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Adsorption to *Clostridium botulinum* Cultures of Phage Controlling Type C Botulinum Toxin Production (40284)

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Toxin production by *Clostridium botulinum* types C and D is closely associated with bacteriophage infection. Cultures become nontoxigenic when cured of a specific temperate phage while nontoxigenic strains can be converted to toxigenicity when infected with the phage (3–6, 8–10). However, the conversion does not occur with all combinations of phage and cultures; only certain phage-culture pairings are productive of conversion. The needed specificity was explained as being due to differences in the adsorption of phages to cells when three antigenic groups were identified among the converting phages (12). The possibility of other explanations has been raised by a later report (6) which shows that the same culture can be made toxigenic by antigenically distinct phages.

The present communication further examines the phage-culture specificity needed for toxigenicity conversion by comparing the adsorption of one of the type C toxin-converting phages to several *C. botulinum* types C and D cultures and their nontoxigenic derivatives.

Materials and methods. Table I shows the origin of the cultures used (9–11). Nontoxigenic (C)-A02 and (D)-139 can be lysogenized routinely with c-st phage (from C-Stockholm) so as to produce type C toxin.

Cultures were maintained in Bacto-Cooked Meat Medium (Difco Lab., Detroit, MI). For the tests, they were grown in LYG medium of pH 7.2 made of 1% lactalbumin (Sigma Chemical Co., St. Louis, MO), 2% yeast extract (Difco), 0.5% glucose and 0.15% cysteine–HCl. Plating medium was Bacto-Brain Heart Infusion Agar (Difco) containing 10% (v/v) whole human blood obtained from a blood bank. Plated cultures were incubated in anaerobic jars but other cultures were incubated in tightly closed screw-capped tubes. All cultures were incubated at 37°.

Filtrates of C-Stockholm cultured overnight in LYG contained c-st phage titer of 10^{3-4} plaque forming units (pfu)/ml when plated with indicator culture (C)-A02. The phage was purified by three successive cycles of incubating a transferred plaque for 4 hr with (C)-A02 actively growing in 5 ml of LYG, filtering culture lysate through Millipore membrane of 450 nm pores, and replating.

Two ml of the broth culture from the third passage were added to 15 ml of a young culture (A_{520} = 0.2) of (C)-A02 and the culture incubated until lysis occurred during the next 3–4 hr. The lysate, clarified by centrifugation and subsequent filtration through a Millipore membrane, had a titer of about 10^7 pfu/ml but the titer decreased during storage of more than one week at 4°. The titer was regained when the phage stock was treated as in the last passage used in its preparation.

Adsorption tests were done in T2 buffer made of 0.4% NaCl, 0.5% K_2SO_4, 0.15% KH_2PO_4, 0.3% Na_2HPO_4, 1 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, and 0.001% gelatin (7). This buffer was used because of convenience rather than superiority over other media. Preliminary tests have shown that c-st phage adsorbs to (C)-A02 equally well in systems using LYG, T2 buffer, or T2 buffer containing 40 μg tryptophane/ml.

Cells for adsorption tests were collected from overnight incubated cultures by centrifugation at 3000*g* for 10 min and washed three times with T2 buffer. Suspensions of 1×10^8 cells/ml were made on the basis of counts made on a Petroff-Hausser counting chamber.

The phage preparation was diluted 1:10 with T2 buffer. After holding separately in an ice water bath for 5 min, 1.8 ml cell and 0.2 ml phage preparations were combined and held at 4°. After the desired adsorption

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time, the suspension was centrifuged for 10 min at 4° and 6000*g*. Unadsorbed phage was quantified by plating 0.1 ml of the decimal dilution series of the supernatant with the indicator strain.

The frequency of conversion was determined by examining isolated colonies. After cell-phage contact, the cells were collected by low speed centrifugation (1000*g*, 10 min) and plated to obtain isolated colonies. Of the colonies developing during 2 days incubation, 20 random picks were subcultured separately for 3 days in tubes of Cooked Meat Medium. The presence of type C botulinum toxin in these cultures were determined by challenging mice ip with 0.5 ml of culture fluid.

Results. Adsorption curves of c-st phage reacting with (C)-A02 were not different from those reported for most other phage systems. Phage adsorption depended on the multiplicity of infection (MOI): starting with 4.0×10^5 pfu and 1.8×10^8 cells/ml, 98% of phage was adsorbed in 10 min while 50% was adsorbed when the cell concentration was 5.5×10^6/ml.

Adsorption of c-st phage to cells of different cultures during 20 min contact at 4° is shown in Table II. Several controls showed the reduction in free phage was due to specific adsorption. As part of the first experiment of

TABLE I. CULTURE STRAINS USED.

Strain[a]	Type toxin produced	Origin
C-Stockholm	C	wild type
D-1873	D	wild type
(C)-AO2(c-st)	C	(C)-AO2 infected with c-st phage
(D)-139(c-st)	C	(D)-139 infected with c-st phage
(C)-AO2	—	AO[b] treatment of C-Stockholm
(C)-N71	—	NG[b] treatment of C-Stockholm
(C)-6813	—	spontaneously from wild type C-6813
(C)-6814	—	spontaneously from wild type C-6814
(D)-139	—	AO treatment of D-1873
(D)-151	—	AO treatment of D-1873
(D)-SA	—	spontaneously from wild type D-South African

[a] For toxigenics, letter indicate type of toxin produced; letter in ( ) indicates toxin type of parent from which nontoxigenic was derived.

[b] AO = acridine orange; NG = nitrosoguanidine.

TABLE II. ABSORPTION OF c-st PHAGE TO TYPE C AND D STRAINS AND NONTOXIGENIC STRAINS DERIVED FROM TOXIGENIC PARENTS. 1×10^8 CELLS/ml; FREE pfu AFTER 20 min CELL-PHAGE CONTACT AT 4°C.

	pfu/ml of supernatant fluid		
Strain	Expt. 1	Expt. 2	Expt. 3
No cells	4.3×10^5	1.8×10^5	1.2×10^5
(C)-AO2	4.0×10^3	6.0×10^3	3.4×10^3
(C)-6813		1.7×10^5	
(C)-6814		1.5×10^5	
(C)-N71			7.0×10^3
(D)-139		2.8×10^4	
(D)-151	1.4×10^4		
(D)-SA		1.5×10^4	
C-Stockholm			4.0×10^3
D-1873	1.0×10^4		
(C)-AO2(c-st)			3.8×10^3
(D)-139(c-st)			1.3×10^4

Table II, possible adsorption to a nonproteolytic *C. botulinum* type B culture (QC strain) and a type E (Morai strain) was examined. The respective titers of 4.5×10^5 and 4.0×10^5 pfu/ml after the adsorption treatments showed that c-st did not adsorb to these cells nor was it adversely affected by them. Phage inactivating factors were not produced by cells since titers of 5.2×10^5 and 4.4×10^5 pfu/ml, respectively, were found after treating the phage suspension with cell-free culture fluids of (D)-151 and (D)-1873.

Some quantitative differences were found in retesting the same cultures, but the conclusion can be drawn that c-st adsorbed to all cultures except (C)-6813 and (C)-6814. The phage adsorbed best to indicator strain (C)-A02, parent toxigenic C-Stockholm, and converted (C)-A02(c-st). The phage adsorbed to a slightly less degree to D toxin producer D-1873 and nontoxigenics derived from parents producing this type of toxin.

As reported previously (11), the phage lysed broth cultures of only (C)-A02 and (D)-139. When the lysates were subcultured in Cooked Meat Medium, type C toxin was formed (12). The phage produced plaques on lawns of these two cultures but not on those of others. However, by degrees of clearing of broth cultures and numbers of plaques formed, the phage was more overtly active against (C)-A02 than (D)-139.

(C)-A02 and (D)-139 differed also in their rates of conversion to toxigenicity (Table III); with optimum conditions of cell-phage con-

TABLE III. CONVERSION RATES OF (C)-AO2 AND (D)-139 STRAINS BY c-st PHAGE WITH OPTIMUM INCUBATION TIMES (4 hr) AND IN PRESENCE OF 2% NaCl.

Strain	NaCl	MOI[a]	Incubation min[b]	Toxic colonies among 20 tested
(C)-AO2	−	0.1	240	19
(C)-AO2	−	0.5	40	4
(C)-AO2	+	0.5	40	4
(D)-139	−	0.1	240	3
(D)-139	−	0.5	40	0
(D)-139	+	0.5	40	0
(D)-139	−	5.0	40	8
(D)-139	+	5.0	40	9

[a] Multiplicity of infection.
[b] Phage-cell contact time at 37°.

tact (MOI = 0.1, 4 hr), the conversion to toxigenicity of (C)-A02 was significantly higher than (D)-139. Raising MOI to 5.0 increased the conversion rate for (D)-139. Using 2% NaCl in the adsorption system in attempts to increase conversion to toxigenicity (5) did not favor greater conversion of (D)-139.

Converted isolates of the two culture strains produced approximately the same levels (10^{3-4} LD_{50}/ml) of toxin. When nontoxigenic isolates from the first treatment were subjected to a second conversion test, the conversion rate of (D)-139 was again lower than for (C)-A02.

A test was made of the possibility that phage in converted strain (D)-139(c-st) might be a modified form that could convert (D)-139 at a higher rate than the c-st phage obtained directly from C-Stockholm. The cell-free filtrate of an overnight incubated (D)-139(c-st) culture was added to separate, actively growing cultures of (D)-139 and (C)-A02. After 4-hr cell-phage contact, the mixtures were plated and 20 resulting colonies selected for toxicity tests. None of the (D)-139 isolates produced toxin although 13 of the (C)-A02 subcultures had been converted to toxigenicity.

Discussion. (C)-A02 and (D)-139 were both converted to type C toxigenicity by c-st phage, although the conversion frequency was significantly higher for (C)-A02. The difference in the conversion rates is related to the more effective phage adsorption to (C)-A02 as measured by comparative adsorption results and lysis of broth cultures.

Several reasons are involved in only certain phage-cell pairings being productive of conversion to toxigenicity. Included are cases where the cells lack receptors for phage attachment. This situation is illustrated by (C)-6813 and (C)-6814 to which c-st phage did not adsorb.

Since c-st adsorbed to some extent to all other cultures used, the conversion or nonconversion of these cultures is not determined by phage adsorption only. (C)-N71 is already lysogenized by a nonconverting phage. Since this phage has the same host spectrum and antigenicity as c-st (11), its presence in the cells would confer immunity against the converting c-st phage. The result would be nonconversion to toxigenicity in spite of adsorption of c-st to the cells.

This nonconverting phage could not be demonstrated in the remaining cultures to which c-st phage adsorbs without converting to toxigenicity. It is possible that some of these cultures carry a defective phage that confers immunity against c-st phage; in others, host controlled restriction (1, 2) may be important in preventing conversion.

Summary. C-st phage which governs production of type C botulinum toxin was mixed at 4° with cells of *C. botulinum* type C and D cultures and nontoxigenics derived from them. The phage adsorbed to all three cultures producing type C toxin, the one type D toxin producer, 2 of 4 nontoxigenics from type C parents and the three nontoxigenics originating from type D toxin producers. The phage adsorbed to some cultures without converting to toxigenicity. The two nontoxigenic which could be converted to toxigenicity differed in degrees of phage adsorption and conversion rates.

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