The Stability of Toxigenicity in *Clostridium botulinum* Types C and D

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SUMMARY

Several type C and D strains of *Clostridium botulinum*, which had been converted to the toxigenic state by phages, were serially transferred through cooked meat medium with and without specific anti-phage serum. Most of the converted strains lost their toxigenicity even during transfer without antiserum, and the non-toxigenic variants that appeared were resistant to lysis and conversion by the original phage. However, in some combinations of phage and host bacteria toxigenicity was stable after ten transfers, though it showed a transient decrease, and the non-toxigenic variants that arose remained sensitive to lysis and conversion. When converted strains were transferred in medium containing anti-phage serum, toxigenicity was lost more rapidly than in the absence of serum and the non-toxigenic variants that appeared remained sensitive to lysis and conversion by the parent phage.

Filtrates of the supernatants of culture fluids of strains transferred without anti-phage serum converted non-toxigenic strains to toxigenicity at varying rates. However, a non-converting phage was demonstrated in one of these filtrates. This phage was identical to the original converting phage in its morphology, host range and antigenicity. Indicator strains treated with this phage acquired resistance to lysis by the parent phage.

The results suggest that re-infection and conversion to toxigenicity occurred in combinations showing stable toxigenicity after ten transfers. However, in those combinations that lost toxigenicity, re-infection with a non-converting mutant of the original phage may have occurred with the result that non-toxigenic variants became resistant to the converting phage. This appears to be one of the causes of the loss of toxigenicity which is common in some type C and D strains of *C. botulinum*.

INTRODUCTION

Phage conversion to toxigenicity in *Clostridium botulinum* types C and D was first reported by Inoue & Iida (1970, 1971) and verified and extended by Eklund *et al.* (1971) and Eklund, Poysky & Reed (1972) using phages purified from plaques. Eklund *et al.* (1971, 1972) and Eklund & Poysky (1974) considered that the relationship between bacteriophage and host was pseudo-lysogeny because of the simultaneous loss of phage and toxigenicity after successive transfers in the presence of anti-phage serum or after passage through the spore state. The facts that non-toxigenic strains cured of their phages were obtained by treatment with acridine orange, and that many toxigenic strains became non-toxigenic during transfer in the laboratory, suggested that the gene controlling toxin production of types C and D was not always integrated into the bacterial chromosome.

Non-toxigenic strains that were capable of being converted to the toxigenic state by infection with specific bacteriophages were also lysed by the same phages. However, other

Table 1. *Properties of strains of C. botulinum*

Strain	Type	Toxin production	Source
Stockholm	C	+	Stored in the author's laboratory
203	C	+	M. E. Macheak, USDA, Ames, Iowa, U.S.A.
1873	D	+	Stored in the author's laboratory
AO2	C	—	Isolated from C-Stockholm, after treatment with acridine orange
139	D	—	Isolated from D-1873, after treatment with acridine orange
151	D	—	Isolated from D-1873, after treatment with acridine orange
N71	C	—	Isolated from C-Stockholm after treatment with nitrosoguanidine

Table 2. *Properties of phages used in experiments*

Phage	Source	Toxigenic conversion	Antigenicity
c-st	C-Stockholm	+ (C type)	Serologically identical
c-n71	C-N71	—	
d-1873	D-1873	+ (D type)	Serologically identical
c-203	C-203	+ (C type)	

non-toxigenic strains, isolated spontaneously or obtained by various methods, were neither lysed nor converted by the phages. A non-toxigenic but lysogenic strain was also isolated from a toxigenic strain by nitrosoguanidine treatment. From the induced lysate of this strain, a non-converting but lytic phage was obtained (Oguma, Iida & Inoue, 1975). Lytic spectrum, antigenicity and the morphology of this phage were the same as those of the converting phage obtained from the parent toxigenic strain. A non-toxigenic indicator strain infected with this non-converting phage exhibited immunity to the converting phage.

This report describes the loss of toxigenicity in converted toxigenic strains through serial transfers with or without anti-phage serum and the nature of the non-toxigenic strains thus obtained.

METHODS

Strains of Clostridium botulinum. Table 1 lists the strains used in these experiments. They were maintained in cooked meat medium (CM) and, when necessary, were transferred to freshly prepared LYG medium pH 7.2, containing (% w/v): lactalbumin, 1; yeast extract, 2; glucose, 0.5; cysteine-HCl, 0.15.

Phages. The four phages used in these experiments (Table 2) were induced from the respective toxigenic strains, C-Stockholm, C-203, D-1873 and the non-toxigenic strain C-N71 by treatment with mitomycin C as described by Inoue & Iida (1968). Lysates were filtered through membrane filters of average pore diameter (a.p.d.) 450 nm. Since we were unable to observe plaque formation with these phages because of the difficulty of making confluent lawns of indicator strains, the filtrates of induced lysates were used as the source of phage.

Phage preparations used in lysis and conversion tests were prepared by incubating filtrates with suitable host strains as described by Oguma *et al.* (1975). Strain C-AO2 was

the indicator for phages c-st and c-n71, and strain D-151 was the indicator for phages d-1873 and c-203 (Oguma, Iida & Inoue, 1973).

Procedure of serial transfer of converted toxigenic strains. Portions (2.5 ml) of young cultures of non-toxigenic strains were mixed with 0.5 ml of phage. Strains C-A02 and D-139 were mixed with phage c-st and are described as A02 (c-st) and 139 (c-st); strain D-151 was mixed with phages d-1873 and c-203, and these are described as 151 (d-1873) and 151 (c-203). After incubating at 37 °C for 4 h the mixtures were centrifuged at 1000 g for 10 min and sediments streaked on to blood-agar plates which were incubated at 37 °C for 2 days in an anaerobic jar. About 20 colonies were picked from the plates on to CM medium and incubated at 37 °C for 3 days. A 0.5 ml sample of each supernatant fluid was injected intraperitoneally into mice to test toxicity. The conversion rate was expressed as the ratio of toxigenic colonies to total colonies tested. One toxic tube was selected as the origin, and 0.2 ml of culture fluid was inoculated from it into CM medium. After incubating at 37 °C for 18 h, the culture was streaked on to blood-agar plates and about 20 colonies were picked on to CM medium and incubated at 37 °C for 3 days. The supernatants were tested for toxicity and, at the same time, 0.2 ml of the culture was inoculated into fresh CM medium as a second transfer. The same procedure was carried out for a total of ten transfers.

Antiserum to phage c-n71, which neutralized the lytic and converting activities of phage c-st at a dilution of 1/128, was added to CM medium to a final dilution of 1/50. Antiserum to phage d-1873, which neutralized phages d-1873 and c-203 at a dilution of 1/32, was added to CM medium to a final dilution of 1/10.

Lysis and conversion tests. Non-toxigenic variants isolated during transfer of converted strains were tested for their susceptibility to lysis and conversion by the original converting phage. A sample (0.5 ml) of the appropriate phage was mixed with 2.5 ml of actively-growing culture. The mixture was incubated at 37 °C for 3 to 6 h and the turbidity assayed, using the Hitachi photometer at 520 nm wavelength, to estimate the extent of lysis. At the same time, 0.2 ml of the mixture was transferred to 10 ml of CM medium and incubated at 37 °C for 3 days. The culture supernatant was diluted ten times to minimize the effect of toxin transferred with the phage preparation, and 0.5 ml was injected intraperitoneally into mice to detect toxin production.

RESULTS

The stability of the toxigenicity of converted strains

During serial transfer, loss of toxigenicity was observed at varying rates with different combinations of host and phage (Table 3). In only one strain, 151 (d-1873), was toxigenicity stable after ten transfers. With the other four strains, most colonies isolated at the 10th transfer were non-toxigenic. Loss of toxigenicity occurred more rapidly in the presence of anti-phage serum. If one assumes that the proportion of non-toxigenic variants arising in the presence or absence of antiserum is the same, this implies that, in the absence of antiserum, many non-toxigenic variants are re-infected with the converting phage and restored to the toxigenic state. Therefore the nature of the non-toxigenic variants obtained during transfer was examined.

The nature of non-toxigenic variants that appeared during serial transfer

Lysis and conversion tests were carried out on the non-toxigenic variants that arose during transfer with phages c-st, c-203 and d-1873 (Table 4). Non-toxigenic variants from converted toxigenic strains A02 (c-st) and 139 (c-st) and from the toxigenic strain C-Stockholm, grown

Table 3. *Appearance of non-toxicogenic colonies during serial transfer*

Converted toxigenic strains were transferred through cooked meat medium (CM) with or without anti-phage serum. Culture fluid of each transferred strain was streaked on blood-agar plates and incubated anaerobically at 37 °C for 2 days. About 20 colonies were picked from the plates to CM medium and incubated at 37 °C for 3 days. Toxicity was tested by injecting 0.5 ml of each supernatant into mice intraperitoneally, and the ratio of toxigenic colonies to total colonies tested was calculated.

		Proportion of colonies originally toxigenic	Proportion of toxigenic colonies after transfer for the following no. of times				
	Con- version rate		1	2	4	8	10
Without anti-phage serum							
C-Stockholm	—	20/20	—	18/20	17/20	2/20	5/20
A02 (c-st)	15/20*	18/20	—	20/20	10/20	8/20	2/20
I39 (c-st)	4/20*	19/20	—	5/5	1/20	2/20	0/10
I51 (d-1873)	10/10†	20/20	—	19/20	15/20	19/20	20/20
I51 (c-203)	10/10†	3/10	17/20	9/20	4/20	—	2/20
With anti-phage serum							
C-Stockholm	—	20/20	—	7/10	3/10	0/10	0/10
A02 (c-st)	15/20	18/20	—	10/10	6/10	0/10	0/10
I51 (d-1873)	10/10	20/20	—	18/20	15/20	8/20	6/20
I51 (c-203)	10/10	3/10	1/20	0/20	0/20	—	0/20

* Conversion rate with filtrate of induced lysate.

† Conversion rate with filtrate of passaged lysate.

Table 4. *The nature of non-toxicogenic variants that appeared during serial transfer: Lysis and conversion tests*

Portions (2.5 ml) of young broth culture of non-toxicogenic variant that appeared during transfer were mixed with 0.5 ml of the respective phage. After incubating at 37 °C for 4 h, the turbidity of the mixture was assayed. At the same time 0.2 ml was inoculated into CM medium and incubated at 37 °C for 3 days. Toxicity was tested in mice.

		Phages					
Non-toxicogenic variants	No. of transfers	c-st		d-1873		c-203	
		Lysis	Con- version	Lysis	Con- version	Lysis	Con- version
		Without anti-phage serum					
C-Stockholm	2	—	s				
	10	—	s				
A02 (c-st)	4	—	s				
	10	—	s				
I39 (c-st)	4	—	s				
	10	—	s				
I51 (d-1873)	4			+	d		
	8			+	d		
I51 (c-203)	0					+	d
	1					+	d
	2					—	s
	10					—	s

s, Survived; d, died.

Table 5. *The converting ability of culture fluid of each transfer*

A portion (0.5 ml) of filtrate of each supernatant from transferred cultures was mixed with 2.5 ml of an actively growing suitable host strain and incubated at 37 °C for 4 h. A sample (0.2 ml) of the mixture was transferred and incubated in CM medium at 37 °C for 3 days and then tested for toxicity. Twenty colonies were picked from several CM cultures and the ratio of toxigenic colonies to total colonies was calculated.

Strains	No. of transfers:				
	2	4	6	8	10
Without anti-phage serum					
C-Stockholm	d	d	d	d	d(17/20)*
AO2 (c-st)	d(18/20)*	d	d	d	d
151 (d-1873)	d(10/10)*	d	d	d	d(0/20)†
151 (c-203)	d(0/20)†	d(0/20)†	—	—	—
With anti-phage serum					
AO2 (c-st)	s (0/20)	s	s	s	s
151 (c-203)	s	s	s	s	s (0/20)

s, Survived; d, died. The numbers in parentheses indicate the proportion of cultures that were toxigenic.

* The toxin titre of these CM cultures was 2000 MLD (minimum lethal dose)/ml.

† The toxin titre of these CM cultures was 20 MLD/ml.

in the absence of antiserum, were neither lysed nor converted to toxigenicity by phage c-st. On the other hand, all of the non-toxigenic variants obtained during transfer of strain 151 (d-1873) were lysed and converted by phage d-1873. In strain 151 (c-203), the non-toxigenic variants from the origin and the first transfer were sensitive to phage c-203, but those obtained after two transfers were resistant.

All the non-toxigenic variants obtained from cultures containing anti-phage serum were sensitive to their respective phages, indicating that these strains were cured of their phages.

The converting ability of culture supernatants from each transfer

Resistance to lysis and conversion of the non-toxigenic variants in cultures of C-Stockholm, AO2 (c-st), 139 (c-st) and 151 (c-203) suggested that these strains might have been rendered immune to the original converting phage by infection with a non-converting mutant phage. To test for this, supernatants from transferred cultures were filtered and the filtrates tested for their ability to convert to toxigenicity.

Filtrates were mixed with appropriate indicator strains, and after incubating at 37 °C for 4 h, 0.2 ml was inoculated into CM medium, incubated for 3 days at 37 °C and toxin production tested in mice. In addition, 20 colonies were picked from each culture and tested for toxigenicity. The results are summarized in Table 5.

Filtrates from culture fluids of C-Stockholm, AO2 (c-st), and 151 (d-1873) retained the ability to convert non-toxigenic strains, thus indicating the presence of the original converting phages. Non-toxigenic survivors were sensitive to the respective converting phages. However, the filtrates of the second and fourth transfers of strain 151 (c-203) rarely converted the indicator strain D-151 to toxigenicity although they caused obvious lysis. The non-toxigenic survivors proved to be resistant to lysis and conversion by phage c-203. It appeared, therefore, that these filtrates contained a lytic agent, presumably a mutant of phage c-203, which had lost the ability to convert to toxigenicity. When the filtrate of supernatant from the fourth transfer of culture 151 (c-203) was incubated with strain D-151, the lytic titre increased but the converting ability of this filtrate disappeared. This phage, designated c-203 Tox⁻ to

Table 6. *Lysis and conversion by phage c-203 Tox⁻ and phage d-1873*

Filtrates of phages d-1873 and c-203 Tox⁻ were diluted in 10-fold steps with LYG medium. A 0.5 ml portion of each dilution was mixed with 2.5 ml of a young culture of strain D-151, and incubated at 37 °C for 4 h. The extinction value of the mixtures was assayed and 0.2 ml was transferred in CM medium and incubated at 37 °C for 3 days. Toxicity was tested in mice (Expt 1). The non-toxicogenic strain treated with filtrate c-203 Tox⁻ was inoculated into fresh LYG medium and incubated at 37 °C. A 2.5 ml portion of actively-growing cells was mixed with 0.5 ml of either d-1873 or LYG medium (cells alone), and lysis and conversion tests were carried out (Expt 2).

Phage dilution	Expt 1				Expt 2		
	d-1873		c-203 Tox ⁻		Cells alone	d-1873	
	<i>E</i>	Con-version	<i>E</i>	Con-version		<i>E</i>	Con-version
Undiluted	0.06	d	0.19	s	1.60	1.55	s
10 ⁻¹	0.12	d	0.22	s	1.54	1.51	s
10 ⁻²	0.10	d	0.40	s	1.56	1.52	s
10 ⁻³	0.26	d	0.80	s	1.57	1.56	s
10 ⁻⁴	0.30	d	1.02	s	1.59	1.52	s
10 ⁻⁵	0.54	d	1.17	s	1.53	1.48	s
10 ⁻⁶	0.98	d	1.18	s	0.21	0.26	s
10 ⁻⁷	1.14	d	1.16	s	1.61	1.56	s
10 ⁻⁸	1.18	s	1.18	s	1.61	0.15	d
10 ⁻⁹	1.16	s	1.15	s	1.57	0.18	d
Control (phage-infected)						0.13	d
D-151 alone*	1.16				1.58		

s, Survived; d, died.

* The extinction values of the indicator strain were 0.18 and 0.22 at the beginning of Expts 1 and 2 respectively.

Table 7. *Effects of various treatments on phages d-1873, c-203 and c-203 Tox⁻*

A 0.2 ml. portion of each filtrate was mixed separately with 1.0 ml of trypsin (100 µg/ml, pH 8.2), DNAase (20 µg/ml, pH 7.2) and anti-phage serum or LYG medium (as control), and then incubated at 37 °C for 1 h. A 1:6 dilution of each filtrate was heated at 80 °C for 10 min or filtered through membrane filters with a pore size of 5 nm. A 0.5 ml portion of these treated filtrates was mixed with indicator strain and lysis and conversion tests were carried out.

Treatments	d-1873		c-203		c-203 Tox ⁻	
	<i>E</i>	Conversion	<i>E</i>	Conversion	<i>E</i>	Conversion
DNAase	0.10	d	0.07	d	0.08	s
Trypsin	0.13	d	0.10	d	0.09	s
Anti-c-171-phage serum	0.65	d	0.26	d	0.24	s
Anti-d-1873 phage serum	1.74	s	1.70	s	1.74	s
80° C for 10 min	1.51	s	1.60	s	1.65	s
Filtration (5 nm)	1.40	s	1.30	s	1.32	s
Controls						
Phage-infected	0.10	d	0.07	d	0.08	s
Cell alone	1.75					

s, Survived; d, died.

Fig. 1. Electron micrograph of the phage, c-203 Tox⁻. (a) Phages attaching to cell debris. (b) A complete phage obtained from the filtrate of lysed culture, 151 (c-203 Tox⁻).

distinguish it from its presumed parent, c-203 Tox⁺, was investigated further, and its properties compared with those of phage c-203 Tox⁺ and the closely related phage d-1873.

Lysis and conversion by phage c-203 Tox⁻ compared with phage d-1873

Filtrates of lysates of 151 (c-203 Tox⁻) and 151 (d-1873) were diluted in 10-fold steps with LYG medium. Portions (0.5 ml) of each dilution were mixed with a young broth culture of strain D-151 and lysis and conversion tests carried out. As shown in Table 6 (Expt 1), both phages lysed the indicator but only phage d-1873 converted it to toxigenicity. The lytic titre of phage d-1873 was invariably 10 to 1000 times less than the converting titre.

The non-toxicogenic cultures treated with phage c-203 Tox⁻ were then transferred to LYG medium and incubated at 37 °C. Cultures in exponential-growth phase were challenged with phage d-1873 and their susceptibility to phage infection was investigated by both lysis and conversion tests (Expt 2). Those cultures treated with phage c-203 Tox⁻ to a dilution of 10⁻⁷ were resistant to infection with phage d-1873.

The lytic spectra of these two filtrates were determined on a number of different strains by the lysis test described above. The results clearly showed that the lytic spectra of these two phages were identical.

Effect of various treatments on phages c-203 Tox⁻, c-203 Tox⁺ and d-1873

Filtrates of the three phages were tested for their susceptibility to inactivation by trypsin, DNAase, antisera to phages c-n71 and d-1873, exposure to 80 °C for 10 min, and their ability to pass through membrane filters with an a.p.d. of 5 nm (Table 7). All three phages survived treatment with trypsin, DNAase and antiserum to phage c-n71. They were inactivated by antiserum to phage d-1873 and by heating at 80 °C for 10 min, and they failed to pass through the membrane filter. The results provide strong support for the view that phage c-203 Tox⁻ represents a mutant of phage c-203 Tox⁺.

Electron microscopy of phage c-203 Tox⁻

An electron micrograph of a complete phage (Fig. 1) was obtained from the filtrate of passaged lysate, 151 (c-203 Tox⁻), by the procedure of negative staining. The morphology of this phage seems to be the same as that of phage d-1873 reported previously (Inoue & Iida, 1968).

DISCUSSION

The ability to produce toxin in various phage-converted strains of *C. botulinum* and in the wild-type strain C-Stockholm was lost during serial transfer in CM medium in the presence of anti-serum specific for the converting phages. Non-toxicogenic variants that appeared during transfer were apparently cured of their phages and remained sensitive to lysis and conversion. This confirms the results of Eklund *et al.* (1971, 1972) and Eklund & Poysky (1974).

When the same strains were transferred in the absence of anti-phage serum they lost the ability to produce toxin at varying rates but the loss was always less rapid than was found for the same combination of host bacterium and phage in the presence of anti-phage serum. This implies that, in the absence of anti-serum, toxigenic strains lose their phages but can be re-infected and converted to toxin production.

However, many non-toxicogenic variants isolated in the absence of anti-serum were resistant to lysis and conversion by the original converting phage. It was considered possible that these strains were made resistant (immune) by infection with non-converting phages

mutated from the converting phage (Oguma *et al.* 1975). Such a mutant phage was demonstrated in the supernatants of transfers of culture 151 (c-203). Non-toxigenic strains infected with this mutant phage acquired resistance to the original converting phage and remained non-toxigenic. Attempts to demonstrate a similar phage in other non-toxigenic variants have so far been unsuccessful. It is possible that these strains have lost the receptor for the converting phages, and studies to clarify this point are being carried out at the present time.

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