Taxonomic Relationships among *Clostridium novyi* Types A and B, *Clostridium haemolyticum* and *Clostridium botulinum* Type C

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The present study was undertaken to examine the genetic relationships among the closely related species, *Clostridium novyi* types A and B, *C. haemolyticum* and *C. botulinum* type C. These species were tested for DNA–DNA homology and thermostability of DNA duplexes and sorted into three genetically related groups: I, *C. novyi* type A; II, *C. novyi* type B, *C. haemolyticum* and one *C. botulinum* type C strain (Stockholm); III, the remaining *C. botulinum* type C strains. A few biochemical criteria corresponding to the genetic differences were recommended to differentiate each group. These studies imply that *C. haemolyticum* might be considered as *C. novyi* type D and that there are two genetically different groups in *C. botulinum* type C.

INTRODUCTION

Clostridium novyi was divided into four types A, B, C and D on the basis of the production of eight different soluble antigens and to some extent on the disease that they produce in man and animals (Oakley & Warrack, 1959; Oakley *et al.*, 1947; Willis, 1964). Types A, B and D are pathogenic, and type C is generally recognized as non-pathogenic to laboratory animals. Although *C. novyi* type D is similar in cultural characteristics and the beta, eta and theta toxins are shared with *C. novyi* type B, it is also considered by some as a separate species, *C. haemolyticum* (Smith, 1975; Smith & Hobbs, 1974), because the pathogenesis is not related to the production of alpha toxin, which is the lethal toxin of virulent strains of *C. novyi* types A and B, and the disease produced by *C. novyi* type D is quite unlike that produced by *C. novyi* types A and B. *Clostridium novyi* type A is frequently involved in gas gangrene in man and animals, type B causes infectious necrotic hepatitis, a disease found frequently in sheep, and type D (*C. haemolyticum*) is the causal organism of bacillary haemoglobinuria in cattle (Smith, 1975).

Some strains of *C. botulinum* type C are culturally indistinguishable from strains of *C. novyi* type A (Holdeman & Brooks, 1970), except for toxin production. This close relationship is strengthened further by the fact that a non-toxigenic, cured culture of *C. botulinum* type C can be infected with specific phage and induced to produce alpha toxin of *C. novyi* type A (Eklund *et al.*, 1974), thus becoming totally indistinguishable from *C. novyi* type A.

The purpose of this study was to examine the genetic relationship among the closely related species, *C. botulinum* type C, *C. novyi* types A and B, and *C. haemolyticum* by DNA–DNA *in vitro* molecular hybridization.

METHODS

Bacterial strains. Bacterial strains used in this study were as follows: toxigenic *Clostridium novyi* type A strains 140 and 77101 (Nishida & Nakagawara, 1965; Taguchi & Ikezawa, 1975); non-toxigenic *C. novyi* type A strains 3-1116 and 4-1102 (Nishida & Nakagawara, 1964); *C. novyi* type B strains 990, GR 2B, GR 8B and GR 11B (Nakamura *et al.*, 1975*b*; Rutter & Collee, 1969); *C. haemolyticum* strains 4507, 6700, 6705 and Shinketsu (Nakamura *et al.*, 1975*b*); *C. botulinum* type C strains Stockholm (Eklund & Poysky, 1972; Inoue & Iida, 1970),

85-8 and 95-2 (Serikawa *et al.*, 1977), all of which produce both C_1 and C_2 toxins; and *C. botulinum* type C strains 85-14 and 98-1 (Nakamura *et al.*, 1978) that produce C_2 but not C_1 toxin.

Preparation of DNA. *Clostridium novyi* type A strains were grown at 37 °C in a medium containing (% w/v): proteose peptone no. 2 (Difco), 2.0; yeast extract (Difco), 0.5; glucose, 1.0; NaCl, 0.5; L-cysteine. HCl, 0.05 (PYG medium, pH 7.2). *Clostridium novyi* type B 990, GR 8B and GR 11B were grown in a medium containing (% w/v): trypticase (BBL), 3.0; yeast extract (Difco), 2.0; glucose, 0.5; $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 0.2; L-cysteine. HCl, 0.05 (TYG medium, pH 7.2). *Clostridium haemolyticum* 4507, 6700 and 6705, and *C. botulinum* type C 85-14 were grown in TYG medium with a concentration of 1.0% (w/v) yeast extract (Difco). *Clostridium botulinum* type C Stockholm, 85-8, 95-2 and 98-1 were grown in a medium containing proteose peptone no. 2 (Difco) 1.0% (w/v), yeast extract (Difco) 1.0% (w/v), glucose 0.5% (w/v), NaCl 0.5% (w/v), L-cysteine. HCl 0.05% (w/v) and liver infusion 15% (v/v) (PYGL medium, pH 7.2). Liver infusion was prepared as follows: 10 g liver extract (Kyokuto, Tokyo, Japan) was dissolved in 170 ml distilled water, kept at 50 °C for 60 min, and then boiled for 5 min. The solution was adjusted to pH 7.2 with NaOH and filtered through Toyo filter paper no. 2 (Toyo, Tokyo, Japan). The filtrate was used as liver infusion. Penicillin G (50 $\mu\text{g ml}^{-1}$) was added in the late-exponential phase of growth and cultures were incubated for a further 30 min at 37 °C. The organisms were then harvested by centrifugation, resuspended in 0.15 M-NaCl/0.01 M-EDTA solution at pH 8.0 and lysed with 3 to 5% (w/v) SDS. *Clostridium novyi* type B GR 2B and *C. haemolyticum* Shinketsu were grown at 37 °C for 24 h in TYG medium. The organisms were then harvested by centrifugation, suspended in NaCl/EDTA solution and lysed with 3% (w/v) SDS.

For the preparation of labelled DNA from *C. novyi* type A 140 and type B 990, *C. haemolyticum* 6700, and *C. botulinum* type C Stockholm and 85-8, [^3H]thymidine [10 μCi (370 kBq); Japan Isotope Association, Tokyo, Japan] was added to 100 ml of the medium used for the isolation of unlabelled DNA of each strain. The organisms were incubated, treated and lysed by the procedure used for the preparation of unlabelled DNA.

In all cases DNA was extracted from the lysate by the method of Marmur (1961).

Test for DNA-DNA homology and the thermostability of DNA duplexes. The percentage DNA-DNA homology and the $T_{m(e)}$ value (the temperature at which 50% of homologous or heterologous DNA duplexes were dissociated) was estimated as described previously (Nakamura *et al.*, 1975a).

Sugar fermentation tests. These were performed in pre-reduced anaerobically sterilized (PRAS) medium according to the method described by Holdeman & Moore (1975). Sugars tested were adonitol, arabinose, cellobiose, fructose, galactose, glucose, inositol, inulin, maltose, mannose, melezitose, melibiose, raffinose, ribose, salicin, sorbose, sucrose, trehalose and xylose. The final sugar concentration employed was 1% (w/v) except for inulin and salicin which were used at the final concentration of 0.5%. The medium (5 ml) was inoculated with 0.1 ml of an overnight culture in liver broth and incubated under oxygen-free CO_2 for 3 d at 37 °C. The pH values of cultures were measured with a Beckman Zeromatic SS-3 pH meter (Beckman-Toshiba, Tokyo, Japan). Further details of evaluation for fermentation are described in the Results.

Other biochemical tests. Tests for reduction of nitrate, production of indole, digestion of milk and casein were performed by the methods of Holdeman & Moore (1975). Liquefaction of gelatin was examined by the methods of Nakamura *et al.* (1973) and Holdeman & Moore (1975). Production of lecithinase and lipase was examined by using Nagler's modified VF blood agar (Nagler's m-VFBA) (Nagler, 1944).

Lecithinase neutralization test. This test was performed on half-antitoxin Nagler's m-VFBA according to the method of Oakley *et al.* (1947). The antitoxin sera of *C. novyi* types A and B were purchased from Wellcome.

RESULTS

DNA-DNA homology

A total of 17 strains of *C. novyi* types A and B, *C. haemolyticum*, and *C. botulinum* type C were examined for DNA-DNA homology with reference DNA from *C. novyi* type A 140, *C. novyi* type B 990 or *C. botulinum* type C 85-8. All of strains tested showed more than 80% homology with one of the above-mentioned three reference DNAs and separated into three genetically related groups: strains of 80 to 100% homology with reference DNA from *C. novyi* type A 140 (group I), strains of 84 to 100% homology with reference DNA from *C. novyi* type B 990 (group II) and strains of 91 to 100% homology with DNA from *C. botulinum* type C 85-8 (group III) (Table 1). Group I comprised all of four *C. novyi* type A strains. All of four *C. novyi* type B, all of four *C. haemolyticum* and one *C. botulinum* type C strain (Stockholm) were included in group II. Group III comprised *C. botulinum* type C 85-8, 95-2, 98-1 and 85-14. The group II and III organisms showed 26 to 49% homology with reference DNA from *C. novyi* type A 140 (group I), whereas the group III organisms showed 68 to 70% homology with reference DNA from *C. novyi* type B 990 (group II) and reciprocally the group II organisms showed 44 to 62% homology with

Table 1. Polynucleotide sequence relationships among *C. novyi* types A and B, *C. haemolyticum* and *C. botulinum* type C

Source of labelled DNA ...		Strain 140		Strain 990		Strain 85-8	
Group	DNA source	Homology (%)	$\Delta T_{m(e)}$ (*) (°C)	Homology (%)	$\Delta T_{m(e)}$ (*) (°C)	Homology (%)	$\Delta T_{m(e)}$ (*) (°C)
I	<i>C. novyi</i> Type A 140	100	0	44	10.0	33	13.3
	<i>C. novyi</i> Type A 3-1116	95					
	<i>C. novyi</i> Type A 77101	91					
	<i>C. novyi</i> Type A 4-1102	80	0.5	45		36	
II	<i>C. novyi</i> Type B 990	28	11.0	100	0	50	6.5
	<i>C. novyi</i> Type B GR 2B	32		99	0	59	
	<i>C. novyi</i> Type B GR 11B	49		94		62	
	<i>C. novyi</i> Type B GR 8B	37		93		59	
	<i>C. haemolyticum</i> Shinketsu	38		92	0.3	45	
	<i>C. haemolyticum</i> 6705	32		92		48	
	<i>C. haemolyticum</i> 6700	26	10.5	91	0.4	44	6.5
	<i>C. botulinum</i> type C Stockholm	42	9.8	85	0.4	54	5.6
	<i>C. haemolyticum</i> 4507	33		84		47	
III	<i>C. botulinum</i> type C 85-8	38	12.0	68	6.5	100	0
	<i>C. botulinum</i> type C 95-2	38		68		100	
	<i>C. botulinum</i> type C 98-1	36		68	5.8	92	0
	<i>C. botulinum</i> type C 85-14	37		70		91	

* $\Delta T_{m(e)}$ is the difference between the temperatures at which there is 50% dissociation of homologous and heterologous DNA duplexes.

Table 2. Polynucleotide sequence relationships among *C. novyi* type B, *C. haemolyticum* and *C. botulinum* type C

Source of labelled DNA ...		Strain 6700		Strain Stockholm	
Group	DNA source	Homology (%)	$\Delta T_{m(e)}$ (*) (°C)	Homology (%)	$\Delta T_{m(e)}$ (*) (°C)
II	<i>C. novyi</i> Type B 990	95	0	96	0.2
	<i>C. novyi</i> Type B GR 2B	95		86	0.2
	<i>C. novyi</i> Type B GR 11B	91		91	
	<i>C. novyi</i> Type B GR 8B	89		92	
	<i>C. haemolyticum</i> Shinketsu	89	0.2	85	0.5
	<i>C. haemolyticum</i> 6705	93			
	<i>C. haemolyticum</i> 6700	100	0	89	0.5
	<i>C. botulinum</i> type C Stockholm	84	0.3	100	0
	<i>C. haemolyticum</i> 4507	100		86	
III	<i>C. botulinum</i> type C 85-8	59		74	5.8
	<i>C. botulinum</i> type C 95-2	67		69	
	<i>C. botulinum</i> type C 98-1	61		69	6.0
	<i>C. botulinum</i> type C 85-14	64		66	

* See Table 1.

reference DNA from *C. botulinum* type C 85-8 (group III). These data suggest that the strains in group II were more closely related to the strains in group III than those in group I.

Further tests were performed to determine the thermostability of the duplexes formed between DNA from *C. novyi* type A 140 (group I), *C. novyi* type B 990 (group II) or *C. botulinum* type C 85-8 (group III) and DNAs from a few strains arbitrarily selected from groups I, II and III. The $T_{m(e)}$ values for the DNA duplexes between group II strains and *C. novyi* type B 990 (group II) were similar to that of homologous DNA duplexes of *C. novyi* type B 990 ($\Delta T_{m(e)}$ 0 to 0.4 °C). The $T_{m(e)}$ values for the DNA duplexes between group II strains and *C. novyi* type A 140 (group I) were 9.8 to 11.0 °C lower than that of homologous DNA duplexes of *C. novyi* type A 140. The $T_{m(e)}$ values for the DNA duplexes between group II strains and *C. botulinum* type

Table 3. *Biochemical properties for differentiation of strains in groups I, II and III*

Strains were differentiated into groups based on DNA-DNA homology (see Table 1). The results show the no. of strains positive/no. of strains tested.

Property	Group I	Group II	Group III
Fermentation of:			
Glucose	4/4	9/9	4/4
Ribose	4/4	9/9	4/4
Fructose	2/4	9/9	4/4
Inositol	2/4	9/9	4/4
Maltose	4/4	8/9	4/4
Mannose	0/4	9/9	4/4
Melibiose	0/4	0/9	4/4
Galactose	0/4	0/9	4/4
Liquefaction of gelatin:			
2% (w/v)	4/4	9/9	4/4
10% (w/v)	3/4	0/9	3/4
Production of indole	3/4	9/9	0/4
Digestion of milk	3/4	9/9	0/4
Digestion of casein	3/4	0/9	0/4

Table 4. *Lecithinase neutralization of Nagler's m-VFBA*

Strains were differentiated into groups based on DNA-DNA homology (see Table 1).

Group	Lecithinase neutralization by antitoxin serum against <i>C. novyi</i> types	
	A	B
I	+	-
II	-	+
III	-	+

C 85-8 (group III) were 5.6 to 6.5 °C lower than that of homologous DNA duplexes of *C. botulinum* type C 85-8. These data again confirmed the closer genetic relationship between groups II and III.

A further study with labelled DNAs from *C. botulinum* type C Stockholm and *C. haemolyticum* 6700 confirmed that there were two genetically different groups in *C. botulinum* type C and that *C. haemolyticum*, *C. novyi* type B and *C. botulinum* type C Stockholm were genetically homologous (Table 2).

Biochemical properties

Biochemical properties were examined to find the differential criteria corresponding to the genetic differences mentioned above (Table 3). In the test for sugar fermentation, the pH of the 3 d cultures without sugar added was usually 6.3 or 6.4. Although some of the strains, in the presence of a certain sugar, exhibited only a slight pH fall, these strains were evaluated as being fermentation-positive because they showed distinctly abundant growth in the presence of the sugar, relative to that in the absence of the sugar. Final pH in these fermentation-positive cases usually ranged between 5.7 and 6.1. Useful criteria for differentiation were as follows: none of group I strains fermented mannose, while all strains of groups II and III fermented this sugar; none of group II strains fermented melibiose and galactose, while all strains of group III fermented the two sugars; none of group III strains produced indole nor digested milk, while all strains in group II were positive for these two characters.

No distinct difference, except for lipase production, was found among strains of group II, although they were designated by different names, i.e. *C. novyi* type B, *C. haemolyticum* and *C. botulinum* type C. In the test for lipase production on Nagler's m-VFBA, *C. botulinum* type C Stockholm (group II) was positive but all the *C. novyi* type B and *C. haemolyticum* strains were negative. All strains of *C. botulinum* type C in group III were positive in the lipase test.

Fig. 1. Lecithinase reaction of *C. novyi* type B 990 (a) and *C. botulinum* type C 85-8 (b) on half-antitoxin Nagler's m-VFBA spread with the antitoxin serum of *C. novyi* type B.

Lecithinase neutralization test

A neutralization test for lecithinase reaction of *C. botulinum* type C, *C. novyi* types A and B, and *C. haemolyticum* was performed on half-antitoxin Nagler's m-VFBA spread with the antitoxin serum of *C. novyi* type A or B. The antitoxin serum of *C. novyi* type A specifically suppressed the lecithinase reaction of *C. novyi* type A, whereas that of *C. novyi* type B suppressed the lecithinase reaction of *C. botulinum* type C in groups II and III as well as that of *C. novyi* type B and *C. haemolyticum* (Table 4, Fig. 1).

DISCUSSION

The present study on DNA-DNA homology showed that *C. novyi* types A and B, *C. haemolyticum*, and *C. botulinum* type C strains were genetically divided into groups I (*C. novyi* type A), II (*C. novyi* type B, *C. haemolyticum* and one *C. botulinum* type C strain) and III (*C. botulinum* type C). Smith & Hobbs (1974) found that *C. haemolyticum* and *C. novyi* type B are separate species, although the major lethal toxin of *C. haemolyticum* is identical with the beta toxin of *C. novyi* type B (Oakley & Warrack, 1959; Oakley *et al.*, 1974) and the cultural properties of the two species resembled each other very closely (Holdeman & Moore, 1975; Nakamura *et al.*, 1975b; Roberts *et al.*, 1970; Rutter, 1970). The present study shows that *C. haemolyticum* and *C. novyi* type B comprise one genetically homologous group (group III). In PAGE analysis of soluble cellular proteins of the two species, Cato *et al.* (1982) also demonstrated that the patterns of *C. novyi* type B and *C. haemolyticum* were indistinguishable. As for the alpha toxin, the main difference between the two species, it has already been demonstrated that the alpha toxigenesis in *C. novyi* type B and *C. haemolyticum* was intimately related with phage infection; *C. novyi* type B, when cured of a certain phage, ceased to produce its alpha toxin and again produced it when the phage-sensitive cultures were reinfected with the phage (Eklund *et al.*, 1976), while *C. haemolyticum*, when infected with phage of *C. novyi* type A, produced the alpha toxin (Schallehn & Eklund, 1980). It appears, therefore, that *C. haemolyticum* should be considered as *C. novyi* type D as Oakley & Warrack (1959) suggested.

The present test for DNA-DNA homology revealed that *C. botulinum* type C Stockholm was genetically different from the remaining type C strains (group III) that were isolated from

marine sediments (Serikawa *et al.*, 1977). For practical purposes, however, the name *C. botulinum* is still preferable for the strain Stockholm because the toxicity of this strain is neutralizable by the common antitoxin serum for *C. botulinum* type C but not by that for *C. novyi* type B or *C. haemolyticum*.

Some comments may be needed concerning the differences in reciprocal crosses of DNA-DNA homology values (%) among reference DNAs of groups I, II and III, although the data are scant. The difference between groups I and III was small (strain 140 $\times$ strain 85-8 = 38%; strain 85-8 $\times$ strain 140 = 33%; difference 5%), while the differences were 15 to 24% between groups II and III, and 16% between groups I and II. One explanation for these larger differences could be that the genome size of group II may be smaller than those of the two other groups.

Although carbohydrate fermentation properties of *C. novyi* types A and B, and *C. haemolyticum* vary somewhat from one strain to another and also from one laboratory to another (Holdeman & Moore, 1975; Roberts *et al.*, 1970; Rutter, 1970), Holdeman & Moore (1975) stated that mannose was fermented by most of *C. novyi* type B and *C. haemolyticum* strains but not by any of *C. novyi* type A strains, when the test was performed in the PRAS medium. Our results also confirmed this observation; none of group I strains (*C. novyi* type A) fermented mannose but all of group II strains did ferment this sugar. The *C. botulinum* type C strain in group III differed from group II strains in fermentation of melibiose and galactose. Fermentation patterns of the *C. botulinum* type C strains in group III were similar to those of marine strains of *C. botulinum* type C described by Segner *et al.* (1971), who stated that the marine and terrestrial strains of *C. botulinum* type C were different from each other in carbohydrate fermentation.

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