Correlation of Toxic and Non-toxic Strains of *Clostridium botulinum* by DNA Composition and Homology

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SUMMARY

Various toxic and non-toxic *Clostridium botulinum* strains can be grouped according to DNA binding homology but not according to DNA composition. The guanine + cytosine (GC) contents of DNA from six strains were 28 to 30 %. DNA binding with radioactive DNA from *C. botulinum* type E, MINN strain, revealed that the non-proteolytic strains type B, 17, type F, 202 and type E, DETROIT are 100 % homologous to the E, MINN strain. *Clostridium botulinum* type E, 32, the boticin producing S 5, and the non-toxic 24 NT, 26 NT and 5 i had 58 to 78 % homology with the MINN strain. *Clostridium botulinum* type A, 62 and 78, type B, 32, type C, 573, and non-toxic OS/E 1, OS/E 7 and OS/MINN strains had only 6 to 14 % homology with type E, MINN. Competition experiments with unlabelled DNA confirmed these results. The studies indicate that strains which are closely related serologically and biochemically are also closely related genetically, and *vice versa*. The data do not support the idea of a common origin of the non-toxic OS strains and *C. botulinum* type E. The toxic type E strains and the toxic type A, B and C strains of *C. botulinum* are genetically heterogenous.

INTRODUCTION

The species *Clostridium botulinum* includes heterogenous strains that produce toxins types A to F. Based on physiological and serological characteristics, the species can be divided into the proteolytic A group, the non-proteolytic E group, and the C group (Smith & Holdeman, 1968, see Table 2). In recent years, non-proteolytic *C. botulinum* types B and F with characteristics of the type E group have been reported (Eklund & Poysky, 1967). Many non-toxic strains, such as 24 NT and 26 NT isolated from the North Sea (Cann *et al.* 1965) and the boticin producing S 5 and 28-2 isolated from the Great Lakes (Kautter, Harmon, Lynt & Lilly, 1966), were reported to have identical characteristics with type E. Non-toxic OS (opaque sporulating) strains were closely associated with type E cultures (Dolman, 1957). The OS strains have different fermentation, serological reactions and spore structure than the type E strains (Hobbs, Roberts & Walker, 1965; Hodgkiss, Ordal & Cann, 1966; Walker & Batty, 1967).

Non-toxic *Clostridium botulinum* strains are difficult to classify because this species is solely defined by toxin formation. Thus all non-toxic *C. botulinum* strains are named by numbers and numerals like 24 NT or 5 i. The characteristics of the strains tested are summarized in Table 1.

The purpose of this study is to examine the genetic relationship of various toxic and non-toxic *Clostridium botulinum* strains with the type E strains. The difference in DNA

base composition can be used as a measure of genetic relatedness (Marmur & Doty, 1962; Schildkraut, Marmur & Doty, 1962; Hill, 1966). McCarthy & Bolton (1963) measured the genetic relatedness of organisms by the binding of complementary DNA base sequences *in vitro*. The DNA composition and the degree of common DNA base sequence of 22 toxic and non-toxic *C. botulinum* strains were measured in this study.

Table 1. *General characteristics of Clostridium botulinum groups A, C and E*
(Smith & Holdeman, 1968; Roberts & Hobbs, 1968)

Group	Serological reaction of vegetative cells	Proteolysis	Gas formation in carbohydrate fermentation	Minimum growth temperature	Resistance of spores to heat
A	A, B and F	Strong	Weak	> 10° (type F to 4°)	> 100° 10 min.
C	C and D	Weak	Weak	> 10°	~ 90° 10 min.
E	E and non-proteolytic B and F	Weak	Strong	3°	~ 60° 30 min.
(OS)*	OS	Weak	Weak	5°	~ 90° 10 min.

* (Ajmal & Hobbs, 1967).

METHODS

Cultures. Toxic cultures *Clostridium botulinum* A, 62, A, 78, B, 32, C, 573, E, MINNESOTA (MINN) and E, DETROIT were obtained from K. Ito (National Canners Assoc., Berkeley, Calif.) and *C. botulinum* E (Wellcome # 3324/61) was obtained from A. Johannsen (Lund, Sweden). Non-proteolytic strains *C. botulinum* B, 17 and F, 202 were obtained from M. W. Eklund (Eklund & Poysky, 1967). *C. botulinum* F, LANGE LAND was obtained from V. Møller (State Serum Institute, Copenhagen, Denmark). *Clostridium sporogenes* 213 was obtained from L. S. McClung (Indiana U., Bloomington, Ind.). Non-toxic strains 24 NT, 26 NT were isolated by G. Hobbs (Torry Research Station, Aberdeen, Scotland). The boticin producing non-toxic strains s 5 and 28-2 were isolated by Kautter *et al.* (1966). Non-toxic strains OS/E 1 and OS/E 7 were obtained from C. E. Dolman (1957). Non-toxic OS/MINN and 5i were picked from the *C. botulinum* type E, MINN culture in our laboratory.

Medium and growth conditions. Unlabelled DNA from all strains was isolated from organisms grown in 1 to 2 l. of medium: proteose peptone 20 g., yeast extract 15 g. (Difco), soluble starch 1 g., mercaptoethanol 0.5 ml. K_2HPO_4 1.85 g./l., pH adjusted to 7.2 with NaOH. Mercaptoethanol and $FeSO_4$ generate a Eh of -275 mv in solution (Wood, 1966). Glucose 10 g./l. was sterilized separately. Organisms were grown at 37° for 7 to 24 hr under 25 in. vacuum in a Duravac plastic desiccator (Ace Glass Inc., Vineland, N.J.) which had been flushed with a gas mixture (N_2 88%, CO_2 10%, H_2 2%). *Clostridium botulinum* C, 573 grew only in the following medium: liver infusion 35 g., meat extract (Difco) 15 g., cysteine-HCl 1 g., and glucose (sterilized separately) 6.7 g./l. Radioactive DNA of *Clostridium botulinum* E, MINN was prepared from organisms grown in 1 l. of Edamin medium: Edamin S (Sheffield Chemicals, Norwich, N.J.) 40 g., soluble starch 1 g., mercaptoethanol 0.5 ml., Triton $\times$ 155 (Rohm & Hass, Philadelphia, Pa.) 0.4 g./l., pH adjusted to 7.4 with KOH. Glucose 10 g./l. was sterilized separately. The growth conditions were as described above.

After 4 hr of growth, 385 μC of thymidine- ^3H , which was placed in a floating small plastic cup, was mixed into the culture medium with a magnetic stirrer. The organisms were harvested 2 hr later.

DNA isolation. Lysis of *Clostridium botulinum* can be difficult to accomplish by standard methods but the following procedure was effective in recovering some DNA from all the strains tested. Acetone dried organisms were sonicated for about 6 min. in ethanol in the cold, dried and suspended in 10 to 20 ml. saline+EDTA buffer (NaCl 0.15 M, Na_2EDTA 0.1 M, pH 8.0). The suspension was treated with about 1 mg lysozyme/ml. at 40° for $\frac{1}{2}$ hr. Then about 1 mg. pronase (Calbiochem, Los Angeles, Cal.)/ml. and 1 % sodium lauryl sulphate (SLS) were added and incubated for 1 to 5 hr. The time of lysis of the various strains differed and lysis could occur at any stage of the treatment. The treatment was terminated when the solution became highly viscous. The released DNA was purified by Marmur's (1961) method for Tm and buoyant density analysis and by Miura's (1967) method for the binding and competition studies. *Clostridium perfringens* DNA was purchased from Worthington Biochem. Corp., Freehold, N.J.

Analytical methods. DNA was determined chemically by Burton's (1968) method and spectrophotometrically by increase in optical density upon heating (Hotchkiss, 1957). Molecular weight was measured by the method of Zimm & Crothers (1962) in a Couette viscometer. The GC content of DNA was determined in duplicate samples by the Tm method (Marmur & Doty, 1962) and checked by the buoyant density method with *Micrococcus lysodeikticus* DNA (Miles Lab., Erkhart, Ind.) as reference (Schildkraut *et al.* 1962). DNA binding was assayed by the method of Denhardt (1966). DNA (30 $\mu\text{g.}$) was applied to each 25 mm. Millipore HA filter and annealed with 2 $\mu\text{g.}$ DNA- ^3H (10,000 c.p.m.) at 66° for 12 hr. For competition experiments, 875 $\mu\text{g.}$ of *Clostridium botulinum* type E, MINN DNA was applied to a 90 mm. Millipore HA filter. Small 11 mm. circles were punched from the 90 mm. filter. Two 11 mm. filters were annealed with 250 $\mu\text{g.}$ of various unlabelled DNA and 1 $\mu\text{g.}$ DNA- ^3H (5,000 c.p.m.) in 0.3 or 0.5 ml. double strength citrate (SSC) at 66° for 12 hr. Thermal denaturation of duplex DNA formed on filters was measured by the radioactivity released from two 11 mm. filters during heating in 1 ml. SSC. The heated solution and 1 ml. rinsing SSC were combined and the radioactivity determined in 15 ml. scintillation solution: Toluene 2 l., Triton $\times 100$ (Rohm & Haas, Philadelphia, Pa.) 1 l., Spectrofluor (Nuclear-Chicago, Des Plaines, Ill.) 100 ml. (Peterson and Greene, 1965). The radioactivity of dry filters were also counted in 15 ml. of the same scintillation solution.

RESULTS

DNA composition. The GC contents of 6 *Clostridium botulinum* strains are presented in Table 2. The thermal denaturation curve of *C. botulinum* E, MINN DNA is shown in Fig. 1. No satellite band was observed in the buoyant density photographs of DNA from *C. botulinum* E, MINN which produces type E toxin, or DNA from s 5, which produces boticin.

DNA molecular weight, binding and competition. The molecular weight of isolated DNA varied from strain to strain. The mol. wt of DNA from *Clostridium botulinum* type E, 3324/61, which was easy to isolate, was 1.1×10^7 . *Clostridium botulinum* type E, MINN DNA degraded during lysis. Heating the cell suspension to 70° for 15 min.,

addition of 0.1 M-EDTA and 1 % SLS did not prevent DNA breakdown. The mol. wt of the unlabelled type E, MINN DNA used in these experiments was 4.2×10^6 , and in one batch it was only 2.4×10^6 . Strains 5 i and *C. botulinum* F, 202 DNA degraded very rapidly. Lysis of these two strains was accomplished by adding 1 % SLS to unwashed cells in ice for 5 min. and then phenol was added to prevent DNA breakdown. The

Table 2. *The GC content of toxic and non-toxic Clostridium botulinum strains*

Group	Strain	Tm method	Buoyant density method
A	<i>C. botulinum</i> A, 62	28.5 %	—
A	<i>C. botulinum</i> B, 32	29.3	—
E	<i>C. botulinum</i> E, MINN	27.9*	28.8 %
E	s5 (boticin)	28	29.6
E	24 NT	29	—
E?	OS/E 7	30	—

* Average of 4 runs.

Table 3. *Percentage binding and competition of DNA from various strains relative to the Clostridium botulinum E MINN strain*

Group	Toxic strains	Source	Binding*	Competition†
E	<i>C. botulinum</i> E, MINN	Fish, Great Lakes	100 %	100 %
E	<i>C. botulinum</i> E, DETROIT	Fish, Great Lakes	100	99
E	<i>C. botulinum</i> E, 3324/61	Fish, Baltic Sea	68	100
A	<i>C. botulinum</i> A, 62	Cow, Nevada	11	0
A	<i>C. botulinum</i> A, 78	Spinach, U.S.	14	20
A	<i>C. botulinum</i> B, 32	Horse	11	3
E	<i>C. botulinum</i> B, 17	Pacific mud, U.S.	100	100
C	<i>C. botulinum</i> C, 573	Horse, France	11	—
E	<i>C. botulinum</i> F, 202	Pacific mud U.S.	100	100
A	<i>C. botulinum</i> F, LANGE LAND	Liver, Denmark	19	52
Non-toxic strains				
E	s5, Boticin	Fish, Lake Erie	78	80
E	28-2, Boticin	Mud, Lake Huron	43	89
E	24NT	Mud, North Sea	69	95
E	26NT	Mud, North Sea	58	65
E?	5 i	Toxic E MINN	73	—
E?	OS/E1	Toxic E culture	8	0
E?	OS/E7	Toxic E culture	6	0
E?	OS/MINN	Toxic E culture	9	0
—	<i>C. perfringens</i>	—	23	0
A?	<i>C. sporogenes</i> 213	U.S.	12	13
A?	P. A. 3679	U.S.	11	—
—	<i>Micrococcus lysodeikticus</i>	—	4	—

* Average of six replications. † 1 to 2 replications.

binding of DNA from various strains relative to type E, MINN DNA is presented in Table 3. From 1 to 250 μ g. of type E, MINN and s5 DNA was applied to each filter and maximum binding was observed at about 20 μ g. level. The DNA duplexes formed on filters were stable and had a similar thermal denaturation curve to the native DNA, but the Tm was lowered by 4° (Fig. 1). The average count and its standard deviation

for 16 replicates of type E, MINN DNA was 2341 ± 481 counts/min. The results of the competition experiments are presented in Table 3. In these experiments the binding was 1000 counts/min. without the competing DNA and the binding was reduced to 100 counts/min. with 250 μ g. of its own competing DNA. The competition experiment was limited to 1 or 2 replicates because of the limited supply of DNA.

Fig. 1. Thermal denaturation of *Clostridium botulinum* E, MINN native DNA (○) and radioactively labeled duplex annealed on filters at 66° (●).

DISCUSSION

The DNA composition of the examined *Clostridium botulinum* strains (Table 2) was similar to that of *C. perfringens* and *C. sporogenes* (Hill, 1966). Since the six strains tested have similar GC composition, the analysis has no taxonomic value with respect to *C. botulinum* strains.

Results of the DNA binding and competition tests were more revealing. They agreed with the characteristics of three groups of *Clostridium botulinum*. The toxic *C. botulinum*, E, DETROIT, E, 3324/61, B, 17 and F, 202 (Eklund, Poysky & Wieler, 1967) strains were reported to have the characteristics of the E group. These strains had 70 to 1000% DNA binding with the E, MINN strain and hence are closely related (Table 3). The non-toxic s5, 28-2, 24 NT and 26 NT were also reported to be identical to the toxic type E group (Kautter *et al.* 1966; Cann *et al.* 1965). These non-toxic strains have 40 to 80% DNA binding with E, MINN DNA and hence are also closely related to the toxic E group. Both 5i and OS/MINN were isolated from *C. botulinum* E, MINN culture. If E, MINN were the parent of 5i and OS/MINN strains, all three strains should have nearly all common DNA base sequences and nearly 100% DNA binding. The binding of 5i DNA to E, MINN was only 73% (Table 2). The DNA of 5i was always degraded during isolation and this might reduce its binding from the expected 100% to a value of 73%. The binding of OS/MINN to E, MINN DNA was only 8% (Table 3). This means that OS/MINN, like the unrelated control *Micrococcus lysodeikticus* (4%), have

very little common DNA base sequence with the E, MINN (Table 2). No mutations are known to change DNA base sequence so completely. Based on present knowledge, it seems improbable that OS/MINN could have originated by mutation from the E, MINN. The same reasoning applies to the other OS strains and their genetic relatedness to *Clostridium botulinum* type E. The other OS strains had only 8% homology (Table 3) with the E, MINN DNA and thus are not related to the E group. Although these OS strains have close association with the *C. botulinum* type E (Dolman, 1957), many differences in their fermentation serology and structure have been noted (Hobbs *et al.* 1965; Walker & Batty, 1967; Hodgkiss *et al.* 1966).

Many of the characters of toxic *Clostridium botulinum* groups A and C are different from the E group. The binding of *C. botulinum* types A, 62, A, 78, B, 32 and C, 573 DNA to E, MINN DNA was 11 to 14% and therefore we concluded that they are not closely related to the E group (Table 3). *Clostridium botulinum* type F, Langeland DNA appeared to have little more common DNA base sequence with E, MINN than the toxic type A, B and C strains tested. Generally the DNA homology among the groups of *C. botulinum* is low compared to the genera of Enterobacteriaceae. The DNA homology of the genera *Escherichia*, *Aerobacter*, *Salmonella* and *Shigella* were found to be 50 to 70% (McCarthy & Bolton, 1963). The species *C. botulinum* seems to include genetically heterogenous strains which are classified together only because they form toxins with similar pharmacological action.

The non-toxic *Clostridium sporogenes* and P.A. 3679 have some characteristics of the toxic *Clostridium botulinum* group A (Kindler, Mager & Grossowicz, 1956; Smith & Holdeman, 1968); *C. sporogenes* 213 and P.A. 3679 had only 10% DNA homology with E, MINN, and so are not related to the E group. We plan to study the DNA homology of *C. botulinum* type A, B and F strains and their related non-toxic strains like *C. sporogenes* and P.A. 3679. Eventually a systematic study of *Clostridium* DNA homology could lead to a better classification of this genus.

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