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Proteolytic Mutants Obtained from *Clostridium botulinum* Type E

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Proteolytic mutants were isolated from toxigenic strains of *Clostridium botulinum* type E after several transfers. When these cultures were plated on blood agar, almost all of the colonies obtained were proteolytic, and there were fewer toxigenic colonies than nontoxigenic colonies. The proteolytic mutants and nonproteolytic original strains were different in their biological properties.

There are both proteolytic and nonproteolytic strains among *Clostridium botulinum* types B and F. It has also been suggested that all toxigenic type E strains are nonproteolytic and that they are similar to nonproteolytic strains of types B and F in their biochemical properties (6, 8, 9). However, Dolman (2) and Dolman and Murakami (3) reported that a transparent and proteolytic mutant, which lost its toxigenicity, arose from toxigenic strains of *C. botulinum* type E. We also isolated proteolytic mutants of *C. botulinum* type E that produced toxin. In this communication, we report some properties of the proteolytic mutants thus obtained.

C. botulinum type E strains used are as follows: toxigenic strains Abashiri, Yakumo, Kamiyama, Tankawa, and Iwanai and nontoxigenic strain Morai. For cultivation, cooked meat medium (BBL) was used.

The meat showed definite proteolysis in strains Abashiri TOX (toxigenic), Yakumo TOX, and Kamiyama TOX after incubation at 33°C for 3 days in cooked meat medium. These cultures were streaked on blood agar plates and incubated anaerobically at 33°C for 48 h. Twenty colonies from each plate were inoculated onto cooked meat medium. The tubes were incubated at 33°C for 7 days, and, after incubating the culture supernatant fluids with 1.0% trypsin (1:250, Difco) in 0.1 M phosphate buffer (pH 6.0) at 37°C for 1 h, their toxigenicity was tested by intraperitoneal injection into mice. At the same time, protease activity was detected by gelatin digestion (5), and amidase activity on *N*-benzoyl-DL-arginine-*p*-nitroanilide (BAPA) was assayed by a method described for the trypsin assay (4). The reaction mixture contained 1.25 ml of 1 mM BAPA in 0.05 M phosphate buffer (pH 6.0), 0.15 ml of 0.1 M 2-mercaptoethanol, and 0.10 ml of the clarified culture supernatant. The reaction was carried out at 35°C for 30 min and then was stopped by the addition of 0.25 ml of 30% acetic acid. Liberated *p*-nitroaniline was determined spectrophotometrically ($E_{410\,nm}$ = 8,800). Hydrolysis of 1 μmol of BAPA per min was taken as 1 U of enzyme activity.

As shown in Table 1, the numbers of toxigenic colonies obtained were less than those of nontoxigenic colonies in all three strains. On the other hand, almost all of the colonies selected were proteolytic, except that one nontoxigenic colony was isolated from strain Abashiri TOX. Proteolytic and toxigenic colonies demonstrated toxicities of 20 minimal lethal doses/ml before trypsin treatment and at least 2,000 minimal lethal doses/ml after trypsinization. Amidase activity of the proteolytic mutants was variable (0 to 0.066 U/ml). However, no difference in amidase activity was observed between toxigenic and nontoxigenic colonies.

TABLE 1. *Toxigenicity and proteolysis of colonies obtained from toxigenic strains of C. botulinum type E* [a]

Strain	Toxigenicity	No. of colonies	
		Proteolytic	Nonproteolytic
E-Abashiri TOX	Toxigenic	6	1
	Nontoxigenic	13	0
E-Yakumo TOX	Toxigenic	4	0
	Nontoxigenic	16	0
E-Kamiyama TOX	Toxigenic	8	0
	Nontoxigenic	12	0

[a] Proteolysis was detected by the gelatin-digestion method and amidase activity was assayed by a method for trypsin assay with BAPA as a substrate.

The carbohydrate fermentation of proteolytic mutants and nonproteolytic original strains was investigated. These results are summa-

TABLE 2. *Biochemical properties of the proteolytic mutants obtained from toxigenic strains of C. botulinum type E*[a]

Strain and colony no.	Toxigenicity	Amidase activity (U/ml)	Carbohydrate fermentation reaction				
			Glucose	Mannose	Sucrose	Glycerol	Salicin
Abashiri							
no. 4	+	0.047	AG[b]	AG	AG	AG	AG
no. 7	−	0.006	AG	−[c]	AG	AG	AG
no. 15	−	0	AG	−	AG	AG	AG
no. 16	−	0.066	AG	−	AG	AG	AG
Yakumo							
no. 11	+	0.021	AG	AG	AG	AG	AG
no. 15	+	0	AG	AG	AG	AG	AG
no. 8	−	0.040	AG	−	AG	AG	AG
no. 12	−	0.005	AG	−	AG	AG	AG
Kamiyama							
no. 1	+	0.028	AG	−	−	AG	AG
no. 11	+	0.033	AG	AG	AG	AG	AG
no. 6	−	0.047	AG	−	−	AG	AG
no. 16	−	0.024	AG	−	AG	AG	AG

[a] Carbohydrate fermentation was determined as follows: peptone water (1% proteose peptone [Difco], 0.5% NaCl, 0.1% sodium thioglycolate, pH 7.0) was used as the nutrient base. The basal medium was sterilized in 3.5-ml quantities in 10- by 105-mm test tubes containing Durham fermentation tubes. The various sugars, which were sterilized by filtration through membrane filters (pore size, 450 nm; Millipore), were added to give final concentrations of 1.0%. The cells from a logarithmic-phase culture in 20 ml of LYG medium (1% lactalbumin, 2% yeast extract, 0.5% glucose, 0.15% *l*-cysteine-HCl, pH 7.2) were collected by centrifugation and suspended in 5 ml of the nutrient base. A 0.2-ml portion of the cell suspensions was inoculated into each tube and incubated at 33°C for 6 days. Each culture was examined for gas production and acid formation by the addition of methyl red. All colonies tested in this table showed negative reaction with arabinose. Gelatin digestion for the detection of proteolysis was positive in all mutants described.

[b] Acid and gas.

[c] No acid or gas.

TABLE 3. *Carbohydrate fermentation reactions of nonproteolytic strains of C. botulinum type E*[a]

Strain	Carbohydrate fermentation reaction					
	Glucose	Mannose	Sucrose	Glycerol	Salicin	Arabinose
Abashiri colony no. 17 NT	AG[b]	AG	AG	A[c]	A	A
Tankawa TOX	AG	AG	AG	A	A	−[d]
Iwanai TOX	AG	AG	AG	A	A	−
Morai NT	AG	AG	AG	A	AG	A

[a] A detailed method is described in Table 2.

[b] Acid and gas.

[c] Acid only.

[d] No acid or gas.

rized in Tables 2 and 3. All proteolytic mutants tested produced gas and acid with glycerol and salicin. Four of the toxigenic colonies examined also produced gas and acid with mannose, though none of the nontoxigenic colonies fermented mannose (Table 2). Fermenting ability was not demonstrated with arabinose, whereas gas and acid production was observed with mannose and sucrose in nonproteolytic strains, and only acid formation was observed with glycerol and salicin (Table 3). Acid was formed with arabinose in a nonproteolytic colony of Abashiri TOX and strain Morai NT (nontoxigenic). Many additional carbohydrates were tested. All the strains showed positive reactions with fructose, maltose, sorbitol, and trehalose and negative reactions with galactose, inositol, mannitol, xylose, lactose, raffinose, and amygdalin.

From the results presented above, a striking difference between toxigenic and nontoxigenic proteolytic mutants is the failure of the latter to ferment mannose. It is equally surprising that the proteolytic mutants and nonproteolytic original strains differ regarding gas production. The mutants produce gas with glycerol and salicin, whereas the wild type do not. Toxigenicity seems to be lost after acquiring the

proteolytic property (Table 1). We are now attempting to elucidate the participation of protease produced by these proteolytic mutants in the activation of botulinum toxins in *C. botulinum* types A, B, and F (1, 7).

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