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Relationship of Bacteriophages to Alpha Toxin Production in *Clostridium novyi* Types A and B

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The relationship of specific bacteriophages to the production of the lethal alpha toxin in *Clostridium novyi* types A and B was investigated. When type A strain 5771 reverted to the phage-sensitive state, it ceased to produce alpha toxin but continued to produce the gamma and epsilon antigens. This "nontoxigenic" culture, therefore, more closely resembled *C. botulinum* types C and D than the other *C. novyi* types. Phage-sensitive type B strains also ceased to produce the alpha toxin but continued to produce the beta toxin, and therefore very closely resembled *C. novyi* type D (*C. haemolyticum*). Alpha toxin was again produced when the phage-sensitive cultures were reinfected with the respective tox^+ phages. Alpha toxin production could also be induced in the "nontoxigenic" phage-sensitive derivatives from type B strain 8024 by tox^+ phages isolated from other strains of type B. tox^- phages were also isolated, but they did not affect alpha toxin production. The tox^+ phages also caused a marked change in the colonial morphology of type B strains. In this report we present evidence that alpha toxin production by *C. novyi* type A strain 5771 and type B strain 8024 depends upon the continued presence and participation of specific bacteriophages designated as $NA1^{tox+}$ and $NB1^{tox+}$, respectively.

Based upon the production of eight different soluble antigens, the species *Clostridium novyi* is divided into four types designated by the letters A, B, C, and D (16, 20, 21, 26, 27, 30, 32). The three pathogenic types include the classical type A, which is frequently involved in gas gangrene infections in man and animals; type B, the etiological agent of infectious necrotic hepatitis (black disease) that has been observed in sheep and other animals; and type D (*C. haemolyticum*), the causal organism of bacillary hemoglobinuria in cattle (2, 26, 27, 31, 32). Type C is generally regarded as nonpathogenic to laboratory animals. Types A and B are the only types that produce the highly lethal alpha toxin.

In this report, we present evidence that alpha toxin production by *C. novyi* type A strain 5771 and type B strain 8024 depends upon the continued participation of specific bacteriophages designated $NA1^{tox+}$ and $NB1^{tox+}$, respectively. Bacterial strains 5771 and 8024 cease to produce alpha toxin when they revert to the phage-sensitive state. Reinfection of these phage-sensitive derivatives with the respective phages from the toxigenic parent culture results in a concomitant production of alpha toxin and immunity to the homologous phage.

MATERIALS AND METHODS

Cultures. The source of the *C. novyi* bacterial cultures and the designations of their phages are given in Table 1. Nontoxigenic *C. botulinum* type C strain HS37 was isolated from toxigenic strain 162 (5).

In addition to producing alpha toxin and other minor antigens, *C. novyi* types A and B each produce a major lecithinase designated as gamma and beta toxin, respectively. The use of the word "nontoxigenic," therefore, will refer hereafter to *C. novyi* strains that have ceased to produce only the alpha toxin. Nontoxigenic, phage-sensitive strains of *C. novyi* were isolated from spores that were heated at 70°C for 15 min to inactivate any exogenous phage, or from vegetative cells cultured in TYG medium (4) containing 5 to 10 μg of acridine orange per ml at pH 7.4. After treatment, the spores or vegetative cells were diluted in TYG broth, plated with TYG agar, and incubated in Brewer anaerobic jars for 48 h at 33°C. Isolated colonies were cultured in cooked-meat (CM) medium (10) for 3 to 5 days and tested for alpha toxin production by the mouse assay and for sensitivity to the phages of the toxigenic parent culture by the agar-layer procedure (1). Strains HS10, HS36, HS37, HS71, HS88, and HS93 are nontoxigenic phage-sensitive derivatives that were isolated from *C. novyi* type A strain 5771. Strains AO26 and AO52 are nontoxigenic derivatives that were isolated from type B strain 8024 cultured in TYG broth

TABLE 1. *Source and designation of phages from C. novyi types A and B*[a]

Source of phage	Strain received from:	Phage[b]
Type A strain 5771	L. DS. Smith	$NA1^{tox+}$
Type A strain 5771	L. DS. Smith	$NA2^{tox-}$
Type B strain 8024	L. DS. Smith	$NB1^{tox+}$
Type B strain 190	M. Macheak	$NB2^{tox+}$
Type B strain KZ391	S. Nishida	$NB3^{tox+}$
Type B strain KZ391	S. Nishida	$NB4^{tox-}$
Type B strain KZ394	S. Nishida	$NB5^{tox+}$
Type B strain KZ394	S. Nishida	$NB6^{tox-}$
Type B strain KZ395	S. Nishida	$NB7^{tox+}$
Type B strain KZ395	S. Nishida	$NB8^{tox-}$
Type B strain KZ396	S. Nishida	$NB9^{tox+}$

[a] *C. novyi* type B strains KZ392 and KZ393 and type D strains KZ406, KZ409, KZ410, KZ411, KZ413, and KZ414, received from S. Nishida, did not produce phages that would infect nontoxigenic type B strain AO52.

[b] The NA series of phage infects nontoxigenic type A strain HS10, and the NB series of phage infects nontoxigenic type B strain AO52.

containing acridine orange. All of the *C. novyi* strains were maintained on CM medium, and nontoxigenic *C. botulinum* type C strain HS37 was maintained on SFEM medium (24) containing 0.5% glucose and 0.5% $(NH_4)_2SO_4$.

Preparation of phage stocks and phage assays. Phages $NA1^{tox+}$ and $NA2^{tox-}$ were isolated from *C. novyi* type A strain 5771. All of the nontoxigenic derivatives from strain 5771 were sensitive to phage NA1 but continued to carry phage NA2. Phage NB1 was isolated from *C. novyi* type B strain 8024.

Phages were isolated from an 18-h TYG broth culture of the toxigenic parent strain. The broth was centrifuged at 6,000 × *g* for 10 min and sterilized by filtration. Phages NA1 and NB1 were purified by five successive single-plaque isolations on the nontoxigenic, phage-sensitive strains HS10 and AO52, respectively. Phage NA2 was purified and propagated on strain HS37 (a nontoxigenic derivative from *C. botulinum* type C). Phage stocks were produced by propagating the purified phage with the respective indicator strain in TYG medium. Purified phages from an 18-h broth culture filtrate was added to the bacterial culture at a ratio of 3 ml of filtrate to 15 ml of actively growing indicator strain. The mixture was incubated for 5 to 6 h at 33°C, and phage titers of 10^5 to 10^6 plaque-forming units per ml were obtained. Phage NA1 was very unstable even during overnight storage at 5 or 25°C and was therefore used the same day as prepared. Phage NB1 was more stable and could be stored for at least 24 h. Bacteriophage stocks were treated with 40 μg of crystalline deoxyribonuclease II (Sigma) per ml for 1 h at 30°C and filter sterilized before use.

Filtrates from overnight cultures of other type B cultures were also tested to determine whether they contained phages that would infect strain AO52. These phages were purified on strain AO52, using the same procedures described for phage NB1.

All filtrates were checked to be bacteria free by inoculating TYG broth or CM medium and incubating for several weeks at 33°C. Electron micrographs of the phages were prepared according to the procedures of Eklund et al. (6).

All experiments were carried out with bacteria-free filtrates. The general phage assay procedures were the same as previously reported for *C. botulinum* (5). The base agar used in the agar-layer procedure contained Trypticase, peptone, yeast extract, and glucose (TPGY). The overlay was 3.3 ml of TPGY soft agar (0.7%) to which the following additions were made immediately before plating: 0.4 ml of 20% sodium chloride; 0.2 ml (500 μg/ml) of catalase (3,600 U/mg; Sigma); and 1 ml of culture. Cysteine hydrochloride at a final concentration of 0.1% was used as the reducing agent in all media except CM.

Toxin assay, neutralization tests, and antiserum production. The cultures were grown in CM and TYG media for 3 to 5 days at 33°C and tested for their ability to produce alpha toxin by assaying the supernatant fluids by the mouse intraperitoneal route. The mouse neutralization test was used to identify the toxin present in the culture supernatant fluids. Pairs of mice were inoculated with 0.6 ml of a mixture composed of a ratio of 0.5 ml of toxic fluid and 0.1 ml of *C. novyi* type A or B antiserum, both of which contain the same alpha antitoxin component. Unprotected mice were inoculated with 0.5 ml of the toxic fluid.

The guinea pig protection test was used in addition to the mouse assay to determine whether low levels of alpha toxin were produced (27). Guinea pigs weighing approximately 300 g were inoculated intraperitoneally with 1 ml of *C. novyi* type A or B antiserum. After 24 h, guinea pigs were inoculated intramuscularly with 1.0 ml of an 18-h CM culture of nontoxigenic strains of *C. novyi* or nontoxigenic culture reinfected with the respective tox^+ phage. Unprotected animals were similarly inoculated with the same cultures. After 48 and 72 h, animals were examined postmortem for the characteristic colorless gelatinous edema that is induced by the alpha toxin.

Nontoxigenic isolates were also examined to determine whether any intracellular alpha toxin could be detected. Cells (not producing alpha toxin in test tubes) from 2-, 4-, and 6-day-old TYG broth cultures and cultures grown in cellophane tubes bathed in broth (28) were lysed by sonification and assayed for alpha toxin before and after concentration (800 ml to 50 ml) by dialysis against polyethylene glycol (15). Nontoxigenic strains of type B produced lethal levels (1 to 4 minimal lethal doses [MLD]/ml) of beta toxin that was detectable in unconcentrated TYG broth after 2 and sometimes 3 days but not after 4 days of incubation. Type B strains, therefore, were incubated for 4 to 6 days, and toxic samples were neutralized with type A antiserum, which does not contain the beta component. The beta toxin was identified by neutralization with *C. haemolyticum* (*C. novyi* type D) and type B antisera.

The production of gamma and epsilon antigens by type A strains and beta antigen by type B strains was tested by using egg yolk agar plates and egg yolk emulsions (20, 21, 32). The blockage of the reactions of gamma and beta antigens was tested by

using antisera against types A and B. *C. novyi* type A and B antisera were obtained from Burroughs Wellcome Research Laboratories, and *C. novyi* type D antiserum was received from M. Macheak, Department of Agriculture, Ames, Iowa.

Antiserum against type B phage NB1 was produced in New Zealand rabbits starting with 0.5 ml of phage (10^9 plaque-forming units) concentrated by ultracentrifugation (50,000 × *g*) and increasing the inoculum in increments of 0.5 ml up to a total volume of 2.5 ml. The animals were immunized by a combination intraperitoneal and subcutaneous route. The animals were immunized every 3 days for a total of six injections. When trial bleedings indicated a satisfactory level of antibody, the rabbits were bled by cardiac puncture.

Relation of phages to alpha toxin production. Dilutions of filter-sterilized phages were plated with each of the nontoxigenic phage-sensitive cultures by using the agar-overlay procedure. Material from the center of isolated phage plaques was transferred into TYG broth and incubated at 33°C for 3 to 5 days and assayed for alpha toxin production. The sensitivity of these cultures was tested by spotting the surface of the base agar with the respective phage and by adding the indicator culture to the overlay agar. The production of phage was tested in the same manner, except that the lysates were from the cultures arising from plaque material and the indicator strain was strain HS10 for type A phages and AO52 for type B phages. Cultures arising from plaque material were permitted to sporulate, and the spores were heated at 70°C for 15 min to inactivate any exogenous phage, diluted, and plated on TYG agar. Isolated colonies were tested for immunity to phage NA1 or NB1 and for alpha toxin and phage production. Nontoxigenic phage-sensitive isolates were again plated with phage NA1 or NB1 and material from plaques transferred into TYG broth, and were assayed for phage and alpha toxin production. These cultures were also permitted to sporulate, and heated spores were again cultured in TYG broth and tested for alpha toxin production, phage sensitivity, and phage production.

Phages from other type B strains were tested to determine whether they would induce alpha toxin production in nontoxigenic type B strain AO52. Materials from phage plaques were transferred into TYG broth and tested for alpha toxin production and phage immunity by the same procedures used for NB1 phage.

RESULTS

Isolation and demonstration of nontoxigenicity of phage-sensitive isolates. (i) Isolation of nontoxigenic strains of types A and B. Of the 100 isolated colonies selected from heat-treated spores of type A strain 5771, 94 continued to produce alpha toxin (100 to 200 MLD/ml) and phage $NA1^{tox+}$ and were immune to phage NA1. The supernatant fluids of the remaining six isolates (HS10, HS36, HS57, HS71, HS88, and HS93), however, did not contain lethal levels of the alpha toxin and, in addition, were sensitive to phage NA1 from the toxigenic parent strain 5771 (Table 2).

Nontoxigenic, phage-sensitive isolates could not be isolated from the heated spores of type B strain 8024. When strain 8024 was cultured in TYG containing acridine orange, two of the 100 isolates (designated as AO26 and AO52) were nontoxigenic and sensitive to phage $NB1^{tox+}$ from the toxigenic parent strain 8024 (Table 2). The remaining cultures were immune to phage NB1 and also continued to produce alpha toxin. The phage-sensitive isolates from both types A and B remained nontoxigenic through 40 transfers over a 1.5-year period in TYG or CM medium with and without fermentable carbohydrates (0.1 and 0.4% glucose or 1% maltose).

(ii) Toxin assay of lysed cultures and concentrated fluids. Cells from 3-, 4-, 5-, and 10-day-old cultures of the nontoxigenic isolates HS10 (from type A) and AO26 (from type B) were lysed by sonification, but detectable levels of intracellular alpha toxin could not be demonstrated. Supernatant fluids of nontoxigenic type A isolates concentrated by dialysis against polyethylene glycol contained very low titers (2 MLD/ml) of a lethal toxin that was not the alpha toxin. This toxin was neutralized by undiluted type A but not by a 1:10 dilution of type A or undiluted type B antisera. This toxin, however, was not detected in unconcentrated fluids of the nontoxigenic isolates cultured for 1 to 7 days at 33°C in either CM medium or TYG broth. Nonetheless, in order to prevent the possible confusion of low levels of this toxin with low levels of alpha toxin, all toxic culture fluids from type A were neutralized with type B antiserum, which contains the alpha component.

Unconcentrated and concentrated supernatant fluids from 2- and 3-day-old TYG and CM cultures of nontoxigenic type B strain AO52

TABLE 2. *Isolation of phage-sensitive cultures of C. novyi type A and type B that cease to produce alpha toxin*

Bacterial strain	Culture treatment[a]	No. of colonies	
		Phage sensitive and nontoxigenic	Tested
Type A, 5771	HS	6	100
Type B, 8024	HS	0	120
Type B, 8024	AO	2	100

[a] Cultures were tested for phage sensitivity and alpha toxin production after the following treatments: (AO) culture grown in TYG broth containing 5 to 10 μg of acridine orange per ml; (HS) sporulated cultures heated at 70°C for 15 min to inactivate free phage.

contained lethal levels of beta toxin but did not contain detectable levels of alpha toxin. The beta toxin was not neutralized by type A antiserum but was neutralized by *C. novyi* type B and type D (*C. haemolyticum*) antisera, which contain the beta component. Hemoglobinuria was evident after 24 to 48 h in some mice that had been inoculated with supernatant filtrates containing the beta toxin of type B strain AO52.

(iii) Guinea pig test. The guinea pig protection test was used in addition to the other tests to determine whether the alpha toxin was being produced at levels not detectable by the mouse assay (27). Inoculum levels of 1 ml of an 18-h CM culture of nontoxigenic type A strain HS10 with and without $CaCl_2$ were not lethal to guinea pigs. Postmortem examination of guinea pigs 2 and 3 days after inoculation did not show any evidence of the typical colorless gelatinous edema caused by alpha toxin. The same inoculation level of type B strain AO52, however, was lethal to guinea pigs within 48 h. Postmortem examination of guinea pigs inoculated with strain AO52 did not show evidence of the typical thick, colorless, gelatinous edema of alpha toxin but did show a moderate amount of hemorrhagic subcutaneous edema, and extreme redness of the muscles was observed. This toxin was neutralized by both type B and D antisera.

The results of these studies, therefore, show that the nontoxigenic, phage-sensitive isolates of *C. novyi* type A strain 5771 and type B strain 8024 do not produce detectable levels of the alpha toxin. The nontoxigenic type A isolates continued to produce the characteristic opalescence (epsilon toxin) seen on and around colonies on egg yolk agar, and also the lecithinase activity in egg yolk emulsion (gamma toxin). Nontoxigenic type B isolates continued to produce the beta toxin (lecithinase) reaction in egg yolk emulsions and on egg yolk agar plates. This reaction was blocked by both type B and D antisera. These nontoxigenic type B cultures are therefore very similar to *C. novyi* type D.

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

Bacteriophages and toxin production by type A strain 5771. (i) Relationship of phages $NA1^{tox+}$ and $NA2^{tox-}$ to alpha toxin production. All nontoxigenic isolates of strain 5771 continued to carry phage $NA2^{tox-}$, and repeated efforts to cure these bacteria of phage NA2 were unsuccessful. No evidence was found to suggest a role of phage NA2 in alpha toxin production. As further evidence for its lack of involvement in alpha toxin production, the NA2 phage from each of the six nontoxigenic derivatives would infect a nontoxigenic derivative of *C. botulinum* type C but would not induce alpha toxin production.

Filter-sterilized fluids containing phage $NA1^{tox+}$ were very unstable even during overnight storage at 25 or 5°C. Numerous attempts to stabilize this phage by varying cultural conditions or by filtration in a nitrogen atmosphere were unsuccessful; therefore, all experiments were performed the same day that the filtrates were prepared. Phage NA1 was purified by five successive single-plaque isolations on indicator strain HS10, one of the six nontoxigenic isolates. Filtration of the plaque material between transfers ruled out the passage of alpha toxin-producing cells during purification. This was confirmed in each transfer by sterility tests in TYG broth and CM medium. After each purification step, material from 10 isolated plaques was picked into TYG broth, and all of these produced phage NA1 and alpha toxin and in turn were immune to phage NA1. After the fifth purification step, a single isolated plaque was selected for further studies.

Phage NA1 was plated on each of the six nontoxigenic isolates, and isolated plaques were transferred into TYG broth. All cultures arising from plaque material produced phage NA1 and, in addition, were immune to phage NA1 and produced alpha toxin. As a control, areas of the soft-agar plates devoid of plaques were picked into TYG broth. These cultures remained phage sensitive and nontoxigenic.

To determine whether the continued participation of phage NA1 was necessary to maintain the toxigenic characteristic, the toxigenic culture HS10(NA1) was permitted to sporulate, and the spores were washed and then heated to 70°C for 15 min to eliminate any free phage. The spores were diluted and plated on TYG agar, and 10 isolated colonies were selected at random and transferred into TYG broth. One of the toxigenic phage-producing isolates was again permitted to sporulate, and spores were treated with heat and plated on TYG agar. Of the 50 colonies selected from this treatment, 25 continued to produce alpha toxin and phage NA1 and were immune to infection by NA1. The remaining isolates were nontoxigenic and also lost their immunity to phage NA1. These 25 isolates were again plated with filtrates of phage NA1, and cultures from the plaque material were toxigenic and immune to phage NA1. These cultures were transferred over 12 times during a period of 1 year in CM medium, and they continued to produce alpha toxin and phage NA1.

Alpha toxin production by strain HS10(NA1)

was also confirmed by the production of the characteristic gelatinous edema in guinea pigs. This reaction was blocked by the alpha component in both type A and B antisera.

(ii) Effect of heat treatment on loss of prophage and alpha toxin production. Nishida and Nakagawara (19) reported that a strain of *C. novyi* type A produced less alpha toxin as the spores were subjected to higher temperatures. Cultures from a spore inoculum heated at 100°C for 10 and 100 min produced 10 and 0 MLD/ml of alpha toxin, respectively. In comparison, cultures from an unheated spore inoculum produced 10^5 MLD/ml. We repeated this experiment in part with spores of *C. novyi* strain 5771. Of the 20 colonies selected from the unheated spores, 19 continued to produce alpha toxin and phage NA1, whereas one culture was sensitive to phage NA1 and did not produce alpha toxin. Heating the spores for 10 min at 100°C yielded 17 toxic isolates and one phage-sensitive nontoxigenic culture. The treatment at 100°C for 100 min inactivated all of the spores. Both the unheated and heated (100°C for 10 min) spore suspensions were cultured in CM medium to determine whether toxin titers varied. After 5 days of incubation at 33°C, both cultures produced approximately 200 MLD of alpha toxin per ml of broth. In these particular experiments, heat treatment of the spores did not appear to have any major effect on alpha toxin production or curing of prophage NA1. The differences in the results of our studies and those of Nishida and Nakagawara (19) may be peculiar to the different strains used in each of the studies.

(iii) Morphology of phage $NA1^{tox+}$ and $NA2^{tox-}$. Bacteriophage NA1 produced colony-centered plaques (0.5 to 1.0 mm in diameter surrounded by a turbid halo) on the nontoxigenic strains. In electron micrograph preparations, NA1 exhibited a polyhedral head 65 nm in diameter and a tail 160 nm long and 5 nm in diameter and surrounded by a sheath 68 nm long and 20 nm in diameter (Fig. 1). Phage NA2 formed turbid plaques (0.5 to 1.0 mm in diameter) when filtrates of NA2 phage were plated on a nontoxigenic strain HS37 of *C. botulinum* type C. Bacteriophage NA2 also exhibited a polyhedral head 55 nm in diameter and a tail 145 nm long and 4 nm wide (Fig. 2). The morphologies of the NA1 and NA2 phages are very

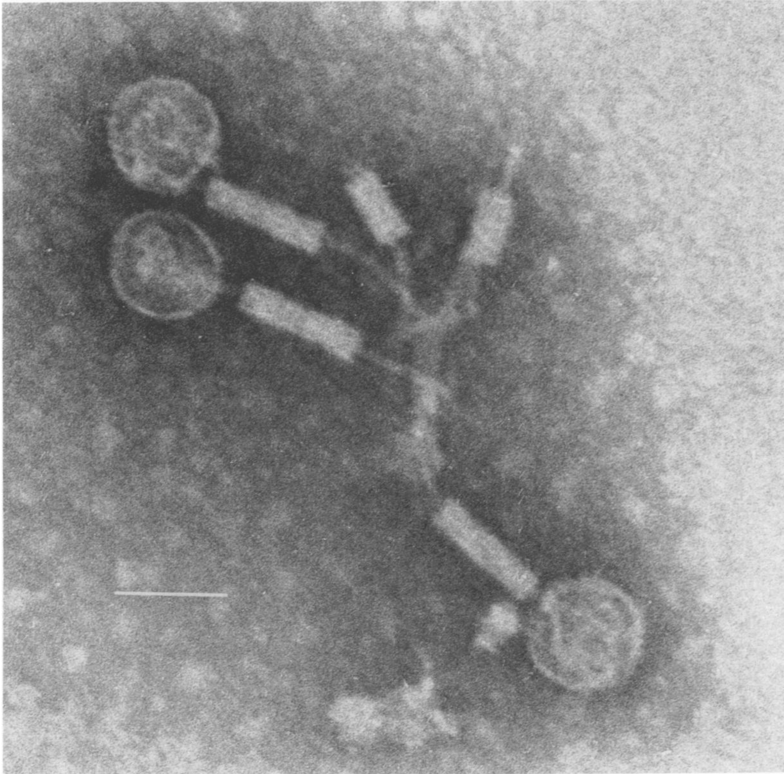

FIG. 1. *Bacteriophage $NA1^{tox+}$ from lysate of C. novyi type A, strain 5771, which induces alpha toxin production (×250,000). Bar = 60 nm.*

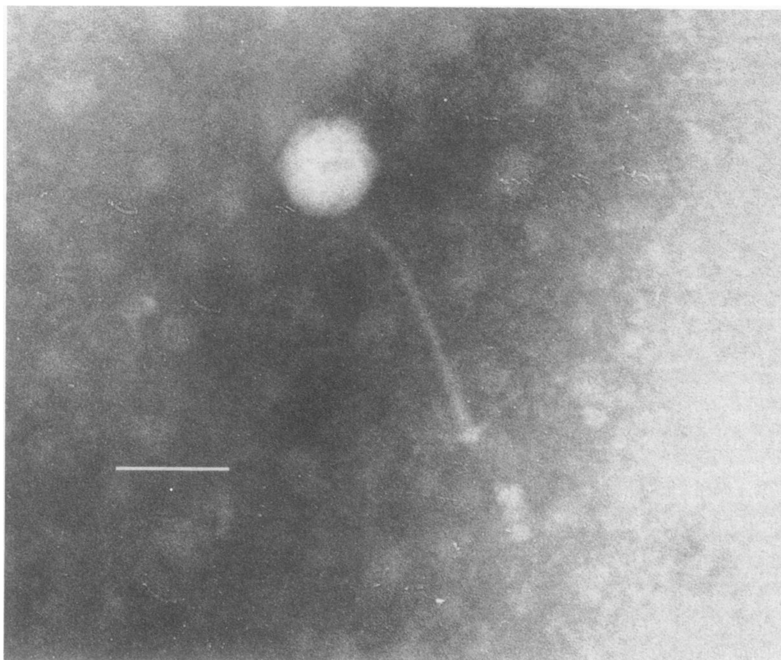

FIG. 2. *Bacteriophage NA2^{tox-} from lysate of C. novyi type A, strain 5771, which does not induce alpha toxin production (×250,000). Bar = 60 nm.*

similar to those reported by Schallehn and Lenz (23) and Smirnova et al. (25). NA1 phage also closely resembles *tox*$^+$ phages from *C. botulinum* types C and D (3, 5, 8, 9, 12–14, 29).

Bacteriophages and toxin production by type B strain 8024. (i) Relationship of phage NB1^{tox+} to alpha toxin production. Nontoxigenic derivatives of strain 8024 were treated with mitomycin C at concentrations of 0.1, 0.5, and 1.0 μg/ml to determine whether any additional phages could be induced. Supernatant fluids were sedimented by ultracentrifugation and examined with the electron microscope (6). Lysis of the culture did not occur, nor could phages be detected in the preparations.

Strains AO26 and AO52 were phage-sensitive cultures that simultaneously ceased to produce the alpha toxin. The relation of phage NB1 to alpha toxin production was studied by using two procedures. In the first experiments, material from isolated plaques on lawns of both AO26 and AO52 were transferred into TYG broth and assayed for alpha toxin and phage production (Table 3). All of the cultures that arose from plaque material were immune to phage NB1 and produced phage NB1 and 20 to 1,000 MLD of alpha toxin per ml, which was neutralized by type A antiserum.

In a second set of experiments, phage NB1 was mixed with actively growing cells of strain AO52 at a ratio of two phage to one bacterium.

TABLE 3. *Relationship of phage NB1^{tox+} to alpha toxin production by nontoxigenic derivatives of C. novyi type B*

Nontoxigenic phage-sensitive strain	No. of cultures			Origin of broth culture
	Toxigenic and immune to phage NB1	Nontoxigenic and phage sensitive	Tested	
AO52	88	0	88	Plaques[a]
AO26	12	0	12	Plaques[a]
AO52	18	80	98	Colonies[b]

[a] Broth cultures arising from plaque material.
[b] Cultures were exposed to phage NB1 at a ratio of 2 phage to 1 bacterium for 30 min, and isolates were tested for alpha toxin production and immunity to phage NB1.

After a 30-min exposure, the culture was centrifuged at 5,000 × *g*, and the sedimented cells were resuspended in freshly heat-exhausted TYG broth, diluted, and plated. Of the 98 colonies selected at random, 18 were immune to phage NB1 and produced phage NB1 and alpha toxin. The remainder of the colonies continued to be phage sensitive and nontoxigenic. The nontoxigenic isolates were each plated with phage NB1, and TYG cultures started from the plaque material produced alpha toxin after 5 days at 33°C.

One of the toxigenic cultures that originated from plaque material was permitted to sporulate, and the spores were heated at 70°C for 15

min to inactivate free phage. None of the 100 isolated colonies from TYG agar were phage sensitive. When this same toxigenic culture was grown in TYG broth containing acridine orange, 23 of the 120 colonies selected were phage sensitive and nontoxigenic. These cultures followed the typical pattern of either being (i) phage sensitive and nontoxigenic or (ii) toxigenic and immune to phage NB1 and able to produce phage NB1. Culture AO52 did not produce the characteristic subcutaneous gelatinous edema in guinea pigs, but when culture AO52 was infected with phage NB1, the typical gelatinous edema caused by the alpha toxin was observed.

(ii) Relationship of phages from different type B cultures to alpha toxin production. Nontoxigenic type B strain AO52 was also used as an indicator strain for phages of other type B strains. Filtrates of cultures 190. KZ391. KZ394, KZ395, and KZ396 all yielded *tox*$^+$ phages that infected AO52 and also induced alpha toxin production. Cultures KZ391, KZ394, and KZ395 each produced a *tox*$^-$ phage that infected AO52 but did not induce alpha toxin production. Other type B cultures did not produce phages that would infect strain AO52 (Table 1).

(iii) Phenotypic alterations in colonial morphology by *tox*$^+$ phages. Smith (26) reported that deep-agar colonies may vary in appearance from lenticular colonies to filamentous colonies resembling snowflakes or having the appearance of bursting grenades. We have observed in our studies that after 3 days of incubation at 33°C the nontoxigenic strain AO52 produced the lenticular colonies and that AO52(NB1) produced the filamentous colonies. The filamentous colonies occasionally showed evidence of "nibbling," and effect probably due to phage NB1. This phenotypic alteration in the colonies has been used to advantage in the isolation of nontoxigenic strains. The filamentous colonies were infected with phage NB1, and the majority of the lenticular colonies were phage sensitive. When strain AO52 was infected with the *tox*$^+$ phages from other type B strains, the same filamentous colonies were produced (Fig. 3). Colonies of AO52 infected with the different *tox*$^-$ phages were of the same morphology as strain AO52 (Fig. 4).

When *C. novyi* type A or B antiserum was added to the agar (at a ratio of 0.3 ml of antiserum to 10 ml of agar), the culture AO52(NB1) produced lenticular colonies during the first 5 days of incubation, but after 7 days of incubation the colonies began to spread into the filamentous snowflake-like appearance. This same colonial morphology phenomenon observed with cultures AO52 and AO52(NB1) was not

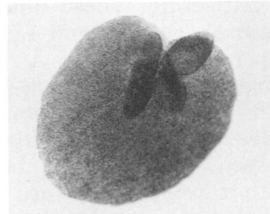

FIG. 4. *Colony of nontoxigenic C. novyi type B strain AO52 infected with phage NB6^{tox-} (×30).*

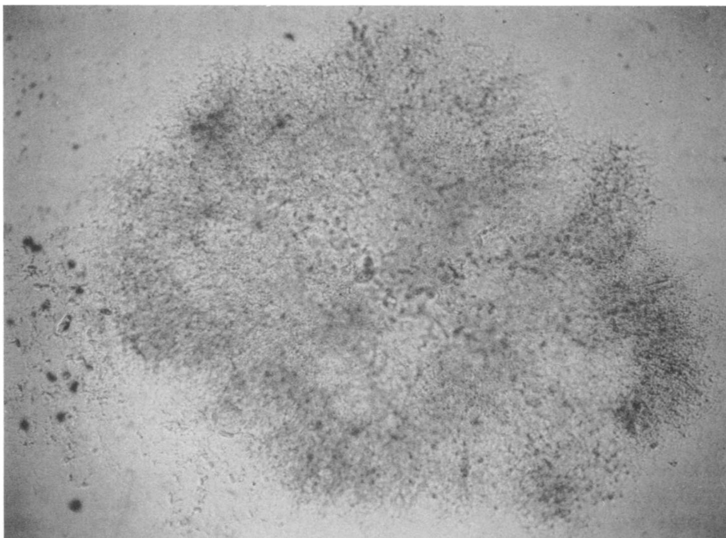

FIG. 3. *Colony of C. novyi type B strain AO52 infected with phage NB1^{tox+} (×30).*

observed in type A cultures HS10 and HS10(NA1). The colonial morphology of AO52 and AO52(NB1) growing on the surfaces of egg yolk agar plates did not appear to be different. The cultures would grow only on the surface of agar plates that contained 0.1% cysteine hydrochloride and were prereduced in anaerobic jars before use. We have observed a similar requirement of cysteine or sodium thioglycolate in agar plates for the growth of certain strains of *C. botulinum* (10). Moore (17) also reported that cysteine and dithiothreitol in agar plates were necessary for the growth of *C. novyi* type B.

(iv) Effect of antiserum against phage NB1^{tox+} on phage sensitivity and alpha toxin production. Antiserum produced against phage NB1 was mixed with filtrates containing phage NB1, and after 1 h the mixture was added to actively growing cultures of nontoxigenic strain AO52. The antiserum against NB1 inactivated the phages, and the cultures remained sensitive and nontoxigenic. To a second set of actively growing cells of strain AO52, filtrates were added that were not treated with NB1 phage antiserum. These untreated cultures produced phage NB1 and 1,000 MLD of alpha toxin per ml.

C. novyi type B strain 8024 was transferred twice a day in TYG broth containing antiserum against phage NB1 to determine whether phage-sensitive cultures could be isolated. After the sixth transfer, the culture was diluted and plated with TYG agar. On the 194 colonies examined from one of the plates, 175 were of the filamentous type and 19 were of the lenticular type. The lenticular colonies had each lost immunity to phage NB1 and also ceased to produce alpha toxin. These data are compatible with the idea that the carrier state in media without antisera is maintained entirely by reinfection and that a pseudolysogenic relationship exists between phage NB1 and host similar to that observed in *C. botulinum* types C and D (5, 8, 9).

(v) Beta toxin production by *C. novyi* type D and nontoxigenic type B. Type D strains of *C. novyi* have been reported to produce larger amounts of beta toxin than strains of type B (18, 22). In our studies, we therefore compared the beta toxin production of type D strains KZ414 to that of the closely related nontoxigenic type B strain AO52 or strain AO52 infected with either phage NB1^{tox+} or phage NB6^{tox-}. These strains were incubated in TYG broth for 6, 15, 24, 48, 72, and 120 h at 33°C, and the supernatant fluids were diluted in twofold dilutions and assayed for beta toxin, using egg yolk emulsions (20, 21) and mouse assay. Results from these studies showed that after 6 h of incubation the type D cultures produced about 32 times more beta toxin (lecithinase) than strain AO52 or strain AO52 infected with either the *tox*$^-$ or *tox*$^+$ phages (end points of lecithinase activity: type D = 1:512, type B strain AO52 = 1:16). After 6 h of incubation, type D cultures contained 40 MLD of beta toxin per ml. Lethal levels of the beta toxin were not detectable in strain AO52 until after 15 h of incubation, at which time a maximum titer of 4 MLD/ml was obtained. Lethal levels of the beta toxin were not detectable in any of the cultures after 72 h of incubation. Lecithinase activity as measured in the egg yolk emulsions also decreased rapidly during incubation, and after 120 h of incubation only the 1:2 dilution of the supernatant fluids showed any activity.

(vi) Morphology of phage NB1^{tox+}. Phage NB1 exhibited an elongated head 65 nm wide and 125 nm long. The tail of this phage was 7 nm wide and 140 nm long and was surrounded by a sheath 130 nm long and 20 nm wide (Fig. 5). This phage produced a slightly turbid plaque 1 to 2 mm in diameter on lawns of nontoxigenic strain AO52. Sometimes these plaques were surrounded by a turbid halo or small satellite plaques.

DISCUSSION

The species *C. novyi* includes a heterogenous group of organisms that is divided into types A, B, C, and D on the basis of the production of eight different soluble antigens (16, 20, 21, 26, 32) and somewhat on the basis the diseases that they produce in man and animals. The main antigen that unites types A and B is the lethal alpha toxin, which is neutralized by both type A and B antisera. Even though the phage-sensitive type A strain HS10 used in these studies ceased to produce alpha toxin, it continued to produce the gamma antigen (hemolytic, lecithinolytic, and necrotizing) and epsilon antigen (causes opalescence on and around its colonies on egg yolk agar). The gamma and epsilon antigens are not produced by the other types of *C. novyi*. The cultural characteristics of type A cultures therefore more closely resemble those of *C. botulinum* types C and D than the other *C. novyi* types (11). This close relationship is strengthened further by the fact that a nontoxigenic, cured culture of *C. botulinum* type C can be infected with specific phages and induced to produce *C. botulinum* type C or D toxin or alpha toxin of *C. novyi* (7). When this nontoxigenic *C. botulinum* type C culture is infected with phages from *C. novyi* type A and produces alpha toxin, it produces the characteristic sub-

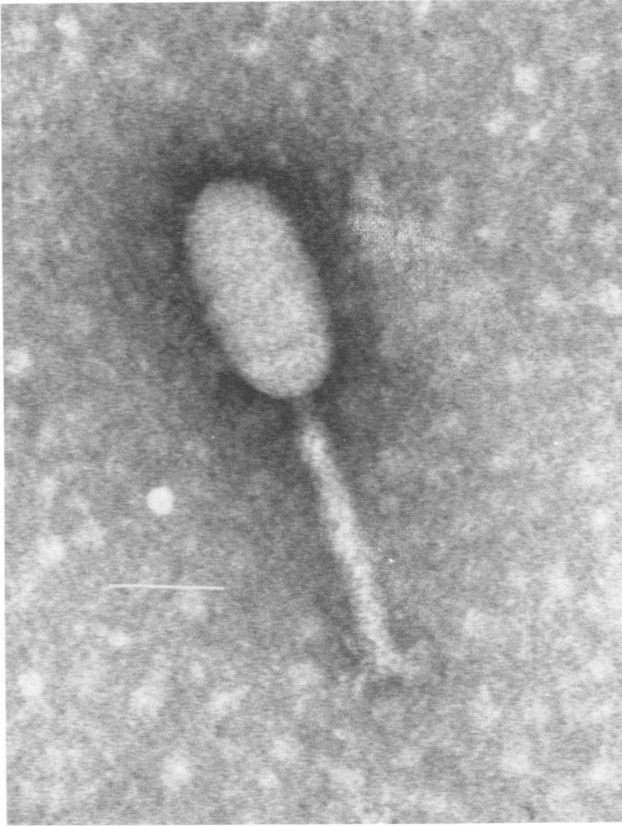

FIG. 5. *Bacteriophage NB1^{tox+} from lysate of C. novyi type B, strain 8024, which induces alpha toxin production (×250,000). Bar = 60 nm.*

cutaneous, gelatinous edema reaction in guinea pigs and is indistinguishable from *C. novyi* type A.

It was recently suggested that *C. haemolyticum* be placed in the *C. novyi* species and be referred to as *C. novyi* type D (20) because its major lethal toxin is identical with the beta toxin of *C. novyi* type B. In fact, Oakley et al. (21) questioned whether *C. haemolyticum* is a member of type B or a separate type. Types B and D are the most closely related types of the *C. novyi* species and differ mainly in that only type B produces the lethal alpha toxin.

The data presented in our studies show that the type B strain very closely resembles type D strains when it is cured of phage NB1^{tox+} and ceases to produce alpha toxin. Both the nontoxigenic type B derivatives and type D cultures produced the lethal beta toxin (necrotic, lecithinolytic, and hemolytic), which was neutralized by type B and D antisera. Type D strains, however, produced larger amounts of beta toxin than type B strain 8024 or phage-sensitive type B strain AO52 that ceased to produce alpha toxin. Both *tox*$^+$ and *tox*$^-$ phages did not appear to affect a change in the amount of beta toxin produced by strain AO52. The possibility that *tox*$^+$ and *tox*$^-$ phages might induce lysis and therefore release larger quantities of beta toxin in nontoxigenic strain AO52 was not established in these studies. This does not, however, rule out the possibility that the beta toxin production by *C. novyi* type D may be induced by another specific phage. This difference between the quantity of beta toxin produced by types B and D strains agrees with the results of Rutter and Collee (22) and Nakamura et al. (18).

Nontoxigenic type B cultures were observed in our studies to occasionally produce hemoglobinuria in mice, a characteristic of *C. novyi* type D, which is responsible for hemoglobinuria disease in cattle. Based upon the very close relationship of types B and D, it is therefore quite feasible that phages will eventually be isolated that will infect type D and induce alpha toxin production, thereby converting it to type B. Since the same antigenically related

alpha toxin production is induced by bacteriophages in both types A and B, it is also quite probable that a single tox^+ phage will be isolated that will infect both nontoxigenic type A and B strains and induce them to produce alpha toxin. Bacteriophages and alpha toxin production, therefore, are the main factors that unite types A and B or differentiate types B and D. In the current studies, nontoxigenic strains of type A continue to produce the gamma and epsilon antigens and nontoxigenic type B strain continues to produce the beta toxin. *C. novyi* type C, however, is not considered pathogenic to laboratory animals, nor does it produce any of the eight different soluble antigens of types A, B, or D. The origin and relationship of type C strain to the other *C. novyi* types is therefore of interest.

The tox^+ bacteriophages from the different type B strains not only induced alpha toxin production in nontoxigenic strain AO52 but also caused a marked change in the morphology of the colonies. Strain AO52 appears to be less motile, and in deep agar, lenticular colonies are produced. This colonial morphology remains the same when AO52 is infected with the tox^- phages from other type B strains. In contrast, when strain AO52 is infected with one of the tox^+ phages from type B strains, the colonies become filamentous and spread into a snowflake-like appearance. This change in colonial morphology in type B, therefore, is a very important characteristic in the isolation of either toxigenic or nontoxigenic cultures of type B.

The ability to isolate type A and B cultures that cease to produce the lethal alpha toxin also introduces a procedure for studying the other biologically active substances in the different *C. novyi* diseases that were previously masked by the lethal alpha toxin. These studies show that the production of alpha toxin depends upon the continued participation and presence of specific tox^+ phages designated as $NA1^{tox+}$ in type A strain 5771 and $NB1^{tox+}$ in type B strain 8024. The phage-host relationship in types A and B appears to be pseudolysogenic, similar to that observed in *C. botulinum* types C and D (5, 8, 9).

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