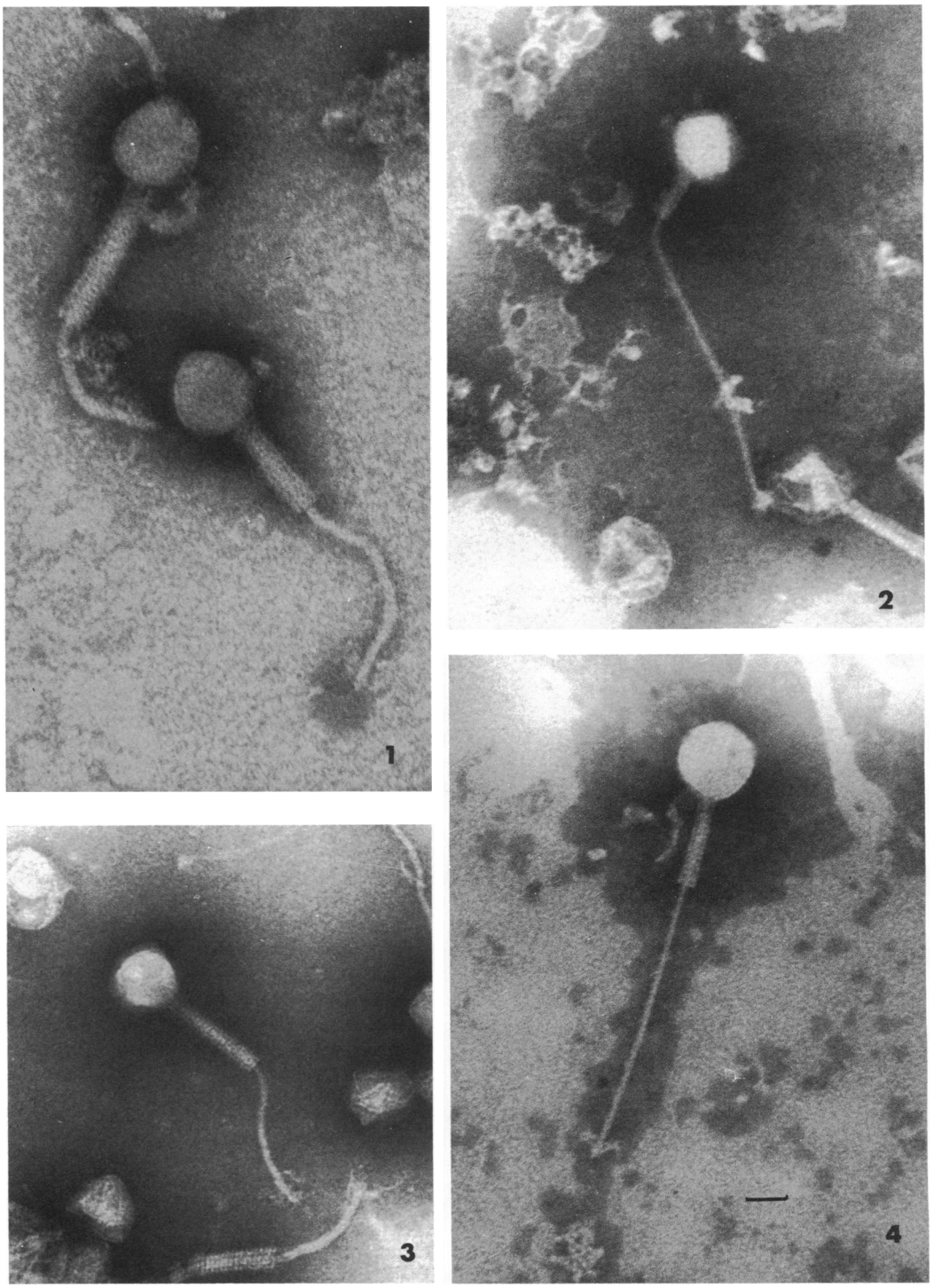

FIG. 3. *Phages of C. botulinum types C and D: (1) 3C^{tox+}; (2) 4C^{tox+}; (3) 1D^{tox+}; and (4) 2D^{tox+}. ×142,600. Bar marker represents 50 nm.*

TABLE 5. *Relation of phages 2D and 4C to the toxigenicity of AOA113 and HS15*

Nontoxigenic[a] cured cultures	Phage	No. of cultures		Toxin neutralized by antiserum of
		Toxigenic and phage producers	Tested	
AOA113	2D	20	20	Type D
AOA113	4C	37	37	Type C
HS15	2D	20	20	Type D
HS15	4C	20	20	Type C

[a] Cultures ceased to produce C_1 and D toxins but continued to produce C_2 toxin, which is toxic only after trypsin treatment.

plaques on cultures AOA113 and HS15. Plaques produced by phage 2D ranged from 0.5 to 1.0 mm in diameter, whereas those produced by phage 4C ranged from 1.0 to 2.0 mm.

Phages 2D and 4C were very similar in morphology and size (Fig. 3). Both exhibited polyhedral heads measuring 100 nm in diameter and long narrow tails 455 nm in length and 7 nm in diameter. The sheath surrounding the tail of phage 2D was 107 nm long and 22 nm wide, whereas the sheath of phage 4C was 115 nm long and 22 nm wide. Phages comparable to these phages in size and morphology have been observed in lysates of other strains of types C and D (5, 10, 15, 24).

DISCUSSION

Bacteriophages play an active role in the production of several bacterial toxins. Freeman's (12) remarkable observations on the participation of the phage in the production of diphtheria toxin paved the way for what has been learned concerning phage-host relationships in certain bacterial toxins. Subsequent reports (1, 13) pointed out the complex nature of this relationship and established that specific phages governed the toxigenicity of *Corynebacterium diphtheriae*.

In 1961, Blair and Carr (2) showed that nontoxigenic staphylococcal strains could be converted to alpha-toxin producers by lysogenizing them with phages from toxigenic strains. Similarly, non-enterotoxigenic strains could be converted to enterotoxin A producers when they were lysogenized with phage from toxigenic strains (3). In contrast, when staphylococcal cultures producing beta-toxin were lysogenized with specific phages, the bacteria then ceased to produce toxin (4). These cultures, however, would produce the beta-toxin when they again became nonlysogenic.

Conversion of nontoxigenicity to toxigenicity has also been reported in group A streptococci (27). Curing the streptococci of their prophages resulted in nontoxigenic cells. Infection of these cured cultures with phages from known scarlatinal toxin strains resulted in lysogenic and toxigenic strains.

The relationship of phages to toxigenicity has also recently been shown in the anaerobic bacterium. *Clostridium botulinum* (9, 10, 16, 17).

The species *C. botulinum* includes a very heterogeneous group of strains that are divided into types A through G based upon the antigenic specificity of the neurotoxins that are produced. The strains of these seven types can be separated into four groups according to their deoxyribonucleic acid homologies and biochemical, physiological, and serological characteristics (11, 14, 19, 22, 23, 25, 26). The members of these groups are as follows: group I, proteolytic types A, B, and F; group II, types C and D; group III, nonproteolytic types B, E, and F; and group IV, the recently isolated type G. Types C and D are further related in that they may produce three toxic components: C_1, C_2, and D (7, 18, 20). Type C strains produce mainly C_1 toxin but may produce small amounts of C_2 and D toxins. Type D may produce, in addition to D toxin, small amounts of C_1 and C_2 toxins.

In the current studies, when types C and D cultures that also produced the C_2 toxin were cured of their prophages, they ceased to produce the C_1 and D toxins and became indistinguishable in that they produced only the C_2 toxin. The production of the C_2 toxin was not governed by the phages used in these studies. On the other hand, when types C and D cultures that did not

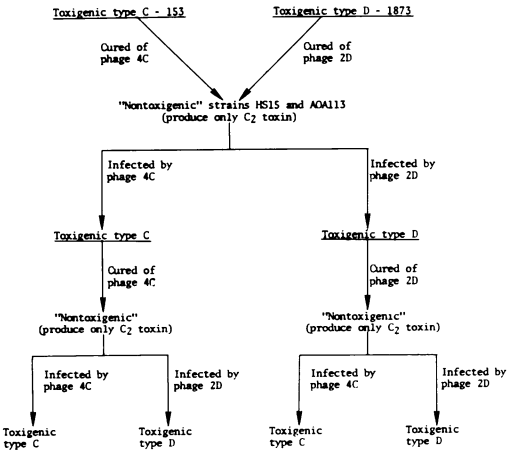

FIG. 4. *Relation of phages $2D^{tox+}$ and $4C^{tox+}$ to the toxigenicity and type of toxin produced by cured cultures of type C strain 153 and type D strain 1873.*

produce the C_2 toxin were cured of their prophages, they ceased to produce any toxin.

Further studies with the two different groups of types C and D cultures showed that types C and D can arise from a common cured bacterial strain. The dominant type of toxin (C_1 or D toxin) produced by the cured cultures of types C and D required the continued participation of specific tox^+ phages. This, however, does not preclude the fact that small amounts of D or C_1 toxin may be produced when infection is by the types C and D phages, respectively.

Previous studies in this laboratory (9, 10) have shown that the relation between the phages and types C and D bacteria is probably pseudolysogeny. The instability of this relationship through the spore state of the organisms used in this current study also suggests that a pseudolysogenic relationship exists. Because of this pseudolysogenic state, it is therefore conceivable that toxigenic types C and D strains in nature could lose their prophages and become nontoxigenic. These nontoxigenic isolates could then be converted to type C by type C phage or to type D by type D phage. Types C and D and nontoxigenic cultures resembling *C. botulinum* have been reported from many parts of the world (22). In fact, the 1873 and South African strains of type D used in these studies were isolated in South Africa, and the type C strain 153 was isolated from poultry in England.

Some of the types of *C. botulinum* other than types C and D that we have studied have been cured of their prophages but still remain toxigenic. It is possible either that they carry other phages or that not all *C. botulinum* toxins are induced by phage.

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