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Antagonistic Effect on *Clostridium botulinum* Type E by Organisms Resembling It

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ABSTRACT

KAUTTER, D. A. (Food and Drug Administration, Washington, D.C.), S. M. HARMON, R. K. LYNT, JR., AND T. LILLY, JR. Antagonistic effect on *Clostridium botulinum* type E by organisms resembling it. Appl. Microbiol. **14**:616–622. 1966.—A bacteriocin-like substance, active against strains of *Clostridium botulinum* type E, is produced by certain nontoxic organisms whose biochemical properties and morphological characteristics are similar to type E. The substance, for which the name "boticin E" is proposed, is bacteriolytic for vegetative cells and bacteriostatic for spores of type E. Its spectrum of activity is somewhat strain-specific. Of the clostridial species tested, only *C. botulinum* type E and, to a lesser extent, *C. perfringens* and *C. acetobutylicum*, but not *C. botulinum* types A, B, or F, are sensitive. Irreversibly resistant variants originating from both vegetative cells and spores of certain strains were obtained. The active substance is heat-stable and dialyzable, and is not inactivated by chloroform but is digested by trypsin. Ethyl alcohol and acetone precipitates are fully active, whereas trichloroacetic acid precipitates are only partially active. Other nontoxic organisms producing similar antagonistic substances are discussed.

The lethal effect of a growth product of one strain of *Escherichia coli* on another was first demonstrated by Gratia (10). Since then, the production of bacteriocins has been observed in many strains of enterobacteria, and is prevalent in other groups as well. A review of bacteriocins has recently been published by Reeves (19).

Bacteriocins differ considerably in their sensitivity to heat, *p*H, and proteolytic enzymes. Generally, they are soluble in water, are precipitated by organic solvents and ammonium sulfate, and are resistant to ultraviolet light and nucleases (1). Although all bacteriocins kill the sensitive bacteria, their physical and chemical natures differ considerably even among those produced by closely related organisms. Bordet (5) has shown, for example, that *E. coli* V gives rise to a variant which produces a colicin differing from that of the parent strain. One produces a wide zone of activity, is heat-stable, and is dialyzable, whereas the other produces a narrow zone, is heat-sensitive, and is nondialyzable. Methods have been developed for purification and concentration of some bacteriocins (9, 12, 14).

Fredericq (8) has shown that colicins could be divided into a number of groups having characteristic spectra of activity, and that variations in the response of susceptible strains suggest differences in the nature of the colicins and their mode of action. Although similar agents may be produced by several genera of enterobacteria and may cross genus and species lines in their range of activity, pyocins are limited in their activity to other members of the genus *Pseudomonas* and megacins to other strains of *Bacillus megaterium* (13).

More recently, a bacteriocin which has been designated pesticin has been isolated from cultures of *Pasteurella pestis* by Ben-Gurion and Hertman (3), and bacteriocin-like substances have been found in cultures of *Clostridium sporogenes* by Betz and Anderson (4).

Studies for evaluating methods of detecting the toxin of *C. botulinum* type E, and for isolating the organism, suggested that certain natural contaminants from mud and the viscera of fish from the Great Lakes prohibited the production of toxin. In addition, this prohibition in some cultures could be correlated with the presence of a nontoxic organism morphologically and culturally identical to *C. botulinum* type E. The purpose of this report is to describe the effect of a bacteriocin-like substance produced by these nontoxic organisms on *C. botulinum* type E.

MATERIALS AND METHODS

Cultures. The *C. botulinum* type E strains used in this investigation represented a wide geographical distribution. Strains Beluga, VH, and Alaska, originating in Alaska or Northwestern Canada, and strain 8-E, the original Russian isolate, were obtained from C. F. Schmidt, Continental Can Co., Chicago, Ill. Strain Salmon A, also originating in Alaska, was isolated in this laboratory from canned "hard smoked" salmon. Strains Minnesota, 070, Kalamazoo, and 066B originated in the Great Lakes, and were isolated by the authors from toxic smoked fish involved in the 1960 and 1963 botulism outbreaks (2, 17). Strains 1304 and Iwanai were received from H. Iida, Hokkaido Institute of Public Health, Sapporo, Japan. Strain D8 was isolated from tuna fish involved in the 1963 outbreak (15).

The nontoxic organisms resembling type E were designated S5, 28-2, and GB-3. They were obtained, respectively, from viscera of a Lake Erie Smelt, mud of Lake Huron, and viscera of a Green Bay Sucker, and were isolated by us. *C. botulinum* types A and B, *C. bifermentans*, *C. sporogenes*, *C. sordelli*, *C. novyi*, *C. chauvoei*, *C. perfringens*, and *C. acetobutylicum* were from the Food and Drug Administration's stock culture collection. *C. botulinum* type F was obtained from the Communicable Disease Center, Atlanta, Ga.

Propagation of strains. Trypticase peptone glucose (TPG) broth of Schmidt, Nank, and Lechowich (21), with 0.1%, in place of 0.2%, sodium thioglycolate, was used for propagation of all strains. Filtrates of the nontoxic organisms were prepared from 16-hr broth cultures, and tested for activity in TPG. Spore suspensions of the type E strains were prepared from 72-hr TPG cultures incubated at 28 C. They were centrifuged, and the spores were washed and resuspended in distilled water. The viable count was determined on Liver Veal Agar (Difco) containing 4% egg yolk.

Morphological and biochemical characteristics of strains. Colonial morphology and characteristics were compared on Liver Veal Agar and Brain Heart Infusion Agar (Difco). Opalescence was determined in Liver Veal Agar containing 4% egg yolk, and hemolysis was detected on Brain Heart Infusion Agar to which 5% human blood was added. Ability to ferment carbohydrates was tested in Trypticase peptone broth base to which 0.5% of the appropriate carbohydrate was added, and, after 3 days at 28 C, the cultures were tested for acid production by adding bromthymol blue indicator. Lipolytic activity was detected by growth on spirit-blue agar (23). Tests for urease production were made in Urea Broth (Difco), which was modified by increasing the amount of yeast extract to 1% and supplementing with 0.1% sodium thioglycolate and 0.4% glucose. Sensitivity to 16 antibiotics was determined by placing sensitivity discs containing known quantities of antibiotics on seeded Liver Veal Agar plates. The degree of sensitivity to 14 dyes or chemical inhibitors was determined by a serial tube dilution technique in TPG. All other tests were performed and interpreted according to the instructions of the *Manual of Methods for Pure Culture Study of Bacteria* (22).

Cultural antagonism. The antagonistic effect of organisms resembling type E on strains of type E was investigated by inoculating TPG with spores of both in various ratios. After 3 days of incubation at 28 C, the cultures were tested for the presence of toxin and plated to select type E colonies. Sterile culture filtrates were prepared by Seitz filtration, and a portion was treated with trypsin (7). Serial dilutions of both the trypsin-treated and untreated filtrates were tested for toxicity by intraperitoneal injection into 18- to 20-g Swiss Webster mice. Death was recorded over a 48-hr period. Concurrently, portions of the above cultures were streaked on liver-veal-egg-agar plates. After 2 days of anaerobic incubation in a Case Anaero jar under a nitrogen atmosphere at 35 C, single colonies were picked into TPG, and after 3 days of growth they were assayed for toxicity in mice.

Effect on toxin. The effect of organisms resembling type E on preformed type E toxin was determined by making dilutions of Beluga toxin in TPG, and inoculating with 0.1 ml of a 3-day-old culture of the antagonistic strain. After 3 days of growth at 28 C, sterile culture filtrates were prepared and assayed for toxin.

Antagonistic agent. The antagonistic strains S5, 28-2, and GB-3 were grown in TPG for 16 hr at 28 C, the cells were removed by centrifuging at 2,000 $\times$ *g* for 45 min, and the supernatant fluid was filtered through a 0.45-μ Millipore filter. The filtrates were tested for sterility, and held frozen until tested for antagonistic activity against type E strains.

Spot tests. As an initial screening procedure, the degree of antagonistic activity of filtrates was determined by spotting dilutions on a sensitive lawn of type E spores. The lawn was prepared by spreading 0.1 ml of a type E spore suspension containing 10^8 spores per milliliter on the surface of a Liver Veal Agar plate. Twofold serial dilutions of the filtrate to be tested were made in 0.0003 M phosphate buffer (*p*H 7.2), and dropped on the lawn by means of a Pasteur pipette. After drying, the plates were incubated anaerobically for 24 hr at 35 C. They were then examined for zones of inhibition. A similar procedure was used to test the sensitivity of spores of other clostridia to the active filtrate.

Activity on vegetative cells. Twofold serial dilutions of the antagonistic filtrates, ranging from 1:10 to 1:320, were made in TPG. They were tested in duplicate against serial 10-fold dilutions of vegetative cells in checkerboard fashion. One series of cultures contained no filtrate, and served as a means of determining the number of vegetative cells used in the test. The inoculated tubes were incubated at 28 C and observed periodically for growth, usually for a 72-hr period, but, in some experiments, the tubes were observed for 1 week. The type E vegetative cells used in these experiments were grown in TPG at 28 C for 16 to 18 hr from an inoculum of approximately 10 spores. This usually produced moderate turbidity and incipient gas formation by this time. A similar procedure and medium were used to test the sensitivity of vegetative cells of other clostridia to the active

filtrate. To demonstrate the absence of spores in the vegetative-cell cultures, a sample was treated with alcohol (16) and subcultured in TPG.

Activity on spores. Twofold serial dilutions, ranging from 1:20 to 1:10,240, of antagonistic filtrates to be tested were made in duplicate tubes of TPG. Each tube was inoculated with either 100 or 100,000 spores of the type E strain to be tested. The inoculated tubes were incubated at 28 C and observed periodically for growth, usually for a 72-hr period, although in some experiments the tubes were observed for 1 week. Similar cultures without filtrate served as controls. The viability of spores after exposure to the filtrate was determined by subculturing serial dilutions in fresh TPG.

Physical and chemical properties; filtrate stability. Preliminary data indicated that the antagonistic activity was lost when filtrates were held at room temperature or refrigerated for an extended period of time.

To test the stability of the filtrate to temperature extremes, equal volumes of filtrate were subjected to the following conditions: 25 C for 72 hr, boiling for 30 min, and freezing at −70 C.

To test the combined effect of temperature extremes, frozen filtrate was thawed and divided into two portions. One portion was boiled for 30 min and then subdivided into two parts: one part was refrozen, and the other part was held at 25 C for 72 hr.

The unheated portion of the thawed filtrate was also subdivided into two parts: one part was refrozen, and the other part was held at 25 C for 72 hr.

The treated filtrates were titrated for activity by the procedure described in *Activity on spores.*

Dialysis. Active filtrates were dialyzed overnight in the cold, with constant stirring, against an equal volume of TPG, by use of cellulose dialyzer tubing. Both the contents of the sack and the material which dialyzed through were tested for activity by spotting dilutions of each on a sensitive type E lawn.

Chloroform treatment. A 1-ml amount of active filtrate was treated with 0.1 ml of chloroform at room temperature for 1 hr with occasional shaking. After this treatment, the chloroform was bubbled off with nitrogen, and the treated filtrate was tested for activity.

Alcohol and acetone precipitation. One volume of ice-cold filtrate was treated with 2 volumes of absolute ethyl alcohol or acetone at −20 C. After 2 hr, the precipitate which had formed was removed by centrifugation in the cold and redissolved in 1 volume of ice-cold saline. The solution, which was clear, was treated with another 2 volumes of alcohol or acetone as before and left overnight. The resulting precipitate was redissolved in the original volume of saline and tested for activity.

Trypsin treatment. An active filtrate was adjusted to *p*H 8.0 with sterile 10% sodium bicarbonate solution. Enough 10% trypsin solution was added to give a final concentration of 1%, and the mixture was incubated at 37 C for 1 hr. After boiling for 10 min to inactivate the trypsin, the precipitate which formed was centrifuged out and the supernatant fluid was tested for activity.

Precipitation by trichloroacetic acid. Portions (5 ml) of active filtrate were treated with an equal amount of ice-cold 10% trichloroacetic acid and held in crushed ice for 4 hr. The resulting precipitate was removed by centrifugation, redissolved in ice-cold saline, neutralized with 1 N KOH, and tested for activity.

RESULTS

The common morphological and physiological properties of typical strains of *C. botulinum* type E and those organisms resembling it, designated S5, 28-2, and GB-3, are presented in Table 1. The only outstanding difference among them is the production of toxin by strains of type E. Some variation in the size and shape of colonies of typical type E is characteristic; however, this same variation was also observed with nontoxic organisms, and may be due to the age and moisture content of the plating medium. Both the strains of type E and those organisms resembling it were resistant to the following antibiotics contained in sensitivity discs: 30 μg of kanamycin, 30 μg of neomycin, 10 μg of streptomycin, 15 μg of colistin, and 48 units of polymyxin. They were sensitive to 10 μg of chloramphenicol, 2 μg of oleandomycin, 10 μg of tetracycline, 5 μg of erythromycin, 10 μg of novobiocin, 10 units of penicillin, 10 μg of ristocetin, 5 μg of vancomycin, 2 units of bacitracin, 0.25 ppm of tylosin, and 100 ppm of nisin, when tested in the same manner. Both the toxic and nontoxic organisms exhibited the same levels of sensitivity to the following: brilliant green, malachite green, crystal violet, methylene blue, acridine orange, thionin, basic fuchsin, eosin Y, iodine, potassium tellurite, tetrazolium salts, sulfadiazine, potassium cyanide, and bile salts. Thus, these nontoxic organisms which so closely resemble type E may be either nontoxic variants or closely related strains which are indistinguishable by these tests.

The antagonistic effect of S5 on toxin production by strains of *C. botulinum* type E are presented in Table 2. No toxin was produced when mixtures of spores having a type E-S5 ratio of 1:10 or 1:100 were used as the inoculum. When the inoculum consisted of an equal number of each, small amounts of toxin were produced by strains Minnesota and 070, but, if there were 10 times as many type E as S5 spores in the inoculum, moderate to large amounts of toxin were produced by strains Minnesota, 070, and Beluga, although not by D8 or Iwanai. There is a direct correlation, then, between the ratio of type E to S5 in the inoculum and the production of toxin.

Trypsin-treated and untreated cultures pro-

TABLE 1. *Properties of Clostridium botulinum type E and organisms resembling type E*

Property	Reaction[a] of			
	C. botulinum type E	S5	28-2	GB-3
Fermentation of				
Glucose	AG[a]	AG	AG	AG
Fructose	AG	AG	AG	AG
Maltose	AG	AG	AG	AG
Sucrose	AG	AG	AG	AG
Ribose	AG	AG	AG	AG
Mannose	AG	AG	AG	AG
Sorbitol	AG	AG	AG	AG
Trehalose	AG	AG	AG	AG
Adonitol	AG	sl. AG	AG	AG
Sporulation[b]	++++	+	+	++
Opalescence in egg yolk	+	+	+	+
Pearly layer on egg yolk	+	+	+	+
Hemolysis	Beta	Beta	Beta	Beta
Growth at 10 C	+	+	+	+
Colonial characteristics on				
Liver Veal Agar	Mosaic	Mosaic	Mosaic	Mosaic
Brain Heart Infusion Agar	Mosaic	Mosaic	Mosaic	Mosaic
Toxicity	+	−	−	−
Urease production	−	−	−	−
Lipolytic	+	+	+	+
Proteolytic	−	−	−	−
Salt tolerance[c]	3.5	3.5	3.5	3.5
Indole production	−	−	−	−
Nitrate reduction	−	−	−	−
Gelatin liquefaction	−	−	−	−
Resistant and sensitive to same levels of 16 antibiotics	Same	Same	Same	Same
Sensitive to same levels of 14 dyes and chemical inhibitors	Same	Same	Same	Same

[a] AG, production of acid and gas; sl., slight.
[b] Relative sporulation in Trypticase, peptone, and glucose media.
[c] Highest concentration of NaCl showing no apparent inhibition of growth. In addition, there was no action by the cultures with dextrin, starch, mannitol, lactose, salicin, dulcitol, rhamnose, galactose, xylose, melibiose, raffinose, inulin, inositol, arabinose, or cellobiose.

TABLE 2. *Effect of growth of S5 on toxin production by Clostridium botulinum type E*

Spore inoculum per tube		Ratio of type E to S5	Toxicity[a] produced by trypsin-treated cultures of strain				
Type E	S5		Minnesota	D8	070	Beluga	Iwanai
100	—	—	>20,000	10,000	>20,000	2,000	2,000
1,000	—	—	>20,000	>20,000	>20,000	20,000	2,000
10,000	—	—	>20,000	>20,000	>20,000	20,000	2,000
100	10,000	1:100	<10	<10	<10	<10	<10
100	1,000	1:10	20	<10	<10	<10	<10
100	100	1:1	200	<10	200	20	<10
1,000	100	10:1	2,000	<10	>20,000	1,000	<10
10,000	100	100:1	>20,000	100	>20,000	1,000	20

[a] Expressed as minimal lethal dose per milliliter.

TABLE 3. *Growth of the antagonistic strains S5 and 28-2 in preformed Beluga toxin*

Dilution of Beluga toxin in TPG	Inoculum (strain)	Toxicity after growth of inoculum	
		Nontreated (MLD/ml)[a]	Trypsin-treated (MLD/ml)
1:2	—	50–400[b]	2,000–4,000
1:5	—	100–500	5,000–10,000
1:10	—	200	2,000–20,000
1:2	S5	50–200	2,000–4,000
1:5	S5	100	1,000–10,000
1:10	S5	200	10,000
1:2	28-2	50–200	4,000–20,000
1:5	28-2	50–100	500–5,000
1:10	28-2	100–200	1,000–10,000

[a] Takes into account original dilution of Beluga toxin in TPG.

[b] Toxicity range of several experiments.

duced similar results, although the amounts of toxin assayed were 10- to 100-fold lower, as might be expected, with cultures that were not treated with trypsin. Strains 28-2 and GB-3 had the same effect as S5. Recovery of type E from these cultures was attempted. When the spore ratio of Beluga to S5 was 1:1 and 10:1 in the inoculum, 3 of 153 colonies and 3 of 147 colonies, respectively, gave rise to toxic subcultures.

The adverse effect of S5 on toxin production suggested that either toxin is destroyed by the growth of S5, or toxin formation is prohibited. The effect of the growth of the antagonistic strains S5 and 28-2 on preformed Beluga toxin is presented in Table 3. The untreated and trypsin-treated cultures of each showed the same degrees of toxicity as those cultures not inoculated with the antagonistic strains. These data indicate that toxin is not destroyed by the growth of the antagonistic organisms and, coupled with failure to recover type E from mixed cultures in which both are present, suggest that toxin formation is prohibited because type E does not grow.

Preliminary studies revealed that sterile culture filtrates of the antagonistic organisms contained a substance which adversely affected strains of type E. Additional experiments showed that cultures in their logarithmic-growth phase, after approximately 16 hr of incubation, produced active filtrates of the highest potency. As the age of the culture increased beyond 16 to 20 hr, the potency declined.

The activity of the antagonistic substance against both vegetative cells and spores of type E was investigated. The addition of an undiluted filtrate to cultures containing 10^6 to 10^7 vegetative cells cleared the cultures within 2 to 6 hr, indicating that lysis occurred. Lysis was confirmed by microscopic examination of the sedimented culture. Subsequent subculturing indicated a complete loss of viability.

TABLE 4. *Lysis of vegetative cells by S5 filtrate*

Type E strain	Challenge inoculum	Survivors in the following dilution of S5 filtrate in TPG		
		1:40	1:80	1:160
Beluga	10^7	0	0	10^4
VH	10^7	0	10	10^4
Minnesota	10^6	0	10	10^2
070	10^7	10	10	10^3
Iwanai	10^7	10	10	10^5
Alaska	10^7	10	10^2	10^5
1304	10^7	0	10	10^5
Kalamazoo	10^7	10^2	10^3	10^5
8E	10^7	10	10^2	10^7
D8	10^7	10	10^7	10^7
066B	10^7	10^7	10^7	10^7

A quantitative evaluation of the lytic activity of the S5 filtrate against vegetative cells of type E strains is presented in Table 4. There were no survivors in cultures containing high concentrations of the filtrate (1:10 and 1:20 dilutions) and inoculated with 10^7 vegetative cells. As the concentration of the filtrate decreased, the number of survivors increased. An exception was strain 066B; this strain is less susceptible to lysis by the agent than the other strains tested. Subsequent subculturing of cultures exposed to dilutions preventing outgrowth for 16 to 20 hr indicated a complete loss of viability of the vegetative cells. Extended incubation did not reveal additional outgrowth.

When growth occurred in lower concentrations of the filtrate, the outgrowth time was markedly increased, suggesting a bacteriostatic effect. Resistant variants originating from vegetative cells of strains Beluga and Iwanai were obtained. They were resistant to the lytic activity of the undiluted filtrate, whereas the parent strain was sensitive to a 1:80 dilution of the agent. No reversion to sensitivity has been observed upon repeated subculturing in TPG in the absence of the antagonistic agent.

Similar results were obtained with filtrates of 28-2 and GB-3.

The growth of spores was inhibited by active filtrates (Table 5) in about 10 times the dilution that caused lysis of vegetative cells. The activity against spores was bacteriostatic. When spores which had been held in high concentrations of the active substance for more than 1 week were subcultured, their ability to germinate and grow in the normal length of time was not affected, nor was there a demonstrable loss in the

TABLE 5. *Bacteriostatic titers of filtrates of antagonistic strains against 100 type E spores*

Strain	Filtrate		
	S5	28-2	GB-3
Beluga	2,560[a]	1,280	5,120
VH	5,120	5,120	5,120
Minnesota	5,120	2,560	5,120
070	5,120	1,280	10,240
Iwanai	5,120	5,120	10,240
Alaska	5,120	5,120	5,120
1304	5,120	5,120	5,120
8E	5,120	5,120	5,120
Salmon A	5,120	5,120	10,240
Kalamazoo	5,120	5,120	5,120
066B	80	80	160
D8	5,120	5,120	5,120

[a] Maximal dilution of filtrate in TPG inhibiting outgrowth for more than 48 hr after control exhibited good growth.

number of viable spores. Microscopic examination showed that they retained their refractility when held under these conditions.

The level of stasis remained the same after incubation for 1 week, and was only slightly lower when tested against 1,000 times as many spores.

As with vegetative cells, the spores of strain 066B were less sensitive to the activity of the filtrates than those of the other strains tested. Resistant variants were obtained from spores of Beluga and Iwanai. These variants did not revert to the sensitive type after serially subculturing in TPG without the active substance.

Tests of the activity of S5 filtrates against spores and vegetative cells of the 10 other clostridia showed S5 activity to be limited to type E and only two of the other species tested. The antagonistic agent was not active against either spores or vegetative cells of *C. botulinum* types A, B, or F. However, it was active against *C. perfringens* and *C. acetobutylicum*, although to a much lower degree.

The physical and chemical properties of the active substance are still being studied, but results obtained so far indicate that it is heat-stable. Heating for 1 hr at 100 C does not reduce its potency. Moreover, a heated filtrate will retain the same level of potency for at least 7 days at 25 C, whereas an unheated filtrate loses practically all of its potency in 72 hr under the same conditions. If unheated filtrates are frozen at −70 C, they retain their activity for at least 6 months. Loss of activity at 25 C is consistent with finding the greatest concentration in cultures in their logarithmic-growth phase and the decrease in potency with the age of the culture.

Other results indicate that the substance is dialyzable, is not inactivated by chloroform, and is digested by trypsin. It can be precipitated by ethyl alcohol, acetone, and trichloroacetic acid. The precipitates obtained are readily soluble in physiological saline, but the activity of those prepared with trichloroacetic acid is much lower than the original material, whereas alcohol and acetone precipitates are fully active.

DISCUSSION

The presence of numerous anaerobes resembling *C. botulinum* type E, or possible nontoxic variants of it, in muds, bottom sediments, fish viscera, and other materials in which type E was suspected of being present has been amply pointed out by other workers (6, 11, 18; Cabelli, *personal communication*). It has also been fairly evident that, although some anaerobes might aid in the detection of toxicity through the production of proteolytic enzymes similar in their action to trypsin (20), others have complicated the isolation of type E by masking its presence.

The production of an antagonistic substance which is bacteriolytic for vegetative cells and bacteriostatic for spores of *C. botulinum* type E by organisms resembling type E is reported here. Of the species of *Clostridium* against which it has been tested, its spectrum of activity is limited to *C. botulinum* type E and, to a lesser extent, to *C. perfringens* and *C. acetobutylicum*. The properties and activity of the substance, for which the name "boticin E" is proposed, fall within the rather loosely defined characteristics of bacteriocins, as reviewed by Reeves (19).

Although it is tempting to speculate whether those organisms resembling type E are nontoxic variants or merely part of a group of clostridia possessing similar characteristics, no conclusion of this sort can be drawn until further information is available. Antigenic studies of the nontoxic organisms and *C. botulinum* type E are being made in an effort to determine possible relationships among these organisms. It is conceivable that organisms which once had the ability to produce toxin have, through genetic alteration in their metabolic processes, lost this ability, and produce, instead, a substance toxic for other strains of the same species. However, no substantial evidence is available to date to support this possibility.

The presence of these organisms in certain specimens known to contain type E have rendered the detection of toxin and the isolation

of the organism extremely difficult if not, indeed, impossible. This is so for two reasons: (i) the organisms resembling type E may inhibit the growth of *C. botulinum* type E, and therefore the production of toxin in laboratory media; and (ii) plating to select single colonies is complicated by the indistinguishable appearance of these organisms which are often numerically dominant.

The number of additional strains producing a highly potent "boticin E" was much lower than expected when repeated attempts were made to isolate them from fish viscera and bottom sediments collected in the general areas from which the original strains were obtained. However, numerous other organisms resembling type E and producing somewhat similar antagonistic substances were found. They differed, however, in the lower potency of filtrates, the production of the active substance later in the growth cycle (about 48 hr), and their heat lability. It is possible that a number of bacteriocins may be produced by this group of organisms or that variants may occur which give rise to substances similar in effect but differing in properties, as with *E. coli* V (5). Further work will be necessary to determine the nature of the activity against type E, the specificity, and the physical and chemical characteristics of this group of bacteriocin-like substances.

Work is in progress to concentrate and purify the "bioticin E" produced by strain S5, and further study of the physical and chemical properties of the purified substance is anticipated.

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