Inhibition of *Clostridium botulinum* by Strains of *Clostridium perfringens* Isolated from Soil

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Thirty-one soil samples were examined for the presence of organisms capable of inhibiting growth and toxin production of strains of *Clostridium botulinum* type A. Such organisms were found in eight samples of soil. Inhibiting strains of *C. perfringens* were found in five samples, of *C. sporogenes* in three and of *Bacillus cereus* in three. Three of the *C. perfringens* strains produced an inhibitor effective on all 11 strains of *C. botulinum* type A against which they were tested, seven of eight proteolytic type B strains, one nonproteolytic type B strain, five of nine type E strains and all seven type F strains, whether proteolytic or nonproteolytic. They did not inhibit any of 26 type C strains, 6 type D strains, 4 type E strains, or 24 *C. sporogenes* strains. In mixed culture, an inhibitor strain of *C. perfringens* repressed growth and toxin production by a *C. botulinum* type A strain even though it was outnumbered by the latter by about 40 times. It also repressed growth and toxin production of *C. botulinum* in mixed culture of soils in which this latter organism naturally occurred when cooked meat medium but not when trypticase medium was used.

The demonstration of *Clostridium botulinum* in soil, sediment, or food is usually carried out by inoculating samples of the material under investigation into tubes of suitable media, incubating anaerobically, and testing the culture fluid for botulinum toxin which is identified by neutralization with type-specific antitoxin. Cooked meat medium is best for this purpose; Bott et al. (2) found that the beef heart infusion-cooked meat medium of Johanssen (7) usually gave about 10-fold higher toxin yields than did other media, including Trypticase-peptone-glucose, glucose-peptone-beef infusion, proteose peptone-Trypticase, fish infusion, reinforced clostridial medium, or brain-heart infusion.

This procedure is comparatively simple to carry out and the method is considered superior to the detection of cells of *C. botulinum* with fluorescent antisera (11, 14). Reliable results, however, are dependent upon the absence of any anaerobic organism that might interfere with the growth or toxin production of *C. botulinum*. *C. botulinum* type E is inhibited by a bacteriocin produced by a non toxigenic organism closely resembling it (1, 5, 8). Consequently, the absence of type E cells from soil or sediment specimens can be accepted only if it has been shown that no factors are present that inhibit the growth of type E cells. Bott et al. (2) found that organisms inhibitory to type E were present in 10% of the samples of soil and sediment that they examined. Some of these were so inhibitory that it was necessary to add as many as 10^6 spores of *C. botulinum* to 1-g samples of soil or sediment to be able to demonstrate type E toxin in the ensuing culture. The inhibition of type F in soil cultures by *Bacillus licheniformis* has also been shown (15).

*C. botulinum* type A has its principal habitat in the soil, particularly in the western part of the United States, and usually has been detected by the method described above, no provision being made for any inhibiting factor, for none has been known. The present paper describes the results of a search for such inhibiting bacteria in soil, the determination of the susceptibility of strains of the various toxin types of *C. botulinum*, and the inhibiting effect of such organisms on type A strains in mixed culture.

**MATERIALS AND METHODS**

**Isolation of inhibiting organisms.** Thirty-one samples of soil from a variety of sources were investigated by a “sandwich” plating technique for bacteria capable of inhibiting *C. botulinum* type A. Comparative trials in preliminary work showed that brain-heart infusion agar (Difco) and Schaedler agar (BBL) were better than tryptic soy agar (Difco), Brucella agar (Aalimi), blood agar base (BBL), and Columbia broth (BBL) for this purpose. Approximately 15 ml of
brain-heart infusion medium made up to contain 2.5% agar was poured into petri dishes and allowed to harden. Soil specimens were weighed out and serially diluted in sterile anaerobic dilution fluid (6), and 1-ml portions were added to brain-heart infusion agar which was poured over the base layer of agar in the petri dishes. These were prepared in triplicate and were incubated anaerobically in Brewer anaerobe jars or aerobically at 30 C for 2 to 4 days. Plates on which isolated colonies were apparent, usually those inoculated with 10^6 or 10^7 dilutions, were overlaid with brain-heart infusion agar containing about 10^5 C. botulinum type A (strain 7124) spores per ml. After solidification, the plates were incubated anaerobically for 2 days. Colonies of the soil organisms that were surrounded by zones of inhibition of the C. botulinum lawn (Fig. 1) were picked to cooked meat medium and streaked on egg yolk agar for purification.

Such strains were further tested for the production of factors inhibitory to type A strains of C. botulinum by inoculating them in a single streak across plates of brain-heart infusion agar, incubating overnight, streaking strains of C. botulinum at right angles to the streak of the inhibitory strain, and reincubating aerobically. If the growth of C. botulinum was inhibited close to the streak of the potential inhibitory organism, the latter was retained.

Preparation of spore suspensions. Suspensions of spores of C. botulinum A were prepared by growing C. botulinum on beef infusion agar medium for 7 days at 30 C and washing with sterile saline by centrifuging, and were stored in the cold. Suspensions of spores of C. perfringens 9078 were prepared by growing that strain in Duncan-Strong sporulation medium (4) for 2 days at 37 C and washing with sterile saline by centrifuging, and were stored in the cold. Both spore suspensions were heated for 10 min at 80 C immediately before use to stimulate spore germination. These spore suspensions were stable for at least 10 months.

Inhibition of strains of C. botulinum by C. perfringens culture fluid. The inhibition of strains of the various toxin types of C. botulinum and of strains of C. sporogenes by culture fluid of strains of C. perfringens was carried out by growing strains of C. perfringens in Columbia broth (BBL) overnight at 37 C and centrifuging. The supernatant fluid was kept in the cold. Strains of C. botulinum and C. sporogenes were grown overnight in cooked meat-glucose medium and 0.1 ml of the cultures used to inoculate agar for individual plates of Brucella agar (Aalbini Laboratories). After the agar had hardened, 0.5-in. (1.27 cm) antibiotic-free “penicillin” disks (A. H. Thomas) were placed on the surface of the agar, lightly tapped so that even contact with the agar was made, and 0.05 ml of C. perfringens culture fluid was pipetted onto the disk. The plates were incubated in Brewer jars anaerobically overnight and, after removal of the disks, the zone of inhibition was measured. When the fluid at test was comparatively weak against the strain being tested, the zone of inhibition would sometimes be smaller than the 13-mm diameter of the disk itself.

Inhibition of growth and toxin production of C. botulinum by C. perfringens in mixed culture. The ability of C. perfringens 9078 to inhibit C. botulinum 7124 was tested by inoculating them together into cooked meat-glucose medium, and by incubation at 30 C for 3 days. The cultures were then frozen and stored in the frozen state overnight, thawed, and centrifuged, and the supernatant fluid of each culture was tested for toxin by inoculating 0.3 ml intraperitoneally into each of two 18- to 22-g white Swiss mice. After this demonstration of toxin in each of the culture fluids, an effort was made to neutralize the toxin with C. perfringens antitoxin. Those culture fluids whose toxicity could not be neutralized with C. perfringens antitoxin were then treated with C. botulinum type A antitoxin.

Inhibition of toxin production of C. botulinum in soil cultures. Two samples of soil that had previously been found to contain C. botulinum type A (12) were used in these experiments. Sample 14 came from a fallow wheatfield in Idaho; sample 22 came from range land in Wyoming. Ten 1-g specimens of each of these samples were weighed out and placed into tubes of cooked meat-glucose medium with or without spores of C. perfringens 9078. Incubation was at room temperature (20 to 22 C), 30, or 37 C for the period of time given in the text.

In two experiments, a medium composed of 2% Trypticase, 0.5% yeast extract, and 0.5% glucose, pH 7.0 to 7.2, was used with or without the addition of a sterile solution of commercial trypsin (Difco; 1:250) to make a final concentration of 0.1%.

RESULTS AND DISCUSSION

Inhibiting organisms were found in 8 of 31 soil samples examined. Four soil samples contained C. perfringens as the only inhibiting organism, one contained an inhibiting strain of C. sporogenes. ...
genes, two contained inhibiting strains of *C. sporogenes* and an organism resembling *Bacillus cereus*, and one contained *B. cereus* and *C. perfringens*. The strains of *B. cereus* were obtained from those plates that had been incubated aerobically before being overpoured with agar inoculated with *C. botulinum*. Inhibiting strains were not found in any soil samples in which *C. botulinum* had been demonstrated in a previous study (12).

The inhibiting effect of culture fluid from three strains of *C. perfringens* was tested on 69 strains of *C. botulinum* of various types (Table 1), as well as 34 strains of *C. sporogenes*. All 11 strains of type A were inhibited, as were eight of nine strains of type B. Seven of eight type B strains were cultural Group I strains, biochemically indistinguishable from type A strains or from strains of *C. sporogenes* (13). One type B strain (1731) was cultural Group II. Twenty-six Group III strains of type C and six strains of type D were resistant to the inhibitory factor. The reactions of Group II strains of type E were variable and could not be correlated with their cultural or toxigenic characteristics. A lack of correspondence between cultural characteristics and susceptibility was shown by the type F strains, all of which were susceptible to the inhibitor. Three of these, VPI, 4404, and 4257, are proteolytic Group I strains; the others belong to Group II. The type G strain, which is culturally unlike any of the others, was also inhibited. All of 34 strains of *C. sporogenes* were unaffected, a somewhat surprising finding since they are culturally indistinguishable from Group I strains of *C. botulinum*, share antigens with them (10), and exhibit high deoxyribonucleic acid homology (9).

Production of inhibitor effective against *C. botulinum* type A is not common among strains of *C. perfringens*. We could not demonstrate it among any of twenty strains of this species in the VPI collection that had been isolated from clinical specimens or fourteen that had been isolated from soil.

The inhibitor produced by strain 9078 is similar in its properties to a bacteriocin produced by a strain of *B. atrophaeus* (3) in being inactivated by trypsin, heat stable, pH indifferent, and adsorbing on cellulose to such an extent that most of the activity is lost on membrane filtration. It can be quantitatively

| Strain of *C. botulinum* | Zone of inhibition (mm)* | Strain of *C. botulinum* | Zone of inhibition (mm)* |
|--------------------------|--------------------------|--------------------------|--------------------------|
| Type A                   |                          | Type D (6 strains)       |                          |
| L-362-1                  | 13                       | 1543, 1615, 2140, 4132, 6793, 6794 |                          |
| 1545A                    | 15                       | 1543, 1615, 2140, 4132, 6793, 6794 | 10                       |
| 1550                     | 20                       | 1543, 1615, 2140, 4132, 6793, 6794 | 14                       |
| 1513-0                   | 20                       | 1543, 1615, 2140, 4132, 6793, 6794 | 16                       |
| 1513-5C                  | 15                       | 1543, 1615, 2140, 4132, 6793, 6794 | 16                       |
| 2085                     | 25                       | 1543, 1615, 2140, 4132, 6793, 6794 | 15                       |
| 2130                     | 34                       | 1543, 1615, 2140, 4132, 6793, 6794 | 15                       |
| 2137                     | 19                       | 1543, 1615, 2140, 4132, 6793, 6794 | 15                       |
| 7124                     | 10                       | 2154, 2160-1, 4664        |                          |
| 8765                     | 17                       | 2154, 2160-1, 4664        |                          |
| 8869                     | 16                       | 2154, 2160-1, 4664        |                          |
| Type B                   |                          | Type E (nontoxic)         |                          |
| 0558                     | 14                       | 2158, 2159, 2160-1, 4664  |                          |
| 1541                     | 21                       | 2158, 2159, 2160-1, 4664  |                          |
| 1618                     | 23                       | 2158, 2159, 2160-1, 4664  |                          |
| 1624                     | 9                        | 2158, 2159, 2160-1, 4664  |                          |
| 1731                     | 14                       | 2158, 2159, 2160-1, 4664  |                          |
| 1750-A                   | 14                       | 2158, 2159, 2160-1, 4664  |                          |
| 2131                     | 20                       | 2158, 2159, 2160-1, 4664  |                          |
| 3801                     | 12                       | 2158, 2159, 2160-1, 4664  |                          |
| 8917                     | 16                       | 2158, 2159, 2160-1, 4664  |                          |
| Type C (27 strains)      |                          | Type G                   |                          |
| Bell-X-20, 1628, 2337, 2429A, 3109, | 1549                     | 6714                     |
| 3903-1, 3903A, 3904, 4529, 4530, | 1549                     | 6714                     |
| 5339, 6462, 6789, 6790, 6791, | 1549                     | 6714                     |
| 6792, 7162, 7199, 7200, 7218, | 1549                     | 6714                     |
| 7221A&B, 7373, 7376, 7377, | 1549                     | 6714                     |
| 7378, 7445               | 1549                     | 6714                     |

* C. perfringens culture fluid.

* No inhibition.
removed from such a membrane, however, by extraction with 0.1% Triton X-100.

When tubes of cooked meat-glucose medium were inoculated with spores of *C. botulinum* type A as well as those of an inhibiting strain of *C. perfringens*, there was marked inhibition of toxin production by *C. botulinum* (Table 2). All cultures inoculated with this mixture of organisms were toxic, but the cultures containing the largest number of *C. perfringens* spores contained only *C. perfringens* alpha toxin. The tube inoculated only with *C. botulinum* spores and those inoculated with small numbers of *C. perfringens* spores contained only botulinum toxin.

Since the inhibitory action of *C. botulinum* was manifest in mixed culture in broth, it seemed desirable to investigate the possibility of the inhibition of toxin production in soil cultures similar to those used in surveys of the incidence of *C. botulinum* in soil. For this purpose, two soil samples were available that naturally contained *C. botulinum* type A (12). Soil 14 contained 17 type A spores per gram (most-probable-number count) and soil 22 contained 170 type A spores per gram.

When $7.9 \times 10^8$ spores of *C. perfringens* 9078 were added to 10 tubes of cooked meat-glucose medium and 1-g portions of soil 22, the number of cultures in which toxin was demonstrated dropped from 9 or 10 to 2 (Table 3). The temperature of incubation, from 20 to 37 C, did not seem appreciably to affect the inhibition of the growth of *C. botulinum*. When soil 14 was used, the production of botulinum toxin in mixed cultures was again inhibited by *C. perfringens* 9078. However, an appreciable number of *C. perfringens* cells was necessary, since reducing the number of *C. perfringens* spores in the inoculum from $7.9 \times 10^8$ to $7.9 \times 10^6$ markedly reduced the inhibition of *C. botulinum* growth and toxin production. Since the soil from which the inhibiting strain was isolated contained it at a level somewhat higher than $10^4$ per g, the addition of $7.9 \times 10^8$ spores did not seem excessive.

The inhibition of the growth of *C. botulinum* in the soil culture was related to the medium; when a medium composed of Trypticase, yeast extract, and glucose was used instead of the cooked meat medium, there was no inhibition of toxin production of the *C. botulinum* in soil sample 14 (Table 3). This medium was used because preliminary experiments had shown

### Table 3. Inhibitory effect of *C. perfringens* on development of botulinum toxin in soil cultures

| Soil   | Temp of incubation | Time of incubation (days) | No. of *C. perfringens* spores per tube | No. of cultures containing toxin out of 10 inoculated |
|--------|--------------------|---------------------------|----------------------------------------|--------------------------------------------------|
| 22     | 20-22              | 5                         | None                                   | 9                                                |
| 20-22  | 5                  | None                      | 9                                      |
| 20-22  | 5                  | $7.9 \times 10^8$         | 2                                      |
| 30     | 3                  | None                      | 10                                     |
| 30     | 3                  | $7.9 \times 10^8$         | 10                                     |
| 37     | 2                  | None                      | 10                                     |
| 37     | 2                  | $7.9 \times 10^8$         | 2                                      |
| 37     | 3                  | $7.9 \times 10^8$         | 2                                      |
| 14     | 20-22              | 5                         | None                                   | 10                                               |
| 20-22  | 5                  | None                      | 10                                     |
| 20-22  | 5                  | $7.9 \times 10^8$         | 2                                      |
| 30     | 3                  | None                      | 10                                     |
| 30     | 3                  | $7.9 \times 10^8$         | 1                                      |
| 30     | 3                  | $7.9 \times 10^8$         | 9                                      |
| 30     | 5                  | None                      | 10                                     |
| 30     | 5                  | $7.9 \times 10^8$         | 10                                     |
| 30     | 5                  | $7.9 \times 10^8$         | 9                                      |

* Trypticase medium.
* Trypticase medium with added trypsin.

### Table 2. Inhibition of growth of *C. botulinum* type A by *C. perfringens* in mixed culture

| *C. perfringens* (9078) (spores per tube) | *C. botulinum* (7124) (spores per tube) | Unprotected mice | Mice protected with: |
|-----------------------------------------|-----------------------------------------|------------------|----------------------|
|                                          |                                         |                  | *C. perfringens* antitoxin | *C. botulinum* antitoxin |
| 0                                       | 92                                      | D, D*            | ND*                  | S, S*                |
| 2                                       | 920                                     | D, D*            | D, D                | S, S                |
| 7                                       | 920                                     | D, D*            | D, D                | S, S                |
| 24                                      | 920                                     | D, D*            | D, D                | S, S                |
| 72                                      | 920                                     | D, D*            | D, D                | S, S                |
| 240                                     | 920                                     | D, D*            | D, D                | S, S                |
| 720                                     | 920                                     | D, D*            | D, D                | S, S                |

* Abbreviations: D, dead; S, survived; and ND, not done.
that the inhibitor was inactivated by trypsin
and this medium allowed the use of trypsin to
inactivate the inhibitor. However, no inhibitor
was produced, either in the medium that con-
tained trypsin or in that which did not, with
either three or five days of incubation.

It is apparent that soil does contain organ-
isms that can interfere with the demon-
stration of C. botulinum types A, B, and F just as
has been reported for type E (2, 8). Detection
of such inhibitor organisms can be accomplished
by the method of Bott et al. (2), i.e., by
inoculating cultures of soil in which C. botu-
linum could not be demonstrated with spores of
a toxigenic strain of this organism, incubating,
and testing for toxin. If toxin has not been
produced, the presence of an inhibiting strain
can be assumed. Fortunately, strain specificity
seems to be rare among strains of cultural
Group I organisms, and spores of a single strain
of C. botulinum type A would probably be
adequate to test for the presence of inhibitory
organisms in a variety of soil samples. It is
unfortunate that the cooked meat medium
which has been found to be best for the demon-
stration of C. botulinum in soil is also a medium
in which the inhibitory action of some strains of
C. perfringens can be manifest. Nevertheless, it
seems clear that the absence of C. botulinum
types A, B, and F, as well as type E, from a soil
or sediment sample cannot be taken as proved
unless the absence of inhibiting organisms has
been demonstrated.

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