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# Phage Conversion to Hemagglutinin Production in *Clostridium botulinum* Types C and D

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Five toxigenic strains of *Clostridium botulinum* types C and D were incubated at 37°C for 7 days in 15 ml of the following media: LYG medium, cooked-meat medium, egg meat medium, and N-Z-amine medium. The supernatants of these cultures were tested for hemagglutinin production with 1% erythrocytes obtained from mice, guinea pigs, chickens, sheep, monkeys, and humans. Four toxigenic strains produced hemagglutinin. The highest hemagglutinin titer was obtained with a combination of human erythrocytes and cultures incubated in LYG medium. When the same experiment was carried out with many nontoxigenic strains, hemagglutination was observed in only one strain, C-N71. Strains producing hemagglutinin also produced phages. The phages obtained from toxin- and hemagglutinin-producing strains converted nontoxigenic indicator strains to produce both toxin and hemagglutinin. The phage obtained from a toxin-positive hemagglutinin-negative strain could only induce cultures to produce toxin, and the phage from a toxin-negative hemagglutinin-positive strain could only induce production of hemagglutinin. These studies suggest that the production of hemagglutinin by *C. botulinum* types C and D is governed by bacteriophages and that hemagglutinin production can be transmitted separately or concomitantly with toxin production.

It has been reported that toxigenic strains of *Clostridium botulinum* types A, B, and E produce hemagglutinin (HA) (11). Lowenthal and Lamanna suggested that toxin and HA are different proteins, and that they are independent particles in alkaline solutions and make a complex in acid solutions (12). Das Gupta and Boroff (3) separated toxin and HA by diethylaminoethyl-cellulose and verified these findings. Recently, Boroff et al. (2) proposed a very interesting model for type A toxin and HA: they propose that the toxin strand may fit within the coil of HA helix. In types C and D, HA was also demonstrated in cultures of toxigenic strains (1, 17, 18; C. Lamanna and W. I. Jensen, Bacteriol. Proc., p. 108, 1952).

Inoue and Iida suggested the phage conversion to toxigenicity in types C and D (9, 10), and this phenomenon was verified and extended by Eklund et al. (5-7). Eklund and Poysky also reported that many type C and D strains that were cured of their prophages and ceased to produce $C_1$ and D toxins continued to produce $C_2$ toxin (4). This $C_2$ toxin was demonstrated when "nontoxigenic" strains were incubated in egg meat (EM) medium and activated by trypsin treatment.

In this paper, we report that both $C_1$ and D toxins and HA production are in some manner related to phages of *C. botulinum* types C and D.

## MATERIALS AND METHODS

**Strains and media.** The bacterial strains used were described previously (13, 14). The following four media were used for HA production. Cooked-meat (CM) medium was obtained from Difco Laboratories. EM medium was made as reported by Eklund and Poysky (4). LYG medium, containing 1% lactalbumin, 2% yeast extract, 0.5% glucose, and 0.15% cysteine hydrochloride, pH 7.2, was prepared by us. N-Z-amine medium was made by mixing 2% N-Z-amine type B, 1% proteose peptone, 0.7% meat extract, 0.3% yeast extract, 0.5% glucose, and 0.1% sodium thioglycolate, pH 7.2.

**Hemagglutination and toxigenicity tests.** Many toxigenic and nontoxigenic strains, maintained in CM medium at 4°C, were transferred to freshly prepared LYG medium and incubated at 37°C overnight. A 0.1-ml portion of culture fluid was inoculated into 15 ml of each of the four media. After incubation at 37°C for 7 days, the culture fluids were clarified by centrifugation at 4,000 rpm for 20 min. Each 0.25 ml of supernatant was diluted in serial twofold steps with physiological saline solution (pH 6.0) to which was added normal rabbit serum at a final concentration of 0.1% to make the hemagglutination reaction stable. Each dilution was mixed with an equal volume of 1% erythrocytes in the same saline and examined for hemagglutination after 2 h

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of incubation at 37°C and again after overnight incubation at 4°C.

The toxin titer was determined by intraperitoneally injecting two mice with 0.5 ml of culture fluid diluted in 10-fold steps with gelatin buffer (pH 6.0). Gelatin buffer was not used as a diluent for the hemagglutination reaction because pseudo-hemagglutination was observed.

**Cellophane-tube procedure.** Some strains were incubated by the cellophane-tube method in order to confirm the production of toxin and HA. A 150-ml amount of physiological saline solution (pH 7.2) and 150 ml of double-strength LYG medium were dispensed inside and outside of a dialysis tube, respectively. The young cells were inoculated into the inside of a cellophane tube and incubated at 37°C. A 5-ml amount was sampled from each culture at various time intervals. After centrifugation at 4,000 rpm for 10 min, the supernatant was tested for toxin and HA production. Also, the turbidity of the culture fluids was determined by using a Hitachi photometer at 520-nm wavelength before centrifugation.

**Phage conversion experiment.** The overnight cultures of toxigenic strains C-Stockholm, D-1873, and D-South African (D-SA Tox) and nontoxigenic strain C-N71 (C-N71 NT) were filtered through a membrane filter with a pore size of 450 nm. The filtrate was incubated with the suitable host strain two or three times to increase the phage titer. The passaged phage were sterilized by filtration and plated on solid medium to make plaques with the respective indicator strain which produced neither toxin nor HA. Plaque formation was performed according to the procedure of Eklund et al. (6, 7). The morphology and characteristics of these phages were reported previously (8, 15, 16). Phages c-st and c-n71, obtained from strains C-Stockholm and C-N71, respectively, were mixed with indicator strain C-A02. Phages d-1873 and d-sa, obtained from D-1873 and D-SA Tox strains, were mixed with nontoxigenic strains D-151 and D-SA NT, respectively. About 10 plaques obtained from each combination were transferred by micropipettes into 10 ml of fresh LYG medium. As controls, C-A02, D-151, and D-SA NT were also transferred from control plates without phages. After incubation at 37°C for 7 days, toxin and HA titers of each culture fluid were determined.

## RESULTS

**Production of HA in different media.** The culture fluids of toxigenic and nontoxigenic strains incubated at 37°C for 7 days in the four media were tested for toxin and HA production (Table 1). The hemagglutination reaction was performed at 37°C with 1% erythrocytes obtained from a guinea pig. Toxin titers were comparable in the four media, although EM medium consistently showed good toxin production, whereas the HA titers were generally

TABLE 1. *HA production in different media*[a]

| Strain | Medium | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | LYG | | CM | | EM | | N-Z-amine | |
| | Toxin (MLD/ml)[b] | HA[c] | Toxin (MLD/ml) | HA | Toxin (MLD/ml) | HA | Toxin (MLD/ml) | HA |
| C-Stockholm | $2 \times 10^4$ | 16 | $2 \times 10^4$ | 4 | $2 \times 10^4$ | 4 | $2 \times 10^4$ | 4 |
| C-468 | $2 \times 10^4$ | 4 | $2 \times 10^4$ | 4 | $2 \times 10^4$ | 4 | $2 \times 10^3$ | 4 |
| C-203 | $2 \times 10^4$ | 8 | $2 \times 10^3$ | 2 | $2 \times 10^4$ | 4 | $2 \times 10^4$ | 8 |
| D-1873 | $2 \times 10^4$ | HL[d] | $2 \times 10^5$ | HL | $2 \times 10^5$ | HL | $2 \times 10^4$ | HL |
| D-SA | $2 \times 10^3$ | HL | $2 \times 10^3$ | <2 | $2 \times 10^4$ | 4 | $2 \times 10^3$ | 2 |
| C-N71 | 0 | 8 | 0 | 4 | 0 | 2 | 0 | 4 |
| C-A02 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| C-468-U16 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| C-468-U31 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| C-203 NT | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| C-203-U28 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| D-134 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| D-139 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| D-151 | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |
| D-SA NT | 0 | <2 | 0 | <2 | 0 | <2 | 0 | <2 |

[a] Several toxigenic and nontoxigenic strains were incubated at 37°C for 7 days in 15 ml of each medium. The supernatant of each culture fluid was tested for toxin and HA production. The toxin titer was determined by intraperitoneally injecting into two mice 0.5 ml of culture diluted in serial 10-fold steps with gelatin buffer. The hemagglutination reaction was performed by mixing 0.25 ml of sample diluted in twofold steps with physiological saline solution and an equal volume of a 1% guinea pig erythrocyte suspension. The tubes were examined for hemagglutination after incubation at 37°C for 2 h and again after overnight incubation at 4°C.

[b] MLD, Minimal lethal dose.

[c] Expressed as end point of dilution that showed complete hemagglutination.

[d] HL, Hemolysis.

higher and more distinct in LYG than in the other media. Four of five toxigenic strains produced HA, whereas only the nontoxigenic strain C-N71 did so. Complete hemolysis, the titer of which was 2 to 8, was demonstrated with the culture of strain D-1873 at 37°C for 2 h. Hemolysis sometimes appeared in the other toxigenic and nontoxigenic strains only after standing at 4°C overnight.

The hemagglutination test was also performed with culture fluids incubated in LYG medium and with erythrocytes obtained from mice, chickens, sheep, monkeys, and humans (Table 2). The highest HA titer was obtained with human erythrocytes, and the ABO group of cells showed no effect on HA titer. Thereafter, human erythrocytes were used in the hemagglutination test.

**Production of $C_2$ toxin.** All of the nontoxigenic strains were tested for $C_2$ toxin production. The supernatant was treated with trypsin (Difco, 1:250) at a final concentration of 0.25% and incubated at 37°C for 1 h. The treated sample was diluted in serial 10-fold and 2-fold steps for toxigenicity and hemagglutination tests, respectively. Strains C-N71, D-SA NT, and D-134 produced no $C_2$ toxin. The other nontoxigenic strains produced 20 to 200 minimal lethal doses of $C_2$ toxin per ml when the culture fluids of EM and CM media were treated with trypsin. $C_2$ toxin was demonstrated only after trypsin treatment. The culture fluids of LYG medium showed no toxin production either with or without trypsin treatment. No positive hemagglutination reaction was observed even after nontoxigenic strains were treated with trypsin. Also, the HA titer of the toxigenic strains and of strain C-N71 was not increased by trypsin treatment. It seems that there is no relationship between $C_2$ toxin production and HA production.

**Production of toxin and HA by the cellophane-tube procedure.** Six cellophane tubes were prepared to confirm toxin, HA, and hemolysin (HL) production. Portions (15 ml) of actively growing nontoxigenic strain C-A02 were inoculated separately into three cellophane tubes. The first and second tubes were mixed with 3 ml of phages c-st and c-n71, respectively; these tubes will be referred to as C-A02(c-st) and C-A02(c-n71), respectively. A 15-ml amount of toxigenic strain C-Stockholm was inoculated into the fourth tube. Into the fifth and sixth tubes was inoculated 2 ml of toxigenic strain D-1873 and nontoxigenic strain D-151, respectively. A 5-ml sample of culture fluid was pipetted at 2, 6, and 18 h and at 2, 3, 4, 5, 6, and 7 days of incubation, and toxin, HA, and HL titers were determined (Fig. 1 and 2). Strains D-1873, C-A02, and D-151 produced no HA, and toxigenic strain C-Stockholm and phage-infected cells C-A02(c-st) produced both toxin and HA. The growth curve of C-A02 cells infected with phage c-n71 closely resembled that of C-A02(c-st). The former, however, produced only HA. As reported previously, it was strongly suggested that the cells growing after infection by phages were lysogenic (or pseudo-lysogenic) cultures because these cells acquired immunity to the parent phage infection, and that these cells produced phages with infection spectra the same as those of the parent phages (14, 15). Therefore, these results suggest that indicator strain C-A02 was converted to produce both toxin and HA by phage c-st and to produce only HA by phage c-n71. The highest toxin and HA titers were obtained with culture fluids incubated for more than 4 days. On the other hand, hemolysis was demonstratedwith culture fluids in logarithmic stage, and its activity decreased rapidly in C-Stockholm and C-A02(c-st). In D-1873 and D-151, however, the cultures sampled at 4 and 7 days showed complete and partial hemolysis, respectively (shown in parentheses in Fig. 2) at 4°C overnight, although little hemolysis appeared at 37°C for 2 h.

**Phage conversion to HA production.** We conclude from the above results that strains C-Stockholm, D-SA Tox, and C-N71 produce HA and that strain D-1873 does not. These four strains produced phages, and indicator strains C-A02, D-151, and D-SA NT produced no HA.

TABLE 2. *Hemagglutination reaction with erythrocytes obtained from various animals*[a]

| Strain | Erythrocytes from: | | | | | | |
|---|---|---|---|---|---|---|---|
| | Humans | | | Monkeys | Sheep | Chickens | Mice |
| | A | B | O | | | | |
| C-Stockholm | 32 | 32 | 32 | 2 | 2 | 4 | 4 |
| C-203 Tox | 64 | 64 | 64 | 4 | 2 | 8 | 4 |
| D-1873 | <2 (2)* | <2 (4)* | <2 (4)* | HL (4) | HL (4) | <2 (2)* | <2 (4)* |
| D-SA Tox | 4 | <2 | 4 (4)* | HL (4) | HL (4) | <2 (2)* | 8 (8)* |
| C-N71 | 64 | 32 | 64 | 4 | 2 | 8 | 4 |

[a] Each supernatant of culture fluids incubated in LYG medium was mixed with erythrocytes obtained from the indicated animals, and the hemagglutination test was carried out. Symbols: ( ), HL titer obtained at 37°C for 2 h. ( )*, HL titer obtained only after standing overnight at 4°C.

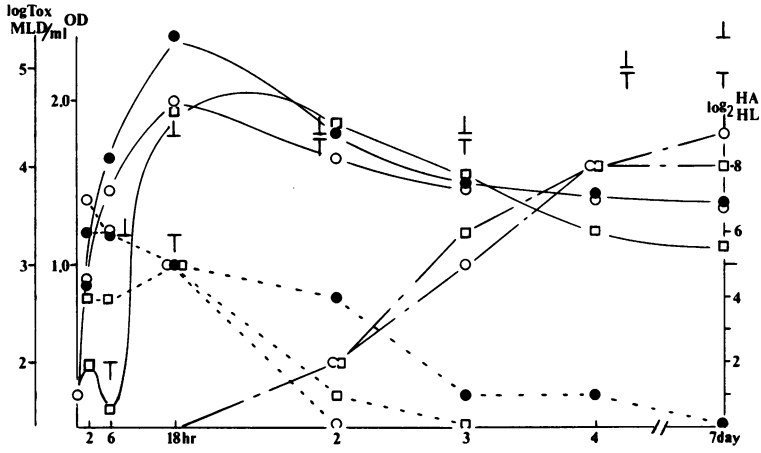


FIG. 1. *Production of toxin, HL, and HA and growth curve of C-Stockholm, C-A02, and C-A02(c-st) by the cellophane-tube method. Fifteen-milliliter portions of young C-Stockholm and C-A02 cells were inoculated separately into the inside of a dialysis tube. C-A02 cells infected with 3 ml of phage c-st were also prepared. A 5-ml amount was sampled from each culture at various times during incubation, and the optical density (OD) values and toxin and HA titers were determined. Symbols: (○——○) OD value of C-Stockholm; (○-----○) HL titer of C-Stockholm; (○- - -○) HA titer of C-Stockholm; (●——●) OD value of C-A02; (●-----●) HL titer of C-A02; (□——□) OD value of C-A02(c-st); (□-----□) HL titer of C-A02(c-st); (□- - -□) HA titer of C-A02(c-st); (⊥) toxin titer of C-Stockholm; (T) toxin titer of C-A02(c-st).*

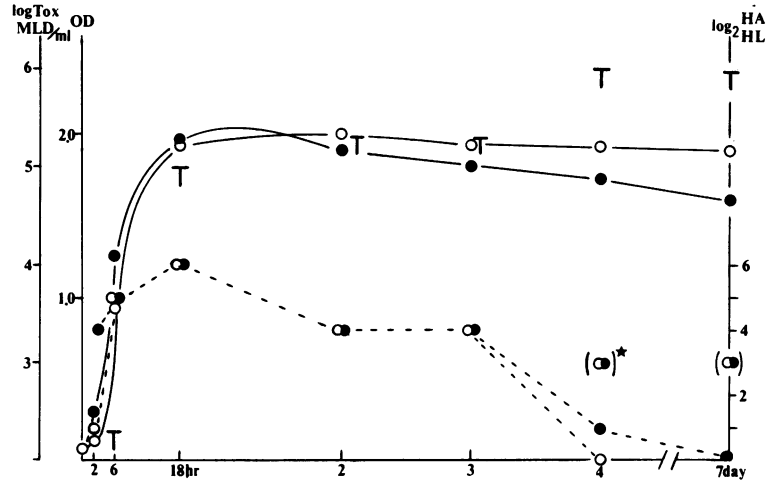


FIG. 2. *Production of toxin and hemolysin and growth curve of D-1873 and D-151 by the cellophane-tube method. Two-milliliter portions of young D-1873 and D-151 cells were inoculated and incubated by the cellophane-tube method. The culture fluids sampled were assayed for their turbidity and then tested for toxin, HA, and HL production. Symbols: (○——○) optical density (OD) value of D-1873; (○------○) HL titer of D-1873; (●——●) OD value of D-151; (●-----●) HL titer of D-151; (T) toxin titer of D-1873; ( )*, complete (at 4 days) and partial (at 7 days) hemolysis appeared only after incubation overnight at 4°C.*

Therefore, phage conversion to toxin and HA production was attempted. The cultures from plaque materials C-A02(c-st) and D-SA NT(d-sa) produced both toxin and HA; the culture from D-151(d-1873) produced only toxin; and the culture from C-A02(c-n71) produced only HA (Table 3). These results strongly suggest that HA production can be transmitted by the specific bacteriophages either separately or concomitantly with toxin production.

The toxin titer of several converted cultures was determined to clarify the relationship between toxin and HA production. The supernatants were diluted with gelatin buffer as follows: 10, $5 \times 10$, $10^2$, $5 \times 10^2$, $10^3$, $5 \times 10^3$, $10^4$, $5 \times 10^4$, $10^5$, and $5 \times 10^5$; then 0.5 ml of each dilution

TABLE 3. *Phage conversion to toxin and HA production* [a]

| Cultures from plaque materials | HA titer | Toxin titer (MLD/ml)[b] |
|---|---|---|
| C-A02(c-st) | | |
| 1 | 256 | $10^5$ |
| 2 | 256 | $10^5$ |
| 3 | 64 | $10^4$ |
| 4 | 64 | d |
| 5 | 64 | d |
| 6 | 64 | d |
| 7 | 64 | d |
| 8 | 32 | $10^4$ |
| 9 | 32 | $2 \times 10^3$ |
| 10 | 32 | d |
| C-A02(c-n71) | | |
| 1 | 64 | s |
| 2 | 32 | s |
| 3 | 32 | s |
| 4 | 32 | s |
| 5 | 16 | s |
| 6 | 16 | s |
| D-SA NT(d-sa) | | |
| 1 | 8 | $10^4$ |
| 2 | 8 | $2 \times 10^3$ |
| 3 | 8 | d |
| 4 | 4 | $2 \times 10^3$ |
| 5 | 4 | d |
| 6 | 4 | d |
| 7 | 4 | d |
| 8 | 2 | $2 \times 10^2$ |
| D-151(d-1873) | | |
| 1 | <2 | $2 \times 10^4$ |
| 2 | <2 | $2 \times 10^4$ |
| 3 | <2 | $2 \times 10^4$ |
| 4 | <2 | $10^4$ |
| 5 | <2 | $10^4$ |
| 6 | <2 | d |
| 7 | <2 | d |
| 8 | <2 | d |
| 9 | <2 | d |
| 10 | <2 | d |
| Controls | | |
| C-A02 | <2 | s |
| D-151 | <2 | s |
| D-SA NT | <2 | s |

[a] The indicator strains that produced neither toxin nor HA were infected with phages obtained from strains producing toxin or HA, or both. About 10 plaques demonstrated in each combination were transferred by micropipette into 10 ml of LYG medium and incubated at 37°C for 7 days. The supernatant of culture fluid was diluted 1:10 with gelatin buffer and tested for toxin production in mice. Also, each supernatant was diluted in serial twofold steps with physiological saline solution and then mixed with an equal volume of 1% human erythrocytes to determine HA production. In several converted strains, the toxin titer was obtained.

[b] MLD, Minimal lethal dose; d, died; s, survived.

was injected intraperitoneally into two mice. A high toxin titer was demonstrated with the culture fluids that showed a high HA titer.

**Neutralization test for toxin and HA.** Antigenicity of HA and $C_2$ toxin was observed by mixing equal volumes of culture supernatant and 5 U of anti-C toxin serum, distributed by the Center for Disease Control, Atlanta, Ga. The converted toxigenic strain C-A02(c-st) and nontoxigenic strains D-151 and C-203-U28, incubated in EM medium, were used. HA was inhibited completely by anti-C toxin serum (Table 4). $C_2$ toxin, however, was not neutralized by this serum.

## DISCUSSION

Toxin and HA production was observed in several toxigenic and nontoxigenic strains of *C. botulinum* types C and D. Four of five toxigenic strains tested produced HA, and nine of ten nontoxigenic strains produced no HA. All the strains used in this experiment, however, showed β-hemolysis on the blood-agar plates, and HL production was demonstrated in early culture fluids of these strains. The results of the cellophane-tube procedure strongly suggested that HL is produced by young growing cells and that its activity is decreased by incubation at 37°C. This was also suggested by the following experiment: a culture of actively growing cells was centrifuged, and the supernatant was divided into two parts, which were then stored at 4 and 37°C, respectively. Hemo-

TABLE 4. *Neutralization test for toxin ($C_1$ and $C_2$) and HA*[a]

| Sample | Anti-C | | Saline | |
|---|---|---|---|---|
| | Toxin | HA | Toxin | HA |
| C-A02(c-st) | | | | |
| Toxin (200 MLD/ml) | s | | d | |
| HA (256) | | <2 | | 128 |
| D-151 | | | | |
| $C_2$ toxin (20 MLD/ml) | d | <2 | d | <2 |
| C-203-U28 | | | | |
| $C_2$ toxin (20 MLD/ml) | d | <2 | d | <2 |

[a] The supernatant of culture C-A02(c-st), which showed high titers of toxin ($10^5$ minimal lethal doses [MLD]/ml) and HA (256), was mixed with an equal volume of antitoxin serum and physiological saline solution (as control) either after diluting the culture to 200 MLD/ml or without dilution. Five units of anti-C toxin serum, distributed by the Center for Disease Control, was used. Also, the supernatants of cultures D-151 and C-203-U28, incubated in EM medium, were mixed with the same toxin serum after these cultures were treated with trypsin. The tubes were incubated at 37°C for 1 h with frequent mixing, and then toxigenicity and hemagglutination tests were performed. Abbreviations: d, died; s, survived.

lytic activity disappeared in the sample stored at 37°C for 2 days, whereas the activity of the sample stored at 4°C for 7 days showed only a slight decrease.

$C_2$ toxin was demonstrated in all nontoxigenic strains except C-N71, D-SA NT, and D-134 only after the culture fluids incubated in EM and CM media were treated with trypsin. Strain C-N71 was the only nontoxigenic strain to produce HA. The HA titer was not increased by trypsin treatment, which indicates that HA and $C_2$ toxin production are not related.

HA production, however, was closely related to $C_1$ and D toxin production: it was transmitted by the specific bacteriophages either with toxin production concomitantly or separately. A high HA titer was demonstrated with the cultures that showed a high toxin titer. These results suggest that there might be some linkage between these two properties. We are now trying to isolate HA-positive, toxin-negative or HA-negative, toxin-positive mutants from converted HA- and toxin-positive strains. We are also carrying out experiments on the stability of HA production in converted strains.

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