Compound Inhibitory to Clostridium botulinum Type E
Produced by a Moraxella Species

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A Moraxella strain, A-43, produced a compound inhibitory to the outgrowth of Clostridium botulinum type E spores. The inhibitor could be produced in various laboratory media, and the outgrowth of germinated spores was inhibited by a 1/6th dilution of the A-43 spent medium. Germination was not affected. Molecular weight of the inhibitor was estimated at 800 to 1,000. The inhibitor was dialyzable and could be concentrated by lyophilization. It was stable at 37, 25, and 5°C, but was 70% inactivated when heated at 65°C for 10 min. The inhibitor was not volatile and could not be vacuum-distilled at 40°C. Solutions of acids with pH values below 2.0 destroyed the activity. The A-43 inhibitor appears to be similar, in molecular weight and inhibition characteristics, to tylosin.

Effects of aerobic microbial growth on Clostridium botulinum have been variously described as stimulatory or inhibitory. Salmonella typhimurium and C. perfringens (D. T. Munsey, B. Boucher, and G. J. Silverman, Abstr. Annu. Meet. Amer. Soc. Microbiol., p. 19, 1973) and some lactic acid bacteria (2) enhanced the growth of C. botulinum by lowering the redox potential and supplying growth factors.

Lactic acid bacteria in fermented foods inhibited C. botulinum (19). Free fatty acids produced by Brevibacterium linens (8), nisin produced by Streptococcus lactis (7), and biotin in produced by type E-like C. botulinum (6) were inhibitory to C. botulinum.

This report describes an antibiotic-like substance, produced by a Moraxella species, that inhibited the out-growth of C. botulinum type E.

MATERIALS AND METHODS

Microorganisms. C. botulinum type E Beluga strain was selected as the test microorganism for its high spore yield. The culture was obtained from Keith Ito of the National Canners Association, Berkeley, Calif. The spores were germinated by heat shock at 65°C for 10 min and grown anaerobiically at 30°C.

The organism is a nonmotile gram-negative coccobacillus. It possesses a cytochrome oxidase, is resistant to 3.0 IU of penicillin G, and does not ferment carbohydrates. It could be classified as either Moraxella or Acinetobacter species, depending on the classification scheme used (1, 23).

C. botulinum type E spores. The biphasic culture system of Bruch et al. (4) was used for the vegetative growth and sporulation of C. botulinum type E. It consisted of a layer of TPG agar of Schmidt et al. (20), overlaid with distilled water containing a 1% suspension of a 48-h culture of C. botulinum cells. The maximal sporulation of approximately 90% of the vegetative cells was accomplished after 48 h at 30°C.

The spores were collected by a combination of the methods of Hitzman et al. (10) and Bruch et al. (4). The overlying liquid was centrifuged, and the sediments containing spores, sporangia, and vegetative cells were washed five times with sterile, distilled water. After the final wash, the sediment was suspended in distilled water and refrigerated at 5°C for 3 days to allow autolysis of the vegetative cells. After this period, the cell debris had collected at the bottom while the spores remained in suspension. The supernatant was then siphoned off, and the spores were collected by centrifugation at 44 × g and washed 10 times with sterile distilled water. Viable spore counts were made by plating the heat-shocked spores on TPG agar. The spore concentration was adjusted to 2.3 × 10⁸ spores per ml, which, when 0.15 ml was added to 9.85 ml of medium in a screw-capped tube (Kimax 71350-4; 16 by 125 mm), would give an optical density reading at 630 nm of approximately 0.35 on a Bausch and Lomb Spectronic 20.

Inhibitor production. Moraxella species were cultured in tryptone-peptone-extract medium (TPE; 5), reinforced clostridia medium (RCM; 9) minus agar and sodium thioglycolate, and Perigo medium (PM 17) minus sodium thioglycolate and bromocresol purple. Since PM supported the maximal production of inhibitory substance by Moraxella A-43 and was equally suitable for C. botulinum type E (with 0.1% sodium thioglycolate), most inhibition studies were conducted with this medium.

Moraxella A-43 was grown in PM for 48 h at 25°C in a New Brunswick G 24 Environmental Incubator-Shaker, and the spent medium (SM) was collected by centrifugation. The pH of the SM was adjusted to 7.0 and sterilized by filtration through a membrane filter.
Growth study of A-43 in different media was conducted in Belco side-arm flasks, and the optical density of the cultures was followed spectrophotometrically at 600 nm.

**Inhibition study.** Inhibition study was carried out in PM minus bromocresol purple indicator. Unless otherwise stated, all inhibition studies were conducted with a 1:1 dilution of SM to compensate for any depletion of nutrient by *Moraxella* species. Accordingly, control study was conducted with 1/2 strength of PM.

The pH of SM was adjusted to 7.0 with 0.5 M NaOH, and 1.0 ml of this was pipetted into a screw-capped tube containing 8.85 ml of fresh PM. The 0.15 ml of distilled water suspension of *C. botulinum* type E spores was inoculated into this medium immediately after heat shock, and the germination and outgrowth of spores was followed spectrophotometrically at 630 nm during 30 C incubation. Some cultures in which inhibition was apparent were examined with a microscope after staining with 1% violet for 30 s (10).

**Inhibitor characterization:** (i) **Dialysis and lyophilization.** The A-43-SM was dialyzed (Scientific Product catalog no. D 1615-1, regenerated cellulose, average pore radius 2.4 nm) overnight against running tap water at 2 C, and the inhibitory activity of the nondialyzable fraction was measured. Also, the dialysate collected in a given volume of water after 7 days at 2 C was concentrated by lyophilization and tested for inhibitory activity.

(ii) **Vacuum distillation.** The lyophilized dialysate, which retained the inhibitory activity, was dissolved in distilled water and vacuum-distilled at 40 C. The distillate and the residue were then examined for inhibitory activities against *C. botulinum* type E spores.

(iii) **Ultrafiltration.** The A-43-SM was passed through Millipore-Pellican ultrafiltration membranes (PSED and PSAC) with the respective molecular weight exclusion limits of 25,000 and 1,000. The filtrates and the residue were tested for the inhibitory activity.

(iv) **Stability testing.** The heat stability of the inhibitory compound produced by *Moraxella* A-43 was tested after autoclaving at 121 C for 15 min, heating at 65 and 85 C for 10 min, and storing at 37 and 25 C for 12 days. The inhibitor stored at 5 C was also examined periodically for 2 months.

The effect of acid was measured by treating SM with 0.5 M HCl to give final pH values of 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0, and left for approximately 1 h at 25 C. The pH of the treated SM, however, was readjusted to 7.0 with 0.5 M NaOH prior to testing for the inhibitory activity.

(v) **Characterization.** The dialysate concentrated by lyophilization was fractionated by two different columns, Bio-Gel P-2 and Bio-Rex 70X. The Bio-Gel P-2 column is essentially a molecular sieve, and the Bio-Rex 70X column separates peptides or compounds with free amino groups according to the ionic strength.

Bio-Gel P-2 resin (Bio-Rad Laboratories, 200 to 400 mesh) was packed in a column (2.5 by 80 cm) to a height of 50 cm. The sample was eluted by gravity with distilled water at room temperature at a rate of approximately 30 ml/h, and the eluate collected in 10-ml fractions. The collected fractions were pooled according to reactions with a 0.5 M AgNO₃ solution, and the pooled fractions were tested for the inhibitory activity.

The Bio-Rex 70X ion exchange resin (Bio-Rad Laboratories, 200 mesh) was prepared by the methods of Stevens and Bergstrom (22). It was packed to a height of 80 cm in a column (2.5 by 120 cm). The sample was eluted by gravity at 2 C with a 0.2 M potassium phosphate buffer (pH 6.2). The rate of elution was 30 ml/h. The presence of peptides in the eluate, collected in 10-ml fractions, was detected by the absorbance at 280 nm. The different fractions were pooled according to their reactions with ninhydrin and tested for the inhibitory activity.

Fractions obtained from Bio-Rex 70X column were spotted on filter paper and tested for the presence of peptides and lipids with ninhydrin (3) and Rhodamine 6G (16) prepared by the methods described by Johnston (12).

**RESULTS**

**Inhibitor production.** Among several strains of *Moraxella* tested, only strain A-43 produced inhibitor effective at a 1/20 dilution. Inhibitory effects of other *Moraxella* SM were lost when diluted 1/100 with fresh medium.

The germination and out-growth of heat-shocked spores of *C. botulinum* type E, Beluga strain, in the presence of a 1/20 dilution of three different A-43-SM is shown in Fig. 1. Germination, measured by the loss of refractibility at 630 nm, was not affected by the presence of A-43-SM, but the outgrowth in SMs of PM and RCM was completely inhibited. Inhibition by TPE-SM was partial. This was later determined as due to slow growth of A-43 in TPE and correspondingly lower concentration of the inhibitor produced in this medium.

The inhibitor production in PM during the growth of A-43 is illustrated in Fig. 2. After 6 h or at the early log phase, the presence of the inhibitor was already detectable. The inhibitor concentration steadily increased until the culture reached the stationary phase at 34 h and the maximum was obtained at 48 h. In contrast, the maximal concentration of the inhibitor produced by *Moraxella* A-44 after 48 h was still less than that produced by A-43 in 6 h (Fig. 2).

The inhibition was always on the outgrowth of germinated spores or during the vegetative growth of *C. botulinum* type E. Other strains of *C. botulinum* type E, Minnesota and VH, were similarly affected by the inhibitor.

**Partial characterization of inhibitor.** Some
of the physicochemical characteristics of the inhibitor tested are as follows: dialyzable; estimated molecular weight of 800 to 1,000; non-volatile; stable at 5, 25, and 37 C; 70% inactivated at 65 C, 10 min; inactivated by strong acid (pH \( \leq 2.0 \)); active fraction contained peptide(s), phosphatidic amino acid(s), and lipid(s).

The inhibitor activity was recoverable from the dialysate by lyophilization, and no activity remained in the nondialyzable fraction. When reconstituted in distilled water, the inhibitor readily passed through a membrane filter of 1,000 molecular weight exclusion limits (Millipore PSAC) but remained with the residue when vacuum distilled at 40 C.

Acidification sufficient to produce pH of 2.0 or below destroyed the activity. Ten percent trichloroacetic acid not only failed to precipitate the inhibitor, but the acidity was sufficient to inactivate the activity of the solution.

The inhibitor was stable at 37 and 25 C for at least 12 days, and no loss of activity was observed after 2 months of storage at 5 C. Mild heat, however, destroyed activity. Heating at 65 C for 10 min resulted in 70% loss of inhibitory activity, and heating at 85 C for 10 min resulted in a complete loss.

The estimated molecular weight of the inhibitor was further confirmed to be 800 to 1,000 by the elution time of the active fraction from Bio-Gel P-2 column. The active fraction formed a purple precipitate with 0.5 M AgNO\(_3\) and a deep purple color with ninhydrin solution. The active fraction also showed ultraviolet light absorption at 280 nm.

The inhibitor did not separate cleanly on the Bio-Rex 70X column, and the activity was recovered from several fractions. The fractions that showed inhibitory activity on C. botulinum type E, however, stained purple with ninhydrin and yellow with a purple hue with Rhodamine 6G.

**DISCUSSION**

The physicochemical reactions indicate that the inhibitor produced by *Moraxella* A-43 appears to be a peptide(s) containing phosphatidic amino acids and maybe lipid. The estimated molecular weight of 800 to 1,000, the mode of action on *C. botulinum* type E spores, and the sensitivity to acid were all similar to those
reported for tylosin (15). The inhibitor appeared to be more sensitive to heat than tylosin (21).

C. botulinum type E spores germinated in the presence of the inhibitor appeared to be phase opaque with no sign of elongation or lysis. This was similar to the action of nisin on C. butyricum reported by Ramseier (18). The lack of immediate lysis may be explained in that antibiotics having surfactant properties such as tyrocidin do not cause immediate cell lysis, and the delayed lysis that follows could not be distinguished from autolysis (11).

It is not known to what extent the antibiotic-like substances, such as that produced by Moraxella A-43, play a role in competition with C. botulinum in nature. It is interesting to note, however, that Moraxella species which are common in many seafoods could not be recovered in some smoked fish (14).

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