The Genetic Relatedness of Proteolytic *Clostridium botulinum* Strains

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SUMMARY

DNA binding and competition tests indicate that the species *Clostridium botulinum* is composed of three groups with only 4 to 18% DNA homology with each other. One group includes the proteolytic *C. botulinum* and *C. sporogenes* strains. The DNA isolated from seven proteolytic *C. botulinum* A, B, F, and six *C. sporogenes* strains was 50 to 100% homologous to the *C. botulinum* A62 DNA. *Clostridium botulinum* c and d strains form another group. *Clostridium botulinum* C8613 and D8625 strains have 38% homology with the *C. botulinum* C573 strain. The last group comprises the non-proteolytic *C. botulinum* B, E, and F strains reported previously. All the *C. botulinum* strains tested were related to only one of the three groups and no intermediate strain was detected.

INTRODUCTION

The relatedness of bacteria can be measured by phage infectivity, genetic transfer mechanisms and also by the degree of common DNA base sequence. Jones & Sneath (1970) reviewed all these criteria with regard to bacterial taxonomy. Until now *Clostridium botulinum* genetics has not been developed and the relatedness of various toxic and non-toxic *Clostridium* strains is open to question. Smith & Holdeman (1968) divided *C. botulinum* into three related groups, I, II, and III, on the basis of physiological and serological characteristics. Group I includes the proteolytic *C. botulinum* toxin types A, B and F strains. Group II includes the non-proteolytic *C. botulinum* toxin types B, E and F strains. Group III includes the *C. botulinum* toxin types c and d strains.

In our previous paper (Lee & Riemann, 1970) the relatedness of various toxic and non-toxic strains of the group II of *Clostridium botulinum* was studied. We found that the *C. botulinum* strains have very similar guanosine+cytosine (GC) content (28 to 29%) but they could be distinguished by DNA homology and competition. Kindler, Mager & Grossowicz (1956) stated that the nutritional and biochemical requirements of *C. sporogenes* were indistinguishable from the proteolytic *C. botulinum* strains. In this paper the relatedness of toxic *C. botulinum* and *C. sporogenes* was tested. We also tested the relatedness of three *C. botulinum* c and d strains and the relatedness between the three groups of *C. botulinum*.

METHODS

Cultures. The sources of many of the cultures have been described (Lee & Riemann, 1970). Additional strains of *Clostridium botulinum* A79, B213, B13983, and *C. sporogenes* PA3679 were received from K. Ito (National Canners Association, Berkeley, California). *Clostridium botulinum* C8613 was obtained from W. P. Segner (Continental Can Co., Chicago, Illinois) and *C. botulinum* D (NCTC8265) was supplied by the National Collection of Type Cultures (Central Public Health Laboratory, Colindale, London, N.W. 9). *Clostridium sporogenes* 175 was obtained from L. S. McClung (Indiana University, Bloomington, Indiana) and *C. sporogenes* SC4131 and SC4679 were obtained from L. D. S. Smith (Virginia Polytechnic Institute, Blacksburg, Virginia). *Clostridium sporogenes* 10-1026 was supplied by V. R. Dowell (Anaerobic Bacteriology Laboratory, National Communicable Disease Center, Atlanta, Georgia). All the cultures received were re-isolated by streaking on blood agar and all the toxic isolates typed with specific botulinum toxin antiserum before they were grown for DNA isolation.

Medium and growth. Cells for DNA isolation were grown in the proteose-peptone and yeast extract (Difco) medium under conditions described (Lee & Riemann, 1970). *Clostridium botulinum* C8613 and D8265 grew well in the above medium provided that the inoculum was grown in a fresh liver infusion (clear infusion from 500 g. liver boiled in 1000 ml. H₂O, pH 8.5 with NaOH plus 10 g. Tryptose (Difco), 1 g. soluble starch, 1 g. K₂HPO₄, 1.4 g. NaHCO₃ and 0.5 ml. mercaptoethanol). Radioactive DNA of *C. botulinum* A62 was prepared from cells grown in 450 ml. Edamin S medium without Triton X155 (Lee & Riemann, 1970) and with 1.75 mC of [³H]thymidine added. *Clostridium botulinum* C573 did not grow in Edamin S medium. The radioactive DNA of *C. botulinum* C573 was obtained from cells grown in 200 ml. of trypticase medium (50 g./l. trypticase (BBL), 1 g./l. soluble starch, 1.4 g./l. NaHCO₃, 0.25 ml./l. Triton X155, 1 mg./l. resazurin, 0.5 ml. mercaptoethanol, pH 7.2 with KOH). Growth was initiated by adding 3 ml. of inoculum grown in fresh liver infusion for 16 h. at 37°. After 3 h. of visible growth, 1 mCi of [³H]thymidine and 1 mCi of [³H]-adenosine were added, and the cells harvested 2 h. later. Strict anaerobic technique (Cato *et al.* 1969), such as the use of inert gas to exclude air and prereduced medium, was necessary for the good growth of the C and D strains.

DNA isolation. Lysis and DNA isolation from *Clostridium botulinum* C and D strains were difficult and variable. *Clostridium botulinum* D8265 was treated with 50 µg./ml. of penicillin G and D-cycloserine 2 h. before harvest, and the cells were lysed in ice cold tris-SDS buffer (0.1 M-tris (hydroxymethyl) aminomethane, 1 % Na dodecyl sulphate, (SDS) 0.1 M-NaCl, pH 9.0 with HCl). The DNA of *C. botulinum* C8613 was obtained by grinding phenol-treated cells with alumina in tris-SDS buffer. The radioactive DNA of the *C. botulinum* C573 was obtained from cells treated with 1 mg./ml. of Pronase and lysozyme (Calbiochem, Los Angeles, California) in saline-EDTA buffer (NaCl 0.15 M, Na₂ ethylenediaminetetra-acetate (Na₂ EDTA) 0.1 M, pH 9.0) for 30 min. at 37° followed by addition of 1 % SDS. The proteolytic *C. botulinum* and *C. sporogenes* strains always lysed by the above Pronase and lysozyme treatment and the DNA recovery was good.

The DNA was purified by the procedure of Miura (1967) including the isopropanol precipitation step. *Clostridium perfringens* DNA was purchased from Worthington

Biochemical Corporation, Freehold, New Jersey. *Escherichia coli* DNA was a gift of P. Packnaud.

Analytical methods. DNA assay, binding, competition, T_m and thermal denaturation procedures have been described (Lee & Riemann, 1970) but certain modifications were introduced. DNA binding and competition were assayed by a modified method of Denhardt's (1966). Thirty $\mu\text{g.}$ of DNA were applied to each 25 mm. Millipore HA filter. A length of 0.75 inch solid glass rod was inserted into the 1 ml. vial to reduce the liquid volume to 0.5 ml. and increase binding (Johnson & Ordal, 1968). In the binding experiment, 0.45 ml. of preincubation mixture (PM) (Denhardt, 1966) and 0.05 ml. of $0.1 \times \text{SSC}$ ($\text{NaCl } 0.15 \text{ M}$, sodium citrate 0.015 M , pH 7.2) containing the radioactive DNA were added to each vial. Two $\mu\text{g.}$ (8000 c.p.m.) of *Clostridium botulinum* A62 DNA, or 2 $\mu\text{g.}$ (8800 c.p.m.) of *C. botulinum* E MINN, or 0.3 $\mu\text{g.}$ (25,000 c.p.m.) of *C. botulinum* C573 were added to each vial. In the competition experiment, 0.5 ml. of PM was removed after preincubation, and 0.28 ml. $0.1 \times \text{SSC}$ containing 250 $\mu\text{g.}$ unlabelled, 1 $\mu\text{g.}$ homologous radioactive *C. botulinum* A62 DNA together with 0.12 ml. of $10 \times \text{SSC}$ was added to each vial. *Clostridium botulinum* C573 competition was carried out with 250 $\mu\text{g.}$ of unlabelled DNA and 0.3 $\mu\text{g.}$ of homologous radioactive C573 DNA. Seven replicates were used in the binding experiments but each competition experiment was made only once or twice due to a limited amount of DNA available. Incubation was carried out at 66° (T_m 81°) for 12 h.

RESULTS

DNA binding and competition. The optimum conditions for DNA duplex formation on filters were tested. Maximum binding (c.p.m.) or saturation of the filter was reached when 15 $\mu\text{g.}$ of *Clostridium botulinum* A62 or A78 DNA was applied to each filter. To be safe, 30 $\mu\text{g.}$ of DNA was used. DNA duplex formation was directly proportional to the amount of radioactive *C. botulinum* A62 DNA added to the solution up to 3 $\mu\text{g.}$ level. [^3H]thymidine uptake by *C. botulinum* A62 was very poor and was not enhanced by the addition of Triton X155 detergent. The best preparation contained only 4000 c.p.m./ $\mu\text{g.}$ of DNA. The DNA duplex of *C. botulinum* on filters was stable and showed a thermal denaturation curve similar to that of the native DNA. The T_m of the radioactive duplex on filters was 76.6° as compared to the T_m of 81° of the native DNA.

Results of percent DNA binding and competition of 28 strains relative to the *Clostridium botulinum* A62 DNA are presented in Table 1. The average of A62 homologous DNA binding was 4150 c.p.m., and the standard deviation of each set was 5 to 26 %. In competition experiments the binding was reduced from 3940 to 1130 c.p.m. with 250 $\mu\text{g.}$ of unlabelled homologous A62 DNA.

Maximum binding was achieved when 30 $\mu\text{g.}$ of *Clostridium botulinum* C573 or D8265 DNA was applied to each filter, but 60 $\mu\text{g.}$ of C8613 DNA was used. Very little (40 $\mu\text{g.}$) of radioactive *C. botulinum* C573 DNA was isolated because of extensive degradation of the DNA during lysis of the cells. The amount of DNA recovered was just adequate for the experiment presented in Table 2. The average count of seven replicates of C573 homologous DNA binding was 3633 ± 923 c.p.m. The binding was reduced from 5030 to 1060 c.p.m. with 250 $\mu\text{g.}$ of unlabelled homologous C573 DNA in competition experiments.

Optimum conditions for binding with *Clostridium botulinum* E MINN were tested previously (Lee & Riemann, 1970). The relatedness of the C and D strains with the E MINN strains is presented in Table 3.

Table 1. *Percent binding and competition of DNA of various strains relative to [³H]DNA of Clostridium botulinum A62*

Group	Toxic strains	Source	Binding (%)	Competition (%)
I	A62	Cow, Nevada, U.S.A.	100	100
I	A78	Spinach, U.S.A.	100	100
I	A79	Berlin, no. 95	74	—
I	B32	Horse	83	41
I	B213	Onions, U.S.A.	69	—
I	B13983	Asparagus, U.S.A.	85	—
II	B17	Pacific mud, U.S.A.	8	0
III	C573	Horse, France	5	0
III	C8613	Marine mud, Maryland, U.S.A.	12	0
III	D8265	South Africa	13	0
II	E DETROIT	Fish, Great Lakes, U.S.A.	9	0
II	E MINNESOTA	Fish, Great Lakes, U.S.A.	6	13
II	E3324/61	Fish, Baltic Sea	6	—
II	F202	Pacific mud, U.S.A.	15	0
I	F LANGE LAND	Liver, Denmark	100	100
Non-toxic strains				
I	PA 3679	U.S.A.	100	90
I	175	U.S.A.	100	51
I	213	U.S.A.	86	80
I	SC4131	Goat, U.S.A.	52	—
I	SC4679	Chicken, U.S.A.	60	—
I	10-1026	Luccheon meat, U.S.A.	48	—
II	S5	Mud, Great Lakes, U.S.A.	4	0
II	(E BOTICIN)			
II	28-2	Mud, Great Lakes, U.S.A.	5	—
II	(E BOTICIN)			
II	24NT	North Sea	10	—
II	26NT	North Sea	4	—
	OS/EI	Toxic E culture	5	—
	OS/MINN	Toxic E culture	7	—
	<i>C. perfringens</i>	—	18	0
	<i>E. coli</i>	—	12	—

Table 2. *Percent binding and competition of DNA from various strains relative to [³H]DNA of Clostridium botulinum C573 and their GC contents*

Group	Strains	GC (%)	Binding (%)	Competition (%)
III	C573	27.6	100	100
III	C8613	27.8	38	34
III	D8265	27.2	39	35
I	A62	28.5*	18	—
II	E MINN	27.9*	5	—
	<i>C. perfringens</i>	—	24	—

* Reported previously.

Table 3. Percent binding of *Clostridium botulinum* C and D strains relative to [³H]DNA of *Clostridium botulinum* E MINN

Group	Strains	Binding (%)
II	<i>C. botulinum</i> {	E MINN 100
III		C 573 11
III		C 8613 12
III		D 8265 11

DISCUSSION

The above results confirm the observations of Kindler *et al.* (1956) that *Clostridium sporogenes* and the proteolytic group I or *C. botulinum* are genetically related. Three of the *C. sporogenes* strains tested have 86 to 100 % common base sequence with the *C. botulinum* A62 strain and the results of competition experiments generally confirm the DNA binding data (Table 1). We will attempt to determine if there are measurable DNA binding error or differences between these highly related *C. sporogenes* and *C. botulinum* strains. Both group I and II (Lee & Riemann, 1970) of *C. botulinum* have 50 to 100 % DNA homology within the group.

The three strains of group III of *Clostridium botulinum* showed limited (38 %) homology with each other (Table 2). The cytosine plus guanine content of their DNA is very similar to the *C. botulinum* A62 and E MINN strains (Table 2). Many non-toxic variants of *C. botulinum* C and D strains have been reported (Smith & Holdeman 1968; Inoue & Iida, 1970) and most probably group III of *C. botulinum* also contains related toxic and non-toxic strains.

The relatedness between the groups, I, II, and III is very limited and all the strains tested were related to only one of the group (Tables 1 and 2) (Lee & Riemann, 1970). The DNA binding between groups was only 4 to 18 %. By comparison the binding of *Clostridium botulinum* A62 DNA to the completely unrelated *Escherichia coli* DNA was 12 % (Table 1) and that of the *C. botulinum* E MINN to *Micrococcus lysodeikticus* was 4 % (Lee & Riemann, 1970). Walker & Batty (1967) found serological cross-reactions within the three groups of *C. botulinum* but not between the groups. It is apparent from our studies and the study of others that the three groups of *C. botulinum* are not related to each other as a single species and they have little common DNA base sequence.

The ribosomal RNA (rRNA) base sequence is conserved in bacterial evolution and rRNA/DNA homology is used as a broader measurement of bacterial relatedness (Doi & Igarashi, 1965). In view of the heterogeneity we have observed, the relatedness of various *Clostridium* species may be best determined by rRNA/DNA binding tests. The relatedness of *Clostridium* and *Bacillus* species has not been tested. It is possible that *Clostridium* is an oxygen-sensitive mutant of *Bacillus*, analogous to the oxygen-sensitive hemin-deficient *Escherichia coli* mutant isolated by Beljanski & Beljanski (1957). However, the GC content of *C. botulinum* DNA is 28 % (Table 2), which is lower than the minimum *Bacillus* GC content of 32 % (Hill, 1966). This difference in DNA composition makes it difficult to relate *C. botulinum* with any *Bacillus* species.

The relatedness of *Clostridium sporogenes* and *C. botulinum* group I strains naturally raises the question of possible transfer of toxigenesis from the toxic to non-toxic strains. Inoue & Iida (1970) reported the conversion of a non-toxic strain to a toxic *C. botulinum*

c strain by filterable phage-like particles. Lytic phages of *C. sporogenes* but not of the proteolytic *C. botulinum* have been reported (Betz & Anderson, 1967).

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